Video Article

Chromatin Immunoprecipitation from Human Embryonic Stem Cells

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Abstract

The functional and structural complexity of the myriad of cells in metazoan organisms arises from a small number of stem cells. Stem cells are characterized by two fundamental properties: self-renewal and multipotency that allows a stem cell to differentiate into virtually any cell type [1]. The progression stem cell to differentiated cell is characterized by loss of multipotency, structural and morphological changes and the hierarchic activity of transcription factors and signaling molecules, whose activities establish and maintain cell-type specific gene expression patterns. At the molecular level, cell differentiation involves dynamic changes of the structure and composition of chromatin and the detection of those dynamic changes can provide valuable insights into the functional features of stem cells and the cell differentiation process [2,3]. Chromatin is a highly compacted DNA-protein complex that forms when cells package chromosomal DNA with proteins, mainly histones [4]. Stemcellness and cell differentiation has been correlated with the presence of specific arrays of regulatory proteins such as epigenetic factors, histone variants, and transcription factors [2,3,5].

Chromatin immunoprecipitation (ChIP) provides a valuable method to monitor the presence of RNA, proteins, and protein modifications in chromatin [6,7]. The comparison of chromatin from different cell types can elucidate dynamic changes in protein-chromatin associations that occur during cell differentiation.

Chromatin immunoprecipitation involves the purification of *in vivo* cross-linked chromatin. The isolated chromatin is reduced to smaller fragments by enzymatic digestion or mechanical force. Chromatin fragments are precipitated using specific antibodies to target proteins or protein and DNA modifications. The precipitated DNA or RNA is purified and used as a template for PCR or DNA microarray based assays. Prerequisites for a successful ChIP are high quality antibodies to the desired antigen and the availability of chromatin from control cells that do not express the target molecule. ChIP can correlate the presence of proteins, protein and RNA modifications, and RNA with specific target DNA, and depending on the choice of outread tool, detects the association of target molecules at specific target genes or in the context of an entire genome. The comparison of the distribution of proteins in the chromatin of differentiating cells can elucidate the dynamic changes of chromatin composition that coincide with the progression of cells along a cell lineage.

Video Link

The video component of this article can be found at http://www.jove.com/video/780/

Protocol

Thawing ES cells (Not Featured in Video)

ES cells are frozen in medium containing 10% DMSO. Since DMSO can induce the differentiation of ES cells, it can be possible to thaw the cells late in the day and so to change the medium the following morning to minimize the effects of residual DMSO.

1. Coat a 6-well tissue culture plate with 0.1% gelatin for at least 15 min and aspirate off immediately before to plate cells on.
2. Thaw ES cells (approximately 5×10^6 cells, equivalent to one confluent 6-well) in a 37°C water bath and dilute into 10 ml of prewarmed ES cell medium.
3. Pellet the cells by spinning for 10 minutes at 1000 rpm in a bench-top clinical centrifuge.
4. Aspirate off medium and gently resuspend cells in 10 ml of 37°C prewarmed medium.
5. Transfer cell suspension to the 6-well plate and grow at 37°C in a humidified 5% CO_2 incubator.
6. Change medium the following day to remove dead cells and residual DMSO.

Passage and expansion of ES cell cultures

ES cells are routinely passaged every 2/3 days, and the medium is changed on alternate days. Thus, ES cells require daily attention. In our experience, feeder-independent ES cells grow rapidly and quickly acidify the medium, turning it yellow. Allowing the cells to acidify the medium (by not changing the media every day or by passaging the cells at too low a dilution) will cause the cells to undergo crisis, triggering excess differentiation and cell death, after which their totipotency cannot be guaranteed. Plating cells at too low a density, insufficient dispersion of cells during passage, or uneven plating can cause similar problems, as the cells will form large clumps before reaching confluence and the cells within

these clumps will differentiate or die. Germline transmission is a significantly reduced in cells that have been mistreated, even when they appear healthy at the time of injection.

