ChIP-IT High Sensitivity®

(version A6)

Catalog No. 53040

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Please Read Before Starting:

The ChIP-IT High Sensitivity® Kit contains an optimized protocol and buffer system to ensure the highest level of sensitivity and lowest background when working with limited sample amounts or low abundance transcription factor targets. It is important to follow our protocol and recommendations throughout the entire manual as many steps may differ from traditional ChIP protocols. Please pay particular attention to our notes regarding the processing of input chromatin for agarose gel analysis prior to the chromatin immunoprecipitation reaction.

Overview

Chromatin Immunoprecipitation (ChIP) is a powerful tool for studying protein/DNA interactions, including transcription factors, co-regulatory proteins, modified histones, chromatin-modifying enzymes and polymerases because it enables identification of the localization of proteins bound to specific DNA loci. When used in combination with whole-genome analysis such as ChIP-Seq or ChIP-chip, insights are possible into gene regulation, gene expression, mechanisms of chromatin modification and pathway analysis.

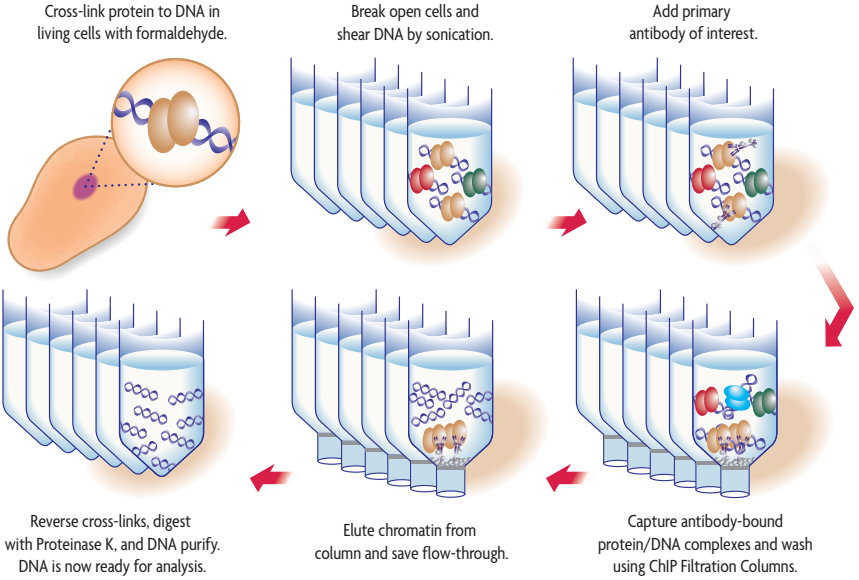
However, ChIP can be technically demanding. The method requires high-quality antibodies to recognize the fixed, target-bound proteins of interest, and an efficient means to precipitate the antibody/chromatin complex (usually protein A or G beads). In addition, specialized buffers, inhibitor cocktails and blocking reagents are required to minimize non-specific enrichment and reduce protein degradation.

Active Motif's ChIP-IT High Sensitivity® Kit is designed to overcome these obstacles and provide the highest quality ChIP enriched DNA. The ChIP-IT High Sensitivity protocol can be used with challenging antibodies that do not give signal with other ChIP methods and is sensitive enough to detect specific binding of even low abundance transcription factors. One important component of the increased sensitivity are the optimized ChIP buffers which reduce the presence of non-specific DNA to reduce background levels and allow better enrichment analysis. Additionally, the kit has demonstrated excellent reproducibility across multiple sample types and immunoprecipitation reactions can be performed from as little as 1,000 cells. The increased sensitivity and reduced background levels of ChIP-IT High Sensitivity are ideal for use in ChIP-Seq. So before investing time and money into expensive downstream genome analysis, ensure that your enriched DNA is of the highest quality.

The ChIP-IT High Sensitivity Kit contains sufficient reagents to perform 16 chromatin preparation and immunoprecipitation reactions. A chromatin preparation is defined as one 15 cm cell culture plate or 100 mg tissue sample. We recommend the use of Active Motif's ChIP-IT® qPCR Analysis Kit in combination with the ChIP-IT High Sensitivity Kit for complete data analysis. To learn about available ChIP-IT® Control Kits, control qPCR primer sets, ChIP-Seq validated antibodies, or Active Motif's EpiShear™ sonication devices, please visit our website at **www.activemotif.com/chip**.

product	format	catalog no.
ChIP-IT High Sensitivity®	16 rxns	53040
ChIP-IT® qPCR Analysis Kit	10 rxns	53029

Flow Chart of Process



Flow Chart of the ChIP-IT High Sensitivity Assay.

In ChIP-IT High Sensitivity, intact cells are fixed with a specially formulated formaldehyde buffer, which cross-links and preserves protein/DNA interactions. DNA is then sheared into small fragments using sonication and incubated with an antibody directed against the DNA-binding protein of interest. The antibody-bound protein/DNA complexes are immunoprecipitated through the use of Protein G agarose beads and washed via gravity filtration. Following immunoprecipitation, cross-links are reversed, the proteins are removed by Proteinase K, and the DNA is recovered and purified. ChIP enriched DNA can be used for either gene-specific or whole-genome analysis.

Kit Performance and Benefits

ChIP-IT High Sensitivity Advantages:

- Ideal for low abundance transcription factors or antibodies with low binding affinities
- Sensitive enrichment of DNA from as little as 1,000 cells for high abundance target proteins and as little as 50,000 cells for low abundance transcription factors
- Optimized reagents reduce background levels caused by non-specific binding events
- Filtration based washes are the easiest wash method available and result in increased consistency in multi-sample experiments
- Highly robust procedure has been validated across multiple sample types with proven performance in both qPCR and ChIP-Seq analysis

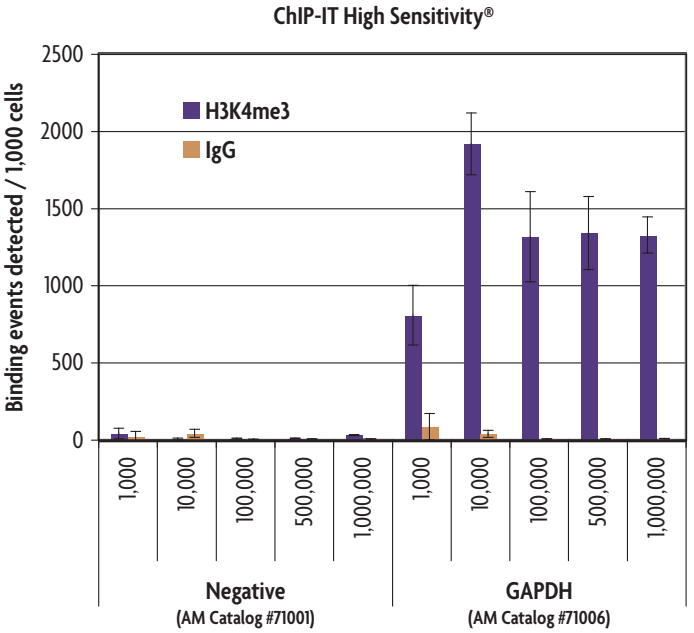


Figure 1: Immunoprecipitation sensitivity of ChIP-IT High Sensitivity Kit.

MCF-7 chromatin was prepared according to the manual using between 100,000 to 4 million cells per chromatin preparation. The immunoprecipitation reaction was then performed using the indicated number of cells with Active Motif's Histone H3K4me3 antibody (Catalog No. 39915) and a negative control IgG. Following enrichment, qPCR was performed using the ChIP-IT qPCR Analysis Kit (Catalog No. 53029) in order to normalize the data and allow for direct comparison of the results. The ChIP-IT High Sensitivity Kit shows strong enrichment of H3K4me3 DNA binding from as little as 1,000 cells, while little to no non-specific binding is detected with the negative control IgG. Untr12 is a gene desert on chromosome 12 that should not show any H3K4me3 enrichment, while GAPDH is an actively transcribed gene that is associated with the presence of H3K4me3. Data is expressed as binding events detected per 1,000 cells which represents the average of the raw data triplicates adjusted for the amount of chromatin in the reaction, the resuspension volume and the primer efficiency. This calculation provides consistency in data analysis and allows direct comparison across samples and experiments. To convert this scale to the percent of ChIP input recovered, divide the values by 1,000.

