

Myers Lab ChIPSeq Protocol

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Introductory information

This protocol is the one currently in use by the Myers lab at Stanford University. We use published methods, mostly based on those from Peggy Farnham and her lab at UC Davis (<http://www.genomecenter.ucdavis.edu/farnham/>) that we have revised during our work on the ENCODE Project. These procedures have worked well for our group for assaying the occupancy of more than a dozen transcription factors, RNA polymerase, TAF250 and methyl binding protein in living cells. During the past year, the major approach we have used for analyzing the ChIP'd DNA is with ultra-high throughput sequencing, or short-read sequencing, which we have called "ChIPSeq" (see Johnson et al., 2007, *Science* **316**:1497-1502).

For each ChIPSeq experiment, we typically perform separate 4 ChIP reactions on chromatin prepared from about 2×10^7 cells, then pool the four sets of ChIP'd DNAs to construct libraries for the Solexa / Illumina sequencing platform. Only 30-50 pg of this DNA is loaded on the sequencing machine. We begin with a large number of cells because our approach for making the libraries involves a gel purification step, and we have had better success by having large, visibly stainable fragments on the gel. Libraries prepared at a final DNA concentration of about 100 ng/ μ l or higher sequence best. However, it is likely that the experiments will work with smaller numbers of cells; we will modify this protocol if we are able to implement such an improvement.

Control or "mock" libraries are made from sonicated chromatin (see Section V, steps 4-12). These contain total reverse cross-linked DNA from the selected cell type. Other types of mock IP, such as DNA eluted from the magnetic beads without primary antibody, contain very little DNA and do not provide a good baseline for a peak calling control.

I. Growth of cells

Note: ChIP works best when cells are healthy and in “log phase” growth. Do not grow suspension cells to maximum density or to high acidity, and, for adherent cells, do not use plates that are completely confluent.

A. Suspension cells [K562, Jurkat, GM06990]

1. Grow cells to log phase growth (10^6 cells/ml maximum density).

For ChIPSeq library construction, the best results use 4 tubes of chromatin prepared from 4×10^7 cells/tube for diploid or near diploid cells (Jurkat, GM06990), and 2×10^7 cells/tube for aneuploid lines (K562). One liter of Jurkat cells yields 25 tubes of chromatin fragments and one liter of K562 cells yields 50 tubes of chromatin.

2. Count cells and measure viability by staining an aliquot of the cells with trypan blue.

If viability, as measured by exclusion of the blue dye, is $>90\%$, proceed to the next steps. If viability is lower, discard the experiment and start over with new cells.

B. Adherent cells [HepG2, HCT-116]

1. Plates are easier to process than flasks for adherent cells. Prepare one additional plate for counting. Cells in 150 mm dishes should be grown to a final cell density of $2-5 \times 10^7$ per dish.

2. Trypsinize one tissue culture plate. Count and test for viability by removing a small aliquot and staining with trypan blue.

If viability, as measured by exclusion of the blue dye, is $>90\%$, proceed to the next steps. If viability is lower, discard the experiment and start over with new cells.

[Database entry: Cell log / Liquid N₂ inventory]

II. Cross-linking of chromatin in living cells, harvest, and storage

Note: Suspension and adherent cells are cross-linked and collected differently.

Reagents:

20% formaldehyde [36.5% Formaldehyde for molecular biology Sigma F87750] rt

2.5M glycine rt

1XPBS 4°C

Farnham Lysis buffer: 5 mM PIPES pH 8.0 / 85 mM KCl / 0.5% NP-40
(adherent cells) + Roche Protease Inhibitor Cocktail Tablet
(Complete 11836145001 for 50 ml
or mini tablets 11836153001 for 10 ml)
4°C [add protease inhibitor tablet just before use]

A. Suspension cells

1. Transfer cells and media from the tissue culture flasks into either 50-ml conical tubes or centrifuge bottles.
2. Add formaldehyde to a final concentration of 1%, mix gently, and incubate at room temperature for 10 minutes.
3. Stop the cross-linking reaction by adding glycine to a final concentration of 0.125 M and mix gently.
4. Pellet cells at 2,000 rpm for 5 minutes at 4°C.
5. Place cells on ice and remove supernatant. Add equal volume cold (4°C) 1XPBS and gently resuspend cells, then centrifuge again.
6. Carefully remove supernatant and either proceed to sonication step or snap-freeze in liquid nitrogen and store at -80°C or in liquid nitrogen.

