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Using Chromatin Immunoprecipitation in Toxicology: A Step-by-Step Guide to Increasing Efficiency, Reducing Variability, and Expanding Applications

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Abstract

Histone modifications work in concert with DNA methylation to regulate cellular structure, function, and the response to environmental stimuli. More than 130 unique histone modifications have been described to date and chromatin immunoprecipitation (ChIP) allows for the exploration of their associations with the regulatory regions of target genes and other DNA/chromatin-associated proteins across the genome. Many variations of ChIP have been developed in the 30 years since its earliest version came into use, which makes it challenging for users to integrate the procedure into their research programs. Further, the differences in ChIP protocols can confound efforts to increase reproducibility across studies. The streamlined ChIP procedure presented here can be readily applied to samples from a wide range of in vitro studies (cell lines and primary cells), and clinical samples (peripheral leukocytes) in toxicology. We also provide detailed guidance on the optimization of critical protocol parameters, such as chromatin fixation, fragmentation, and immunoprecipitation, to increase efficiency and improve reproducibility. Expanding toxicogenomic studies to more readily include histone modifications will facilitate a more comprehensive understanding of the role of the epigenome in environmental exposure effects and the integration of epigenetic data in mechanistic toxicology, adverse outcome pathways, and risk assessment.

Keywords

Epigenome; chromatin; histone; immunoprecipitation; leukocytes; transcription factors

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INTRODUCTION

The genomic DNA of every eukaryotic cell is organized on nucleosomes, repeating heterooctameric structures containing two copies of histone 2A (H2A), H2B, H3 and H4, collectively referred to as chromatin. While the genome contains the blueprint for cellular structure and function, the accessibility and use of this information is regulated by covalent modifications to the DNA and its histone protein scaffolding known as the epigenome. In contrast to DNA, histones are subject to a broad range of post-translational modifications, including methylation, acetylation, phosphorylation, ubiquitination, and SUMOylation, among others (Kouzarides, 2007). To date more than 130 discrete histone modifications have been identified (Tan *et al.*, 2011), and along with DNA methylation these modifications are analogous to an individual letters in a complex alphabet that encodes the instruction manual for the regulation of gene expression. This epigenetic code is “read” by specific binding domains in chromatin-associated proteins, which control chromatin accessibility and the recruitment of the transcriptional machinery (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Cedar and Bergman, 2009). The epigenome is dynamic and responsive to both chemical and non-chemical aspects of an individual’s environment (Baccarelli and Bollati, 2009; Burris and Baccarelli, 2014). While environmentally-induced changes in some epigenetic modifications are relatively transient, others are persistent and have the potential to impact cellular function and organismal health throughout an organism’s lifetime and across generations. Further, pre-exposure levels of certain histone modifications have been shown to correlate with the magnitude of post-exposure gene induction (McCullough *et al.*, 2016), suggesting that baseline histone modification levels have potential as predictors of exposure outcomes. Furthering our understanding of interactions between the environment and the epigenome will provide novel insight into inter-individual variability in susceptibility, mechanisms underlying exposure-related disease, multi- and trans-generational health effects, and modifiable factors that can be leveraged to mitigate exposure effects.

The NIH Roadmap Epigenetics Consortium recently highlighted the variation in genome-wide patterns of histone modifications across gene regulatory regions and between cell and tissue types (Roadmap Epigenetics Consortium). Chromatin immunoprecipitation (ChIP) allows the researcher to explore the distribution and abundance of specific histone modifications either globally (via ChIP-Seq) or at specific loci of interest (via ChIP-qPCR). While **Basic Protocol 1** describes the protocol for ChIP-qPCR, this protocol can be easily adapted for ChIP-Seq experiments. The ChIP protocol presented here is generally applicable to cells grown in culture and peripheral leukocytes isolated from whole blood (**Support Protocol 2**) and provides guidance on optimizing critical parameters of the protocol for use with other cell lines/types and target histone modifications/genomic loci of interest. This protocol can also be adapted for use with tissue or biopsy specimens. Specific details are presented here for conducting ChIP in primary bronchial epithelial cells grown in air-liquid interface (ALI) culture and the monocytic leukemia cell line THP-1 for specific histone modifications within the regulatory regions of four genes (*IL-8*, *IL-6*, *COX2*, and *HMOX1*) that are commonly considered in toxicological studies.

STRATEGIC PLANNING

It is important to optimize the ChIP protocol for the cell type/line of interest and carefully plan studies that will involve ChIP experiments. Optimization of critical aspects of the protocol, such as the use of a sufficient number of cells per sample for the histone modification and genomic locus of interest, sample fixation conditions, chromatin fragmentation parameters, and choice of antibody, facilitates assay success and consistency. After conditions have been optimized, the number of ChIP assays for each antibody target should be estimated and used to calculate the total amount of antibody required for all the technical and biological replicates in the study. The required amount of antibody should be obtained, combined, and separated into single use aliquots. Doing so will increase reproducibility across experiments within the study (explained further in Critical Parameters below). Care should be taken at all steps to limit technical variability thus increasing reproducibility.

BASIC PROTOCOL 1

Quantifying the Abundance of Epigenetic Modifications Within the Regulatory Regions of Target Genes—This protocol describes the use of chromatin immunoprecipitation (ChIP) in cultured cells to quantify the relative abundance of specific epigenetic modifications, transcription factors, and other chromatin-associated proteins within target areas of the genome. The protocol can be used for either adherent cells (cell lines or primary cells), cells grown in suspension, or peripheral leukocytes isolated from whole blood. Chromatin within a given genomic region typically contains a variety of epigenetic modifications; however it is important to note that not all epigenetic modifications exist within the regulatory regions of all genes and thus may not be universally detectable. The ChIP protocol described below has been optimized for the detection of histone H3 lysine 4 trimethylation (H3K4me3), H3K27me3, H3K27ac, pan-acetylated histone H4 (H4ac), total H3, and 5-hydroxymethylcytosine (5-hmC) within the promoter regions of interleukin-8 (*IL-8*), *IL-6*, cyclooxygenase 2 (*COX2* also known as *PTGS2*), and hemeoxygenase 1 (*HMOX1*); however, it can serve as a starting point for the quantification of other histone modifications within additional regulatory regions of interest. An overview of the ChIP workflow is shown in Figure 1. The protocol described here represents a traditional approach to ChIP that is suitable for analyzing interactions between target histone modifications and/or chromatin-associated proteins (*e.g.* histone modifying enzymes, transcription factors, and co-activators/co-repressors) and genomic loci of interest; however, there are alternatives such as native ChIP (O'Neill and Turner, 2003) and microchip (Dahl and Collas, 2008) that have more specialized applications, which will not be discussed here.

Materials

Cells in culture

ChIP grade antibodies (see Table 2 for antibodies that we have used successfully with this protocol)

Dulbecco's phosphate buffered saline (DPBS, room temperature)

0.25% Trypsin (for adherent cultures)

DPBS-TI (for adherent cultures)

Cell strainer (70 μ m pore)

16% paraformaldehyde (in single-use ampules)

2.5M glycine

Protease inhibitor solution (PIS; ice-cold; see recipe)

Hemocytometer

Liquid nitrogen

Sonicator with micro probe tip (Fisher Scientific Sonic Dismembrator Model 500)

End-over-end rotator

Digital temperature controlled dry bath

ChIP Lysis Buffer (see recipe)

ChIP Dilution Buffer (see recipe)

Low Salt Wash Buffer (see recipe)

High Salt Wash Buffer (see recipe)

LiCl Wash Buffer (see recipe)

TE Buffer (see recipe)

Elution Buffer (see recipe)

Elution Addition Buffer (see recipe)

3 M sodium acetate, pH 5.2 (see recipe)

100% ethanol

Protein A/G polyacrylamide beads

10 mg/mL Proteinase K (see recipe)

Phenol:chloroform:isoamyl alcohol (25:24:1)

Chloroform:isoamyl alcohol (1:1)

10 mg/mL glycogen (see recipe)

Protocol Steps

Cell Collection—Adherent cells in submerged culture

1. Following the completion of the desired exposure, aspirate the medium (for submerged cells) and wash with DPBS.
2. Add 0.25% trypsin and incubate at 37 °C to detach cells from the culture dish.
 - a. **OPTIMIZATION:** the duration of trypsinization required to remove cells from the culture dish will vary between cell types. Determine the optimal trypsinization time for your cell type(s) of interest as under-trypsinization will reduce the cell yield and over-trypsinization will result in cell clumping. Depending on the cell type a less stringent digestion, for example using Accutase (Sigma), may be preferred.
 - b. **REPRODUCIBILITY:** the activity of enzyme solutions changes with each warming and cooling cycle. Prepare single use aliquots of the trypsin solution to decrease variability due to differential trypsinization.
 - c. **VARIATION:** if using cells in air-liquid interface cultures then add trypsin to the apical and basolateral compartments of the Transwell insert. Incubate at 37 °C for 5–10 minutes, or until cells detach.
 - i. For 24 mm inserts use 500 µL and 1500 µL, respectively.
 - ii. For 12 mm inserts use 250 µL and 750 µL, respectively
 - iii. For 6.5 mm inserts use 100 µL and 500 µL, respectively
 - iv. **NOTE:** Cell detachment should be monitored closely to prevent over-trypsinization, which will reduce reproducibility between replicates.
 - d. **VARIATION:** if using THP-1 then trypsinization is not required. Instead, transfer the medium containing cells to a suitably-sized conical tube, wash the plate with fresh growth medium, and add the wash medium to the same conical tube. Proceed to step #5.
3. Immediately after cells become detached, add an equal volume of DPBS-TI.
4. Collect the trypsinized cells by adding ice-cold DPBS-TI and triturating to dissociate clumped cells.
 - a. **REPRODUCIBILITY:** if multiple culture dishes are being used to generate sufficient starting material for each treatment condition then combine them into a single conical tube.
5. Pellet cells by centrifugation at $1,000 \times g$ for 4 minutes at 4 °C.
6. Carefully aspirate the supernatant, resuspend the cell pellet in 1 mL of room temperature DPBS, then add DPBS to give a final cell density of 1×10^6 cells/mL.