1. For a confluent 6-well plate of cells aspirate medium off and wash with 2-3 ml of 37°C prewarmed PBS, pipetting it away from the cells.
2. Cover cells with 1 ml of 1× trypsin solution for 3-4 minutes or until cells are uniformly dispersed into small clumps.
3. Add 1 ml of medium to inactivate the trypsin.
4. Collect trypsinized cells and plate cells (usually 2/5 of well) to a freshly gelatinized 6-well plate.

Freezing ES cells

1. Trypsinize a confluent 6-well plate (approximately 1 × 10^7 cells) as described above.
2. Collect trypsinized cells in 9 ml of medium and pellet for 5 minutes at 1,000 rpm.
3. Aspirate off medium and resuspend cell pellet in 1 ml of freshly prepared freezing medium. Aliquot 0.5 ml of cells into two cryotubes.
4. Freeze the vials at -80°C overnight and transfer to liquid nitrogen for long-term storage.

ChIP-on-chip PROCEDURE

Immunoprecipitation

1. Formaldehyde crosslinking cells (for suspension cells)
    1. Use 5 x 10^7 – 1 x 10^8 cells for each immunoprecipitation.
    2. Add fresh Formaldehyde to the cell suspension at a concentration of 1%.
    3. Incubates cells with Formaldehyde Solution for 10 minutes at room temperature.
    4. Add 1/10 volume of 1.25 M glycine to quench Formaldehyde.
    5. Rinse cells twice with 10 ml of 1x PBS.
    6. Pool cells in 50 ml conical tubes and spin at 700g for 5 min at 4°C. Discard the supernatant and resuspend pellet in 1.5 ml of Lysis Buffer. Transfer cells in a 1.5 ml microfuge tube.
2. Flash-freeze three times cells in liquid nitrogen and use a tissue grinder to break cells. Once cells are crosslinked, they may be stored frozen at -80°C indefinitely.

Preparing magnetic beads (Steps are all performed at 4°C)

1. Add 50 µl of Dynal beads to 1.5 ml low retention microtube. Set up 1 tube of beads per immunoprecipitation. Add 1 ml Block Solution.
2. Collect beads using Dynal MPC. Place tubes in magnetic rack. Allow beads to collect on side of tube. This should take approximately 15 s. Invert rack twice to help to collect beads. Remove supernatant with pipettor.
3. Add 1 ml Block Solution and gently resuspend beads in removing the magnetic strip from the rack and inverting the rack, with the tubes still in place – either 10-20 times, or until the beads are evenly resuspend. Collect beads as above (Step 2). Remove supernatant with pipettor.
4. Wash beads in 1.5 ml Block Solution, as in Step 3, one more time.
5. Resuspend beads in 750 µl Block Solution and add 10 µg of antibody. Incubate at 4°C for a minimum of 6h , or overnight, on a rotator.
6. Wash beads three times in 1 ml Block Solution, as described in Step 3.

Cell sonication

1. Remove frozen cell pellets from -80°C and transfer cells to tubes for sonication. We currently prefer to use the bottom of a standard polupropylene, 15 ml conical tube for sonication. We cut the tube in two pieces at the 5 ml mark and discard the upper half. Tubes can be covered with parafilm or with the tube cap while setting up.
2. Using a microfuge tube rack, position tube so the sonicator probe sits approximately 0.5-1.0 cm above the bottom of the tube. Take care that the probe is centered and does not contact the sides of the tube. (Probe positioning can affect whether the solution foams or not during sonication. Typically, foaming indicates that the sonicated DNA will be poorly sheared.)
3. Sonicate suspension 6 times during 20 s. Samples should be kept in an ice-water bath during the sonication. To decrease foaming, initially set output power to 0 and increase manually to final power during first burst. (If there is significant foaming, all bubbles can be removed by centrifugation at 20,000g followed by gentle resuspension of all material, leaving no foam bubbles.)
4. Spin at 20,000g for 10 min at 4°C to pellet debris and harvest the supernatant in a 1.5 ml tube.
5. Save 50 µl of cell lysate from each sample as input DNA. Store at -20°C. At least one input DNA aliquot should be kept per batch of sonicate lysate. Note that the effects of the effects of the sonication and the resulting distribution of fragment sizes can only be checked after crosslink reversal and purification of DNA.

