Agilent Mammalian ChIP-on-chip

Protocol

Version 10.2, March 2011

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Agilent Technologies

Notices

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What's New in Version 10.2?

- The expected specific activity values for the Cyanine-3 and Cyanine-5 labeled DNA are updated.

What's New in Version 10.12?

- Agilent Genomic DNA Enzymatic Labeling Kit (5190-0449) replaces Agilent Genomic DNA Labeling Kit PLUS (5188-5309).

What's New in Version 10.11?

- Centrifuge speeds are corrected for Sample Labeling.

What's New in Version 10.1?

This protocol differs from the previous release of the Mammalian ChIP-on-chip Protocol in the following ways:

- Millipore's Microcon YM-30 columns are replaced by the Amicon 0.5-Ultra 30kDa columns for the clean-up of the labeled genomic DNA.
- The 2100 Bioanalyzer, in conjunction with the Agilent High Sensitivity DNA kit, is incorporated as a QC step for analysis of the sheared genomic DNA.

Contents

1 Sample Preparation

The steps in this protocol and the estimated amounts of time required are listed in Table 1 on page 8.

The reagents, enzymes, buffers and equipment that are used in this protocol are listed in "Reagents, Enzymes, Buffers and Equipment" on page 66.

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 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

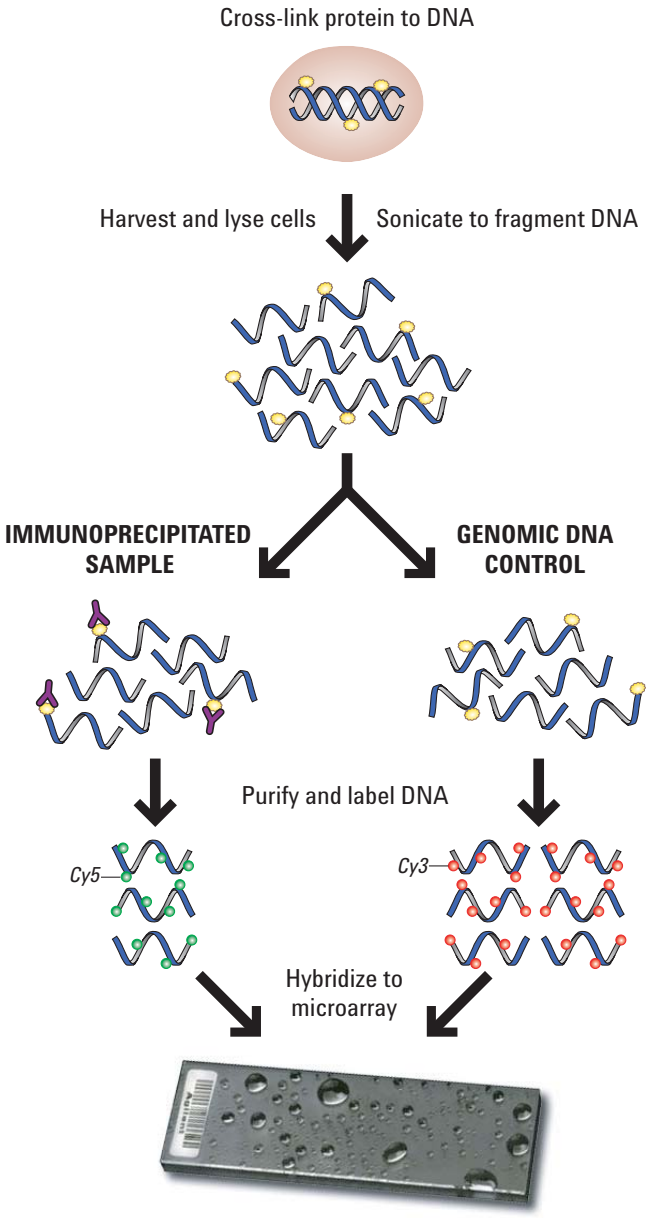


Figure 1 ChIP-on-chip overview

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

Use 5 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 11) 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 1X PBS. Add another 5 mL of 1X PBS and Harvest cells using a silicone 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. 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 2 on page 11) 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 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 of 1x PBS per 10^8 cells. Transfer 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 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 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

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 Dynal (Life Technologies) magnetic beads. Dynal beads will have settled during storage.
2. Add the 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.
3. Add 1 mL Block Solution (see Table 3 on page 13.)
4. Gently mix the beads in Block Solution.
5. Put tubes into a magnetic device such as the Dynal small-volume magnetic particle concentrator (Life Technologies).
6. Remove the supernatant.
7. 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.
8. Resuspend the beads in 250 µL 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 Block Solution (as described in step 7 above).

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

12 Resuspend the beads in 100 µL Block Solution.

Table 3 Block Solution

Stock	For 100 mL	Final Concentration
10x PBS	10 mL	1X
BSA	500 mg	0.5% BSA (w/v)
ddH_2O	90 mL	
Total	100 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 15). 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 15). 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 15).
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.

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 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% Igepal	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 12 to the 15 mL conical tube containing the cell lysate from "Step 3. Lyse the cells" on page 14.
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 7 on page 17). 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 with a pipette.

Elution and Reversing Cross-Links

1. Add 210 µL of elution buffer and resuspend beads.
2. Elute by placing 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 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 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 8), and mix. Reverse the cross-links by incubating in a water bath 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% Igepal	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 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 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 in a water bath 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 (Eppendorf, NY) tube for each IP and WCE sample by spinning tube at 14,000 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 14,000 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% ice-cold EtOH.
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 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 dd$H_2$0 to 40 µM.