- a. **REPRODUCIBILITY:** the cell suspension does not need to be quantified at this point since the added time may augment the epigenetic modifications of interest; however, an average number of cells per culture dish for each treatment condition should be determined prior to conducting ChIP experiments. Consistent cell density during fixation will increase the reproducibility of the procedure.
7. Remove cell clumps by passing the cell suspension through a 70 μ m pore cell strainer.
8. Crosslink the chromatin by adding 16% paraformaldehyde to the strained cell suspension to a final concentration of 1% and mix by gentle inversion. Incubate at room temperature for 5 minutes without agitation.
 - a. **REPRODUCIBILITY:** paraformaldehyde ampules are preferable and paraformaldehyde should only be used within 24 hours of the ampule being opened. The use of larger volume commercial preparations of formaldehyde solution is discouraged since it will reduce chromatin yield when “older” fixative solutions are used.
9. Quench the fixation reaction by adding 2.5 M glycine to a final concentration of 125 mM and mixing by gentle inversion. Incubate for 5 minutes at room temperature without agitation.
10. Pellet crosslinked cells at $1,000 \times g$ for 4 minutes at room temperature.
11. Aspirate the supernatant and wash the fixed cells by resuspending in 1 mL of ice-cold PIS then adding 9 mL of additional ice-cold PIS and mixing by gentle inversion.
12. Take an aliquot (dilute if desired) and enumerate the cells with a hemocytometer.
13. Pellet the cells at $1,000 \times g$ for 4 minutes at room temperature.
14. Aspirate the supernatant and resuspend the cells at a concentration that is twice the desired number of cells per immunoprecipitation (IP) per milliliter (*e.g.* if 1×10^6 cells per IP is desired then prepare a cell suspension of 2×10^6 cells/mL).
 - a. **NOTE:** the number of cells that should be used for each IP may vary depending on the target and genomic locus of interest and should be empirically determined.
15. Aliquot 1 mL of the cell suspension into individual Eppendorf tubes, pellet the cells at $1,000 \times g$ for 4 minutes at 4 °C, and aspirate the supernatant.
 - a. **STOPPING POINT:** if desired the cell pellets can be flash frozen in liquid nitrogen and stored at -80 °C. Freezing in liquid nitrogen is optimal for preserving protein modifications.
 - b. **REPRODUCIBILITY:** if pellets are frozen prior to continuing, then ensure that all replicates/samples are similarly frozen before proceeding to the fragmentation step.

Chromatin Fragmentation and Immunoprecipitation

16. If cell pellets were frozen after step #15 then thaw cell pellets on ice.
17. Resuspend the cell pellet in 300 μ L of room temperature ChIP Lysis Buffer and incubate on ice for 10 minutes.
 - a. **NOTE:** ChIP Lysis Buffer has a high concentration of SDS, which will precipitate if the buffer gets too cold. To prevent precipitation, lyse cells by placing tubes transversely on top of ice.
18. Fragment the chromatin.
 - a. **NOTE:** This protocol uses a probe-tip sonicator but enzymatic techniques are available, as are other sonication instruments (*e.g.* Bioruptor).
 - b. **NOTE:** Place each tube on ice for at least 90 seconds between each sonication cycle. Excess heating will compromise the samples and reduce IP efficiency.
 - c. Sonication conditions for primary bronchial epithelial cells when using the Fisher Scientific Sonic Dismembrator Model 500
 - i. Sonicate the fixed cell pellets for 7 cycles, each consisting of 10 pulses (0.9 seconds on and 0.1 seconds off) at 20% output.
 - d. Sonication conditions for THP-1 cells when using the Fisher Scientific Sonic Dismembrator Model 500
 - i. Sonicate the fixed cell pellets for 6 cycles, each consisting of 10 pulses (0.9 seconds on and 0.1 seconds off) at 20% output.
 - ii. Figure 2 demonstrates the effect of sonication cycle number on chromatin fragmentation in fixed THP-1 cells.
 - e. **OPTIMIZE:** the duration, power, and number of sonication cycles must be empirically determined for each cell type that will be used in the ChIP protocol. Further, the sonication conditions will depend on the wattage of the sonicator being used. The optimal number of cycles can be determined using **Support Protocol 1**. Using the data shown in Figure 2, optimal fragmentation was accomplished with six cycles of sonication.
 - f. **REPRODUCIBILITY:** if multiple pellets from the same sample are being used to achieve the required amount of starting material then combine the fragmented chromatin into a single pre-chilled Eppendorf tube prior to proceeding.
19. Clarify the fragmented chromatin by centrifugation at $20,000 \times g$ for 10 minutes at 10 °C.
 - a. **NOTE:** centrifugation results in the collection of any insoluble material at the bottom of the tube.

20. Transfer the supernatant (clarified chromatin) to a pre-chilled conical tube on ice.
21. Dilute the clarified chromatin with nine volumes of ice-cold ChIP Dilution Buffer.
22. Take a sample of the diluted chromatin to be used as the “input” sample and store at -80°C until step #30.
 - a. The input sample is typically either 1% or 5% of the diluted chromatin volume. Make note of the percentage of the diluted chromatin taken for the input sample(s) as it will be needed during data analysis. If the chromatin is pooled prior to beginning the IPs then only one input sample for each treatment condition (instead of each sample) is required.
23. Divide the diluted chromatin evenly into individual snap-lock Eppendorf tubes (one tube for each IP).
 - a. **NOTE:** snap-lock Eppendorf tubes are used to prevent the caps from opening during the subsequent antibody and bead binding steps.
24. Add the antibody targeting the epigenetic modification of interest to each tube.
 - a. **OPTIMIZE:** the amount of antibody used for each IP must be determined empirically for each antibody and cell type. Typically, an IP from 1×10^6 cells will require between 1 and 15 μg of antibody.
 - b. **REPRODUCIBILITY:** there is considerable lot-to-lot variability in antibody IP efficiency, especially in polyclonal antibodies. Avoid directly comparing data from IPs conducted with different antibodies and antibody preparations from different production lots. It is recommended that the user either acquire a sufficient amount of antibody preparation to complete the study from a single lot or pool different lots of the same antibody and prepare single-use aliquots before beginning the project. When possible, use monoclonal antibodies to reduce the variability in avidity that can occur between lots of polyclonal antibodies. When possible, use purified antibody preparations to reduce interactions between non-specific antibodies and the IP reaction.
25. Incubate overnight at 4°C on an end-over-end rotator.

Capture antibody-chromatin complexes

26. Prepare protein A/G polyacrylamide beads.
 - a. For n immunoprecipitations, transfer $(n + 2) \times 50 \mu\text{L}$ of Protein A/G polyacrylamide bead slurry into a pre-chilled conical tube.

32. Elute immunoprecipitated material from the beads by adding 265 μL of SDS Elution Buffer, vortexing, and incubating at 65 $^{\circ}\text{C}$ for 5 minutes.
33. Pellet beads at $3,000 \times g$ for 2 minutes at room temperature and transfer 250 μL of the supernatant to a new Eppendorf tube.
34. Repeat steps #30 and 31 and combine the two elution volumes (now referred to as the “elution”) in a single Eppendorf tube.

Crosslink Reversal, Proteinase Digestion, and DNA Purification

35. Add SDS Elution Buffer to input samples (thawed in step #30) to a total volume of 500 μL .
36. Add 50 μL of Elution Addition Buffer to the diluted input (500 μL) and pooled elution (500 μL) from each IP and vortex.
37. Add 2 μL of 10 mg/mL proteinase K to each sample and incubate at 65 $^{\circ}\text{C}$ for 4 hours.
 - a. **NOTE:** if desired, this incubation step can be extended to overnight; however, to ensure reproducibility between samples the same incubation duration should be used for the entire study.
38. Extract immunoprecipitated DNA by adding an equal volume (550 μL) of phenol/chloroform/isoamyl alcohol (PCI; 25:24:1 v/v ratio), vortexing thoroughly, and centrifuging at $13,000 \times g$ for 3 minutes at room temperature.
 - a. **NOTE:** after vortexing the solution will have an opaque white appearance, which will be separated into aqueous (upper) and an organic (lower) phases during centrifugation.
39. Transfer 500 μL of the aqueous (top) phase to a new Eppendorf tube.
 - a. **NOTE:** the aqueous phase contains the immunoprecipitated DNA.
 - b. **NOTE:** the use of filter tips will reduce dripping of the aqueous phase during transfer.
 - c. **NOTE:** do not attempt to collect the entire aqueous phase as doing so will result in the carryover of phenol, which will interfere with PCR reactions and will impair accurate quantification of immunoprecipitated DNA.
40. Back extract the aqueous phase by adding an equal volume (500 μL) of 1:1 (v:v) chloroform:isoamyl alcohol solution, vortexing thoroughly, and centrifuging at $13,000 \times g$ for 3 minutes at room temperature.
 - a. **NOTE:** ensure that the 1:1 chloroform:isoamyl alcohol solution is prepared fresh before use.
41. Transfer 450 μL of the aqueous (top) phase to a new Eppendorf tube.

