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Genome-Wide Identification of Transcription Factor-Binding Sites in Quiescent Adult Neural Stem Cells

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Abstract

Transcription factors bind to specific DNA sequences and control the transcription rate of nearby genes in the genome. This activation or repression of gene expression is further potentiated by epigenetic modifications of histones with active and silent marks, respectively. Resident adult stem cells in the hematopoietic system, skin, and brain exist in a non-proliferative quiescent resting state. When quiescent stem cells become activated and transition to dividing progenitors and distinct cell types, they can replenish and repair tissue. Thus, determination of the position of transcription factor binding and histone epigenetic modification on the chromatin is an essential step toward understanding the gene regulation of quiescent and proliferative adult stem cells for potential applications in regenerative medicine. Genome-wide transcription factor occupancy and histone modifications on the genome can be obtained by assessing DNA-protein interaction through next-generation chromatin immunoprecipitation sequencing technology (ChIP-seq). This chapter outlines the protocol to perform, analyze, and validate ChIP-seq experiments that can be used to identify protein-DNA interactions and histone marks on the chromatin. The methods described here are applicable to quiescent and proliferative neural stem cells, and a wide range of other cellular systems.

Keywords

Chromatin sonication; DNA-protein crosslinking; ChIP-seq; ChIP-qPCR; Transcription factor genome-wide DNA occupancy; Genome-wide histone modification; Bioinformatics

1 Introduction

DNA-protein interactions are required for DNA replication, chromatin remodelers, transcriptional regulation, and DNA repair. The binding of transcription factors modulates and coordinates gene expression in response to external stimuli. Adult mammals have resident stem cells that predominantly exist in a resting non-proliferative quiescent state, which preserves the stem cell pool over the lifetime of the animal [1, 2]. In response to extrinsic and intrinsic factors, adult stem cells exit from quiescence and proliferate and differentiate into cells that maintain and repair the local tissue [3–7]. Adult resident stem cells provide an opportunity to harness them for regenerative medicine and stem cell therapy. Quiescent and proliferative adult stem cells possess distinct transcriptional profiles and epigenetic modifications, which govern distinct cell fate decisions [3, 4, 8]. Understanding

the transcriptional regulatory network of DNA-protein interactions is essential to decipher the molecular basis of stem cell activation and differentiation in response to external stimuli.

Chromatin-immunoprecipitation (ChIP) gives us the capability to determine occupancy of proteins on DNA from intact cells in contrast to the earlier in vitro technique of electrophoretic mobility shift assays (EMSA) [9–11]. In ChIP, the protein (such as a transcription factor) is cross-linked to the DNA and then the DNA is fragmented mechanically by sonication or enzymatically using DNase. The DNA fragment left bound to the protein is then pulled down with a specific antibody and when the chromatin-immunoprecipitated (ChIP-ed) DNA sequence is compared to genome sequence of the organism, it reveals the occupancy of the transcription factor. Thus, the size of DNA post-fragmentation determines the resolution of all ChIP methods, usually it is optimally maintained around 250–300 bp. The advances in the field of DNA-protein ChIP assays have primarily focused on developing better DNA fragmentation techniques and improved means to identify the chromatin-immunoprecipitated DNA.

Fragmentation of DNA or sonication of chromatin can be achieved by both enzyme-based methods and water bath-based methods. Among the water bath-based methods Bioruptor and Covaris sonicators are most popular. Bioruptor uses longer unfocused wavelengths without thermal control, while Covaris sonicators use shorter focused wavelengths with thermal control. As sonication efficiency and protein-DNA binding depends on temperature, Covaris sonicators that have thermal control are gaining popularity in the field [12]. The ChIP-on-chip method identifies protein DNA occupancy genome-wide using microarray technology with probes on the array representing only the promoter regions of the genome, as whole genome probe arrays are cost inhibitive [13]. The onset of next-generation sequencing, Solexa platform and Illumina HiSeq platform, allowed sequencing of all DNA with transcription factor occupancy across the genome without promoter bias [13].

In this chapter, we describe optimized methods for ChIP-seq and ChIP-qPCR in quiescent and proliferating adult hippocampal neural stem cells for the transcription factor REST, histone mark H3K27Ac, acetylation at the 27 lysine residue of the histone H3, and RNA Pol II. This protocol will be generally applicable to other types of biological cells and tissue. We also provide an overview of ChIP-seq data analysis.

2 Materials

2.1 Common Reagent Stocks

Buy commercially molecular grade or make in the laboratory.

1. PBS without Ca^{2+} Mg^{2+} .
2. 8 M LiCl.
3. 1 M Tris-HCl, pH 8.0.
4. 1 M HEPES pH 6.8 to 8.2 adjust pH with KOH as required.
5. Tris-EDTA buffer (TE buffer).

6. 0.5 M EDTA and 0.5 M EGTA.