NOTE Neither oligo requires 5' phosphorylation.

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.

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

Step 8. Blunt the DNA ends and ligate the linkers

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 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 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 x 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 x g for 5 minutes at room temperature.

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

14 Add 250 µL of 100% 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 ice-cold 70% EtOH.

18 Dry the pellets for 10 minutes with a vacuum desiccator, 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 (Life Technologies)	10.0 µL	1x
15 µM linkers (see "Step 7. Prepare linkers for LM-PCR" on page 19)	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 of 100% 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 ice-cold 70% EtOH.

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

Step 9. 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 JW102 (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 Add 45 µL of PCR mix to each reaction tube.

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
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 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 ice-cold 70% EtOH.

16 Dry the pellets for 10 minutes with a vacuum desiccator, 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.

2 Sample Labeling

The Agilent Genomic DNA Enzymatic Labeling Kit (Agilent p/n 5190-0449) uses random primers and the exo-Klenow fragment to differentially label genomic DNA samples with fluorescent-labeled nucleotides. For Agilent's Oligo ChIP-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 in the green channel and the IP is labeled in the red channel.

Step 1. Fluorescent Labeling of DNA

NOTE Cyanine 3-dUTP and cyanine 5-dUTP are light sensitive and are subject to degradation by multiple freeze thaw cycles. Minimize light exposure throughout the labeling procedure.

For 1x, 2x and 4x Array Formats

1. Equilibrate water baths to 95°C, 37°C and 65°C. You can also use a thermocycler for these heating steps.
2. Place 2 µg of amplified WCE or IP DNA to a 1.5 mL heat-sustainable nuclease-free microfuge tube per reaction. Bring the volume up to 26 µL with nuclease free water.
3. Add 5 µL of Random Primers (supplied with Agilent Genomic DNA Enzymatic Labeling Kit) to each reaction tube containing 26 µL of amplified DNA to make a total volume of 31 µL. Mix well by gently pipetting up and down.
4. Transfer sample tubes to a circulating water bath or heat block at 95°C. Incubate at 95°C for 3 minutes, then move to ice and incubate for 5 minutes.
5. Prepare the Labeling Master Mix by mixing the components in Table 15 on ice in the order indicated.

Table 15 Preparation of Labeling Master Mix (for 1x, 2x and 4x array formats)

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

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

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

8 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, then move to ice.

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

For 8x Array Format

1 Add 2.5 µL of Random Primers (supplied with Agilent Genomic DNA Enzymatic Labeling Kit) to each PCR tube containing 13 µL of amplified WCE or IP DNA to make a total volume of 15.5 µL. Mix well by pipetting up and down gently.

2 Transfer sample tubes to a PCR machine. Program the PCR machine according to the following table and run the program:

Step	Temperature	Time
Step I	95°C	3 minutes
Step II	4°C	hold

3 Remove sample tubes from PCR machine, and place them on ice for 5 minutes.

4 Prepare the Labeling Master Mix by mixing the following components in Table 16 on ice in the order indicated.

Table 16 Preparation of Labeling Master Mix (for 8x array format)

Component	Per reaction (µL)	x 8 rxns (µL) (including excess)
5X Buffer	5.0	45.0
10X dNTP	2.5	22.5
Cyanine 3-dUTP (1.0 mM) or Cyanine 5-dUTP (1.0 mM)	1.5	13.5
Exo-Klenow fragment	0.5	4.5
Final volume of Labeling Master Mix	**9.5**	**85.5**

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

6 Transfer sample tubes to a PCR machine. Program the machine according to the following table and run the program:

Step	Temperature	Time
Step I	37°C	2 hours
Step II	65°C	10 minutes
Step III	4°C	hold

7 Remove sample tubes from PCR machine, and place them on ice.

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

NOTE The above steps can also be performed in 1.5 mL heat-sustainable nuclease-free microfuge tubes in water baths.

Step 2. Clean-up of Labeled Genomic DNA

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

1. Transfer samples to a 1.5 mL heat-sustainable nuclease-free microfuge tube.
2. Add 430 µL of 1X TE (pH 8.0) to each reaction tube.
3. For each labeled sample, place a Amicon 30kDa filter into a 1.5-mL microfuge tube (supplied) and load each labeled amplified WCE or IP sample into the filter. Spin 10 minutes at 14,000 × g in a microcentrifuge at room temperature. Discard the flow-through.
4. Add 480 µL of 1X TE (pH 8.0) to each filter. Spin for 10 minutes at 14,000 × g in a microcentrifuge at room temperature. Discard the flow-through.

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

For 1x Array Format

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

Keep the filter until you are certain that you have collected enough sample for the next step.

6 Measure and record volume (µL) of each eluate. If sample volume exceeds 80.5 µL, return sample to its filter and spin 1 minute at 14,000 × g in a microcentrifuge at room temperature. Discard the flow-through.

7 Repeat step 5 and step 6 until each sample volume is ≤80.5 µL.

8 Bring total sample volume to 80.5 µL with 1X TE (pH 8.0).

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

- DNA yield > 5 µg per reaction
- Cyanine 5 > 15 pmol/µg
- Cyanine 3 > 18 pmol/µg

10 Combine the appropriate cyanine 5-labeled sample and cyanine 3-labeled sample for a total mixture volume of 158 µL in a new 1.5-mL heat-resistant microfuge tube.

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

For 2x array format

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

Keep the filter until you are certain that you have collected enough sample for the next step.