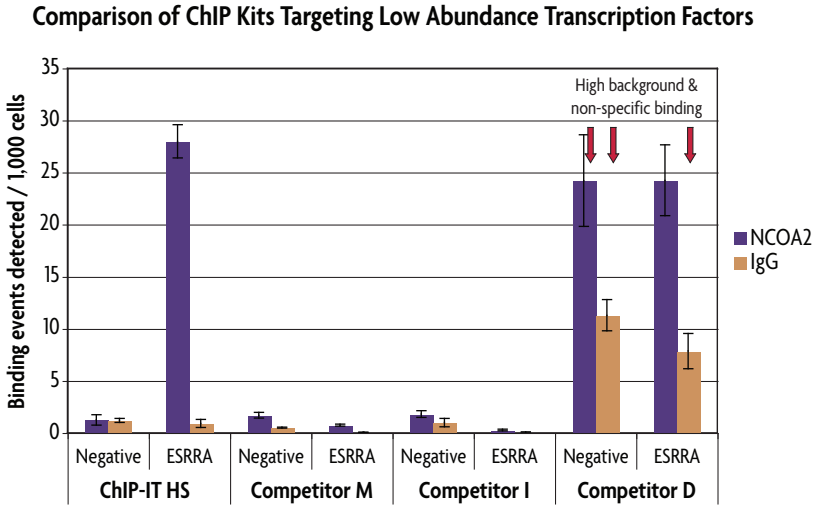


Figure 2: ChIP-IT High Sensitivity can detect low abundance protein targets.

MCF-7 chromatin was prepared according to each manufacturer's recommendations for their ChIP assay from 1.5 x 10^6 cells. ChIP was performed with the optimal amount of chromatin as suggested by the protocol for each manufacturer using an antibody for the low abundance nuclear co-activator 2 (NCOA2) protein and a negative control IgG. Following enrichment, qPCR was performed using the ChIP-IT qPCR Analysis Kit (Catalog No. 53029) in order to normalize the data for chromatin amounts and ChIP volume to allow for direct comparison of the competitor kit results. NCOA2 is considered a difficult antibody for ChIP, yet using ChIP-IT High Sensitivity, NCOA2 binding was detected at the estrogen-related receptor alpha (ESRRA) promoter and enrichment was approximately 20-fold higher than the negative control primer set and the IgG. NCOA2 was either not enriched at all (Competitors M & I) or the enrichment was non-specific with high background visible in the negative primer set and IgG (Competitor D). The data represents triplicate values expressed as Binding events detected per 1,000 cells. To convert this scale to the percent of ChIP input recovered, divide the values by 1,000.

Protocol Overview and Time Table

	Required Time
Cell or Tissue Fixation and Lysis	1.5 hours
Chromatin Sonication	15 minutes per sample
Assessment of Chromatin Size*	4.5 hours for cell culture Overnight for tissue
Immunoprecipitation	Overnight incubation
Binding to Protein G agarose Beads	3 hours
Wash Immune Complexes	20 minutes
Reversal of Cross-links	2.5 hours
DNA Purification	15 minutes
qPCR Analysis	2 hours

* The protocol varies between cell culture and tissue samples.

Kit Components and Storage

Please store each component at the temperature indicated in the table below. **Do not re-freeze the Protein G Agarose Beads. Once thawed, Protein G beads should be stored at 4°C.**

Reagents	Quantity	Storage
RNase A (10 µg/µl)	40 µl	-20°C
Proteinase K (10 µg/µl)	200 µl	-20°C
Blocker	100 µl	-20°C
5 M NaCl	400 µl	RT
100 mM PMSF	500 µl	-20°C
Protease Inhibitor Cocktail (PIC)	500 µl	-20°C
Precipitation Buffer	1.5 ml	-20°C
Carrier	35 µl	-20°C
TE pH 8.0	2 x 1.5 ml	RT
Detergent	25 ml	RT
10X PBS	120 ml	-20°C
Fixation Buffer	2 x 1.5 ml	4°C
Stop Solution	20 ml	RT
Chromatin Prep Buffer	85 ml	RT
ChIP Filtration Columns	16 ea	RT
ChIP Buffer	35 ml	RT
Wash Buffer AM1	100 ml	RT
Elution Buffer AM4	2 x 1.5 ml	RT
Protein G Agarose Beads*	500 µl	4°C
DNA Purification Binding Buffer	50 ml	RT
3 M Sodium Acetate	500 µl	RT
DNA Purification Wash Buffer**	10 ml	RT
DNA Purification Elution Buffer	5 ml	RT
DNA Purification Columns	16 ea	RT

* The Protein G Agarose Beads are shipped on dry ice and can be stored frozen until their first use. Once thawed, the Protein G beads **should not be re-frozen** by the customer. Protein G Agarose Beads should be stored at 4ºC.

**Requires the addition of ethanol before use.

Additional materials required

- A ChIP-validated antibody directed against the protein of interest
- Dounce homogenizer with a small clearance pestle (*e.g.* Active Motif Catalog Nos. 40401 & 40415) with the tight-fitting "A" pestle). Use of a homogenizer is necessary for shearing chromatin.
- 37% formaldehyde solution with 10-15% methyl alcohol to prevent polymerization (e.g. Sigma Aldrich Catalog No. 252549). Do not use paraformaldehyde.
- For tissue preparations you will need phenol, saturated (DNA Purification, Molecular Biology Grade, Amresco Catalog No. 0945)
- For tissue preparations you will need chloroform/isoamyl alcohol (24:1) (DNA Purification, Molecular Biology Grade)
- 100% ethanol (absolute)
- 70% ethanol
- DNase-free H_2O
- Rocking platform for culture plates
- Apparatus to rotate tubes end-to-end at 4°C (*e.g.* a Labquake from Barnstead/Thermolyne with a tube holder for 1.5 ml microcentrifuge tubes)
- Microcentrifuge (table top centrifuge 4°C) and microcentrifuge tubes
- 250 µl PCR tubes
- Thermocycler
- 15 and 50 ml conical tubes
- Spectrophotometer for DNA quantitation
- Pipettors and tips (filter tips are recommended)
- Sonicator (*e.g.* Active Motif's EpiShear™ Sonicator with a 1/8" probe (Catalog No. 53051))
- Agarose gel electrophoresis apparatus
- Razor blades (for tissue preparations)
- Hand-held homogenizer for tissue preparations (e.g. Biospec Products Tissue-Tearor)
- Cell scraper (rubber policeman)
- (Optional) ChIP-IT® qPCR Analysis Kit (Catalog No. 53029)
- (Optional) Gene-specific qPCR primer pairs for enrichment analysis; see Appendix Section I
- (Optional) SYBR Green qPCR master mix (Bio-Rad Catalog No. 170-8882)

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Cell Growth Recommendations

When planning an experiment, calculate the number of chromatin preparations you will require and determine the number of ChIP reactions you plan to perform on each chromatin preparation. Be sure to include the appropriate positive and negative control ChIP reactions in your calculations. Also, note that if you wish to analyze the effect of particular compounds or culturing conditions on transcription factor/DNA interactions, you should prepare chromatin from control (untreated) cells as a reference sample. The minimum recommended number of cells that should be used for the preparation of chromatin is 100,000 cells.

	24-well plate	12-well plate	6-well plate	60 mm dish	100 mm dish	150 mm dish
Seeding Density	0.05 x 10^6	0.1 x 10^6	0.3 x 10^6	0.8 x 10^6	2.2 x 10^6	5.0 x 10^6
Cells at 70-80% Confluency*	0.15 x 10^6	0.3 x 10^6	0.9 x 10^6	2.4 x 10^6	6.6 x 10^6	15.0 x 10^6
Growth Medium Volume	1 ml	2 ml	3 ml	5 ml	10 ml	20 ml
Cell Fixative Solution	100 µl	200 µl	300 µl	500 µl	1 ml	2 ml
Stop Solution	55 µl	110 µl	165 µl	275 µl	550 µl	1.1 ml
PBS Wash Buffer (used per wash)	500 µl	500 µl	1 ml	2 ml	5 ml	10 ml
Chromatin Prep Buffer	500 µl	500 µl	1 ml	2 ml	5 ml	5 ml
ChIP Buffer	500 µl	500 µl	500 µl	500 µl	500 µl	500 µl

* The number of cells on a confluent plate or dish will vary with cell type. For this table, HeLa cells were used. Please adjust as needed based on your particular cell type.

