Probing chromatin modifications in response to ERK signaling

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i. Summary: Chromatin immunoprecipitation (ChIP) is a technique used to determine the association of proteins or histone modifications with chromatin regions in living cells or tissues, and is used extensively in the chromatin biology field to study transcriptional and epigenetic mechanisms. Increasing evidence points to an epigenetic coordination of signaling cascades, such as ERK, that regulate key processes in development and disease, revealing novel principles of gene regulation. Here we describe a detailed protocol for performing chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) for probing histone modifications regulated by ERK signaling in mouse ESCs.

ii. Key Words: Chromatin immunoprecipitation, Histone modifications, Polycomb, ERK signaling, Spike-in normalization, ChIP-Western blot, ChIP-qPCR.

1. Introduction

Chromatin is the basic regulatory unit of the eukaryotic genetic material comprising of repeating arrays of nucleosomes. Each nucleosome particle contains 147 bp of DNA wrapped around a histone octamer made of two dimers of H2A and H2B and a tetramer of H3 and H4. Beyond a scaffolding role in packaging the genome, nucleosomes play critical roles in many genetic transactions, including transcription, DNA replication and chromosome segregation, by regulating DNA accessibility *(1)*. Importantly, all histones are subjected to various posttranslational modifications, most extensively at the N-terminal tails, which are highly unstructured and protrude out from the histone cores. These modifications include phosphorylation, methylation and acetylation, amongst many others, and are imposed by different classes of epigenetic enzymes *(2)*. The presence of these modifications may directly alter the structural properties of the nucleosome particle, or recruit downstream effector proteins that recognize the modifications. Chromatin may henceforth be viewed as a 'hub', integrating extra- and intracellular signaling pathways with a network of transcription factors and epigenetic factors, connecting cellular experience to gene expression changes *(3,4)*.

The extracellular signal-regulated kinase (ERK) signaling pathway is one of the most critical signaling cascades that participates in multiple physiological events and is often deregulated in diseased states *(5,6)*. ERK belongs to the mitogen-activated protein kinase (MAPK) signaling pathway, and is generally activated by growth factor signals. The conventional view is that activated ERK translocates into the nucleus, and brings about

changes in gene transcription, in part through phosphorylation of transcription factors *(7,8)*. Yet recent studies show that ERK is able to bind to DNA as well as phosphorylate histones and RNA Polymerase II, highlighting an instructional mechanism how ERK signaling can directly impact gene expression by converging on chromatin *(9-12)*. ERK can also partner with different chromatin complexes, both activating and repressive, to regulate transcription outputs *(10)*. Notably, ERK signaling is able to alter the DNA methylome by controlling the expression levels of DNA methyltransferases in different cellular contexts *(13-18)*. Collectively, these findings firmly establish a role of ERK signaling in regulating epigenetic changes. The clinical relevance of this signaling-to-chromatin nexus becomes apparent when considering that unrestrained ERK hyperactivation, for e.g., driven by oncogenic Ras signaling, is a potent driver of tumorigenesis, and that persistent ERK activation can trigger extensive enhancer remodeling that supports the oncogenic state *(19)*. Understanding how ERK signaling co-opts chromatin regulators to bring about epigenetic changes may therefore open up new therapeutic options.

Among the many functions of ERK signaling, it is known to also play a central role in embryonic stem cell (ESC) pluripotency. It is requisite for human ESC self-renewal but promotes lineage priming in mouse ESCs *(20)*. We and others have recently shown that ERK binds to a large number of regulatory elements in the ESC genome, effecting changes in nucleosome and transcriptional dynamics *(21,11)*. Furthermore, in mouse ESCs, ERK activation instructs Polycomb Repressive Complex 2 (PRC2) deposition on developmental promoters in the self-renewing state *(11)*. PRC2 is a chromatin complex responsible for catalyzing the repressive histone H3 lysine 27 tri-methylation mark, H3K27me3 *(22)*. That

activation of ERK is intimately tied to PRC2 deposition in ESCs not only exemplifies the highly plastic nature of PRC2 mediated repression, but also further emphasizes the importance of signal-induced chromatin remodeling during developmental transitions *(23)*. Such epigenetic coupling may account for how transient signaling experience (e.g., during normal developmental patterning or during periods of environmental stress) can potentially lead to long-lasting and heritable phenotypic changes, in both normal and pathological states.

