

## *Protocols for* **Chromatin Immunoprecipitation and Amplification**

### Outline

This protocol describes the process for immunoprecipitation and amplification by ligation mediated PCR (LM-PCR) of samples derived from cultured eukaryotic cells. Included here are detailed protocols for ChIP sample preparation, including cross-linking, shearing, IP, enrichment, and amplification.

Background information on the immunoprecipitation and amplification process, developed in the Ren and Farnham labs can be found online at:

[http://www.protocol-online.org/prot/Molecular\\_Biology/Protein/Immunoprecipitation/Chromatin\\_Immunoprecipitation\\_ChIP\\_Assay/](http://www.protocol-online.org/prot/Molecular_Biology/Protein/Immunoprecipitation/Chromatin_Immunoprecipitation_ChIP_Assay/)

and

<http://www.sciencemag.org/cgi/content/full/290/5500/2306/D1>

### Protocol Information & Safety

- Wear gloves and take precautions to avoid sample contamination.
- This protocol requires the use of formaldehyde as a cross-linker. Formaldehyde is a known carcinogen, suspect mutagen, and irritant, particularly at air concentrations above 20ppm. All work with formaldehyde must be performed in a chemical fume hood. The odor threshold for formaldehyde is 1ppm.
- Store all ChIP samples at -20°C, and keep on ice when in use.

### Required Apparatus & Labware\*

\* See last page for a full supplier list

- Thermocycler
- Refrigerated clinical centrifuge with tube adaptors compatible with 15ml and 50ml conical (Falcon) tubes
- Dynal Magnetic Particle Concentrator
- Cold box or refrigerator (4°C) equipped with rotating platform
- Rotating platform at room temperature
- Branson 450 Sonifier equipped with microtip
- Heat block(s) at 37°C, 45°C, and 95°C.
- Spectrophotometer
- Speed-Vac
- Liquid nitrogen

## Experimental Plan

Listed below is a recommended timeline for completing the required steps for ChIP enrichment and two rounds of amplification. The entire process from cross-linking to final PCR purification requires five days.

	Protocol Step	Page
Day One	<b>Step 1.</b> Formaldehyde cross-linkng	3
	<b>Step 2.</b> Antibody binding to Dynabeads	4
Day Two	<b>Step 3.</b> Chromatin extract preparation	4
	<b>Step 4.</b> Chromatin immunoprecipitation reactions	6
Day Three	<b>Step 5.</b> Washing & elution of Dynabeads	6
	<b>Step 6.</b> Linker Annealing	7
Day Four	<b>Step 7.</b> Post IP sample workup	
	Protease digestion of IP samples & controls	
	Phenol and chloroform extractions	8
	RNase A treatment	
	QIAquick PCR purification	
	<b>Step 8.</b> Blunting of DNA ends	9
Day Five	Extraction & precipitation of blunted DNA	
	<b>Step 9.</b> Ligation of Linkers	10
Day Five	<b>Step 10.</b> First round LM-PCR	10
	QIAquick PCR purification	
	<b>Step 11.</b> Second round LM-PCR	12
	QIAquick PCR purification	

## Step 1. Formaldehyde Cross-Linking

### Important!

Formaldehyde is a known carcinogen. It also causes irritation and can trigger allergic reactions.

Perform these steps in a fume hood.

1. Prepare 4ml crosslinking solution for each  $10^9$  sample being processed. Prepare this solution just prior to use with fresh (less than two months old) formaldehyde.

Crosslinking	
Formaldehyde	11% (v/v)
NaCl	0.1M
Disodium EDTA	1mM
Disodium EGTA	0.5mM
Hepes, pH 8.0	50mM

2. Prepare 100ml stock of 1X phosphate buffered saline for each  $10^9$  cells being processed (PBS) and chill on ice:

1X PBS	
NaCl	137mM
Na <sub>2</sub> HPO <sub>4</sub>	10mM
KH <sub>2</sub> PO <sub>4</sub>	2mM

3. Harvest  $10^9$  mammalian cells from T-flask or spinner flask by gentle centrifugation and resuspend in 40ml of growth medium. Transfer to a 50ml conical (Falcon) tube and place on ice for 10 minutes.
4. Add 1/10 (4ml) volume of crosslinking solution directly to each tube and mix gently by inversion.
5. Place conical tube on ice and incubate for 10 minutes.
6. Prepare a stock of 2.5M glycine by combining 1.88g in 10ml water. Vortex to completely dissolve the glycine before use.

