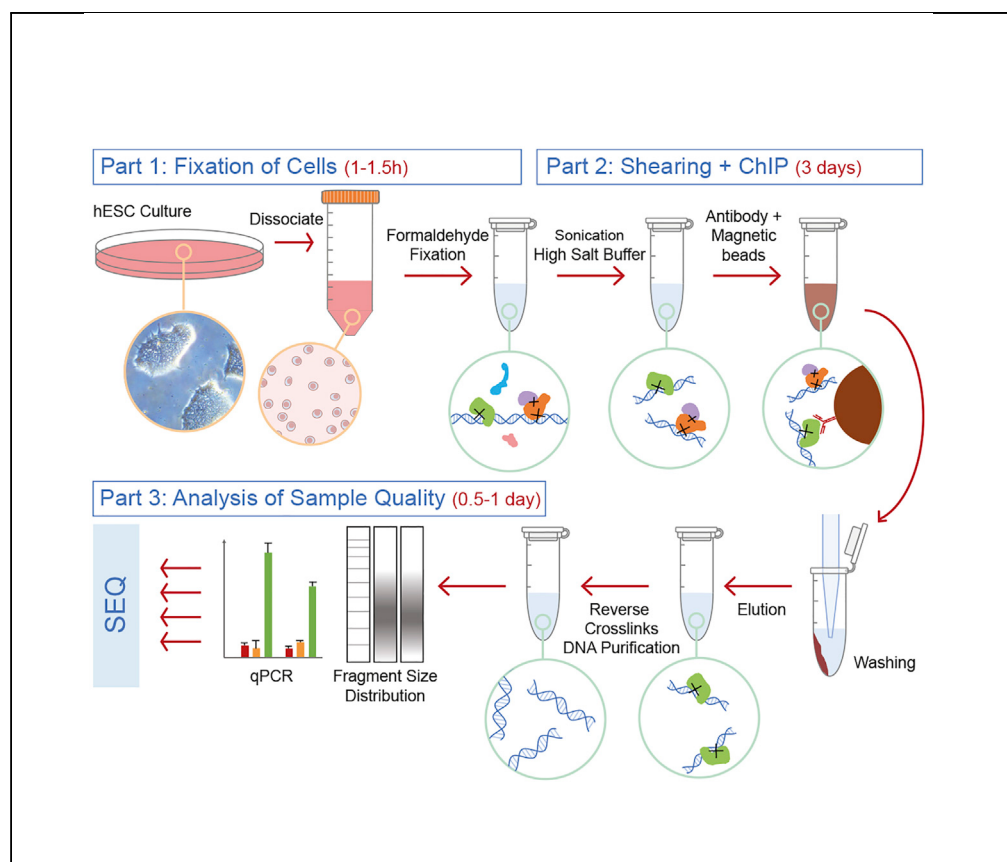


Protocol

An Optimized Protocol for ChIP-Seq from Human Embryonic Stem Cell Cultures



Chromatin immunoprecipitation (ChIP) followed by next-generation sequencing is a powerful technique that characterizes the genome-wide DNA-binding profile of a protein of interest. The general ChIP-seq workflow has been applied widely to many sample types and target proteins, but sample-specific optimization of various steps is necessary to achieve high-quality data. This protocol is specifically optimized for cultured human embryonic stem cells (hESCs), including steps to check sample quality and non-specific enrichment of “hyper-ChIPable” regions prior to sequencing.

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HIGHLIGHTS

ChIP-seq is used to characterize genome-wide DNA-binding profile for a protein of interest

Conventional ChIP protocols produce poor or highly variable results in hESC cultures

ChIP can be optimized for hESC cultures to ensure high-quality sequencing data

Optimized ChIP includes improved chromatin fragmentation and sample quality checkpoints

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Protocol

An Optimized Protocol for ChIP-Seq from Human Embryonic Stem Cell Cultures

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SUMMARY

Chromatin immunoprecipitation (ChIP) followed by next-generation sequencing is a powerful technique that characterizes the genome-wide DNA-binding profile of a protein of interest. The general ChIP-seq workflow has been applied widely to many sample types and target proteins, but sample-specific optimization of various steps is necessary to achieve high-quality data. This protocol is specifically optimized for cultured human embryonic stem cells (hESCs), including steps to check sample quality and non-specific enrichment of “hyper-ChIPable” regions prior to sequencing.

For complete details on the use and execution of this protocol, please refer to Gunne-Braden et al. (2020).

BEFORE YOU BEGIN

We have found that various existing ChIP protocols produced poor or highly variable results when applied to hESC cultures, primarily in regard to the efficiency and fragment size distribution pattern of shearing chromatin via sonication. This is likely due to the non-standard nature of the cell cultures – hESCs are small in size with a large nucleus and little cytoplasm, which grow in very dense colonies (Orozco-Fuentes et al., 2019). Chromatin fragmentation is particularly important for ChIP experiments that will be analyzed using next-generation sequencing (NGS), as fragment size will affect the resolution of enriched regions that indicate protein binding sites. Therefore, a major improvement in this protocol is the use of dissociation and a high salt sonication buffer to reliably produce a high yield of appropriately sized fragmented chromatin (100 bp to 500 bp).

Another advantage of this protocol is the expanded guidelines for testing sample quality before submission for sequencing, as NGS is usually the most time-consuming and costly step of ChIP-seq. In particular, we propose that checking for non-specific enrichment of hyper-ChIPable regions is a quick and simple step to address an issue (Teytelman et al., 2013) that seems to be rarely acknowledged.

Design Primers

⌚ Timing: 1 h

1. Design and test quantitative PCR (qPCR) primers, aiming for an annealing temperature of 60°C and an amplicon length of roughly 80–90 bp, to amplify a region of DNA that is:
 - a. Bound by your protein of interest (positive control)

- b. Not bound by your protein of interest (negative control)

Note: For a guide on designing qPCR primers, including a list of available design software, see (Rodríguez et al., 2015). It may be that a positive control site has not previously been identified for the protein of interest in hESCs, but ChIP or ChIP-seq data from other human cell lines exists (e.g., on NCBI Gene Expression Omnibus; <https://www.ncbi.nlm.nih.gov/geo/>). Good candidates for a positive control are binding sites that are occupied by the protein in other cell contexts, and/or within proximity of known target genes that are also regulated in hESCs. A corresponding negative control may be a region some distance (e.g., 3 kb) away from the positive control, or in the promoter region of a gene not regulated by the protein of interest. For a database of previously verified ChIP-qPCR primers, see (Kurtenbach et al., 2019).

Prepare Solutions

⌚ Timing: 2 h:0 min

2. Prepare the buffers listed below, making sure to adjust the pH and/or temperature if indicated. See [Materials and Equipment](#) for buffer recipes.
 - a. Dulbecco's Phosphate Buffered Saline (DPBS), pH 7.1–7.5 (4°C) (also have another stock ready at room temperature; i.e., 20°C–21°C)
 - b. 1.25 M Glycine (4°C)
 - c. High Salt Lysis/Sonication Buffer (4°C)
 - d. Chromatin Dilution Buffer (4°C)
 - e. Wash Buffer A (pH 7.9, 4°C)
 - f. Wash Buffer B (pH 7.9, 4°C)
 - g. Wash Buffer C (pH 8.0, 4°C)
 - h. TE Buffer (4°C)
 - i. Elution Buffer

Prepare hESC Cultures

⌚ Timing: 2–5 days

3. This protocol has been used with hESC lines (WA01; (Thomson, 1998)) grown as colonies cultured in mTeSR1 media on GFR Matrigel either as pluripotent cells or differentiated with BMP4 (Bone Morphogenetic Protein 4) by adding fresh mTeSR1 supplemented with BMP4 (50 ng/mL) daily for up to 3 days. This protocol could also be reasonably applied to other growth conditions that involve serum-free media and an attachment matrix, i.e., a different coating. See [Troubleshooting 1](#) for tips on culture density.