Note: Frozen cross-linked cells appear to be stable indefinitely. Therefore, this is a convenient step in the protocol to collect many samples for testing multiple antibodies, biological replicates, controls, etc.

B. Adherent cells

1. To adherent cells growing on tissue culture plates, add formaldehyde directly to the media to a final concentration of 1%, swirl gently, and incubate at room temperature for 10 minutes.
2. Stop the cross-linking reaction by adding glycine to a final concentration of 0.125 M and swirl gently to mix.
3. Remove media from plates and wash cells with equal volume cold (4°C) 1XPBS.
4. Aspirate PBS and add 5-8 ml cold (4°C) Farnham lysis buffer + protease inhibitors.

3. Scrape the cells off the plate with a cell scraper and transfer into 50-ml conical tubes on ice.
4. Pellet cells at 2,000 rpm for 5 minutes at 4°C.
5. Place cells on ice. Carefully remove supernatant and either proceed to sonication step or snap-freeze in liquid nitrogen and store at -80°C or in liquid nitrogen.

Note: Frozen cross-linked cells appear to be stable indefinitely. Therefore, this is a convenient step in the protocol to collect many samples for testing multiple antibodies, biological replicates, controls, etc.

[Database entry: Cell pellets / -80°C inventory]

III. Sonication

Reagents:

Farnham Lysis buffer: 5 mM PIPES pH 8.0 / 85 mM KCl / 0.5% NP-40 (filtered 0.2 - 0.45 micron filter unit) + Roche Protease Inhibitor Cocktail (complete) 4°C [add protease inhibitor tablet just before use]

RIPA Buffer: 1XPBS / 1% NP-40 / 0.5% sodium deoxycholate / 0.1% SDS (filtered 0.2 - 0.45 micron filter unit) + Roche Protease Inhibitor Cocktail 4°C [add protease inhibitor tablet just before use]

1. Resuspend each fresh or frozen pellet (10^8 cells) on ice in 5 ml Farnham Lysis Buffer + protease inhibitors and mix gently by flicking the test tube.

Two tubes of 10^8 cells will yield 10 tubes of chromatin fragments from K562 cells. Use 0.5 buffer volumes for Jurkat cell pellets.

Note: This treatment breaks the cells while keeping the nuclei mostly intact. The Farnham lab includes a douncing step here to ensure that the cells are completely broken. We have found that douncing has not been necessary, but it is possible that it will increase yields and/or purity with some cell types.

2. Collect the crude nuclear prep by at 2,000 rpm at 4°C for 5 minutes.
3. Resuspend pellet to 5 ml with RIPA Buffer + protease inhibitors at 4°C and transfer 1 ml aliquots to 1.5-ml microfuge tubes (2×10^7 cells/ml equivalent).

Do not vortex the tubes and try to avoid bubbles. Bubbles will cause popping and loss of samples during sonication.

4. Sonicate each 1.0 ml ChIP sample on ice at Power Output 5 watts 4 times for 30 seconds each, with at least 30 second cooling on ice between each 30-second sonication.

Note: For ChIPSeq, it is critical that the average length of the sheared chromatin is about 250 bp or less, with a range of about 100-600 bp. If the average size range is much higher, there will be very little DNA in the portion of the gel from which the 100-250 bp fragments will be extracted.