42. Precipitate the immunoprecipitated DNA from the back extracted aqueous phase by adding 44 μL of 3 M sodium acetate, pH 5.2 (see recipe), 2 μL of 10 mg/mL glycogen, and 1 mL of 100% ethanol, vortexing, and incubating at $-80\text{ }^{\circ}\text{C}$ overnight
 - a. **NOTE:** the addition of glycogen increases the precipitation of small amounts of low molecular weight DNA.
 - b. **STOPPING POINT:** the precipitation can be left at $-80\text{ }^{\circ}\text{C}$ for up to several days if necessary.
43. Pellet precipitated DNA at $20,000 \times g$ for 15 minutes at $4\text{ }^{\circ}\text{C}$.
44. Carefully aspirate the supernatant with a 25 ga needle.
 - a. **NOTE:** the DNA pellet is not always visible following centrifugation. To prevent unintended sample loss place all of the Eppendorf tubes in the same orientation in the centrifuge rotor so the pellet will form in a predictable location in all tubes.
45. Wash the pellets by adding 1 mL of 70% ethanol (chilled to $-20\text{ }^{\circ}\text{C}$) to each tube and vortexing.
46. Re-pellet the washed DNA by centrifugation at $20,000 \times g$ for 10 minutes at $4\text{ }^{\circ}\text{C}$.
47. Carefully aspirate the supernatant with a 25 ga needle, leaving approximately 10–15 μL volume to prevent the accidental aspiration of DNA.
48. Dry the DNA pellets in a speed-vac, fume hood, or laminar flow hood until all of the residual ethanol has evaporated.
 - a. **NOTE:** ensure that all of the residual ethanol has evaporated before continuing. Residual ethanol will interfere with PCR reactions and will impair accurate quantification of immunoprecipitated DNA.
49. Resuspend the DNA in 50 μL of TE Buffer and store at $-20\text{ }^{\circ}\text{C}$.
 - a. **NOTE:** depending on the number of target loci to be evaluated by qPCR it may be necessary to dilute the input DNA. If so, then dilute the input DNA in TE Buffer and account for the dilution factor in the calculations used to determine % input values for immunoprecipitated DNA.

Quantify Immunoprecipitated DNA by qPCR

50. Use 2 μL of the purified DNA from step #49 per 20 μL qPCR reaction.
 - a. **NOTE:** if desired, the purified DNA can be diluted prior to use in qPCR reactions; however, this must be determined empirically for each genomic locus to be assessed as the distribution and abundance of epigenetic modifications varies between genomic regions. If targeting a low-abundance epigenetic modification then a greater volume of

purified DNA may be required in each qPCR reaction for reproducible quantification.

- b. **NOTE:** recommendations on ChIP-qPCR data analysis are given in the *Expected Results* section below.

SUPPORT PROTOCOL 1

Determine Optimal Sonication Conditions for ChIP—The target locus specificity and reproducibility of ChIP relies on the use of fragmentation conditions that consistently generate chromatin fragments that average ~500 bp with a range of ~250 – 750 bp (Figure 2). The use of chromatin fragments within this size range allows for the reliable detection of association between target histone modifications and discrete genomic loci by ChIP-qPCR. Larger chromatin fragments reduce the specificity of detection (target histone modifications could be occurring on more distant nucleosomes that remain linked to target genomic loci) and smaller fragments can hinder the detection of target genomic loci by qPCR. The sonication conditions required to achieve this level of fragmentation will vary based on several parameters, including cell type/cell line, sonicator wattage, sonicator probe tip, and buffer conditions. This protocol describes the optimization of chromatin fragmentation by sonication. During early protocol development prepare a large number of chromatin pellets to determine the sonication/fragmentation conditions. Then test the conditions across several chromatin preparations to ensure that the sonication/fragmentation is reproducible across replicates.

Materials

Cells in culture

Dulbecco's phosphate buffered saline (DPBS; room temperature)

0.25% Trypsin (for adherent cultures)

Soybean trypsin inhibitor (for adherent cultures)

Cell strainer (70 µm pore)

16% paraformaldehyde (in single-use ampules)

2.5M glycine (see recipe)

Protease inhibitor solution (PIS; ice-cold; see recipe)

Hemocytometer

Liquid nitrogen

Sonicator with micro probe tip (Fisher Scientific Sonic Dismembrator Model 500)

Digital temperature controlled dry bath

ChIP Lysis Buffer (see recipe)

ChIP Dilution Buffer (see recipe)

TE Buffer (see recipe)

3 M sodium acetate, pH 5.2 (see recipe)

100% ethanol

10 mg/mL Proteinase K (see recipe)

RNaseA (20 mg/mL)

Phenol:chloroform:isoamyl alcohol (25:24:1)

Chloroform:isoamyl alcohol (1:1, v:v)

10 mg/mL glycogen (see recipe)

3% agarose/TBE gel (see recipe)

TBE Buffer (see recipe)

10 mg/mL ethidium bromide

100bp DNA ladder

6X Purple gel loading dye

Ultraviolet light box

Protocol Steps

Determine Optimal Chromatin Fragmentation Parameters

1. Complete steps #1–15 of **Basic Protocol 1** to prepare fixed chromatin.
2. Fragment the chromatin using an increasing duration of sonication.
 - a. **NOTE:** Start with four cycles at 10 pulses (0.9 sec on and 0.1 sec off) and increase the number of cycles. Be sure to place each tube on ice for at least 90 seconds between each cycle. This is important because excessive heat generated by sonication may compromise sample integrity, decreased IP efficiency, and increased variability.
 - b. **NOTE:** Before beginning the sonication time course, determine the most rigorous sonication conditions for your particular sonicator and sample. The goal of this step is to identify the highest output that can be used consistently without causing foaming of the chromatin samples. Foaming should be avoided because the bubbles reduce the transmission of energy from the sonicator probe tip to the sample thus limiting sonication efficiency. If using a 500 Watt sonicator (as described here), begin by sonicating in cycles of 10 pulses (0.9 sec on

and 0.1 sec off) at 20% output. Using 30% output on a 500 W sonicator can reduce the total number of pulses/cycles required to achieve the desired chromatin fragmentation; however, using a higher output increases the likelihood of foaming in chromatin samples of a relatively low volume, such as those described here. The likelihood of foaming at higher output can be reduced in samples of larger volume, or when the microprobe tip is able to project into the sample (e.g. sonication of chromatin in a volume of 300 μ L is more successfully executed in a 0.5 mL microcentrifuge tube than a 1.5 mL Eppendorf tube). If using a sonicator with a lower power output then a greater percentage of output may be required to achieve the desired level of chromatin fragmentation. Consult the user's manual provided with your sonicator to avoid exceeding the recommended maximum output for a microprobe tip.

3. Clarify the fragmented chromatin by centrifugation at $20,000 \times g$ for 10 minutes at 4 °C.
4. Transfer the clarified chromatin to a new Eppendorf tube.
5. Add SDS Elution Buffer to each sample to give a total volume of 500 μ L then add 50 μ L of Elution Addition Buffer and vortex.
6. Add 2 μ L of 10 mg/mL proteinase K to each sample and incubate at 65 °C for 4 hours.
 - c. **NOTE:** if desired, this incubation step can be extended to overnight; however, to ensure reproducibility between samples the same incubation duration should be used for the entire study.
7. Extract DNA from the fragmented chromatin samples by adding an equal volume (550 μ L) of phenol/chloroform/isoamyl alcohol (PCI; 25:24:1 v/v ratio), vortexing thoroughly, and centrifuging at $13,000 \times g$ for 3 minutes at room temperature.
8. Transfer 500 μ L of the aqueous (top) phase to a new Eppendorf tube.
 - d. **NOTE:** the use of filter tips will reduce dripping of the aqueous phase during transfer.
9. Back extract the aqueous phase by adding an equal volume (500 μ L) of 1:1 (v:v) chloroform:isoamyl alcohol solution, vortexing thoroughly, and centrifuging at $13,000 \times g$ for 3 minutes at room temperature.
 - e. **NOTE:** ensure that the 1:1 chloroform:isoamyl alcohol solution is prepared fresh before use.
10. Transfer 450 μ L of the aqueous (top) phase to a new Eppendorf tube.
11. Precipitate the immunoprecipitated DNA from the back extracted aqueous phase by adding 44 μ L of 3 M sodium acetate, pH 5.2 (see recipe), 2 μ L of 20 mg/mL