2.2 Preparation of Nuclei and DNA-Protein Crosslinking

1. 16% formaldehyde methanol. Once glass ampule is opened use within 1–2 weeks.
2. 10× Fixation buffer: Add 10 ml of 1 M HEPES pH 8.0, 4 ml of 5 N NaCl, 0.4 ml of 0.5 M EDTA, and 0.2 ml of 0.5 M EGTA to about 100 ml nanopure H₂O. Then, adjust volume to 200 ml with nanopure H₂O.
3. 1.25 M glycine (crosslinking quenching buffer): Dissolve 0.938 g of glycine in 10 ml nanopure H₂O.
4. Cell wash buffer B: Add 10 ml of 1 M HEPES-KOH pH 7.6, 5.6 ml of 5 M NaCl, 0.4 ml of 0.5 M EDTA pH 8.0, 20 ml of 100% Glycerol, 1 ml of 100% NP-40, 0.5 ml of 100% Triton-X100 to about 100 ml nanopure H₂O. Then, adjust volume to 200 ml with nanopure H₂O. Optional: add a tablet with protease inhibitors.
5. Cell rinse buffer C: Add 2 ml of 1 M Tris-HCl pH 8.0, 8 ml of 5 M NaCl, 0.4 ml of 0.5 M EDTA pH 8.0, 0.4 ml of 0.5 M EGTA pH 8.0 to about 100 ml nanopure H₂O. Then, adjust volume to 200 ml with nanopure H₂O. Optional: add a tablet with protease inhibitors.

2.3 Chromatin Shearing or Sonication

1. Sonication or shearing buffer D: Add 2 ml of 1 M Tris-HCl pH 8.0, 2 ml of 10% SDS, 0.4 ml of 0.5 M EDTA pH 8.0 to about 100 ml nanopure H₂O. Then, adjust volume to 200 ml with nanopure H₂O. Optional: add a tablet with protease inhibitors.
2. For Covaris sonication: Covaris milliTUBE 1 ml AFA fiber Part #520135 Covaris.

2.4 Chromatin Immunoprecipitation (ChIP)

1. Immunoprecipitation (IP) buffer or ChIP dilution buffer: To about 100 ml sonication or shearing buffer D add 20 ml of 10% Triton-X100 and 6 ml of 5 M NaCl. Then adjust volume to 200 ml with nanopure H₂O. Optional: add a tablet with protease inhibitors.
2. Low salt wash buffer I: Add 4 ml of 1 M Tris-HCl pH 8.0, 2 ml of 10% SDS, 1.6 ml of 0.25 M EDTA pH 8.0, 20 ml of 10% Triton-X100, 6 ml of 5 M NaCl to about 100 ml nanopure H₂O. Then adjust volume to 200 ml with nanopure H₂O. Optional: add a tablet with protease inhibitors.
3. High salt wash buffer II: Add 4 ml of 1 M Tris-HCl pH 8.0, 2 ml of 10% SDS, 1.6 ml of 0.25 M EDTA pH 8.0, 20 ml of 10% Triton-X100, 16 ml of 5 M NaCl to about 100 ml nanopure H₂O. Then adjust volume to 200 ml with nanopure H₂O. Optional: add a tablet with protease inhibitors.

4. LiCl wash buffer III: Add 2 ml of 1 M Tris-HCl pH 8.0, 20 ml of 10% Deoxycholate, 0.8 ml of 0.25 M EDTA pH 8.0, 20 ml of 10% NP-40, 12.8 ml of 8 M LiCl to about 100 ml nanopure H₂O. Then adjust volume to 200 ml with nanopure H₂O. Optional: add a tablet with protease inhibitors.
5. Mild wash buffer: Add 2.5 ml of 20% NP-40, 0.5 ml of 20% SDS, 0.8 ml of 0.5 M EDTA, 4 ml of 1 M Tris-HCl, 10 ml of 5 M NaCl to 100 ml of nanopure H₂O. Adjust volume to 200 ml with nanopure H₂O. Optional: add a tablet with pro-tease inhibitors.
6. Elution buffer: Add 1 ml of 10% SDS and 84 mg of NaHCO₃ to nanopure H₂O and make volume up to 10 ml.
7. No-stick SNAPLOCK microcentrifuge tubes: Eppendorf 1.5 ml tubes (e.g., Company Light Labs). Any other no-stick Eppendorf tubes may also be used.
8. Protein G Dynabeads or Protein A Dynabeads: Use Protein G Dynabeads for Rabbit host generated ChIP-grade antibody.
9. Magnetic rack for 1.5 ml eppendorf tubes.

2.5 DNA-Protein Reverse Crosslinking, DNA Isolation, and DNA Estimation of ChIP-ed DNA

1. Qiaquick PCR purification kit or general DNA isolation protocol.
2. DNA estimation kit Qubit (nano drop is not accurate for small amounts of DNA).