### Tip

Glycine is acting as a primary amine donor to absorb the excess crosslinking capacity of the formaldehyde present in the reaction and "quench" the crosslinking.

Working stocks can be stored at room temperature for several weeks if prepared under sterile conditions. Solution can be 0.2 $\mu$ m filtered or autoclaved

7. Add 1/20 (44ml/20 = 2.2ml) volume of a 2.5M glycine solution to each tube to stop the cross-linking reaction.
8. Centrifuge at 2000 x g for 10 minutes at 4°C.
9. Gently resuspend cell pellet in 40ml ice cold PBS.
10. Repeat steps 8 and 9.
11. Centrifuge washed cells at 2000 x g for 10 minutes at 4°C and carefully decant supernatant.
12. Snap freeze cell pellet in liquid nitrogen, and store at -80°C.

## Step 2. Antibody Binding to Dynabeads

In Step 2, the paramagnetic Dynabeads used for immunoprecipitation are blocked with BSA and complexed with the specific IgG selected for immunoprecipitation. The choice of antibody will depend on the specific modified DNA binding proteins being studied. Antibodies used for immunoprecipitation (IP) should be IP qualified. A range of commercially available antibodies has been tested for IP.

**Note:** The specified Dynabead Sheep anti-Mouse IgG is specific only for IgG1 and IgG2 subclasses and will not bind IgG3. Antibodies used with this protocol must be of the IgG1 or 2 subclasses.

### Important!

Excessive vortexing of protein solutions can lead to irreversible denaturation of the protein. Prevent excessive foaming when mixing the BSA solution.

Prepare this solution just prior to use.

1. Prepare a 100ml stock of BSA Blocking Solution by combining 0.5g BSA with 100ml 1X PBS. Mix gently until the BSA is fully dissolved. Do not vortex.
2. Transfer 600µl of Sheep anti-Mouse IgG Dynabeads to a 15ml conical tube.
3. Centrifuge for 5 minutes at 2000 x g, 4°C. Remove supernatant with a pipette.
4. Resuspend the Dynabeads in 10ml PBS Blocking Solution.
5. Use Dyal Magnetic Particle Concentrator (MPC) to capture Dynabeads.
6. Gently decant the supernatant, retaining Dynabeads.
7. Repeat steps 4 through 6 three more times.
8. Capture Dynabeads with MPC, decant supernatant and resuspend the Dynabeads in 1000µl BSA Blocking Solution
9. Add 300µl (60µg for 6 ChIP reactions) IP-qualified IgG antibody to the Dynabeads and mix gently.
10. Incubate overnight on a rotating platform at 4°C. Rotate at a low enough speed to prevent significant foaming.

## Step 3. Preparation of Buffers

1. Prepare the following buffers just prior to use:

L1 (Lysis Buffer)	Stock	Volume for 60mL 2X	
50mM Hepes KOH, pH 7.5	1M	3ml	Combine the indicated components and mix. Add 1 protease inhibitor cocktail tablet (Roche Complete No. 1 697 498).
140mM NaCl	5M	1.68ml	
1mM EDTA	0.5M	120µl	
10% Glycerol	100%	6ml	
0.5% NP-40	100%	3ml	
0.25% Triton X-100	100%	0.15ml	
dH <sub>2</sub> O		46.05ml	
<b>Total</b>		<b>60ml</b>	

# Chromatin Immunoprecipitation and Amplification

L2 Buffer	Stock	Volume for 50mL 2X	
200mM NaCl	5M	2ml	Combine the indicated components and mix. Add 1 protease inhibitor cocktail tablet (Roche Complete No. 1 697 498).
1mM EDTA, pH 8.0	500mM	100µl	
0.5mM EGTA, pH 8.0	500mM	50µl	
10mM Tris, pH 8.0	1M	500µl	
dH <sub>2</sub> O		47.35ml	
<b>Total</b>		<b>50ml</b>	

L3 Buffer	Stock	Volume for 10mL	Volume for 20mL
1mM EDTA, pH 8.0	500mM	20µl	40µl
0.5mM EGTA, pH 8.0	500mM	10µl	20µl
10mM Tris, pH 8.0	1M	100µl	200µl
1 Complete tablet in 1ml H <sub>2</sub> O	50X	200µl	400µl
dH <sub>2</sub> O		9.76ml	19.34ml
<b>Total</b>		<b>10ml</b>	<b>20ml</b>

