

Myers Lab ChIP-seq Protocol v011014

Modified January 10, 2014

Contact information:

Dr. Florencia Pauli Behn
HudsonAlpha Institute for Biotechnology
601 Genome Way
Huntsville, AL 35806
Telephone: 256-327-5229
Email: fpauli@hudsonalpha.org

Dr. Richard Myers
HudsonAlpha Institute for Biotechnology
601 Genome Way
Huntsville, AL 35806
Telephone: 256-327-0431
Email: rmyers@hudsonalpha.org

This protocol is currently in use by the Myers lab at HudsonAlpha Institute for Biotechnology for performing ChIP-seq, a method for identifying genome-wide occupancy sites of DNA binding proteins in the nuclei of living cells (see Johnson et al., 2007, Science 316: 1497-1502). Here we describe the experimental details of how we perform the steps of ChIP-seq in human cells, including cross-linking and harvesting cultured cells, fragmenting chromatin, immunoprecipitating the desired TF:DNA complexes by ChIP, and preparing the library for sequencing on the Illumina HiSeq platform. We also describe the preparation of a control library from DNA recovered after reversing the chromatin cross-links. This control is critical and is used to filter out regions of the genome, particularly those at open chromatin, that fragment more readily than others and can be over-represented in the sequencing step giving the false conclusion that they are sites of TF occupancy. By performing the reverse-crosslink control, these false signals can be subtracted on a region-by-region basis. Indeed, the peak-calling algorithms for ChIP-seq take this issue into account, as most of them require a control experiment to identify TF binding sites.

I. Notes on cell culture for ChIP-seq

Suspension cells – grow to log phase (10^6 cells/ml maximum density) and avoid high acidity. 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.

Adherent cells – grow on tissue culture plates rather than in flasks to facilitate cell harvest and prepare an additional plate for counting the cells. Grow cells to a final cell density of $2-5 \times 10^7$ per 150 mm dish. Do not grow cells to confluency. Trypsinize one tissue culture plate, count the number of cells, 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.

II. Chromatin cross-linking, Cell harvest, and Storage

Reagents:

All reagents are at room temperature unless otherwise noted.

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

2.5 M glycine

1X PBS at 4°C

Farnham Lysis buffer at 4°C: 5 mM PIPES pH 8.0 / 85 mM KCl / 0.5% NP-40

Filter the buffer with a 0.2-0.45 micron filter unit and add a protease inhibitor cocktail tablet (Roche 11836145001 or 11836153001) just before use. Store buffer at 4°C for no more than 4-6 weeks.

Before cross-linking, cells should be counted and checked for viability by greater than 90% exclusion of trypan blue. Since chromatin fragmentation and ChIP are performed in aliquots of 2×10^7 cells, we recommend that cells be stored in 2×10^7 cell aliquots after the final collection described below for suspension and adherent cells. Alternatively, they can be stored as larger pellets and then divided into 2×10^7 cell aliquots at the fragmentation step (depending on the method used) or at the immunoprecipitation step. 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.

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. To stop the cross-linking reaction, add 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 cold (4°C) 1X PBS roughly equal in volume to the cell pellet, gently resuspend cells, and then centrifuge again as in step 4.
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. As described above, cells may be divided into 2×10^7 cell aliquots at this step, prior to freezing.

B. Adherent cells

1. Remove culture plates from the incubator and place at room temperature on the bench.
2. Add formaldehyde to a final concentration of 1% directly to the media of adherent cells growing on tissue culture plates, swirl 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 swirl gently to mix.

4. Remove media from plates and wash cells with equal volume cold (4°C) 1X PBS.
5. Aspirate the PBS and add 5-8 ml cold (4°C) Farnham lysis buffer.
6. Scrape the cells off the plate with a cell scraper and transfer into 15-ml conical tubes on ice.
7. Pellet cells at 2,000 rpm for 5 minutes at 4°C.
8. 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. As described above, cells may be divided into 2×10^7 cell aliquots at this step, prior to freezing.