5 Measure and record volume (µL) of each eluate. If sample volume exceeds 41 µL, return sample to its filter and spin 1 minute at 14,000 × g in a microcentrifuge at room temperature. Discard the flow-through.

6 Repeat step 5 and step 6 until each sample volume is ≤41 µL.

7 Bring total sample volume to 41 µL with 1X TE (pH 8.0).

8 Take 1.5 µL of each sample to determine yield and specific activity by using the NanoDrop ND-1000 UV-VIS Spectrophotometer.

- DNA yield > 5 µg per reaction
- Cyanine 5 > 15 pmol/µg
- Cyanine 3 > 18 pmol/µg

9 Combine the appropriate cyanine 5-labeled sample and cyanine 3-labeled sample for a total mixture volume of 79 µL in a new 1.5-mL heat-resistant microfuge tube.

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

For 4x Array Format

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

Keep the filter until you are certain that you have collected enough sample for the next step.

5 Measure and record volume (µL) of each eluate. If sample volume exceeds 21 µL, return sample to its filter and spin 1 minute at 14,000 × g in a microcentrifuge at room temperature. Discard the flow-through.

6 Repeat step 5 and step 6 until each sample volume is ≤ 21 µL.

To achieve the desired volume, use a vacuum concentrator (such as a SpeedVac concentrator) to concentrate the sample.

7 Bring total sample volume to 21 µL with 1X TE (pH 8.0).

8 Take 1.5 µL of each sample to determine yield and specific activity by using the NanoDrop ND-1000 UV-VIS Spectrophotometer:

- DNA yield > 5 µg per reaction
- Cyanine 5 > 15 pmol/µg
- Cyanine 3 > 18 pmol/µg

9 Combine the appropriate cyanine 5-labeled sample and cyanine 3-labeled sample for a total mixture volume of 39 µL in a new 1.5-mL heat-resistant microfuge tube.

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

For 8x Array Format (standard)

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

Keep the filter until you are certain that you have collected enough sample for the next step.

5 Measure and record volume (µL) of each eluate. If sample volume exceeds 10 µL, return sample to its filter and spin 1 minute at 14,000 × g in a microcentrifuge at room temperature. Discard the flow-through.

6 Repeat step 4 and step 5 until each sample volume is ≤ 10 µL.

To achieve the desired volume, use a vacuum concentrator (such as a SpeedVac concentrator) to concentrate the sample.

7 Bring the total sample volume to 10 µL with 1X TE (pH 8.0).

8 Take 1.5 µL of each sample to determine yield and specific activity by using the NanoDrop ND-1000 UV-VIS Spectrophotometer:

- DNA yield > 5 µg per reaction
- Cyanine 5 > 15 pmol/µg
- Cyanine 3 > 18 pmol/µg

9 Combine the appropriate cyanine 5-labeled sample and cyanine 3-labeled sample by removing 8 µL of each sample from its respective tube and placing it into a new 1.5-mL heat-resistant microfuge tube for a total mixture volume of 16 µL.

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

For 8x Array Format (with vacuum concentrator)

4 Invert the filter into a fresh 1.5-mL microfuge tube (supplied). Spin for 1 minute at 1,000 x g in a microcentrifuge at room temperature to collect purified sample.

Keep the filter until you are certain that you have collected enough sample for the next step.

5 Place sample tubes in a vacuum concentrator, such as a SpeedVac, with tops open, and evaporate to dryness. This may take 20 to 40 minutes depending on sample volume. If possible, cover the speed vac to reduce light exposure to the samples.

6 Reconstitute each sample with 1X TE (pH 8.0) to a final volume of 10 µL.

7 Take 1.5 µL of each sample to determine yield and specific activity by using the NanoDrop ND-1000 UV-VIS Spectrophotometer:

- DNA yield > 5 µg per reaction
- Cyanine 5 > 15 pmol/µg
- Cyanine 3 > 18 pmol/µg

8 Combine the appropriate cyanine 5-labeled sample and cyanine 3-labeled sample by removing 8 µL of each sample from its respective tube and placing it into a new 1.5-mL heat-resistant microfuge tube for a total mixture volume of 16 µL.

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

3
Hybridization and Wash

In this section, you hybridize and wash ChIP-on-chip microarrays.

Hybridization and Wash with SureHyb Hybridization Chamber

This section contains information to help you hybridize and wash your microarray using the SureHyb Hybridization Chamber. As an alternative, you can use the Tecan Pro HS Hybridization Station to hybridize and wash your microarrays.

Step 1. Hybridize the microarray

1 Prepare the 10X Blocking Agent:

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

   b Check that the Blocking agent is completely reconstituted before use. You may need to incubate at room temperature for 60 minutes to reconstitute sample before use or storage.

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

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

The next steps are an abbreviated version of the Agilent aCGH array hybridization protocol. Please refer to aCGH protocol for details, notes, and warnings.

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

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

Table 17

	8x Array Format	4x Array Format	2x Array Format	1x Array format
Cy5-labeled Samples	1.25 to 2.5 µg	2.5 to 5.0 µg	4 to 5 µg	5 µg
Cy3-labeled Samples	1.25 to 2.5ug	2.5 to 5.0 µg	4 to 5 µg	5 µg
Total Volume with ddH_20	**16 µL**	**39 µL**	**79 µL**	**158 µL**

NOTE The input mass should be balanced for each channel, e.g. 2.5 µg of Cy3 labeled DNA and 2.5 µg of Cy5 labeled DNA.

