



Judd Rice Laboratory

at the USC/Norris Comprehensive Cancer Center

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X-ChIP Protocol with LM-PCR Amplification

Revised by S Houston
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Procedure

A method for obtaining ChIP'd DNA for microarray analysis.

Isolate Chromatin

1. Trypsinize HeLa cells, and count to 1×10^7 cells per individual IP.
2. Wash cells 1 X in PBS.
3. Resuspend the cell pellet in media with 1 % formaldehyde and rotate at room temp for 10 min.
4. Quench reaction by adding 1M glycine to a final conc. of 0.125 M and rotate at room temp for 5 min.
5. Spin down cells.
6. Wash 2 X with PBS.
7. Resuspend cell pellet in nuclear isolation buffer and incubate on ice for 10 min.
Spin 8 min at 600 g.
8. Resuspend nuclei pellet in Farnham nuclei lysis buffer to a conc of 10^8 nuclei per mL.
Incubate on ice for 10 min.
9. Sonicate chromatin for 2 X 20 sec at output level of 4.0 (to obtain approx 500-1000bp).
Sonication conditions must be determined for each cell type.
10. Spin sonicated chromatin at 14000 rpm for 10 min at 4 degrees. Keep supernatant.

Immunoprecipitation

1. Place 600 microliters of pro-A Dynabeads in 1.5 mL tube.
Wash 3 times with PBS + 5 mg/ml BSA.
2. Resuspend in 600 microliters PBS + BSA. Split beads as follows:
 - 400 microliters beads plus Specific Antibody (approx 40 micrograms) plus 500 microliters PBS+BSA
 - 100 microliters beads plus 300 microliters PBS + BSA
 - 100 microliters beads plus 15 microliters non-specific IgG plus 300 microliters PBS + BSA
3. Incubate rotating O/N at 4 degrees.
4. Wash beads 3 X with PBS.
5. Resuspend beads plus antibody in 400 microliters of Farnham IP dilution buffer.
Resuspend No Ab and IgG beads in 100 microliters of IP dilution buffer each.
6. Set up IPs as follows:
 - Beads plus (or minus) Ab: 100 microliters
 - Sonicated Chromatin: 100 microliters
 - IP dilution Buffer: 1000 microliters
 - 1200 microliters total
7. Rotate O/N at 4 degrees.

Washing and Eluting

Make 10 % DOC and RIPA buffer immediately before use.

1. Save 1st supernatant.
2. Wash beads 8 times with 1 mL RIPA buffer
3. Wash beads once with 1 mL TE
4. Spin for 3 min at 3000 rpm and remove remaining supernatant.
5. Add 60 microliters of TE + 1% SDS and incubate at 65 degrees for 10 min.
Vortex every two minutes.
6. Repeat with 120 microliters of TE + SDS.
7. Pool supernatants and add 200 microliters of TE. Add 15 microliters of 5 M NaCl and 1 microliter of 10 mg/mL RNase A. (Take 100 microliters of total input and treat similarly)
8. Incubate at 65 degrees O/N to reverse cross-links.
9. Add 2.5 vol of EtOH and precipitate O/N

10. Spin samples for 15 min at 4 degrees.
Let pellets dry.
11. Dissolve each pellet in 135 microliters of TE. Add 15 microliters of 10 X ProK buffer and 1.5 microliters of Pro K.
12. Incubate in 37 waterbath for 2 hours.
13. Adjust conc. to 400 microliters.
14. Phenol:chloroform extract samples.
15. Add 5 micrograms of glycogen to each sample.
16. Add 1000 microliters EtOH. Precipitate O/N.
17. Resuspend in 30 microliters of 10 mM tris pH 7.5

Blunting and LM-PCR

oligo JW102 5' gcggtgacccgggagatctgaattc 3'
oligo JW103 5' gaattcagatc 3'

1. Form a unidirectional linker.
 - 1.1. Combine 6.7 microliters of 100 micromolar JW102 and JW103 and 86.6 microliters of H₂O.
 - 1.2. Boil this mixture for 5 min. in a water bath and then allow to slowly cool to room temperature.
 - 1.3. Store at -20°C.
2. To each experimental sample, IgG sample, and NoAb sample, add the following:
 - 11 microliters T4 DNA polymerase buffer,
 - 1.1 microliters of 100 X BSA,
 - 5 microliters 2 mM dNTPs,
 - 1 microliter T4 DNA polymerase
 - H₂O to 110 microliters.
3. Place sample at 37 degrees for 1 hr.
4. Phenol:Chloroform extract.
5. Resuspend in 30 microliters of 10 mM tris.

6. For each blunting reaction, add the following:
 - 27 microliters of blunted chromatin
 - 10.3 microliters H₂O
 - 5 microliters 10X T4 DNA ligase buffer
 - 6.7 microliters annealed linker
 - 1.0 microliters T4 DNA ligase.
7. Place at 16 degrees O/N.
8. Clean each sample with Qiaquick PCR purification kit, eluting with 30 microliters of elution buffer.

Test PCR

Do test PCR of experimental sample vs. IgG of a known binding site. Do not proceed if Experimental sample does not show much more amplification than IgG. If you do not have a known binding site, look to see that you have more overall amplification with your experimental samples than with IgG. PCR reactions using 2-2.5 microliters of oligo JW102 as primer. Purify using Qiaquick PCR purification columns.

For total input sample, just reverse crosslinks, proK digest and P:Cl extract leftover sonicated nuclei.

Solutions

Farnham Nuclei Lysis Buffer

50 mM Tris pH 8
10 mM EDTA
1% SDS

RIPA Buffer

50 mM Hepes pH8
1 mM EDTA
1 % NP-40
0.7% NaDOC
H₂O
0.5 M LiCl

Add components in this order

Nuclear Isolation Buffer

150 mM NaCl
10 mM Hepes pH7.4
1.5 mM MgCl₂
10 mM KCl
0.5% NP-40 (This may need to be decreased, depending on cell type)
0.5 mM DTT
Protease inhibitors