** Please refer to the descriptions below for complete details on buffer preparations

Buffer Preparation

Complete Cell Fixation Solution

Buffer should be prepared fresh before each experiment. For every 20 ml of cell growth medium used, prepare 2.5 ml of Complete Cell Fixation Solution by adding 180 µl Fixation Buffer to 1.57 ml sterile water in a 15 ml conical tube. Using appropriate precautions (*i.e.* safety glasses, gloves and lab coat), add 750 µl 37% formaldehyde to the tube and vortex to mix. Use 1/10 growth medium volume per plate. Complete cell fixation solution can be added to the growth medium in the presence or absence of serum.

Complete Tissue Fixation Solution

Buffer should be prepared fresh before each experiment. Prepare 10 ml of Tissue Fixation Solution for each tissue sample to be processed by adding 1 ml 10X PBS to 8.7 ml sterile water in

a 15 ml conical tube. Using appropriate precautions (*i.e.* safety glasses, gloves and lab coat), add 280 µl 37% formaldehyde to the tube and vortex to mix.

Stop Solution

Is provided ready to use. Use 1/20 media volume per cell culture plate or 515 µl per 10 ml Complete Tissue Fixation Solution.

PBS Wash Buffer

Prepare 25 ml PBS Wash Buffer for every 15 cm plate or tissue sample. To a 50 ml conical tube add 21.25 ml sterile water, 2.5 ml 10X PBS and 1.25 ml Detergent. Mix by inverting. Place PBS Wash Buffer on ice to chill. PBS Wash Buffer can be prepared in large quantities and stored at 4ºC for 6 months.

100 mM PMSF and Protease Inhibitor Cocktail (PIC)

Thaw the PMSF and the PIC at room temperature until fully dissolved, which takes about 30 minutes. Vortex gently and spin down briefly before use, then add to the buffers immediately before use.

Chromatin Prep Buffer

Is supplied ready to use.

ChIP Buffer

Is supplied ready to use.

DNA Purification Wash Buffer

The DNA Purification Wash Buffer requires the addition of ethanol before use. The final concentration of ethanol should be 80%. Add 40 ml of fresh 100% ethanol to the DNA Purification Wash Buffer bottle. Invert repeatedly. The buffer can be stored at room temperature after the addition of ethanol. The ethanol only needs to be added before the first use, after that the Wash Buffer is ready for use.

3M Sodium Acetate

It is important to check the sodium acetate before use to ensure that the salts have not precipitated out of solution. Once the sodium acetate is in solution it should be stored at room temperature.

Protein G Agarose Beads

The supplied agarose beads require washing prior to use. Follow the instructions in the manual to wash the beads for use in the ChIP reactions. There is no need to pre-block the beads or pre-clear the sample. For best results, gently shake and invert the tube to resuspend the agarose beads. The beads settle quickly, and therefore should be resuspended just before pipetting. We recommend cutting 2 mm from the end of a pipet tip prior to pipetting to prevent the tip from becoming clogged. **Protein G Agarose Beads are shipped on dry ice and can be stored frozen until their first use. Once thawed, beads should not be re-frozen by the customer. Protein G Agarose Beads should be stored at 4ºC.**

Recommendations

ChIP-validated Antibody

We recommend using 4 µg antibody per ChIP reaction in a maximum volume of 30 µl. However, this will vary according to the affinity of the antibody and the quality of the chromatin; you may need to use more of a particular antibody. ChIP antibodies must recognize fixed, native protein that is bound to DNA and/or complexed with other proteins. Many antibodies that perform well in other applications do not perform in ChIP. Thus, ChIP performed with an antibody that has not been ChIP-validated must include appropriate controls (such as Active Motif's ChIP-IT Control qPCR Kits, Catalog Nos. 53026, 53027 and 53028) to validate the chromatin preparation and the ChIP methodology. To see a list of available ChIP-validated antibodies available from Active Motif, please visit www.activemotif.com/chipabs.

Chromatin Shearing Tips

We suggest using a probe sonicator (*i.e.* Active Motif's EpiShear Probe Sonicator) which employs a direct sonication method to prepare chromatin for use in the ChIP-IT High Sensitivity Kit. Indirect sonication systems may require longer sonication times to achieve optimal chromatin shearing. ChIP experiments usually require chromatin that has been sheared to a size of 200-1200 bp. In general, shearing efficiency is improved through the use of a small shearing volume and a V-bottom tube rather than a round-bottom tube. Also, note that shearing is inefficient if the chromatin sample becomes emulsified with air bubbles. To determine the appropriate shearing level for your sample, set up a "practice" tube containing only ChIP Buffer. Slowly increase the sonication amplitude until foaming starts to occur. Reduce the amplitude setting down slightly and mark this as the highest possible intensity to use without foaming. If a chromatin preparation becomes emulsified inadvertently, discontinue shearing and centrifuge the sample at maximum speed for 4 minutes at 4ºC in a microcentrifuge to remove trapped air. Finally, to prevent overheating and denaturation of chromatin, samples should be kept on ice as much as possible during shearing, and shearing should be performed discontinuously (*i.e.* sonicate for 20 seconds, then place on ice/water for 30 seconds, sonicate again for 20 seconds, *etc.*). If possible, shear while on ice to help regulate sample temperature.

Chromatin Quantity

It is recommended to use 10-30 µg chromatin per IP reaction (1.5-4.5 million cells). However, if chromatin is in limited supply lower amounts may be used due to the high quality of the DNA obtained from the ChIP-IT High Sensitivity Kit. We recommend at least 1,000 cells per IP for high abundance targets and 50,000 cells per IP for low abundance target (assume 1.5×10^6 cells will yield 10 µg chromatin). If ChIP-Seq will be performed following enrichment, please read the recommendations listed in Section H on page 22 prior to starting the protocol.

Safety Precautions

Formaldehyde and PMSF are highly toxic chemicals. Appropriate safety precautions (*i.e.* safety glasses, gloves and lab coat) should be used. Also, formaldehyde is highly toxic by inhalation and should be used only in a ventilated hood. Finally, chromatin sonication should be performed in a biosafety hood if the chromatin is extracted from biohazardous or infectious materials.

Section A: Cell Fixation Starting with Cultured Cells

This protocol describes cell fixation and chromatin preparation from one 15 cm plate (approximately 1.5 x 10^7 cells). We recommend using 20 ml growth medium per 15 cm plate. Please refer to page 7 for information on scaling the protocol for use with other amounts of cells. The minimum cell number to be used for the preparation of chromatin is 100,000 cells.

1. Prepare 15 cm plates for each cell line to be tested. Grow the cells to 70-80% confluency. Stimulate cells as desired to activate the pathway of interest.
2. Freshly prepare Complete Cell Fixation Solution for each 15 cm plate. The volumes listed below are enough to process one 15 cm plate. Please refer to the chart on page 7 to scale the solution volumes.
3. To fix cells, add 1/10 growth medium volume of freshly prepared Complete Cell Fixative Solution to the existing culture media for the cells (*e.g.* 20 ml growth medium would get 2 ml Complete Cell Fixation Solution). Shake gently at room temperature for 15 minutes.
4. Stop the fixation reaction by adding 1/20 media volume of Stop Solution to the existing culture media for the cells (*e.g.* 20 ml growth medium would get 1.1 ml Stop Solution). Swirl to mix and incubate at room temperature for 5 minutes.
5. Following the incubation, hold the plate at an angle and using a rubber policeman scrape cells down to collect them at the bottom edge of the plate. Use a pipette to transfer the cells to a 50 ml conical tube on ice.
6. Pellet the cells from step 5 by centrifugation for 3 minutes at 1,250 x g at 4°C.
7. Remove the supernatant and discard. Resuspend the pellet(s) in 10 ml ice-cold PBS Wash Buffer by pipetting up and down. Keep samples ice-cold for the remainder of the procedure.
8. Centrifuge for 3 minutes at 1,250 x g at 4°C. Remove the supernatant and discard. Wash the pellet(s) a second time in 10 ml ice-cold PBS Wash Buffer by pipetting up and down. Centrifuge for 3 minutes at 1,250 x g at 4°C. Remove the supernatant and discard. (Cell pellets may be stored at -80°C at this stage).
9. Resuspend each pellet(s) in 5 ml Chromatin Prep Buffer supplemented with 5 µl PIC and 5 µl 100 mM PMSF. Pipet up and down to mix.
10. Incubate on ice for 10 minutes.
11. Transfer the resuspended pellets individually to a chilled dounce homogenizer on ice. Use the tight fitting pestle (Type A) to homogenize the sample for 30 strokes. Transfer the contents to a new 15 ml conical tube and centrifuge for 3 minutes at 1,250 x g at 4°C.

