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Epigenome analysis of pluripotent stem cells

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Summary

Mis-regulation of gene expression due to epigenetic abnormalities has been linked with complex genetic disorders, psychiatric illness and cancer. In addition, the dynamic epigenetic changes that occur in pluripotent stem cells are believed to impact regulatory networks essential for proper lineage development. Chromatin immunoprecipitation (ChIP) is a technique used to isolate and enrich chromatin fragments using antibodies against specific chromatin modifications, such as DNA binding proteins or covalent histone modifications. Until recently, many ChIP protocols required millions of cells for each immunoprecipitation. This severely limited analysis of rare cell populations or post-mitotic, differentiated cell lines. Here, we describe a low cell number ChIP protocol with next generation sequencing and analysis, that has the potential to uncover novel epigenetic regulatory pathways that were previously difficult or impossible to obtain.

Keywords

Chromatin immunoprecipitation; ChIP-sequencing; Histone Modifications; Epigenetics; Pluripotent Stem Cells

Introduction

Epigenetics is the study of non DNA sequence-related heredity (1). The strictest definition labels epigenetics as any meiotically or mitotically heritable change in gene function that is not explained or results from changes in the underlying primary DNA sequence (2,3,4). Recently, this definition has been expanded to include epigenetic modifications that may not be heritable, but still result in gene expression changes (2,3). The modifications can be broadly classified into DNA methylation, covalent modifications of histone tails, and mechanisms involving non-coding RNAs (3,4,5,6). The presence of these modifications is of great interest because of the wide range of biological and clinical implications resulting from epigenetic mechanisms. Mis-regulation of gene expression due to epigenetic abnormalities has been linked with complex genetic disorders, psychiatric illness and cancer (1,7). In addition, the dynamic epigenetic changes that occur in pluripotent stem cells are believed to impact regulatory networks essential for proper lineage development (8).

Methods to observe the chromatin state in pluripotent stem cells has provided an unparalleled understanding of chromatin status before most lineage restriction pathways are

induced. Tracking these marks over time during differentiation into multiple lineages may assist in predicting the phenotypic potential of embryonic stem (ES) or induced pluripotent stem (iPS) cell lines. With the recent revolution of iPS technology where somatic cells are reprogrammed to an embryonic cell like state, epigenetic analysis is useful to assess reprogramming status. There have been reports of partial reprogramming where the epigenetic state of both DNA methylation and/or histone modifications were quite different than ES cells. These can have devastating effects from tumor formation to inappropriate tissue differentiation (9,10,11). Therefore, effective epigenetic diagnostics can highlight inconsistencies from improper reprogramming that may have been overlooked using morphology and gene expression assays.

Researchers first interrogated isolated gene sequences for various histone modifications using chromatin immunoprecipitation (ChIP) (12,13). Chromatin consists of DNA bound with histones and non-histone proteins that promote the proper packaging of DNA by altering its underlying structure and thus influencing gene regulation (7,14). ChIP is a technique used to isolate and enrich chromatin fragments using antibodies against specific chromatin modifications. These modifications can be DNA binding proteins, such as transcription factors, or a particular covalent histone modification. Chromatin is sheared into small fragments (200–500 bp) to allow for proper genomic resolution. Enriched regions can be identified using PCR or quantitative PCR (qPCR) with specific primers designed for a selected genomic region. The enrichment level of various modifications is then used to describe and correlate with the transcriptional activity of the interrogated gene. Although this method can be precise, it is limiting because of the specificity needed to probe each genomic region.

To overcome these limitations, this technique has been scaled to produce genome wide maps of chromatin state. Combining ChIP with microarrays (ChIP-Chip) or next generation deep sequencing methods (ChIP-Seq) has enabled the global analysis of chromatin state for the entire genome, defined as the epigenome (15,16,17,18,19). The epigenome is the combination of all epigenetic modifications in a particular cell. In contrast to the genome, each organism contains multiple epigenomes that display extensive differences per tissue type, developmental stage, and environmental influences (20). As opposed to ChIP, genome-wide epigenetic analysis is independent of hypotheses, allowing the detection of previously unknown patterns in cancer, development, and disease.

