High-throughput chromatin immunoprecipitation for genome-wide mapping of *in vivo* protein-DNA interactions and epigenomic states

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Dynamic protein binding to DNA elements regulates genome function and cell fate. Although methods for mapping *in vivo* protein-DNA interactions are becoming crucial for every aspect of genomic research, they are laborious and costly, thereby limiting progress. Here we present a protocol for mapping *in vivo* protein-DNA interactions using a high-throughput chromatin immunoprecipitation (HT-ChIP) approach. By using paramagnetic beads, we streamline the entire ChIP and indexed library construction process: sample transfer and loss is minimized and the need for manually labor-intensive procedures such as washes, gel extraction and DNA precipitation is eliminated. All of this allows for fully automated, cost effective and highly sensitive 96-well ChIP sequencing (ChIP-seq). Sample preparation takes 3 d from cultured cells to pooled libraries. Compared with previous methods, HT-ChIP is more suitable for large-scale *in vivo* studies, specifically those measuring the dynamics of a large number of different chromatin modifications/transcription factors or multiple perturbations.

INTRODUCTION

Transcription factors (TFs) are key players in regulation of gene expression and cellular outcome[1]. Binding of TFs to DNA determines both long-term gene expression programs, such as development and differentiation, and immediate programs, such as responses to environmental changes[1–4]. One of the major challenges in genomics is to understand how different TF networks bind in a dynamic fashion to specific *cis* elements in the genome to regulate gene expression in diverse cells and conditions[5]. A variety of approaches are commonly used to decipher the principles of gene regulatory networks. These approaches include studies of TF networks in simple model organisms[6–8], small-scale *in vitro* experiments[9–11] and genome-wide genetic approaches[12,13].

An alternative approach is direct measurement of the *in vivo* binding of proteins to specific genomic regions; the most commonly used method is ChIP[14]. Conventional ChIP assays, as first described by Solomon *et al.*[14], consist of cross-linking cells or tissue to stabilize protein-DNA interactions, followed by immunoprecipitation (IP) of the proteins of interest and detection of the bound DNA by radioactive labeling or, more recently, by quantitative real-time PCR (qPCR) and ChIP-chip[15–18]. Recent progress in sequencing technologies has opened the door for ChIP followed by DNA sequencing (ChIP-seq), a technology for unbiased and accurate genome-wide characterization of TF binding[19–23]. Nevertheless, ChIP and ChIP-seq are both limited to handling only a few samples at a time, precluding research involving a large number of factors, as well as studies of the dynamics of binding following diverse developmental process, stimuli, time points, malignancies and perturbations. The HT-ChIP method streamlines the entire ChIP and indexed library construction process; thus, it allows the investigation of the complexity and dynamics of epigenomic modifications and TF binding in mammalian cells under various physiological and pathological conditions[24–27], thereby accelerating our understanding of the underlying principles of the regulatory code.

Overview of the procedure

Here we present a protocol for HT-ChIP, a user-friendly high-throughput approach for ChIP-seq, which allows the handling of dozens of samples in parallel, generating reproducible genome-wide data. We have used this protocol to uncover the transcriptional landscape of mammalian cells by following the dynamics of the chromatin state and the binding of over two dozen TFs at four time points after the response of primary innate immune cells to pathogen challenge[28]. The HT-ChIP method can be easily implemented on most automated liquid handling robots or set up as a manual version using upgrades to standard laboratory equipment. Owing to improved molecular biology techniques, the cell number required for analysis can be markedly reduced. This may be particularly notable when working with *in vivo* low-abundance cells. The overall process can be divided into two modules: the ChIP module (PROCEDURE Steps 1–28) and the sequencing library module (PROCEDURE Steps 29–57). See **Figure 1** for a schematic illustration of the protocol. In both modules magnetic beads are used, eliminating laborious manual steps and enabling sample processing in the same well of a 96-well plate, thus greatly increasing throughput and sensitivity. As the entire process is based on standard enzymes from various vendors and the addition of 96 bar-coded adapters, the cost per ChIP-seq sample (including sequencing) is reduced compared with samples prepared with commercially available ChIP-seq kits, and it ranges from a few dozen to a few hundred US dollars, thereby enabling large-scale projects to be carried out at an affordable cost.

The ChIP module consists of the following steps: cell sample preparation (e.g., in temporal studies stimulating for various time points, cross-linking and washing the cells); lysis and sonication of cell pellets to shear the DNA into smaller fragments; IP of protein-DNA complexes; extensive washes to eliminate unspecific interactions; and, finally, reverse cross-linking and proteinase K treatment to dissociate protein-DNA complexes and degrade the proteins.

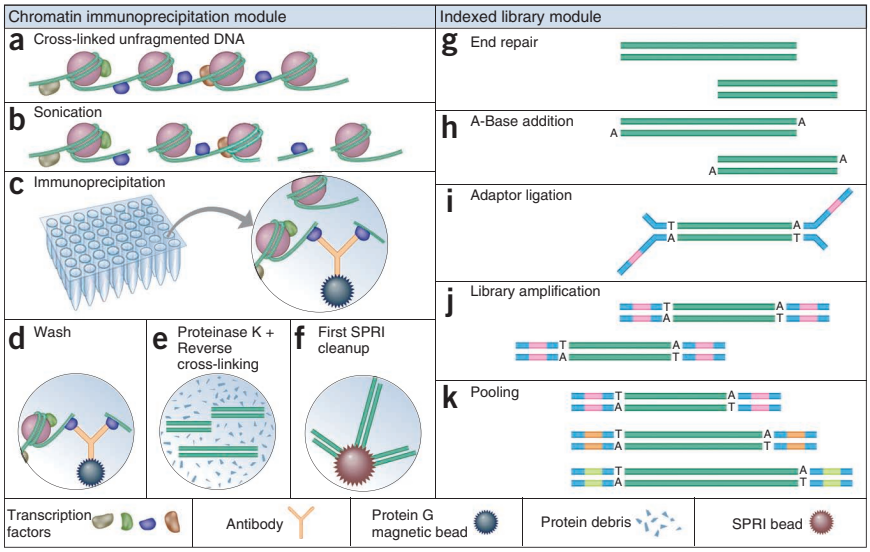

Figure 1 | Schematic description of the two modules of the HT-ChIP method. (**a**) The ChIP module begins with cross-linking the cells, which results in stabilizing DNA-protein interactions. (**b**,**c**) Cross-linked DNA is then sheared by sonication (**b**) and the protein of interest is immunoprecipitated using a specific antibody coupled to magnetic beads (**c**). (**d**,**e**) Beads are washed to reduce unspecific interactions (**d**) and the sample is eluted from the beads, treated with RNase and proteinase K and is then reverse cross-linked (**e**). (**f**) Paramagnetic SPRI beads are used for purification of the immunoprecipitated DNA, while washing out protein debris. (**g**) Sequencing library module begins with repairing the ends of the DNA precipitated in the ChIP module to generate blunt-end fragments. (**h**,**i**) An 'A' nucleotide is then added to the 3′ end of each DNA strand (**h**) to which Y-shaped bar-coded adapters containing a T-overhang can be ligated (**i**). (**j**) Next, each DNA molecule that contains the adapters is amplified by a PCR reaction. (**k**) Finally, several libraries (each containing a unique barcode) are pooled together and sequenced.

The sequencing library module consists of four enzymatic reactions to introduce Illumina sequencing platform–compatible indexed adapters to allow for multiplexing and to amplify the signal. Fragmented DNA ends are first repaired by T4 polymerase, as many of them contain single-stranded overhangs. T4 polymerase has both 5′–3′ polymerase activity, which fills in 5′ overhangs, as well as 3′–5′ exonuclease activity, which removes 3′ overhangs. The result is blunt-ended fragments. Next, T4 polynucleotide kinase adds a phosphate group at the 5′ ends. An adenosine base is then added to the blunt-ended fragments. This A-base is used for efficient ligation, using T4 quick ligase, of sequencing adapters, which contain a T-overhang. Finally, the chromatin immunoprecipitated DNA is amplified by a PCR reaction, which also introduces the Illumina-P5 adapter at one end of the molecule.

DNA is purified after each enzymatic step with the solid-phase reversible immobilization (SPRI) method[29,30] using carboxylic acid–coated superparamagnetic beads (referred to in this manuscript as SPRI beads). These beads reversibly bind nucleic acids in the presence of high salt (>0.7 M NaCl) and polyethylene glycol (PEG) as a crowding agent. Therefore, most enzymatic reactions can be performed while keeping SPRI beads in the tube, provided that the reaction mixture contains no PEG, with the advantage of reducing both cost and material loss during sample transfer. The ratio of SPRI buffer to aqueous solution determines the size of DNA fragments bound by the beads. The lower the ratio, the higher the cutoff size (for example, at a 2× ratio, beads will mostly bind fragments that are ~100 bp and larger, whereas at a 0.6× ratio beads will bind DNA fragments that are 300 bp and larger)[30]. This feature is used throughout the protocol to purify relevant DNA fragments while discarding the unnecessary components for the following stage. Every SPRI cleanup is composed of three steps: binding of DNA to the beads; ethanol wash; and elution of DNA. Superparamagnetic SPRI beads replace the former use of columns to purify DNA and the use of gels for size selection; as a consequence, they markedly reduce sample loss, which is crucial in ChIP and any other genomic enrichment assay that initially uses 1 ng or less DNA as input. As the enzymatic processes in these low concentrations are sensitive, it is essential to follow instructions carefully. At the end of library generation, samples can be pooled and sequenced together because of the use of bar-coded adapters in this protocol. In a final step that is performed with the pooled sample before sequencing, SPRI beads are used to generate accurate size cutoff of DNA fragments between 200 and 400 bp (eliminating both small and large fragments), a step that increases the amount of aligned usable reads from the same samples by more than 50%. After HT-ChIP, sequencing and data analysis can be performed according to standard procedures [28,31].