## Important!

Thaw frozen cell pellet slowly on ice water. This process can take as long as 1 hour

2. Thaw the frozen cell pellets prepared in Step 1 by incubation in an ice water bath.
3. Resuspend cell pellets in 30ml of L1 Buffer. Incubate on rotating platform at 4°C for 10 minutes.
4. Centrifuge at 3000 rpm for 10 minutes at 4°C.
5. Carefully remove supernatant using a micropipette .
6. Resuspend each cell pellet in 24ml of L2 Buffer.
7. Incubate on rotating platform at room temperature for 10 minutes.
8. Pellet nuclei in a clinical centrifuge by spinning at 3000 rpm for 10 minutes at 4°C.
9. Carefully remove supernatant using a micropipette.
10. Resuspend pelleted nuclei in 5ml L3 Buffer. Transfer to a 15mL conical tube.
11. Set Branson Sonifier to the following settings:
  - Microtip
  - Power setting = 4 (25% output)
  - Constant output

## Important!

Wear hearing protection when operating the sonicator.

Sonicators may vary. It is important to sonicate until the particulate chromatin suspensions are no longer visible and the solution becomes significantly less opaque. This may mean sonicating for additional cycles.

12. Place 15ml conical tube in a 50ml conical tube filled with ice and sonicate 8 times for 20 seconds, allowing the suspension to cool on ice between pulses.
13. Centrifuge for 15 minutes at 4000 rpm, at 4°C to remove debris.
14. Dilute a small aliquot of the supernatant 1:50 in water, and measure the A<sub>260</sub> of each sample.
15. Adjust the final glycerol concentration to 10% with 80% glycerol stock solution.
16. Adjust the final DNA concentration to 2mg/ml by adding L3 Buffer.
17. Freeze chromatin at -80°C in 2mg aliquots (1mL).
18. Store chromatin samples at -80°C.

## Step 4. Chromatin Immunoprecipitation Reactions

1. Prepare a stock of 10% deoxycholate by dissolving 0.3g deoxycholate in 3ml dH<sub>2</sub>O.
2. Prepare a stock of 5mg/ml BSA by dissolving 0.2g BSA in 40ml 1X PBS.
3. Retrieve antibody complexed Dynabeads prepared in Step 2. Use the MPC to capture the Dynabeads, and remove the supernatant with a pipet.
4. Resuspend in 1ml 1X PBS and mix gently. Capture beads with the MPC and remove the PBS supernatant. Repeat the Dynabead wash step two additional times for a total of three washes.
5. Resuspend antibody beads in 600µl 1X PBS, 5mg/ml BSA prepared in Step 4.2.

### Important!

Be sure to reserve 25µl of the sonicated chromatin extract used for the immunoprecipitation reactions. This will be used as a control sample later in the protocol

6. Thaw the sonicated chromatin extract samples, and reserve a 25µl sample of each for later use. Assemble chromatin reactions by combining the following components in a separate 15ml conical tube for each sample:

	Stock	Final Conc.	Per Reaction
Sonicated chromatin extract	2mg/ml	1.54mg/ml	975µl
Triton X-100	10%	1%	130µl
deoxycholate	10%	0.1%	13µl
1 Complete tablet in 1ml H <sub>2</sub> O	50X	1X	26µl
10X TE	10X	1X	131µl
Antibody complexed Dynabeads			100µl
	<b>Total</b>		<b>1.3ml</b>

7. Incubate at 4 °C overnight on a rotating platform.

## Step 5. Washing & Elution of Dynabeads

### Important!

The order of addition of components is critical for this buffer. Add water prior to LiCl to prevent precipitation of detergents.

1. Prepare 50ml RIPA Buffer immediately prior to use according to the following recipe. **Add components in the order listed to prevent precipitation of detergents.**

RIPA Buffer	Stock	Final conc.	Volume for 50mL 6X
50mM Hepes, pH 8.0	1M	50mM	2.5ml
500mM EDTA, pH 8.0	0.5M	1mM	100µl
10% NP-40	10%	1%	5ml
10% DOC	10%	0.7%	3.5ml
dH <sub>2</sub> O			34.8ml
8M LiCl	8M	0.5M	3.125ml
Complete solution	50X	1X	1ml
	<b>Total</b>		<b>50ml</b>

2. Use a magnet MPC-S to capture the beads, and save the first supernatant. Store reserved supernatant samples at -80 °C.
3. Resuspend the beads in 1ml RIPA Buffer and gently mix to resuspend the beads. Capture beads with the MPC, and remove the supernatant. Repeat this washing cycle a total of 8 times.