Until recently, many ChIP protocols required millions of cells for each immunoprecipitation. This severely limited analysis of rare cell populations or post-mitotic, differentiated cell lines. This barrier can be overcome by using Invitrogen's MAGnify™ kit. This method utilizes magnetic beads to recover antibody-bound chromatin fragments, requiring far fewer cells per immunoprecipitation (IP) than previously reported protocols. There are many benefits to using a low cell number ChIP kit such as the ability to IP multiple histone marks or transcription factors using the same biological sample. In addition, it also provides the ability to investigate chromatin profiles from differentiated cultures containing limited quantities. By combining the benefits of low cell number ChIP with next generation sequencing, this protocol can be used to uncover novel epigenetic regulatory pathways that were previously difficult or impossible to obtain.

Materials

MAGnify™ ChIP Kit Module #1. Store at 4°C

MAGnify™ ChIP Kit Module #2. Store at 4°C

MAGnify™ ChIP Kit Module #3. Store at –20°C

MAGnify™ ChIP Kit Module #4. Store at –20°C

2.1 Cell Culture

1. Dissociation media: 0.25% Trypsin/EDTA (see Note 1) - Store at –20°C
2. Trypsin Inhibitor - 0.25 mg/ml - GibcoBRL #17075-011 - Store at –20°C
3. Formaldehyde - 37% Molecular Biology Grade
4. Glycine –1.25M – MAGnify™ ChIP Kit Module #1
5. Phosphate Buffered Saline (PBS) (1×)

2.2 Cell Lysis

1. Ice
2. Lysis Buffer - MAGnify™ ChIP Kit Module #4
3. Protease Inhibitors - MAGnify™ ChIP Kit Module #3
4. Vortex mixer

2.3 Chromatin Shearing

1. Sonicator - Bioruptor® - Diagenode
2. 1.5–2% Agarose Gel
3. 1kilobase pair DNA Ladder - Promega #G571A - Store at –20°C
4. 250 base pair DNA Ladder –Lonza FlashGel DNA Marker #57033 - Store at –20°C
5. DNA Dye as Loading Buffer - EZ-Vision™ Three – AMRESCO #N313-Kit
6. Ice
7. 4°C Microcentrifuge
8. Proteinase K MAGnify™ ChIP Kit Module #1
9. Heating Block

2.4 Dynabeads Conjugation

1. Primary Antibody(s) of interest

¹Depending on the sensitivity of your cells, a gentler dissociative enzyme may be used such as TrypLE™. We have also observed that if you are planning to harvest cells for separate RNA analysis, using TrypLE™ yielded higher quality RNA. If using TrypLE™, there is no need for trypsin inhibitor, just dilute with culture media. Crosslinking can also be performed on adherent cells directly in a dish as an alternative to crosslinking the cells in suspension.

2. Isotype Control Antibody
3. Protein A/G Dynabeads MAGnify™ ChIP Kit Module #1
4. 200 µl PCR Tubes/Strips wells
5. DynaMag™-PCR Magnet
6. Rotating mixer
7. Dilution Buffer - MAGnify™ ChIP Kit Module #4

2.5 Chromatin Dilution/Binding/Washing

1. Dilution Buffer- MAGnify™ ChIP Kit Module #1
2. Protease Inhibitors - MAGnify™ ChIP Kit Module #3
3. IP Buffer 1 - MAGnify™ ChIP Kit Module #2
4. IP Buffer 2 - MAGnify™ ChIP Kit Module #2

2.6 Reverse Crosslinking

1. Reverse Crosslinking Buffer - MAGnify™ ChIP Kit Module #1
2. Proteinase K - MAGnify™ ChIP Kit Module #1
3. Thermal mixer or thermal cycler

2.7 DNA purification and Elution

1. DNA Purification Magnetic Beads - MAGnify™ ChIP Kit Module #1
2. DNA Purification Buffer - MAGnify™ ChIP Kit Module #1
3. DNA Wash Buffer - MAGnify™ ChIP Kit Module #2
4. DNA Elution Buffer - MAGnify™ ChIP Kit Module #2
5. Thermal mixer or thermal cycler