Prepare Equipment

⌚ Timing: 30 min

4. All centrifugation prior to the elution step will be performed at 4°C, so make sure that centrifuges have been pre-chilled.

Note: Before performing the full protocol, it is recommended to confirm the optimal number of cycles of sonication used to shear the chromatin as this may vary depending on the sonicator used. See [Troubleshooting 4](#) for an example workflow.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Pierce 16% Formaldehyde (w/v), Methanol-free	Life Technologies	28908
TrypLE Express Enzyme (1×) Phenol Red	Life Technologies	12605010
Dulbecco's Phosphate-Buffered Saline (DPBS) (pH 7.1–7.5)	Sigma	D8537
Glycine	Sigma	G8898
Hydrochloric Acid (HCl)	Sigma	H1758
Sodium hydroxide (NaOH)	Sigma	S8045
Sodium chloride (NaCl)	Sigma	S3014
Tris base	Millipore	648310
EDTA	Sigma	E9884
Triton X-100	Sigma	T8787
Sodium dodecyl sulfate (SDS)	Sigma	71725
NP-40 Alternative	Millipore	492016
HEPES	Sigma	H3375
Sodium deoxycholate	Sigma	30970
Lithium chloride (LiCl) solution, 8 M	Sigma	L7026
Halt Protease Inhibitor Cocktail (100×) EDTA-free	Thermo Fisher	78437
RNase A	Thermo Fisher	EN0531
Proteinase K	Thermo Fisher	AM2546
BMP4 Recombinant Human Protein	Life Technologies	PHC9534
DMEM/F12 Media	Gibco	11320033
mTeSR1	STEMCELL Technologies	85850
GFR Matrigel Matrix	Corning	354230
Critical Commercial Assays		
Dynabeads Protein G (or Protein A) for Immunoprecipitation	Thermo Fisher Scientific	10003D (OR 10008D)
Zymo ChIP DNA Clean & Concentrator	Zymo Research	D5205
iTaq Universal SYBR Green Supermix	BioRad	172-5122
Experimental Models: Cell Lines		
H1 hESC (WA01)	WiCell	RRID:CVCL_9771
Oligonucleotides		
hs tRNA-Gln F: CCAGGATGGAATGAGAAAGG	N/A	tRNA-Gln F
hs tRNA-Gln R: GACCCAGTCTGCTGTTCAAG	N/A	tRNA-Gln R
Other		
Bioruptor Plus 1.5 mL TPX Sonication Tubes	Diagenode	DIAG-C30010010
Protein LoBind PCR Clean 2 mL Tubes	Eppendorf	0030108132
QuantStudio6 Real-Time PCR Machine	Applied Biosystems	4485692
PureProteome Magnetic Stand	Millipore	LSKMAGS08

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
INCU-Line IL23 Digital incubator	VWR	390-0482
Diagenode Bioruptor Plus Sonication System	Diagenode	B01020001
Diagenode Water Cooler	Diagenode	BioAcc-cool
Agilent TapeStation 4200 System	Agilent	G2991AA
Qubit 3.0 Fluorometer	Life Technologies	Q33216

MATERIALS AND EQUIPMENT

Equipment

- Diagenode Bioruptor Plus Sonication System + Diagenode Water Cooler

Alternatives: Other sonicator system used for chromatin shearing (see [Troubleshooting 3](#) to optimize)

- Agilent TapeStation System + High Sensitivity DNA ScreenTape Analysis Reagents

Alternatives: DNA gel electrophoresis equipment (a less sensitive/quantitative alternative)

- Qubit Fluorometer

Alternatives: Nanodrop 3300 Fluorospectrometer or other quantification technology with a dsDNA lower detection limit < 0.1 ng/μL

- QuantStudio6 Real-Time PCR Machine

Alternatives: Any qPCR machine

- VWR INCU-Line IL23 Incubator

Alternatives: Any incubator or oven capable of 45°C - 65°C temperature

- PureProteome Magnetic Stand

Alternatives: Any magnetic holder for 1.5 mL and 2.0 mL microfuge tubes

Other

- A primary antibody against your protein of interest (ChIP-verified)

Note: A control ChIP done in parallel is recommended – this may be a ChIP from a hESC sample that does not express your protein of interest (recommended), or a ChIP from an identical hESC sample using an equivalent amount (ng) of a type-matched non-specific antibody. See [Troubleshooting 6](#)

- Growth media for hESC culture (e.g., mTeSR1, STEMCELL Technologies #85850)

Alternatives: Other hESC growth media

STAR Protocols

Protocol



- Attachment matrix for coating plates in hESC culture (e.g., GFR Matrigel diluted 1:10 in DMEM/F12 media)

Alternatives: Other attachment matrix used in hESC culture (e.g., laminin)

- Dry ice/Ethanol bath for snap freezing

Alternatives: Liquid nitrogen

Buffers

Alternatives: The chemicals listed in the [Key Resources Table](#) can all be replaced with identical chemicals from different suppliers, provided that they are of the same grade.

Note: All stock solutions and buffers are pH adjusted with either 1 M HCl or 1 M NaOH solutions as required.

1.25 M Glycine (Store at 4°C)

Stock Chemical Solutions (for use in making up buffers, below)

- 0.5 M EDTA, pH 8 (14.612 g EDTA, to 100 mL with ddH₂O)

Note: Adjust pH of solution with NaOH while stirring in order to dissolve EDTA powder.

- 1 M Tris, pH 7.5 (12.114 g Tris base, to 100 mL with ddH₂O)
- 4 M NaCl (23.376 g NaCl, to 100 mL with ddH₂O)
- 1 M HEPES pH 7.9 (23.83 g HEPES, to 100 mL with ddH₂O; store at 4°C)

High Salt Lysis/Sonication Buffer (Store at 4°C)

Reagent	Final Concentration	Amount (g or mL) in 100 mL
4 M NaCl	800 mM	20 mL
1 M Tris pH 7.5	25 mM	2.5 mL
0.5 M EDTA pH 8	5 mM	1 mL
Triton X-100	1% v/v	1 mL
SDS	0.1% w/v	0.1 g
Sodium deoxycholate	0.5% w/v	0.5 g
ddH ₂ O	n/a	To 100 mL
Protease Inhibitor Cocktail ^a	1×	

^aAdd fresh before use

Chromatin Dilution Buffer (Store at 4°C)

Reagent	Final Concentration	Amount (g or mL) in 100 mL
1 M Tris pH 7.5	25 mM	2.5 mL
0.5 M EDTA pH 8	5 mM	1 mL
Triton X-100	1% v/v	1 mL
SDS	0.1% w/v	0.1 g
ddH ₂ O	n/a	To 100 mL
Protease Inhibitor Cocktail ^a	1×	