## Chromatin Immunoprecipitation and Amplification

4. Capture beads and discard the final supernatant. Resuspend the beads in 1 ml 1X TE and mix gently to wash. Transfer each reaction to a sterile 1.6ml microcentrifuge tube.
5. Prepare 50ml of Elution Buffer by the assembling the following components:

Elution Buffer	Stock	Final Conc.	Volume for 50mL
1M Tris, pH 8.0	1M	10mM	500µl
500mM EDTA	0.5M	1mM	100µl
10% SDS	10%	1%	5ml
dH <sub>2</sub> O			44.35ml
<b>Total</b>			<b>50ml</b>

6. Capture beads with the MPC and remove the TE supernatant. Transfer tubes to the microcentrifuge and spin at 3,000 rpm for three minutes. Remove any excess buffer with a micropipette, being careful not to disturb the pelleted beads.
7. Add 50µl of Elution Buffer to each tube. Vortex briefly to resuspend the beads, and incubate at 65°C for 10 minutes, vortexing briefly every 2 minutes during the incubation.
8. Spin for 30 seconds at maximum speed, and transfer supernatant to a clean 1.6ml tube.
9. To each recovered supernatant, add 120µl Elution Buffer.
10. To each reserved input chromatin sample (set aside in Step 4.6), add 145µl Elution Buffer.
11. Transfer all tubes (immunoprecipitated samples and input controls in Elution Buffer) to a 65°C block and incubate overnight (16 hours) to reverse the crosslinking reaction.

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### Step 6. Linker Annealing

Two oligos are annealed in this step to form a linker/adaptor molecule. The two required oligos are:

- oJW102 (5'-GCGGTGACCCGGGAGATCTGAATTC-3', HPLC purified)
- oJW103 (5'-GAATTCAGATC-3', HPLC purified)

Oligos are typically ordered from commercial sources and are synthesized on a 1µMole scale.

1. Using the molar yield information provided on the certificate of analysis, dissolve the two oligos (oJW102 and oJW103) in dH<sub>2</sub>O to a final concentration of 40µM each.
2. Assemble the following components in a 1.6ml microcentrifuge tube:

Component	Volume
1M Tris, pH 7.9	250µl
40µM oJW102	375µl
40µM oJW103	375µl
<b>Total</b>	<b>1ml</b>

## Chromatin Immunoprecipitation and Amplification

3. Aliquot in 100µl volumes in sterile 1.6ml microcentrifuge tubes.
4. Place the tubes in 95°C heat block for 5 minutes.
5. Transfer samples to 70°C heat block.
6. Remove the block from the heater, and place it at room temperature. Allow it to cool to 25°C.
7. Transfer the block to 4°C, and incubate overnight.
8. Store annealed linker aliquots at –20°C.

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### Step 7. Post-IP Sample Workup

1. Prepare the Proteinase K Mix. Select the scale of mix appropriate to the number of samples you are processing, including all input controls.

Proteinase K Mix Component	Number of Samples			
	1	10	20	35
1 XTE	140µl	1.4ml	2.8ml	4.9ml
10 mg/ml Glycogen	3µl	30µl	60µl	105µl
Proteinase K 20mg/ml	7µl	70µl	140µl	245µl
<b>Total</b>	<b>150µl</b>	<b>1.5ml</b>	<b>3ml</b>	<b>5.25ml</b>

2. Add 150µl Proteinase K Mix to each tube from Step 5. Mix by gentle vortexing.
3. Incubate for 2 hours at 37°C.
4. Extract 2X with 300µl phenol (equilibrated with TE, pH 8.0), and retain the aqueous phase.
5. Extract once with 300µl chloroform:isoamyl alcohol (24:1), and retain the aqueous phase.
6. The recovered aqueous volume of each reaction should be approximately 320µl. To each tube add 13µl of 5M NaCl (200mM NaCl final).
7. Add 700µl 100% EtOH, and vortex to mix.
8. Incubate at –80°C for 15-30 minutes.
9. Spin at 14,000 rpm for 15 minutes at 4°C.
10. Remove supernatant and wash pellet with 500µl cold 70% EtOH. Vortex, and centrifuge at 4°C for 5 minutes at 14,000 rpm.
11. Decant final supernatant, spin briefly and remove all excess liquid with a micropipette. Air dry the pellet for 5 minutes.
12. Prepare a stock solution RNase TE by combining 16.5µl 10mg/ml RNase A with 483.5µl dH<sub>2</sub>O.
13. Resuspend each sample in 30µl RNase TE and incubate for 2 hours at 37°C.
14. Purify the DNA using the QIAquick PCR purification kit, and elute in 50µl of the elution buffer provided with the kit.