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

Table 18

Stock	8x Array Format (µL)	4x Array Format (µL)	2x Array Format (µL)	1x Array format (µL)
Human Cot-1 DNA (1.0 mg/mL) *	2	5	25	50
Agilent Blocking Agent (10x)†	4.5	11	26	52
Agilent Hybrdization Buffer (2x)*	22.5	55	130	260

* Use the Cot-1 DNA for the species you are studying if it is available.

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

5 Mix contents and quick spin to collect.

6 Heat samples for 3 minutes at 95°C.

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

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

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

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

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

8x format	40 µL/array (8 individual samples of 40 µL each)
4x format	100 µL/array (4 individual samples of 100 µL each)
2x format	240 µL (2 individual samples of 240 µL each)
1x format	490 µL

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

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

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

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

14 Place assembled slide chamber in rotisserie hybridization oven set to 65°C. Hybridize as follows:

4x or 8x formats	Hybridize at 20 RPM for 24 hours
1x or 2x formats	Hybridize at 20 RPM for 40 hours

Step 2. Prewarm Oligo aCGH/ChIP-on-chip Wash Buffer 2

Warm the **Oligo aCGH/ChIP-on-chip Wash Buffer 2** to 31°C as follows:

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

Step 3. Prepare the equipment

Always use clean equipment when doing the hybridization and wash steps. Designate and dedicate dishes to two-color experiments. The acetonitrile wash is only necessary if the staining dishes, racks and stir bars were used in previous experiments with the Agilent Stabilization and Drying Solution. Otherwise proceed to "Milli-Q water wash" on page 44.

Acetonitrile wash

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

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

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

6 Discard the acetonitrile as is appropriate for your site.

7 Repeat steps step 1 through step 6.

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

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

Milli-Q water wash

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

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

2 Empty out the water collected in the dish.

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

4 Discard the Milli-Q water.

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

Step 4. Wash the microarray slides

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

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

Before you begin, determine which wash procedure to use:

- If you are washing slides in an ozone-controlled environment, and the ozone level is 5 ppb or less, then use the standard wash procedure, which *does not* include the optional steps listed in Table 19.
- If you are washing slides in an environment in which the ozone level exceeds 5 ppb, do the optional 3rd and 4th washes listed in Table 19. These steps are described in "Stabilization and Drying Solution (optional)" on page 54.

Table 19 Wash conditions

	Dish	Wash Buffer	Temperature	Time
Disassembly	1	Oligo aCGH/ChIP-on-chip	Room temperature	
1st wash	2	Oligo aCGH/ChIP-on-chip	Room temperature	5 minutes
2nd wash	3	Oligo aCGH/ChIP-on-chip	31°C	5 minutes
3rd wash (optional)	4	Wash Buffer: ACN	Room temperature	10 seconds
4th wash (optional)	5	Wash Buffer: Stabilization and Drying Solution	Room temperature	30 seconds

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

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

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

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

4 Prepare the hybridization chamber disassembly.

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

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

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

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

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

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

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

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

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

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

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

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

8 During this wash step, remove Oligo aCGH/ChIP-on-chip Wash Buffer 2 from the 31°C water bath and pour into the slide-staining dish #3.

9 Approximately 1 min before the end of Wash1, put the prewarmed 1.5 L glass dish filled with water and containing slide-staining dish #3 on a

magnetic stir plate with heating element. Fill the slide-staining dish #3 approximately three-fourths full with Oligo aCGH/ChIP-on-chip Wash Buffer 2 (warmed to 31°C). Add a magnetic stir bar. Turn on the heating element and maintain temperature of Oligo aCGH/ChIP-on-chip Wash Buffer 2 at 31°C. Monitor using a thermometer.

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

11 If you are washing slides in an environment in which the ozone level exceeds 5 ppb, continue at "Stabilization and Drying Solution (optional)" on page 54.

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

13 Repeat step 1 through step 12 for the next group of eight slides using fresh Oligo aCGH/ChIP-on-chip Wash Buffer 1 and Oligo aCGH/ChIP-on-chip Wash Buffer 2 pre-warmed to 31°C.

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

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

Hybridization and Wash with Tecan HS Pro Hybridization Station

Follow these steps to hybridize the microarray with the Tecan HS Pro Hybridization Station. This is an alternative to "Hybridization and Wash with SureHyb Hybridization Chamber" on page 40

Step 1. Test and prepare equipment for first use

CAUTION Before you begin, make sure you have read and understand operating, maintenance and safety instructions for using your Tecan hybridization station. Refer to the documentation that came with your hybridization station.

1. Set up the hybridization station, including computer, all tubes, reagents, waste, and pressure bottles, power, and software.
2. Carefully check O-rings for damage.
3. Clean the hybridization chambers with water and then dry with compressed nitrogen.
4. Insert hybridization chambers into chamber frames.
5. Place the adapter with dummy slides onto the heating plates and close the chamber carefully.
6. Flush the delivered reagent bottles with distilled water before use.
7. Run a rinsing procedure with the Cleaning and Conditioning Solution, instead of water.

   To prepare the Cleaning and Conditioning Solution:

   - In the Tecan wash bottle, add 1 mL of the Agilent Microarray Wash Additive (5190-0401) to 1 L of the Agilent Oligo Wash Buffer 2. Mix thoroughly by shaking.

NOTE Use the solution to rinse the instrument at regular intervals of approximately every 2 weeks to coincide with the replacement of the hybridization chamber O-rings.

8 Run a rinsing procedure with distilled water.

9 For conditioning the hybridization chambers, start a hybridization run (for example 12 hours) at the temperature used in the protocol (67°C) with 1X Hybridization buffer and dummy glass.

