ChIP-IT® ChIP-Seq

(version A3)

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Active Motif North America
1914 Palomar Oaks Way, Suite 150
Carlsbad, California 92008, USA
Toll free: 877 222 9543
Telephone: 760 431 1263
Fax: 760 431 1351

Active Motif Europe
Avenue Reine Astrid, 92
B-1310 La Hulpe, Belgium
UK Free Phone: 0800 169 31 47
France Free Phone: 0800 90 99 79
Germany Free Phone: 0800 181 99 10
Telephone: +32 (0)2 653 0001
Fax: +32 (0)2 653 0050

Active Motif Japan
Azuma Bldg, 7th Floor
2-21 Ageba-Cho, Shinjuku-Ku
Tokyo, 162-0824, Japan
Telephone: +81 3 5225 3638
Fax: +81 3 5261 8733

TABLE OF CONTENTS **Page**

Overview 1

Flow Chart of Process 2

Kit Performance and Benefits 3
Protocol Overview and Time Table 3

Kit Components and Storage **4**
Reagents for Chromatin Preparation and Immunoprecipitation 4
Reagents for ChIP-IT qPCR Analysis 6
Reagents for Sequencing Library Construction 7

Protocols – Chromatin Preparation and Immunoprecipitation **8**
Cell Growth and Recommendations 8
Reagent Information 9
Recommendations 10
Section A. Cell Fixation Starting with Cultured Cells 12
Section B. Chromatin Sonication of Cultured Cells 13
Section C. Cell Fixation Starting with Fresh or Frozen Tissue 15
Section D. Chromatin Sonication of Tissue 16
Section E. Immunoprecipitation 18
Section F. Reversal of Cross-links and DNA Purification 20

Protocols – ChIP-IT qPCR Analysis **21**
Reagent Information 22
Section G. qPCR Plate Set Up 24
Section H. Using the Analysis Spreadsheet 26
Section I. Data Calculations 27
Section J. Data Interpretation 28

Protocols – Sequencing Library Construction **30**
Reagent Information 30
Recommendations 30
Section K. Perform End Repair 31
Section L. Add 'A' Overhang to 3' end of DNA Fragments 31
Section M. Ligate Adapters to DNA Fragments 32
Section N. Size Select the Library 32
Section O. Enrich the Adapter-Modified DNA Fragments by PCR 33
Section P. Validate the Library 34

Appendix
Section Q. qPCR Primer Design and Data Analysis 35
Section R. Troubleshooting Guide 37
Section S. Related Products 40

Technical Services 42

Overview

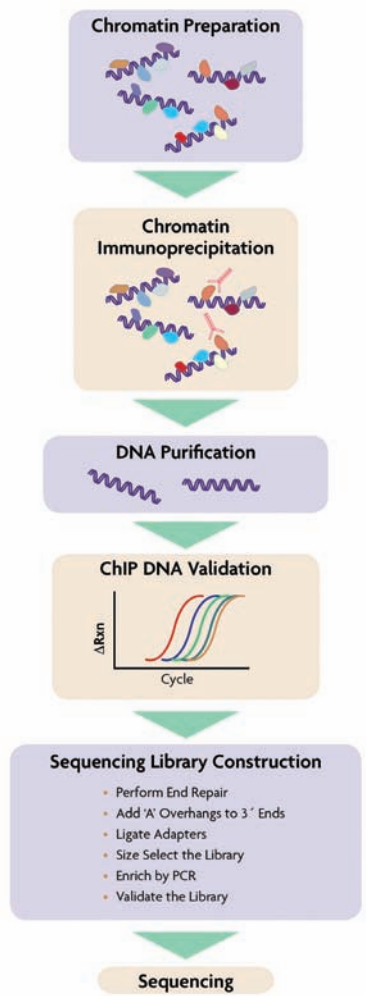
Chromatin Immunoprecipitation (ChIP) is a powerful tool for studying protein/DNA interactions, including transcription factors, co-regulatory proteins, modified histones, chromatin-modifying enzymes and polymerases because it enables identification of the localization of proteins bound to specific DNA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

Active Motif's ChIP-IT® ChIP-Seq Kit is designed to provide the highest quality ChIP-enriched DNA for use in sequencing on Next-Generation platforms, such as Illumina® Genome Analyzer II, HiSeq and MiSeq systems. The ChIP-IT ChIP-Seq protocol is robust enough for use 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, resulting in lower background levels and better enrichment. Better enrichment translates into larger ChIP-Seq peaks and more accurate peak calling since a larger percentage of the sequence reads are mapped to binding sites rather than background DNA regions. The ChIP-IT ChIP-Seq Kit has been validated across multiple sample types with excellent reproducibility. For successful ChIP-Seq, we recommend using at least 30 µg chromatin (or 4.5 million cell equivalents) per ChIP reaction.

The ChIP-IT ChIP-Seq Kit provides validated reagents and protocols to perform 16 chromatin preparation and immunoprecipitation reactions and construct 10 sequencing libraries. Oligonucleotide primers and adapters **are not provided** in the kit, please refer to your instrument system manufacturer's guidelines for specific sequences for single end, paired end or barcoded library construction. A chromatin preparation is defined as one 15 cm cell culture plate or 100 mg tissue sample. The ChIP-IT ChIP-Seq Kit also includes Active Motif's ChIP-IT® qPCR Analysis Kit for use in assessing the quality of ChIP enriched DNA prior to library construction. 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® ChIP-Seq	10 libraries	53041

Flow Chart of Process



Flow Chart of ChIP-IT ChIP-Seq Process.

ChIP-Seq is a multi-step process that includes chromatin preparation, immunoprecipitation, DNA purification, ChIP DNA validation and sequencing library construction. First, intact cells or fresh or frozen tissue are fixed with a specially formulated formaldehyde buffer, which cross-links and preserves protein/DNA interactions. DNA is then sheared into fragments using sonication and incubated with a ChIP-validated 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 using ChIP filtration columns. Following immunoprecipitation, cross-links are reversed, the proteins are removed by Proteinase K and the DNA is recovered and purified. The ChIP-enriched DNA is then validated by qPCR to confirm the quality of the DNA prior to use in sequencing library construction. Once the ChIP DNA is confirmed to be of high quality, the DNA ends are modified and adapters are ligated to the DNA. The adapter-modified DNA is then run on an agarose gel and the library is size selected and purified. High-fidelity PCR is used to amplify the library, which is validated for quantity and purity before use in sequencing on the Illumina platforms.

Kit Performance and Benefits

ChIP-IT ChIP-Seq Advantages:

- Works with histone and transcription factors antibodies
- Optimized reagents reduce background levels caused by non-specific binding events providing high quality DNA for sequencing analysis
- 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
- Includes Active Motif's ChIP-IT® qPCR Analysis Kit for validation of ChIP enriched DNA prior to library generation
- Easy-to-use protocols for entire workflow from chromatin preparation through library preparation for Next-Generation sequencing

Protocol Overview and Time Table

The ChIP-IT ChIP-Seq Kit requires multiple days to complete. It is strongly advised to read the entire protocol before starting and to plan your experiments in advance.