2.6 Determination of Sonicated DNA Size, Bioanalyzer Quality Check, and ChIP-seq

1. BIO-33062 EZ Ladder I: Any DNA ladder with range from 100 bp to 1 kb.

3 Methods

3.1 Preparation of Nuclei and DNA-Protein Crosslinking (Fig. 1)

3.1.1 Cell Preparation and Crosslinking

1. Grow cells up to 80–90% confluency as a monolayer in a 10 cm plate.
2. Remove the medium and add 9 ml fresh medium (DMEM or medium in which the cells were growing) to a 10 cm plate. All the reagents are at room temperature in this step.
3. Add to above 1 ml of 10 Fixation Buffer A and 625 µl of 16% of methanol-free formaldehyde (1% final concentration). All reagents must be at room temperature in this step.
4. Place the cells on a shaker at room temperature and shake for 10 min to allow for efficient crosslinking (maximum 15 min).
5. Quench crosslinking by adding to above 10 ml volume 1.12 ml freshly prepared 1.25 M Glycine stock at room temperature and shake for 5 min (125 mM final concentration).

6. Completely aspirate the solution from the plate and wash twice with 10 ml of ice-cold PBS without Ca_2^+ and Mg_2^+ on a shaker at room temperature or 4 °C. First wash for 1 min and second wash for 3 min.
7. Harvest cells in fresh 5 ml or 10 ml ice-cold PBS by scrapping.
8. Spin down the pellet at $300 \times g$ for 2 min at room temperature or 4 °C and discard the supernatant.

3.1.2 Nuclei Preparation

1. Resuspend cell pellet of maximum 30 million cells in 10 ml of ice-cold cell wash buffer B.
2. Rotate at 4 °C on a rabbit-ear rotor for 10 min.
3. Spin down the nuclear pellet at $1500 \times g$ for 2 min at room temperature or 4 °C and discard the supernatant.
4. Resuspend the nuclear pellet in 10 ml of ice-cold cell-rinse buffer C.
5. Immediately, spin down the nuclear pellet at $1500 \times g$ for 2 min at room temperature or 4 °C and discard the supernatant.

3.2 Chromatin Shearing or Sonication (Fig. 1)

1. Resuspend maximum 15 million cells worth nuclear pellet in 1 ml sonication or shearing buffer D.
2. Rotate at 4 °C on a rabbit-ear rotor for 30 min or for a minimum of 10 min.
3. Covaris sonication: Now take the Covaris milliTUBE 1 ml AFA fiber to Covaris sonicator for sonication. One tube can be reused for up to 8 sonications (6 min 30 s each). Alternative option Bioruptor: Add 1 ml of sample to no-stick eppendorf and wrap cap with parafilm. Sonicate in ice-cold Bioruptor for five times 7 min each with 30 s on and 30 s off cycle. To maintain cold temperature, replace ice bath each time.
4. Covaris S-series sonicator settings: Duty cycle 5%, Intensity 4, Cycle per burst 200, Temperature 4 °C, Power mode frequency Sweeping, Degassing mode (default), AFA Intensifier (default), Water level (enough until base of black cap, use the Covaris black/white holder).
5. Sonicate 1 million cells in 1 ml volume for 6 min 30 s.
6. Spin sonicated chromatin, more than 15 min at $18,000 \times g$ to pellet debris, then transfer the supernatant into a fresh no-stick snaplock eppendorf tube.
7. To convert shearing buffer D to IP (immunoprecipitation buffer) add Triton-X100 to 1% final concentration and NaCl 150 mM final concentration. Example: To 8.70 ml of sonicated chromatin solution add 1 ml of 10% Triton-X100 and 300 μl of 5 M NaCl.