Applications of HT-ChIP

Chromatin-state maps and TF-binding profiles have tremendous significance to almost every biological research field, from development and organogenesis to cancer research and from immunological studies to trials of new therapeutic drugs. Although ChIP is becoming crucial for every aspect of genomic research, it is laborious, insensitive to low cell numbers and costly. The protocol presented here overcomes many of these technical issues, making the assay much more user friendly, less complicated, cheaper and high throughput. Out of the many possible applications of the HT-ChIP protocol, we present here a few examples (**Fig. 2**).

Having a ChIP-grade antibody is essential to any successful ChIP assay. Unfortunately, it is difficult to predict antibody quality on the basis of other applications, such as western blotting or IP. Indeed, precious time, reagents and samples are exhausted while working with unsuitable antibodies. The HT-ChIP protocol allows simultaneous screening of multiple antibodies for each TF or chromatin modification, and thus allows the most effective ChIP-seq antibodies to be selected for future research. In a recent screen (R.B.-G, Z.B.-I., I.A. and S. Bonnette, unpublished data), we tested four different antibodies for over 15 TFs. Although only 15% of the antibodies were of ChIP grade, almost all of them performed well in western blotting or IP (**Fig. 2a**). It is notable that precipitation products of all of these antibodies

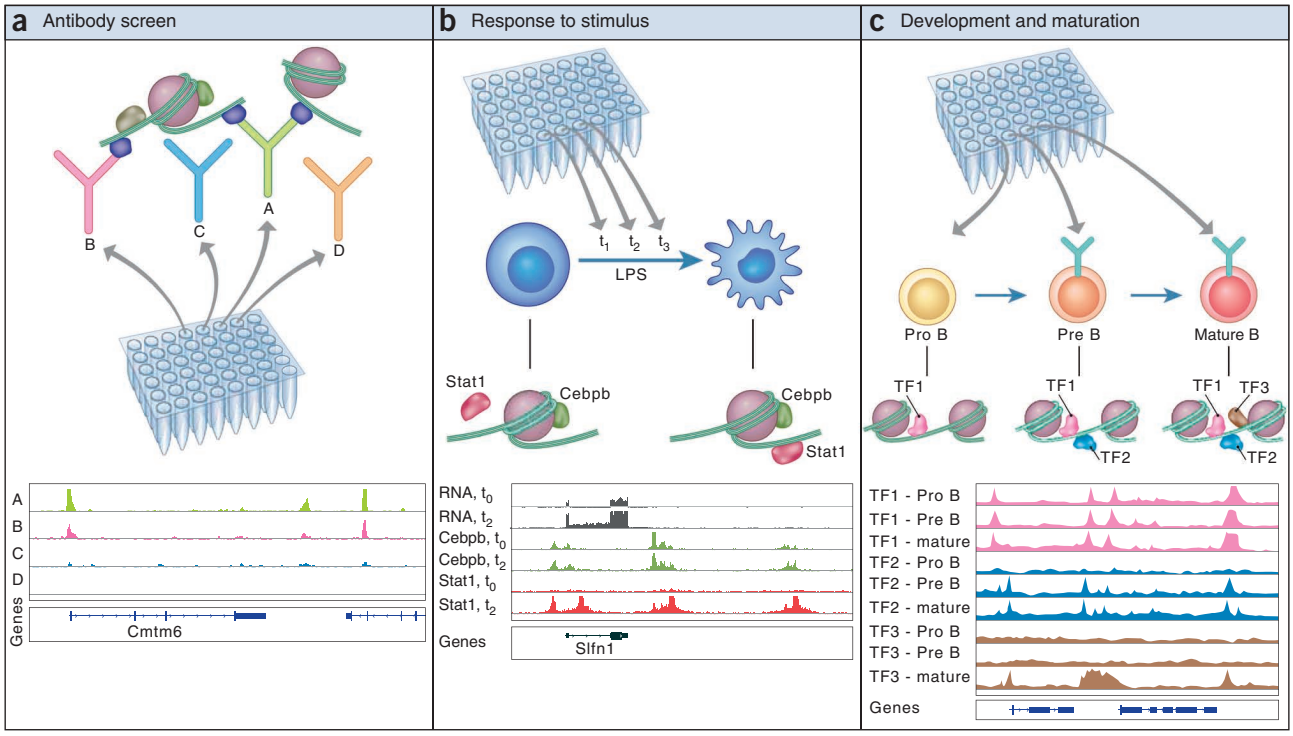

Figure 2 | Potential applications for the HT-ChIP protocol. (**a**–**c**) Schematic showing potential applications for HT-ChIP: antibody screening (**a**) and temporal studies of TF binding (**b**,**c**).

were assessed both by sequencing and by carefully tested qPCR primers. In general, it seems that sequencing provides a much better indication than qPCR of antibody quality for ChIP-seq, as it tests the global binding pattern rather than data for a few particular loci. From our experience, two antibodies may seem to have equal efficiency when looking at a specific locus in qPCR; however, sequencing data often reveal a major difference in the number of peaks or in genome-wide binding between the two antibodies. An example is presented in **Figure 3**. As antibody screening does not require much sequencing depth (in most cases, 5×10^6 aligned reads), 30–40 different antibodies can be efficiently screened in a single lane. This markedly reduces screening costs, and thus thorough antibody screening is highly recommended before proceeding with the biological research.

Activation of immune cells is a multistep process that encompasses numerous changes in the epigenetic landscape and in dynamic binding of TFs to specific genomic loci[28]. These changes are stimulation dependent and alter over time, and thus related studies must include examination of the temporal changes of the genomic landscape. HT-ChIP offers the ability to do so and to measure association of dozens of factors over several crucial time points. An example of such an assay is presented in **Figure 2b**, in which binding profiles of key immune TFs were generated in dendritic cells activated with lipopolysaccharides (LPS) for several time points. As presented in **Figure 2b**, some factors (such as Stat1) are very dynamic, whereas others (such as Cebpb) are more static, and this feature is correlated with their biological function[28].

As in the case of cellular activation in response to stimulus, cellular differentiation and maturation is a dynamic process that unfolds over time. Dynamic changes in TF binding lead to alterations in the epigenetic landscape, followed by robust gene expression changes, and these in turn produce the conversion in cell phenotype as it differentiates[32,33]. From a technical point of view, in this sort of research there is a need for a high-throughput technique that allows detection of multiple factors and time points in parallel. Moreover, the distinct populations of differentiating cells are often quite small, and thus there is a need for a method sensitive enough for low cell numbers. **Figure 2c** illustrates such a possible approach, in which the TF and epigenetic landscape of developing B cells (pre B, pro B and mature naive B cells) is investigated. This sort of research can shed light on key questions such as the role of specific TFs in maturation and chromatin modifications that are unique to particular cellular stages. Moreover, HT-ChIP can be combined with methods for batch isolation of tissue-specific chromatin for IP[34] in order to study developmental epigenomic modifications. Similarly, monitoring alterations in chromatin state and TF binding during neoplasia can elucidate genomic and epigenomic mechanisms that lead to malignancy[27].

The examples briefly discussed above are only a fraction of possible research questions that can be studied using HT-ChIP. Others include the following: differences in TF binding across species[35,36] and conservation of enhancers[37]; binding of multiple factors to elucidate cooperatives[7,38,39]; activity of small molecules; cellular response to environmental changes; systematic profiling of chromatin modifications and remodeling[40,41]; and research on signaling pathways.