	Required Time
Cell or Tissue Fixation and Lysis	1.5 hours
Chromatin Sonication	20 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
ChIP DNA Purification	15 minutes
qPCR Analysis of ChIP enriched DNA	2 hours
Perform End Repair	45 minutes
Add 'A' Overhang to 3 ´ Ends of DNA fragments	45 minutes
Ligate Adapters to DNA Fragments	30 minutes
Size Select the Library	1.5 hours
PCR Amplify Adapter-Modified Library	1 hour
Validate the Library	Variable

* The protocol varies between cell culture and tissue samples.

Kit Components and Storage

The ChIP-IT ChIP-Seq Kit is for research use only. Not for use in diagnostic procedures. The kit contains multiple modules to help organize your experiments. One module is for chromatin preparation and immunoprecipitation, another module contains Active Motif's ChIP-IT qPCR Analysis Kit for ChIP DNA validation and a third module includes the reagents needed for library construction for Next-Generation sequencing. Reagents within each module may contain multiple storage temperatures. All components can be stored at -20°C prior to first use. Then we recommend storing each component at the temperatures indicated in the tables below. **Do not re-freeze the Protein G Agarose Beads once they have been thawed for use.**

Reagents for Chromatin Preparation and Immunoprecipitation

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

* The Protein G Agarose Beads are shipped on dry ice, but **should not be re-frozen** by the customer. Upon receipt of this kit, the beads should be stored at 4°C.

**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 efficient chromatin preparation.
- 37% formaldehyde solution with 10-15% methyl alcohol to prevent polymerization. Do not use paraformaldehyde.
- For tissue preparations you will need Phenol/chloroform TE saturated pH 8 Phenol/chloroform (50:50) or Phenol/chloroform/isoamyl alcohol (25:24:1) (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) with the EpiShear™ Cooled Sonication Platform (Catalog No. 53080))
- 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)

Reagents for ChIP-IT® qPCR Analysis Kit

Reagents	Quantity	Storage
DNA Standard AM1	100 µl	-20°C
DNA Standard AM2	100 µl	-20°C
DNA Standard AM3	100 µl	-20°C
Standard Curve Primer Pair (2 µM)	400 µl	-20°C
Human Negative Control Primer Set 1 (2.5 µM)	400 µl	-20°C
Human Positive Control Primer Set GAPDH-2 (2.5 µM)	400 µl	-20°C
Mouse Negative Control Primer Set 1 (2.5 µM)	400 µl	-20°C
Mouse Positive Control Primer Set Gapdh-2 (2.5 µM)	400 µl	-20°C
2 mM Tris-HCl, pH 8.0	2 x 1 ml	RT

Additional materials required

- 96-well PCR plate or PCR tubes
- Thermocycler
- Pipettors and tips (filter tips are recommended)
- qPCR Primer Pairs to study the gene locus of interest in the ChIP DNA. Positive and negative control primers are recommended. Primers should be designed to anneal optimally at 58°C with a recommended length of 18-22 bp. See Reagent Information on page 22 for complete details on primer design.
- SYBR Green qPCR master mix (Bio-Rad Catalog No. 170-8882)
- Active Motif's ChIP-IT® qPCR Analysis sheet which can be downloaded from the Active Motif website at www.activemotif.com/qPCRanalysis.

Reagents for Sequencing Library Construction

Reagents	Quantity	Storage
DNA Polymerase I Klenow (5 U/µl)	5 µl	-20°C
T4 DNA polymerase (3 U/µl)	10 µl	-20°C
T4 Polynucleotide Kinase (10 U/µl)	10 µl	-20°C
Klenow Fragment (3'-5' exo-) (5 U/µl)	10 µl	-20°C
10X Reaction Buffer AM3	50 µl	-20°C
T4 DNA Ligase (2,000 U/µl)	15 µl	-20°C
10X T4 DNA Ligase Buffer	50 µl	-20°C
2X Quick Ligation Buffer	150 µl	-20°C
dNTP Mix, 10 mM each	35 µl	-20°C
1 mM dATP	100 µl	-20°C
Q5® High-Fidelity DNA Polymerase (2 U/µl)	5 µl	-20°C
5X Q5 Reaction Buffer	100 µl	-20°C

Additional materials required

- Illumina® adapters for single end, paired end or barcoded library preparation
- Amplification primers (12.5 µM each)
- Purified ChIP DNA and Input DNA for each sample to be tested
- QIAquick PCR Purification Kit (QIAGEN Cat. No. 28104)
- MinElute PCR Purification Kit (QIAGEN Cat. No. 28004)
- QIAquick Gel Extraction Kit (QIAGEN Cat. No. 28704)
- Agarose gel electrophoresis apparatus
- Razor blades (for gel extraction)
- Agencourt® AMPure® XP beads (Beckman Coulter Cat. No. A63880)
- Nanodrop or equivalent method to determine DNA concentration
- qPCR Primer Pairs to study the gene locus of interest
- 2X SYBR Green qPCR Master Mix (e.g. Bio-Rad Catalog No. 170-8882)
- 250 µl PCR tubes
- Thermocycler

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

The ChIP-IT ChIP-Seq Kit requires multiple days to complete. It is strongly advised to read the entire protocol before starting and to plan your experiments in advance. For ease-of-use the manual is divided into sections to represent the three different modules provided: Chromatin Preparation and Immunoprecipitation, ChIP-IT qPCR Analysis Kit and Sequencing Library Construction. Please refer to the kit contents and additional materials required section of the manual (pages 4-7) to identify the components that are needed for each step of the process.

Protocols – Chromatin Preparation and Immunoprecipitation

Please refer to pages 4-5 of the manual for the kit components and additional materials required to complete the chromatin preparation and immunoprecipitation protocols.

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 4.5 million cells.

	60 mm dish	100 mm dish	150 mm dish
Seeding Density	0.8 x 10^6	2.2 x 10^6	5.0 x 10^6
Cells at 70-80% Confluency*	2.4 x 10^6	6.6 x 10^6	15.0 x 10^6
Growth Medium Volume	5 ml	10 ml	20 ml
Cell Fixative Solution	500 µl	1 ml	2 ml
Stop Solution	275 µl	550 µl	1.1 ml
PBS Wash Buffer (used per wash)	2 ml	5 ml	10 ml
Chromatin Prep Buffer	2 ml	5 ml	5 ml
ChIP Buffer	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

Reagent Information

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 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 4°C 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.

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, but should not be re-frozen by the customer. The beads should be stored at 4°C.**

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.

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

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 4°C 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 or use Active Motif's EpiShear Cooled Sonication Platform (Catalog No. 53080) to help regulate sample temperature.