Here we describe a detailed Chromatin Immunoprecipitation protocol for probing epigenetic changes downstream of ERK signaling in mouse ESCs. Specifically, we focus on histone modifications such as H3K27me3. We refer readers to other reports examining ERK induced alterations in DNA methylation, nucleosome occupancy and higher order chromatin organization *(24,25)*. We have successfully used this protocol to probe for different histone modification changes in mouse ESCs, as a function of ERK activity *(11)* (**Fig. 3)**. This protocol can be readily applied to other cell types and cellular contexts.

2. Materials

2.1 Cell Culture and Media Preparation

1. Cell type: Feeder free E14 mouse ESCs.
2. Conventional ESC medium: Knockout DMEM (Thermofisher, Cat. No. 10829-018), supplemented with ESC certified fetal bovine serum (FBS; Atlanta Bio, Cat.

No. S10250; 15% final), leukemia inhibitory factor (LIF; Millipore, Cat. No. ESG1107; 1000U/mL final), non-essential amino acids (NEAA; Thermofisher, Cat. No. 11140-076; 1X final), L-glutamine (Thermofisher, Cat. No. 25030-081; 2 mM final), 2-mercaptoethanol (Sigma; Cat. No. M3148; 0.1 mM final) and penicillin/streptomycin (Sigma; Cat. No. P0781; 1X final).

3. Naïve ESC medium: Serum-free N2B27 base medium (Stem Cell Science; Cat. No. SCS-SF-NB-02), supplemented with LIF and small-molecule inhibitors against ERK signaling, PD0325901 (Stemgent; Cat. No. 04-0006; final 1 mM), and GSK3 signaling, CHIR99021 (Stemgent; Cat. No. 04-0004; final 3 mM).

2.2 Reagents for cross-linking and sonication

1. Formaldehyde (FA; Fisher Scientific, Cat. No. F79-500)
2. Glycine (2.5 M stock, Roche Applied Science, Cat. No. 03117251001)
3. Polystyrene 15 mL falcon tubes (Corning, Cat. No. 352099)
4. Bioruptor (Diagenode).

2.3 Chromatin extraction Solutions

All the solutions are prepared with DNase-free milli-Q water. The following inhibitors are added right before use to the solutions when stated: Leupeptin 1 μg/mL, Pepstatin 1 μg/mL, Aprotinin 1 μg/mL, 0.2 mM PMSF, 10 mM Sodium butyrate, 10 mM Sodium fluoride, 1 mM Sodium orthovanadate. All solutions can be prepared in large quantities and stored at 4°C unless otherwise specified (*see* **Note 1**).

1. Lysis Buffer 1 (LB1): 50 mM HEPES (pH 7.5 at 4°C), 140 mM NaCl, 1 mM EDTA, 10% Glycerol, 0.5% NP40, 0.25% Triton X. (Add inhibitors)
2. Lysis Buffer 2 (LB2): 10 mM Tris (pH 8 at 4°C), 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA. (Add inhibitors)
3. Lysis Buffer 3 (LB3): 10 mM Tris (pH 7.5 at 4°C), 1 mM EDTA, 0.5 mM EGTA, 0.5% N-Lauroylsarcosine sodium salt. (Add inhibitors)

2.4. ChIP Solutions

1. Blocking solution for magnetic beads: 0.05 g of BSA in 15 mL 1X PBS. (Prepare fresh)
2. Incubation Buffer: 3% Triton X, 0.3% Deoxycholic Acid Sodium Salt, 15 mM EDTA. (Add inhibitors)
3. RIPA Buffer: 50 mM HEPES (pH 7.5 at 4°C), 0.7% Deoxycholic Acid Sodium Salt, 1 mM EDTA, 1% NP40, 500 mM LiCl. (Add inhibitors)
4. High SDS Elution Buffer: 50 mM Tris (pH 8 at RT), 10 mM EDTA, 1% SDS. (Prepare fresh)
5. TE: 10 mM Tris (pH 8 at RT), 1 mM EDTA.
6. TE + 50 mM NaCl: 10 mM Tris (pH 8 at RT), 50 mM NaCl, 1 mM EDTA.