Limitations of HT-ChIP

Although the number of cells required for HT-ChIP is markedly lower than the number of cells required for the alternative approaches mentioned above, it is still high, ranging from ~10^4 cells for histone modification to hundreds of thousands or millions

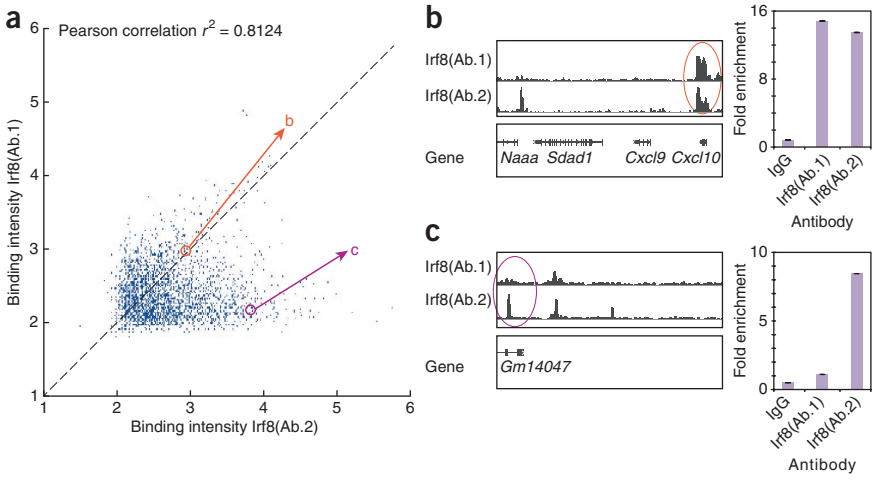

Figure 3 | Validation of antibody screening. HT-ChIP was performed with two Irf8-specific antibodies under the same conditions. Precipitated DNA was sent for sequencing and tested by qPCR. (**a**) Correlation of the peak intensities obtained with the two antibodies in sequencing. Dots b and c correspond to panels **b** and **c**, respectively. (**b**,**c**) Magnification of two selected peaks in **a**, as represented by sequencing and in qPCR.

of cells per sample when using antibodies directed against TFs. This may be specifically challenging when investigating rare cell populations. In addition, the method relies on finding ChIP-grade antibodies for each regulatory protein, a task that requires a thorough examination of a wide range of antibodies. Moreover, some antibodies require slightly different conditions than others (for example, cross-linking and washing conditions), which cannot be achieved in a 96-well format; however, we feel that the ability to test several antibodies for each factor in one experiment outweighs providing custom conditions for each antibody. Another limitation is the difficulty in assessing the quality of the assay before sequencing. Although DNA concentration should be measured at the end of the IP module, it is not indicative of assay quality. In other words, the fact that a ChIP assay ended with a detectable amount of DNA gives no indication of the specificity of the DNA that co-precipitated. qPCR is also a limited means for assessing assay quality, as primer design is difficult when performing ChIP on TFs that have no previous data on binding targets, nor is it indicative of the global efficiency of the antibody (**Fig. 3**). Finally, this protocol uses probe sonication for DNA shearing. Acoustic fragmentation using a probe is considered to be an unbiased means to fragment DNA as it breaks the DNA at random sites; however, it also limits the number of different samples that can be handled together. Other alternatives are available for high-throughput fragmentation, including the Bioruptor and Covaris acoustic sonicators, as well as enzymatic DNA fragmentation methods[42].

Future improvements

We aim to improve the HT-ChIP protocol so that fewer cells will be needed by testing other cross-linking conditions and new approaches to bar-coding the DNA and amplifying the small amount of precipitated DNA[43,44]. We are also constantly seeking new suitable antibodies for a large panel of regulatory proteins. The use of an overexpressed tagged TF as reference for an endogenous TF, the use of more than one antibody for each TF and the use of RNAi or TF knockout mice will help in generating a set of ChIP-seq–validated antibodies for future research and diagnostics.

Experimental design

Although we have made major efforts to generate a user-friendly protocol, even for non-experts in ChIP and sequencing, there are several crucial steps that need to be optimized for different cell types in order to increase the final signal and reduce unspecific interactions. The following section describes the parameters that should be optimized, discusses the suggested controls and emphasizes the crucial steps of the HT-ChIP protocol.

Cell number. The number of cells required for each IP can markedly change according to the antibody used, the abundance of the factor to be precipitated and the number of its genomic binding sites. For example, when immunoprecipitating histone modifications it is usually sufficient to use as few as 10^4–10^5 cells. This is because there are thousands of genomic loci bound by such histones, they are tightly bound to the DNA (enabling efficient cross-linking), and there are excellent ChIP-grade antibodies that can be used. In contrast, most TFs are loosely bound to the DNA, have far fewer genomic binding sites and often only lower-quality antibodies are available. Therefore, more cells must be used for each IP, and their numbers can range from 10^5 to 10^7 cells, depending on the factor, the antibody and the cell type.

Cross-linking conditions. We suggest cross-linking the cells for 10 min with 1% (wt/vol) formaldehyde. These conditions are commonly used for many cell types and are considered to be suitable for stabilizing transient interactions but mild enough to prevent unspecific cross-linking[45]. Nevertheless, it is possible to optimize cross-linking conditions for specific research questions; for example, reducing the percentage of formaldehyde should result in cross-linking mostly tightly bound direct DNA-interacting proteins and may be desired when you are interested only in those interactions. For factors that are not directly bound to DNA, it often helps to add a protein-protein cross-linker such as disuccinimidyl glutarate in addition to formaldehyde[46].

Cell lysis and sonication. Lysis and sonication conditions in this protocol are optimized for bone marrow–derived dendritic cells. For optimal results with other cell types, lysis and sonication conditions must first be fine-tuned[47]. For some cell types it is enough to lyse the entire cell, whereas for others it is better to first isolate the nuclei and then lyse the nuclei alone. In this protocol, we offer two alternative lysis options: direct lysis of cells and lysis of isolated nuclei. Other lysis buffers can be used; however, for optimal IP, SDS concentration at this step must not exceed 0.1% (wt/vol). Therefore, if a higher SDS concentration has to be used for sonication, the buffer should be diluted before proceeding to the IP. There are two major points that must be adhered to in order to obtain good and reproducible sonication of samples: preventing the sample from overheating and keeping the same sonication conditions in each and every experiment. During sonication, samples tend to heat up,

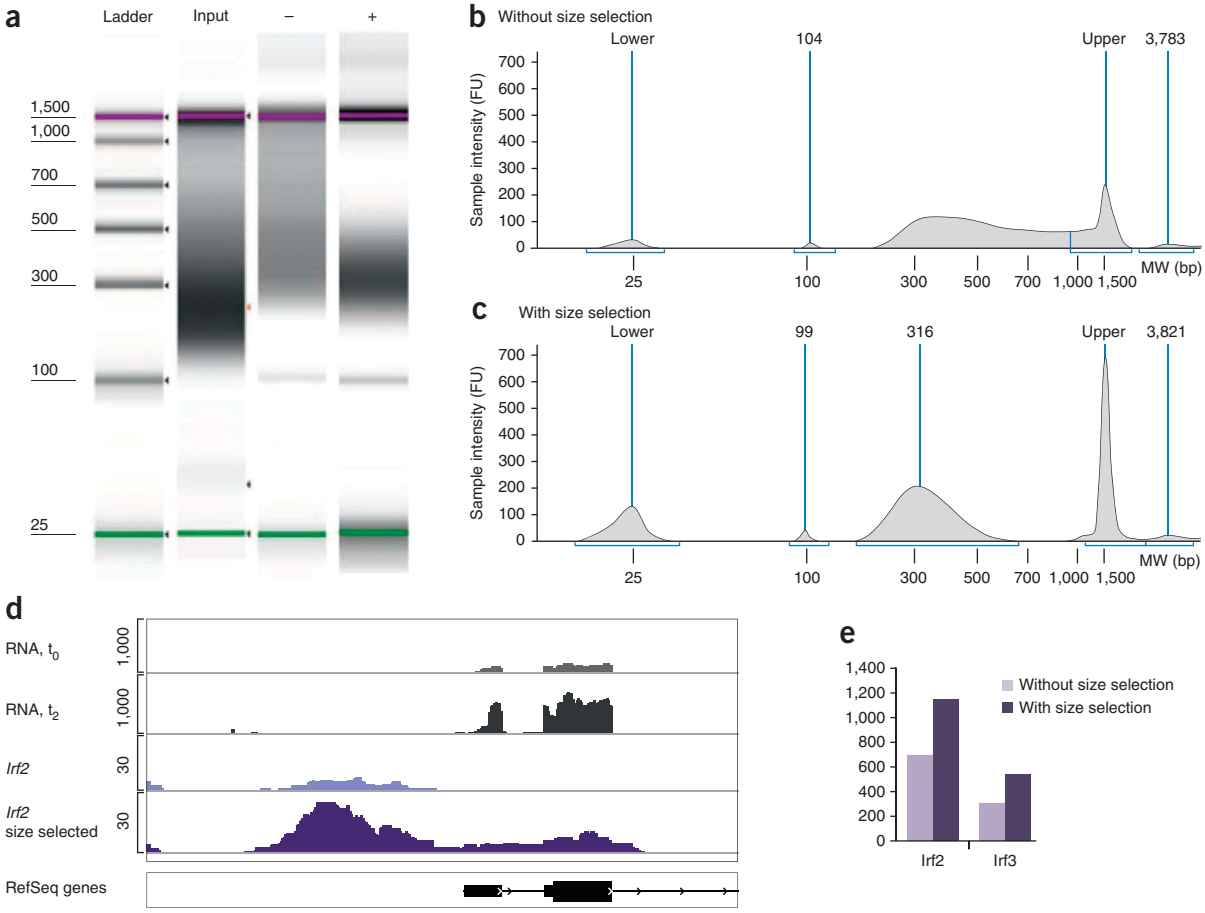

Figure 4 | Size selection of pooled libraries improves sequencing results. (**a**–**c**) Gel electrophoresis measured using a TapeStation assay of pooled libraries, before and after the final size selection: (**a**) gel image (purple and green lines are intrinsic upper and lower markers, respectively). Input, fragmented DNA; (–) and (+) indicate before and after size selection, respectively. (**b**,**c**) Histogram representation of the results from panel **a**. (**d**) Example of sequencing results with and without final size selection (chromosome 11: 59,355000-59,357,200). Peaks presented are summary of reads that markedly differ from the input. (**e**) The number of peaks obtained by sequencing libraries with and without final size selection (HT-ChIP data for Irf2 and Irf3 are shown as an example).

which may lead to protein denaturation and severely damage IP. To avoid such damage, we place sample tubes in a cooling block connected to a cooling system (for more details see: http://www.weizmann.ac.il/immunology/AmitLab/data-and-method/iChIP). Block temperature is set to −4 °C. We also use short pulses for sonication, which are interspersed with longer pauses. To maintain reproducibility of sonication, the sonicator probe should always be placed in the exact same position inside the sample tube. We find that the key to reproducible DNA fragmentation is consistency in probe positioning and not necessarily the exact position itself. Optimal sonication will result in DNA fragments of approximately 200–500 bp. We suggest optimizing sonication conditions once, and then following the same conditions in all experiments using the same cell type. Other methods for DNA fragmentation, such as enzymatic digestion, may be used, but they are considered to have more bias.

