

Chromatin Immunoprecipitation (ChIP)

Required Solutions

10x PBS

80 g NaCl

2 g KCl

17.4 g Na₂HPO₄·7H₂O

2.4 g KH₂PO₄

Adjust pH to 7.4 and add H₂O to 1 l

Lysis buffer

1% SDS

10 mM EDTA pH 8.0

50 mM Tris-HCl pH 8.0

add before use:

1 mM PMSF

1 µg/ml leupeptin

1 µg/ml Aprotinin

1 µg/ml Pepstatin

or replace Leupeptin/Aprotinin/Pepstatin with Roche protease inhibitor tablets (cOmplete, EDTA-free for 50 ml [#11697498001]; cOmplete mini, EDTA-free for 10 ml [#04693159001])

IP dilution buffer

0.01% SDS

1.1% Triton X-100

1.2 mM EDTA

16.7 mM Tris-HCl pH 8.0

150 mM NaCl

add before use:

1 mM PMSF

1 µg/ml leupeptin

1 µg/ml Aprotinin

1 µg/ml Pepstatin

or replace Leupeptin/Aprotinin/Pepstatin with Roche protease inhibitor tablets (cOmplete, EDTA-free for 50 ml [#11697498001]; cOmplete mini, EDTA-free for 10 ml [#04693159001])

IP Wash A

0.1% SDS

1% Triton X-100

2 mM EDTA pH 8.0

20 mM Tris-HCl pH 8.0

150 mM NaCl

add before use:

1 mM PMSF

1 µg/ml leupeptin

1 µg/ml Aprotinin

1 µg/ml Pepstatin

or replace Leupeptin/Aprotinin/Pepstatin with Roche protease inhibitor tablets (cOmplete, EDTA-free for 50 ml [#11697498001]; cOmplete mini, EDTA-free for 10 ml [#04693159001])

IP Wash B

0.1% SDS

1% Triton X-100

2 mM EDTA pH 8.0
20 mM Tris-HCl pH 8.0
500 mM NaCl

IP Wash C

0.25 M LiCl
1% NP-40
1% Na-Deoxycholate
1 mM EDTA pH 8.0
10 mM Tris-HCl pH 8.0

TE (4th IP wash)

10 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0

SDS-Na Carbonate

1% SDS
0.1 M NaHCO₃
Make fresh from 10% SDS and 1 M NaHCO₃

Denaturing solution

1.5 M NaCl
0.5 M NaOH

Neutralizing solution

3 M NaCl
0.5 M Tris-HCl pH 7.0

1 M NaPi pH 7.2

In 2.3 liters (orange capped roller bottle filled to shoulder).
308 g Na₂HPO₄
9.2 ml H₃PO₄
add ddH₂O to close to final volume and adjust pH to 7.2 with H₃PO₄
adjust volume to 2.3 l

Church Mix

500 ml 1 M NaPi pH 7.2 (see below)
2 ml 0.5 M EDTA pH 8.0
70 g SDS
10 g BSA
ddH₂O to 1 l
Heat to 55°C to dissolve. Filter through a 0.45 µm filter.

10x OLB (10x oligo nucleotide labeling buffer)

0.5 M Tris-HCl pH 6.8
0.1 M MgOAc
1 mM DTT
0.5 mg/ml BSA
for 1 ml of 10x OLB add 6 µl of 100 mM dGTP, dATP, TTP
make small aliquots (nucleotides are unstable) and store at -80°C, avoid freeze and thaw

ChIP-Grade Protein G Magnetic Beads (Cell signaling, #9006)

Ready to use

Hybond-N membrane (GE Healthcare)

DNA templates for labeling reaction

- Telomeric probe: 800 bp [TTAGGG]_n Sty11 insert, (~20 ng/μl)
Isolate fragment from pSP73.Sty11x #21 digested with EcoRI on an agarose gel, use [CCCTAA]₃ oligo (1ng/μl) for labeling reaction
- BamHI repeat probe for mouse DNA: 1 kb insert, (~20 ng/μl)
Isolate 1 kb fragment from plasmid #7320 (pBLUE KS- backbone) digested with EcoRV on an agarose gel, use random hexamer primers for labeling reaction (Applied Biosystems, S06405, 50 μM)
- Alu repeat probe for human DNA: ~ 2 kb insert, (~20 ng/μl)
Isolate ~ 2 kb fragment from plasmid #53 (pBLUE KS- backbone) digested with XbaI and HindIII on an agarose gel, use random hexamer primers for labeling reaction (Applied Biosystems, S06405, 50 μM)

ChIP timeline:

Day 1: Prepare lysate and do the IP o/n

Day 2: Wash the IPs, reverse crosslinks and extract the DNA

Day 3: Dot blot and hybridize o/n or for 6 hours

Day 4: Wash the membrane and expose

Preparing the lysate

1. Grow cells to subconfluence. Typically, harvest 1-2 Ø 15 cm dishes per condition. For TRF1/ TRF2/TIN2/Rap1 IP or overexpression constructs, one 10 cm plate can be split for 3-4 pulldowns.
2. Crosslinking:
 - a. Crosslinking done on cells on plate
 - i. Remove medium and add 15 ml PBS/1% Formaldehyde (from 37% solution).
 - ii. Swirl on platform at RT 10-30 min
 - iii. Add 2 ml 1.5 M Glycine (final concentration 0.2 M) to stop crosslinking
 - iv. Swirl at RT for 5 min
 - v. Wash 2 times with cold PBS
 - vi. Scrape cells in ~ 10 ml PBS into 50 ml conical tube. Spin down cells. (for the estimation of cell numbers, count cells on a plate harvested in parallel by trypsinization)
 - b. Crosslinking done on trypsinized cells
 - i. Harvest cells by trypsinization; collect in a 50 ml tube, add sufficient medium with serum to inactivate the trypsin (15 ml medium/ 1.5 ml trypsin and count cells), count cells and spin down
 - ii. Add 15 ml PBS/1% Formaldehyde (from 37% solution).
 - iii. Crosslink at RT for 10-30 min on a nutator
 - iv. Add 2 ml 1.5 M Glycine (final concentration 0.2 M) to stop crosslinking
 - v. Mix on a nutator for 5 min
 - vi. Wash 2 times with cold PBS, spin down cells in between the washes.
3. Combine cell pellets in cold PBS with (optional) 1 mM PMSF. Spin again. Place cell pellet on ice or store at -80°C.

4. Resuspend pellet in lysis buffer + protease inhibitors to a concentration of 2×10^7 cells/ml. The buffer needs to be at RT, but keep the lysate on ice from this point on. The lysate can be more concentrated if necessary, but no more than 5×10^7 cells/ml.
5. Incubate for 15 min on ice.
6. Sonicate in ice/water using the Bioruptor (standard conditions: 1.5 ml tubes, 100-300 μ l per tube, power setting H (high), sonication cycle 30 sec ON, 30 sec OFF, sonication time 10 min. If other tubes are used, choose the right adapters and optimize sonication conditions.) The lysate should clear up. Check between cycles to make sure that the lysate remains ice-cold. Optional: Check for sonication on a gel. To do this, take an aliquot before and after sonication, reverse crosslinks and extract the DNA as outlined below. Bulk DNA should be sheared to 0.8-1 kb (This is sufficient for regular ChIPs. For other applications, such as BrdU IP, stronger sonication can be performed to effectively bring the size of the DNA fragments down to 300-500 bp).
7. After sonication, spin down at max speed for 10 min at RT (pool supernatants if necessary).

Immunoprecipitations

1. Use 100-200 μ l lysate (1×10^6 - 2×10^6 cells [to immunoprecipitate shelterin proteins in MEFs 500'000 cells are enough]) per IP. On ice, add 1 ml of IP dilution buffer + protease inhibitors (fresh). Mix by gently inverting the tubes a few times, let sit on ice for 10 min, and add the antibody. Incubate o/n at 4°C on a rotator.
 For our rabbit polyclonal sera, use crude serum and include the pre-immune controls. Usually 15 μ l – 20 μ l of crude serum is good. 1-2 μ g of purified antibodies can be used a reference amount. Optimal conditions have to be tested.
 aTRF1: human - #371, 20 μ l; mouse - #1448/1449, 15 μ l
 aTRF2: human - #647, 20 μ l; mouse - #1254/1255, 15 μ l
 aRAP1: human - #765, 20 μ l; mouse - #1252/1253, 15 μ l
 aTIN2: human - #864, 20 μ l; mouse - #1446/1447, 15 μ l
 aPOT1: human – Abcam Ab124784, 4 μ l; mouse not available
 aTPP1: human - #1151, 20 μ l; mouse not available
2. Remember: Keep one aliquot of 50 μ l lysate (50% of amount used for immunoprecipitation) o/n at 4°C, as 'input' fraction, to be processed the next day. Inputs will be processed with the other samples after the immunoprecipitation step.
3. The next day: Add 30 μ l magnetic beads (Cell signaling, #9006) to each IP. Incubate another 1 hr at 4°C on the rotator.
4. Wash the beads once with 1 ml of each of the buffers: A, B, C, and TE. Add protease inhibitors only to buffer A. Vortex and place on ice in between washes.
5. Elute the immunoprecipitated material as follows: At the end of the last wash, re- pellet the beads, remove as much buffer as possible, and resuspend beads with 60 μ l (250 μ l) of 1% SDS/0.1 M NaHCO₃ (freshly diluted) by vortexing. Incubate for 10 min at RT. Pellet the beads, transfer supernatant to a fresh tube, and wash the beads again with 60 μ l (250 μ l) of SDS-carbonate. Incubate at RT 10 for min, pellet beads, and pool supernatant with the previous eluate. (Use the volume in brackets for EtOH precipitation).
6. Important: this is the step where the input samples start to get processed. For the input samples bring the volume up to 120 μ l (500 μ l) with SDS-carbonate. (Use the volume in brackets for EtOH precipitation).
7. To all the samples, add 4.8 μ l (20 μ l) of 5 M NaCl, mix, and incubate at 65°C for 4 to 6 hours to reverse the crosslinks. (Use the volume in brackets for EtOH precipitation).
8. After 4-6 hours at 65°C, add 2.4 μ l (10 μ l) of 0.5 M EDTA and 4.8 μ l (20 μ l) 1M Tris-HCl pH 6.5. (Use the volume in brackets for EtOH precipitation).
9. Add 20 μ g RNase A (2 μ l of 10 mg/ml, heat inactivated, DNase-free), incubate at 37°C for 30 min.