III. Chromatin fragmentation by sonication

We have used several methods for fragmenting chromatin, including a Sonics VibraCell sonicator, a Bioruptor XL (Diagenode), and a Bioruptor Twin (Diagenode) sonicator. This protocol describes sonication with a Bioruptor Twin. All of these methods are suitable for chromatin fragmentation, but 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 a titration experiment with cross-linked chromatin be performed to determine the appropriate conditions (time and intensity) for fragmenting chromatin before attempting a ChIP experiment (see “Checking the size of sonicated fragments” below). Regardless of the fragmentation method, samples should be kept as close to 4°C as possible during sonication with an ice water bath in a cold room or a circulator chiller water bath.

Reagents:

Farnham lysis buffer at 4°C: 5 mM PIPES pH 8.0 / 85 mM KCl / 0.5% NP-40

Filter the buffer with a 0.2-0.45 micron filter unit and add a protease inhibitor cocktail tablet (Roche 11836145001 or 11836153001) just before use. Store buffer at 4°C for no more than 4-6 weeks.

RIPA buffer at 4°C: 1X PBS / 1% NP-40 / 0.5% sodium deoxycholate / 0.1% SDS

Filter the buffer with a 0.2-0.45 micron filter unit and add a protease inhibitor cocktail tablet (Roche 11836145001 or 11836153001) just before use. Store buffer at 4°C for no more than 4-6 weeks.

Chromatin Fragmentation with the Bioruptor Twin (Diagenode):

1. Resuspend each fresh or frozen pellet (containing 2×10^7 cells) on ice in 1 ml Farnham lysis buffer and mix gently by flicking the test tube.
2. Pass the lysate through a 20 gauge needle 20 times, avoiding air bubbles. This treatment breaks the cells while keeping the nuclei mostly intact.
3. Collect the crude nuclear prep by centrifuging at 2,000 rpm at 4°C for 5 minutes.
4. Remove the supernatant and resuspend the pellet to 300 μ l with RIPA buffer.
5. Process samples in Bioruptor Twin (Diagenode) with circulating water bath according to the manufacturer’s instructions for temperature (less than 4°C) and speed of circulation (<500 ml per minute) at high setting. We perform a minimum of two 10-minute rounds of 30 seconds ON, 30 seconds OFF, but these conditions should be determined empirically for each cell

type (see “Checking the size of sonicated fragments” below).

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

When processing more than one tube of the same type of chromatin (i.e. same cell type and biological replicate), we recommend mixing all the sonicated chromatin at this step, removing 50 ul to prepare a reverse crosslink library (see below), and then re-aliquoting into equal aliquots of $\sim 2 \times 10^7$ -cell equivalents. For example, process 12 tubes of 2×10^7 cell aliquots on the Bioruptor Twin. After the centrifugation step, mix the contents of the 12 tubes, remove and save 50 ul, then divide the remaining chromatin back out into 12 equal aliquots. We implement this procedure to reduce sample to sample variability in sonication. The reverse crosslink chromatin control library made from the 50 ul aliquot is used as the control for all of the ChIPs performed from the mixed batch of chromatin.

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

IV. Checking the size of sonicated fragments

In order to determine the correct sonication conditions for each cell line, we carry out a shearing time course in the Bioruptor Twin. We typically process the chromatin for several (2-5) 10-minute cycles of 30 sec ON, 30 sec OFF and then assess the size of the fragments after reversing the crosslinks, isolating the DNA, and visualizing it on a gel and a BioAnalyzer. The shearing time must be determined empirically for each cell line before proceeding with immunoprecipitation.

- Load 1 ug of DNA from each sonication time point from samples prepared as described in Step 3.e-j of Section V: Immunoprecipitation on a 1% agarose gel. Load and run a 100 bp ladder size marker (NEB) along with the samples.
- After electrophoresis, examine the gel and compare the midpoint of the sample’s size distribution to 100 bp ladder size markers (NEB). The ideal condition should yield an enrichment of fragments in the 100 to 500 bp range, but larger fragments should still be present. We typically choose the shortest time point that shows noticeable enrichment in the 100 to 500 bp range. We find that over-fragmentation of chromatin causes ChIP-seq to fail, possibly due to damage caused to the protein epitope targeted by the antibody. Figure 1 shows the results of sonicating H1-hesc derived neuron cells for 2, 3, and 4 10-minute rounds of 30 sec ON, 30 sec OFF. Based on this gel, we selected to sonicate this cell line for 2 10-minute rounds since it is the shortest sonication time that showed fragments in the 100-500 bp range.