Selection of antibodies. As mentioned in the overview section, using a high-quality ChIP-grade antibody is important for assay performance. We strongly recommend testing several antibodies for each target (or using a formerly validated antibody). We also advise including two control antibodies in every assay: nonimmunized serum as a negative control and anti-RNA polymerase II antibody as a positive control (see Reagents).

The use of input DNA for normalization. The analysis of sequencing results includes determining what is true signal and what is random unspecific 'noise' generated through the ChIP-seq process. We find that the most representative unspecific pattern is generated by a sample of input DNA, which should be included in every experiment. This negative control sample is chromatin-treated identically to the IP samples, except that the IP steps (PROCEDURE Steps 14–21) are omitted (and therefore, the input sample should not be enriched for specific genomic loci). Ensure that you have an input sample for every cell type or condition used in the experiment. As this sample contains much more DNA than samples after IP, make sure not to exceed 30 ng when entering the library module, as this will cause nonlinear amplification.

Validation of the ChIP module. At the end of the ChIP module, after the first SPRI cleanup, the IP can be validated by measuring the concentration of the precipitated DNA and by performing qPCR with primers designed for genomic regions of interest. If the positive control anti-RNA-Pol II sample does not precipitate

~5 ng of DNA and no enrichment is detected using qPCR at known polymerase-bound loci, it is possible that there was a problem with the IP steps; you should consider repeating the assay rather than continuing with library construction and sequencing.

Library module. This module is specifically designed for low amounts of input DNA (sub-nanograms of DNA). As the module is composed of sensitive enzymatic reactions, it is essential to follow instructions carefully. We strongly advise using a multichannel pipette (even when working on a relatively small number of samples) for maintaining accuracy and consistency.

Multiplexing. Multiplexing allows sequencing of multiple samples as a pool in one lane. One key step is to accurately mix equal molar ratios of the samples. As fragment size should be similar for all samples (ranging from 200 to 500 bp), there is no need to calculate sample molarity and it is sufficient to pool equal quantities from each sample. The number of samples that can be pooled depends on the sequencing depth required; in antibody screening, a large number of samples can be multiplexed, as shallow sequencing (~5×10^6 aligned reads per sample) is sufficient to assess the quality of the antibody. Once a high-quality antibody is obtained, deeper sequencing is performed (~20×10^6 aligned reads per sample) for data collection.

Pool size cutoff. Despite the fact that sonication has been optimized in the ChIP module to generate fragments of 200–500 bp, there are always some larger fragments that may interfere with sequencing. We found that the removal of these large fragments improves the number of peaks obtained from sequencing (**Fig. 4**), and therefore we suggest performing an additional SPRI cleanup in which large fragments are discarded. From our own experience using Illumina HiSeq2000, the biggest effect of excluding the large fragments is not the loss of true positives but improved cluster generation.

MATERIALS

REAGENTS

▲ **CRITICAL** All reagents must be nuclease free. Unless indicated otherwise, all reagents should be stored at room temperature (20–25 °C).

ChIP reagents

- Starting material: ~10^4 cells per IP of chromatin modifications or approximately 10^6–10^7 cells per IP of TFs
- Formaldehyde ampoules, 16% (wt/vol) (Pierce Biotechnology, cat. no. 28908) ! **CAUTION** Formaldehyde is toxic on inhalation, on contact with skin or if swallowed. Use a chemical hood, lab coat and gloves when handling formaldehyde. Formaldehyde is flammable and must be kept away from heat and fire. ▲ **CRITICAL** Avoid repeated use of open formaldehyde; discard any leftover formaldehyde.
- Dulbecco's PBS without Ca^{2+}, Mg^{2+} (Beit Haemek Biological Industries, cat. no. 02-023-1)
- Water, molecular biology grade (Sigma-Aldrich, cat. no. W4502)
- Glycine, molecular biology grade (Calbiochem, cat. no. 357002)
- Tris buffer, 1 M, pH 8.0, molecular biology grade (Calbiochem, cat. no. 648314)
- EDTA, 0.5 M, pH 8.0, molecular biology grade, DEPC treated (Calbiochem, cat. no. 324506)
- EGTA, molecular biology grade (Calbiochem, cat. no. 324626)
- *N*-Lauryl-sarcosine 20% (vol/vol), molecular biology grade (Sigma-Aldrich, cat. no. L7414)
- Sodium deoxycholic acid (NA-DOC; Calbiochem, cat. no. 264103)
- Sodium chloride (NaCl; Calbiochem, cat. no. 567441)
- Triton X-100, molecular biology grade (Calbiochem, cat. no. 648466)
- SDS, 20% solution (wt/vol; Calbiochem, cat. no. 428018)
- Lithium chloride (LiCl), 8 M, molecular biology grade (Sigma-Aldrich, cat. no. L7026)
- NP-40 alternative (Calbiochem, cat. no. 492016)
- HEPES buffer, 1 M, molecular biology grade (Calbiochem, cat. no. 375368)
- Glycerol, molecular biology grade (Calbiochem, cat. no. 356352)
- BSA (MP Biomedicals, cat. no. 0216006980X). Store at 4 °C
- Tween-20, molecular biology grade (Calbiochem, cat. no. 655204)
- Complete protease inhibitors cocktail tablets (Roche, cat. no. 04-693-124-001). Store at 4 °C
- RNase, DNase free, 500 μg ml^{-1} (Roche, cat. no. 11-119-915-001). Store at −20 °C
- Proteinase K (Invitrogen, cat. no. 25530-049). Store proteinase K at −20 °C
- Glycogen (Roche, cat. no. 10-901-393-001). Store glycogen at −20 °C
- Dynabeads protein G for IP (Invitrogen, cat. no. 100-04D). Store Dynabeads protein G at 4 °C
- Quant-iT 500 dsDNA high-sensitivity (HS) assay kit (Invitrogen, cat. no. Q32854)
- Tris-HCl buffer, 1M, pH 8.0, molecular biology grade (Calbiochem, cat. no. 648314)
- KOH solution, 1M, molecular biology grade (Sigma, cat. no. 319376)
- Anti-RNA polymerase II antibody, clone CT4H8 (Covance, cat. no. MNS-128P); aliquot the antibody and store it at −20 °C. This antibody, conjugated to protein G magnetic beads (**Box 1**), serves as a good positive control for ChIP-seq assays
- Normal rabbit serum (Jackson ImmunoResearch, cat. no. 011-000-120); aliquot and store the serum at −20 °C. The normal rabbit serum conjugated to protein G magnetic beads (**Box 1**) is used as a negative control
- ChIP-grade antibodies of choice: antibodies should be conjugated to magnetic protein G beads, as described in **Box 1**. For a list of antibodies we have used successfully for data shown in this manuscript, please see **Supplementary Table 1**

Library reagents

- Agencourt AMPure XP (SPRI beads; Beckman Coulter, cat. no. A63881). Store the beads at 4 °C
- PEG-8,000 (Sigma-Aldrich, cat. no. P5413)
- Ethanol, 100%
- T4 polynucleotide kinase (T4 PNK) 10 U μl^{-1} (New England BioLabs, cat. no. M0201). Store T4 PNK at −20 °C
- T4 DNA polymerase, 3 U μl^{-1} (New England BioLabs, cat. no. M0203). Store T4 DNA polymerase at −20 °C
- T4 ligase buffer, 10× (New England BioLabs, cat. no. B0202). Store T4 ligase buffer at −20 °C ▲ **CRITICAL** Avoid repeated thawing and freezing.
- BSA, 10 mg ml^{-1} (New England BioLabs, cat. no. B9001). Store BSA at −20 °C
- ATP, 10 mM (New England BioLabs, cat. no. P0756). Store ATP at −20 °C ▲ **CRITICAL** Avoid repeated thawing and freezing.
- Klenow fragment (3′–5′ exo−; New England BioLabs, cat. no. M0212). Store Klenow fragment at −20 °C
- NEB buffer 2 (New England BioLabs, cat. no. B7002). Store NEB buffer 2 at −20 °C
- dATP, 100 mM (New England BioLabs, cat. no. N0440). Aliquot and store dATP at −20 °C ▲ **CRITICAL** Avoid repeated thawing and freezing.
- Quick ligase (New England BioLabs, cat. no. M2200—part of the quick ligation kit). Store the ligase at −20 °C
- Quick ligation buffer, 2× (New England BioLabs, cat. no. M2200—part of the quick ligation kit). Store the buffer at −20 °C
- Pfu Ultra II fusion (Agilent Technologies, cat. no. 600670). Store the Pfu at −20 °C
- Pfu Ultra buffer, 10× (Agilent Technologies, cat. no. 200532). Store the buffer at 4 °C
- dNTP solution set (100 mM; 25 mM each; New England BioLabs, cat. no. N0446). Aliquot and store at the solutions −20 °C ▲ **CRITICAL** Avoid repeated thawing and freezing.
- Illumina compatible universal adapter: 5′-ACACTCTTTCCCTACACGACG CTCTTCCGATC*T-3′, * indicates phosphorothioate modification.