10. Add 40 µg of Proteinase K (4 µl of 10 mg/ml freshly made stock). Incubate for 1 hour at 37°C.
11. Use the ChIP DNA Clean & Concentrator kit (Zymo) to purify the DNA.

(Alternatively) Extract the samples with 0.5 ml phenol/chloroform/isoamylalcohol. Transfer the aqueous phase to an Eppendorf tube and add 20 µg of glycogen (from 20 mg/ml stock) and mix. Add 1 ml 100% of EtOH to precipitate the DNA. Precipitate o/n at -20°C. Note: In order to keep the volume low and to do the precipitation step in 1.5 ml Eppendorf tubes (2 ml tubes don't work well because of their shape), we only add 2 volumes of EtOH instead of 2.5 volumes. This EtOH concentration is suboptimal (66% instead of 75% EtOH).

Spin down for 20 min in microfuge at max speed at 4°C. Remove all EtOH after second spin down. Resuspend pellet in 10 mM Tris-HCl pH 7.5.

Dot Blotting and hybridization

1. For dot blotting, first prepare the manifold and membranes. Then boil DNA samples for 5 min, snap cool on ice, quick spin in microfuge, and load.
2. Wash the dot blot manifold thoroughly with H₂O or H₂O₂ to avoid DNA contamination. With the dot blot manifold, use two 3MM Whatman papers and Hybond-N membrane (11 cm x 7.5 cm), all saturated with 2x SSC (pre-wet membrane first by floating it on top of H₂O, then soak in 2x SSC). Assemble Whatman papers and membrane on the manifold and ensure that the vacuum is working well by loading 2x SSC on a few wells. Then switch the vacuum off until you are ready to load. Beware to not let the membrane dry out.
3. For loading, pipet 200 µl of 2x SSC in each well. Then load the samples and mix with the 2x SSC (alternatively mix 2x SSC and sample in Eppendorf tubes). This ensures an even distribution of the sample on the membrane.
4. Load 30-50 µl (80 µl for IMR90 cells) on dot blot to probe for telomeric DNA with the Sty11 insert, load 10-20 µl on membrane to probe for Alu (human cells) or BamHI repeats (mouse cells). When ready, switch on the vacuum. It is important to load at least 3 input concentrations to confirm the linear range for each sample.
5. Denature the membrane by placing it on a 3MM Whatman saturated with denaturing solution, DNA side up. Denature for 10 min.
6. Neutralize the membrane by placing it on a 3MM Whatman saturated with neutralizing solution for 10 min, DNA side up.
7. Remove excess liquid by gently blotting on dry paper. Crosslink the DNA to the membrane immediately in the Stratagene UV crosslinker, DNA side up (Auto crosslink program). Rinse in 2x SSC.
8. Prehybridize in medium size Hybaid bottle with 20 ml Church buffer for 30 min at 65°C.
9. Add filtered mixture of Church mix and probe (20 ml – for details on probe preparation see also Telomere Blot protocol) and hybridize o/n at 65°C. 1/4 of the Sty11 probe prepared for 20 cm x 20 cm membranes is enough for the ChIP membrane hybridization.
 - 2.5 µl DNA template [20ng/µl]
 - 1.25 µl oligo
 - 6 µl ddH₂O
 - Mix, boil 5 min, spin for 5 sec to get condensation down, cool on ice, and add:
 - 1.25 µl 10xOLB with dATP, dGTP, dTTP
 - 1.25 µl 32P-alpha-dCTP (3000 Ci/mmol)
 - 0.25 µl Klenow polymerase
 - Mix, incubate 90 min at RT (or longer), add ddH₂O up to 50 µl and purify over a G-50 column, add to Church mix and filter through 0.45 µm filter.
10. Washes: wash the membrane in 2x SSC at RT for 5 min. Wash 2-3 times. If necessary, perform the following washes, but carefully monitor the blot after each wash. Expose as soon as non-specific counts appear to have disappeared (e.g. in the corners): 2x SSC/RT, 0.2x SSC/RT

(usually this is enough), 2x SSC 0.1%SDS/RT, 0.2x SSC 0.1% SDS/RT. Perform same sequence of washes at 65°C if necessary.

11. Expose on to a phosphorImager screen (usually a few hours is enough).