Chromatin Quantity

It is recommended to use 30 µg chromatin per IP reaction (4.5 million cell equivalents). However, if chromatin is in limited supply and the target protein is a highly abundant histone, lower chromatin amounts (10 µg or 1.5 million cell equivalents) may be used due to the high quality of the DNA obtained from the ChIP-IT ChIP-Seq Kit.

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 8 for information on scaling the protocol for use with other amounts of 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 in the protocol below are enough to process one 15 cm plate. Please refer to the chart on page 8 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.
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 in combination with an EpiShear™ Cooled Sonication Platform to maintain probe height and temperature consistency between samples. We do not recommend sonication of samples containing less than 100,000 cells and/or 350 µl volume.

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. 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 (which is equivalent to 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 Section B, 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 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 on an agarose gel. Store the remaining Input DNA at -20°C.

14. Analyze each chromatin preparation on an agarose gel.

    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 a 100 bp DNA ladder to analyze chromatin size. DNA should appear as a smear anywhere between 200-1200 bp.

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.

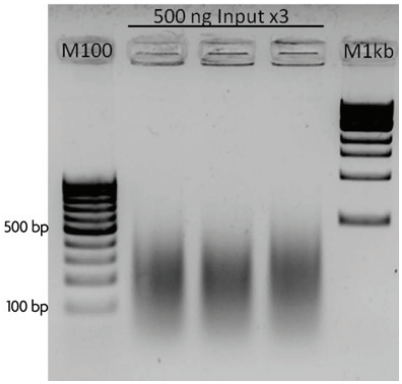


Figure 1: Validation of chromatin shearing efficiency.

Three chromatin preparations of MCF-7 cells were fixed and sonicated using the EpiShear™ Probe Sonicator and EpiShear™ Cooled Sonication Platform from Active Motif. The chromatin was prepared according to the instructions in the manual and 500 ng each was run on a 1.5% agarose gel. The three samples show a DNA smear concentrated around 200-500 bp.

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 9) 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.
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 in combination with the EpiShear™ Cooled Sonication Platform to maintain probe height and temperature consistency between samples. We do not recommend sonication of samples containing less than 50 mg tissue and/or 350 µl volume.

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. 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 (which is equivalent to 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 Section D, 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 on an agarose gel. Store the remaining Input DNA at -20°C.
17. Analyze each chromatin preparation on an agarose gel.
    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 a 100 bp DNA ladder to analyze chromatin size. DNA should appear as a smear anywhere between 200-1200 bp.
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.

Section E. Immunoprecipitation

Successful chromatin immunoprecipitation depends on the quality of the ChIP antibody and the abundance of the target protein. We suggest using 30 µg chromatin (4.5 x 10^6 cell equivalents) per immunoprecipitation reaction to ensure good ChIP DNA recovery and successful library preparations, although 10 µg may be used for highly abundant target proteins.

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 30 µg chromatin (4.5 million cell equivalents) per ChIP reaction, although less can be used (10 µg) if chromatin yields are limiting and the target protein is a high abundance histone protein.
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 above)	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 2 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: Reversal of Cross-links and DNA Purification.

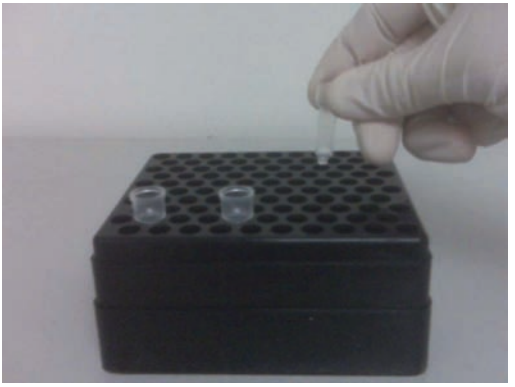


Figure 2: Using the ChIP Filtration Columns.
Remove the tab from the bottom of the ChIP Filtration Columns and place columns in an empty 1 ml pipet tip box to perform the wash steps.

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 37 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 10 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. Pre-warm the required elution buffer volume at 37°C for 5 minutes prior to use.
10. 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.
11. Discard column. Purified DNA may be stored at -20°C for future use.

Protocols – ChIP-IT qPCR Analysis Kit

Please refer to page 6 of the manual for the components and additional materials required to complete the qPCR validation of the ChIP enriched DNA using the ChIP-IT qPCR Analysis Kit.

Prior to generating the libraries for sequencing, we recommend performing gene-specific quantitative PCR (qPCR) for verification of the quality of the enriched ChIP DNA. 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.

To validate the quality of the ChIP enriched DNA, the ChIP-IT ChIP-Seq Kit includes Active Motif's 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. Using the ChIP-IT qPCR Analysis strategy provides consistency in data analysis and allows direct comparison across samples and experiments. Quality DNA that is suitable for use in ChIP-Seq should show a minimum of 5-fold enrichment of known binding sites over negative control primer sets (See Figure 3 on page 29). If the qPCR enrichments are satisfactory then the remaining ChIP DNA can be used for library generation. If the ChIP-IT qPCR Analysis strategy is not performed then qPCR data normalization and graphing can be done using the methods described in Section Q.

Reagent Information

DNA Standards AM1, AM2 and AM3

The DNA Standards provided in the kit can be used to generate a standard curve. Each standard vial contains a known amount of genomic DNA. Since the DNA mass in each standard is known, the number of copies of the genome present in each standard can be calculated (assuming that each cell contains 6.6 pg of DNA). DNA Standards are provided ready to use. Duplicate qPCR reactions are prepared for each DNA Standard.

Standard Curve Primer Pair

The Standard Curve Primer Pair is designed to be used with the standard curve generated from the DNA Standard samples. The primer pair is provided at 2 µM.

ChIP DNA

qPCR reactions with ChIP DNA should be performed in triplicate for each primer pair used. Prepare a dilution by adding 6 µl ChIP DNA to 94 µl of 2 mM Tris-HCl, pH 8.0. Use 5 µl diluted ChIP DNA per qPCR reaction. It is necessary to run the included Negative Control Primer Pair (human or mouse) in triplicate with the ChIP DNA. This helps with data interpretation as deviation from the expected output may indicate potential problems in the ChIP reaction.

Input DNA

Input DNA for each sample type should be diluted to a final concentration of 2.5 ng/µl using 2 mM Tris-HCl pH 8.0. In experiments with multiple Input DNAs prepared from multiple samples, a DNA pool should be made and used for testing. Combine the various Input DNAs at their diluted 2.5 ng/µl concentration into a single tube. Input DNA should be tested in triplicate.

2 mM Tris-HCl, pH 8.0

2 mM Tris-HCl is provided ready to use.