## Important!

Do not measure  $A_{260}$  for IP samples. The DNA yield is too low for a meaningful measurement at this point.

15. Measure the  $A_{260}$  for input control samples only and calculate the concentration of DNA.
16. For each input DNA sample, prepare a 50 $\mu$ l sample diluted to a concentration of 20ng/ $\mu$ l.
17. Store all samples at  $-20^{\circ}\text{C}$ .

## Step 8. Blunting DNA Ends

1. In separate 200 $\mu$ l PCR tubes, add 40 $\mu$ l ChIP DNA or 20ng (1 $\mu$ l) input DNA and bring the total volume of each sample to 100 $\mu$ l with dH<sub>2</sub>O. Store the remaining DNA at  $-20^{\circ}\text{C}$ .
2. Prepare the Blunting Mix scaled to the number of reactions you are processing, according to the following table. Maintain the mix and samples on ice during reaction assembly.

Blunting Mix	Number of Samples			
	1	10	20	35
10X T4 DNA polymerase buffer NEB #007-203	11 $\mu$ l	110 $\mu$ l	220 $\mu$ l	385 $\mu$ l
10mg/ml BSA NEB #007-BSA	0.5 $\mu$ l	5 $\mu$ l	10 $\mu$ l	17.5 $\mu$ l
20mM dNTP	0.5 $\mu$ l	5 $\mu$ l	10 $\mu$ l	17.5 $\mu$ l
T4 DNA polymerase 3U/ $\mu$ l NEB #203L	0.2 $\mu$ l	2 $\mu$ l	4 $\mu$ l	7 $\mu$ l
<b>Total</b>	<b>12.2<math>\mu</math>l</b>	<b>122<math>\mu</math>l</b>	<b>244<math>\mu</math>l</b>	<b>427<math>\mu</math>l</b>

3. Add 12.2 $\mu$ l of Blunting Mix to each reaction.
4. Mix by pipetting, and incubate at  $12^{\circ}\text{C}$  for 20 minutes in thermocycler.
5. Transfer blunted DNA to sterile 1.6ml tubes, and place on ice.
6. Prepare the NaOAc/Glycogen mix scaled to the number of reactions you are processing, according to the following table. Maintain the mix and samples on ice during reaction assembly.

NaOAc/Glycogen Mix	Number of Samples			
	1	10	20	35
3M NaOAc	11 $\mu$ l	110 $\mu$ l	220 $\mu$ l	385 $\mu$ l
20mg/ml Glycogen, Roche #85343271-31	1.0 $\mu$ l	10 $\mu$ l	20 $\mu$ l	35 $\mu$ l
<b>Total</b>	<b>12<math>\mu</math>l</b>	<b>120<math>\mu</math>l</b>	<b>240<math>\mu</math>l</b>	<b>427<math>\mu</math>l</b>

4. Add 12 $\mu$ l of NaOAc/Glycogen mix to each tube, and vortex to mix.
5. Add 120 $\mu$ l of phenol:chloroform:isoamyl alcohol (25:24:1, Sigma P-3803). Vortex for 1 minute.
6. Centrifuge for 5 minutes at maximum speed.
7. Transfer 110 $\mu$ l of the aqueous supernatant to a new 1.6ml microcentrifuge tube and add 230 $\mu$ l cold 100% EtOH, and vortex. Store at  $-80^{\circ}\text{C}$  for 15-30 minutes. Spin for 14,000 rpm for 15 minutes at  $4^{\circ}\text{C}$ .
8. Remove supernatant, and wash the pellet with 500 $\mu$ l cold 70% EtOH.
9. Spin for 5 minutes at  $4^{\circ}\text{C}$ .

## Chromatin Immunoprecipitation and Amplification

10. Remove supernatant with micropipette, spin briefly and remove any remaining liquid with pipette. Allow the pellet to dry for 5 minutes.
11. Resuspend pellet in 25µl dH<sub>2</sub>O, and place on ice.