Box 1 | Coupling the antibodies to magnetic protein G beads ● TIMING ~1 h

▲ CRITICAL To save time, coupling antibodies to the magnetic protein G beads can be done in parallel with cell lysis and sonication (Steps 10–12 of main PROCEDURE). Timing here is not crucial—coupling the antibodies to the beads should be done for at least 1 h (room temperature), but longer incubations can be performed without affecting the results.

1. Transfer 75 µl of magnetic protein G beads per IP into a microcentrifuge tube (it is possible to reduce the amount of beads when using lower cell numbers). Place the tube on the DynaMag-2 magnet for 30 s. Discard the supernatant by vacuum aspiration.
2. Remove the top rack of the magnet. Add 300 µl of binding/blocking buffer to the tube and mix the beads by pipetting. Place the top rack with the tube back on the DynaMag-2 magnet for 30 s and discard the supernatant by vacuum aspiration. Repeat this step once again.
3. Add 75 µl of binding/blocking buffer per IP. Aliquot to several microcentrifuge tubes according to the number of IPs to be performed.
4. To each microcentrifuge tube, add the desired antibody (we recommend using 10 µg per IP of antibodies directed against transcription factors and 3 µg per IP of antibodies directed against histone modifications). Add 300 µl of binding/blocking buffer and mix by flicking the tube.
5. Incubate the mixture for 1 h or more while rotating at room temperature.
6. Place the microcentrifuge tubes on the DynaMag-2 magnet. Allow the beads to separate from the supernatant for 30 s. Discard the supernatant by using vacuum aspiration.
7. Wash the beads with 300 µl of blocking/binding buffer, as described in step 2. Proceed immediately with Step 14 of the main PROCEDURE.

▲ CRITICAL STEP Wash the beads immediately before using them for IP (immediately before adding the sonicated lysates). It is better to couple the beads to antibodies for more than 1 h than to let them dry out while sonicating the cells.

Store Illumina compatible universal adapter at −20 °C (Integrated DNA Technologies—custom order)
- Illumina compatible Indexed Adapter (NNNNNN = bar code for multiplexing). Bar codes are specified in **Supplementary Table 2**
- 5′-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**NNNNNN**-NATCTCGTATGCCGTCTTCTGCTTG-3′ (Integrated DNA Technologies—custom order). Store at −20 °C ▲ CRITICAL Always spin before opening the indexed adapter plates, to avoid contamination of adapters between wells.
- Reverse amplification primer: 5′-CAAGCAGAAGACGGCATACG AGAT-3′ (Integrated DNA Technologies, custom order). Store the primer at −20 °C
- Forward amplification primer: 5′-AATGATACGGCGACCACCGAGAT CTACACTCTTTCCCTACACGAC-3′ (Integrated DNA Technologies, custom order). Store the primer at −20 °C

EQUIPMENT
- Branson digital sonifier (Branson Ultrasonics, cat. no. 33995-592)
- Sonicator probe (Branson Ultrasonics, cat. no. 33996-243)
- Cooling unit (RTE-7 D1; Thermo Scientific, cat. no. 271103200000)
- Cooling plate (Mecour, bottom: cat. no. 99-401 CB-LS, top: cat. no. 00-60/24)
- Tubing for cooling unit (VWR, cat. no. 60985-544)
- DynaMag-2 magnet (Invitrogen, cat. no. 123-21D)
- DynaMag-96 magnet (Invitrogen, cat. no. 120-27)
- HulaMixer sample mixer (Invitrogen, cat. no. 159-20D)
- Twin.Tec PCR plate 96, skirted (Eppendorf, cat. no. 0030128648)
- Vacuboy multichannel vacuum aspirator (Integra Biosciences, cat. no. 155500)
- Disposable reservoir inserts, 6 ml (Labcyte, cat. no. ALL031-01)
- Disposable reservoir holder, 3 × 6 ml (Labcyte, cat. no. ALL032-01)
- Thermal cycler (Eppendorf MasterCycler Pro, cat. no. 950040015)
- Adhesive PCR film (ABgene, cat. no. AB-0558)
- Filter tips, 200–1,200 µl (Rainin, cat. no. RT-L1200F)
- Filter tips, 20–200 µl (Rainin, cat. no. RT-L200F)
- Filter tips, 1–20 µl (Rainin, cat. no. RT-L10F)
- Multichannel pipette, 2–20 µl (PipetLiteXLS LTS, Rainin, cat. no. L12-20XLS)
- Multichannel pipette, 20–200 µl (PipetLiteXLS LTS, Rainin, cat. no. L12-200XLS)
- Tecan Infinite 200 Pro plate-reader
- Magnetic stirrer
- Petri dishes
- Tissue culture dishes
- Rotary shaker
- Microcentrifuge tubes

REAGENT SETUP

Glycine, 2.5 M Weigh 18.76 g of glycine and add molecular biology–grade water up to a volume of 100 ml. Heat the mixture to 65 °C, stir it on a magnetic stirrer until glycine dissolves and filter the solution. The solution should be stored at room temperature for no more than 1 month. ▲ CRITICAL The pH of glycine solution tends to change over time, and therefore the solution should not be stored for more than 1 month.

TE, 1× Mix 10 mM Tris-HCl (pH 8.0) with 1 mM EDTA (pH 8.0). The solution is prepared with molecular biology–grade water. Filter the solution and store it at room temperature for 6 months.

Na-DOC, 5% (wt/vol) Dissolve Na-DOC in molecular biology–grade water to a concentration of 5% (wt/vol). Filter the solution and store it at room temperature for 6 months.

RIPA buffer Mix 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 140 mM NaCl, 1% (vol/vol) Triton X-100, 0.1% SDS (vol/vol, use 20% SDS solution) and 0.1% Na-DOC (wt/vol, use 5% Na-DOC solution). Prepare the solution with molecular biology–grade water. Filter and store it at 4 °C for 6 months.

RIPA-500 buffer Mix 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 500 mM NaCl, 1% (wt/vol) Triton X-100, 0.1% SDS (vol/vol, use 20% SDS solution) and 0.1% Na-DOC (wt/vol, use 5% Na-DOC solution). Prepare the solution with molecular biology–grade water. Filter and store it at 4 °C for 6 months.

LiCl wash buffer Mix 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 250 mM LiCl, 0.5% (vol/vol) NP-40 and 0.5% Na-DOC (wt/vol, use 5% Na-DOC solution). Prepare the solution with molecular biology–grade water. Filter and store it at 4 °C for 6 months.

Direct elution buffer Mix 10 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 300 mM NaCl and 0.5% SDS (vol/vol, use 20% SDS solution). Prepare the solution with molecular biology–grade water. Filter the buffer and store it at room temperature for 6 months. ▲ CRITICAL Always store the buffer at room temperature (in cooler temperature SDS will precipitate) and ensure that there are no precipitants of SDS before use.

Binding/blocking buffer Mix 0.5% (wt/vol) BSA and 0.5% (vol/vol) Tween-20 in PBS (without Ca^{2+}, Mg^{2+}). Filter the buffer and store it at 4 °C for 3 months.

Lysis buffer 1 Mix 50 mM HEPES-KOH, 1 mM EDTA (pH 8.0), 140 mM NaCl, 0.25% (vol/vol) Triton X-100, 0.5% (vol/vol) NP-40 and 10% (vol/vol) glycerol. Prepare the solution with molecular biology–grade water. Filter the buffer and store it at 4 °C for 6 months.

Lysis buffer 2 Mix 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 200 mM NaCl and 0.5 mM EGTA (pH 8.0). Prepare the solution with molecular biology–grade water. Filter the buffer and store it at 4 °C for 6 months.
Lysis buffer 3 Mix 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl, 0.5 mM EGTA (pH 8.0), 0.1% Na-DOC (wt/vol, use 5% Na-DOC solution) and 0.5% *N*-lauryl-sarcosine (wt/vol, use a 10% *N*-lauryl-sarcosine solution). Filter the buffer and store it at 4 °C for 6 months.
SPRI buffer (20% (wt/vol) PEG, 2.5 M NaCl) Mix 2.5 M NaCl and 20% (wt/vol) PEG-8,000 in molecular biology–grade water. Stir the buffer on a magnetic stirrer until all solids dissolve. Adjust the pH to 5.5 with a concentrated HCl solution; filter the solution and store it at room temperature for 6 months. ▲ **CRITICAL** Proper SPRI buffer preparation is crucial for each and every step of library preparation. Ensure that you prepare it accurately.