Important note: *Our lab uses a Sonics VibraCell sonicator. Different sonicator models and even different individual sonicators of the same model may vary in the settings and conditions needed for fragmenting DNA to a particular size range. We strongly recommend that each group do a titration experiment with cross-linked chromatin to determine conditions for shearing to an average of 250 bp before attempting a ChIP experiment. See detailed discussion of sonication below.*

5. Spin the sonicated mixture at 14,000 rpm in a microfuge for 15 minutes at 4°C and collect the supernatant.

6. Snap-freeze the sample in liquid nitrogen and store at -80°C, or do not freeze and continue with the immunoprecipitation steps below.

[Database entry: Chromatin Preps / -80°C inventory]

IV. Immunoprecipitation

Reagents:

Magnetic beads: Dynal / Invitrogen Dynalbeads (sheep anti-rabbit or anti-mouse IgG)

PBS/BSA: 1XPBS + 5 mg/ml BSA (fraction V) (freshly-prepared and filtered 0.2-0.45 micron filter unit) 4°C + Roche Protease Inhibitor Cocktail [add protease inhibitor tablet just before use]

LiCl IP Wash Buffer: 100 mM Tris pH 7.5 / 500 mM LiCl / 1% NP-40 / 1% sodium deoxycholate (filtered 0.2 - 0.45 micron filter unit) 4°C

TE: 1XTE (10 mM Tris-HCl pH 7.5 + 0.1 mM Na₂EDTA) 4°C

IP Elution Buffer: 1% SDS / 0.1 M NaHCO₃ (filtered through 0.2 - 0.45 micron filter unit) rt

1. Perform all steps in an ice bucket or in the cold room at 4°C. Couple the primary antibody for each transcription factor or chromatin protein to magnetic beads as follows:
 - a. Add 50 µl resuspended magnetic bead slurry to a 1.5 ml microfuge tube on ice containing 1 ml PBS/BSA. Vortex briefly to mix well.
 - b. Place the microfuge tubes on the magnet and remove supernatants.
 - c. Resuspend the beads in 1 ml PBS/BSA.
 - d. Repeat Steps b and c 3 times.
 - e. Add 1 ml PBS/BSA to beads.
 - f. Add 5 µg primary antibody (25 µl 200 µg/ml from Santa Cruz).
 - Do not vortex beads with bound antibody.*
 - g. Gently mix on a rotator platform overnight at 4°C.
 - h. Wash beads 3X (steps b-c) resuspend beads by inverting the tubes.
 - i. Resuspend in 100 µl PBS/BSA, and proceed to Step 2.
 2. Add 100 µl coupled antibody to each 1ml chromatin preparation (from Step 6 of Sonication protocol) and incubate at 4°C overnight on a rotator.
 3. Perform all steps in the cold room at 4°C. Collect beads containing immuno-bound chromatin by placing the microfuge tube on the magnet.
 4. Remove and discard supernatant.
 5. Wash beads 5 times with LiCl Wash Buffer, mixing 3 minutes for each wash on a rotator.
 6. Wash bead pellet with 1 ml TE Buffer. Mix 1minute on rotator and place tubes on magnet.
 7. Discard supernatant. Resuspend the bead pellet in 200 µl IP Elution Buffer rt. Vortex to mix.
- [Database entry: Record date and use of Cells or Chromatin Preps / -80°C inventory]

V. Reverse Cross-linking and recover DNA ChIP and Control DNA

Reagents:

Phenol/chloroform/isoamyl alcohol (25:24:1)