    Monitor Cell Lysis: To ensure cell lysis, take 10 µl of the cell lysate from the dounce and look at it under a phase contrast microscope using a hemocytometer to verify that the nuclei have been released. It is often helpful to look at the cells before and after the lysis step as this makes it easier to identify the nuclei versus whole cells. Intact cells should have a dark central region (nucleus) surrounded by a halo of less dense cytoplasm. In lysed cells,

the nuclei will appear as dots surrounded by asymmetric debris. If the cells are not lysed, then dounce on ice with an additional 10 strokes, or until the cells are lysed.

12. Remove the supernatant and discard. Resuspend each pellet in 500 µl ChIP Buffer supplemented with 5 µl PIC and 5 µl 100 mM PMSF. Transfer the contents to a new 2 ml microcentrifuge tube.

13. Incubate on ice for 10 minutes. Proceed to Step B: Chromatin Sonication of Cultured Cells.

Section B. Chromatin Sonication of Cultured Cells

The section below describes the fragmentation of chromatin using sonication. Sonication results may vary depending on cell type and sonication device being used. This protocol has been validated using Active Motif's EpiShear™ Probe Sonicator with samples on ice and the probe height maintained between samples to ensure consistency in processing. We do not recommend sonication of samples containing less than 100,000 cells and/or 350 µl volume.

The Chromatin Prep Buffer has been optimized for immunoprecipitation performance, however, due to its unique composition optimization of sonication conditions may be required. To maintain the high sensitivity of the assay, we recommend using our buffer system and altering the sonication time and/or amplitude of your sonication system to achieve the desired fragmentation (*e.g.* some systems may require as much as a three-fold increase in sonication time to improve chromatin shearing). Please pay particular attention to our protocol regarding the processing of input chromatin for agarose gel analysis prior to the chromatin immunoprecipitation reaction as many steps may differ from traditional ChIP protocols and failure to follow the outlined procedure may lead to artifacts in the gel images as shown in Figure 3 on page. 13

1. Place the 2 ml microcentrifuge tube containing the chromatin into the tube cooler or packed ice. Open cap and submerge the microtip into the liquid until the microtip is approximately 5 mm from the bottom of the tube. Sonicate according to optimized settings for the cell type being used (see Recommendations on page 9). A recommended starting range for cultured cells is: 25% amplitude, pulse for 30 seconds on and 30 seconds off for a total sonication "on" time of 10 minutes (or 20 minutes elapsed time).

2. Spin tubes at 4°C in a microcentrifuge at maximum speed for 2 minutes to pellet the cellular debris.

3. Transfer 25 µl of each chromatin preparation into a 250 µl PCR tube for analysis of shearing efficiency and chromatin quantification. This sample will be used to generate the Input DNA.

4. Aliquot the remainder of each chromatin preparation into 1.5 ml microcentrifuge tubes. We recommend making aliquots of 150 µl volume and storing at -80°C.

Note: The size of the chromatin sonication should be verified before proceeding to the immunoprecipitation step.

Input Preparation

5. To each 25 µl chromatin preparation from Step 3 above, add 175 µl TE pH 8.0 and 1 µl RNAse A. Cap the PCR tubes and vortex to mix
6. Incubate in a thermocycler at 37°C for 30 minutes.
7. Add 2 µl Proteinase K to each tube and vortex. Incubate tubes in a thermocycler at 55°C for 30 minutes and then increase the temperature to 80°C for 2 hours.
8. Transfer each chromatin input to a 1.5 ml microcentrifuge tube. Add 83 µl Precipitation Buffer, 2 µl Carrier and 750 µl absolute ethanol. Vortex to mix and chill at -80°C for 30 minutes to overnight.
9. Spin tubes at 4°C in a microcentrifuge at maximum speed for 15 minutes.
10. Carefully remove the supernatant taking care not to disturb the pellet. Wash the pellet with 500 µl 70% ethanol and spin at 4°C in a microcentrifuge at maximum speed for 5 minutes.
11. Carefully remove the supernatant taking care not to disturb the pellet. Remove residual ethanol with a pipet tip. Leave the tubes uncapped and air dry for 10-15 minutes.
12. When the pellets are dry, add 25 µl DNA Purification Elution Buffer to each tube. Incubate at room temperature for 10 minutes. Then vortex to ensure the pellet is completely resuspended. This solution contains your Input DNA.
13. Read the absorbance of each sample on a NanoDrop or other spectrophotometer at 260 nm to determine the DNA concentration of each chromatin preparation. Set aside 500 ng of DNA for analysis as described in Step 14. Store the remaining Input DNA at -20°C.
14. Analyze each chromatin preparation on an agarose gel by following the instructions below.
    a. Prepare 500 mM NaCl by adding 2 µl 5M NaCl to 18 µl sterile water. Vortex to mix.
    b. Transfer 500 ng of Input DNA to a 250 µl PCR tube and add 1 µl 500 mM NaCl. Adjust the final volume to 10 µl with sterile water if needed.
    c. Heat samples in a thermocycler at 100°C for 20 minutes followed by ramping the temperature down to 50°C.
    d. Remove tubes from the thermocycler and incubate at room temperature for 5 minutes.
    e. Add gel loading buffer to each sample and run on a 1.5% agarose gel. Include 100 bp and 1 kb DNA ladders to analyze chromatin size. DNA should appear as a smear anywhere between 200-1200 bp.

Note: Chromatin prepared using the ChIP-IT High Sensitivity protocol may look different on an agarose gel compared to chromatin prepared using traditional ChIP methods. However, this will not affect the sensitivity of the assay or increase background signal. Please follow the protocol as listed above for preparing Input DNA. Use of an alternative reverse cross-linking method or omitting the 20 minute incubation at 100°C in NaCl is not recommended as this will cause artifacts that make the DNA appear larger. As long as the chromatin falls within the recommended 200-1200 bp range, proceed with the ChIP reaction. If fragments do not fall within this range sonication conditions should be further optimized.

15. If chromatin preparations were successful, the aliquots stored at -80°C from Section B, Step 4 can be used to perform the ChIP reactions in Section E.

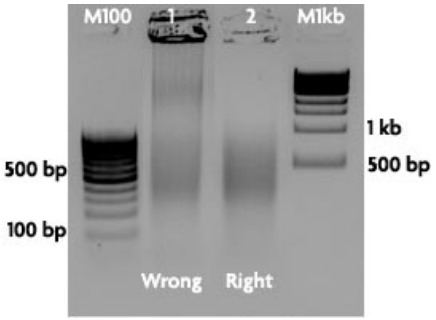


Figure 3: Validation of chromatin shearing efficiency following reversal of cross-links at 80°C for 2 hours.
Chromatin preparations of MCF-7 cells were fixed and sonicated using the EpiShear™ Probe Sonicator. Input DNA was prepared in duplicate according to Section B, Steps 5-13 in the manual. In Sample 1, Step 14 was not performed and 500 ng of input DNA was loaded directly onto a 1.5% agarose gel without receiving the addition of NaCl and incubation at 100°C. The omission of Step 14 has caused a buffer artifact that makes the DNA appear larger on a gel. The duplicate sample, Sample 2, was processed according to the manual instructions and included the addition of NaCl and incubation at 100°C as stated in Step 14. Analysis of 500 ng of this input DNA on a 1.5% agarose gel shows the expected fragmentation between 200-1200 bp. The difference in DNA sizing on the gel between the two samples illustrates the importance of following the protocol recommendations regarding the processing of input chromatin for agarose gel analysis prior to chromatin immunoprecipitation. Omission of key steps can lead to inaccurate analysis of chromatin shearing efficiency. If the protocol steps were followed and the DNA fragments fall outside of the recommended range, sonication conditions should be further optimized.

Section C: Cell Fixation Starting with Fresh or Frozen Tissue

This protocol describes cell fixation and chromatin preparation from 100-400 mg fresh or frozen animal tissue. If performing chromatin preparation on multiple tissue samples, we recommend completing Steps 1-7 for each sample before processing the next sample.