glycogen, and 1 mL of 100% ethanol, vortexing, and incubating at -80°C overnight

- f. STOPPING POINT:** the precipitation can be left at -80°C for up to several days if necessary.
- 12.** Pellet precipitated DNA at $20,000 \times g$ for 15 minutes at 4°C .
- 13.** Carefully aspirate the supernatant with a 25 ga needle.
 - g. NOTE:** the DNA pellet is not always visible following centrifugation. To prevent unintended sample loss place all of the Eppendorf tubes in the same orientation in the centrifuge rotor so the pellet will form in a predictable location in all tubes.
- 14.** Wash the pellets by adding 1 mL of 70% ethanol (chilled to -20°C) to each tube and vortexing.
- 15.** Re-pellet the washed DNA by centrifugation at $20,000 \times g$ for 10 minutes at 4°C .
- 16.** Carefully aspirate the supernatant with a 25 ga needle.
- 17.** Air dry the DNA pellets in a fume hood or laminar flow hood until all of the residual ethanol has evaporated.
 - h. NOTE:** ensure that all of the residual ethanol has evaporated before continuing. Residual ethanol will interfere with PCR reactions and will impair accurate quantification of immunoprecipitated DNA.
- 18.** Resuspend the DNA in 50 μL of TE Buffer.
- 19.** Add 20 μg of RNaseA and incubate at 37°C for 30 minutes.
 - a. STOPPING POINT:** samples can be stored at -20°C until use.
- 20.** Separate the fragmented DNA on a 3% agarose/TBE gel.
 - a.** Add ethidium bromide to a final concentration of 0.4 $\mu\text{g/mL}$ to the molten agarose prior to casting the gel.
 - b.** If starting with 1×10^6 cells/condition then load 24 μL per well of a mixture containing 20 μL of the resuspended DNA with 4 μL a suitable 6X loading buffer.
 - c.** Use a 100bp ladder as a basis for assessment of average chromatin fragment size.
- 21.** After running the gel for the desired amount of time, destain the gel in TBE Buffer in 15–20 minute intervals (use fresh TBE Buffer for each interval) to remove unbound ethidium bromide from the gel and image on a UV light source.

SUPPORT PROTOCOL 2

Isolation of leukocytes from peripheral whole blood—Peripheral blood samples can be readily collected during toxicology studies; however, whole blood is not suitable for ChIP. This support protocol describes a rapid method for the isolation of leukocytes from peripheral whole blood for use in ChIP experiments.

Materials

ACK Lysis Buffer (see recipe)

Cell Resuspension Solution (see recipe)

Hemocytometer

Protocol Steps

1. Transfer fresh whole blood from each collection tube into individual 50 mL conical tubes on ice.
 - a. **NOTE:** blood should be used immediately after collection in tubes with either EDTA or sodium citrate as an anti-coagulant.
2. Add 5 volumes of ACK buffer and mix gently, but thoroughly prior to incubating on ice for 5 minutes.
 - a. **NOTE:** incubation with ACK Lysis Buffer lyses erythrocytes, but not leukocytes.
3. Pellet intact cells by centrifugation at $500 \times g$ for 4 minutes at 4 °C
4. Carefully aspirate the supernatant.
 - a. **NOTE:** the aspirate should be treated as potentially biohazardous material until decontaminated with bleach (final concentration of 10%) or other suitable decontaminating agent.
5. Resuspend the cell pellet in one volume (original blood volume transferred in step #1) of ACK Lysis Buffer and transfer to a new 15 mL conical tube and incubate on ice for 2 minutes.
 - a. **NOTE:** A single ACK lysis step is often insufficient to lyse all of the erythrocytes in the blood sample. This second ACK lysis step ensures thorough erythrocyte lysis.
6. Centrifuge at $500 \times g$ for 4 minutes at 4 °C and aspirate the supernatant.
7. Wash the pellet by resuspending in 1 mL of Cell Resuspension Buffer and transfer the cell suspension to a 15 mL conical tube. Add Cell Resuspension Buffer to a final volume of 10 mL.
8. Enumerate cells with a hemocytometer.
9. Pellet cells at $500 \times g$ for 4 minutes at 4 °C and aspirate the supernatant.

10. Resuspend the pellet with room temperature Cell Resuspension Buffer to the desired cell density.
11. Continue ChIP by starting at step #8 of **Basic Protocol 1**.
 - a. **NOTE:** the relative abundance of different cell populations should be considered when using total leukocytes from whole blood as starting material. Ideally, a white blood cell differential count should be conducted to determine the composition of the leukocyte population in the sample (or blood collected at the same time as the sample).

SUPPORT PROTOCOL 3

TaqMan Quantitative PCR of ChIP Samples—TaqMan qPCR can be used to quickly and accurately quantify the amount of specific gDNA loci isolated by the ChIP procedure. Multiplexing of TaqMan qPCR probes can allow for the quantification of multiple loci in each qPCR reaction; however, the pairing and concentration of TaqMan probes for multiplex qPCR reactions should be optimized to ensure proper detection and reaction efficiency.

Materials

Bio-Rad iTaq Universal Probes 2X Master Mix (#172–5134)

Nuclease-free water

Eppendorf tubes

Kimwipes

Primers and probes resuspended at 100 μ M in 10mM Tris, pH 8.0, 0.1mM EDTA and used to prepare working stocks at 6.0 μ M (primers) and 6.8 μ M (probes)

PCR plate and optical film compatible with desired real-time qPCR apparatus

One aliquot of undiluted genomic DNA (gDNA) to use for the preparation of a standard curve. Can be input material from another ChIP or generated from Support Protocol 1.

Protocol Steps

Prepare a standard curve

1. Thaw a single-use aliquot of standard curve gDNA. This gDNA will serve as the undiluted standard.
2. Label five Eppendorf tubes B through F and aliquot 90 μ L of nuclease-free water into each tube.
3. Add 10 μ L of the undiluted standard to tube B and vortex thoroughly.
4. Add 10 μ L of the Standard B to tube C and vortex thoroughly.
5. Add 10 μ L of the Standard C to tube D and vortex thoroughly.
6. Add 10 μ L of the Standard D to tube E and vortex thoroughly.

7. The water aliquoted into tube F will serve as the non-template control.

Prepare the TaqMan qPCR Reaction Master Mix

8. Mix reagents as described below. The recipe below is designed for multiplexing two targets, which are detected using TaqMan probes labeled with compatible dyes. The optimal probe concentration for TaqMan qPCR detection may vary between probes and multiplexing conditions. Each ChIP sample should be assayed in technical triplicate. Prepare a sufficient volume for the number of wells required with additional volume to compensate for volume lost during pipetting.

	Per reaction
2X iTaq Universal Probes Mix	10 μ L
Target 1-F (6.0uM)	0.67 μ L
Target 1-R (6.0uM)	0.67 μ L
Target 1-Probe (6.8uM)	0.66 μ L
Target 2-F (6.0uM)	0.67 μ L
Target 2-R (6.0uM)	0.67 μ L
Target 2-Probe (6.8uM)	0.66 μ L
Water	4 μ L
Total	18 μ L

Load the plate

9. Vortex the master mix and pipette 18 μ L into the allotted wells of a 96-well PCR plate.
10. Add 2 μ L of gDNA standard or sample to be assayed into its allotted wells using a new pipette tip for each well.
 - a. **NOTE:** Check the pipette tip following the addition of each standard and sample to ensure that volume is not retained in the tip, which can be common when pipetting small volumes. Not doing so can result in high variability between technical replicates.

Prepare and read plate

11. Cover the loaded plate with adhesive optical film ensuring firm adhesion of the film to the plate and rub the film with Kimwipes to ensure a uniform fit without transferring oil or debris from gloves onto the film.
12. Wrap the plate in Saran wrap and vortex thoroughly. Wrapping the plate prevents the transfer of oil and debris from the vortex to the plate and thus into the qPCR apparatus.
13. Unwrap the plate and collect the reaction volume in the bottom of each well by spinning the plate at $1,000 \times g$ for four minutes at room temperature in a tabletop centrifuge.
14. Place the plate in the machine and proceed with the quantitative PCR. The following general conditions may be used but may have to be adjusted for specific primer/probe sets or if alternative qPCR master mixes are used:

	Temp	Duration (min)
Step #1	95°C	5:00
Step #2	95°C	0:15
Step #3	60°C	0:45
Go to Step #2 (x39)		

REAGENTS AND SOLUTIONS

Use distilled and deionized water (or equivalent) for all reagents and buffers. For common stock solutions, see *APPENDIX*; for suppliers, see *SUPPLIERS APPENDIX*.

ACK Lysis Buffer

150 mM ammonium chloride

10 mM potassium bicarbonate

500 mM EDTA

Prepare immediately before use and keep on ice

Cell Resuspension Solution

Hank's Balanced Salt Solution

1X cOmplete protease inhibitor cocktail (Roche)

20 mM sodium butyrate

25 mM sodium fluoride

1 mM sodium pyrophosphate

0.1% bovine serum albumin

Prepare immediately before use and keep on ice

DPBS-TI

Dulbecco's phosphate buffered saline

30mg/mL Soybean trypsin inhibitor

Protease Inhibitor Solution (PIS)

Dulbecco's phosphate buffered saline (Gibco)

1X cOmplete protease inhibitor cocktail (Roche)

25 mM sodium fluoride

1 mM sodium pyrophosphate

20 mM sodium butyrate

Prepare the day of use and keep on ice

ChIP Lysis Buffer

50 mM Tris, pH 8.1 (see recipe)

10 mM EDTA (see recipe)

1% SDS (w/v)

1 mM phenylmethylsulfonyl fluoride (PMSF; see recipe)

1X cOmplete protease inhibitor cocktail (Roche)

Prepare the day of use and keep at room temperature.