^aAdd fresh before use

Wash Buffer A (Adjust to pH 7.9, Store at 4°C)

Reagent	Final Concentration	Amount (g or mL) in 100 mL
4 M NaCl	140 mM	3.5 mL
1 M HEPES pH 7.9	50 mM	5 mL
0.5 M EDTA pH 8	1 mM	0.2 mL
Triton X-100	1% v/v	1 mL
SDS	0.1% w/v	0.1 g
Sodium deoxycholate	0.1% w/v	0.1 g
ddH ₂ O	n/a	To 100 mL

Wash Buffer B (Adjust to pH 7.9, Store at 4°C)

Reagent	Final Concentration	Amount (g or mL) in 100 mL
4 M NaCl	500 mM	12.5 mL
1 M HEPES pH 7.9	50 mM	5 mL
0.5 M EDTA pH 8	1 mM	0.2 mL
Triton X-100	1% v/v	1 mL
SDS	0.1% w/v	0.1 g
Sodium deoxycholate	0.1% w/v	0.1 g
ddH ₂ O	n/a	To 100 mL

Wash Buffer C (Adjust to pH 8.0, Store at 4°C)

Reagent	Final Concentration	Amount (g or mL) in 100 mL
1 M Tris pH 7.5	20 mM	2 mL
0.5 M EDTA pH 8	1 mM	0.2 mL
8 M LiCl	250 mM	3.125 mL
NP-40 Alternative	0.5% v/v	0.5 mL
Sodium deoxycholate	0.5% w/v	0.5 g
ddH ₂ O	n/a	To 100 mL

TE Buffer (Store at 4°C)

Reagent	Final Concentration	Amount (g or mL) in 200 mL
1 M Tris pH 7.5	10 mM	2 mL
0.5 M EDTA pH 8	1 mM	0.4 mL
ddH ₂ O	n/a	To 200 mL

Elution Buffer (Store at room temperature (i.e., 20°C–21°C))

Reagent	Final Concentration	Amount (g or mL) in 20 mL
1 M Tris pH 7.5	10 mM	0.2 mL
0.5 M EDTA pH 8	1 mM	0.04 mL
SDS ^a	1% w/v	0.2 g
ddH ₂ O	n/a	To 20 mL

^aAdd fresh before use

Formaldehyde Stock Solution (16% Formaldehyde (w/v), Methanol-free; Pierce, 28906)

Alternatives: Prepare a fresh stock solution from paraformaldehyde powder. Do not use formaldehyde solution that contains methanol, which can also act as a fixative and may produce artifacts in chromatin shearing.

⚠ **CRITICAL:** Formaldehyde is acutely toxic. Wear full personal protective equipment (PPE) when handling and dispose of waste appropriately.

STEP-BY-STEP METHOD DETAILS

Fixation of Cells

⌚ **Timing:** 1–1.5 h

Figure 1 This part dissociates hESCs and immediately fixes them with formaldehyde to capture proteins on the DNA. At the time of fixation, pluripotent hESC colonies should be dense monolayers with tight borders, but not overgrown. Differentiated hESCs (for example, with Bone Morphogenetic Protein 4, BMP4) may have changed morphology but should likewise not be overgrown. See [Figure 2](#) for examples of recommended hESC cultures at the time of fixation.

Note: Volumes given in brackets are for a 6-well plate. A 6-well plate that is approximately 70% confluent will provide roughly $7E+06$ cells. This is sufficient for 2 aliquots of $3E+06$ cells, with each aliquot used for 1 ChIP sample.

1. Remove media and wash once with room temperature (20°C – 21°C) room-temperature Dulbecco's Phosphate Buffered Saline (DPBS) (2 mL per well)
2. Add just enough TrypLE Express Enzyme to cover the cells (250 μL per well)
3. Incubate at 37°C until the cells appear to be mostly single cells under a microscope. Tap the edge of the tray every 2–3 min to help shift the cells.

⚠ **CRITICAL:** ESCs grow in dense colonies which, if insufficiently dissociated, can lead to high levels of heterogeneity in chromatin shearing with sonication and hence impact data quality. However, cells will lyse if incubated with TrypLE for too long; 4–5 min is typical for pluripotent cells. See [Troubleshooting 2](#).

Part 1: Fixation of Cells (1–1.5 hrs)

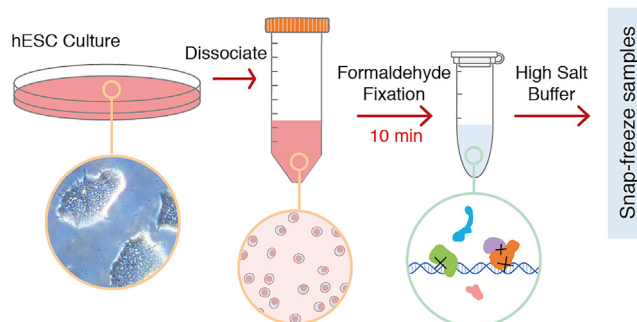


Figure 1. Schematic Summary of Part 1: Fixation of Cells, Which Starts with hESC Cultures and Ends with Snap-Frozen, Formaldehyde-Crosslinked Samples that Can Be Stored at -80°C

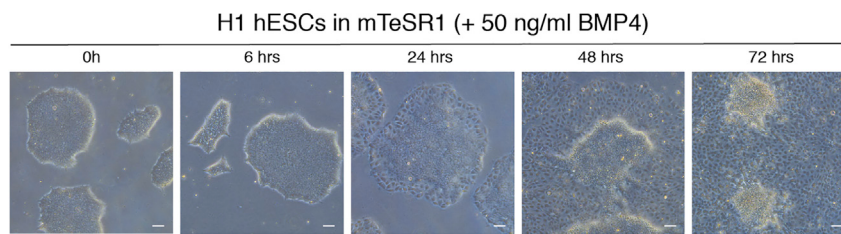


Figure 2. Example Images of hESC Cultures Used for ChIP-Seq

This protocol has been used successfully with the hESC line H1 as pluripotent cells, or differentiated with BMP4 (50 ng/mL) for 6 h, 24 h, 48 h, and 72 h. Scale bar represents 100 μ m.

4. Add growth media (e.g., mTeSR1) to 3 $\times$ the volume of TrypLE (750 μ L per well), and briefly pipette the suspension up and down against the base of the plate to break up remaining clumps. Wash off any cells still stuck to the tray.
5. Pipette cell suspension into a screw-cap tube, pooling cells that have been grown under the same conditions (6 wells = 6 mL). Immediately add fresh, methanol-free formaldehyde to a final concentration of 1% (375 μ L of 16% stock) and shake to mix.

⚠ **CRITICAL:** Add fresh media to the cells in TrypLE without pelleting and resuspension before adding formaldehyde, to minimize the time that cells spend in suspension before fixation, in order to prevent changes in expression profiles.

⚠ **CRITICAL:** Do not add formaldehyde directly to the 6-well culture plate, as it would result in very heterogeneous shearing patterns with both high and low molecular weight fragment smears. This may be due to cells in a dense colony potentially having different exposure to the fixative depending on their position within the colony, or it may be a result of heterogeneous clumps of cells in the sonication reaction since fixed colonies become very difficult to lyse and dissociate. For this reason, we use dissociation prior to fixation.