1. For tissue fixation, transfer 10 ml Complete Tissue Fixation Solution (see Buffer Preparation on page 7) to a 60 mm petri dish. Place the dish on ice.
2. Add 100-400 mg fresh or frozen tissue sample to the petri dish and ensure that the sample is fully immersed. Cut the tissue sample into small pieces (approximately 1 mm cubes) using a razor blade.
3. Transfer the sample plus the Complete Tissue Fixation Solution to a 15 ml conical tube and rotate at room temperature for 15 minutes.
4. Stop the fixation reaction by adding 515 µl Stop Solution to the conical tube and rotate at room temperature for 5 minutes.
5. Place the conical tube on ice and homogenize the contents with a hand-held tissue homogenizer set at 30,000 rpm for 45 seconds.
6. Pellet the cells from step 5 by centrifugation for 3 minutes at 1,250 x g at 4°C.
7. Remove the supernatant and discard. Resuspend the pellet in 10 ml ice-cold PBS Wash Buffer by pipetting up and down. Keep samples ice-cold for the remainder of the procedure.
8. Centrifuge for 3 minutes at 1,250 x g at 4°C. Remove the supernatant and discard. Wash the pellet(s) a second time in 10 ml ice-cold PBS Wash Buffer by pipetting up and down. Centrifuge for 3 minutes at 1,250 x g at 4°C. Remove the supernatant and discard. (Cell pellets may be stored at -80°C at this stage).
9. Resuspend each pellet in 5 ml Chromatin Prep Buffer supplemented with 5 µl PIC and 5 µl 100 mM PMSF.
10. Incubate on ice for 10 minutes.
11. Transfer the resuspended pellet(s) individually to a chilled dounce homogenizer on ice. Use the tight fitting pestle (Type A) to homogenize the sample for 30 strokes. Once finished, transfer the contents to a new 15 ml conical tube.

    Monitor Cell Lysis: To ensure cell lysis, take 10 µl of the cell lysate from the dounce and look at it under a phase contrast microscope using a hemocytometer to verify that the nuclei have been released. It is often helpful to look at the cells before and after the lysis step as this makes it easier to identify the nuclei versus whole cells. Intact cells should have a dark central region (nucleus) surrounded by a halo of less dense cytoplasm. In lysed cells, the nuclei will appear as dots surrounded by asymmetric debris. If the cells are not lysed, then dounce on ice with an additional 10 strokes, or until the cells are lysed.
12. Centrifuge for 3 minutes at 1,250 x g at 4°C.
13. Remove the supernatant and discard. Resuspend each pellet in 500 µl - 1 ml ChIP Buffer supplemented with PIC and 100 mM PMSF. (For 500 µl add 5 µl PIC and 5 µl PMSF. For 1 ml

add 10 µl PIC and 10 µl PMSF.) Transfer the contents to a new 2 ml microcentrifuge tube.

14. Incubate on ice for 10 minutes. Proceed to Section D: Chromatin Sonication of Tissue.

Section D. Chromatin Sonication of Tissue

The section below describes the fragmentation of chromatin using sonication. Due to the increased concentration of protein and cellular debris present in animal tissue, we recommend following this protocol for the preparation of chromatin and input DNA from tissue. Sonication results may vary depending on tissue type and sonication device being used. This protocol has been validated using Active Motifs EpiShear™ Probe Sonicator with samples on ice and the probe height maintained between samples to ensure consistency in processing. We do not recommend sonication of samples containing less than 50 mg tissue and/or 350 µl volume.

The Chromatin Prep Buffer has been optimized for immunoprecipitation performance, however, due to its unique composition optimization of sonication conditions may be required. To maintain the high sensitivity of the assay, we recommend using our buffer system and altering the sonication time and/or amplitude of your sonication system to achieve the desired fragmentation (*e.g.* some systems may require as much as a three-fold increase in sonication time to improve chromatin shearing). Please pay particular attention to our protocol regarding the processing of input chromatin for agarose gel analysis prior to the chromatin immunoprecipitation reaction as many steps may differ from traditional ChIP protocols and failure to follow the outlined procedure may lead to artifacts in the gel images as shown in Figure 4 on page 17.

1. Place the 2 ml microcentrifuge tube containing the chromatin into the tube cooler or packed ice. Open cap and submerge the microtip into the liquid until the microtip is approximately 5 mm from the bottom of the tube. Sonicate according to optimized settings for the tissue type being used (see Recommendations on page 9). A recommended starting range for tissue samples is: 25% amplitude, pulse for 30 seconds on and 30 seconds off for a total sonication "on" time of 10 minutes (or 20 minutes elapsed time).
2. Spin tubes at 4°C in a microcentrifuge at maximum speed for 2 minutes to pellet the cellular debris.
3. Transfer 25 µl of each chromatin preparation into a 250 µl PCR tube for analysis of shearing efficiency and chromatin quantification. This sample will be used to generate the Input DNA.
4. Aliquot the remainder of each chromatin preparation into 1.5 ml microcentrifuge tubes. We recommend making aliquots of 150 µl volume and storing at -80°C.

Note: The size of the chromatin sonication should be verified before proceeding to the immunoprecipitation step.

Input Preparation

5. To each 25 µl chromatin preparation from Step 3 above, add 175 µl TE pH 8.0 and 2 µl RNAse A. Cap the PCR tubes and vortex to mix.
6. Incubate in a thermocycler at 37°C for 1 hour.
7. Add 5 µl Proteinase K to each tube, vortex and incubate in a thermocycler at 37°C for 3 hours.
8. Add 10 µl 5 M NaCl, vortex and incubate at 65°C for 6-16 hours to reverse cross-links.
9. Remove tubes from the thermocycler and add 250 µl phenol and 125 µl chloroform:isoamyl alcohol (24:1). Vortex vigorously and spin tubes in a room temperature microcentrifuge at maximum speed for 2 minutes.
10. Transfer each upper aqueous layer to a new 1.5 ml microcentrifuge tube and add 250 µl chloroform:isoamyl alcohol (24:1). Vortex vigorously and spin tubes in a room temperature microcentrifuge at maximum speed for 2 minutes.
11. Transfer the upper aqueous layer to a new 1.5 ml microcentrifuge tube. Add 83 µl Precipitation Buffer, 2 µl Carrier and 900 µl absolute ethanol. Vortex to mix and chill at -80°C for 30 minutes to overnight.
12. Spin at 4°C in a microcentrifuge at maximum speed for 15 minutes.
13. Carefully remove the supernatant taking care not to disturb the pellet. Wash the pellet with 500 µl 70% ethanol and spin at 4°C in a microcentrifuge at maximum speed for 5 minutes.
14. Carefully remove the supernatant taking care not to disturb the pellet. Remove residual ethanol with a pipet tip. Leave the tubes uncapped and air dry for 10-15 minutes.
15. When the pellets are dry, add 25 µl DNA Purification Elution Buffer to each tube. Incubate at room temperature for 10 minutes. Then vortex to ensure the pellet is completely resuspended. This solution contains your Input DNA.
16. Read the absorbance of each sample on a NanoDrop or other spectrophotometer at 260 nm to determine the DNA concentration of each chromatin preparation. Set aside 500 ng of DNA for analysis as described in Step 17. Store the remaining Input DNA at -20°C.
17. Analyze each chromatin preparation on an agarose gel by following the instructions below.
    a. Prepare 500 mM NaCl by adding 2 µl 5M NaCl to 18 µl sterile water. Vortex to mix.
    b. Transfer 500 ng of Input DNA to a 250 µl PCR tube and add 1 µl 500 mM NaCl. Adjust the final volume to between 10 µl with sterile water if needed.
    c. Heat samples in a thermocycler at 100°C for 20 minutes followed by ramping the temperature down to 50°C.
    d. Remove tubes from the thermocycler and incubate at room temperature for 5 minutes.
    e. Add gel loading buffer to each sample and run on a 1.5% agarose gel. Include 100 bp and 1 kb DNA ladders to analyze chromatin size. DNA should appear as a smear anywhere between 200-1200 bp.

Note: Chromatin prepared using the ChIP-IT High Sensitivity protocol may look different on an agarose gel than chromatin prepared using traditional ChIP methods. However, this will not affect the sensitivity of the assay or increase background signal. Please follow the protocol as listed above for preparing Input DNA. Use of an alternative reverse cross-linking method or omitting the 20 minute incubation at 100°C in NaCl is not recommended as this will cause artifacts that make the DNA appear larger. As long as the chromatin falls within the recommended 200-1200 bp range, proceed with the ChIP reaction. If fragments do not fall within this range sonication conditions should be further optimized.

18. If chromatin preparations were successful, the aliquots stored at -80°C from Section D, Step 4 can be used to perform the ChIP reactions in Section E.