1. Incubate in a 65°C water bath for 1 hour, shake or vortex every 15 minutes, to elute the immunobound chromatin from the beads.
2. Spin at 14,000 rpm in a microfuge at rt for 3 minutes. Collect the supernatant, which contains the ChIP'd DNA. The tubes can be placed on the magnet to facilitate supernatant recovery.
3. Incubate the supernatant containing the ChIP'd DNA in a 65°C water bath overnight to complete the reversal of the formaldehyde cross-links.
4. To prepare a Reverse Cross-link Total Chromatin library, thaw a 1-ml chromatin tube and divide into two 500 µl aliquots and incubate in a 65°C water bath overnight to reverse the cross-links in that sample.
5. Extract the ChIP'd each with 200 µl phenol/chloroform/isoamyl alcohol and Total DNA samples with 500 µl, vortex thoroughly, and separate the phases by spinning in a microfuge at 14,000 rpm rt for 3 minutes.
6. Collect the aqueous supernatants from each sample.
7. Back-extract the phenol/chloroform/isoamyl alcohol phase from each ChIP sample with 50 µl dH₂O and add to the extracted aqueous supernatants from Step 13 above.
8. Add 750 µl Qiagen Buffer PM (PCR cleanup kit) to each extracted supernatant.
9. Add about half (~500 µl) of the solution to a Qiagen PCR Cleanup column, centrifuge, and then repeat with other half to bind the 1-ml sample on a Qiagen column.
10. Wash the column with 750 µl Qiagen Buffer PE, centrifuge, empty, and centrifuge the column, which contains the DNA, to dry.
11. Elute the DNA from the column with 2 50-µl aliquots of warmed (~55°C) Qiagen Buffer EB.
12. Combine the 4 100µl aliquots into one tube. Dry down in a Speedvac.
13. Resuspend the dried DNA in 50µl water.
12. Measure DNA concentration by using the Nano-drop device.

NRSF ChIP recovery is about 1-5 μg DNA from 4 1-ml chromatin preps from 2×10^7 cells and total chromatin recovery is about 20 μg . Reserve 5 μl for qPCR. To this, add 45 μl TE Buffer, and store at -80°C .

DNA is ready for end-repair and ligation according to Solexa library construction protocol.

VI. Checking size of sonicated ChIP'd fragments.

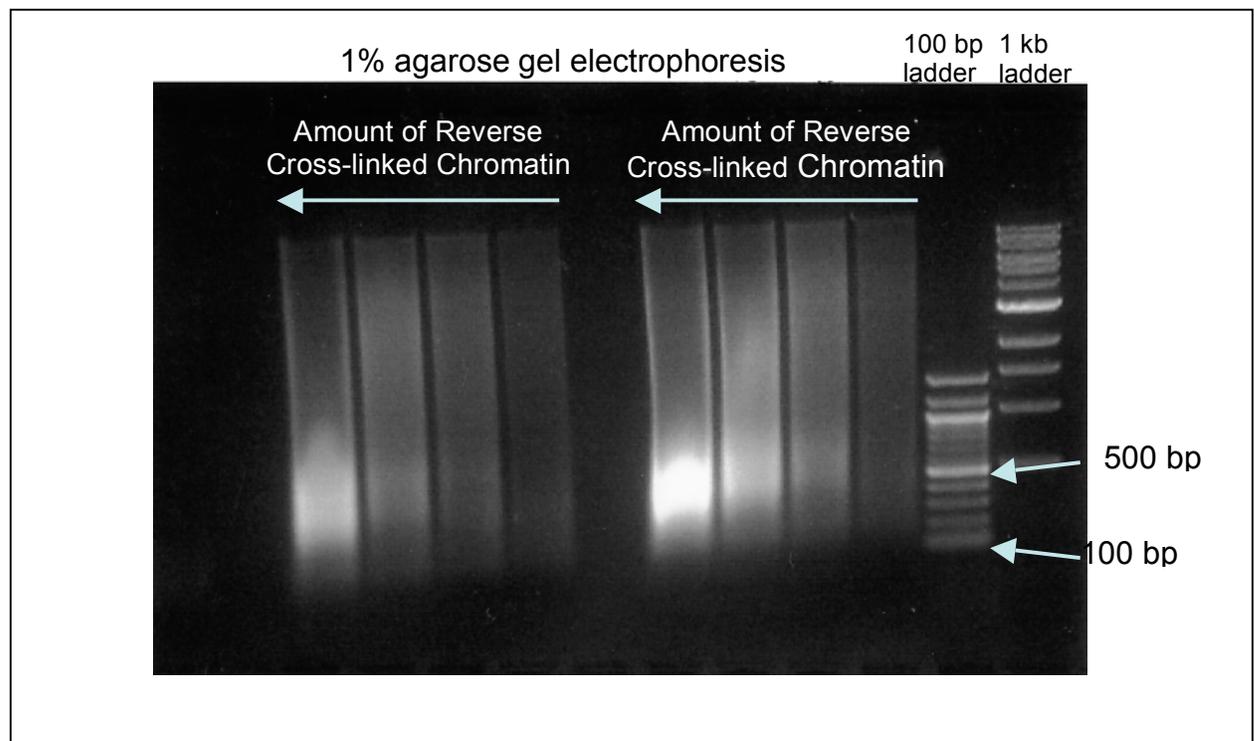
We perform this step only on the total chromatin DNA, as both are prepared from the same sonicated sample. This test should be done on each experiment prior to constructing the Solexa sequencing library.