Gene-specific qPCR Primer Pair

When designing PCR primer pairs for use in qPCR analysis of the ChIP DNA, it is recommended to design primers that generate amplicons 75-150 bp long. Primers should also be designed to perform optimally at an annealing temperature of 58°C with a recommended length of 18-22 bp each. The Primer3 program (Primer3 at http://frodo.wi.mit.edu/) is a good resource for designing primers to meet these criteria. We suggest preparing a mix of both primers at a concentration of 2.5 µM each for qPCR reactions. Gene-specific primer sets should be tested with 12.5 ng Input DNA in a qPCR reaction. Usable primer pairs will have CT values between 23-27. If primers have CT values greater than 27, the primers should be redesigned.

Human Negative Control Primer Set1

The negative control primer set is designed to serve as a universal negative ChIP control when performing chromatin immunoprecipitation with human samples. If your test ChIP sample is human, run triplicate qPCR reactions using the Human Negative Control Primer Set in addition to a positive gene-specific qPCR primer pair. When the Human Negative Control primer set is used in

the analysis spreadsheet the signal should fall within an expected range. For more information on data interpretation, please refer to Section J of the manual. The primer pair is provided at 2.5 µM.

Note: The universal negative control primers could give a positive readout for repressive histone marks such as H3K27me3. In this case we recommend using the supplied positive control primer set for GAPDH-2 as the negative control.

Human Positive Control Primer Set GAPDH-2

The positive control primer set is designed to serve as a positive ChIP control when performing chromatin immunoprecipitation with human samples. The Human Positive Control Primer Set GAPDH-2 amplifies a region of the metabolic gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) associated with intron 1 and has been validated to work with a multitude of ChIP antibodies including: H3K9ac, H3K14ac, H3K4me2, H3K4me3, H4K5ac, H4K8ac, H4K12ac, H4K16ac, Total RNA pol II, and RNA pol II phospho Ser5. If your test ChIP sample is human, run triplicate qPCR reactions using the Human Positive Control Primer Set GAPDH-2 in addition to the gene-specific qPCR primer pair for the gene of interest. The primer pair is provided at 2.5 µM.

Mouse Negative Control Primer Set 1

The negative control primer set is designed to serve as a universal negative ChIP control when performing chromatin immunoprecipitation with mouse samples. If your test ChIP sample is mouse, run triplicate qPCR reactions using the Mouse Negative Control Primer Set in addition to a positive gene-specific qPCR primer pair. When the Mouse Negative Control primer set is used in the analysis spreadsheet the signal should fall within an expected range. For more information on data interpretation, please refer to Section J of the manual. The primer pair is provided at 2.5 µM.

Note: The universal negative control primers could give a positive readout for repressive histone marks such as H3K27me3. In this case we recommend using the supplied positive control primer set for Gapdh-2 as the negative control.

Mouse Positive Control Primer Set Gapdh-2

The positive control primer set is designed to serve as a positive ChIP control when performing chromatin immunoprecipitation with mouse samples. The Mouse Positive Control Primer Set Gapdh-2 amplifies the promoter region of the metabolic gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and has been validated to work with a multitude of ChIP antibodies including: H3K9ac, H3K14ac, H3K4me2, H3K4me3, H4K5ac, H4K8ac, H4K12ac, H4K16ac, Total RNA pol II, and RNA pol II phospho Ser5. If your test ChIP sample is mouse, run triplicate qPCR reactions using the Mouse Positive Control Primer Set Gapdh-2 in addition to the gene-specific qPCR primer pair for the gene of interest. The primer pair is provided at 2.5 µM.

Section G. qPCR Plate Set Up

Following ChIP, qPCR reactions should be performed in triplicate for each primer pair used with the ChIP and Input samples. The DNA Standard curve should be performed in duplicate using the provided Standard Curve Primer Pair.

An example 96-well plate qPCR set up including the DNA Standards and standard curve primers along with six different ChIP DNA samples and four different PCR primer pairs is provided below. The top table shows the layout of the DNA samples and the bottom table shows the layout of the PCR primer pairs. Please adjust your plate layout based on the number of samples and primer sets to be analyzed.

	1	2	3	4	5	6	7	8	9	10	11	12
A	–	Std AM1	Std AM1	Std AM2	Std AM2	Std AM3	Std AM3	–	–	–	–	–
B	ChIP 1	ChIP 1	ChIP 1	ChIP 1	ChIP 1	ChIP 1	ChIP 1	ChIP 1	ChIP 1	ChIP 1	ChIP 1	ChIP 1
C	ChIP 2	ChIP 2	ChIP 2	ChIP 2	ChIP 2	ChIP 2	ChIP 2	ChIP 2	ChIP 2	ChIP 2	ChIP 2	ChIP 2
D	ChIP 3	ChIP 3	ChIP 3	ChIP 3	ChIP 3	ChIP 3	ChIP 3	ChIP 3	ChIP 3	ChIP 3	ChIP 3	ChIP 3
E	ChIP 4	ChIP 4	ChIP 4	ChIP 4	ChIP 4	ChIP 4	ChIP 4	ChIP 4	ChIP 4	ChIP 4	ChIP 4	ChIP 4
F	ChIP 5	ChIP 5	ChIP 5	ChIP 5	ChIP 5	ChIP 5	ChIP 5	ChIP 5	ChIP 5	ChIP 5	ChIP 5	ChIP 5
G	ChIP 6	ChIP 6	ChIP 6	ChIP 6	ChIP 6	ChIP 6	ChIP 6	ChIP 6	ChIP 6	ChIP 6	ChIP 6	ChIP 6
H	Input	Input	Input	Input	Input	Input	Input	Input	Input	Input	Input	Input

Note: Input DNA contains a mixture of Input material associated with all 6 ChIP reactions.

	1	2	3	4	5	6	7	8	9	10	11	12
A	–	Standard Curve PCR Primer Pair						–	–	–	–	–
B	Negative Control Primer Set			Positive Control Primer Set			Gene-specific PCR Primer Set 1			Gene-specific PCR Primer Set 2		
C												
D												
E												
F												
G												
H												

1. Prepare DNA for use in qPCR reactions.
   a. **DNA Standards**: Use 5 µl DNA Standard per qPCR reaction. Also use 5 µl of the provided 2 µM Standard Curve Primer Pair per qPCR reaction.
   b. **ChIP DNA:** The ChIP DNA will need to be diluted prior to use in qPCR due to the re-

duced 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.

c. **Input DNA**: Input DNA should be adjusted to 2.5 ng/µl concentration. Use 5 µl per qPCR reaction.

2. Set up qPCR reactions as follows:

Reagent	20 µl PCR reactions
2X SYBR Green qPCR master mix	10 µl
PCR primer pair (2.5 µM each)	5 µl
DNA sample (ChIP or Input)	5 µl
Total volume	**20 µl**

3. Place the PCR plate in a real time PCR instrument. Using the software for your qPCR machine, assign a value of 3788 to DNA Standard AM1, a value of 378.8 to DNA Standard AM2 and a value of 37.88 to DNA Standard AM3. These numbers represent copy numbers for any unique genomic region of DNA contained in the Standard Curve qPCR reactions. These numbers are used to calculate copy numbers for the genomic regions detected in the test reactions on the qPCR plate.