End-repair buffer Mix 6.7 µl of 10× T4 DNA ligase buffer (contains 10 mM ATP), 0.67 µl of BSA (10 mg ml^{-1}), 0.67 µl of dNTPs (10 mM) and 16.96 µl of nuclease-free water. Add 25 µl of this buffer per 40 µl of sample volume to yield the following final concentrations: 1× T4 DNA ligase buffer, 0.1 mg ml^{-1} BSA and 0.1 mM dNTPs. The stock buffer can be stored in 500-µl aliquots at −20 °C for 12 months. Avoid repeated thawing and freezing.
A-base addition buffer Mix 6 µl of 10× NEB buffer 2, 0.1 µl of dATP (100 mM) and 10.9 µl of nuclease-free water. Add 17 µl of this buffer per 40-µl sample volume to yield the following final concentrations: 1× NEB buffer 2 and 167 µM dATP. The stock buffer can be stored in 500-µl aliquots at −20 °C for 12 months. Avoid repeated thawing and freezing.
Adapter ligation buffer Use 2× DNA quick ligase buffer, 29 µl per reaction. Store the buffer at −20 °C according to the manufacturer's instructions.

PROCEDURE

Cell stimulation and cross-linking ● TIMING 1–2 h (for cross-linking and washes)

1| Seed ~10^7 cells in 10 ml of growth medium in a 10-cm Petri dish or a tissue culture dish. Incubate the cells at 37 °C until the next day.

2| If desired, stimulate the cells with the appropriate stimulant.

3| *Cross-linking*. Place the dishes on a rotary shaker inside a chemical hood and start shaking it slowly. Add formaldehyde to a final concentration of 1% (wt/vol) (for 10 ml of medium, add 667 µl of 16% formaldehyde) while shaking. Incubate the dishes for exactly 10 min at room temperature.
! **CAUTION** Formaldehyde is toxic on inhalation, on contact with skin or if swallowed. Always wear a lab coat and gloves and work in a chemical hood. All formaldehyde waste must also be kept inside a chemical hood or in sealed containers.
▲ **CRITICAL STEP** Cross-linking time is crucial for a successful assay. Do not cross-link for more than 10 min.

4| Stop the cross-linking reaction by adding glycine to a final concentration of 0.15 M (add 520 µl of 2.5 M glycine). Shake the reaction mixture for 5 min at room temperature.
▲ **CRITICAL STEP** Glycine acts as a quencher. Make sure to use fresh glycine solution (no more than 1 month old), as its pH tends to change over time. When the pH of glycine is correct, and when cells are cultured in a medium containing phenol red pH indicator, the medium will change color to yellow.

5| Scrape the cells and collect them in a 15- or a 50-ml tube.
▲ **CRITICAL STEP** From this step forward, keep the samples and buffers ice cold.

6| Pellet the cells by centrifuging at 290*g* for 5 min at 4 °C. Discard the supernatant.

7| To wash the cells, resuspend the pellet in 10 ml of ice-cold PBS and centrifuge the mixture at 290*g* for 5 min at 4 °C. Discard the supernatant. Repeat Step 7 one more time.

8| Resuspend the cells in 1 ml of ice-cold PBS and transfer them into a 1.5-ml tube. Centrifuge the mixture for 5 min at ~850*g* at 4 °C. Discard the supernatant.
■ **PAUSE POINT** At this point, cells can be flash-frozen at −80 °C for up to 12 months.

Cell lysis ● TIMING 0.5–1 h

▲ **CRITICAL** Coupling of antibodies to magnetic protein G beads (**Box 1**) can be carried out in parallel to cell lysis and sonication (Steps 9–13), in order to save time.

9| If cells were frozen after Step 8, thaw the cells on ice before proceeding with lysis. To lyse the cells without isolating nuclei, follow option A. Use option B if nuclear isolation is required before lysis. Note that other lysis conditions may be required for different cell types.

(A) Direct cell lysis

(i) Add 1 ml of ice-cold RIPA buffer supplemented with protease inhibitors (PIs; one tablet of PIs is suitable for 10 ml of buffer) per up to 7.5×10^7 cells. Incubate the mixture on ice for 10 min.

(ii) Proceed with Step 10.

(B) Isolation and lysis of nuclei

(i) Add 1 ml of ice-cold lysis buffer 1 supplemented with PI per 7.5×10^7 cells. Incubate the mixture on ice for 10 min at 4 °C. Spin the mixture for 10 min at ~850*g*, 4 °C. Discard the supernatant.

(ii) Add to the pellet 1 ml of ice-cold lysis buffer 2 supplemented with PI. Incubate the mixture on ice for 10 min. Spin the mixture for 10 min at ~850*g*, 4 °C. Discard the supernatant. The pellet contains nuclei.

(iii) Add to the nuclei 1 ml of ice-cold lysis buffer 3 supplemented with PI. Incubate the mixture on ice for 10 min and proceed to sonication (Step 10).

Sonication ● TIMING variable

10| For bone marrow–derived dendritic cells, sonicate the cells at 40% amplitude for 5.20 min: 0.7 s on, 1.3 s off. The cooling system should be set to −4 °C.

▲ **CRITICAL STEP** Sonication conditions may need to be optimized for different cell types and different equipment setups.

11| Centrifuge samples at ~20,000*g* for 10 min at 4 °C. Transfer the supernatant to a new microcentrifuge tube.

12| Adjust the volume to 200–500 µl of lysate per IP (if necessary, add RIPA buffer supplemented with PI).

13| Take out 30 µl from each sample and freeze at −20 °C until required at Step 22 the next day.

▲ **CRITICAL STEP** This sample will be used for normalization purposes as the input for each sample (input meaning the DNA composition of each sample, without undergoing IP).

IP ● TIMING overnight

14| Aliquot the remaining sonicated material (from Step 12) into microcentrifuge tubes containing DynaBeads protein G coupled to the specific antibody (**Box 1**). Tumble the tubes overnight at 4 °C.

15| On the next morning, place each tube on the DynaMag-2 magnet and allow the beads to adhere to the magnet for 30 s. Discard the supernatant by vacuum aspiration. Add 180 µl of ice-cold RIPA buffer supplemented with PIs. Mix by pipetting two or three times and transfer the beads and supernatant to a prechilled 96-well PCR plate.

16| Place the 96-well plate on the DynaMag-96 magnet and allow the beads to adhere to the magnet for 30 s. Discard the supernatant by using vacuum aspiration.

▲ **CRITICAL STEP** Adjust the vacuum level to avoid aspirating the beads together with the supernatant. When you are using an aspirator, never touch the beads.

Washing the beads ● TIMING 30 min for one 96-well plate

▲ **CRITICAL** From this step forward, in order to increase accuracy and reproducibility, it is strongly advisable to use a multichannel pipette and a multichannel vacuum aspirator.

▲ **CRITICAL** For all washing steps, work fast and keep all wash buffers cold. Do not mix by pipetting, but by moving the plate on the DynaMag-96 magnet one column to the right and then to the left again. This will make the beads move from one side of the well to the other side and then back, and it will result in good washing of the beads without pipetting.

17| Wash the beads five times with 180 µl of ice-cold RIPA buffer. Do this (and subsequent washes) by moving the plate onto the DynaMag magnet one column to the right; wait for a few seconds and then move the plate one column to the left again. After 30 s, discard the supernatant by vacuum aspiration.

18| Wash the beads twice with 180 µl of ice-cold RIPA-500 buffer.

19| Wash the beads twice with 180 µl of ice-cold LiCl wash buffer.

20| Wash the beads once with 180 µl of ice-cold 1× TE buffer. Discard the supernatant and leave the beads as dry as possible.

Elution ● TIMING 2–5 min for one 96-well plate

21| To elute protein-DNA complexes from the beads, remove the plate from the magnet and add 50 µl of direct elution buffer (room temperature). Mix by pipetting once.

RNase and proteinase K treatments ● TIMING 2.5 h

▲ **CRITICAL** From this step forward, include the input sample(s) taken at Step 13.

22| Prepare a mix of 3 µl of direct elution buffer and 2 µl of RNase (DNase free). Add 5 µl of the mix to each well (1 µg of RNase per well). Seal the plate with an adhesive film and incubate it for 30 min at 37 °C. To 0.1–30 ng of input sample, add direct elution buffer to a final volume of 50 µl (in order to keep the buffer composition similar to the IP samples). Place the mixture into a well in the 96-well plate and process together with the IP samples.

23| Prepare a mix of 2.5 µl of proteinase K, 1 µl of glycogen and 1.5 µl of direct elution buffer per well. Add 5 µl of the mix to each well (1 U of proteinase K and 20 µg of glycogen per well). Incubate the mixture for 2 h at 37 °C.

Reverse cross-linking ● TIMING overnight

24| Incubate the plate at 65 °C overnight (or at least 4 h).