ChIP Dilution Buffer

16.7 mM Tris, pH 8.1 (see recipe)

167 mM NaCl

1.2 mM EDTA (see recipe)

1.1% Triton X-100

0.01% SDS (w/v)

1 mM phenylmethylsulfonyl fluoride (PMSF; see recipe)

1X cOmplete protease inhibitor cocktail (Roche)

Low Salt Wash Buffer

20 mM Tris, pH 8.1

150 mM NaCl

2 mM EDTA

1% Triton X-100

0.1% SDS

High Salt Wash Buffer

20 mM Tris, pH 8.1

500 mM NaCl

2 mM EDTA

1% Triton X-100

0.1% SDS

Lithium Chloride Wash Buffer

10 mM Tris, pH 8.1

1 mM EDTA

1% IGEPAL CA-630

250 mM LiCl

1% sodium deoxycholate

TE Buffer

10 mM Tris, pH 8.0

1 mM EDTA

Elution Buffer

100 mM NaHCO₃

1% SDS

Elution Addition Buffer

400 mM Tris, pH 6.5

2.0 M NaCl

100 mM EDTA

Proteinase K (10 mg/mL)

20 mM Tris, pH 8.0

40% glycerol

1 mM CaCl₂

Use this buffer to resuspend powdered proteinase K in the manufacturer's container to a concentration of 10 mg/mL then prepare 50 µL aliquots and store at –20 °C.

Tris Borate Electrophoresis (TBE) Buffer

8.9 mM Tris base

8.9 mM sodium borate

2.0 mM EDTA, pH 8.0

COMMENTARY

Background Information

Chromatin immunoprecipitation is a powerful technique for exploring the role of the epigenome in environmental exposure-mediated disease and susceptibility, which has been adapted and improved over the last three decades (reviewed in Carey, 2009). An early precursor of the procedure was pioneered by Gilmour and Lis (1984) to examine interactions between RNA polymerase and specific regions within the *Escherichia coli* and *Salmonella spp* genomes. While these bacteria lack the chromatin structure found in eukaryotes that would ultimately be the namesake of the technique, by identifying specific RNA polymerase binding sites within the bacterial genome Gilmour and Lis (1984) demonstrated that protein-DNA interactions could be fixed by covalent crosslinking, purified by immunoprecipitation, and mapped by hybridization. They transitioned these principles to a eukaryotic model by mapping the distribution of RNA polymerase II (RNAPII) in *Drosophila melanogaster* (Gilmour and Lis, 1985; 1986). These early ChIP protocols relied on UV light to crosslink DNA and closely associated proteins (Gilmour and Lis, 1984; 1985; 1986); however, the utility and efficacy of UV crosslinking are limited. The ability of formaldehyde to form DNA-protein and protein-protein crosslinks had been previously characterized (Brutlag et al., 1969; Ilyin and Georgiev, 1969; Van Lente, et al., 1975; Jackson, 1978), applied to whole-cell fixation to study chromatin dynamics (Jackson and Chalkley, 1981; Solomon and Varshavsky, 1985), and replaced UV light as a crosslinking agent in ChIP (Solomon et al., 1988). Nearly a decade later, the traditional probe-based blotting methods used for quantifying immunoprecipitated DNA was replaced with a PCR-based detection method (Hecht et al., 1996). The introduction of formaldehyde and PCR facilitated the emergence and popularization of ChIP (reviewed in Carey, et al., 2009). ChIP has continued to evolve with subsequent improvements in discrete aspects of the procedure to become more sensitive, accurate, and reproducible. In more recent years, the quantification of immunoprecipitated DNA by semi-quantitative PCR was replaced with SYBR green-based quantitative PCR (qPCR) or the more target-specific TaqMan qPCR. Specificity and reproducibility have also been increased by the increased availability of monoclonal antibodies and the emerging use of recombinant antibodies as a replacement for polyclonal antibodies to target proteins and histone modifications of interest.

While ChIP is a complex and potentially intimidating procedure, with careful optimization of key steps it can easily become a reliable way to readily explore the role of the epigenetic modifications in toxicology studies. Given the central role that the epigenome plays in regulating gene expression, as well as DNA replication and damage repair among other functions, understanding the relationship between environmental exposures and histone modifications will provide critical insight into the mechanisms underlying exposure effects and provide novel biomarkers of susceptibility to exposure-related disease. Here we have described a streamlined protocol for quantifying the relative abundance of specific histone modifications within regulatory regions of four genes (*IL-8*, *IL-6*, *COX2*, and *HMOX1*) that are commonly studied in the field of toxicology. This protocol is directed toward examining the relationship between histone modifications and the regulatory regions of four toxicity-related genes; however, using the principles outlined here the protocol can be readily

adapted to examine the relative abundance of target histone modifications, transcription factors, and other chromatin-associated proteins within the regulatory regions of any gene(s) of interest.

Critical Parameters and Troubleshooting

The ChIP procedure relies on several critical parameters that play an important role in both the success of the assay and reproducibility between replicates. The intrinsic variability in this complex procedure can be minimized by thoughtful attention to detail and careful optimization of several key steps. Further, it is important to consider that nucleosomal density (Figure 4A) and the distribution of histone modifications vary between target genomic loci.

Experimental Treatment Conditions—As with all experiments, it is critically important to carefully control the conditions under which the cells being used are grown, exposed, and harvested. We recommend plating cells at a calculated and consistent cell density during regular passage and in preparation for experimental treatments. Further, we recommend plating cells for experiments in a consistent manner across biological replicates (*e.g.* plating cells for an experiment the same number of days after being passaged). When possible, the experimental treatment agent should be prepared in batch and frozen in single-use aliquots prior to use.

Buffer Preparation—Buffers can be prepared ahead of conducting the ChIP protocol; however, aliquots should be removed and treated with the indicated protease (PMSF and cOmplete protease inhibitor cocktail), phosphatase (sodium fluoride and sodium pyrophosphate), and histone deacetylase (butyric acid) inhibitors immediately prior to use. Protease inhibitors are intentionally omitted from all of the wash buffers because residual carryover can hinder the subsequent proteinase K digestion step. Further, if histone deacetylase inhibitors are desired in the binding and washing buffers then butyrate should be used in place of butyric acid in the PIS because the use of butyric acid alters the buffers' pH sufficiently to hinder antibody-antigen binding and thus immunoprecipitation efficiency (Figure 3).

Fixation—Treatment of cells with formaldehyde/paraformaldehyde results in the formation of inter- and intra-molecular crosslinks between DNA and proximal proteins (discussed in detail by Hoffman *et al.*, 2015). The optimal fixation time for ChIP differs among cell types/lines and can vary from 1 to 30 minutes. While over-fixation can impair chromatin fragmentation and interfere with antibody binding by deforming epitopes on the coordinate antigen, under-fixation can reduce ChIP efficiency by failing to covalently link DNA with its associated proteins. Thus the length of fixation should be determined empirically for each application by testing chromatin fragmentation and ChIP efficiency across a range of fixation times. The fixation described in this protocol is conducted at room temperature, but fixation can also be conducted at 37 °C or on ice. Once optimal conditions are determined, the fixation conditions should be closely followed to limit the intrinsic variability in fixation between samples and replicates (Figure 4B)

Sonication—Chromatin fragmentation is a critical aspect of successful ChIP experiments. Reproducible sonication conditions should be determined empirically as described here in **Support Protocol 1**. Under-fragmented chromatin has limited solubility and will reduce ChIP efficiency and the resolution of detection for target genomic loci. Excessively fragmented chromatin will reduce qPCR detection of target genomic loci following immunoprecipitation. While not discussed here, it should be noted that chromatin can also be fragmented by micrococcal nuclease (MNase) digestion. While MNase digestion can be used to fragment fixed chromatin, it is more commonly used in the fragmentation of native (un-fixed) chromatin as discussed by O'Neill and Turner (2003).

IP antibody—Choice of immunoprecipitation antibody is instrumental to the success of the ChIP protocol. While many antibodies are recommended for use in other immunodetection assays (*e.g.* Western blotting, ELISA, immunofluorescence staining), this does not ensure that they will work well in ChIP. Check datasheets provided by the antibody manufacturer to ensure that a specific antibody preparation has been validated for use in ChIP. Further, conduct an antibody titration to determine that a sufficient quantity of antibody is being used for the immunoprecipitation. Polyclonal antibodies are readily available for most histone modifications; however, while their sensitivity of detection is lower, monoclonal antibody preparations are more consistent and their use can increase the reproducibility of results across replicates in ChIP experiments. Experiments should be carefully planned such that a sufficient amount of each antibody for all replicates in a study can be obtained prior to beginning. Ideally, a single lot should be used as inter-lot variability can introduce variability in the assay results (Figure 4C); however, if not practical, several lots of the same antibody can be pooled before beginning the study. Regardless of the number of lots used, the tubes of antibody preparation should be pooled, distributed into single-use aliquots, and stored according to the manufacturer's recommendation before beginning the study. Antibodies that we have used successfully with the protocol described here are listed in Table 2.