4. If the gene-specific PCR primer pairs were designed with an optimal annealing temperature of 58°C, follow the amplification conditions listed below. The provided primers have been optimized for use with Bio-Rad SYBR Green qPCR Master Mix (Catalog No. 170-8882). Other master mixes could affect PCR performance and melt curves. If necessary, optimize conditions based on the SYBR Green master mix reagent and PCR instrument used.

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

5. Include and inspect the melt curve based on the protocols recommended by the qPCR instrument manufacturer to ensure that primer pairs amplify only a single product.

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. Using the Analysis Spreadsheet

1. Download Active Motif's ChIP-IT® qPCR Analysis sheet from the Active Motif website at www.activemotif.com/qPCRanalysis. This file is an Excel template that contains formulas to perform all the calculations necessary to normalize sample data according to primer pair efficiency, the amount of chromatin in each reaction and the resuspension volume of the ChIP DNA. The spreadsheet template is designed to accommodate data from multiple 96-well PCR plates in a single analysis.
2. In column A of the template, fill in the primer name on the first line of each box. Then in columns B-I, copy the numbers calculated by the qPCR instrument software for Standard Quantity into the Analysis spreadsheet. In column J, enter the Standard Quantity values for the Input. (See example below)

	A	B	C	D	E	F	G	H	I	J
	Primer Names	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**	**Sample 7**	**Sample 8**	**Input**
10	Negative	Triplicate 1								Triplicate 1
11		Triplicate 2								Triplicate 2
12		Triplicate 3								Triplicate 3
13	Positive	Triplicate 1								Triplicate 1
14		Triplicate 2								Triplicate 2
15		Triplicate 3								Triplicate 3
16	GAPDH	Triplicate 1								Triplicate 1
17		Triplicate 2								Triplicate 2
18		Triplicate 3								Triplicate 3

3. Enter the amount of chromatin used in the ChIP reaction into the template worksheet in cell D2. This value should be recorded in micrograms (µg).
4. Enter the resuspension volume of the ChIP DNA in cell D4. This value should be recorded in microliters (µl). Since the ChIP DNA was diluted in Section G, Step 1b, the final resuspension volume must be adjusted to account for the dilution factor. The equivalent resuspension volume to enter into cell D4 is 600 µl.

   (100 µl total dilution volume) / (6 µl ChIP DNA for dilution) = 16.67 dilution factor

   (16.67 dilution factor) x (36 µl elution volume) = 600 µl equivalent resuspension volume

   Note: This step is critical for accurate quantification of the DNA.

Section I. Data Calculations

The spreadsheet will perform the calculations to normalize the data based on Binding events detected per 1,000 cells as follows. The rationale behind the calculations is explained below.

Standard Curve

a. The qPCR instrument will generate a standard curve based on the known quantities of DNA that were assigned to each DNA standard. The standard curve primer set is assigned a primer efficiency value of 1.

b. Based on the values of the standard curve, the qPCR instrument will assign a Standard Quantity value to each of the ChIP and Input samples. These values are used for the normalization calculations.

Primer Efficiency

a. Primer efficiency ratios for the gene-specific primers and positive and negative control primers are calculated by dividing the average input value by the expected copy number in the input. The average input value is the average of the 3 qPCR values that are generated by the primer set when 12.5 ng of Input DNA are amplified. The expected copy number is calculated assuming 6.6 pg of DNA per cell.

Input DNA: (2.5 ng/µl) * (5 µl per PCR reaction) = 12.5 ng Input DNA amplified

$$\frac{\text{12,500 pg Input DNA}}{\text{6.6 pg DNA per cell}} * \text{2 DNA copies per cell} = \text{3,788 copies}$$

Data Normalization

a. The average of the 3 qPCR values generated by the primer set when each ChIP sample is amplified in triplicate is calculated. This value represents the number of copies in the 5 µl ChIP sample that was amplified.

b. The above value is then multiplied by the resuspension volume and divided by 5 to calculate the number of copies in the entire ChIP reaction.

c. To normalize the values per 1,000 cells, the value is then multiplied by the ratio of 1,000 divided by cell equivalents in the ChIP reaction.

d. Finally, the value is divided by the primer efficiency ratio.

e. The complete formula for normalization is:

$$\frac{\text{average qPCR value} * \left(\frac{\text{resuspension volume}}{5}\right) * \left(\frac{1000}{\text{cell equivalents in ChIP}}\right)}{\text{primer efficiency ratio}}$$

Section J. Data Interpretation

The following recommendations are provided to evaluate the success of the ChIP reactions and the quality of the ChIP DNA. These recommendations are based on the use of the chromatin preparation and immunoprecipitation protocols provided in the ChIP-IT ChIP-Seq Kit. Use the values obtained for Binding events detected per 1,000 cells to perform the data interpretation. See Figure 3 on page 29 for an example of good and poor results.

1. Evaluate the signal of the included Negative Control PCR primer set
    a. Values should be less than 2 Binding events detected per 1,000 cells. Values higher than 2 could indicate a poor quality ChIP reaction. The ChIP reaction should be repeated, especially if the ChIP DNA will be used for ChIP-Seq.
    b. High background levels can sometimes be associated with certain chromatin preparations. If high background persists in ChIP, we recommend making new chromatin.
    c. Some antibodies show signal as high as 1,000 Binding events detected per 1,000 cells. Antibodies that give extremely high signal may have negative control values above 2. In these cases, values above 2 are acceptable.
    d. The negative control primer may actually serve as a positive control for repressive histone marks such as H3K27me3. If testing a repressive histone, we recommend using the included GAPDH primer set as the negative control.
2. Signal strength of the positive control and gene-specific primer sets
    a. Antibodies for high abundance targets, such as histone antibodies, will routinely give signal between 100 and 1,000 Binding events detected per 1,000 cells.
    b. Antibodies for low abundance targets, such as transcription factor antibodies, will routinely give signal between 10 and 100 Binding events detected per 1,000 cells.
    c. ChIP reactions that give signals between 5 and 10 Binding events detected per 1,000 cells can still perform well in downstream applications such as ChIP-Seq, however, fold enrichment over the negative control primer set becomes the critical variable. If fold enrichments are greater than 5, the ChIP reactions may still be of high enough quality to perform ChIP-Seq.
    d. ChIP reactions that give signals of less than 5 Binding events detected per 1,000 cells are usually not of high quality. The ChIP should be repeated or a different antibody should be used.
3. Enrichment levels over the negative control primers
    a. The negative control primers serve as a measure of the background of the ChIP reactions and are an important indicator of the quality of the ChIP DNA. The negative control primers are an alternative to using an IgG or non-specific antibody as a control. The advantage is that the negative control primers measure the background in the ChIP reaction containing the actual antibody of interest while the IgG control is a separate reaction that may not have much relevance to what is occurring in the reaction of interest. Fold enrichment refers to signal from positive control or gene-specific primers

divided by signal from negative control primers.

b. A successful ChIP reaction will have a minimum of 5-fold higher signal with positive control primers as compared to negative control primers.

c. When comparing data from multi-sample ChIP experiments it is best not to express the data as fold enrichment since background levels (measured by the negative control primer sets) can fluctuate across multiple ChIP reactions. When data is expressed as fold enrichment, a two-fold increase in background could be interpreted as a 2-fold change in binding when comparing two samples. Instead, use background levels as a measure of the quality of the experiment. When comparing multiple samples, changes in actual binding can be evaluated by directly comparing the Binding events detected per 1,000 cells.