6. Place on rocker at room temperature (20°C–21°C) for 10 min.

⚠ **CRITICAL:** The duration of fixation before quenching is important, as over- or under-fixation will affect the results. See [Troubleshooting 4](#).

7. Quench the formaldehyde by adding 1:10 volume (640 μ L) of cold 1.25 M Glycine. If the growth media contains phenol red, the suspension should become bright yellow. Let it rock at room temperature (20°C–21°C) for 5 min.

Note: After this step, cells should always be kept on ice unless otherwise indicated.

8. Pellet cells by spinning at 760 $\times$ g for 5 min at 4°C. Carefully tip off supernatant and resuspend pellet in an equivalent volume of cold DPBS with gentle pipetting (6 mL).
9. Pellet cells by spinning at 760 $\times$ g for 5 min at 4°C. Repeat wash step with cold DPBS (6 mL).
10. Remove supernatant and resuspend pellet in 1 mL of cold DPBS with gentle pipetting, then transfer to a 1.5 mL microfuge tube on ice.
11. Take 5 μ L of cell suspension and perform a cell count to determine concentration. You may need to dilute this sample to bring the concentration within a range to be accurately counted.

Note: The cell count (and subsequent aliquots of 3E+06 cells) are to control the concentration of the cell suspension used in the sonication reaction, as cell density can affect the efficiency of shearing. In this protocol, we also use cell count to determine an equal input between samples as a simpler option in comparison to measuring the specific amount of chromatin. Freezing a

defined number of cells per aliquot can also make it easy to thaw exactly enough cells for your experiment.

12. From your 1 mL suspension, pipette aliquots of 3E+06 cells into 1.5 mL microfuge tubes on ice.

Note: DNA yield from a ChIP experiment is dependent on various things, such as the efficacy of the primary antibody and the abundance and activity of your protein of interest – a ChIP for a modified histone, for example, will yield a lot more DNA than a ChIP for a transcription factor with relatively few binding sites. We have recommended 3E+06 cells as a default amount in this protocol because this gave at least 1 ng of DNA for all of the transcription factors we tested (SMAD1, SMAD2, GATA3), which is a good input amount for sequencing library generation. For highly abundant targets (e.g., pluripotency transcription factors POU5F1, SOX2, NANOG) we speculate that 1E+06 or even 5E+05 cells will still yield sufficient DNA.

13. Pellet cells by spinning at 380 × g for 5 min at 4°C. Remove the supernatant with a pipette, taking care not to disturb the pellet.
14. Resuspend cells in 300 µl of High Salt Lysis/Sonication Buffer with freshly added Protease Inhibitor Cocktail.
15. Snap freeze cells in a dry ice and ethanol bath, and store at –80°C.

Note: Snap freezing helps preserve the integrity of the samples by preventing ice crystal formation.

▮▮ **Pause Point:** Snap-frozen fixed cells may be kept at –80°C for several months.

Shearing and Immunoprecipitation of Chromatin

⌚ **Timing:** 3 days

Figure 3 This part of the protocol will shear the fixed chromatin into a size-range suitable for sequencing, then use a specific antibody to pulldown fragments of chromatin that are cross-linked to your protein of interest. The crosslinks will then be reversed and the DNA fragments purified.

16. Day 1: Shearing of chromatin and incubation with primary antibody
 - a. Incubate primary antibody with Dynabeads (Protein G or Protein A, depending on the species of your primary antibody) by doing the following:
 - i. Resuspend Dynabead slurry with pipetting and gentle swirling as beads will have settled out during storage.
 - ii. Aliquot 20 µl of Dynabead slurry per ChIP and place in a microfuge tube (0.2 mL or 0.5 mL)
 - iii. Add 2–5 µg of primary antibody per ChIP to the Dynabeads and add Chromatin Dilution Buffer to a final volume of 40 µl
 - iv. Incubate with rotation or gentle agitation at room temperature (20°C–21°C) for 3 h.

Note: 20 µl of Protein G Dynabeads has a binding capacity of roughly 4.8 µg of IgG. Check the binding capacity and slurry concentration of your Dynabeads stock to confirm.

Note: Incubating the antibody with the Dynabeads prior to incubation with the chromatin sample (a Direct IP) is recommended for conserving antibody. For lower affinity antibodies, it may help to incubate the sample with the antibody first, followed by the Dynabeads (an Indirect IP). See [Troubleshooting 4](#) for more details.

Part 2: Shearing + ChIP (3 days)

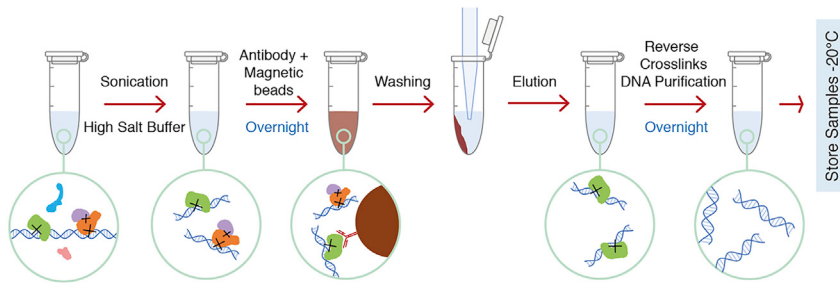


Figure 3. Schematic Summary of Part 2: Shearing and Immunoprecipitation of Chromatin, Which Starts with Thawed Formaldehyde-Crosslinked Samples and Ends with Purified, ChIP-ed DNA that Can Be Stored at -20°C

- △ **CRITICAL:** The amount of primary antibody required for good signal:noise in a ChIP is usually 2–5 μg , but this varies and may need to be tested and optimized for each antibody. See [Troubleshooting 4](#).
- b. Thaw 1 aliquot of $3\text{E}+06$ fixed cells per ChIP experiment on ice for at least 30 min. While samples are thawing, start the Diagenode Bioruptor and water bath system to give it time to cool to 4°C .
- c. Transfer the cell aliquot to a Diagenode TPX tube (1.5 mL) on ice for sonication.
- d. Sonicate samples in the Diagenode Bioruptor for 30 s on, 30 s off on High. We have found 10–12 cycles to be best for a size range of 100 bp to 500 bp from $3\text{E}+06$ cells.
- △ **CRITICAL:** The optimal number of cycles can vary with sonicator make and model, so it is highly recommended that this is tested first. The range of chromatin fragment sizes is critical for good coverage and resolution of data, without losing material due to over-fragmentation. See [Troubleshooting 3](#).
- e. Add 1 mL of cold Chromatin Dilution Buffer with fresh Protease Inhibitor Cocktail to each sonicated sample, and spin at $13,600 \times g$ for 30 min at 4°C . This will pellet any insoluble material.
- f. Remove the supernatant containing soluble chromatin and place in a protein low-bind tube (2 mL).
- g. Take 65 μl of soluble chromatin as a 5% Input sample and store at -20°C .

Note: 5% Input will provide plenty of chromatin for all tests described in this protocol, but a larger or smaller input sample could also be taken as required.