10 Run a rinsing procedure with the **Final System Drying** check box marked to dry the chambers.

This step is described in the Tecan operating guide.

Step 2. Set up reagent bottles

- Prepare the six Wash Bottles according to Table 20.

Table 20

Wash Bottle	Reagent	Heated
1	Agilent Prehybridization Buffer	Yes
2	Oligo aCGH Wash Buffer 1	No
3	Oligo aCGH Wash Buffer 2 w/ 0.01% Wash Buffer Additive*	Yes
4	Empty	N/A
5	Water	No
6	Cleaning and Conditioning Solution	No

* Spike the Wash Buffer Additive into the Oligo CGH Wash Buffer 2 (5188-5222) at the ratio of 100 µL per 1 liter of wash buffer

WARNING **Never add Acetonitrile or Agilent Stabilization and Drying Solution to a Tecan reagent bottle for use inside the HS Pro Hybridization Station. These solutions are incompatible with the Tecan HS Pro Hybridization Station.**

Step 3. Prepare labeled genomic DNA for hybridization

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

a Add 135 µL of nuclease-free water to the vial containing lyophilized 10X Blocking Agent (supplied with Agilent Oligo aCGH Hybridization Kit).

NOTE

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

Reconstitution volume of 10X Blocking Agent for hybridization with the Tecan HS Pro Hybridization Station differs from that for the SureHyb Hybridization Chamber.

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

2 Prepare the samples for hybridization:

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

b Add the components in Table 21 in the order indicated in a nuclease-free tube.

Table 21 Assembly of hybridization samples

Component	Volume (µL) per hybridization (for 1x array format)	Volume (µL) per hybridization (for 2x array format)
Cyanine 5- and cyanine 3-labeled gDNA mixture	55	25
Cot-1 DNA (1.0 mg/mL)*	5	5
Agilent 10X Blocking Agent†	5	5
Agilent 2X Hybridization Buffer†	65	35
Final Hybridization Sample Volume	130	70

* Use Cot-1 DNA from the appropriate species.

† Supplied with Agilent Oligo aCGH Hybridization Kit

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

d Transfer sample tubes to a circulating water bath or heat block at 95°C. Incubate at 95°C for 3 minutes.

e Immediately transfer sample tubes to a circulating water bath or heat block at 37°C. Incubate at 37°C for 30 minutes.

f Remove sample tubes from the water bath or heat block. Spin 1 minute at 17,900 × g in a microcentrifuge to collect the sample at the bottom of the tube.

Step 4. Tecan Hybridization

In this step, you hybridize the microarrays with the Tecan HS Pro hybridization station. For support on the Tecan HS Pro hybridization station, contact your Tecan representative.

1 Set up the Tecan HS Pro hybridization station. Follow the instructions in *Instructions for use for HS 4800/HS400 Pro Hybridization Station* to:

- Set up the reagent bottles (see Table 20 on page 49).
- Load slides.
- Inject samples.

Note that the Tecan segmented chamber labeled “B” corresponds to the array closest to the barcode or number 1 in Agilent’s Feature Extraction software. The chamber labeled “A” is number 2 in Agilent's Feature Extraction software.

2 Open the Tecan HS Pro Control Manager software, then run the Agilent Oligo ChIP-on-chip program.

You can download the Agilent Oligo ChIP-on-chip program from http://www.opengenomics.com/Hardware.aspx.

The Oligo ChIP-on-chip Program contains the steps in Table 22.

Table 22 Oligo aCGH Program

Step Number	Program Step	Step Action
1	Wash	Agilent Prehybridization Buffer at 67°C
2	Sample Injection	Sample Loading (120 µL with A1X244k or 65 µL with A2x105K)
3	Hybridization	67°C for 24 hours
4	Wash	Oligo aCGH/ChIP-on-chip Wash Buffer 1 at Room Temp
5	Wash	Oligo aCGH/ChIP-on-chip Wash Buffer 2 at 31°C
6	Drying	2 minutes at 30°C

3 Turn on the reagent bottle heating.

4 Click **Go**.

5 Follow the instructions in *Instructions for use for HS 4800/HS400 Pro Hybridization Station* to inject the samples.

6 When the program is finished, remove the MTP Slide adapter from the instrument.

You can now remove the slides to scan in the Agilent scanner.

At this point, you can process the slides with the Agilent Stabilization and Drying Solution to protect the dyes against ozone degradation. See "Stabilization and Drying Solution (optional)" on page 54.

After you remove the MTP slide adapter from the instrument, a small amount of adhesive or ink from the barcode may remain on the heating block. Remove the adhesive and ink with a 70% ethanol solution.

Stabilization and Drying Solution (optional)

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

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

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

Step 1. Prewarm Stabilization and Drying Solution

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

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

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

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

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

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

2 Gently shake the container to obtain a homogenous solution.

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

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

Step 2. Wash microarray with Stabilization and Drying Solution

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

NOTE

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

7. Scan slides immediately to minimize impact of environmental oxidants on signal intensities. If necessary, store slides in original slide boxes in a N_2 purge box, in the dark.
8. Dispose of acetonitrile and Stabilization and Drying Solution as flammable solvents.
9. If you are hybridizing with the SureHyb Hybridization Chamber, return to step 13 on page 47.

4
Scanning and Feature Extraction

In this section, you scan and extract data from ChIP-on-chip microarrays.