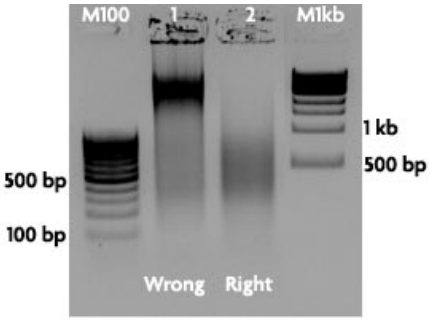


Figure 4: Validation of chromatin shearing efficiency following reversal of cross-links overnight at 65°C.
Chromatin preparations were fixed and sonicated using the EpiShear™ Probe Sonicator. Input DNA was prepared in duplicate according to Section D, Steps 5-16 in the manual. In Sample 1, Step 17 was not performed and 500 ng of input DNA was loaded directly onto a 1.5% agarose gel without receiving the addition of NaCl and incubation at 100°C. The omission of Step 17 has caused a buffer artifact that makes the DNA appear larger on a gel. The duplicate sample, Sample 2, was processed according to the manual instructions and included the addition of NaCl and incubation at 100°C as stated in Step 17. Analysis of 500 ng of this input DNA on a 1.5% agarose gel shows the expected fragmentation between 200-1200 bp. The difference in DNA sizing on the gel between the two samples illustrates the importance of following the protocol recommendations regarding the processing of input chromatin for agarose gel analysis prior to chromatin immunoprecipitation. Omission of key steps can lead to inaccurate analysis of chromatin shearing efficiency. If the protocol steps were followed and the DNA fragments fall outside of the recommended range, sonication conditions should be further optimized.

Section E. Immunoprecipitation

Successful chromatin immunoprecipitation depends on the quality of the ChIP antibody and the abundance of the target protein. For high abundance proteins as little as 1,000 cells can be used for each immunoprecipitation reaction. For lower abundance proteins as little as 50,000 cells can be used for each immunoprecipitation reaction. (1.5 x 10^6 cells is equivalent to 10 µg chromatin)

1. Thaw sonicated chromatin on ice. Spin chromatin at 4°C in a microcentrifuge at maximum speed for 2 minutes.
2. Set up the ChIP reactions by adding the components in the order shown in Table 1 below to 1.5 ml microcentrifuge tubes. Be sure to use the DNA concentration that was determined for your sonicated chromatin sample to calculate the volume to use. We recommend using 10-30 µg chromatin (1.5 -4.5 million cells) per ChIP reaction, although less can be used if chromatin yields are limiting.
3. In a 1.5 ml microcentrifuge tube prepare the antibodies to be used in the ChIP reactions. Use a separate tube for each antibody. To the tube add 5 µl Blocker and 4 µg ChIP antibody. (Antibody volume should not exceed 30 µl per reaction). Incubate Antibody/Blocker mix for 1 minute at room temperature and then add to the ChIP reactions.

Table 1

Reagent	1 reaction
Sheared Chromatin (10 - 30 µg)	X µl
ChIP Buffer	adjust up to 200 µl
Protease Inhibitor Cocktail (PIC)	5 µl
Antibody/Blocker mix (from Step 3)	not to exceed 35 µl
Maximum Volume Allowed	**240 µl**

4. Cap tubes and incubate on an end-to-end rotator overnight at 4°C.
5. The Protein G agarose beads require washing before use. Transfer 30 µl Protein G agarose beads for each IP reaction to a 1.5 ml microcentrifuge tube. Add an equal volume of TE, pH 8.0 and invert to mix. Spin at 1250 x g in a microcentrifuge for 1 minute. Remove the supernatant equivalent to the volume of TE added to the agarose beads.

   Note: Before pipetting the Protein G agarose beads, they should be fully resuspended by inverting the tube. When pipetting the beads, cut 2 mm from the end of a pipet tip to prevent the tip from becoming clogged.

6. Wash the beads a second time with the same volume of TE, pH 8.0. Invert to mix. Spin at 1250 x g for 1 minute in a microcentrifuge. Remove the supernatant equivalent to the

volume of TE added to the agarose beads. The beads are now ready to use.

7. Spin the ChIP reactions at 1250 x g for 1 minute to collect liquid from the inside of the cap.
8. Using a cut pipet tip, add 30 µl washed Protein G agarose beads to each immunoprecipitation reaction. Cap tubes and incubate on an end-to-end rotator at 4°C for 3 hours.
9. Label a ChIP Filtration Column for each ChIP reaction. Remove the tab from the bottom of the column and place in an empty 1 ml pipet tip box as a holder (see Figure 5 below).
10. Remove ChIP reactions from rotator and spin at 1250 x g for 1 minute to collect liquid from inside of the cap.
11. Add 600 µl ChIP Buffer to each ChIP reaction then transfer the entire reaction (including the protein G agarose beads) to its labeled column. Allow flow-through to occur by gravity.
12. During the gravity flow, transfer 100 µl per ChIP reaction of Elution Buffer AM4 to a 1.5 ml microcentrifuge tube and allow to pre-warm at 37°C during the wash steps.
13. Wash each column with 900 µl Wash Buffer AM1. Let stand for 3 min.
14. Repeat Step 13 four more times for a total of five washes.
15. Transfer columns to a new 1.5 ml microcentrifuge tube and spin in a room temperature microcentrifuge at 1250 x g for 3 minutes to remove residual Wash Buffer.
16. Following the spin, transfer the ChIP Filtration Columns to new 1.5 ml microcentrifuge tubes. Add 50 µl 37°C Elution Buffer AM4 to each column. Incubate at room temperature for 5 minutes. Spin in a room temperature microcentrifuge at 1250 x g for 3 minutes.
17. With columns remaining in the same microcentrifuge tube, add another 50 µl 37°C Elution Buffer AM4 to each column. Incubate at room temperature for 5 minutes and spin in a room temperature microcentrifuge at 1250 x g for 3 minutes.
18. Discard the ChIP Filtration Columns. The flow-through (~100 µl volume) contains the ChIP DNA. Proceed to Section F: DNA purification.

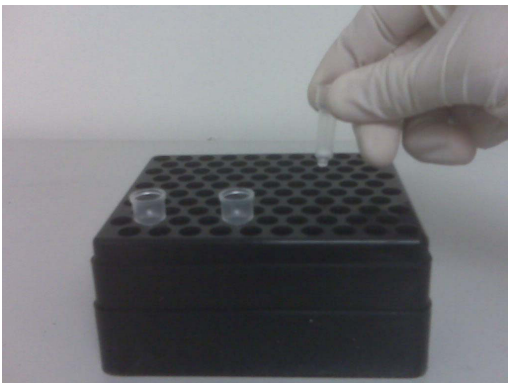


Figure 5: Using the ChIP Filtration Columns.

Remove the tab from the bottom of the ChIP Filtration Columns and place columns in an empty 1 ml pipet tip box to perform the wash steps.

Section F. Reversal of Cross-links and DNA Purification

1. Transfer each eluted ChIP DNA to a 250 µl PCR tube and add 2 µl Proteinase K. Vortex to mix and heat in a thermocycler at 55°C for 30 minutes and then increase the temperature to 80°C for 2 hours.
2. Transfer the DNA to a 1.5 ml microcentrifuge tube and add 5 volumes (500 µl) DNA Purification Binding Buffer to each tube and vortex to mix. Adjust the pH with 5 µl 3M Sodium Acetate. The sample should be bright yellow in color to indicate a proper pH. If your sample is not bright yellow, please refer to the Troubleshooting guide in the Appendix on page 26 for details to adjust pH prior to loading the sample into the purification column.
3. For each sample, place a DNA purification column (AM #103928) in the collection tube and add each pH adjusted sample to its own column. Close the cap on each column, place them with the collection tubes in a microcentrifuge and spin them at 14,000 rpm for 1 minute.
4. Remove the column from the collection tube, then remove and discard the flow through from the collection tube. Return the column to the collection tube.
5. Prepare DNA Purification Wash Buffer (AM #103497) before the first use. Follow the instruction on page 8 for the addition of ethanol prior to using the solution. Add 750 µl DNA Purification Wash Buffer to each column and cap the column.
6. Spin at 14,000 rpm for 1 minute in a microcentrifuge.
7. Remove the column from the collection tube, then remove and discard the flow through from the collection tube. Return the column to the collection tube.
8. With the column cap open, spin at 14,000 rpm for 2 minutes in a microcentrifuge to remove any residual Wash Buffer from the column.
9. Transfer the column to a clean microcentrifuge tube. Depending on the method to be used for downstream analysis of the ChIP enriched DNA, use the appropriate elution volume. For ChIP-Seq or ChIP-chip applications, we recommend pre-warming the required elution buffer volume at 37°C for 5 minutes prior to use.
    a. **For qPCR analysis:** add 100 µl of DNA Purification Elution Buffer (AM #103498) to the center of the column matrix and incubate for 1 minute at room temperature. Spin at 14,000 rpm for 1 minute in a microcentrifuge. Add an additional 100 µl DNA Purification Elution Buffer to the column and incubate for 1 minute at room temperature. Spin at 14,000 rpm for 1 minute in a microcentrifuge. Total elution volume is 200 µl.
    b. **For ChIP-Seq or ChIP-chip analysis:** add 36 µl of 37°C DNA Purification Elution Buffer (AM #103498) to the center of the column matrix and incubate for 1 minute at room temperature. Spin at 14,000 rpm for 1 minute in a microcentrifuge.
10. Discard column. Purified DNA may be stored at -20°C for future use.