3. Methods

Overview: In a typical ChIP experiment, cells are grown to the desired confluency and protein-DNA interactions are stabilized *in vivo* via the addition of Formaldehyde, a cell-permeable and reversible cross-linking agent. Glycine is then added to quench unreacted Formaldehyde and to terminate the cross-linking reaction. Cells are harvested, lysed and the DNA is sheared. This is followed by an immunoprecipitation step to enrich for the desired protein-DNA fragment using specific antibodies against the proteins of interest. The cross-links are reversed, and the DNA processed for PCR, Next Generation Sequencing, or other downstream applications. When probing for covalent histone modifications, a variant ChIP procedure known as native ChIP can be adopted where the Formaldehyde cross-linking step is omitted *(26)* (*see* **Note 2**).

3.1. Cell Fixation for ChIP

1. For adherent cells: aspirate the medium and wash the cells with 1X PBS. For suspension cells: spin down first then wash with 1X PBS. Dissociate the cells by treating with trypsin-EDTA solution for 1 - 2 min in a 37°C incubator. Add serum-containing medium to inactivate the trypsin and spin down the cells at 1200 rpm for 3 min. Next, resuspend the cells with fresh medium, count and collect 30 x 10^6 cells into the 15 mL polystyrene falcon tubes that are suitable for use in the Bioruptor (*see* **Note 3**).
2. Cross-link the protein-DNA complexes by adding to the cells 37% Formaldehyde diluted to a 1% final concentration. For adherent cells: medium containing 1% Formaldehyde can be prepared separately and added directly to the tissue culture

dishes (e.g., 5 – 8 mL for a 10 cm tissue culture dish) immediately following the PBS wash. Incubate for 15 min at RT and mix gently on an orbital shaker. For cell suspension: top up to 10 mL with medium, and add 270 µL of 37% Formaldehyde in a drop wise manner (final concentration of 1%). Mix well and incubate for 15 min at RT. (*see* **Note 4**).

3. Quench the Formaldehyde by adding 2.5 M Glycine diluted to a final concentration of 125 mM. Mix well and incubate for 15 min at RT.
4. For adherent cells: discard the Formaldehyde-Glycine mixture and wash twice with ice-cold 1X PBS containing protease and phosphatase inhibitors. For cell suspension: spin down the cells for 5 min at 1200 rpm at 4°C before PBS wash.
5. For adherent cells: scrape the cells in ice-cold 1X PBS containing protease and phosphatase inhibitors using a cell scraper and snap freeze the cell pellet in liquid nitrogen. For cell suspension: snap freeze the cell pellet in liquid nitrogen, then store at -80°C until use.

3.2. Chromatin extraction and sonication

1. Prepare all buffers prior to thawing the cells. Thaw the cells on ice.
2. Add 5 mL of LB1 and resuspend the cell pellet by gentle pipetting.
3. Rock on a shaker in the cold room for 10 min (do not rotate).
4. Spin the cells at 1350 rcf for 5 min at 4°C.
5. Aspirate the supernatant. Flick to dislodge the cell pellet and add 5 mL of LB2 and resuspend by gentle pipetting.

6. Rock on shaker at RT for 10 min (do not rotate).
7. Spin the cells at 1350 rcf for 5 min at 4°C. The pellet should have a glassy appearance, which is typical of a nuclei preparation.
8. Resuspend the nuclei pellet in 1.2 mL LB3 (*see* **Note 5**).
9. Place the tube in the Bioruptor (Diagenode), and sonicate at high power for 10 cycles (30 s ON and 30 s OFF; 1st round), followed by an additional 5 cycles (30 s ON and 30 s OFF; 2nd round). When dealing with multiple chromatin preparations, go through all the tubes in the first round and then do the second round of sonication in the same order. Staggering of the samples prevents over heating and denaturation of the proteins (*see* **Note 6**).
10. Remove 20 µL of the sample, max spin for 1 min and run the supernatant on a 1% agarose gel. The size range of the DNA should be between 500 - 1500 bp before de-cross-linking (**Fig. 1A**)
11. Sonication may be repeated until the desired fragment size is obtained.
12. Max spin the samples for 20 - 30 min at 4°C to pellet the insoluble material.
13. Transfer the supernatant (sheared chromatin preparation) into a new low DNA binding eppendorf tube and proceed with pre-clearing step.