Capture Resin—The choice of capture depends on the desired downstream application of ChIP material and can have a substantial impact of the immunoprecipitation efficiency. The antibody-chromatin capture step in ChIP is typically accomplished through the use of either agarose/sepharose, magnetic, or polyacrylamide beads conjugated to the IgG binding factor protein A, protein G, or both protein A and G. Care should be taken to determine the efficiency with which the binding protein (*e.g.* protein A or protein G) binds the immunoprecipitation antibody. The binding affinity of protein A and G vary depending on antibody host and isotype. These different capture resin formats have individual advantages and disadvantages as shown in Figure 5 and summarized in Table 1. Agarose and sepharose beads have been commonly used in ChIP given their relatively low cost and antibody binding capacity; however, the porous nature of agarose often results in a high background signal. To reduce the background signal agarose beads are typically pre-adsorbed with salmon sperm DNA to reduce the non-specific binding of DNA from ChIP samples during the capture step. While salmon sperm DNA effectively reduces non-specific DNA binding to the beads, it is also carried over into the eluted immunoprecipitated DNA. While salmon sperm DNA is sufficiently divergent from human DNA to prevent it from being amplified with primers/probes targeting human loci used in ChIP-qPCR, it can contaminate

downstream sequencing in ChIP-seq experiments. To avoid salmon sperm DNA contamination, ChIP-seq protocols utilize magnetic beads, which are non-porous and do not readily bind non-specific sample DNA. Magnetic beads also require the use of a magnetic tube rack to collect the beads for binding and wash steps, which precludes the need to collect beads by centrifugation during each of these steps. Despite these benefits, the use magnetic beads in ChIP-qPCR is often limited by higher cost. To overcome the limitations of both agarose/sepharose and magnetic beads, we use polyacrylamide beads because they are low cost, do not require pre-adsorption with salmon sperm DNA, and have an equal/greater binding capacity as magnetic beads.

Controls—Once determined, ChIP conditions should be validated with the use of suitable positive and negative controls. Either a “no antibody” (beads only) or non-specific IgG antibody can be used as a negative control to test the stringency of the ChIP conditions. In contrast, a total H3 antibody should be used, in conjunction with qPCR primers/probes for a genomic locus that is known to contain nucleosomes, as a positive control.

Troubleshooting

Sub-optimal chromatin fragmentation

- Under- or over-fragmentation
 - Increase or decrease the number of sonication cycles or the number/duration of pulses in each sonication cycle.
 - Under fragmentation can also result from over-fixing the sample. Conduct a time course to determine the optimal duration of fixation for the cell type/line being used.
 - Sonication should be optimized as described in **Support Protocol 1** for each cell type/line and set of fixation conditions being used for ChIP.
- Foaming during sonication
 - Improper or variable probe placement during sonication. Ensure that the probe is in a consistent position near the bottom of the sample volume during sonication; however, avoid touching the sides of the tube with the probe. If sonicating in a volume less than 500 μ L use a 0.5 mL microcentrifuge tube instead of a 1.5 mL Eppendorf tube to increase the depth of the sample.
 - Sonicator output is too high. Reduce the sonicator output and/or the number/duration of pulses in each sonication cycle.

Low or no detectable PCR signal

- Poor or no PCR amplification of purified DNA. Perform a controlled test with known material to ensure that the qPCR reaction for each target genomic locus is working efficiently. Include a standard curve for each qPCR primer/probe set on each PCR plate to evaluate PCR function and efficiency.

- Insufficient or no immunoprecipitation of target DNA. Increase the amount of chromatin used for the immunoprecipitation by increasing the number of cells used to prepare the cell pellets in step #15. The distribution of histone modifications varies greatly across the genome and some histone modifications are sparse or absent within target genomic loci.
- Low immunoprecipitation efficiency. Ensure that ChIP-validated antibodies are being used. Many antibodies that are recommended for use in other immunodetection assays (*e.g.* Western blotting, ELISA, immunofluorescence staining) do not work well in ChIP. Check the datasheets provided by the antibody manufacturer to determine whether a specific antibody preparation has been validated for use in ChIP. Further, conduct an antibody titration to determine that a sufficient quantity of antibody is being used for the immunoprecipitation.
- Check reagents and buffers. Ensure that reagents and buffers were made properly and that stock solutions of buffering solutions (*e.g.* Tris) are at the correct pH. Ensure that inhibitors (other than those recommended here) have not altered the pH of buffers used in the protocol. For example, the use of butyric acid as a replacement for its conjugate base sodium butyrate will significantly augment buffer pH and impair the immunoprecipitation (Figure 3).
- Ensure that all of the residual ethanol has evaporated in step #48 prior to resuspension of the precipitated DNA in step #50.

High non-specific background signal

- Prolonged incubation with protein A-acrylamide beads. Limit the incubation with protein A-acrylamide beads in step #27 to one hour or less.
- Insufficient wash stringency. Increase the number of washes or the stringency of the washes by increasing the NaCl or Triton X-100 concentration in wash buffers.
- Ensure that the beads are transferred to a new tube in step #29d(i). Transferring the beads to a new tube in this step reduces the carryover of unbound DNA adhered to the wall of the original tube.

High variability between replicates

- Variability between lots of antibody used for immunoprecipitation. Obtain a sufficient amount of each antibody for all ChIP experiments in a given study from a single lot. After arrival, pool the antibody and prepare single use aliquots prior to storage under the conditions recommended by the antibody supplier. Alternatively, if it is not possible to obtain sufficient antibody for the study from a single lot then obtain the total amount of antibody required from the necessary number of lots and pool and aliquot as described above.

- Utilize fresh single-use ampules of formaldehyde for fixation. The yield of the ChIP protocol decreases with increasing age of the formaldehyde used for fixation in step #8.
- Ensure the consistency of all indicated incubation times across replicates.
- Ensure consistent numbers of cells are used in each ChIP sample. Carefully count the cells in each sample or treatment group in step #12.
- Ensure that fresh phenol:chloroform:isoamyl alcohol is used and prepare the chloroform:isoamyl alcohol solution immediately prior to each use.

Anticipated Results

Data analysis—The data collected from ChIP-qPCR experiments do not reflect an absolute quantification of epigenetic modification or chromatin-associated protein levels, rather they are relative measures that are influenced by the strength of antibody-epitope interactions, the accessibility of the epitope, and the absolute amount of epitope present at the target genomic locus (Milne *et al.*, 2010). While ChIP-qPCR data can be analyzed by a simple C_t comparison of input and immunoprecipitated (IP) values, this method fails to account for the differences in PCR efficiency between primer/probe sets. Further, the PCR reaction efficiency of each primer/probe set varies between PCR runs and accounting for this intrinsic variability is crucial to obtain the most accurate data from ChIP-qPCR reactions. PCR reaction efficiency can be calculated from the slope of the line of a standard curve for each primer/probe set, which should be included for each primer/probe set on every qPCR plate.

$$\text{Efficiency (E)} = 10^{(-1/\text{slope})}$$

For example, if the line resulting from standard curve values for a specific primer/probe set had a slope of -3.425 then the efficiency of the PCR reaction would be determined as follows:

$$E = 10^{(-1/-3.525)}$$

$$E = 10^{(0.283)}$$

$$E = 1.92$$

The efficiency of the PCR reaction is a measure of the number of copies of each template molecule generated during each PCR cycle. A PCR reaction that results in two copies of each template molecule being produced during each amplification cycle (100% efficient reaction) has a slope of -3.3 and an E value of 2.0. In the example described above, an E

value of 1.92 indicates that 1.92 copies are made from each template molecule during each PCR amplification cycle, meaning that the PCR reaction is 92% efficient.

Chromatin immunoprecipitation data are most frequently expressed as the amount of immunoprecipitated genomic locus relative to the total amount of that genomic locus in the input sample. The values obtained from immunoprecipitated samples are expressed as “% input”, which requires the calculation of the total amount of target material in the chromatin sample prior to beginning the immunoprecipitation. This value can be obtained by correcting the raw C_t values obtained for the input samples according to their relative fraction to the original sample (noted in step #22 of **Basic Protocol 1**). The input fraction noted in step #21 is converted to a decimal and used to calculate a “correction value,” which accounts for the $\log_2$ nature of C_t values.

$$\text{Correction value} = -\log_2(\text{input fraction})$$

For example, if 1% of the diluted chromatin was taken as the input sample then convert 1% into decimal form (0.01) and determine $-\log_2(0.01)$.

$$-\log_2(0.01) = 6.644$$

The correction value is then used to correct the raw C_t values of the input samples by subtracting the correction value from the raw C_t value for each input sample. The corrected input C_t values reflect the total amount of quantifiable target in the original chromatin sample prior to beginning the immunoprecipitation.

$$\text{Corrected } C_t = [(\text{raw } C_t) - (\text{correction value})]$$

For example, if a 1% input sample had a raw C_t value of 29.5 then the corrected C_t value would be calculated as follows:

$$\text{Corrected } C_t = [(29.5) - (6.644)]$$

$$\text{Corrected } C_t = 22.856$$

The corrected input values are then used as the basis for determining the relative quantity of the target genomic locus in the immunoprecipitated samples using a comparison of C_t values relative to the efficiency of the qPCR reaction.

$$\text{Immunoprecipitated quantity relative to input} = E^{(C_t \text{ input} - C_t \text{ IP})}$$

For example, if an IP sample from the same chromatin sample used in the examples above had a raw C_t value of 28.0 measured in a PCR reaction with an E value of 1.92 then the fold enrichment would be calculated as follows:

$$\text{Immunoprecipitated quantity relative to input} = 1.92^{(22.856-28.0)}$$

$$\text{Immunoprecipitated quantity relative to input} = 0.0349$$

The immunoprecipitated quantity relative to input can then be converted from a decimal to a percentage to give the % input value.

$$\text{Immunoprecipitated quantity relative to input} \times 100 = \% \text{ input}$$

Using the example above:

$$0.0349 \times 100 = 3.49\% \text{ input}$$

The importance of using observed efficiency values for each qPCR run can be highlighted using the sample data given in the above examples by comparing the result as calculated with an observed efficiency value and as calculated with the efficiency assumed (E = 2.0, 100% efficient PCR reaction) in the simple C_t method.

$$\text{Calculated with observed efficiency: } 1.92^{(22.856-28.0)} = 0.0349 = 3.49\% \text{ input}$$

$$\text{Calculated with assumed efficiency of 100\%: } 2.0^{(22.856-28.0)} = 0.0283 = 2.83\% \text{ input}$$

The baseline relative abundance of histone modifications at target genomic loci can be expressed as % input and treatment-induced changes can be expressed as a fold change (treatment/control). It is important to remember that the distribution of histone modifications can vary considerably between different genomic loci and experimental treatment conditions and thus the % input values can vary dramatically between immunoprecipitation targets and genomic loci of interest. Typically, total H3 can range from <1% to >10% input, while values obtained following immunoprecipitation with histone modification-specific antibodies are regularly ~1% input. It is important to remember that the % input is a relative measure of immunoprecipitated target locus DNA relative to the total amount of the target locus present in the cleared chromatin sample and not an absolute quantification of the interaction between the histone modification of interest and the target genomic locus.