Section G. Quantitative PCR (qPCR)

ChIP DNA can be analyzed on a gene-specific basis using quantitative PCR (qPCR). Positive control and negative control PCR primer pairs should be included in every analysis to determine the fold enrichment. Negative control primers will amplify a region of the genome not bound by the antibody target of interest. Active Motif recommends the use of its ChIP-IT® qPCR Analysis Kit (Catalog No. 53029) for the analysis of qPCR data. The ChIP-IT qPCR Analysis Kit contains positive and negative control primer pairs, standard curve DNA, standard curve primers and a qPCR Analysis spreadsheet to perform the analysis calculations. Active Motif's analysis strategy determines primer efficiencies and the ChIP sample values are normalized according to input, primer efficiency, chromatin amount used in the ChIP reaction and resuspension volume. The ChIP-IT qPCR Analysis Kit provides consistency in data analysis and allows direct comparison across samples and experiments. If not using the ChIP-IT qPCR Analysis Kit, qPCR data normalization and graphing can be done using the methods described in Section I.

1. Below is an example qPCR reaction. Please follow the specific instructions for your qPCR instrument. We recommend using a commercially available SYBR Green qPCR master mix (*e.g.* Bio-Rad Cat # 170-8882) and preparing triplicate reactions.

Reagent	20 µl PCR reactions
2X SYBR Green master mix	10 µl
Primer mix (2.5 µM each primer)*	4 µl
Sterile water	1 µl
DNA sample (ChIP or Input)	5 µl
Total volume	**20 µl**

* We recommend designing primers to perform at an annealing temperature of 58°C so that all qPCR reactions can be performed under identical conditions. An amplicon length of 75-150 bp is recommended. Active Motif offers validated species-specific qPCR primers designed according to these recommendations at www.activemotif.com/chipprimers.

2. Place tubes in a real time PCR instrument and program as below:

   95°C for 2 minutes
   (95°C for 15 seconds, 58°C for 20 seconds, 72°C for 20 seconds) for 40 cycles

3. If using the ChIP-IT qPCR Analysis Kit, please refer to the product manual for analysis instructions. Otherwise, follow the recommendations in Section I.

Notes: The polymerase chain reaction (PCR) process for amplifying nucleic acid is covered by U.S. Patent Nos. 4,683,195 and 4,683,202 assigned to Hoffmann-La Roche. Patents pending in other countries.

Section H. ChIP-Seq

The ChIP-IT High Sensitivity Kit has been extensively validated for whole genome analysis using ChIP-Seq. This process involves the preparation of libraries from ChIP DNA by the addition of adapter sequences to the ends of the DNA fragments. The library is then PCR amplified and validated prior to sequencing.

Commercial kits are available to prepare the ChIP DNA libraries for sequencing. Select a library preparation kit compatible with the sequencing platform to be used.

A. General Recommendations

- We recommend using 30 µg chromatin for the ChIP Reactions, although less may be used if chromatin amounts are limiting or you are enriching for a highly abundant histone protein
- Library generation usually requires 10 ng of ChIP enriched DNA. However, by using the ChIP-IT High Sensitivity Kit the enriched DNA is of such a high quality that lower amounts can be used when working with low abundance transcription factors. (See B. ChIP-Seq Requirements below for more details)
- Sequence library yields should be in the range of 1-2 micrograms
- 30 million sequencing reads is sufficient for most transcription factor and histone modifications
- 36 bp single end reads are sufficient for unique mapping and good ChIP-Seq data, although longer reads can be used
- Input DNA should be sequenced as a control reaction in order to identify false "peaks" and also to reveal regions of the genome that have been duplicated. Subtracting the input peaks from the experimental peaks will help to eliminate false data. Use 50 ng Input DNA (from Section B Step 12 for cultured cell samples or Section D Step 15 for tissue samples) for library generation.

B. ChIP-Seq Requirements

It is commonly recommended that library generation of ChIP DNA requires 10 ng of ChIP enriched DNA. Obtaining 10 ng of ChIP DNA should be attainable when using good antibodies against abundant histone modifications. However, 10 ng is an unrealistic number for a sequence specific DNA binding transcription factor. Using the ChIP-IT High Sensitivity Kit, we have routinely generated high quality libraries from sub-nanogram levels of ChIP DNA due to the high quality of the DNA obtained. For good ChIP-Seq data, quality enrichments with low background is more important than the total quantity of DNA recovered. Therefore, we recommend performing qPCR on known binding sites to verify enrichment levels against a negative control primer set using Active Motif's ChIP-IT qPCR Analysis Kit (Catalog No. 53029) to confirm DNA quality rather than quantifying the DNA.

C. qPCR prior to ChIP-Seq

To perform qPCR for verification of the quality of the enriched ChIP DNA, follow the instructions for qPCR in Section G on page 21 with the following modification. The ChIP DNA will need to be diluted prior to use in qPCR due to the reduced elution volume. Dilute 6 µl of ChIP DNA in 94 µl DNA Purification Elution Buffer (AM #103498). Use 5 µl of the diluted ChIP DNA per qPCR reaction.

Quality DNA that is suitable for use in ChIP-Seq should show enrichment of known binding sites over negative control primer sets of 5-fold (See Figure 6 below). If the qPCR enrichments are satisfactory then the remaining 30 µl of ChIP DNA can be used for library generation.

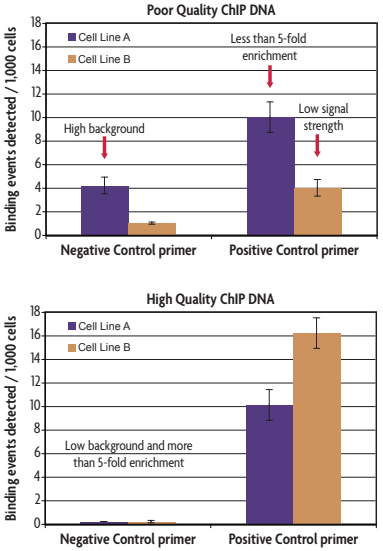


Figure 6: Comparison of qPCR results showing good versus poor enrichment over negative control primers.

Data shows qPCR results analyzed using the ChIP-IT qPCR Analysis Kit with the Human Negative Control Primer as a reference and a gene-specific positive control primer. In the top image the negative control primer set for Cell Line A gives high background levels with Binding events detected per 1,000 cells above a value of 2, while Cell Line B has positive control signal levels below 5 Binding events detected per 1,000 cells. The bottom image shows low background in the negative control primer set with Binding events detected per 1,000 cells below a value of 2. The fold enrichment of the positive control primer set exceeds 5-fold. Only the ChIP DNA from the bottom sample is recommended for use in ChIP-Seq.

D. Library Generation

ChIP DNA from the ChIP-IT High Sensitivity Kit has been extensively validated for ChIP-Seq using the Illumina® platform. ChIP DNA can be used to generate single or paired-end libraries using the standard Illumina® library protocols. Commercially available library preparation kits can be used to generate the NGS library. Select a library preparation kit compatible with the sequencing platform to be used.

Appendix

Section I: qPCR Primer Design and Data Analysis

A. Design of the primers

- Design and analyze your potential primer pairs using an *in silico* PCR program (*i.e.* Primer3 at http://frodo.wi.mit.edu/)
- Primers that dimerize should be avoided, as they will be bound by SYBR Green, which will compromise accurate quantitation.
- Ideally, the amplicons should be 75-150 bp in length.
- For use with the ChIP-IT qPCR Analysis Kit, primers should be designed to anneal optimally at 58°C with a recommended length of 18-22 bp.
- Active Motif offers ChIP Control qPCR primer sets validated to work in our ChIP-IT qPCR Analysis Kit. To see a list of the available species-specific primers, please visit www.activemotif.com/chipprimers.