3.3. Pre-clearing of the chromatin

1. Remove 20 µL of Dynabeads Protein G slurry per sample and wash 3 times with blocking solution. Next, rotate the beads in blocking solution for 1 h. The beads for immunoprecipitation (section 3.5 step 4) may also be pre-cleared at the same time

for use the following day. Typically, 5 µL of bead slurry can be used per 1 µg of antibody.

2. After blocking, wash the beads five times with TE and add them into chromatin. Rotate the chromatin/beads for 1 - 2 h in the cold room for pre-clearing. The pre-cleared beads for immunoprecipitation may be stored in TE until use the following day.
3. Max spin the samples for 1 min and carefully transfer the supernatant into fresh low DNA binding eppendorf tube. Be careful not to carry over any beads.
4. Measure the crude chromatin concentration using Nanodrop. The $OD_{260/280}$ should be about 1.7. For 30 $x10^6$ ESCs, we typically obtain around 1 µg/µL.
5. Proceed immediately with the immunoprecipitation step or snap-freeze the samples (aliquot for single use) in liquid nitrogen and store at -80°C until use.

3.4. Optimization of antibody concentration by ChIP-Western blot

This section outlines the general steps to determine the optimal concentration of antibody used in a ChIP experiment.

1. Set up at least three tubes of 50 µg of pre-cleared chromatin from section 3.3. Top up the volume to 200 µL with LB3 and then add 100 µL of incubation buffer. Set aside 5 µg for input and store it at -80°C (*see* **Note 7**).
2. Test at least three different antibody concentrations (e.g. 0.5 µg, 1 µg and 3 µg), and rotate overnight at 4°C.

3. Next day, add the pre-blocked beads into each of the tubes and rotate for 2 - 3 h in the cold room.
4. Wash the beads five times with RIPA buffer.
5. Perform a quick wash in TE + 50 mM NaCl solution.
6. Spin the beads at 3000 rpm for 2 min, and place into the magnetic rack to remove any residual TE.
7. Add 15 µL of high SDS elution buffer to the beads, pipette to mix, and transfer the bead suspension into the 0.2 mL PCR tubes. Add an additional 15 µL of 2X Laemelli sample buffer to the suspension. Do the same for the inputs.
8. Heat all the samples in the PCR machine at 98°C for 30 min.
9. After heating, max spin the beads and load 25 µL of the supernatant onto the SDS-PAGE gel. Proceed with standard Western blotting.
10. Determine the optimal antibody:chromatin ratio from the Western blot (**Fig. 2**), and scale up accordingly depending on the final amount of chromatin used per ChIP.

3.5. ChIP and reverse cross-linking

1. Use 100 µg of cross-linked chromatin per histone modification ChIP. Greater amounts may be needed for less abundant chromatin associated proteins. Top up the volume to 200 µL with LB3 and then add 100 µL of incubation buffer (*see* **Note 8**).
2. Add the desired amount of antibodies (determined in 3.4) and rotate overnight at 4°C.

3. Set aside 10 µg for input from each sample and store at -80°C.
4. Next day, add the pre-blocked beads and rotate for 2 - 3 h in the cold room.
5. Wash the beads four times with RIPA buffer. Then transfer the mixture into a fresh tube, followed by an additional wash with RIPA buffer.
6. Wash once in TE + 50 mM NaCl solution.
7. Spin the beads at 3000 rpm for 2 min, and put onto magnetic rack to remove the residual TE.
8. Add 125 µL of elution buffer to the beads to elute the antibody-DNA complex (*see* **Note 9**).
9. Elute at 65°C in a thermoshaker, agitating at 1000 rpm for 20 min. Vortex occasionally to ensure even mixing.
10. Briefly spin down the beads and put into the magnetic rack. Carefully transfer the eluate into a fresh low DNA binding eppendorf tube. Also add 125 µL of the elution buffer to the inputs.
11. Reverse cross-link all tubes (IP and inputs) at 65°C in a thermoshaker, with constant agitation for at least 6 h.
12. Add 4 µL of Proteinase K (stock 20 mg/mL; final conc: 0.62 mg/mL) and incubate at 55°C for 2 h in a thermoshaker with constant agitation.
13. Purify the DNA using QIAquick PCR purification kit and elute in 30 µL EB (*see* **Note 10**).
14. Check the size of the input DNA after de-cross-linking on a 1.5% agarose gel. The optimal average size range should be 200 – 400 bp, suitable for ChIP-qPCR and ChIP-Seq library preparations (**Fig. 1B**). Proceed with ChIP-qPCR.