1. Load $\sim 1 \mu\text{l}$, $2 \mu\text{l}$, $4 \mu\text{l}$ and $8 \mu\text{l}$ aliquots of reverse cross-linked DNA (from Step 4-12 in the Immunoprecipitation Protocol (Section V above) in lanes of a 1% agarose gel.

Note: We run this wide range of amounts to be sure we can assess the size range accurately; because the yield and spread of the sizes varies, you may learn that your sonication protocol requires a different range of amounts for this test.

2. After electrophoresis, examine the gel and compare the midpoint of the sample's size distribution to 100 bp ladder size markers (NEB).

3. Ideally, the DNA should be in a relatively tight distribution between about 100 bp and 500 bp, such as in the gel below.



VII. Reagents

Farnham Lysis buffer: 5 mM PIPES pH 8.0
85 mM KCl
0.5% NP-40
*Roche Protease Inhibitor Cocktail

RIPA Buffer: 1X PBS
1% NP-40
0.5% Sodium Deoxycholate
0.1% SDS
*Roche Protease Inhibitor Cocktail

IP Elution Buffer: 1% SDS
0.1 M NaHCO₃

LiCl IP Wash Buffer: 100 mM Tris-HCl pH7.5
500 mM LiCl
1% NP-40
1% Deoxycholate

VIII. Sonication details.

The Myers Lab uses a Sonics VibraCell sonicator for all of our ChIP experiments. We use the stepped microtip #630-0422 (the distal tip of this microtip is a 3-mm diameter cylinder). For the first use and when the microtip is changed, it is important to recalibrate the sonication conditions. Calibrate as follows:

1. Prepare 4 samples of cross-linked chromatin as in Section III (1-34) above.
2. Sonicate for 30 seconds, continuously, on amplitude setting 5 or 6 as follows:
 - a. Sample 1: 2 x 30 seconds
 - b. Sample 2: 4 x 30 seconds
 - c. Sample 3: 6 x 30 seconds
 - d. Sample 4: 8 x 30 seconds

Notes: Dial up slowly to amplitude 5, and start counting to 30 seconds after the sonicator reaches the 5 setting. Watch the dial the entire time to be sure that it does not go below 5; if it does, dial up slowly to 5 again.

The sonicator tip should be submerged about halfway to the bottom of the liquid in the microfuge tube. These conditions should not result in bubbling of the liquid.

Important: Leave microfuge tube on ice for 30 seconds between each 30-second sonication.

3. After sonication, reverse the cross-links as in Section V, steps 4-12.
4. Load 1, 2, 4 and 8 μl of each sample from each sonication condition onto a 1% agarose gel as in Protocol VI above.
5. Determine which sonication condition gives the appropriate size range for ChIPSeq (100-500 bp, with the center of the distribution at 250 bp or lower).

Below is a drawing of the microtips of the Sonics VibraCell sonicator. The stepped microtip on the bottom is the one we use.