Note: It may be necessary to check the fragmentation pattern of the chromatin prior to continuing with the IP. In this case, snap freeze the remaining soluble chromatin sample as in step 15 and continue with the 5% Input sample from steps 17g–18e before checking the fragmentation as described in Test 1 (step 19). Then thaw the chromatin sample on ice before continuing with the protocol. However, if the shearing by sonication has been optimized and appears to be highly reproducible (as we have found), it is recommended to avoid subjecting the chromatin to an extra freeze/thaw cycle.

- h. Add Dynabead + Primary Antibody mix to the soluble chromatin and incubate at 4°C for at least 12 h, with rotation such that the samples mix and the Dynabeads remain suspended (e.g., 15–20 rpm on a circular rotator).
- 17. Day 2: Washing and Elution
 - a. Remove the unbound supernatant from the beads ([Figure 4](#)).

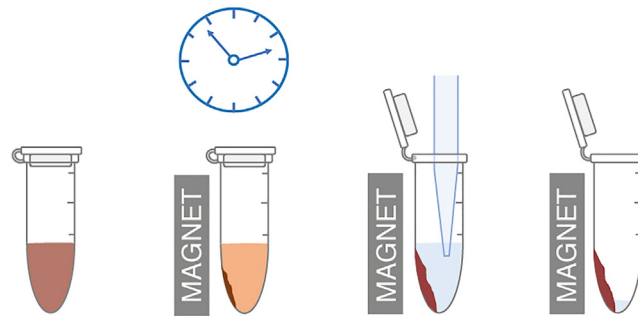


Figure 4. Schematic Showing Separation and Washing of Magnetic Beads with Bound Antibody and Protein-DNA Complexes

When placed in a magnetic holder for approximately 2 min, beads will separate to the side of the tube eventually leaving a clear supernatant. While leaving the tube in the magnetic holder, the supernatant can be removed with a pipette without touching the beads. The next wash should be added immediately to avoid drying the beads.

- i. Place the tubes in the magnetic holder and let sit for 2 min or until all the Dynabeads have separated to the edge of the tube.
- ii. While keeping the tubes in the holder and without touching the beads, carefully remove the supernatant with a pipette.
- b. Wash by adding 1 mL of Wash Buffer to the beads before removing the tubes from the holder and incubating with rotation at 4°C for 5 min, for the following washes:
 - i. Wash Buffer A
 - ii. Wash Buffer B
 - iii. Wash Buffer C
 - iv. TE Buffer (first wash)
 - v. TE Buffer (second wash)
- c. Remove the final wash and add 100 μ L of Elution Buffer to the beads. Briefly vortex to resuspend, then incubate at 65°C for 5 min.

Note: For this step, a water bath or heating block can be used as an alternative to an incubator or oven.

Note: . Incubating for 30 min at 65°C would likely also work, although we would still recommend performing two rounds of elution to improve yield.

- d. Incubate samples at room temperature (20°C–21°C) for a further 15 min with rotation or agitation.
 - e. Separate the Dynabeads using the magnetic holder and remove the eluate to a new 1.5 mL tube.
 - f. Add a fresh aliquot of 100 μ L of Elution Buffer and repeat steps 17c to 17e. Pool the first and second round eluates together (final volume is 200 μ L).
 - g. Add 135 μ L of Elution Buffer to the 65 μ L Input sample from Day 1 (final volume is 200 μ L).
 - h. For both Input and ChIP samples, increase NaCl to 160 mM (add 8 μ L of 4 M stock) and add RNase A to a final concentration of 20 μ g/mL (add 0.4 μ L of a 10 mg/mL stock). Briefly vortex to mix.
 - i. Incubate samples at 65°C for at least 8 h to reverse crosslinks and digest any contaminating RNA.
18. Day 3: Protein Digestion and DNA Purification
- a. For both Input and ChIP samples, increase EDTA concentration to 5 mM (add 2 μ L of 0.5 M stock) and add Proteinase K to a final concentration of 200 μ g/mL (add 2 μ L of 20 mg/mL stock).
 - b. Incubate at 45°C for 2 h to digest proteins.
 - c. Purify ChIP and Input samples using a Zymo ChIP DNA Clean & Concentrator Kit and following manufacturer's instructions. Elute in 26 μ L of Kit Elution Buffer.

Part 3: Analysis of Sample Quality (0.5-1 day)

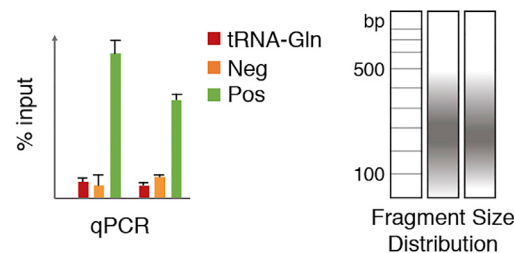


Figure 5. Schematic Summary of Part 3: Checking Quality of ChIP Samples

These steps use a minimal amount of purified, ChIP-ed DNA. The remaining sample can be used to generate sequencing libraries and then sequenced.

- d. Take 0.5 - 1 μ L from each sample for quantification of DNA concentration using a Nanodrop or Qubit fluorometer or equivalent technology.

Note: The concentration of ChIP DNA will vary depending on your antibody and protein of interest but is commonly <2 ng/ μ L. The Input DNA will be many times higher, roughly in the range of 50 ng/ μ L.

- e. Store eluted samples at -20°C .

▮ **Pause Point:** DNA samples may be kept long-term at -20°C or -80°C .

Checking Quality of ChIP Samples

⌚ **Timing:** 0.5–1 day

Figure 5 Before committing to the time and expense of sequencing, it is best to check the quality of your samples for (1) size range of DNA fragments, (2) relative enrichment of areas of DNA known to be bound by your protein of interest, and (3) non-specific enrichment of other areas, including highly transcribed regions that have previously been reported to be enriched by some antibodies ('hyper-ChIPable' regions). These tests will consume a minimal amount of your purified DNA.

19. Test 1: Chromatin Fragmentation

Note: It is important that crosslinks have been reversed and chromatin has been purified before assessing fragmentation patterns, as DNA migration through the gel can be affected

- a. Analyse the fragment size distribution of the purified chromatin samples by taking 1–2 μ L from each sample and running it on an Agilent TapeStation according to manufacturer's instructions ([Figure 6](#)).

Alternatives: Take 5 μ L from the Input sample and run it on a 1.8% Agarose electrophoresis gel at 100 V or less. Make sure to use a DNA ladder with sufficient resolution at the <500 bp range (e.g., NEB 1 kb+ DNA Ladder).

⚠ **CRITICAL:** The majority of DNA should fall between 100 and 500 bp for good resolution in ChIP-seq. See [Troubleshooting 3](#).