Methods

Cell Preparation

1. When culturing cells for ChIP, choose an appropriate tissue culture plate or flask to ensure 1×10^6 - 3×10^6 cells will be harvested per sample.
2. Aspirate the medium and wash cells briefly with $1 \times$ PBS.
3. Aspirate the PBS and then add enough trypsinizing reagent (0.25% Trypsin EDTA) to cover the cells. Immediately rock the plate gently back and forth to mix the trypsinizing reagent throughout the plate (*see* Note 1).
4. Incubate at 37°C for 2 to 5 minutes or until cells detach from their substrate. The timing may depend on the cell type's attachment characteristics and confluency. Once cells have dislodged, add an equal amount of trypsin inhibitor. Transfer the

cell suspension into a 15 ml centrifuge tube. Add additional media or $1\times$ PBS and rinse the plate for additional cells in suspension. Transfer the remaining suspension into the centrifuge tube, up to 15 ml and then gently pipette the cell mixture up and down 5 to 10 times.

5. Take a small aliquot and count the cells using a hemocytometer or an automated cell counter. Aliquot a minimum of 1×10^6 - 3×10^6 cells per sample and spin each cell suspension at $200\times g$ for 5 minutes at room temperature (*see* Note 2).
6. Aspirate the supernatant and lightly flick the tube to gently dislodge the cell pellet before resuspending in 500 μ l of room temperature $1\times$ PBS. Transfer each sample into 1.5 ml Eppendorf tubes (*see* Note 3). To crosslink in suspension, add 13.5 μ l of 37% Formaldehyde to each 500 μ l sample, making the final working concentration 1%. Immediately invert each tube approximately 5 times to mix and incubate for 10 minutes at room temperature. The time course for optimal crosslinking should be discovered empirically (*see* Note 4).
7. Add 57 μ l of room temperature 1.25 M Glycine to quench the crosslinking reaction. Immediately invert each tube approximately 5 times to mix and incubate for 5 minutes at room temperature.
8. From this point forward, keep all samples on ice and spin at 4°C . Spin each crosslinked sample at $200\times g$ for 10 minutes. Aspirate the supernatant leaving approximately 20 to 30 μ l behind as to not disturb the cell pellet.
9. Lightly flick the cell pellet to gently dislodge it before resuspending in 500 μ l cold $1\times$ PBS. Spin at $200\times g$ for 10 minutes. Carefully discard the supernatant. Repeat this one more time.
10. Aspirate the supernatant leaving 10 to 20 μ l behind and either flash freeze the cell pellet in liquid nitrogen and store at -80°C or proceed directly to cell lysis (*see* Note 5).

Cell Lysis and Shearing

11. Thaw the Lysis Buffer and Protease Inhibitors ($200\times$). Mix the Lysis Buffer by vortexing or pipetting up and down. Prepare a fresh master mix by adding 199 μ l Lysis Buffer to 1 μ l Protease Inhibitor. For each experiment, scale the master mix accordingly and use 50 μ l of master mix for every 1×10^6 cells. Resuspend by light vortexing or pipetting, ensuring little to no bubbles. Incubate on ice for at least 5 minutes. Proceed to chromatin shearing or snap freeze with liquid nitrogen and store at -80°C .

²The number of cells per immunoprecipitation should be equal; therefore it is important to count the cells as accurately as possible.

³This tube transfer step will make it easier for cross-linking and for spinning multiple samples in a 4°C centrifuge in later steps.

⁴Too much crosslinking can result in the masking of epitopes, resulting in less than optimal immunoprecipitation, while not enough crosslinking can inefficiently bind the protein of interest to the chromatin.

⁵Sometimes it is necessary to store crosslinked cell pellets before cell lysis and sonication. (For example, when multiple time points are necessary). However, we do not recommend the long term storage of frozen crosslinked cell pellets that have not been lysed as this may negatively affect the yield of chromatin immunoprecipitation.