Sonication time (min): 20 30 40

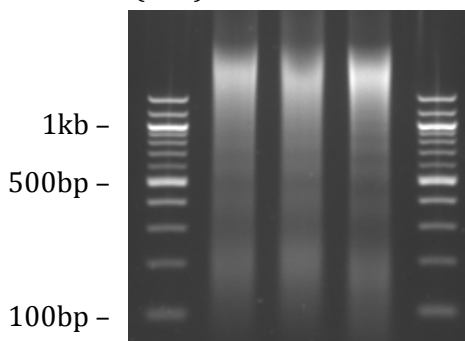


Figure 1: H1-hesc derived neuron cells were crosslinked, harvested, and sonicated with a Bioruptor Twin for 2, 3, and 4 10-minute rounds of 30 sec ON, 30 sec OFF. The chromatin crosslinks were reversed and DNA was purified. One microgram of DNA was loaded on a 1% agarose gel for each sample, along with a 100 bp ladder.

3. Samples can also be visualized on a Bioanalyzer (Agilent Technologies) to confirm fragment size (Fig. 2). Use a High Sensitivity DNA Chip per the manufacturer's instructions. Due to discrepancies and possible size biases on the BioAnalyzer, we recommend that gel electrophoresis be used as the primary way to visualize fragmented DNA.

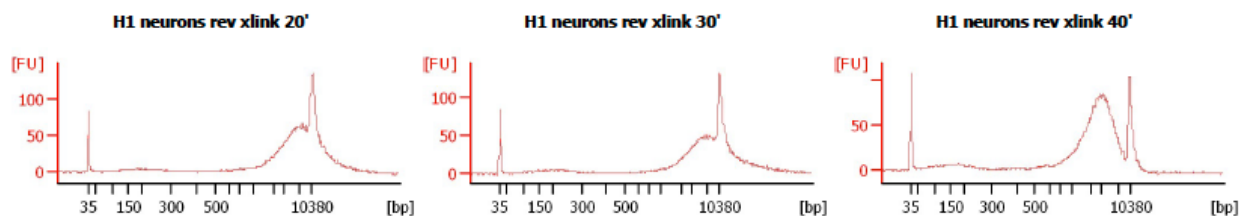


Figure 2: BioAnalyzer traces of H1-hesc derived neuron samples described in Figure 1.

V. Immunoprecipitation

Reagents:

All reagents are at room temperature unless otherwise noted.

Magnetic beads:

Dynabeads M-280 Sheep Anti-Rabbit IgG (Invitrogen 11203D) for antibodies made in rabbit

Dynabeads M-280 Sheep Anti-Mouse IgG (Invitrogen 11201D) for antibodies made in mouse

Dynabeads Protein G (Invitrogen 10003D) for antibodies made in goat

PBS/BSA at 4°C: 1XPBS / 5 mg/ml BSA (fraction V)

Filter the buffer with a 0.2-0.45 micron filter unit and add a protease inhibitor cocktail tablet (Roche 11836145001 or 11836153001) just before use.

LiCl IP Wash Buffer at 4°C: 100 mM Tris pH 7.5 / 500 mM LiCl / 1% NP-40 / 1% sodium deoxycholate.

Filter the buffer with a 0.2-0.45 micron filter unit.

1X TE at 4°C: 10 mM Tris-HCl pH 7.5 / 0.1 mM Na₂EDTA

IP Elution Buffer: 1% SDS / 0.1 M NaHCO₃

Filter the buffer with a 0.2-0.45 micron filter unit.

QIAquick PCR Purification Kit (Qiagen 28104)

Perform all steps in an ice bucket or in the cold room at 4°C.