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### Step 9. Ligation of Linkers

1. Prepare the Ligase Mix scaled to the number of reactions you are processing, according to the following table. Maintain the mix and samples on ice during reaction assembly.

Ligase Mix	Number of Samples			
	1	10	20	35
5X Ligase Buffer	10µl	100µl	200µl	350µl
15µM annealed linkers (Step 6.)	6.7µl	67µl	134µl	234.5µl
T4 DNA ligase NEB #202L	0.5µl	5µl	10µl	17.5µl
dH <sub>2</sub> O	8µl	80µl	160µl	280µl
<b>Total</b>	<b>25.2µl</b>	<b>252µl</b>	<b>504µl</b>	<b>882µl</b>

2. Add 25µl of cold ligase mix to each 25µl blunted DNA sample from Step 8.11.
3. Mix by pipetting and incubate overnight at 16 °C.
4. Following overnight incubation, add 6µl of 3M NaOAc, and 130µl of EtOH to each tube. Vortex to mix.
5. Freeze at –80 °C for 15-30 minutes, and spin at 14,000 rpm for 15 minutes.
6. Wash with 500uL of 70% EtOH, then spin at 14,000 rpm for 5 minutes. Remove supernatant, spin briefly and remove all excess liquid.
7. Air dry for 5 minutes.
8. Resuspend the pellet in 25µl dH<sub>2</sub>O, and transfer to 200µl PCR tubes.

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### Step 10. First Round LM-PCR

1. Place 25µl samples of ligated DNA from Step 9.8 on ice.
2. Prepare the PCR Labeling Mix scaled to the number of reactions you are processing, according to the following table. Maintain the mix and samples on ice during reaction assembly.

PCR Labeling Mix	Number of Samples			
	1	10	20	35
10X ThermoPol Reaction Buffer, NEB	4µl	40µl	80µl	140µl
2.5mM dNTP	5µl	50µl	100µl	175µl
40uM oJW102	1.25µl	12.5µl	25µl	43.75µl
dH <sub>2</sub> O	4.75µl	47.5µl	95µl	166.25µl
<b>Total</b>	<b>15µl</b>	<b>150µl</b>	<b>300µl</b>	<b>525µl</b>

3. Add 15µl of PCR Labeling Mix to each tube.

## Chromatin Immunoprecipitation and Amplification

4. Prepare the Polymerase Mix scaled to the number of reactions you are processing, according to the following table. Maintain the mix and samples on ice during reaction assembly.

Polymerase Mix	Number of Samples			
	1	10	20	35
10X ThermoPol Reaction Buffer, NEB	1µl	10µl	20µl	35µl
<i>Taq</i> Polymerase, 5U/µl Qiagen	1µl	10µl	20µl	35µl
<i>Pfu</i> Turbo Pol. 5U/µl, Stratagene	0.01µl	0.1µl	0.2µl	0.35µl
dH <sub>2</sub> O	8µl	80µl	160µl	280µl
<b>Total</b>	<b>10µl</b>	<b>100µl</b>	<b>200µl</b>	<b>350µl</b>

5. Add 10µl of polymerase mix to each tube, and mix well by pipetting.
6. Program your thermocycler for the following profile:
  1. 55°C for 2'
  2. 72°C for 5'
  3. 95°C for 2'
  4. 95°C for 1'
  5. 60°C for 1'
  6. 72°C for 2'
  7. Go to step 4 for **22** times
  8. 72°C for 5'
  9. 4°C forever
7. Transfer to PCR tubes on ice, place in thermocycler, and start the program.
8. Purify with QIAquick PCR purification kit. Elute in 50µl Qiagen elution buffer.
9. Measure the A<sub>260</sub>, and calculate the DNA concentration for each sample.

## Step 11. Second Round LM-PCR

**Note:** Perform this step only if more LM-PCR DNA is required for labeling, for example when hybridizing to the NimbleGen 38-array whole-genome tiling set.

1. Transfer 200ng each of the first round LM-PCR DNA to sterile 200µl PCR tubes and place on ice. Dilute each to 25µl with dH<sub>2</sub>O.
2. Prepare the PCR Labeling Mix scaled to the number of reactions you are processing, according to the following table. Maintain the mix and samples on ice during reaction assembly.