12. Fragmenting the chromatin can be achieved through sonication or enzymatic digestion using Micrococcal nuclease. Although both can result in preferential shearing, sonication is the recommended method when crosslinking with formaldehyde.
13. Shear the lysed chromatin into random fragments using a sonicator with 30 second on/off pulses using the high power setting on the Bioruptor®. Optimal sonication times should be empirically tested with a sample time course ranging from 10 to 30 pulses, separated in 5 pulse increments. After sonication is complete, confirm that there is minimal foaming and keep the samples on ice (*see* Note 6). If there is a substantial amount of foaming, discard the sample.
14. Spin the sonicated samples at 20,000×g at 4°C for 5 minutes to pellet the cell debris. Transfer the supernatant containing the sonicated chromatin to a new 1.5 ml ependorf tube.
15. The sonicated fragment sizes should be verified by running a small aliquot on a 2% agarose gel. First, take an aliquot of approximately 10 µl of sonicated chromatin and a non-sonicated control. Next, add 1 µl of Proteinase K and incubate at 55°C for 20 minutes. Run both samples on a 2% agarose gel with the appropriate DNA ladders to verify sizes. The sonicated chromatin should appear as a smear (Figure 1). To achieve proper resolution for ChIP-sequencing, the fragmented chromatin sizes should be in the range of 150 to 300 base pair (bp) due to the need for additional adaptor sequences later in the ChIP sequencing protocol.

Chromatin Immunoprecipitation

16. Primary antibodies need to be coupled to the protein A/G Dynabeads before chromatin immunoprecipitation. Thaw the Dilution Buffer and place on ice along with the Dynabeads, ChIP magnet, sample tubes, and all primary antibodies.
17. Resuspend the protein A/G Dynabeads by lightly pipetting up and down. Do not vortex as this can damage the beads.
18. Wash the Dynabeads. Add 100 µl of cold Dilution Buffer to each individual or 8-strip 0.2 PCR tubes. Next add 10 µl of the protein A/G Dynabeads and gently pipette 5 to 10 times (*see* Note 7).
19. Insert each tube or strip into the DynaMag™-PCR Magnet and wait 30 seconds to 1 minute for the beads to pellet against the side of the tube. While the tubes are still in the magnet, carefully remove the liquid and discard without disturbing the pellet. Remove the tubes or strip from the magnet and once again add 100 µl of cold Dilution Buffer to each tube and gently pipette 5 to 10 times.

⁶It is important to always keep the samples cold during the sonication process because excess heat can reverse the crosslinking. Add ice cold water to the sonicator water bath every 5 minutes to ensure the samples remain cool. In addition, ensure that there is no foaming during sonication because this could also disrupt the crosslinking. An additional option is to also save 10 µl of non-sonicated chromatin. This can be run on a 2% agarose gel to verify the size by running alongside the sonicated chromatin samples.

⁷The use of a multichannel pipette is highly recommended for the duration of this protocol for both consistency and efficiency.

20. Add the primary antibody of interest to each sample tube. The amount of antibody used should be determined empirically and based on the manufacturer's recommendations. Generally, 1–10 µg is a standard starting range. It is also important to include a negative control antibody, (i.e. - a mouse or rabbit IgG) based on the isotope of the primary antibody of interest. Do not pipette up and down, instead gently flick and invert the tubes 5 to 10 times to fully resuspend the bead/antibody mix. Place the tubes in a rotator and mix end-over-end at 4°C for at least 1 hour.
21. Dilute the chromatin during the antibody/bead conjugation. Thaw the sheared chromatin and set on ice. Prepare the Dilution Buffer/Protease Inhibitor (200×) master mix similar to step #11 and keep on ice. The final dilution volume for each sample is 100 µl and the ratio of sheared chromatin to Dilution Buffer depends on the number of cells user per immunoprecipitation (IP). For example, the concentration of sheared chromatin was 1×10^6 cells per 50 µl of Lysis Buffer. Therefore, to immunoprecipitate 0.2×10^6 cells, add 10 µl of sheared chromatin to 90 µl of the Dilution Buffer/Protease Inhibitor mix. Make a sample master mix based on the number of IPs per sample plus one extra 100 µl dilution per sample to use as an input control. Mix the sample thoroughly and aliquot 10 µl into a new 0.2 ml PCR tube to use as the input control for each sample (*see* Note 8).
22. Once the primary antibody/bead coupling is complete, remove any liquid in the caps by gently and briefly spinning the sample tubes from step #20 on a tabletop centrifuge capable of holding 0.2 ml PCR tubes. Place the tubes into the DynaMag™-PCR Magnet and wait 30 seconds to 1 minute for the bead pellet to form. Carefully discard the liquid. The beads now contain the conjugated antibodies needed for immunoprecipitation. Add 100 µl of the diluted chromatin master mix, from step #21, to their respective antibody/bead PCR tube. Do not pipette, instead cap the tubes, gently flick and invert 5 to 10 times to fully resuspend the bead complexes. Rotate the tubes as previously described at 4°C for at least 2 hours to overnight.
23. Before proceeding with the wash steps after chromatin binding, the Proteinase K, Reverse Crosslinking Buffer, DNA Purification Beads, and DNA Purification Buffers should all be at room temperature.
24. After the chromatin has been bound to the beads, gently spin all sample tubes briefly and place the tubes into the DynaMag™-PCR Magnet and wait 30 seconds to 1 minute for the bead pellet to form. Remove the liquid carefully without disturbing the pellet. Add 100 µl of cold IP Buffer 1 to each sample tube and gently flick and invert briefly to resuspend the beads. Rotate the tubes at 4°C for approximately 5 minutes. Repeat these steps two more times.