3.3 Chromatin Immunoprecipitation (ChIP) (Fig. 2)

1. For each ChIP use 1 ml or 1.45 ml of the sonicated chromatin IP solution or 150–300 µg of chromatin (DNA concentration can be estimated using nano-drop DNA) and take out 5% input from it 50 µl or 72.5 µl, respectively (*see* Note 1).
2. Optional: If ChIP-seq or ChIP-qPCR shows high background, then preclear the above 1.45 ml or 1 ml sonicated chromatin IP sample with 20 µl of pre-washed Protein G Dynabeads by rotation on a rabbit-ear rotor for 1–2 h at 4 °C. Put the eppendorf on the magnetic rack and use the supernatant to proceed to the ChIP reaction in **step 3**. Pre-wash beads two times with 0.5% of BSA in PBS before three times with Covaris IP buffer washes as described in **step 5**.
3. Add 10 µl of preferably a ChIP-grade antibody (1 µg/µl) to the 1.45 ml of sonicated chromatin in IP or precleared sonicated chromatin in IP above and incubate at 4 °C on a rabbit-ear rotor overnight for up to 18 h and a minimum of 6 h.
4. For each ChIP use about 50 µl of Protein G Dynabeads.
5. Wash Protein G Dynabeads three times with Covaris IP buffer in 1.5 ml eppendorf tubes. Each wash is done by adding, 1 ml Covaris IP buffer and 4 min rotation in rabbit-ear at 4 °C. Then place the eppendorf tube on a magnetic rack for 1 min to allow beads to settle on the magnet. Pipette out and discard the supernatant and then add next wash 1 ml volume, take the tube out of the magnetic rack and invert seven times by hand to resuspend beads, then place in a rabbit-ear rotor and repeat the cycle (*see* Note 2).
6. After the last wash, resuspend the Protein G Dynabeads into 50 µl of IP buffer and add to the ChIP reaction (antibody and sonicated chromatin) eppendorf tube from **step 2**.
7. Incubate this ChIP reaction and Dynabead mix at 4 °C on the rabbit-ear rotor for up to 6 h.
8. Collect beads on the magnetic rack, discard the supernatant, and proceed to washes. Each wash is done by adding 1 ml of the following wash buffers, using the magnetic rack and rabbit-ear rotor as described above in **step 4** (*see* Note 3).
  - a. Wash in low salt wash buffer I for one time.
  - b. Wash in high salt wash buffer II for two times.
  - c. Wash in BC LiCl wash buffer III for five times.
  - d. Wash in Tris-EDTA buffer for one time.

¹For all immunoprecipitations done from the sonicated chromatin IP solution in **step 7** in Subheading 3.2, only one 5% input is required. Proceed to ChIP after ensuring sonication efficiency with 5% input for every experiment even when sonication conditions have been optimized.

²Optional: If ChIP-seq or ChIP-qPCR shows high background, wash beads twice with 0.5% BSA in PBS before the three times with Covaris IP buffer washes.

³Alternatively, wash only with mild wash buffer for five times if target protein does not bind strongly with DNA.

9. After final wash above discard the supernatant and resuspend beads in preheated freshly made elution buffer to 70 °C. Resuspend beads in 250 µl of preheated elution buffer and incubate in 70 °C heating block preferably with shaking. Then add beads on the magnetic rack and keep the supernatant as it contains the immunoprecipitated chromatin. Repeat elution with another 250 µl of elution buffer and combine the supernatants to get 500 µl of total eluted immunoprecipitated chromatin.
10. To the 5% input previously collected in **step 1**, similarly add 500 µl of elution buffer.
11. Now proceed with the ChIP-ed chromatin and input chromatin in elution buffer to the next step of DNA-protein reverse crosslinking, DNA isolation, and DNA estimation of ChIP-ed DNA.

3.4 DNA-Protein Reverse Crosslinking and Isolation of ChIP-ed DNA (Fig. 3)

1. Make all volumes up to 500 µl by adding elution buffer. The 5% input and chromatin-immunoprecipitated DNA are already at 500 µl in elution buffer.
2. Reverse crosslinking: Add 11 µl of 5 M NaCl to the 500 µl DNA in elution buffer to get 0.3 M NaCl final concentration. Incubate on heating block overnight at 65 °C preferably with shaking.
3. RNA digestion: To the above reverse crosslinked DNA solution add 5 µl of RNase to digest RNA. Incubate on a heating block for 1 h at 37 °C preferably with shaking.
4. Protein digestion: To the above reverse crosslinked and RNA digested DNA solution add 5 µl of Proteinase K to digest all proteins. Incubate on a heating block for 1–4 h at 55 °C preferably with shaking.
5. Purify and extract DNA using Qiaquick PCR DNA purification kit or any other DNA column purification and DNA extraction kit following the manufacturer's instructions. Steps are briefly described below for Qiaquick PCR DNA purification kit using buffers from the kit,
  - a. To ~500 µl of DNA solution from **step 4**, add 5× its volume ~2500 µl PB buffer (buffer provided with the kit) and 5 µl of pH indicator. The solution should turn yellow, if orange add more PB buffer and/or 10 µl of 3 M sodium acetate until it turns yellow (*see Note 4*).
  - b. Place 750 µl at a time of the above solution on the binding column that is placed on the empty eppendorf tube and wait for 1–2 min. Centrifuge at room temperature at 18,000 × *g* for 1 min and discard flow-through. Repeat for the remainder of the solution from step a.

⁴Yellow color indicates the solution is at the correct pH for optimal binding of DNA to the column in the next step.