4. Conversion of Binding events detected per 1,000 cells to % input

a. To convert the calculated Binding events detected per 1,000 cells to the more recognizable "% input" scale, simply divide the values by 1,000.

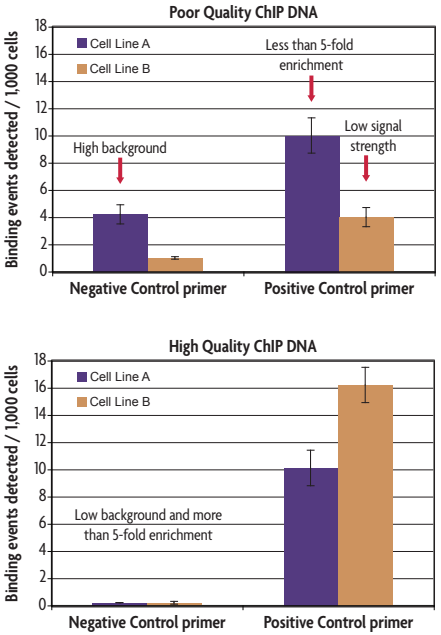


Figure 3: Comparison of qPCR results showing good versus poor enrichment over negative control primers.

Data shows qPCR results analyzed using the ChIP-IT qPCR Analysis strategy 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 set is recommended for use in ChIP-Seq.

Protocols – Sequencing Library Construction

Please refer to pages 7 of the manual for the kit components and additional materials required to complete the library construction.

ChIP DNA from Active Motif's ChIP-IT ChIP-Seq Kit has been extensively validated for ChIP-Seq using the Illumina® sequencing platforms. ChIP DNA can be used to generate single end, paired end or barcoded libraries using the following sequencing library construction protocol. This process involves the preparation of libraries from ChIP DNA by the addition of adapter sequences to the ends of the DNA fragments. Adapter sequences are not included in the ChIP-IT ChIP-Seq Kit and should be obtained from Illumina®. The library is then PCR amplified and validated prior to sequencing on Illumina® platforms such as HiSeq 2000, HiSeq 1000, MiSeq or Genome Analyzer II.

Reagent Information

All library construction reagents are provided ready-to-use. Quick spin the vials before opening.

Enzymes used for library construction are temperature sensitive and should be kept on ice during the entire process and returned to -20°C storage as quickly as possible.

Recommendations

We recommend using 30 µg chromatin for the ChIP reactions (4.5 million cell equivalents), although less chromatin (10 µg) 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, due to the high sensitivity of the chromatin preparation and immunoprecipitation method used in the ChIP-IT ChIP-Seq Kit and the fact that 10 ng is an unrealistic number for a sequence specific DNA binding transcription factor, lower amounts can be used when working with low abundance proteins. 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 (See page 21) to confirm DNA quality rather than quantifying the DNA.

Sequence library yields should be in the range of 0.5-2 micrograms. 30 million sequencing reads is sufficient for most transcription factor and histone modifications and 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 cells or Section D, Step 15 for tissue samples) for Input library generation.

Section K: Perform End Repair

This process is to convert the overhangs into phosphorylated blunt ends.

1. Prepare a fresh 1:5 dilution of the DNA Polymerase I Klenow just before use. Add 0.5 µl DNA Polymerase I Klenow (5 U/µl) to 2 µl sterile dH_2O. Pipet up and down to mix. Discard any unused enzyme dilution.
2. Using 200 µl PCR tubes, prepare end repair reactions on the purified ChIP DNA.

Reagents	**One rxn**
ChIP enriched DNA or Input	30 µl
dH_2O	Up to 40 µl
10X T4 DNA Ligase Buffer	5 µl
dNTP Mix, 10 mM each	2 µl
T4 DNA Polymerase (3 U/µl)	1 µl
Diluted DNA Polymerase I Klenow (5 U/µl) from Step K.1	1 µl
T4 Polynucleotide Kinase (10 U/µl)	1 µl
Total Volume	**50 µl**

3. Incubate the reactions in a thermal cycler for 30 minutes at 20°C.
4. Purify the reactions using QIAquick PCR Purification Kit (QIAGEN Cat. No. 28104) following the suppliers protocol. Elute the DNA using 36 µl Buffer EB.

Section L: Add 'A' Overhang to 3´ Ends of DNA Fragments

This step is designed to add an 'A' base to the 3´ end of the blunt phosphorylated DNA fragments to prepare the DNA fragments for ligation to adapters containing single 'T' base overhang at their 3´ end.

1. Prepare reactions to add an 'A' overhang to the 3´ ends of the DNA fragments.

Reagents	**One rxn**
DNA from Step K.4	34 µl
10X Reaction Buffer AM3	5 µl
1 mM dATP	10 µl
Klenow Fragment (3´-5´ exo-) (5 U/µl)	1 µl
Total Volume	**50 µl**

2. Incubate the reactions in a thermal cycler for 30 minutes at 37°C.

3. Purify the reactions using the MinElute PCR Purification Kit (QIAGEN Cat. No. 28004) following the suppliers protocol. Elute the DNA using 14 µl Buffer EB.

Section M: Ligate Adapters to DNA Fragments

This protocol is for ligation of adapters to the ends of the DNA fragments. Adapters should be obtained from Illumina.

1. Prepare adapters as recommended by the manufacturer.
2. Set up adapter ligation reactions as follows:

Reagents	**One rxn**
DNA from Step L.3	12.5 µl
2X Quick Ligation Buffer	15 µl
5 µM Adapter oligo mix (from Step M.1 above)	1 µl
T4 DNA Ligase (2,000 U/µl)	1.5 µl
Total Volume	**30 µl**

3. Incubate for 20 minutes at room temperature (approximately 24°C).
4. Purify the reactions using MinElute PCR Purification Kit (QIAGEN Cat. No. 28004) according to the suppliers protocol. Elute the DNA using 11 µl Buffer EB.

Section N: Size Select the Library

This process removes excess adapters and selects a size range of DNA templates.

1. Prepare a 2.% agarose TAE gel with 400 ng/ml ethidium bromide.

   Note: Ethidium bromide is a mutagen. Use appropriate precaution when handling.