⁸Non immunoprecipitated input controls are critical for evaluating enrichment. It is not always easy to accurately count cells when harvesting for ChIP. Therefore, obtaining an input control for each sample can be used as a normalizing factor when analyzing enrichment.

25. Continue the washes by repeating step #24 with IP Buffer 2 in place of IP Buffer 1, for a total of 2 washes.
26. Prepare a reverse crosslinking master mix by adding 1 μ l of Proteinase K and 53 μ l of Reverse Crosslinking Buffer for each sample. For example, 8 samples would require 8 μ l of Proteinase K plus 424 μ l of Reverse Crosslinking Buffer. When making the reverse crosslinking master mix, prepare a separate master mix for the input samples that were set aside in step #21. For these samples, add 1 μ l of Proteinase K and 43 μ l of the Reverse Crosslinking Buffer.
27. Following the last wash with IP Buffer 2, put the tubes into the DynaMag - PCR Magnet, wait for the bead pellet and carefully discard the liquid without disturbing the pellet. Remove the tubes from the magnet and add 54 μ l of the reverse crosslinking master mix to each sample tube containing beads. Separately, for each of the 10 μ l diluted input samples, add 1 μ l of Proteinase K to 43 μ l of the Reverse Crosslinking Buffer and scale accordingly. Incubate all sample tubes at 55°C for 15 minutes in a thermal shaker or other heat source available.
28. Following the reverse crosslinking steps, the immunoprecipitated DNA has now been released from the bead complexes and the liquid now contains the sample DNA, and therefore should not be discarded. Place each tube into the DynaMagTM-PCR Magnet and wait for the bead pellet to form. Carefully transfer the liquid without disturbing the pellet to a new 0.2 ml PCR tube. The tubes containing the used magnetic beads can be discarded. Incubate all of the newly transferred sample tubes along with the input samples at 65°C for another 15 minutes.
29. Briefly resuspend the DNA Purification Magnetic beads by light vortexing or pipetting up and down 5 to 10 times. Prepare a DNA purification master mix by adding 50 μ l of DNA purification Buffer to 20 μ l of DNA Purification Magnetic beads for all samples, including input controls. Pipette up and down to mix thoroughly. The DNA Purification Magnetic beads and buffer should be at room temperature before use.
30. After the reverse crosslinking incubation, briefly spin to collect any liquid. Next, set the tubes on ice or let cool to room temperature for 5 minutes.
31. Add 70 μ l of the DNA purification master mix to each sample tube and input controls. Gently mix by pipetting up and down 5 to 10 times. Let stand for at least 5 minutes at room temperature. Place each tube into the DynaMagTM-PCR Magnet and wait for the pellet to form. Remove the liquid carefully without disturbing the pellet (*see* Note 9).

⁹This pellet may be larger than previous pellets with the protein A/G beads so it is acceptable to leave a few microliters of liquid behind in order to not disturb the cell pellets as these will now contain the bound DNA fragments.