- c. Add 750 μ l of PE wash (buffer provided with the kit) buffer to column and centrifuge at room temperature at $18,000 \times g$ for 1 min and discard flow-through.
    - d. Centrifuge again on empty eppendorf to get rid of residual liquid from the column at room temperature at $18,000 \times g$ for 1 min and discard flow-through.
    - e. Put column on a no-stick eppendorf tube, add 30–50 μ l of elution buffer from the kit to the column. Wait for 10–15 min and centrifuge at room temperature at $18,000 \times g$ for 2 min.
    - f. Discard column and save flow-through as it is pure eluted DNA from chromatin-immunoprecipitated DNA or 5% input samples.
  6. Alternatively, purify and extract DNA by the kit-free Phenol/Chloroform method. This method usually gives lower yield than the described column-based method (*see* Note 5).
    - a. Add 500 μ l of phenol/chloroform equilibrated with TE buffer pH 8.0.
    - b. Mix by vortexing.
    - c. Centrifuge at room temperature at $18,000 \times g$ for 10 min.
    - d. Save the aqueous phase and add 500 μ l of chloroform: isoamyl alcohol (24:1).
    - e. Save the aqueous phase as it contains pure eluted DNA from chromatin-immunoprecipitated DNA or 5% input samples.
    - f. Take aqueous phase into a new eppendorf tube and add 2 μ l of glycogen, 50 μ l of 3 M Sodium Acetate pH 5.2, and 900 μ l of isopropanol. Mix well.
    - g. Leave at -20°C for 30 min to 1 h or overnight for DNA to precipitate.
    - h. Centrifuge at $18,000 \times g$ for 15–30 min at 4°C to pellet DNA and discard the supernatant.
    - i. Wash the pellet with 75% ethanol, centrifuge as above, and discard the supernatant. Air-dry the pellet but do not over dry.
    - j. Resuspend DNA pellet in 50 μ l of water.
  7. DNA estimation: Input 5% DNA is usually $>10 \mu\text{g}/\mu\text{l}$ in concentration that is reliably estimated by nanodrop. The chromatin-immunoprecipitated DNA from transcription factors is usually of a much lower concentration and amount (5–10 ng), so the concentration is estimated using Qubit following the manufacturer's instruction. Briefly,

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⁵It should be OK to use for histone modifications and polycomb complexes as these have many binding sites on the chromatin, unlike transcription factors for which the column-based method is preferred for higher yields.

- a. Prepare a master mix for 200 μ l buffer per sample and 1 μ l of dye per sample in regular eppendorf. Vortex to mix.
- b. Use Qubit eppendorf for this step. For samples, add 199 μ l of master mix to 1–2 μ l of sample. For kit standards, add 190 μ l of master mix to 10 μ l of standards.
- c. Vortex to mix and incubate at room temperature in the dark for 2 min.
- d. Now take readings on the Qubit machine for samples and standards in the Qubit eppendorf. Make sure the bottom of the Qubit eppendorf tube is clean. For standard readings, order is important, first read the first standard and then read the second standard.
- e. Record the concentrations. Store these input and ChIP-ed DNA samples at 4 °C or lower temperatures until further use.

3.5 Determination of Sonicated DNA Size, Bioanalyzer Quality Check, and ChIP-seq (Fig. 4)

1. DNA sonication size: Prepare a 1% agarose gel without ethidium bromide. Run ~200 ng of 5% input from the sample and the EZ-Ladder I until the 100 bp to 1 kb ladder bands are resolved in TAE buffer. Incubate gel in ethidium bromide TAE buffer with shaking at room temperature for 20 min to 1 h. Then visualize gel under UV, if bands not visible incubate longer in ethidium bromide TAE buffer. Save the ethidium bromide TAE solution to reuse. Smear of sonicated DNA size range from 100 to 500 bp is best with highest intensity in the 250 bp region.
2. DNA sonication size and concentration Bioanalyzer: Chromatin-immunoprecipitated DNA is usually a small amount (<200 ng), so to determine DNA size distribution of these samples use High Sensitivity Bioanalyzer assay (see Note 6). Submit 5% input samples diluted to pg/ μ l range to confirm DNA size range seen in 1% agarose gel above (optional). Follow the manufacturer's instruction or use sequencing core facility services.
3. ChIP-qPCR validation of known binding sites is optional if there are some known targets for the transcription factor. Alternatively, ChIP-qPCR is performed on chromatin-immunoprecipitated DNA to validate ChIP-seq peaks (see Subheading 3.8).
4. Library preparation: Illumina's TruSeq ChIP library preparation kit or NEB Next Ultra II DNA Library Prep Kit for Illumina. Follow the manufacturer's instruction or use sequencing core facility services.
5. ChIP-seq: For sequencing at least 5–10 ng of chromatin-immunoprecipitated DNA is minimally required. Multiplex and run parallel ChIP reactions to get this

⁶The sensitivity of Bioanalyzer is in pg/ μ l range and thus 1 ng of chromatin-immunoprecipitated DNA in 5 μ l of elution buffer is sufficient to accurately detect size range of pulled down ChIP-ed DNA and accurately measure concentration, to confirm Qubit concentration estimation.

amount of chromatin-immunoprecipitated DNA if necessary. Submit above ChIP-seq sample libraries for sequencing, preferably by Illumina HiSeq at 50 base single-end and at least 10–15 million reads per sample. Include 5% Input sample in the ChIP-seq run and IgG ChIP-ed DNA is optional.