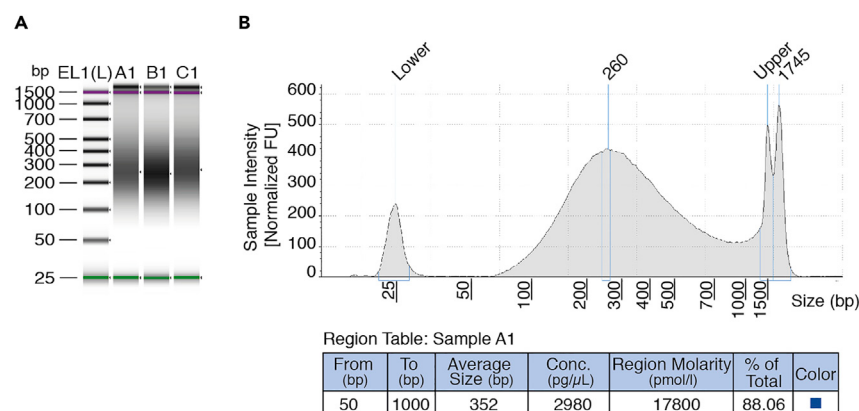


Figure 6. Example Sonicated Chromatin (Input Samples) from H1 hESC Cultured in mTeSR1 on Matrigel, Separated on an Agilent TapeStation System with a High Sensitivity D1000 ScreenTape

Both the pseudo-gel image (A) and the plot of signal intensity vs predicted fragment size (B) show that the majority of the DNA lies in the 100 bp to 500 bp range, with an intensity peak at 260 bp and an average size of 352 bp for signal over the 50–1,000 bp range. Upper (1,500 bp) and Lower (25 bp) size markers are indicated.

20. Test 2: qPCR of Positive and Negative Control Sites

In order to assess the success of the ChIP, it is best if at least one canonical binding site for your protein of interest is known – if not, it is still possible to test that negative control regions are not significantly enriched (ideally C_T value of >30), and Test 3 should still be performed.

- Take 5 μl of the Input Sample and add it to 20 μl of nuclease-free water to make a diluted Input sample that is now roughly 1% of the original total input. This will make analysis easier later.
- Set up a QPCR assay in triplicate using your positive control primers and negative control primers (see Table 1 for reaction mix; note that the amount of sample per reaction can be increased if enrichment is low), for:
 - ChIP sample(s)
 - Diluted 1% Input sample
- Run the assay according to the recommendations of the manufacturer of the qPCR Machine and SYBR Green Mastermix.
- Calculate signal of each product for each ChIP sample as a percentage of total input chromatin using the following equations (primer amplification coefficient is estimated as 2):

$$C_T(\text{Input}) = C_T(1\% \text{ Input}) - \log_2(100)$$

$$\Delta C_T = C_T(\text{Input}) - C_T(\text{ChIP})$$

$$\% \text{ of Input} = 2^{\Delta C_T} \times 100$$

- Compare the enrichment of positive and negative control regions.

Note: The positive control amplicon should be more enriched than the negative control amplicon. For good quality ChIP-seq data, we recommend aiming for a positive: negative ratio (% Input of Positive Region / % Input of Negative Region) of at least 10. Additionally, the negative control ChIP sample reaction should ideally have a C_T value of >30, whereas we expect a 1% Input reaction C_T in the range of 22 - 25, and the positive control ChIP sample reaction C_T ideally <27. See Troubleshooting 4.

Table 1. A qPCR Reaction Mix, to be Made in Triplicate

Reagent	Amount in 1 Reaction (10 μ L)
Sample	0.5 μ L
5 μ M Primer F	1 μ L
5 μ M Primer R	1 μ L
SYBR Green Mastermix	5 μ L
Nuclease-free Water	2.5 μ L

21. Test 3: qPCR of a Highly Transcribed tRNA Gene

We have found that enrichment of highly transcribed regions such as tRNA genes is an issue for some primary antibodies, which has also been reported in the literature (Teytelman et al., 2013) (Park et al., 2013). Indeed, in a prior experiment we discovered that up to 80% of our sequencing reads were mapped to tRNA regions, to the extreme detriment of signal at other sites. We therefore recommend testing for enrichment relative to a negative control at least once for every new antibody used for ChIP. This is important because not only will these regions provide a false impression of the target protein binding profile, this enrichment will consume sequencing reads and reduce signal from sites that are legitimately bound.

- Set up a qPCR assay in triplicate using tRNA-Gln Primers (see Table 1 for reaction mix), for ChIP sample
- Diluted 1% Input sample (test 2, step 20a)
- Run the assay according the recommendations of the manufacturer of the qPCR Machine and SYBR Green Mastermix
- Calculate signal of tRNA for each ChIP sample as a percentage of input chromatin as in test 2, step 20d
- Compare to % enrichment of positive and negative control regions from Test 2 (Figure 7)

△ CRITICAL: Levels of the tRNA-Gln amplicon should be similar to levels of the negative control amplicon. Additionally, the tRNA-Gln ChIP sample reaction should ideally have a C_T value of >30 . If not, see Troubleshooting 5.

EXPECTED OUTCOMES

The expected outcome is ChIP-ed and purified genomic DNA fragments that are expected, based on experimental tests performed as part of this protocol, to yield good quality ChIP-seq data. The next steps required prior to sequencing, including library preparation and quality control, are not covered in this protocol.

DNA Fragment Size Distribution

To provide good sequencing coverage of the genome and resolution of binding sites, it is important to have the majority of DNA fragments in the range of 100–500 bp for ChIP-seq. We have found that by dissociating hESCs and using the High Salt Lysis/Sonication Buffer, we can consistently and efficiently produce fragments in this range when shearing by sonication (Figure 6).

Specific Enrichment of Bound Sites

This protocol provides methods to test the relative enrichment of a site expected to be bound by the protein of interest (positive control) in comparison to a site that is not expected to be bound (negative control) and a site that can potentially be highly enriched in a non-specific manner (tRNA-Gln). This outcome will depend on the binding affinity and specificity of the particular primary antibody used, as well as the relative abundance of the protein of interest. An ideal result would show very

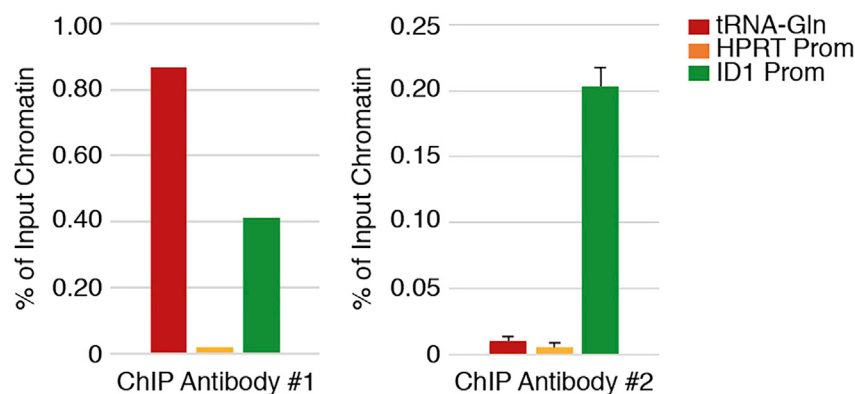


Figure 7. An Example qPCR of ChIP Samples Using Different Antibodies against SMAD1 in H1 hESCs Treated with BMP4 (50 ng/mL) for 1 h

ID1 Promoter (green) is the positive control region, HPRT Promoter (yellow) is the negative control region, tRNA-Gln (red) is a highly transcribed region that is significantly enriched by the first antibody (SMAD1, Cell Signaling Technology #6944) but not the second (phospho-SMAD1/5/9, Cell Signaling Technology #11971). Left histogram shows signal as % Input for $n = 1$ sample, right histogram shows mean signal as % Input for $n = 3$ biological replicates, error is standard deviation.

low levels of negative control and tRNA-Gln products ($C_T > 30$) and a positive: negative ratio of at least 10 (Figure 7). See Troubleshooting 4 and 5 for approaches to improve this result.