32. Remove the tubes from the magnet and add 150 µl of DNA Wash Buffer and mix 5 to 10 times. Place each tube into the magnet and repeat the wash step one more time by removing the liquid and adding 150 µl of DNA Wash Buffer.
33. Add the tubes back into the magnet and wait for the bead pellet to form. Discard the liquid, leaving a few µl behind making sure not to disturb the bead pellet. Remove the tubes from the magnet and add 60 µl of DNA Elution Buffer to all tubes. Pipette gently 5 to 10 times to mix thoroughly. Add all tubes to a thermal shaker or other heat source and incubate for 20 minutes at 55°C.
34. Following the incubation, briefly spin to collect any liquid, put the tubes into the DynaMag™-PCR Magnet and wait for the bead pellet to form. The liquid now contains the purified sample. Gently transfer the liquid to a new 0.2 ml PCR tube without disrupting the bead pellet. Once transferred, the tubes containing the DNA purification beads can then be discarded. (*see* Note 10)
35. The purified DNA can now be temporarily stored at 4°C if immediately analyzed by qPCR or can be stored at –20°C.

ChIP qPCR

36. Before proceeding towards ChIP-sequencing, it is beneficial to run a few real time quantitative PCR (qPCR) quality control assays using the optional 10 µl of each eluted purified DNA that was set aside previously (*see* Note 10), to confirm that reasonable chromatin enrichment was achieved.
37. Design positive and negative primers to regions of the genome where immunoprecipitated enrichment is both expected and not predicted. Amplicons should range from 80 to 150 base pairs (bps) since the optimal range of sheared chromatin was 150–300 base pairs for ChIP-sequencing. Primer amplification efficiency should first be verified by running a qPCR reaction with 10 fold serial dilutions using either input DNA or genomic DNA as standards. A standard curve with a slope of –3.1 to –3.6 is an acceptable efficiency range. This method will produce a relative quantity of each sample's enrichment compared to its own input.

ChIP Sequencing

38. Following the elution of the purified ChIP-enriched DNA, fragment libraries will need to be constructed. Please refer to the SOLiD™ ChIP-Seq Kit Guide for a more-detailed protocol. However, for the purpose of generating barcoded libraries for multiplexing ChIP samples, one should refer to the SOLiD™ ChIP-Seq Library Preparation with Barcodes protocol for additional details. We generally prepare 12–20 barcoded ChIP-seq libraries in parallel for each SOLiD slide to obtain the required sequencing depth ($>20 \times 10^6$ aligned reads per library).

¹⁰It is suggested that 10 µl from the eluted 60 µl samples be set aside for later ChIP-qPCR analysis before or after ChIP sequencing.

39. A starting quantity of 1–10 ng is recommended for library construction. If the enriched ChIP sample is less than 10 ng/μl, quantify the DNA using Invitrogen's Qubit® Fluorometer. The Qubit® dsDNA HS Assay is sensitive and accurate for double stranded DNA and has a linear detection range of 0.2–100 ng.
40. Proceed to library construction. Briefly, the DNA fragments are end repaired, polished, followed by ligation of adaptors to each end of DNA. If you will be multiplexing using barcodes, the P1 adaptor is replaced by the Multiplex Library P1 Adaptor and the P2 adaptor replaced by the Barcode-0XX.
41. Once the barcoded DNA is purified, each sample is nick translated and amplified. If barcoding is used, the Library PCR Primer 1 and PCR Primer 2 will be replaced by Multiplex Library PCR-1 and Multiplex Library PCR-2. During the amplification step, the 250-μL amplified sample is placed on ice after 10 cycles. To determine the optimal number of amplification cycles, remove 20 μL from the 250 μl sample and divide into 5 tubes of 4 μl each. These separate samples are further amplified at 2 cycle increments from 10–18 cycles and then run on a Lonza Gel™ to determine the minimum number needed for amplification. In some cases, where there is lower than 1 ng of enriched chromatin, it may be necessary to increase the amplification cycle increments up to 20 cycles (Figure 2). Run the remaining 230 μl the appropriate number of cycles that was determined from the Lonza Gel™ results. The optimal number of PCR cycles produces a barely-detectable quantity of product in the desired size range.
42. The amplified DNA is then purified and quantified by qPCR. Once complete, the fragment library can be stored in Elution Buffer at –20°C or you may proceed to the next step by referring to the detailed Applied Biosystems SOLiD™ System Templated Bead Preparation Guide for emulsion PCR and sequencing.
43. After sequencing, the reads and quality strings should be aligned to the reference species genome. Bowtie (21) is one example that can be used to identify the single, best-quality match location, selecting the option to produce output in SAM format. Results should be converted to sorted and indexed BAM format using Samtools (22) (*see* Note 11).
44. Duplicate reads aligning with identical genomic positions are likely to be the product of overamplification during library construction. The Java package Picard (22) is useful for removing exact duplicate reads.
45. There are many peak calling programs using various algorithms to highlight chromatin enrichment (23). Find Peaks 4.0 (24) is one example program that compares each sequenced sample against the input control. Using the peak