3.6 Sonication Optimization and Antigen Retention (Fig. 4)

1. To optimize sonication settings, use recommended settings in Subheading 3.2 or default manufacturer's settings for the model of Covaris S or E series. Vary total time of sonication (e.g., 5, 6.5, 8.5, 12.5, 16.5 min) and number of cells (e.g., 0.5, 1, 2, 4, 6 million cells per ml) (see Note 7).
2. From the 1 ml sonication volume remove a 100 μ l aliquot after each sonication trial time and replace the AFA covaris tube with fresh shearing buffer D to keep total volume of sonication always at 1 ml. Divide the 100 μ l aliquot into two 50 μ l parts, use one for DNA fragmentation and sonication check and the other part for antigen retention western blot.
3. DNA sonication or fragmentation: Use 50 μ l of the 100 μ l aliquot from **step 2**, make up to 500 μ l with elution buffer or water and perform reverse crosslinking, RNase digestion, and Proteinase K digestion as described in Subheading 3.4. Visualize fragments on 1% agarose gel as described in Subheading 3.5.
4. Antigen retention: To the other 50 μ l of the 100 μ l aliquot from **step 2** add 50 μ l of elution buffer or water. Then add 5 M NaCl to a final concentration of 0.3 M. Incubate on heating block overnight at 65 °C preferably with shaking. Digest RNA using RNase for 30 min to 1 h at 37 °C preferably with shaking. Now denature samples with LDS reducing buffer and perform western blot to probe with the same ChIP-grade antibody that will be used for ChIP in Subheading 3.4 (see Note 8).

3.7 ChIP-seq Bioinformatics Data Analysis, a Brief Overview (Figs. 5 and 6)

1. ChIP-seq run on Illumina or Solexa generates fastq read files. This section gives a brief overview of how to obtain occupancy of the protein on the DNA and other biologically relevant information from these raw reads. Alternately, ChIP-seq published data may be obtained from ENCODE <https://www.encodeproject.org/> or NCBI GEO <https://www.ncbi.nlm.nih.gov/geo/> and analyzed with the same pipeline described below.
2. Running platform and software installation: Web-based open resource platform Galaxy <https://usegalaxy.org/> is a good starting point. For command line-based analysis, Mac users can use *Terminal* and Windows (UNIX operating system) users can use *cmd* or Cygwin or Ubuntu on Virtual box. For intense computing installing linux ubuntu operating system or cloud computing options may be considered. Many bioinformatics core host Galaxy cluster, which can be

⁷Avoid over sonication of samples as it will reduce DNA size and antigen retention; hence, it will reduce the ChIP signal.

⁸This experiment probing antigen retention is good to do during optimization of sonication conditions, to ensure that efficient DNA fragmentation occurs without loss of the antigen being used to pull down the DNA in ChIP.

accessed through *Terminal* and *Putty* by Mac and Windows users, respectively. Local installation of the FastQC, Bowtie, SAMtools, and HOMER is required for ChIP-seq. For RNA-seq Tophat and Cuffdiff is also required. HOMER tools are described in detail on the website <http://homer.salk.edu/homer/>.

3. In the scripts below it is assumed that the software/packages are available in the path. If need be modify script to specify the path. Also, replace home in the scripts below with the home directory in use for running the pipeline. The script command lines are highlighted in gray.
4. *Quality check and mapping on genome.* The ChIP-seq fastq data after fastq quality check and *FastqGroomer* (tool to convert fastq file to fastqsanger) is mapped to the genome of the organism using Bowtie for ChIP-seq (or Tophat for RNA-seq) [14, 15]. The fastq dataset obtained from ENCODE or NCBI GEO can also be similarly run through fastq quality check and *FastqGroomer* [16–18]. ENCODE and NCBI GEO files are downloadable by ftp transfer in bam and fastq formats. The NCBI GEO fastq file is often available in the sra format, which can be converted to fastq with the command. Example:

```
#!/bin/sh

# request Bourne shell as shell for job

#$ -S /bin/sh

# run the fastq-dump on the specified sra file

/usr/local/bin/sratoolkit/bin/fastq-dump/home/example.sra
```

5. Bam files and sam files of mapped reads can be sorted and interconverted using samtools [19]. Example:

```
#!/bin/sh

# request Bourne shell as shell for job

#$ -S /bin/sh

# run the samtools on the specified bam file to sort bam file by name

/usr/local/bin/samtools sort-n/home/example.bam/home/example.name.sorted
```