LIMITATIONS

This protocol has been used successfully for ChIP-seq of several transcription factors (including SMAD1, SMAD2, GATA3) from pluripotent and differentiated hESC cultures, and should also work for ChIP of other proteins such as histones. However, some optimization by the user for their specific experiment is still required. The number of cycles of sonication required to achieve an optimal fragment size range will vary with the model and equipment setup of the sonicator (see Troubleshooting 3). The specificity of enrichment and ratio of positive: negative enrichment will vary with the primary antibody used, and the abundance and biology of the protein of interest (see Troubleshooting 4).

Additionally, we see consistent results from chromatin shearing of hESCs that have been differentiated for up to 3 days with BMP4 (50 ng/mL). While we have no experience with other differentiation protocols, we suspect that this also holds true for hESCs that have been differentiated with other methods. We also anticipate that this protocol could be applied to human iPSC cultures. We have not tested this protocol with ESC cultures from other species, which have similar morphology to hESC but have various differences in their biology and culture conditions (Schnerch et al., 2010).

TROUBLESHOOTING

Problem 1

The cell culture is overgrown, or too few cells are obtained for the ChIP.

Potential Solution

Overgrown cultures will be more difficult to dissociate to single cells, and may also affect the biology of your protein of interest. If your differentiation protocol requires multiple days of culture, seed the pluripotent stem cells at Day 0 such that they form sparse and relatively small colonies with plenty of room to expand. See Figure 2 for examples of ideal cultures of cells differentiated with BMP4.

If your cultures are not providing enough cells for a ChIP experiment, consider scaling up to a larger culture vessel or pooling multiple cultures together rather than seeding more cells and risking overgrowth of cultures.

Problem 2

The cell culture does not dissociate in 4–5 min.

Potential Solution

Pluripotent cells will take different amounts of time to dissociate with TrypLE depending on the size of the colonies – large, dense colonies will take longer. The dissociation time will also be different if the cells are differentiated and hence have altered morphology and cell-cell and cell-matrix interactions. We recommend checking the cells every few minutes and tapping the edge of the tray to promote dissociation.

Problem 3

The chromatin fragmentation pattern is not within the optimal size range.

Potential Solution

The number of cycles of sonication required to produce fragments in the 100 bp to 500 bp range is likely to vary depending on the sonicator model and setup. It is highly recommended to run a test of shearing efficiency prior to performing the full ChIP-seq protocol. An example optimization protocol is as follows:

1. Perform 'Fixation of Cells' protocol as listed (15 steps) to produce fixed, snap-frozen aliquots of cells in Lysis/Sonication Buffer
2. Follow 'Shearing and Immunoprecipitation of Chromatin' steps 16b and 16c
3. Sonicate the sample for 30 s ON, 30 s OFF on High for 5 cycles
4. After 5 cycles of on/off sonication, pulse vortex the samples to mix and remove 5 μ L of chromatin.
5. Repeat steps 3 and 4 until reaching 30 cycles total (6 $\times$ 5 μ L samples)
6. Add 195 μ L Elution Buffer or TE Buffer to each sample (final volume 200 μ L)
7. Follow 'Shearing and Immunoprecipitation of Chromatin' steps 17h–18b to reverse crosslinks, remove RNA and digest proteins.
8. Purify samples using a Zymo ChIP DNA Clean & Concentrator Kit and following manufacturer's instructions. Elute in 20 μ L of Kit Elution Buffer.
9. Take 10 μ L from each eluted sample and run it on a 1.8% Agarose electrophoresis gel at 100 V or less. Make sure to use a DNA ladder with sufficient resolution at the <500 bp range (e.g., NEB 1 kb+ DNA Ladder). Visualize the DNA distribution and determine the lowest number of cycles required to produce a range of 100–500 bp ([Figure 8](#)).

If your sample requires greater than 10 cycles of sonication, it is recommended to pause and briefly vortex after each 10 cycles in order to make sure that the sample stays homogenous.

Problem 4

The enrichment of the positive control region is too low (< 10-fold above negative control region).

Potential Solution

There are several reasons why enrichment may appear to be low.

Chromatin fragments are over-sheared: If fragments are too small, they may be lost during the column purification step. Additionally, if samples heat up or boil during sonication, crosslinks may be reversed (inspect the sonicated samples for foaming). See [Troubleshooting 3](#) for a strategy to optimize shearing and make sure your samples are kept cold during sonication.

Background enrichment is high: If the negative control ChIP qPCR is shows relatively high enrichment ($C_T < 29$), the ratio of signal to noise (positive: negative) will be lower. This may be due to non-specific binding by the antibody, in which case lowering the amount of antibody used may improve the positive: negative ratio. Altering wash conditions, for example extending wash duration or increasing salt concentration in wash buffers could also improve background.

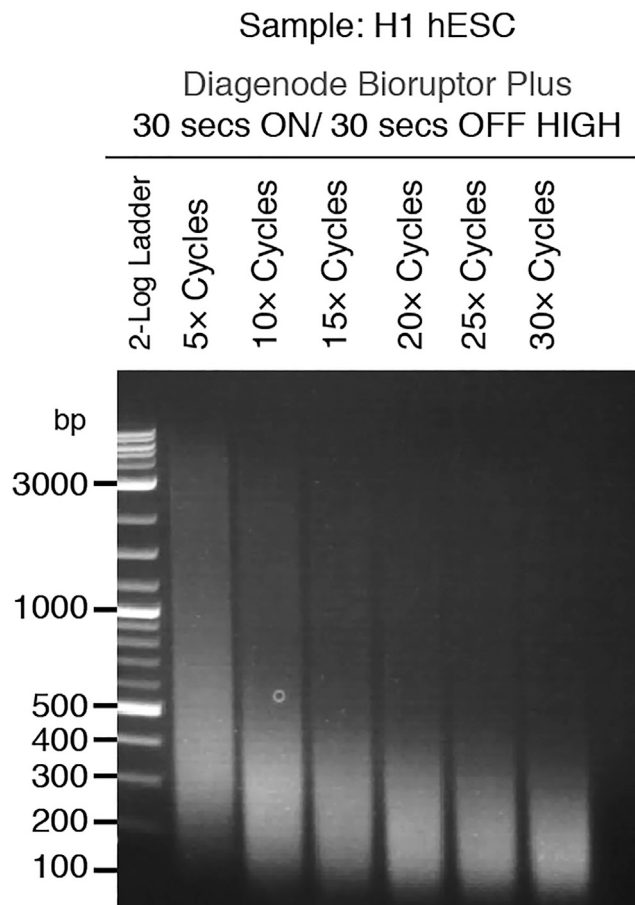


Figure 8. An Example of a Sonication Optimization Experiment, Where Fixed H1 hESCs Were Sheared Using a Bioruptor Plus Sonication System for the Indicated Number of Cycles of 30 s ON/30 s OFF

Sheared DNA was then purified and separated on a 1.8% Agarose Gel. 10 was determined to be the minimum number of cycles required to shear the majority of chromatin into the 100 bp to 500 bp range.