25| Place the microcentrifuge tubes on the DynaMag-96 magnet for 30 s. Transfer the supernatant to a new plate and discard protein G magnetic beads.

■ **PAUSE POINT** After reverse cross-linking, the samples can be frozen at −20 °C for up to 12 months.

DNA purification of ChIP products ● TIMING 20 min

▲ **CRITICAL** We recommend using a multichannel aspirator system, with a needle valve-controlled vacuum and firm tubing, to allow for the fine regulation of the aspiration flow. Gentle liquid removal is important to avoid aspiration of beads.

26| Add 140 µl of SPRI beads to 60 µl of sample (after reverse cross-linking; 2.3× ratio) and mix it by carefully pipetting up and down 25 times with a multichannel pipette. The liquid in each well should look homogenous. Incubate the mixture for 2 min at room temperature. Place the plate on a DynaMag-96 magnet for 4 min to capture the beads. Discard the supernatant.

27| Leave the plate on the magnet and add 100 µl of freshly prepared 70% (vol/vol) ethanol. Incubate the plate for 30 s without disturbing the beads. Discard the supernatant. Repeat this step once more and at the end aspirate ethanol completely. Remove the plate from the magnet and let the SPRI beads air-dry for 4 min at room temperature.

▲ **CRITICAL STEP** It is important to have no ethanol traces in the well before elution. Incomplete ethanol removal may seriously compromise the yield.

28| Add 60 µl of 10 mM Tris-HCl (pH 8.0) and resuspend the beads by pipette-mixing 25 times. Incubate the suspension for 2 min at room temperature. Place the plate back on the magnet for 4 min to separate the beads from the supernatant. Transfer 20 µl of the supernatant to another plate. This 20 µl will be used for backup. Proceed with the remaining 40 µl (including the beads) to the end-repair step (Step 29).

▲ **CRITICAL STEP** It is advisable to roughly evaluate IP quality by measuring DNA concentration (the Qubit DNA HS kit is sensitive enough to detect low DNA concentrations, which are expected in ChIP assay). A qPCR assay may also be performed at this point (see ANTICIPATED RESULTS).

? TROUBLESHOOTING

End repair ● TIMING 45 min

29| Prepare the end-repair reaction mix according to sample number, as indicated in the table below. Add 27 µl of mix to each well (total reaction volume is 67 µl) and pipette-mix 15 times.

Reagent	Volume (µl) per reaction	Final concentration
End-repair buffer	25	1×
T4 PNK (10 U µl^{-1})	1	0.15 U µl^{-1}
T4 polymerase (3 U µl^{-1})	1	0.04 U µl^{-1}
Mix total volume	27	

30| Seal the plate with an adhesive PCR film and incubate it in a thermal cycler using the following program:

Step	Temperature (°C)	Time (min)
1	15	15
2	25	15
3	4	∞

End-repair reaction cleanup ● TIMING 20 min

31| Add 147 µl of SPRI buffer (2.2× ratio) to each well and resuspend the beads by pipette-mixing 25 times. Incubate the suspension for 2 min at room temperature. Place the plate on a DynaMag-96 magnet for 4 min to capture the beads. Discard the supernatant.

32| Leave the plate on the magnet and wash the beads twice with 100 µl of 70% (vol/vol) ethanol without disturbing the beads; discard the supernatant. Remove the plate from the magnet and air-dry SPRI beads for 4 min at room temperature.

33| Elute with 40 µl of 10 mM Tris-HCl (pH 8.0). Pipette-mix 25 times and incubate the mixture for 2 min at room temperature. Proceed to the A-base addition step (Step 34); as in the end-repair step, the reaction will be done in the same well, in the presence of the beads.

A-base addition ● TIMING 45 min

34| Prepare the A-base addition reaction mix according to sample number as outlined in the table below. Add 20 µl of mix to each well (final reaction volume is 60 µl) and pipette-mix 15 times.

Reagent	Volume (µl) per reaction	Final concentration
A-base addition buffer	17	1×
Klenow exo$^-$ (5 U µl^{-1})	3	0.25 U µl^{-1}
Mix total volume	20	

35| Seal the plate with an adhesive PCR film and incubate it at 37 °C for 30 min in the thermal cycler.

A-base reaction cleanup ● TIMING 20 min

36| Add 132 µl of SPRI buffer to each well (2.2× ratio) and resuspend the beads completely by pipette-mixing 25 times. Incubate the suspension for 2 min at room temperature. Place the plate on a DynaMag-96 magnet for 4 min to capture the beads. Discard the supernatant.

37| Leave the plate on the magnet and wash the beads twice with 100 µl of 70% (vol/vol) ethanol without disturbing the beads; discard the supernatant. Remove the plate from the magnet and air-dry the SPRI beads for 4 min at room temperature.

38| Elute with 21 µl of 10 mM Tris-HCl (pH 8.0). Pipette-mix 25 times and incubate the mixture for 2 min at room temperature. Place the plate on the magnet for 3 min and transfer 19 µl of the supernatant containing the purified products into a new well. Discard the SPRI beads.

▲ **CRITICAL STEP** The ligation buffer contains PEG. Therefore, the next reaction (adapter ligation, Step 39) is done without SPRI beads in order to avoid compromising the yield.

Adapter ligation ● TIMING 30 min

39| Thaw the plate containing Y-shaped indexed adapters. Spin the plate for 30 s at maximum speed before removing the adhesive film cover.

▲ **CRITICAL STEP** It is very important to spin down the adapter plate before removing the adhesive film cover in order to prevent cross-contamination of adapters.

40| Prepare the adapter ligation mix according to sample number as outlined in the table below. Add 34 µl of mix to each well and pipette-mix.

Reagent	Volume (µl) per reaction	Final concentration
2× DNA quick ligase buffer	29	1×
DNA ligase	5	
Mix total volume	34	

41| Add 5 µl of adapter (0.75 µM) to each well (the final reaction volume is 58 µl).
▲ **CRITICAL STEP** Most library preparation protocols use adapters at a concentration of 15 µM. As the input DNA from ChIP is very low (sub-nanograms), we reduced the adapter concentration 20 times to increase ligation efficiency and reduce by-products resulting from adapter excess during amplification (Steps 46–47).

42| Seal the plate with an adhesive PCR film, spin down the plate and incubate it for 15 min at 25 °C in a thermal cycler.

Adapter ligation SPRI cleanup ● TIMING 20 min
43| Add 58 µl of 10 mM Tris-HCl (pH 8.0) to 58 µl of the reaction mix from the adapter ligation step (Step 42). Add 56 µl of new SPRI beads and pipette-mix 25 times. Incubate the mixture for 2 min at room temperature. Place the plate on a DynaMag-96 magnet for 4 min to capture the beads. Discard the supernatant.
▲ **CRITICAL STEP** The resulting 0.7× ratio of PEG to sample (some of the PEG is already present in the ligation mix) is lower than former cleanup steps. This low ratio allows for the removal of free adapters by size cutoff.

44| Leave the plate on the magnet and wash the beads twice with 100 µl of 70% (vol/vol) ethanol without disturbing the beads; discard the supernatant. Remove the plate from the magnet and air-dry SPRI beads for 4 min at room temperature.

45| Elute with 42 µl of 10 mM Tris-HCl (pH 8.0). Pipette-mix 25 times and incubate the mixture for 2 min at room temperature. Place the plate on the magnet for 3 min and transfer 40 µl of the supernatant containing the purified product into a new well. Discard the SPRI beads.
■ **PAUSE POINT** The sample can be frozen at −20 °C for up to 12 months.

Amplification ● TIMING 1 h
46| Prepare the amplification PCR mix according to the sample number as tabulated below. Add 10 µl of PCR mix to each well (the final reaction volume is 50 µl) and pipette-mix.

Reagent	Volume (µl) per reaction	Final concentration
Amplification primers (25 µM)	2	0.5 µM each
dNTP mix (100 mM)	0.4	0.8 mM
Pfu Ultra buffer (10×)	5	1×
Pfu Ultra II fusion	1	
Nuclease-free water	1.6	
Mix total volume	10	

47| Seal the plate with an adhesive PCR film and incubate it in a thermal cycler using the following program:

Cycle number	Denature	Anneal	Extend
1	95 °C, 2 min		
2–15	95 °C, 2 min	55 °C, 30 s	72 °C, 1 min
16			72 °C, 10 min

Amplification SPRI cleanup ● TIMING 20 min

48| Add 35 µl of new SPRI beads to the sample (0.7× ratio). Pipette-mix 15 times and incubate the mixture for 2 min at room temperature. Separate the supernatant from the beads by placing the plate on the magnet for 4 min; discard the supernatant.
▲ **CRITICAL STEP** This ratio of 0.7× PEG is necessary to remove the remaining primers and adapter dimers.

49| Leave the plate on the magnet and wash the beads twice with 100 µl of 70% (vol/vol) ethanol without disturbing the beads; discard the supernatant. Remove the plate from the magnet and air-dry the SPRI beads for 4 min at room temperature.

50| Add 40 µl of 10 mM Tris-HCl (pH 8.0) to each well and pipette-mix 15 times. Separate the supernatant from the beads by placing the plate on the magnet for 3 min; transfer the supernatant (which contains the ChIP-seq library) to a new plate. Discard the SPRI beads.