2. Add 2.5 µl GelPilot QIAGEN loading buffer to each reaction.
3. Run the entire adapter ligated DNA from Step M.4 and 500 ng of 100 bp DNA ladder on a 2% agarose gel for size selection of the DNA library. To avoid potential cross contamination of adjacent wells, leave at least one empty lane between DNA ladder, sample and Input wells.
4. Run the gel at 120V for approximately 1 hour or until the tracking dye is 2/3 down the length of the gel.
5. Visualize the DNA using a Dark Reader transilluminator to avoid exposure to UV light. If using a UV light, work quickly when excising the band to minimize direct exposure to UV. Prolonged exposure to UV light can damage DNA.
6. Using a clean razor blade, excise a gel slice in the 200-300 bp range. We recommend to photograph the gel before and after the band is excised.

7. Use a QIAquick Gel Extraction Kit (QIAGEN Cat. No. 28704) to purify the DNA from the agarose. Make the following protocol modifications to the QIAquick Gel Extraction Kit protocol:
    a. Incubate the gel slice in 3 volumes Buffer QG at 37°C for 30 minutes instead of the suggested 50°C for 10 minutes.
    b. Add the recommended extra 0.5 ml Buffer QG to the QIAquick column.
    c. During the wash steps, incubate 2-5 minutes in Buffer PE before centrifugation.
    d. Elute the DNA in 38 µl Buffer EB.

Section O: Enrich the Adapter-Modified DNA Fragments by PCR

This step is designed to PCR amplify the gel-extracted DNA. Use the appropriate PCR primers for the adapter-modified DNA fragments.

1. Using a 200 µl PCR tube, prepare the following reactions. Add components in the order listed in the table below.

Reagents	**One rxn**
PCR primer mix (12.5 µM each)	2 µl
Gel extracted DNA products from N.7d	36 µl
5X Q5 Reaction Buffer	10 µl
dNTP Mix, 10 mM each	1.5 µl
Q5® High-Fidelity DNA polymerase (2 U/µl)	0.5 µl
Total Volume	**50 µl**

2. Amplify the reactions in a thermal cycler as follows:

    95°C for 90 seconds

    (98°C for 10 seconds, 65°C for 30 seconds and 72°C for 30 seconds) for 18 cycles

    72°C for 5 minutes

    Hold at 4°C

3. Purify using Agencourt® AMPure® XP beads (Beckman Coulter Cat. No. A63880). Add 90 µl AMPure beads to the PCR reaction. Mix well by pipetting up an down ten times. Incubate 10 minutes at room temperature.
4. Use magnetic separation to collect the beads. Carefully remove the supernatant.
5. Add 200 µl 70% ethanol without removing tubes from the magnetic stand. Incubate for 30 seconds at room temperature, then carefully remove the ethanol by pipetting.
6. Repeat the ethanol wash from Step 5 above.
7. Let the beads air dry for 10 minutes at room temperature or until all the ethanol has evaporated.

8. Remove from the magnet and add 21 µl QIAGEN Buffer EB and resuspend the beads completely by pipetting up and down.
9. Use magnetic separation to collect the beads. Carefully transfer 20 µl of supernatant to a new tube (leave 1 µl of eluate behind with the beads). The supernatant contains the amplified library.

Section P: Validate the Library

It is recommended to validate the library prior to submitting samples for sequencing: Below are suggested methods to determine the quantity, quality, size and purity of the library.

a. Determine the final concentration of the library using Nanodrop. Typical yield is approximately 25 - 100 ng/µl
b. Confirm the quality of the library using qPCR with the same primer sets that were used to validate the ChIP DNA. Positive primer pairs should still give high signal as compared to negative primers. Dilute 1-2 µl of DNA to a concentration of 2 ng/µl (dilute to 1 ng/µl if amounts are limiting).
c. Check the library using agarose gel electrophoresis with 50 -100 ng DNA on a 2% agarose TAE gel to confirm the library preparation is depleted of free adapters. The ability to visualize a shift in the library size is dependent on the size and type of adapters used.
d. Libraries are now ready for sequencing.

Section Q: 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/ or the UCSC Genome Browser at http://genome.cse.ucsc.edu/cgi-bin/hgPcr).
- Primers that dimerize should be avoided, as they will be bound by SYBR Green, which will compromise accurate quantitation. You can test your primers for self-complementarity and secondary structure at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi.
- 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

We strongly recommend the use of Active Motif's ChIP-IT qPCR Analysis Kit (Cat. No. 53029) to confirm the quality of the ChIP-enriched DNA. The recommendations provided in Section J. Data Interpretation only apply if the ChIP-IT qPCR Analysis Kit was used. 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 36 µl and used 5 µl in the qPCR reaction the formula is 36/5 = 7.2). Then, multiply the average qPCR quantity by this number (*e.g.* qPCR quantity in ng x 7.2).
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 R. 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.
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 sodium 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.
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.

Problem/question	Recommendation
Poor or no enrichment with target antibody.	Too little chromatin. Generally, we recommend using 10 - 30 µg of chromatin per ChIP reaction. 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 IgG, 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.
Do I need to run a standard curve for every primer pair I include on my qPCR plate?	No, the included standard curve and primer provide a mechanism by which the efficiencies of all other primers can be determined. The key is knowing how much DNA is in the standard curve and running qPCR with other primers using input DNA of a known concentration.
Do I have to perform triplicate qPCR reactions?	We recommend triplicate qPCR reactions for ChIP DNA and Input DNA in order to provide more accurate data. However, the ChIP-IT qPCR Analysis Spreadsheet is able to calculate accordingly if only single or duplicate values are entered.
When using the provided Negative Control Primer Set I saw a positive result.	The included Negative Control Primer Sets are intended to be as universal as possible and were designed to serve as negative controls for all activating histone marks and transcription factors. However, these primer sets can give positive results for repressive histone marks such as H3K27me3. If evaluating repressive histone marks, we recommend using the supplied Positive Control Primer Set in place of the negative control primer set to establish a negative result.
Why don't you recommend using an IgG control antibody?	In testing this method across thousands of samples we have found that using primers that target an unbound region is a more stringent control than IgG. The negative control primers serve as an internal control for the ChIP reaction as they are tested using the ChIP DNA generated from the antibody of interest, while IgG is a completely separate reaction using an antibody that may give different non-specific binding than the experimental reaction of interest.

Problem/question	Recommendation
Why am I detecting negative control primer Binding events detected per 1,000 cells values greater than 2?	In most cases this means that there is high non-specific binding in your reaction. Sometimes this can be rectified by repeating the ChIP, but usually it is associated with the chromatin preparation (for reasons that are unknown) and we recommend preparing new chromatin.
	Sometimes antibodies give very high signals and therefore can have correspondingly higher signal from the negative control primers. If you are achieving enrichment levels of the positive control primer set of 5-fold above the negative control primers then the higher background levels are not of critical importance. However, if the downstream application is for ChIP-Seq, then low background levels are critical. For ChIP-Seq you should strive to obtain less than 2 Binding event detected per 1,000 cells for negative control primers.
Can the Binding events detected per 1,000 cells data be converted to percent of input?	Yes, simply divide the output values by 1,000 to achieve percent of input.
Can I use columns to purify my enriched adapter-modified DNA rather than AMPure® XP beads?	We recommend the use of Agencourt® AMPure® XP Beads (Beckman Coulter Cat No. A63880) for purification of the adapter-modified DNA rather than column purification. With column purification we noticed a higher degree of free adapter in the flow through. The AMPure bead purification provides better purity of the library.