Chromatin immunoprecipitation

1. Add the chromatin-equivalent amount of 5 x 10^7 – 1 x 10^8 cells from **Cell sonication,** step 4 to the 50 µl antibody/magnetic bead mix from **Preparing magnetic beads**, Step 6 with a final volume of 1 ml (It can be possible to adjust with Lysis Buffer, if ever). Incubate overnight on rotator at 4°C.

Wash

1. Collect beads using Dynal MPC. Place tubes in rack. Allow beads to collect on side of tube. This should be take approximately 20 s. Invert rack twice to help collect all beads. Remove supernatant with pipettor, changing tips between samples.
2. Add 1 ml Lysis Buffer to each tube and gently resuspend beads. This can be done by removing the magnetic strip from the rack and inverting the rack, with tubes still in place – 10-20 times or until the beads are evenly resuspended. Collect beads. Remove supernatant by pipettor. Repeat this wash 5 more times, changing tips between washes.

3. Wash beads 6 times in 1 ml IP1 Buffer, as described in Step 2.
4. Wash beads 6 times in 1 ml IP2 Buffer, as described in Step 2.
5. Wash beads 6 times in 1 ml TE 8.0 Buffer, as described in Step 2.
6. Spin at 960g for 3 min at 4°C and remove any residual TE Buffer.

Elution

1. Add 210 µl of Elution Buffer and elute material from beads by incubating tubes in a 65°C water bath for 15 min. Vortex briefly every 2 min. This incubation can be extended as long as 30 min, which can help improve recovery of the eluate.
2. Spin down beads at 16,000g for 1 min at room temperature.
3. Remove 200 µl of supernatant and transfer to new tube. Material can be frozen at -20°C and stored overnight.

Crosslink reversal

1. Reverse crosslink the immunoprecipitation DNA from **Elution**, Step 3 by incubating at 65°C for a minimum of 6 h and a maximum of 15 h (Longer times of crosslink reversal usually result in increased noise in the microarray analysis). This incubation can be done in an oven so that the tube is heated evenly and there is less condensation formed.
2. Thaw 50 µl of input DNA reserved after sonication (Step 13), add 150 µl (3 volumes) of elution buffer and mix. Reverse crosslink this input DNA by incubating at 65°C as in **Crosslink reversal**, Step 1. From this point, every tube of immunoprecipitation or input DNA is considered to be a separate tube or sample for later processing steps.

Purification DNA

1. Add 8 µl of 10 mg ml-1 RNaseA (0.2 mg ml-1 final concentration), mix by inverting the tube several times and incubate at 37°C for 2 h.
2. Add 4 µl of 20 mg ml-1 Proteinase K (0.2 µg ml-1 final concentration) and mix by inverting the tube several times and incubate at 55°C for 2 h.
3. Add 400 µl phenol:chloroform:isoamyl alcohol (P:C:IA), vortex and separate phases with 2 ml Heavy Phaselock tube (follow instructions provided by Eppendorf).
4. If the P:C:IA solution is old or is at low pH, there will be degradation of DNA, causing noise in the microarray analysis and loss of detection of valid targets.
5. Transfer aqueous layer to new centrifuge tube containing 16 µl of 5M NaCl (200 mM) final concentration) and 1.5 µl of 20 µ µl-1 glycogen (30 µg total).
6. Add 800 µl EtOH. Incubate for overnight at -20°C or 30 min at -80°C.
7. Spin at 20,000g for 10 min at 4°C to pellet DNA. Wash pellets by adding 500 µl of 80% EtOH, vortexing to resuspend pellet and spinning again at 20,000g for 5 min at 4°C.
8. Remove any remaining 80% EtOH. Spin the tubes briefly to collect any remaining liquid and remove liquid with a pipetteman, avoiding the pellet. Let tubes air dry until pellets are just dry: pellets should still retain a moist appearance. Resuspend each pellet in 70 µl of 10 mM Tris-HCl, pH 8.0.
9. Overdrying of these pellets can make them difficult to resuspend, or liable to flake and peel away from the side of the tube.
10. Optional: Save 15 µl of immunoprecipitation sample for future use. This material can used to perform gene-specific PCR confirmation of microarray results.
11. Quantify concentration by UV absorption (260 nm).