Multiplexing ● TIMING 1 h

51| Measure DNA concentration using the Qubit dsDNA HS kit according to the manufacturer's instructions (a Qubit fluorometer may be used; alternatively, use any plate-reader with similar sensitivity).
▲ **CRITICAL STEP** The expected library concentration is at least 5 ng μl^{-1}. When the DNA concentration is low, it is advisable to run TapeStation or Bioanalyzer assays in order to evaluate the content of the library (the amount of primer dimers that are detected as a ~100 bp band, the range of the DNA size and so on).
? TROUBLESHOOTING

52| Pool equimolar amounts of bar-coded samples. Since fragment size should be similar for all samples, it is sufficient to pool equal quantities from each sample. We suggest pooling 20 ng from each library.
▲ **CRITICAL STEP** When pooling libraries for the first time, it is suggested to test that indeed all samples contain a similar range of fragments. This could be done by running TapeStation or Bioanalyzer assays. In addition, library concentration must be measured directly before pooling, in order to pool as accurately as possible.
? TROUBLESHOOTING

Pool size cutoff ● TIMING 30 min

53| Place the pooled libraries in a microcentrifuge tube. Adjust the pool volume to 100 µl using 10 mM Tris-HCl (pH 8.0). Add 65 µl of new SPRI beads (0.65× ratio), pipette-mix 15 times and incubate the mixture for 2 min at room temperature. Separate the supernatant from the beads by placing the tube on the DynaMag-2 magnet for 4 min.
▲ **CRITICAL STEP** At this ratio, beads bind DNA fragments larger than ~500 bp, leaving shorter fragments in the supernatant. Fragments smaller than 500 bp are the ones suitable for sequencing.

54| Transfer the supernatant to a clean microcentrifuge tube (discard the beads).

55| Add 115 µl of new SPRI beads (1.8× ratio, as the supernatant already contains 65 µl of SPRI buffer), pipette-mix 15 times and incubate the mixture for 4 min at room temperature. Place the tube on the DynaMag-2 magnet and incubate it for 4 min. Discard the supernatant.

56| Wash the beads twice with 100 µl of 70% (vol/vol) ethanol. Remove the tube from the magnet and air-dry it for 4 min at room temperature. Ensure that no ethanol traces remain in the tube.

57| Elute in 25 µl of 10 mM Tris-HCl (pH 8.0), pipette-mix 25 times and incubate the mixture for 5 min at room temperature. Place the tube on the magnet for 4 min. Transfer the supernatant, which now contains the size-selected pooled ChIP-seq libraries, to a clean microcentrifuge tube.
▲ **CRITICAL STEP** It is recommended to use TapeStation or Bioanalyzer assays to verify that the pooled libraries indeed contain the expected fragment range before sequencing.
? TROUBLESHOOTING

58| Sequence the pool containing ChIP-seq libraries on an Illumina sequencing platform. We recommend sequencing 50 bases single-end, ~2×10^6 reads per sample.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
28	No DNA at the end of the ChIP module (after SPRI cleanup)	Sonication conditions used are not optimal for the specific cell type	Adjust sonication conditions. DNA fragments after sonication should range from 200 to 500 bp. Make sure that the samples do not overheat during sonication
		Antibody used is not suitable for ChIP	Test other available antibodies
		Cross-linking was not optimal (fixation time too long, formaldehyde not fresh or concentration too high)	Follow cross-linking instructions strictly. Be sure to use glycine solution that is not older than 1 month
		Problem with the reagents used for the ChIP module	ChIP is a very sensitive assay. Be sure to prepare all reagents accurately, using molecular biology–grade chemicals. Also, make sure to add protease inhibitors as indicated in the protocol
	No enrichment detected by qPCR after the ChIP module	qPCR was conducted without previous purification of the DNA	Run qPCR only after a SPRI cleanup
		Primer design is not optimal	Design other primer sets. Usually primers designed against promoter regions work better for ChIP, as TFs usually bind at these regions
51	Low library concentration	Library enzymatic steps are not optimal	Ensure that fresh dNTP and ATP is used in the buffers
		Product is lost during SPRI cleanup steps	Perform the SPRI cleanup at the exact indicated SPRI buffer ratio. Be sure to remove all ethanol traces after washing
		Poor enrichment due to inefficient end repair, A-base addition or adaptor ligation	Be sure to mix well all reaction components before adding to the sample, and pipette afterwards
52	High levels of adapter dimers in the library	Low DNA input (< < 1 ng)	Adapters can also be amplified during the enrichment step. The lower the sample concentration, the more adaptors remain after cleanup. Perform an extra SPRI purification cycle after the adaptor ligation cleanup by adding SPRI beads at an 0.7× ratio
57	High levels of adapter dimers in the pooled libraries	Insufficient size-selection procedure	Adapter dimers are removed during the pool size cutoff step. Be sure to follow instructions carefully

● TIMING

Steps 1–8, cell stimulation and cross-linking: 1–2 h (for cross-linking and washes)
Step 9, cell lysis: 0.5–1 h
Steps 10–13, sonication: dependent on the cell type and number of samples
Steps 14–16, IP: overnight
Steps 17–20, washing the beads: 30 min for one 96-well plate
Step 21, elution: 2–5 min for one 96-well plate
Steps 22 and 23, RNase and proteinase K treatments: 2.5 h
Steps 24 and 25, reverse cross-linking: overnight
Steps 26–28, DNA purification of ChIP products: 20 min

Steps 29 and 30, end repair: 45 min
Steps 31–33, end-repair reaction cleanup: 20 min
Steps 34 and 35, A-base addition: 45 min
Steps 36–38, A-base reaction cleanup: 20 min
Steps 39–42, adapter ligation: 30 min
Steps 43–45, adapter ligation SPRI cleanup: 20 min
Steps 46 and 47, amplification: 1 h
Steps 48–50, amplification SPRI cleanup: 20 min
Steps 51 and 52, multiplexing: 1 h
Steps 53–58, pool size cutoff: 30 min

ANTICIPATED RESULTS

Step 11: Proper shearing of the DNA should result in fragments ranging from 200 to 500 bp. In some cases, fragments of other sizes may also be seen; however, the majority should match this range, or it will affect subsequent steps (**Fig. 4a**, 'input' lane).

Step 28: Measurement of DNA concentration at the end of the ChIP module may give some information on IP. DNA concentration of the samples (as measured by the Qubit HS DNA kit) can vary depending on the quality of the antibody, the redundancy of the factor precipitated and the number of cells used. Typical results for TFs precipitated from 10^7 cells usually range from 0.1 to 1 ng μl^{-1}. IP of histone modification usually generates a much higher DNA concentration (10–50 ng μl^{-1}). It is notable that DNA concentration is not indicative of the specificity of the DNA precipitated. In other words, a certain antibody may precipitate a moderate amount of DNA and still be of high quality because of its specificity, whereas a second antibody may precipitate many nonspecific DNA fragments and therefore the concentration of DNA measured will be relatively high.

It is possible to perform a qPCR assay after Step 28 in order to obtain some preliminary data regarding the ChIP module. However, this can be done only for factors that have some known binding sites; otherwise, it is practically impossible to design suitable qPCR primers. For normalization purposes, the qPCR assay should always contain the input sample (fragmented DNA that was not immunoprecipitated) and also a set of control primers directed against a gene that is not expressed in the cell type tested (for example, in dendritic cells crystallin (*CRYAA*) is used as the control gene). Results (Ct) are first normalized relative to the input sample ($=2^{(\text{input Ct} - \text{sample Ct})}$) and then normalized again relative to the control gene. The result is the fold enrichment. In general, qPCR may be a limited means to evaluate ChIP quality, as primer design substantially affects results.

Step 51: DNA concentration after library enrichment step is important for both evaluating the quality of ChIP-seq libraries and for pooling samples with the correct ratios. The concentration is expected to be 30- to 100-fold higher than the concentration after the ChIP module (ranging from 5 to 50 ng μl^{-1}). When the concentration after the ChIP module is very high (as happens, for example, with the input samples or with histone modifications samples), the concentration after the library module may not increase substantially.

Step 57: Typical results of the TapeStation HS DNA assay before and after final size selection are presented in **Figure 4a**,**b**. Whereas before size selection the pool is composed of fragments of a large range of fragment sizes (despite the fact that sonication was optimized to produce fragments of 200–500 bp), after the last size-selection step the pool size ranges from 200 to 400 bp. The 100-bp band that appears in both lanes is composed of primer dimers. Molarity of peaks does not notably change after the size-selection process.

Sequencing results: The number of anticipated peaks, representing binding sites of a certain factor, may vary markedly depending on the factor's biology (some factors have many thousands of binding sites, and others have much fewer), the quality of the antibody and the sequencing depth (the number of reads per sample). Thus, there is no rule of thumb that can be provided here, except that we expect at least a few hundred peaks for TFs and a few thousands for chromatin marks. Examples of sequencing results are presented in **Figure 2**. **Figure 4d**,**e** presents the improvement in read number after the final size-selection step, in comparison with sequencing the same pool without size selection.

Note: Supplementary information is available in the online version of the paper.

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