1. Couple the primary antibody for each transcription factor or chromatin protein to magnetic beads:
 - a. Add 200 µl re-suspended magnetic bead slurry to a 1.5 ml microfuge tube on ice containing 1 ml cold PBS/BSA. Vortex briefly to mix well.
 - b. Place the microfuge tubes on the magnet rack and remove supernatants.
 - c. Resuspend the beads in 1 ml cold PBS/BSA.
 - d. Repeat Steps b and c 3 times.
 - e. Add 200 µl PBS/BSA to beads.
 - f. Add 5 µg primary antibody. Do not vortex beads after adding the antibody.
 - g. Gently mix on a rotator platform for at least 2 hours at 4°C.
 - h. Wash beads 3 times (steps b-c), resuspending the beads by inverting the tubes during each wash.
 - i. Resuspend in 100 µl PBS/BSA, and proceed to Step 2.

2. Incubate bead-antibody complex with fragmented, cross-linked chromatin
 - a. Add 100 µl of antibody-coupled beads (from step 1.i above) to each 300 µl chromatin preparation (from Sonication protocol) and incubate on a rotator for one hour at room temperature, followed by one hour at 4°C.
 - b. Collect beads containing immuno-bound chromatin by placing the microfuge tube on a magnet rack.
 - c. Remove and discard supernatant.
 - d. Wash beads 5 times with LiCl Wash Buffer, mixing 3 minutes for each wash on a rotator.
 - e. Add 1 ml TE Buffer to beads. Mix 1 minute on rotator and then place tubes on magnet rack to collect beads and discard supernatant.
 - f. Resuspend the bead pellet in 200 µl IP Elution Buffer (at room temperature). Vortex to mix.

3. Reverse cross-linking and recover ChIP DNA and prepare a control DNA sample
 - a. Incubate bead pellet from step 2.f above in a 65°C water bath for 1 hour, shake or vortex every 15 minutes to elute the immuno-bound chromatin from the beads.
 - b. Spin at 14,000 rpm in a microfuge at room temperature for 3 minutes.
 - c. Collect the supernatant, which contains the ChIP'd DNA. The tubes can be placed on the magnet to facilitate supernatant recovery.
 - d. Incubate the supernatant containing the ChIP'd DNA in a 65°C water bath overnight to complete the reversal of the formaldehyde cross-links.
 - e. To prepare a control Reverse Cross-link Chromatin library, incubate a sample of sheared chromatin representing at least 1×10^6 cells in a 65°C water bath overnight to reverse the cross-links. Bring the volume up to 200 µl with 1X PBS, add 20 µl of Proteinase K (Qiagen 19131) and incubate at room temperature for 2 minutes. Add 4 µl of RNase A (Qiagen 19101), then add 5 volumes of Qiagen buffer PB (QIAquick PCR Purification Kit), and incubate at room temperature for 20 minutes. Skip step f and proceed with step g.
 - f. Add 5 volumes Qiagen Buffer PB (QIAquick PCR Purification Kit) to one volume of ChIP'd DNA. Upon addition of Buffer PB, the sample should be yellow, indicating the correct pH. If the sample is not yellow, the pH should be adjusted with 3M sodium acetate

- as recommended by the manufacturer (Qiagen).
- g. Add half (~600 μ l) of the solution to a QIAquick PCR Purification column, centrifuge per manufacturer's instructions, and then repeat with other half to bind the 1.2 ml sample on a Qiagen column.
 - h. Wash the column with 750 μ l Qiagen Buffer PE, centrifuge per manufacturer's instructions, empty, and centrifuge the column containing the bound DNA a second time allow it to dry.
 - i. Elute the DNA from the column with two 30 μ l aliquots of warmed (~55°C) Qiagen Buffer EB, following the manufacturer's instructions (add buffer, allow to sit on column for 1 minute, spin, and repeat).
 - j. Measure DNA concentration by using a Qubit (Invitrogen) fluorometer.

DNA recovery varies by antibody used for ChIP and ranges from 5-50+ ng from a chromatin prep of 2×10^7 cells. Total chromatin for the control sample recovers about 20 μ g. Store at -80°C. DNA is ready for Illumina library construction.