Example:

```
#!/bin/sh

# request Bourne shell as shell for job
```

```
# $ -S /bin/sh

# run the samtools on the specified bam file to convert bam to sam /usr/
local/bin/samtoolsview-h-o/home/example.name.sorted.sam/home/
example.name.sorted.bam
```

6. *Visualization of mapped ChIP-seq and RNA-seq data on UCSC genome browser.* To visualize the mapped reads on the UCSC genome browser, run HOMER tools makeTagDirectory and makeUCSCfile on bam files [20]. The resultant bedGraph file can then be uploaded and visualized on the UCSC genome browser as a custom track (create free UCSC genome browser login to save these tracks). Once the UCSC custom track is created it can be used to visualize protein binding peaks and can be used for primer design (Fig. 6). This method can also be used on RNA-seq bam files to upload them on the UCSC genome browser.

Example:

```
#!/bin/sh

# request Bourne shell as shell for job

# $ -S /bin/sh

# run the makeTagDirectory on the specified bam file have samtools in path
if needed or change to sam or do interactively with bam

/usr/local/bin/homer/bin/makeTagDirectory/home/example-TagDirectory/home/
example.bam
```

Example:

```
#!/bin/sh

# request Bourne shell as shell for job

# $ -S /bin/sh

# run the makeUCSCfile on the specified tag directory

/usr/local/bin/homer/bin/makeUCSCfile/home/exampleTag-Directory-o/home/
exampleTagDirectory/exampleTagDirec-tory.ucsc.bedGraph
```

7. *ChIP-seq peak calling and motif analysis using HOMER.* There are several methods for ChIP-seq peak calling, such as MACS peak calling, Peak ranger and HOMER. Presently, described is the HOMER-based peak calling and HOMER motif analysis of peaks [20]. In the example below, for genome version input the

same genome version that was used in mapping with Bowtie or Tophat, in this example it is mouse mm9 genome.

Example:

```
#!/bin/sh

# request Bourne shell as shell for job

#$ -S /bin/sh

# run the findPeaks on the specified TagDirectory file both ChIP antibody
and Input

# The output will show up in the home directory as did not specify it for me
its [smukherjee2@mb-galaxy ~]$

/usr/local/bin/homer/bin/findPeaks/home/exampleTagDirectory-stylefactor-
oexample-i/home/exampleTagDirectory
```

Example:

```
#!/bin/sh

# request Bourne shell as shell for job

#$ -S /bin/sh

# run the findmotifgenome on the above output homer peak file

/usr/local/bin/homer/bin/findMotifsGenome.pl/home/exam-
plemotif.txtgenomeversion/home/example/results-preparedDir/home/example/
prepared/
```

8. To obtain a global average view of ChIP peak enrichment over input ngs.plot can be useful [21]. This is however not an essential step for detailed annotation of peaks and assignment to genes.
9. *Annotation of ChIP-seq peaks and intersection with RNA-seq.* To annotate ChIP peaks bed file use ChIP-seek, <http://chipseek.cgu.edu.tw/>. The input bed file should contain the peak genomic coordinates in the format chrN:start-end [22]. From the result summary, download peak location pie chart (global distribution of peaks on different regions of the genome) and annotation table file (each peak assigned to nearest gene).
10. Other useful resources for ChIP-seq peak annotation are <http://sartorlab.ccmb.med.umich.edu/software>

<http://broad-enrich.med.umich.edu/>

<http://manticore.niehs.nih.gov/pavis2/>

11. To determine the gene expression changes associated with ChIP-seq protein-DNA occupancy. First, *Cuffdiff* analysis is performed on RNA-seq bam files generated by using Tophat (**step 4**) [23, 24]. Second, the output Cuffdiff file is intersected or joined with the ChIP-seq gene annotated file from ChIP-seek above (**step 9**) by gene name. This join function can be performed on excel or on Galaxy using the *Join two dataset function*.
12. The resultant file contains protein-DNA interactions and associated gene expression changes. This dataset of upregulated and downregulated genes associated with transcription factor occupancy is very useful for biological interpretation of the data and hypothesis generation. These “target gene” lists can now be used to perform gene ontology using NIH DAVID gene ontology or panther gene ontology or gene ontology analysis tools [25–28].
13. Additional tool: To convert between genomes of species or within species, use UCSC LiftOver, <https://genome.ucsc.edu/cgi-bin/hgLiftOver>. The input file to be converted should be a bed format file of peak genomic coordinates.