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

Step 1. Microarray Scanning using Agilent C, B or A Scanner or GenePix Scanner

Agilent C Scanner Settings

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

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

Agilent A and B Scanner Settings

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

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

2 Put assembled slide holders into scanner carousel.

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

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

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

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

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

GenePix Scanner Settings

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

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

- Refer to the manufacturer's user guide for appropriate scanner settings.
- Refer to "General Microarray Layout and Orientation" on page 81 for appropriate slide layout and orientation in GenePix scanner.

Step 2. Data Extraction using Feature Extraction Software

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

The Feature Extraction (FE) software v9.5 supports extraction of microarray TIFF images (.tif) of Agilent ChIP microarrays scanned on the Agilent Scanner B and GenePix 4000B Scanner.

Feature Extraction version 10.7 or later can automatically download Grid Templates, protocols and QC metrics (QCMT) from eArray if configured appropriately. See "Automatic Download from eArray" on page 64 for configuration.

Figure 2 shows an example of an Agilent 1M microarray image opened in Feature Extraction software v10.5 in both full and zoomed view.

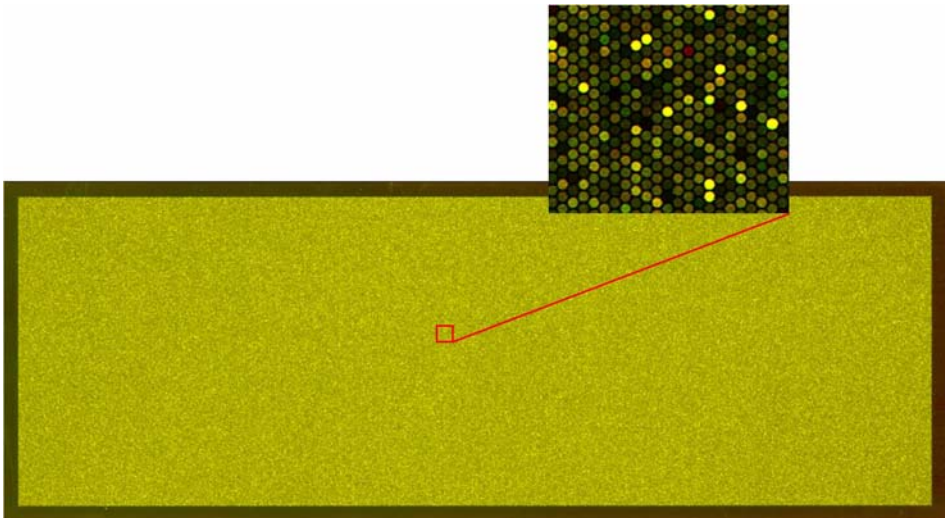


Figure 2 Agilent SurePrint G3 1x1M microarray shown in red and green channels: full and zoomed view

1 Open the Agilent Feature Extraction (FE) program.

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

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

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

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

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

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

3 Set FE Project Properties.

a Select the **Project Properties** tab.

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

c In all other sections, verify that at least the following default settings as shown in Figure 3 below are selected.

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

For FE 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 eArray if configured appropriately. See "Automatic Download from eArray" on page 64 for configuration.

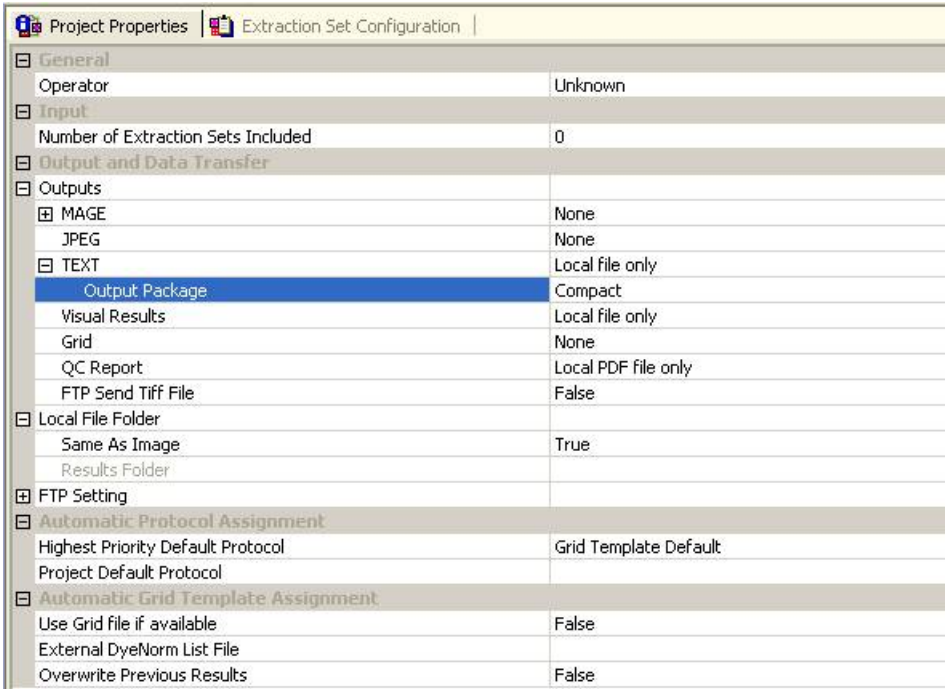


Figure 3 Default settings in FE 10.5

4 Check the Extraction Set Configuration.

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

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

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

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

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

   c Verify that the ChIP-v1_95_May07.xml (in FE 9.5), ChIP_105_Jan09 (in FE 10.5) or ChIP_107_Sep09 (in FE 10.7) protocol is assigned to each extraction set in the **Protocol Name** column.