PCR Labeling Mix	Number of Samples			
	1	10	20	35
10X ThermoPol Reaction Buffer, NEB	4µl	40µl	80µl	140µl
2.5mM dNTP	5µl	50µl	100µl	175µl
40uM oJW102	1.25µl	12.5µl	25µl	43.75µl
dH <sub>2</sub> O	4.75µl	47.5µl	95µl	166.25µl
<b>Total</b>	<b>15µl</b>	<b>150µl</b>	<b>300µl</b>	<b>525µl</b>

3. Add 15µl of PCR labeling mix to each tube.
4. Prepare the Polymerase Mix scaled to the number of reactions you are processing, according to the following table. Maintain the mix and samples on ice during reaction assembly.

Polymerase Mix	Number of Samples			
	1	10	20	35
10X ThermoPol Reaction Buffer, NEB	1µl	10µl	20µl	35µl
Taq Polymerase, 5U/µl Qiagen	1µl	10µl	20µl	35µl
Pfu Turbo Pol. 5U/µl, Stratagene	0.01µl	0.1µl	0.2µl	0.35µl
dH <sub>2</sub> O	8µl	80µl	160µl	280µl
<b>Total</b>	<b>10µl</b>	<b>100µl</b>	<b>200µl</b>	<b>350µl</b>

5. Add 10µl of Polymerase Mix to each tube and mix well by pipetting.
6. Program your thermocycler for the second round LM-PCR:
  1. 55°C for 2'
  2. 72°C for 5'
  3. 95°C for 2'
  4. 95°C for 1'
  5. 60°C for 1'
  6. 72°C for 2'
  7. Go to step 4 for 5 times
  8. 72°C for 5'
  9. 4°C forever
7. Transfer assembled reactions to PCR tubes on ice, place in thermocycler and start program.
8. Purify with QIAquick PCR purification kit. Elute in 50µl Qiagen elution buffer.
9. Measure the A<sub>260</sub> of each sample, and calculate the DNA yield and concentration.
10. Dry samples down in a Speed-Vac, and resuspend in dH<sub>2</sub>O to 500µg/ml.

## Component and Supplier List

Component	Vendor	Package Size	Item Number
Formaldehyde, 36.5-38% v/v solution, methanol stabilized	VWR/JT Baker	150ml or 500ml	JT2106
Dynabeads Sheep anti-Mouse IgG	Dynal Biotech (Invitrogen)	5ml	110.31
BSA, Fraction V	Sigma Aldrich	100g	A3350
10X PBS	Gibco	500mL	70013-073
Deoxycholate	Sigma Aldrich		D-6750
Glycogen	Roche Applied Science	20 mg (1 ml)	10901393001
0.5M EDTA	Sigma Aldrich	100ml	E-7889
EGTA	Sigma Aldrich		E3889
50mM Hepes	Sigma Aldrich		H0887
Na <sub>2</sub> HPO <sub>4</sub>	Sigma Aldrich		S3264
KH <sub>2</sub> PO <sub>4</sub>	Sigma Aldrich		P9791
Glycerol	Sigma Aldrich		G5516
NP-40	Sigma Aldrich		74385
Triton X-100	Sigma Aldrich		93426
8M LiCl	Sigma Aldrich		L7026
chloroform:isoamyl alcohol	Sigma Aldrich		C0549
Absolute Ethanol	Sigma Aldrich	500mL	E702-3
Water, Reagent Grade, ACS, Nonsterile, Type 1	VWR	2.5 gallon	RC915025
Proteinase K, PCR Grade	Roche Applied Science	25mg	03115836001
T4 DNA polymerase	NEB	750U	203L
T4 DNA Ligase	NEB	100,000U	202L
10x ThermoPol Reaction Buffer	NEB	6ml	B9004S
Taq Ploymerase 5U/μl	Qiagen	250U	201203
Pfu Turbo Pol 5U/μl	Stratagene	100U	600135
dNTP	Invitrogen	25μmol	10297-018
Phenol, equilibrated with TE, pH 8.0	Sigma Aldrich		P-4557
1M Tris HCL	Sigma Aldrich	1L	T-2663
5M NaCl	Ambion	500ml	9759
EtOH	Sigma Aldrich	500ml	E702-3
10% SDS	Sigma Aldrich	100ml	L-4522
1M Tris HCL	Sigma Aldrich	1L	T-2663
3M Sodium Acetate	Ambion	100ml	9740
MES, sodium salt	Sigma Aldrich	100g	M 3885