Antibody binding affinity is low: Try increasing the amount of antibody used, for example, to 10 μ g. The amount of Dynabeads used should also be increased accordingly – check the binding capacity of your stock, but as a general rule we use 20 μ L of Dynabead suspension for 5 μ g of antibody. It is also recommended that for lower affinity antibodies, the chromatin sample is incubated with the antibody prior to addition of the Dynabeads. Specifically, skip step 16a of this protocol and add the primary antibody directly to the chromatin sample at step 16h. After incubating for at least 12 h at 4°C, add the Dynabeads to the antibody and chromatin mixture and incubate with rotation at 4°C for at least 1 h. Proceed to step 17a.

Target is low abundance: Try increasing the number of cells used in a ChIP, for example by 2–3 times. Remember that the concentration and volume of cells used in sonication will likely affect the shearing efficiency, so be prepared to re-optimize the number of rounds of sonication required. If you instead choose to shear and pool multiple 300 μ L aliquots of 3E+06 cells in High Salt Lysis/Sonication Buffer, be sure to dilute accordingly with Chromatin Dilution Buffer (at the recommended ratio of 300 μ L High Salt Lysis/Sonication Buffer to 1 mL Chromatin Dilution Buffer) – this step dilutes the levels of salt in the Sonication Buffer, which otherwise may affect antibody binding.

If the target is a post-translationally modified protein, it is recommended to add inhibitors against de-modification enzymes (e.g., phosphatase inhibitors) in addition to Protease Inhibitors to the High Salt Lysis/Sonication Buffer and Chromatin Dilution Buffer.

Table 2. A Resources Table for ChIP of phospho-SMAD1/5/9

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
pSMAD1/5/9 (rabbit, monoclonal)	Cell Signaling Technology	11971; RRID: AB_2797785
Normal Rabbit IgG	Cell Signaling Technology	2729; RRID: AB_1031062
Oligonucleotides		
hs ID1 Prom F: AGCTAGACCAGTTTGTCTCTC	N/A	hs ID1 Prom F
hs ID1 Prom R: TCATAAAACCCGGACGGACTG	N/A	hs ID1 Prom R
hs HPRT Prom F: TGTTTGGGCTATTTACTAGTTG	N/A	hs HPRT Prom F
Hs HPRT Prom R: ATAAATGACTTAAGCCCAGAG	N/A	hs HPRT Prom R

Fixation time is non-optimal: 10 min fixation time at room temperature (20°C–21°C) worked well for all motif-specific transcription factors we tested with this protocol, so we recommend it as a default duration. However, optimal fixation time can be specific to the biology of the cells and protein of interest; longer fixation times may improve capture of protein complexes on the DNA, but will also risk increasing non-specific interactions (Baranello et al., 2016) or potentially mask protein antigens. Try different fixation times between 5 and 15 min. Be aware that this will also affect chromatin shearing efficiency, so be prepared to re-optimize the number of rounds of sonication required.

Problem 5

Enrichment of tRNA genes is too high, or possibly expected to be a legitimate site of binding.

Potential Solution

If enrichment of tRNA-Gln is many times higher than the negative control region, this is a sign that the primary antibody may be non-specifically enriching highly transcribed regions. Alternatively, you may suspect that the protein of interest has a genuine role in regulating tRNA expression. In either scenario, a good option is to try to repeat the ChIP with a different primary antibody against the protein of interest. If the enrichment is non-specific, there is a chance that a different antibody will not be susceptible (Figure 7). If the enrichment is consistent between antibodies and also within a similar range as the positive control region, it may be genuine.

Additionally, if you have not done so already, consider performing a control ChIP in parallel – if possible, using the same antibody but with a hESC line that does not express your protein of interest. This will allow you to correct for non-specific enrichment at the analysis stage.

Problem 6

The protocol may not be working for my antibody/protein of interest

Potential Solution

Unfortunately, not all antibodies are useful for ChIP – a good ChIP antibody needs to recognize the folded protein (unlike in Western Blot) that has been cross-linked (unlike in native IP). To verify that the protocol is otherwise working as intended, we recommend performing a ChIP with an antibody and protein of interest that is known to give good results; for example, here are the details for performing a ChIP for phospho-SMAD1 in BMP4-treated hESCs. Note that for this experiment, a negative control ChIP can be either (1) a ChIP using a control type-matched IgG antibody or (2) a ChIP using the pSMAD1/5/9 antibody with hESCs that have not been treated with BMP4. See Figure 7 (right histogram) for an example of qPCR from ChIPs with these conditions, and Table 2 for details of reagents.

Cultures: H1 hESC (small colonies) treated with 50 ng/mL BMP4 in mTeSR1 for 1 h
3E+06 cells per ChIP
5 μ g Primary Antibody or Control IgG per ChIP (pSMAD1/5/9)
Positive control qPCR Primers: hs ID1 Prom
Negative control qPCR Primers: hs HPRT Prom

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Silvia Santos (silvia.santos@crick.ac.uk).

Materials Availability

This study did not generate any new unique reagents.

Data and Code Availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

A.E.S. optimized the protocol and wrote the manuscript. S.D.M.S. edited the manuscript and supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Baranello, L., Kouzine, F., Sanford, S., and Levens, D. (2016). ChIP bias as a function of cross-linking time. *Chromosome Res.* 24, 175–181.
- Gunne-Braden, A., Sullivan, A., Gharibi, B., Sheriff, R.S.M., Maity, A., Wang, Y.-F., Edwards, A., Jiang, M., Howell, M., Goldstone, R., et al. (2020). GATA3 mediates a fast, irreversible commitment to BMP4-driven differentiation in human embryonic stem cells. *Cell Stem Cell* 26, 693–706.e9.
- Kurtenbach, S., Reddy, R., and Harbour, J.W. (2019). ChIPprimersDB: a public repository of verified qPCR primers for chromatin immunoprecipitation (ChIP). *Nucleic Acids Res.* 47, D46–D49.
- Orozco-Fuentes, S., Neganova, I., Wadkin, L.E., Baggaley, A.W., Barrio, R.A., Lako, M., Shukurov, A., and Parker, N.G. (2019). Quantification of the morphological characteristics of hESC colonies. *Sci. Rep.* 9, 17569.
- Park, D., Lee, Y., Bhupindersingh, G., and Iyer, V.R. (2013). Widespread misinterpretable ChIP-seq bias in yeast. *PLoS One* 8, e83506.
- Rodríguez, A., Rodríguez, M., Córdoba, J.J., and Andrade, M.J. (2015). Design of primers and probes for quantitative real-time PCR methods. *Methods Mol. Biol.* 1275, 31–56.
- Schnerch, A., Cerdan, C., and Bhatia, M. (2010). Distinguishing between mouse and human pluripotent stem cell regulation: the best laid plans of mice and men. *Stem Cells* 24, 175–181.
- Teytelman, L., Thurtle, D.M., Rine, J., and van Oudenaarden, A. (2013). Highly expressed loci are vulnerable to misleading ChIP localization of multiple unrelated proteins. *Proc. Natl. Acad. Sci. U S A* 110, 18602–18607.
- Thomson, J.A. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145–1147.