¹¹Bowtie, Samtools, Picard, and Findpeaks can all be downloaded and run on Windows, Mac, or Linux computers. While a computer cluster is useful for processing many sequencing projects at once, a reasonably powerful desktop computer is adequate for running one at a time. Generally the limiting factor is storage space more than processing power. For occasional ChIP-seq projects, a RAID-capable network attached storage device is adequate. For greater usage, storage area network capacity should be used.

calling algorithm generates both bed position files and wiggle peak tracks, which can then be visualized using a genome browser. For example, using the UCSC Genome Browser (25,26), each peak file can be designated its own track within a user defined browser session and then be simultaneously viewed by stacking each sample track in a defined order.

46. ChIP sequencing has the potential to generate large numbers of peaks throughout the genome. A first step in managing this wealth of data is to annotate each peak based on a set of criteria. We recommend using the ChIPpeakAnno algorithm (27), a plugin from the Bioconductor package within the R Statistical programming environment. The algorithm is designed to annotate enriched peaks based on the nearest gene. Various filtering options are available based on peak distance and overlapping between samples to generate meaningful data. In addition, Gene Ontology (GO) terms that are associated with the nearest genes can be generated.

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References

1. Feinberg AP. Epigenetics at the epicenter of modern medicine. JAMA : the journal of the American Medical Association. 2008; 299:1345–1350.
2. Bird A. Perceptions of epigenetics. Nature. 2007; 447:396–398. [PubMed: 17522671]
3. Hamby ME, Coskun V, Sun YE. Transcriptional regulation of neuronal differentiation: the epigenetic layer of complexity. Biochimica et biophysica acta. 2008; 1779:432–437. [PubMed: 18674649]
4. Wu H, Sun YE. Epigenetic regulation of stem cell differentiation. Pediatric research. 2006; 59:21R–25R. [PubMed: 16326995]
5. Bird A. DNA methylation patterns and epigenetic memory. Genes & development. 2002; 16:6–21. [PubMed: 11782440]
6. Goldberg AD, Allis CD, Bernstein E. Epigenetics: a landscape takes shape. Cell. 2007; 128:635–638. [PubMed: 17320500]
7. Mehler MF. Epigenetics and the nervous system. Annals of neurology. 2008; 64:602–617. [PubMed: 19107999]
8. Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell. 2006; 125:315–326. [PubMed: 16630819]
9. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006; 126:663–676. [PubMed: 16904174]
10. Stadtfeld M, Maherali N, Breault DT, Hochedlinger K. Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse. Cell stem cell. 2008; 2:230–240. [PubMed: 18371448]
11. Yamanaka S. A fresh look at iPS cells. Cell. 2009; 137:13–17. [PubMed: 19345179]
12. Solomon MJ, Larsen PL, Varshavsky A. Mapping protein-DNA interactions in vivo with formaldehyde: evidence that histone H4 is retained on a highly transcribed gene. Cell. 1988; 53:937–947. [PubMed: 2454748]