3.8 ChIP-qPCR Primer Design from ChIP-seq (Fig. 5)

1. The ChIP-ed DNA from Subheading 3.4—reverse crosslinked, RNase and Proteinase K treated, isolated and purified with Qiaamp PCR purification kit, concentration from Qubit—can be directly used for ChIP-qPCR. Use about 250–500 pg of chromatin-immunoprecipitated DNA per qPCR reaction. For input normalization use same amount per qPCR reaction as chromatin-immunoprecipitated DNA (i.e., about 250–500 pg of 5% input or 1% input).
2. Binding site primer: Design qPCR primers around the center of the known binding site or if validating ChIP-seq at highest point, usually at the center of ChIP-seq-binding peak. For internal control and internal normalization, design qPCR primers around sites 3 kb or further away from the binding site or ChIP-seq peak at a region of nonbinding or non-ChIP-seq peak. Additional controls that can be included are IgG and specific antibody chromatin-immunoprecipitated DNA from cells where the antigen being used to pull down has been knockdown or knockout.
3. The qPCR primers should cover 120–200 bp amplicon size on genomic DNA sequence. Primers can be designed using default parameters a NCBI Primer blast tool, <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>, IDT Primer quest tool <https://www.idtdna.com/Primerquest/Home/Index>, <http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm>, <http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi> or any other software.
4. To validate primers, run a dilution series with 5% or 1% input sample, 1:2, 1:5, 1:10, 1:100. An efficient primer should give a straight line for Ct vs

concentration plot and R2 value >90%, at least within 1:2, 1:5, 1:10 of 5% or 1% input dilution series.

5. With validated primers run the qPCR reaction (ChIP-qPCR) with SYBR green using standard protocol or manufacturer's instruction on ABI or Biorad qPCR instruments. Applied Bio-systems 7000 detection system using Biorad iTaq Universal SYBR green supermix (172–5124). Example of a typical reaction mixture, 1 µl of forward primer (10 µM stock), 1 µl of reverse primer (10 µM stock), 12.5 µl of SYBR green (+ROX or other quenchers), 0.5 ng DNA and nano-pure water up to 25 µl.
6. Normalize ChIP-qPCR signal on binding site primer with 5% or 1% input or IgG ChIP-qPCR using Delta Ct (δCt) method. Similarly, normalize to nonbinding site primer signals using the δCt method. Enrichment of at least twofold of antibody ChIP-ed signal over input or IgG or nonbinding site indicates real binding (*see* Note 9). Calculate the δCt value using formula,

$$\delta Ct = (Ct(5\% \text{ Input}) - Ct(\text{ChIP})) \text{ or } \delta Ct = (Ct(\text{IgG}) - Ct(\text{ChIP}))$$

Next, calculate the fold enrichment from the δCt values using the formula, fold enrichment = 2^(-δCt).

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⁹Decreased enrichment of chromatin-immunoprecipitates signal over input or IgG by at least twofold should occur in knockout or knockdown samples of protein being used for ChIP. This validates the specificity of the antibody being used for ChIP.

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Sonication Covaris Flow Chart

Fig. 1. Flowchart of cell to sonication. Related to Subheading 3.1 preparation of nuclei and DNA-protein crosslinking and Subheading 3.2 chromatin shearing or sonication: Covaris sonication

Chromatin-immunoprecipitation Flow Chart

Fig. 2.
Flowchart of ChIP. Related to Subheading 3.3 Chromatin Immunoprecipitation (ChIP)

DNA extraction Flow Chart

Fig. 3.
Flowchart of DNA extraction and purification. Related to Subheading 3.4 DNA-protein reverse cross-linking and isolation of chromatin-immunoprecipitated DNA

Fig. 4. Sonication optimization, antigen retention, and bioanalyzer. **(a)** 1% Agarose gel electrophoresis shows sonication efficiency with varying time and cell number using Covaris sonication system and TAP (proliferating neural stem cells) from rats. Asterix indicates best sonication condition at 6.5 min and preparation from 10–20 million cells. **(b)** Bioanalyzer graph showing peak of ChIP-ed DNA at >300 bp after library preparation. **(c)** Western blots from sonication of 15 million cells for 6.5 min show efficient antigen retention for REST, RNA Pol 2, H3K27Me3, and CREB in QNPs (quiescent hippocampal neural stem cells) and TAPs from rat. Related to Subheading 3.5 determination of sonicated DNA size, Bioanalyzer quality check and ChIP-seq and Subheading 3.6 Sonication optimization and antigen retention

Fig. 5.

Gria2 UCSC view (with primer) and REST ChIP-qPCR. (a) UCSC genome browser view of REST ChIP-seq bedGraph file showing REST occupancy at Gria2 gene promoter.

Doublehead arrows indicate region of genomic DNA suitable for primer design for positive binding and negative no binding sites. (b) ChIP-qPCR validation of REST binding peak at Gria2 promoter in TAPs. Related to Subheading 3.8 ChIP-qPCR primer design from ChIP-seq and Subheading 3.7 ChIP-seq bioinformatics data analysis, a brief overview

Bioinformatics Flow Chart

Fig. 6.

Flowchart of bioinformatics for ChIP-seq and RNA-seq intersection. Related to Subheading 3.7 ChIP-seq bioinformatics data analysis, a brief overview