Time Considerations

Cells from exposure experiments can be fixed, frozen as cell pellets, and stored at -80°C (as indicated in steps #1–15 of **Basic Protocol 1**) at any time prior to completing the remainder of the ChIP protocol. We typically begin the fragmentation and immunoprecipitation (steps #16–25 of **Basic Protocol 1**) in the afternoon of “Day #1” in such a manner that step #24 is completed immediately before leaving for the day. The bead binding step (steps #26–27 of **Basic Protocol 1**) is then started in the morning on Day 2 after preparing all of the required reagents and buffers. Washing and elution (steps #28–34 of **Basic Protocol 1**) are completed by late morning on Day #2 and the crosslinking reversal/proteinase K digestion (steps #35–37 of **Basic Protocol 1**) is completed by early to mid-afternoon. The DNA extraction (steps #38–41 of **Basic Protocol 1**) is completed by late afternoon and the DNA precipitation (step #42) is left overnight. The precipitated DNA is pelleted, washed, and resuspended (steps #43–49 of **Basic Protocol 1**) the following morning, after which the purified DNA is ready to be used in downstream analyses.

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Appendix

50X cOmplete protease inhibitor solution

Dissolve one cOmplete protease inhibitor tablet in 1 mL of water. Store at -20°C in 250 μL single-use aliquots for up to one month.

500 mM EDTA, pH 8.0

93.06 g EDTA disodium salt dehydrate

Water to 500 mL

Adjust pH to 8.0 with NaOH

Store at room temperature

NOTE: EDTA does not dissolve at this concentration below pH 8.0

10 mg/mL ethidium bromide (EtBr)

100 mg ethidium bromide

Water to 10 mL

Store at room temperature

NOTE: light sensitive, cover the tube in aluminum foil

2.5 M glycine

9.38 g glycine

Water to 50 mL

Store at room temperature

10 mg/mL glycogen

Add water directly to the original manufacturer's container to give a final glycogen concentration of 10 mg/mL then prepare 500 μ L aliquots and store at -20°C .

10% IGEPAL CA-630

5 mL IGEPAL CA-630

45 mL water

Filter sterilize (0.22 μ m pore)

Store at room temperature

5 M lithium chloride (LiCl)

105.98 g LiCl

Water to 500 mL

Store at room temperature

100 mM phenylmethylsulfonyl fluoride (PMSF)

174.2 mg PMSF

Methanol to 10 mL

Store at -20°C for up to three days

3 M sodium acetate (NaOAc)

61.52 g sodium acetate

Water to 250 mL

Adjust the pH to 5.3 with glacial acetic acid

Store at room temperature

1.0 M sodium butyrate

2.75 g sodium butyrate

Water to 25 mL

Filter sterilize (0.22 μ m pore)

Prepare 250 μ L aliquots and store at -20°C

500 mM sodium bicarbonate

10.5 g sodium bicarbonate

Water to 250 mL

Filter sterilize (0.22 μm pore)

Store at room temperature

5M sodium chloride

146.1 g sodium chloride

Water to 500 mL

Store at room temperature

10% sodium deoxycholate

- 25 g sodium deoxycholate
- Water to 250 mL
- Filter sterilize (0.22 μm pore)
- Store at room temperature

10% sodium dodecylsulfate (SDS)

25 g SDS

Water to 250 mL

Filter sterilize (0.22 μm pore)

Store at room temperature

500 mM sodium fluoride

525 mg sodium fluoride

Water to 25 mL

Filter sterilize (0.22 μm pore)

Prepare 250 μ L aliquots and store at -20°C

100 mM sodium pyrophosphate

665 mg sodium pyrophosphate

Water to 25 mL

Filter sterilize (0.22 μm pore)

Prepare 250 μ L aliquots and store at -20°C

Soybean trypsin inhibitor (30X stock)

Dissolve powder in water at 30 mg/mL

Freeze in 1 mL aliquots at -80°C

1.0 M Tris, pH 8.1

60.57 g Tris base

Water to 500 mL

Adjust pH to 8.1 with HCl

10% Triton X-100

5 mL Triton X-100

45 mL water

Filter sterilize (0.22 μm pore)

Store at room temperature

SUPPLIER APPENDIX

100 bp DNA ladder (NEB #N3231L)

6X Purple gel loading dye (NEB #B7025S)

Agarose (Sigma #A6013)

Ammonium chloride (Sigma #213330)

Boric acid (Fisher #BP168)

Bovine serum albumin (Sigma #A7906)

Cell strainer (Falcon #352350)

Chloroform (Sigma #650498)

cOmplete protease inhibitor tablets (Roche #11844600)

Dulbecco's phosphate buffered saline (Life Technologies #14190–144)

Ethanol (Sigma #459844)

Ethidium bromide (Sigma #E8751)

Ethyl diaminetetraacetic acid, disodium salt dihydrate (EDTA; Sigma #E5134)

Glycine (Sigma #G8878)

Glycogen (Sigma #G8751)

Hank's balanced salt solution (Life Technologies #14025-092)

IGEPAL CA-630 (Sigma #I3021)

Isoamyl alcohol (Sigma #320021)

Lithium chloride (Sigma #62476)

Paraformaldehyde (16%; Electron Microscopy Sciences #15710)

Phenol:chloroform:isoamyl alcohol (25:24:1; Life Technologies #15593-031)

Phenylmethylsulfonyl fluoride (PMSF; Sigma #78830)

Potassium bicarbonate (Sigma #P9144)

Protein A/G acrylamide beads (Thermo #53133)

Proteinase K (Sigma #P2308)

RNaseA (Life Technologies #12091-021)

Sodium acetate (Sigma #S2889)

Sodium bicarbonate (Fisher #S233-500)

Sodium butyrate (Sigma #B5887)

Sodium chloride (Sigma #S7653)

Sodium deoxycholate (Sigma #D6750)

Sodium dodecylsulfate (Bio-Rad #161-0301)

Sodium fluoride (Sigma #S7920)

Sodium pyrophosphate, tetrabasic (Sigma #P8010)

Soybean trypsin inhibitor (Sigma #T6522)

Tris base (Fisher #BP152)

Triton X-100 (Sigma #T8787)

0.25% Trypsin (Life Technologies #25200-056)

LITERATURE CITED

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SIGNIFICANCE STATEMENT

The rapidly emerging field of toxicoepigenetics has enormous potential to provide new insight into adverse outcome pathways and new biomarkers for risk assessment. To realize this potential we must gain a more comprehensive understanding of interactions between environmental factors, toxic exposures, and the epigenome. Here we provide a streamlined ChIP protocol with detailed information on the optimization of critical parameters. This protocol can be readily applied to samples from a wide range of *in vitro* studies (cell lines and primary cells) and clinical samples (peripheral leukocytes).

Figure 1. Workflow for chromatin immunoprecipitation.

Chromatin immunoprecipitation allows for the relative quantification of histone modifications within the regulatory regions of target genomic loci. Protein-DNA interactions are chemically crosslinked together by treatment with formaldehyde prior to being fragmented by sonication. Fragmented chromatin containing target histone modifications is bound by target-specific antibodies and the antibody-chromatin complexes are captured by protein A-bound beads. After washing, bound chromatin is eluted from the beads, the

crosslinks are reversed, proteins are digested with proteinase K, and the purified DNA is precipitated. Isolated DNA is quantified by qPCR.

Figure 2. Optimization of chromatin fragmentation in THP-1 cells.

The chromatin fragmentation conditions should be optimized for each cell type/line and fixation condition used for ChIP. These results demonstrate the effect of successive cycles of sonication on THP-1 chromatin fragmentation. Samples were generated by fixing THP-1 cells with formaldehyde for five minutes prior to preparing pellets containing 2.0×10^6 cells. Pellets were thawed, resuspended in 600 μ L ChIP Lysis Buffer, and divided into equal fractions so each sonication sample contained 1×10^6 cells in 300 μ L. The 300 μ L samples were then subjected to a range of sonication cycles where each cycle consists of 10 pulses (0.9 second on and 0.1 seconds off) at 20% output on a Fisherbrand Sonic Dismembrator 500. DNA was isolated from fragmented chromatin as described in **Support Protocol 1** and separated on a 3% agarose-TBE gel containing 0.4 μ g/mL ethidium bromide. After running, the gel was destained overnight in TBE and imaged while being subjected to trans-illumination with UV light. These results demonstrate that six cycles of sonication yield chromatin fragments with an optimal size for ChIP (average length of 500 bp with a range of 250–750 bp).