13. Hebbes TR, Thorne AW, Crane-Robinson C. A direct link between core histone acetylation and transcriptionally active chromatin. *The EMBO journal*. 1988; 7:1395–1402. [PubMed: 3409869]
14. Kouzarides T. Chromatin modifications and their function. *Cell*. 2007; 128:693–705. [PubMed: 17320507]
15. Ren B, Robert F, Wyrick JJ, Aparicio O, Jennings EG, Simon I, Zeitlinger J, Schreiber J, Hannett N, Kanin E, Volkert TL, Wilson CJ, Bell SP, Young RA. Genome-wide location and function of DNA binding proteins. *Science*. 2000; 290:2306–2309. [PubMed: 11125145]
16. Lieb JD, Liu X, Botstein D, Brown PO. Promoter-specific binding of Rap1 revealed by genome-wide maps of protein-DNA association. *Nature genetics*. 2001; 28:327–334. [PubMed: 11455386]
17. Johnson DS, Mortazavi A, Myers RM, Wold B. Genome-wide mapping of in vivo protein-DNA interactions. *Science*. 2007; 316:1497–1502. [PubMed: 17540862]
18. Robertson G, Hirst M, Bainbridge M, Bilenky M, Zhao Y, Zeng T, Euskirchen G, Bernier B, Varhol R, Delaney A, Thiessen N, Griffith OL, He A, Marra M, Snyder M, Jones S. Genome-wide profiles of STAT1 DNA association using chromatin immunoprecipitation and massively parallel sequencing. *Nature methods*. 2007; 4:651–657. [PubMed: 17558387]
19. Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K. High-resolution profiling of histone methylations in the human genome. *Cell*. 2007; 129:823–837. [PubMed: 17512414]
20. Schones DE, Zhao K. Genome-wide approaches to studying chromatin modifications. *Nature reviews. Genetics*. 2008; 9:179–191.
21. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome biology*. 2009; 10:R25. [PubMed: 19261174]
22. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 2009; 25:2078–2079. [PubMed: 19505943]
23. Park PJ. ChIP-seq: advantages and challenges of a maturing technology. *Nature reviews. Genetics*. 2009; 10:669–680.
24. Fejes AP, Robertson G, Bilenky M, Varhol R, Bainbridge M, Jones SJ. FindPeaks 3.1: a tool for identifying areas of enrichment from massively parallel short-read sequencing technology. *Bioinformatics*. 2008; 24:1729–1730. [PubMed: 18599518]
25. Mangan ME, Williams JM, Kuhn RM, Lathe WC 3rd. The UCSC genome browser: what every molecular biologist should know. *Current protocols in molecular biology* / edited by Frederick M. Ausubel ... [et al.] Chapter 19. 2009; (Unit19):19.
26. Karolchik D, Hinrichs AS, Kent WJ. The UCSC Genome Browser. *Current protocols in human genetics* / editorial board, Jonathan L. Haines ... [et al.] Chapter 18. 2011; (Unit18):16.
27. Zhu LJ, Gazin C, Lawson ND, Pages H, Lin SM, Lapointe DS, Green MR. ChIPpeakAnno: a Bioconductor package to annotate ChIP-seq and ChIP-chip data. *BMC Bioinformatics*. 11:237. [PubMed: 20459804]

Figure 1. Optimizing sonicated chromatin sizes

Crosslinked neural precursor cells were lysed and fragmented by sonication using the Bioruptor® with 30 second on/off pulses. Optimal sonication times were empirically tested in duplicate, with a time course ranging from 5 to 15 pulses. Each sample was then treated with Proteinase K for 20 minutes at 55°C. Next, 10 µl and 20 µl from each sample were loaded into a 2% agarose gel to verify the fragmented chromatin sizes. The dotted outline represents the optimal chromatin size for ChIP sequencing (~150 to 300 base pair fragments). 15 minutes of sonication was optimal as these samples contained the majority of sheared chromatin within the desired fragmented range.

Figure 2. Optimizing ChIP-sequencing sample amplification

Following DNA purification, each sample was nick translated and amplified. During the amplification step, the 250- μ L amplified sample was placed on ice after 10 cycles. To determine the optimal number of amplification cycles, 20 μ L was removed from the 250 μ L sample and divided into 5 tubes of 4 μ L each. These separate samples were further amplified at 2 cycle increments from 16–20 cycles and then run on a Lonza Gel™ to determine the minimum number needed for amplification. The dotted outline represents the fragmented ChIP sequenced chromatin after the ligation of adaptors to each end of the DNA. For this example, 17 cycles was chosen as the minimum number of cycles needed for sufficient amplification.