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

   Protocols are also available automatically from eArray if configured appropriately. See "Automatic Download from eArray" on page 64 for configuration.

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

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

7 Select **Project > Start Extracting**.

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

Automatic Download from eArray

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 4.

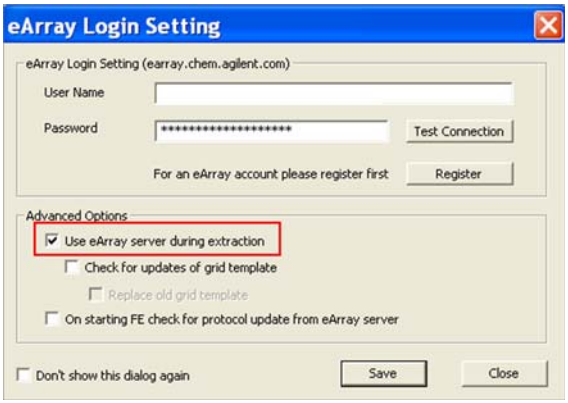


Figure 4 eArray Login Setting

5 Reference

Reagents, Enzymes, Buffers and Equipment

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

Table 23 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 24 Immunoprecipitation

Item	Size	Vendor	Catalog #	Stock Concentration
Dynal Magnetic Beads	various	Life Technologies	Various	
Antibody		Various		
BSA, powder	50 g	Sigma	A7906-50G	0.5% (in PBS)
10X PBS	500 mL	Dynal (Life Technologies)	70013-032	1X

Table 25 Cell Sonication, Reverse Cross-linking and DNA Clean-up

Item	Size	Vendor	Catalog #	Stock Concentration
Amicon Ultra-0.5 30 kDa	96/pk	Millipore	UFC503096	
Protease Inhibitor (Complete Tablets)	20 tablets	Roche	11 697 498	25X
RNase A	10 mL	Life Technologies	12091-021	10 mg/mL
Proteinase K	5 mL	Life Technologies	25530-049	20 mg/mL

Table 25 Cell Sonication, Reverse Cross-linking and DNA Clean-up

Item	Size	Vendor	Catalog #	Stock Concentration
Sonicator machine	n/a	Various (see protocol)		n/a
DynaMag-2 magnet	n/a	Dynal (Life Technologies)	123-21D	n/a

Table 26 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	GE Healthcare	28-4065-51	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	Life Technologies	46300-018	5X
Linkers, see "Step 7. Prepare linkers for LM-PCR" on page 19.				
Taq Polymerase	1000 units/tube	Life Technologies (Applied Biosystems)	N808-0156	5 U/µL
ThermoPol buffer	4 x 1.5 mL	NEB	B9004S	10X

Table 26 DNA Amplification (LM-PCR)

Item	Size	Vendor	Catalog #	Stock Concentration
oligo JW102, see "Step 7. Prepare linkers for LM-PCR" on page 19				
oligo JW103, see "Step 7. Prepare linkers for LM-PCR" on page 19				

Table 27 Cy Labeling, Hybridization, Washing and Scanning

Item	Size	Vendor	Catalog #	Stock Concentration
Agilent Genomic DNA Enzymatic Labeling Kit	50 reactions	Agilent	5190-0449	
Oligo aCGH/ChIP-on-chip Hybridization Kit	25 reactions	Agilent	5188-5220	
Human Cot-1 DNA	500 µg	Life Technologies	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 28 Required equipment

Description	Company and part no.
Tecan HS 400 Pro or Tecan HS 4800 Pro	Tecan Group Ltd.
A1x244K (for 1x1M and 1x244K microarrays), A2x105K (for 2x400K and 2x105K microarrays) *or* A4x44K (for 4x180K and 4x44K microarrays)	Tecan Group Ltd.

Table 29 Required equipment

Description	Company and part no.
Agilent Microarray Scanner Bundle	
for 1x244K, 2x105K, 4x44K or 8x15K, *or*	Agilent p/n G2565BA or G2565CA
for 1x1M, 2x400K, 4x180K or 8x60K	Agilent p/n G2565CA
Hybridization Chamber, stainless	Agilent p/n G2534A
Hybridization Chamber gasket slides, 5-pack (alternative packaging sizes are available)	
for 1x microarrays *or*	Agilent p/n G2534-60003
for 2x microarrays *or*	Agilent p/n G2534-60002
for 4x microarrays *or*	Agilent p/n G2534-60011
for 8x microarrays	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

Table 30 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	Life Technologies	15575-020	0.5 M
EGTA, pH 8.0	100 g	Sigma	E3889	0.5 M
Glycerol	500 mL	Life Technologies	15514-011	50%
Igepal	100 mL	Sigma	I8896	10%
Triton X-100	250 mL	Sigma	T8787	10%
Tris-HCl, pH 8.0	1 L	Life Technologies	15568-025	1 M
Sodium Deoxycholate	100 g	Sigma	D6750	10%
N-Lauroylsarcosine	25 g	Sigma	61743	20%
SDS	4 x 100 mL	Life Technologies	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	Sigma	77617	n/a
MaXtract Low Density Tubes *or* Phase Lock Gel Tubes	200 tubes	Qiagen Fisher	129016 FP2302820	2 mL size
Sodium Acetate	100 mL	Sigma	S7899	3 M
Ammonium Acetate	100 mL	Sigma	A2706	7.5 M

Table 31 Optional Equipment

Item	Size	Vendor	Catalog #
2100 Bioanalyzer System	n/a	Agilent	G2940CA
Agilent High Sensitivity DNA Kit	10 chips	Agilent	5067-4626

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 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.