3.6. Spike-in Normalization (Optional, but strongly recommended)

As described above, ChIP is a multi-step protocol that is subjected to various technical variations such as chromatin fragmentation, immunoprecipitation efficiency and sample processing bias *(27,28)*. Therefore, when comparing experimental signals between samples, it is very important to correct for these variables. The use of 'spike' chromatin can serve as an internal reference for sample-to-sample variations, and this method has been successfully implemented to reveal quantitative differences in histone modification status across different cell populations *(27)*. In this method, foreign chromatin, which is prepared as indicated above, can be spiked in to each reaction as a small fraction of the total chromatin, prior to immunoprecipitation. Therefore any technical variation arising during the ChIP protocol will similarly affect the spiked in chromatin. Given that an exact amount of spike-in chromatin is used across all experimental samples, this allows for an internal normalization factor to be created, to which the experimental signals can be adjusted. We typically use chromatin prepared from *Drosophila melanogaster* S2 cells as foreign chromatin, and an antibody against the *Drosophila* specific histone variant, H2Av, to pull down the spiked in *Drosophila* chromatin. Theoretically, the amount of recovered *Drosophila* chromatin in each sample should be identical, and if not, a normalization factor can be created to eliminate the aforementioned technical variations introduced during the ChIP protocol.

1. Prepare the chromatin from *Drosophila* S2 cell line as indicated above (Steps 3.1, 3.2, and 3.3)
2. After adding the desired amount of antibodies in Section 3.5 step 2, add 2 µg of *Drosophila* chromatin and 0.5 µg of H2Av antibody (Active motif, Cat. No. 61686) per 100 µg of total chromatin (1/50 of the total chromatin) to each ChIP reaction.
3. Proceed with Section 3.5 step 3.

3.7. ChIP-qPCR

To determine the enrichment of the particular DNA sequences associated with the histone modification of interest, ChIP-qPCR method should be performed. However, this method requires *a priori* knowledge of the genomic binding sites in order to design primers to specific regions. For e.g., H3K27me3 can be found at the promoter, TSS, and gene body regions of repressed genes, whereas H3K36me3 is selectively enriched at the gene body of active genes *(29,30)*. If the precise binding sites are unknown, multiple primers that span the entire gene unit can be designed. When probing for a novel chromatin modification and/or factor where the binding profile is completely unknown, ChIP-Seq method should be used.

1. Primer design: To quantify the fragments (200 - 400 bp) after ChIP, we design primers to amplify regions around 80 - 120 bp. Simply retrieve the DNA sequence of interest from USCS Genome Browser *(31)* and insert it to Primer3web primer design tool *(32,33)*. Set the product size ranges section to 80 - 120 and keep all the

other parameters as default. Click on 'pick primers' button. It is important to design primers for both positive and negative binding locations, if available (*see* **Note 11**).

2. qPCR reaction: 1 µL of the ChIP DNA or 1 µL of 1:10 diluted input DNA, 0.5 µL of each forward and reverse primers (10 µM), 5 µL of 2X SYBR (Roche, Cat. No. 04887352001), top up to 10 µL with dH2O.
3. qPCR conditions: 10 min 95°C (to inactivate hotstart), 40 cycles of (10 s 95°C, 30 s 65°C , 30 s 72°C).
4. Calculate the fold enrichment by normalizing the amount of ChIP DNA to input DNA ($2^{-\Delta Ct(Ct\ of\ ChIP\ DNA\ –\ Ct\ of\ input\ DNA)}$) **(Fig. 3).** If spike-in control is used, calculate the fold enrichment of spike-in DNA in each sample using the *Drosophila* specific primers (Scr and/or Ubx) (*see* **Note 12**). Identify the sample with the highest enrichment value, and use this value to create normalization factors for the remaining samples. Finally, multiply each normalization factor by its corresponding ChIP DNA enrichment value to equalize the signals across samples.

4. Notes

1. It is important to add protein phosphatase inhibitors such as sodium fluoride and sodium orthovanadate in all buffers to preserve the protein phosphorylation state in cells and cell lysates. The pH of all buffers should be adjusted accordingly to the temperature stated.