B. Data Analysis

If the data analysis will not be performed using Active Motif's ChIP-IT qPCR Analysis Kit, two other simplified methods of analysis are provided below. Both methods listed require the generation of a standard curve, containing known amounts of DNA, for each primer pair being used in the experiment.

Method 1: Fold enrichment of positive primers relative to negative control primers

1. Produce a standard curve by performing qPCR with your primer set on known DNA quantities of Input DNA (from Section B Step 12 for cultured cell samples or Section D Step 15 for tissue samples) in triplicate. Run three to five samples that are 10-fold dilutions, *e.g.* 0.005 ng, 0.05 ng, 0.5 ng, 5 ng and 50 ng.
2. Run the ChIP and IgG samples along with the dilution series of the Input DNA standards using both positive control primers (known binding sites) and negative control primers (regions of the genome not bound by your protein of interest).
3. Your qPCR instrument will assign values to each qPCR reaction based on the standard curve. If your machine does not average your triplicate reactions automatically, you will need to calculate these averages.
4. Divide the average value from the positive control primer set by the average value of the negative control primer set to obtain your fold enrichment.

Method 2: Express data as a percent of input

1. Produce a standard curve by performing qPCR with your primer set on known DNA quantities of Input DNA (from Section B Step 12 for cultured cell samples or Section D Step 15 for tissue samples) in triplicate. Run three to five samples that are 10-fold dilutions, *e.g.* 0.005 ng, 0.05 ng, 0.5 ng, 5 ng and 50 ng.
2. Run the ChIP and IgG samples along with the dilution series of the Input DNA standards using both positive control primers (known binding sites) and negative control primers (regions of the genome not bound by your protein of interest).
3. Your qPCR instrument will assign values (in ng) to each qPCR reaction based on the standard curve. If your machine does not average your triplicate reactions automatically, you will need to calculate these averages.
4. For each qPCR reaction you will have used a percentage of your total ChIP DNA. In order to calculate the amount in the whole reaction, divide the elution volume of the entire ChIP reaction by the volume used in the qPCR reaction (*e.g.* if you eluted ChIP DNA in 200 µl and used 5 µl in the qPCR reaction the formula is 200/5 = 40). Then, multiply the average qPCR quantity by this number (*e.g.* qPCR quantity in ng x 40).
5. To express data as a percent of input, divide the adjusted values from Step 4 above by the amount of DNA that went into the ChIP reaction and then multiply by 100%. (*e.g.* if 20 µg was used in the ChIP reaction this is equivalent to 20,000 ng of chromatin. The calculation would be the adjusted value from Step 4 divided by 20,000 ng and then multiplied by 100). Typical percent of input recovered values are 0.05% to 1%.

Section J. Troubleshooting Guide

Problem/question	Recommendation
At what points in the protocol can I stop?	The protocol may be stopped and samples stored at the times and temperatures below: 1. After formaldehyde fixation and centrifugation (intact cell pellet), -80°C. 2. After chromatin shearing, -80°C. 3. After DNA clean up, -20°C.
After sonication shearing and centrifugation, a viscous or cloudy layer is visible in the chromatin.	Depending upon the cell type, lipid or glycogen layers may be seen after centrifugation. For example, fatty tissues may have a lipid layer. Avoid such layers when you remove the supernatant. However, if the whole supernatant is cloudy, it should not interfere with the IP reaction.
Poor yield of sheared chromatin.	Insufficient cell numbers were used. Repeat chromatin preparation using a larger number of cells.
	Nuclei not released. It is highly recommended to perform dounce homogenization, even when using sonication. Use a dounce homogenizer with a small clearance pestle (Active Motif Catalog Nos. 40401 & 40415). Monitor cell lysis under a microscope. Generally, the more cells that are lysed, the higher the sheared chromatin yield.
	Sonication samples were emulsified. Avoid emulsification by turning up the power of the sonicator gradually. If a chromatin preparation becomes emulsified inadvertently, discontinue shearing and centrifuge the sample for 4 minutes at 8,000 rpm in a 4°C microcentrifuge to remove trapped air.
	Use fresh formaldehyde when preparing Complete Cell Fixation Solution and Complete Tissue Fixation Solution.
	Buffers were not scaled proportionally to the size of the sample. Use the chart in Cell Growth Recommendations to scale up or down chromatin preparation.
Shearing efficiency is not clear from gel analysis.	Material is stuck in the wells, and smears or streaks are seen from the top to bottom of the lane. The sheared chromatin needs to have the cross-links reversed, protein removed (Proteinase K) and RNA removed (RNase), followed by DNA purification.
	High molecular weight products. Decrease the size of the fragments by re-sonicating the sample. If an alternative reverse cross-linking method was used, or the 20 minute incubation at 100°C in NaCl was omitted prior to running the agarose gel for analysis, please repeat the input chromatin preparation and follow the manual instructions.
Performing ChIP with a large volume of chromatin.	This is not recommended. It is better to set up several small ChIP reactions (240 µl each) and pool the samples at the end, rather than trying to ChIP a single large sample. Do not perform a single scaled-up reaction, as the capture efficiency is lower.
ChIP DNA does not turn bright yellow following the addition of 3 M sodiium acetate	If the color is light orange or violet, this indicates the pH is too high. Add more 3 M sodium acetate 5 µl at a time, mixing after addition until the color is bright yellow. This step is crucial to the success of DNA binding and purification. For a full color image please see the manual for Active Motif's Chromatin IP DNA Purification Kit Catalog No. 58002 available online at our website www.activemotif.com.

Problem/question	Recommendation
High background.	Chromatin not sheared enough. Shearing should produce DNA fragments that are small enough to exclude background from neighboring chromosomal sequences, but still large enough that there is a good possibility your amplicon remains intact. We recommend 200-1200 bp fragments. If the DNA fragments are too large, the background is increased. Consider increasing the number of pulses for sonication. Check the fragment size on a gel to assess your shearing efficiency.
	Antibody issue. Too much antibody relative to the amount of chromatin in the ChIP reaction. Excess antibody will result in more non-specific binding, which will be detected as increased background.
Poor or no enrichment with target antibody.	Too little chromatin. Generally, we recommend using 10 - 30 µg of chromatin per ChIP reaction. For highly abundant, DNA-associated targets such as histones, chromatin from as little as 1000 cells or 6.7 ng can be used. For very low abundance transcription factors, chromatin from as little as 50,000 cells or 333 ng can be used. ChIP reactions should not exceed 50 µg per IP reaction. Be sure to quantitate the concentration of the sheared chromatin sample(s) being ChIP'd to ensure both that adequate chromatin is used per sample, and that equal mass quantities of chromatin are used in each ChIP.
	Antibody is not ChIP validated. The antibody does not efficiently recognize fixed proteins, either because the epitope is destroyed by fixation or because the epitope is masked by other proteins in a larger complex. To assist in ChIP validating an antibody, it is very useful to use a positive control antibody such as Histone H3K4me3 (Catalog No. 39915) and a negative IgG from the same species, and primers that have been proven to work in the type of PCR being used. Active Motif offers species-specific ChIP-IT Control qPCR Kits for antibody validation (Catalog Nos. 53026, 53027 & 53028).
	Low-affinity antibody. Use a different antibody.
	Antibody affinity to protein G is weak. Individual monoclonals have variable binding affinities to protein G, which are pH dependent; the optimal pH may vary for each Ig, For those with low to medium affinity, capture efficiency by protein G can be dramatically improved through use of our Bridging Antibody (Catalog No. 53017). This antibody is a rabbit anti-mouse pAb that recognizes all subclasses of mouse immunoglobulins. If your IgG has a weak/medium affinity to protein A or G, the Bridging Antibody will increase antibody capture by the beads without increasing background.
	Problems with PCR. Confirm the amplified sequence for the positive control primer set is bound by the antibody target. Identify other binding sites.
No PCR products for the ChIP'd samples (but the Input DNA yields the correct PCR product)	Increase the amount of chromatin used in the ChIP reaction, the amount of antibody used, or both.
	Use a different antibody.
No PCR products with real-time PCR	Confirm the species specificity and efficiency of your primers. You may need to redesign your primers. Primers that work in end point PCR do not always work in qPCR.
	No ethanol in DNA Purification Wash Buffer. Make sure that ethanol has been added to the DNA Purification Wash Buffer prior to first use.

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

Active Motif North America

Toll free: 877.222.9543
Direct: 760.431.1263
Fax: 760.431.1351
E-mail: tech_service@activemotif.com

Active Motif Europe

UK Free Phone: 0800/169 31 47
France Free Phone: 0800/90 99 79
Germany Free Phone: 0800/181 99 10
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