Figure 3. The histone deacetylase inhibitor butyric acid lowers buffer pH and inhibits chromatin immunoprecipitation.

Butyric acid and its conjugate base butyrate are commonly used to inhibit deacetylation of histone proteins during ChIP. (A) Butyric acid is effective at inhibiting histone deacetylation; however, its inclusion in buffers used in the ChIP procedure (Low Salt Wash Buffer is shown, which is identical to the buffer condition after dilution of the fragmented chromatin in step #21 of **Basic Protocol 1**) results in a concentration-dependent reduction in buffer pH. Despite being used at similar concentrations, its conjugate base butyrate does not alter ChIP buffers. (B) The inclusion of butyric acid in associated binding and wash buffers inhibits ChIP efficiency. The effect observed in (B) is likely the result of poor antibody-chromatin binding due to the effect of decreased pH in buffers containing 20mM butyric acid on antibody folding. To facilitate optimal immunoprecipitation efficiency, sodium butyrate should be used as a replacement for butyric acid in the ChIP protocol.

Figure 4. Variability in ChIP.

(A) Nucleosomal density, as indicated by the abundance of histone H3, varies between promoters of genes that are commonly examined in toxicology studies. The data shown in A are the mean $\pm$ SD of three independent fixations that were subjected to α H3 ChIP in triplicate, with target quantification of each genomic target by qPCR in triplicate. (B) Once conditions are carefully established, independent fixation replicates should exhibit limited variability; however, variability can increase in samples with a relatively high % input, such as those observed for total H3 abundance at the HMOX1 promoter. The data shown in B are the mean $\pm$ SD of three α H3 ChIP assays using THP-1 cells that were fixed on three different days, frozen as fixed cell pellets (**Basic Protocol 1** steps #1–15), and subjected to α H3 ChIP simultaneously with the quantification of each genomic locus by qPCR in triplicate. (C) Two different lots of the same manufacturer product number that were used in side-by-side ChIP assays from pooled starting material. Data shown in A, B, and C represent α H3 ChIP conducted in THP-1 cells according to the conditions described in Basic Protocol 1 and IL-8, IL-6, COX-2, and HMOX1 promoter primer/probe sets listed in Table 3.

Figure 5. Comparison of capture bead materials.

The use of different capture beads can impact the outcome of ChIP experiments. ChIP experiments were conducted side-by-side using fragmented chromatin from 1.0×10^6 THP-1 cells, α H3 monoclonal antibody (isotype IgG3), and three different capture bead materials according to Basic Protocol 1. Each IP sample contained the equivalent of 50 μ L of resuspended bead slurry as provided by the manufacturer. The immunoprecipitated material was subjected to qPCR with the IL-8 promoter primer/probe set (Table 3). The “beads only” data are shown to demonstrate the non-specific binding capacity of each capture bead material. The data shown represent the mean $\pm$ SD of three independent immunoprecipitations, which were quantified by qPCR in technical triplicate. ND indicates no detectable qPCR signal.

	Per reaction
2X iTaq Universal Probes Mix	10µL
Target 1-F (6.0uM)	0.67µL
Target 1-R (6.0uM)	0.67µL
Target 1-Probe (6.8uM)	0.66µL
Target 2-F (6.0uM)	0.67µL
Target 2-R (6.0uM)	0.67µL
Target 2-Probe (6.8uM)	0.66µL
Water	4µL
Total	18µL

	Temp	Duration (min)
Step #1	95°C	5:00
Step #2	95°C	0:15
Step #3	60°C	0:45
Go to Step #2 (x39)		

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Table 1.

Comparison of Capture Beads Commonly Used in ChIP.

	Protein A Agarose w/Salmon sperm DNA	Protein A Magnetic	Protein A/G Polyacrylamide
Antibody Binding Capacity	+	+++	+++
Cost	\$	\$ \$ \$	\$
Background	Low/Medium	Low	Low
ChIP-qPCR Compatible	Yes	Yes	Yes
ChIP-Seq Compatible	No	Yes	Yes
Bead Collection Method	Centrifugation	Magnetic rack	Centrifugation

Table 2.

Antibodies successfully used in ChIP as described in Basic Protocol 1.

ChIP Target	Mono/polyclonal	Antibody Manufacturer	Product Number
H2BK5ac	Polyclonal	Active Motif	39123
H2BK120ub	Monoclonal	Active Motif	39623
H2BK120ub	Monoclonal	Millipore	17–650
Total H3	Polyclonal	Active Motif	39163
Total H3	Monoclonal	Active Motif	61475
H3K4me1	Polyclonal	Active Motif	61633
H3K4me3	Polyclonal	Active Motif	39915
H3K4me3	Monoclonal	Active Motif	61379
H3K9ac	Monoclonal	Active Motif	61251
H3K27me3	Monoclonal	Active Motif	61017
H3K27me2/3	Polyclonal	Active Motif	39535
H3K27ac	Polyclonal	Active Motif	39133
H3K27ac	Monoclonal	Active Motif	39685
H3K79me1	Polyclonal	Active Motif	39921
H4ac	Polyclonal	Active Motif	39925
H4K20me1	Monoclonal	Active Motif	39727
5-hmC	Monoclonal	Active Motif	39999
RNA PolIII	Monoclonal	Active Motif	39097
RNA PolIII	Monoclonal	Millipore	17–620
RNA PolIII phosphor-Ser5	Polyclonal	Active Motif	39749

Table 3.

Primer and Probe Sequences for Quantification of Epigenetic Modifications within the Regulatory Regions of the Human *IL-8*, *IL-6*, *COX-2*, and *HMOX1* Genes by ChIP.

Genomic Locus Target	Sequence (5' – 3')	Dye/Quencher
IL-8 Promoter Forward	CATCAGTTGCAAATCGTGGA	
IL-8 Promoter Reverse	GAAGCTTGTGTGCTCTGCTG	
IL-8 Promoter Probe	AAAGCCACCGGAGCACTCCA	FAM/BHQ
IL-8 Enhancer 1 Forward	CCTTGCCTTCAAGGAGCCTA	
IL-8 Enhancer 1 Reverse	AAAGCAGTAACTGTGCAGCC	
IL-8 Enhancer 1 Probe	TCACCAGTGGAATGAAACTGCTGTT	JOE/BHQ
IL-8 Enhancer 2 Forward	TGTACCCACCATACGTGAGGA	
IL-8 Enhancer 2 Reverse	TTGTGTCTCCCTTCTGGACG	
IL-8 Enhancer 2 Probe	TCAGACCCTGTTACACAGAGGTCAC	JOE/BHQ
IL-6 Promoter Forward	AGCCTCAATGACGACCTAAGCT	
IL-6 Promoter Reverse	ATTGTGCAATGTGACGTCCTTT	
IL-6 Promoter Probe	CACCTTTCCCTTAGTTGTGTCTTGCCA	FAM/BHQ
IL-6 Enhancer 1 Forward	TGAACCAAGTGGGCTTCAGT	
IL-6 Enhancer 1 Reverse	CCTCCCAGTGAGGACTTTGC	
IL-6 Enhancer 1 Probe	CCCATGCAGGTGCCCCAGTG	JOE/BHQ
IL-6 Enhancer 2 Forward	GTGGGACTCAACCCAGAAG	
IL-6 Enhancer 2 Reverse	CTGGGGGTGGAGATGGATG	
IL-6 Enhancer 2 Probe	TGGCAGCAACTGCAAGTTCCT	FAM/BHQ
IL-6 Enhancer 3 Forward	TCAGCTCTGGATGGGACAGA	
IL-6 Enhancer 3 Reverse	AGTTAGGATGTGCGCAGGAC	
IL-6 Enhancer 3 Probe	ATCCAGCACGTGGCTGGGGA	JOE/BHQ
COX-2 Promoter Forward	CTGGGTTTCCGATTTCTCATT	
COX-2 Promoter Reverse	GTACCCCCACAAATTTTCC	
COX-2 Promoter Probe	TGGGTAAAAAACCTGCCCCACC	FAM/BHQ
HMOX1 Promoter Forward	GACATTTTAGGGAGCTGGA	
HMOX1 Promoter Reverse	TCCCAGAAGGTTCAGAAAGC	
HMOX1 Promoter Probe	CTGATGTTGCCACCAGGCTATTGC	FAM/BHQ
HMOX1 Enhancer 1 Forward	CCCTGCAGAATCGAGCACAT	
HMOX1 Enhancer 1 Reverse	TGAGGAGACCTTGCCTGGAT	
HMOX1 Enhancer 1 Probe	TGGGGACGTCCCCTGCTTGA	FAM/BHQ
HMOX1 Enhancer 2 Forward	CCTAGGGAAGCCATAGCAGC	
HMOX1 Enhancer 2 Reverse	AGGCCGGCCATTTAACAGA	
HMOX1 Enhancer 2 Probe	CGGGGGCGCTGCAACCTAAA	JOE/BHQ