2. Formaldehyde is a small molecule that is highly diffusible. It is one of the most common protein-DNA reversible cross-linker used in molecular biology. For most conventional ChIP protocols, Formaldehyde treatment (1% final concentration) for 10 – 15 min is sufficient to covalently capture protein-DNA interactions. However, for probing of histone modifications, this cross-linking step is optional, and that Native ChIP can be performed. In the latter, nuclei are isolated and the unfixed chromatin fragmented using micrococcal nuclease to achieve >80% mononucleosomes (~150 bp). Given that sonication yields a heterogeneous mixture of DNA sizes (200 – 400 bp), Native ChIP offers a higher resolution compared to conventional cross-link ChIP. This approach can also be used to map nucleosome positioning. When probing for chromatin associated factors that exhibit transient and hyperdynamic binding e.g., coactivator CBP/p300 and RNA Polymerase II, a two-step cross-linking can be performed, involving the sequential use the protein-protein cross-linker, Disuccinimidyl glutarate (DSG), followed by Formaldehyde treatment.

3. This cell number must be determined empirically and can vary depending on the cell types, cross-linking conditions and the sonication parameters. For adherent cells, one plate may be set aside for determining the cell density. The use of polystyrene tubes offers more efficient chromatin shearing over polycarbonate tubes.

4. Cross-linking is time sensitive. Avoid over cross-linking as it affects epitope accessibility and reduces sonication efficiency.

5. This volume may be adjusted based on the cell number. Sonication conditions should be adjusted accordingly.

6. Ensure that the probe is vertical and does not touch the wall of the falcon tube. Depending on the Bioruptor/sonicator setup, multiple samples can be sonicated at once. The sonication conditions described are optimized for 30 x 10^6 mouse ESCs that are cross-linked for 15 min and resuspended in 1.2 mL LB3.

7. The amount of chromatin used for titration can vary depending on the abundance of the protein of interest and/or the efficiency of the antibody. Similarly, the total volume may be adjusted without changing the ratio between LB3 and incubation buffer.

8. We typically usually use 50 – 100 µg of crude chromatin per histone modification ChIP and at least 300 µg for other proteins. The amount of chromatin used must be determined empirically for each protein factor probed and antibody used. Multiple immunoprecipitations may be pooled to obtain the desired amount of DNA required for Next Generation Sequencing or other downstream applications.

9. Elution buffer must be prepared fresh.

10. The elution volume can be varied depending on the amount and efficiency of DNA recovered.

11. We design at least 2 primer sets for each positive and negative binding locations. We also validate the quality of all newly designed primers by melting curve analysis and running the qPCR products on an agarose gel.

12. Primer sequences are: Ubx-Fw; CTGAGACGGGCTAAAAGTCG, Ubx-REV; AGCACAGAAAGCGAGGAAAC, Scr-Fw; TGGCTAGACTTTTGCGTCCT, Scr-REV; TACCAAAAGCCGTCCAAATC.

Figure legends:

Fig. 1. Agarose gel image before and after de-cross-linking of the sonicated chromatin. (a) The optimal chromatin fragments before de-cross-linking are between 500 - 1500 bp as indicated. (b) The optimal DNA fragments after de-cross-linking are between 200 - 400 bp as indicated.

Fig. 2. ChIP-Western blot of H3K27me3 antibody. 50 μg chromatin is pulled down with different concentrations of H3K27me3 antibody (Cell signaling, Cat. No. 9733) and blotted with the same antibody. The pulled down H3K27me3 amount is saturated after 1 μg of antibody. Therefore the optimal antibody amount for 50 μg chromatin would be 1 μg.

Fig. 3. ChIP-qPCR of H3K27me3 in response to ERK and GSK inhibition. Comparing ChIP-qPCR signal enrichment of H3K27me3 histone mark over input in ESCs cultured in conventional or naïve ESC medium containing inhibitors to ERK and GSK3 signaling as indicated in Materials 2.1 step 2 and 3. As expected H3K27me3 is enriched on developmental genes (*Hoxc5* and *Fgf5*) in conventional ESCs and reduced in naïve ESCs *(34)*. However, there is very little enrichment of H3K27me3 mark on pluripotency gene *Oct4* and on a gene desert region, both serving as negative controls.

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