NB: The following steps including library preparation and amplification use a modified GenomePLex WGA kit with 10X Amp Mix from Sigma without dNTPs:

Library preparation

Available in the GenomePLex WGA kit with 10X Amp Mix from Sigma

1. Add 2 µl of 1X Library Preparation Buffer to 10 µl of DNA sample from the **Purification DNA**, Step 11 in a PCR tube.
2. Add 1 µl of Library Stabilization Solution.
3. Vortex thoroughly, consolidate by centrifugation, and place in the thermal cycler at 95°C for 2 minutes.
4. Cool the sample on ice, consolidate by centrifugation by centrifugation, and then return to ice.
5. Add 1 µl of Library Preparation Enzyme, vortex thoroughly, and then centrifuge briefly.
6. Place sample in a thermal cycler and incubate as follows:
    - 16°C for 20 minutes
    - 24°C for 20 minutes
    - 37°C for 20 minutes
    - 75°C for 5 minutes
    - 4°C hold
7. Remove samples from thermal cycler and centrifuge briefly. Samples may be amplified immediately or stored at 20°C for three days.

Amplification

Available in the GenomePLex WGA kit with 10X Amp Mix from Sigma

1. A master mix is prepared by adding the following reagents to the 15 µl reaction from Step 3, below:
    - 7.5 µl of 10X Amp Mix (without dNTPs)
    - 5.0 µl of WGA DNA Polymerase
    - 3.0 µl of a 10 mM dNTP (each) stock (final concentration 0.4 mM)

- Bring final reaction volume to 75 µl with nuclease-free water

2. Vortex thoroughly. Centrifuge briefly, and begin thermocycling.
   - Initial Denaturation 95°C for 3 minutes
   - Perform 14 cycles as follows:
   - Denature 94°C for 15 seconds
   - Anneal/Extend 65°C for 5 minutes

   After cycling is complete, maintain the reactions at 4°C or store at -20°C until ready for analysis or purification.
3. Purify the DNA with PCR Purification® Kit from Quiagen according to the manufacturer's instructions and quantify concentration by UV absorption (260 nm).
4. Perform again a cycle of amplification from **Library Preparation**, Step 1 through **Amplification**, Step 2, with the following adaptations.
   • Concerning the amount of required DNA from Step 3, above, to initiate the fragmentation process: If the concentration of DNA is around 200-300 µg/ml, use 2.5 µl of sample and adjust with water for a final volume of 10 µl.
   If the concentration of DNA is around 50-60 µg/ml, use 5 µl of sample and adjust with water for a final volume of 10 µl.
   • In this second amplification process, dNTPs with dTTPs for the master mix is exchange for a mix including 10 mM dATP, 10 mM dGTP, 10 mM dCTP and 8 mM dTTP and 2 mM dUTP at the same concentration that the previous mix.
5. Measure DNA using UV-vis spectrophotometer (260 nm). Normally, greater than 9 µg of amplified DNA is obtained from each reaction.
   NB: The labelling of the DNA targets is performed with the GeneChip® WT Double-Stranded DNA Terminal Labelling Kit from Affymetrix according to the manufacturer's instructions, as described below:

Fragment amplified targets

1. Fragment the samples using the appropriate table below depending on what array type the target will be hybridized to.
   Table 1. Fragmentation Mix for single arrays

Component Volume/Amount in 1 Rxn
Double-Stranded DNA 7.5 µg
10X cDNA Fragmentation 4.8 µl
UDG, 10 U/µl 1.5 µl
APE 1, 100 U/µl 2.25 µl
Nuclease-free water up to 48 µl

   Available in the GeneChip® WT Double-Stranded DNA Terminal Labelling Kit from Affymetrix
   Table 2. Fragmentation Mix for multi-array sets

Component Volume/Amount in 1 Rxn
Double-Stranded DNA 9 µg
10X cDNA Fragmentation 4.8 µl
UDG, 10 U/µl 1.5 µl
APE 1, 100 U/µl 2.25 µl
Nuclease-free water up to 48

   Available in the GeneChip® WT Double-Stranded DNA Terminal Labelling Kit from Affymetrix
2. Set up fragmentation mix according to either Tables above. Flick-mix and spin down the tubes.
3. Incubate the reactions at:
   - 37°C for 1 h.
   - 93°C for 2 min.
   - 4°C for at least 2 minutes.

4. Flick-mix, spin down the tubes, and transfer 45 µl of the sample to a new tube.
5. Remove 2 µl of each sample for gel-shift analysis.

Label fragmented double-stranded DNA

1. Prepare the double-stranded DNA Labelling Mix as described in the table below:
   Table 3. Composition of double-stranded DNA Labelling Mix

Component Volume/Amount in 1 Rxn
5X TdT Buffer 12 µl
TdT 2 µl
DNA Labelling Reagent, 5 mM 1 µl
Total volume 15 µl

   Available in the GeneChip® WT Double-Stranded DNA Terminal Labelling Kit from Affymetrix
2. Add 15 µl of the double-stranded DNA Labeling Mix to the DNA samples, flick-mix, and spin them down.

3. Incubate the reactions at:
   - 37°C for 60 min.
   - 70°C for 10 min.
   - 4°C for at least 2 min.

4. Remove 2 µl of each sample for gel-shift analysis.

Gel-shift analysis

1. Prepare a 4% agarose gel.
2. Incubate samples from **Fragment amplified targets**, Step 5 and **Label fragmented double-stranded DNA**, Step 4 at 65°C for 2 min.
3. Add 10 µl of Streptavidin (1 mg/ml), and incubate samples at room temperature for 5 min.
4. Run samples from Step 3 on a 4% agarose gel (**Gel-shift analysis**, Step 1) to check the shift migration of the labelling products.

Array hybridization & Array washing

Performed by the Genomic Center of the University of California Riverside.

Array analysis

Performed by computational analysis.

BUFFER COMPOSITIONS:

Block Solution: PBS + 0.5% Bovine Serum Albumin (BSA).

Lysis Buffer: 50 mM HEPES-KOH, pH 7.5
140 mM NaCl
1 mM EDTA
1% Triton X-100
0.1% SDS
1mM PMSF
Final pH 7.5

IP1: Lysis Buffer + 500 mM NaCl

IP2: 10 mM Tris-HCl
250 mM LiCl
1 mM EDTA
0.5% NP-40
0.5% Sodium Dioxycholat
Final pH 8.0

TE: 10 mM Tris, pH 7.4
1 mM EDTA

Final pH 8.0
Elution Buffer: TE Buffer + 1% SDS.

Discussion

Chromatin immunoprecipitation (ChIP) offers a valuable technique for the dissection of chromatin-based processes during cellular differentiation. Prerequisites for the success of this method are good antibodies and the availability of chromatin from control cells or tissues that lack the antigen of interest. By combining ChIP with DNA microarray technology, vast amounts of information can be obtained. The validation of ChIP results depends on the availability of suitable test systems that can link the dynamic association of proteins, protein modifications and/or RNA with the execution of biological processes during cell development.

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