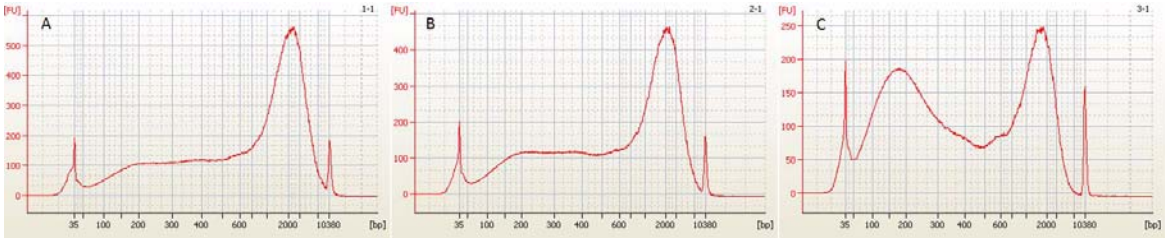


Figure 5 The effect of sonication on DNA fragment size. Samples of whole cell extract (WCE) were sonicated with increasing cycles on the Misonix 3000. Each sonication cycle consisted of: 30 seconds at level 7 followed by 60 seconds off. Panel A: 7 cycles, B: 10 cycles and C: 13 cycles. The resulting 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 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 Agilent's 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).

7a. 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.

7b. Tecan HS Pro Hybridization and Wash Station

Goal Tecan preconditions slides with Pre-hyb solution.

SOP

1. Hybridize for 24 hours at 65°C. Tecan HS Pro automatically hybridizes, washes and dries the slides.
2. Wash slides in buffer including ozone-scavenging reagents to help prevent premature dye degradation.

8. Microarray scanning and storage

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

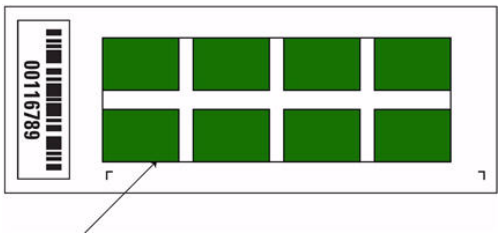
SOP

1. Use default settings on Agilent scanner.
2. Store used slides in N_2 purged vacuum pack.

General Microarray Layout and Orientation

Agilent oligo microarray (8 microarray/slide format) as imaged on the Agilent microarray scanner (G2565CA)

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



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

Agilent microarray slide holder

Figure 6 Agilent microarray slide and slide holder

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

Figure 6 depicts how the Agilent microarray scanner reads the microarrays and how this relates to the "microarray design files" that Agilent generates during the manufacturing process of its *in situ*-synthesized oligonucleotide microarrays. Thus, if you have a scanner that reads microarrays from the "front side" of the glass slide, the collection of microarray data points will be different in relation to the "microarray design files" supplied with the Agilent oligo microarray kit you purchased. Therefore, please take a moment to become familiar with the microarray layouts for each of the Agilent oligo microarrays and the layout information as it pertains to scanning using a "front side" scanner.

Non-Agilent Front Side Microarray Scanners

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

- If the scanner images the microarrays by reading them on the “front side” of the glass slide (“Agilent”-labeled barcode side of the slide) and
- If the microarray image produced by the non-Agilent scanner is oriented in a “portrait” or “landscape” mode, and “Agilent”-labeled barcode is on the left-side, right-side, up or down, as viewed as an image in the imaging software (see Figure 7).

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

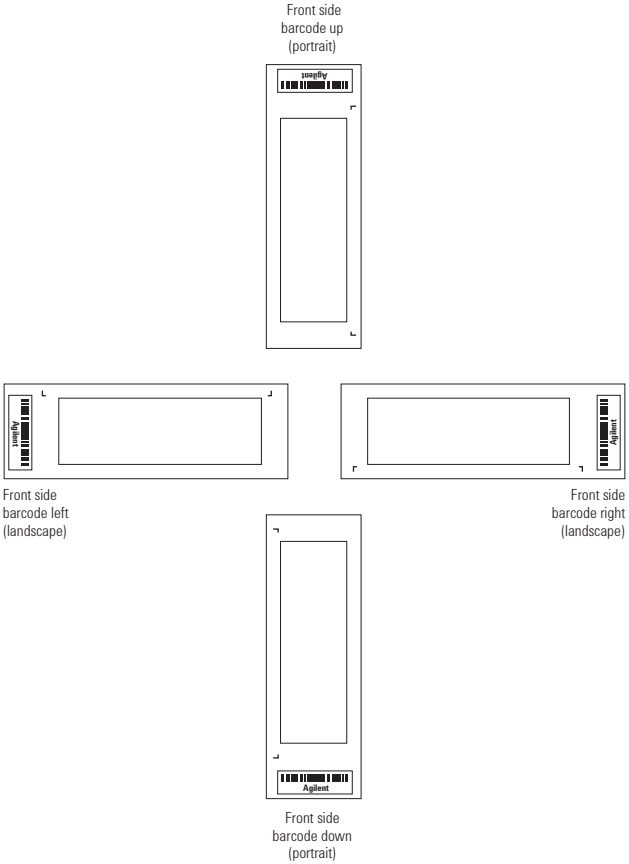


Figure 7 Microarray slide orientation

Array/Sample tracking on a 8x array slide

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

Arrays

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

Barcode Number _____

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

Rbl1/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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