Section S. Related Products

ChIP-IT® Kits	Format	Catalog No.
ChIP-IT® High Sensitivity	16 rxns	53040
ChIP-IT® Express	25 rxns	53008
ChIP-IT® Express Enzymatic	25 rxns	53009
ChIP-IT® Express Shearing Kit	10 rxns	53032
ChIP-IT® Express Enzymatic Shearing Kit	10 rxns	53035
ChIP-IT® qPCR Analysis Kit	10 rxns	53029
ChIP-IT® ChIP-Seq	10 libraries	53041
ChIP-IT® FFPE	16 rxns	53045
ChIP-IT® FFPE Chromatin Preparation Kit	5 rxns	53030
ChIP-IT® Express HT	96 rxns	53018
Re-ChIP-IT®	25 rxns	53016
RNA ChIP-IT®	25 rxns	53024
Chromatin IP DNA Purification Kit	50 rxns	58002
EpiShear™ Probe Sonicator	110 V	53051
EpiShear™ Cooled Sonication Platform, 1.5 ml	1 platform	53080
ChIP-IT® Protein G Magnetic Beads	25 rxns	53014
Protein G Agarose Columns	30 rxns	53039
Siliconized Tubes, 1.7 ml	25 tubes	53036
ChIP-IT® Control qPCR Kit – Human	5 rxns	53026
ChIP-IT® Control qPCR Kit – Mouse	5 rxns	53027
ChIP-IT® Control qPCR Kit – Rat	5 rxns	53028
ChIP-IT® Control Kit – Human	5 rxns	53010
ChIP-IT® Control Kit – Mouse	5 rxns	53011
ChIP-IT® Control Kit – Rat	5 rxns	53012
Ready-to-ChIP HeLa Chromatin	10 rxns	53015
Ready-to-ChIP Hep G2 Chromatin	10 rxns	53019
Ready-to-ChIP K-562 Chromatin	10 rxns	53020
Ready-to-ChIP NIH/3T3 Chromatin	10 rxns	53021
Bridging Antibody for Mouse IgG	500 µg	53017
Dounce Homogenizer	1 ml	40401
Dounce Homogenizer	15 ml	40415

ChIP-validated Antibodies

For an up-to-date list of over 125 ChIP-validated antibodies, please visit **www.activemotif.com/chipabs**.

Whole Genome Amplification	Format	Catalog No.
GenoMatrix™ Whole Genome Amplification Kit	1 kit	58001

Co-Immunoprecipitation	Format	Catalog No.
Nuclear Complex Co-IP Kit	50 rxns	54001
Universal Magnetic Co-IP Kit	25 rxns	54002

Modified Histones Array	Format	Catalog No.
MODified™ Histone Peptide Array	1 array	13001

Histone Modification FP Binding Assay	Format	Catalog No.
HiLite™ Histone H3 Methyl-Lys9 / Lys27 FP Binding Assay	1 kit	57001

Histone ELISAs	Format	Catalog No.
Histone H3 monomethyl Lys4 ELISA	1 x 96 rxns	53101
Histone H3 dimethyl Lys4 ELISA	1 x 96 rxns	53112
Histone H3 trimethyl Lys4 ELISA	1 x 96 rxns	53113
Histone H3 acetyl Lys9 ELISA	1 x 96 rxns	53114
Histone H3 dimethyl Lys9 ELISA	1 x 96 rxns	53108
Histone H3 trimethyl Lys9 ELISA	1 x 96 rxns	53109
Histone H3 phospho Ser10 ELISA	1 x 96 rxns	53111
Histone H3 acetyl Lys14 ELISA	1 x 96 rxns	53115
Histone H3 monomethyl Lys27 ELISA	1 x 96 rxns	53104
Histone H3 trimethyl Lys27 ELISA	1 x 96 rxns	53106
Histone H3 phospho Ser28 ELISA	1 x 96 rxns	53100

Histone Purification & Chromatin Assembly	Format	Catalog No.
Histone Purification Kit	10 rxns	40025
Histone Purification Mini Kit	10 rxns	40026
Chromatin Assembly Kit	10 rxns	53500
HeLa Core Histones	36 µg	53501

Recombinant Methylated, Acetylated and Phosphorylated Histone Proteins

For an up-to-date list of Recombinant Histone Proteins, please visit **www.activemotif.com/recombhis**.

Histone Acetyltransferase and Deacetylase Activity	Format	Catalog No.
HAT Assay Kit (Fluorescent)	1 x 96 rxns	56100
Recombinant p300 protein, catalytic domain	5 µg	31205
Recombinant GCN5 protein, active	5 µg	31204
HDAC Assay Kit (Fluorescent)	1 x 96 rxns	56200
HDAC Assay Kit (Colorimetric)	1 x 96 rxns	56210

Histone Demethylase Activity	Format	Catalog No.
Histone Demethylase Assay (Fluorescent)	48 rxns	53200

DNA Methylation	Format	Catalog No.
hMeDIP	10 rxns	55010
MeDIP	10 rxns	55009
MethylDetector™	50 rxns	55001
MethylCollector™	25 rxns	55002
MethylCollector™ Ultra	30 rxns	55005
HypoMethylCollector™	30 rxns	55004
Hydroxymethyl Collector™	25 rxns	55013
DNMT Activity / Inhibition Assay	96 rxns	55006
Methylated DNA Standard Kit	3 x 2.5 µg	55008
Fully Methylated Jurkat DNA	10 µg	55003
Jurkat genomic DNA	10 µg	55007

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

Active Motif North America
1914 Palomar Oaks Way, Suite 150
Carlsbad, CA 92008
USA
Toll Free: 877 222 9543
Telephone: 760 431 1263
Fax: 760 431 1351
E-mail: tech_service@activemotif.com

Active Motif Europe
Avenue Reine Astrid, 92
B-1310 La Hulpe, Belgium
UK Free Phone: 0800 169 31 47
France Free Phone: 0800 90 99 79
Germany Free Phone: 0800 181 99 10
Telephone: +32 (0)2 653 0001
Fax: +32 (0)2 653 0050
E-mail: eurotech@activemotif.com

Active Motif Japan
Azuma Bldg, 7th Floor
2-21 Ageba-Cho, Shinjuku-Ku
Tokyo, 162-0824, Japan
Telephone: +81 3 5225 3638
Fax: +81 3 5261 8733
E-mail: japantech@activemotif.com

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