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.
Protocol

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

Adrienne E. Sullivan¹,² and Silvia D.M. Santos¹,³,∗

¹Quantitative Cell Biology Laboratory, The Francis Crick Institute, London NW1 1AT, UK
²Technical Contact
³Lead Contact
∗Correspondence: silvia.santos@crick.ac.uk
https://doi.org/10.1016/j.xpro.2020.100062

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 Ominbus; 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 x) 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 (100x) 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 | DS205 |
| 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: GACCCAGTCTGCTGGTTCAGG | 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)
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
· 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)

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

#### 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) | 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                     |
| ddH2O                                           | n/a                 | To 100 mL                 |
| Protease Inhibitor Cocktail*                     | 1 x                 |                           |

*Add fresh before use

| Chromatin Dilution Buffer (Store at 4°C) | 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                     |
| ddH2O                                   | n/a                 | To 100 mL                 |
| Protease Inhibitor Cocktail*            | 1 x                 |                           |

*Add 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% w/v              | 1 mL                      |
| SDS                            | 0.1% w/v            | 0.1 g                     |
| Sodium deoxycholate            | 0.1% w/v            | 0.1 g                     |
| ddH2O                          | 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% w/v              | 1 mL                      |
| SDS                            | 0.1% w/v            | 0.1 g                     |
| Sodium deoxycholate            | 0.1% w/v            | 0.1 g                     |
| ddH2O                          | 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                     |
| ddH2O                          | 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                    |
| ddH2O                          | 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*                           | 1% w/v              | 0.2 g                     |
| ddH2O                          | n/a                 | To 20 mL                  |

*Add 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*. 

---

**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**

---

STAR Protocols 1, 100062, September 18, 2020 7
4. Add growth media (e.g., mTeSR1) to 3× the volume of TrypLE (750 μl 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 μL 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 μl) 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 × 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 × 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 μl 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 pull down 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)**

### b. Thaw 1 aliquot of 3E+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 3E+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 x 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).
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 µl 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 µl of Elution Buffer and repeat steps 17c to 17e. Pool the first and second round eluates together (final volume is 200 µl).

g. Add 135 µl of Elution Buffer to the 65 µl Input sample from Day 1 (final volume is 200 µl).

h. For both Input and ChiP samples, increase NaCl to 160 mM (add 8 µl of 4 M stock) and add RNase A to a final concentration of 20 µg/mL (add 0.4 µl 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 µl of 0.5 M stock) and add Protease K to a final concentration of 200 µg/mL (add 2 µl 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 µl of Kit Elution Buffer.
d. Take 0.5 - 1 μl 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/μL. The Input DNA will be many times higher, roughly in the range of 50 ng/μL.

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 μl from each sample and running it on an Agilent TapeStation according to manufacturer’s instructions (*Figure 6*).

*Alternatives:* Take 5 μl 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**.
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 $CT$ value of >30), and Test 3 should still be performed.

a. 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.

b. 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:

i. ChIP sample(s)
ii. Diluted 1% Input sample

c. Run the assay according the recommendations of the manufacturer of the qPCR Machine and SYBR Green Mastermix.

d. 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):

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

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

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

e. 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 $CT$ value of >30, whereas we expect a 1% Input reaction $CT$ in the range of 22 - 25, and the positive control ChIP sample reaction $CT$ ideally <27. See Troubleshooting 4.
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

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

| Reagent                  | Amount in 1 Reaction (10 µL) |
|--------------------------|------------------------------|
| Sample                   | 0.5 µL                       |
| 5 µM Primer F            | 1 µL                         |
| 5 µM Primer R            | 1 µL                         |
| SYBR Green Mastermix     | 5 µL                         |
| Nuclease-free Water      | 2.5 µL                       |

Reagents are mixed and prepared as follows:

- 0.5 µL of sample
- 5 µM of each primer
- 5 µL of SYBR Green Mastermix
- 2.5 µL of nuclease-free water

For each qPCR reaction:
low levels of negative control and tRNA-Gln products (CT >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 µL of chromatin.
5. Repeat steps 3 and 4 until reaching 30 cycles total (6 x 5 µL samples)
6. Add 195 µL Elution Buffer or TE Buffer to each sample (final volume 200 µL)
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 µl of Kit Elution Buffer.
9. Take 10 µl 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.
Antibody binding affinity is low: Try increasing the amount of antibody used, for example, to 10 μg. 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 μL of Dynabead suspension for 5 μg 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 μL 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 μL 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.
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.

---

**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: AGCTAGACCAAGTTGTCGTC | N/A | hs ID1 Prom F |
| hs ID1 Prom R: TCATAAAACCCGGACGGACTG | N/A | hs ID1 Prom R |
| hs HPRT Prom F: TGTTTGGCATTATCTAGTG | N/A | hs HPRT Prom F |
| Hs HPRT Prom R: ATAAAAATGACCTAAGCCCAGAG | N/A | hs HPRT Prom R |
Cultures: H1 hESC (small colonies) treated with 50 ng/mL BMP4 in mTeSR1 for 1 h
3E+06 cells per ChIP
5 µg Primary Antibody or Control IgG per ChIP (pSMAD1/5/9)
Positive control qPCR Primers: hs ID1 Prom
Negative control qPCR Primers: hs HPRT Prom

RESOURCES 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.

ACKNOWLEDGMENTS

The authors would like to acknowledge and thank the Advanced Sequencing Facility at the Francis Crick Institute, in particular Deborah Jackson and Robert Goldstone. We also thank Stephan Sauer from Lymphocyte Development Lab at the London Institute of Medical Sciences (LMS) and Philip East from BABS at the Francis Crick Institute for valuable advice. Work in the Santos lab at the Francis Crick Institute is supported by the Cancer Research UK (FC001596), the Medical Research Council (FC001596) and the Wellcome Trust (FC001596).

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). ChiPyrmsgDB: 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., Bhupindesinhg, G., and Iyer, V.R. (2013). Widespread misinterpretable ChiP-seq bias in yeast. PLoS One 8, e83506.

Rodriguez, A., Rodriguez, M., Cordoba, J.J., and Andrade, M.J. (2015). Design of primers and probes for quantitative real-time PCR methods. Methods Mol. Biol. 1275, 31–56.

Schnerrch, 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.