

Tag-CHIP-IT®

(version A2)

Catalog No. 53022

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

Active Motif China

787 Kangqiao Road

Building 10, Suite 202, Pudong District

Shanghai, 201315, China

Telephone: (86)-21-20926090

Hotline: 400-018-8123

Copyright 2017 Active Motif, Inc.

Information in this manual is subject to change without notice and does not constitute a commitment on the part of Active Motif, Inc. It is supplied on an “as is” basis without any warranty of any kind, either explicit or implied. Information may be changed or updated in this manual at any time.

This documentation may not be copied, transferred, reproduced, disclosed, or duplicated, in whole or in part, without the prior written consent of Active Motif, Inc. This documentation is proprietary information and protected by the copyright laws of the United States and international treaties.

The manufacturer of this documentation is Active Motif, Inc.

© 2017 Active Motif, Inc., 1914 Palomar Oaks Way, Suite 150; Carlsbad, CA 92008. All rights reserved.

All trademarks, trade names, service marks or logos referenced herein belong to their respective companies.

TABLE OF CONTENTS	Page
Overview	1
Flow Chart of Process	2
Kit Performance and Benefits	3
Protocol Overview and Time Table	4
Kit Components and Storage	
Tag-ChIP-IT® Kit	5
Additional Materials Required	6
Protocols – Experimental Set Up	
Chromatin Preparation	7
Buffer Preparation	7
Recommendations	9
Protocols – Preparation of Sheared Chromatin	
Section A. Cell Fixation Starting with Cultured Cells	10
Section B. Chromatin Sonication of Cultured Cells	11
Protocols – Chromatin Immunoprecipitation	
Section C. Immunoprecipitation	14
Protocols – ChIP DNA Purification	
Section D. Reversal of Cross-links and DNA Purification	16
Protocols – ChIP DNA Analysis	
Section E. Quantitative PCR (qPCR)	17
Section F. ChIP-Seq	18
Appendix	
Section G. Prepare AM-tag Fusion Protein Construct	20
Section H. Transfection of Cells with AM-tag Construct	20
Section I. qPCR Primer Design and Data Analysis	22
Section J. Troubleshooting Guide	24
Section K. Related Products	26
Technical Services	28

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.

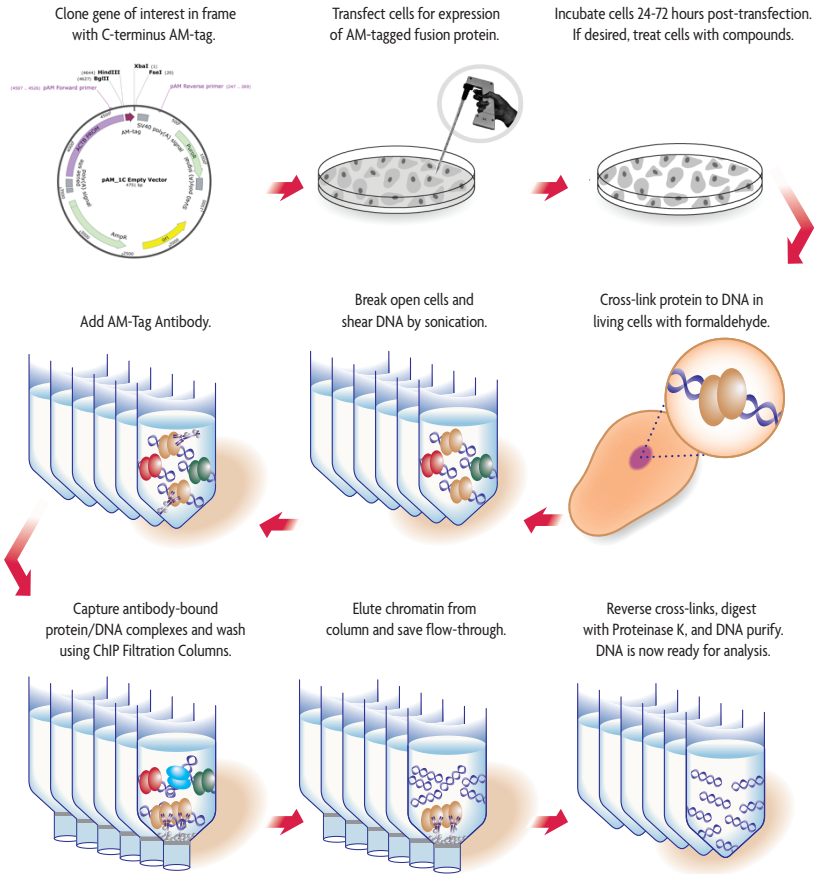
However, ChIP can be technically demanding. The method requires high-quality antibodies to recognize the fixed, target-bound proteins of interest. For many transcription factors, suitable ChIP-seq validated antibodies are not available or have low binding affinity. Additionally, ChIP antibodies are often unable to distinguish between protein isoforms, thereby making it difficult to study the regulatory effects of sequence variants and truncations.

Active Motif's Tag-ChIP-IT® Kit is designed to overcome these obstacles enabling chromatin immunoprecipitation of transcription factors, sequence variants and truncated proteins that have been expressed using Active Motif's unique AM-tag. The AM-tag is specifically designed to minimize cross-reactivity with mammalian samples, thereby reducing background signal. Another benefit of the AM-tag is that the sequence is unstructured which allows the tag to protrude from the protein of interest for maximum exposure during the immunoprecipitation. This increases enrichment efficiency by allowing even low abundance transcription factors to be detected. Simply clone the AM-tag in frame with the C-terminus of your protein of interest in the expression vector of your choice. Following transfection and expression of your tagged protein, the Tag-ChIP-IT Kit can be used to isolate chromatin and perform immunoprecipitation using an antibody directed against the AM-tag.

The Tag-ChIP-IT Kit contains sufficient reagents to perform 16 chromatin preparation and immunoprecipitation reactions. A chromatin preparation is defined as one 100 mm cell culture dish. We recommend the use of Active Motif's ChIP-IT® qPCR Analysis Kit in combination with the Tag-ChIP-IT Kit for complete data analysis. Active Motif also offers the pAM_IC_JunD vector for use as a control. To learn about available qPCR primer sets or Active Motif's EpiShear™ sonication devices, please visit our website at www.activemotif.com/chip.

product	format	catalog no.
Tag-ChIP-IT®	16 rxns	53022
pAM_IC Empty Vector	20 µg	53023
pAM_IC_JunD Vector	50 µg	53044
ChIP-IT® qPCR Analysis Kit	10 rxns	53029

Flow Chart of Process



Flow Chart of the Tag-ChIP-IT Assay.

Prior to using the Tag-ChIP-IT Kit, the protein of interest is cloned into an expression vector containing the C-terminal AM-tag, such as Active Motif's pAM_1C Empty Vector (Catalog No. 53023). Following sequence verification of the cloned insert, cells are transfected for expression of the AM-tagged fusion protein. Once transfection and protein expression conditions have been optimized, chromatin is prepared from the transfected cells using the Tag-ChIP-IT Kit. 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 AM-tag. 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

Tag-ChIP-IT Advantages:

- Ideal for proteins lacking CHIP-validated antibodies or antibodies with low binding affinities
- Study the effects of sequence variants, mutations or truncations on gene regulation
- High exposure of AM-tag allows for efficient enrichment of low abundance proteins
- AM-tag has little to no cross-reactivity with mammalian systems while 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
- Reproducible results with proven performance in both qPCR and ChIP-Seq analysis

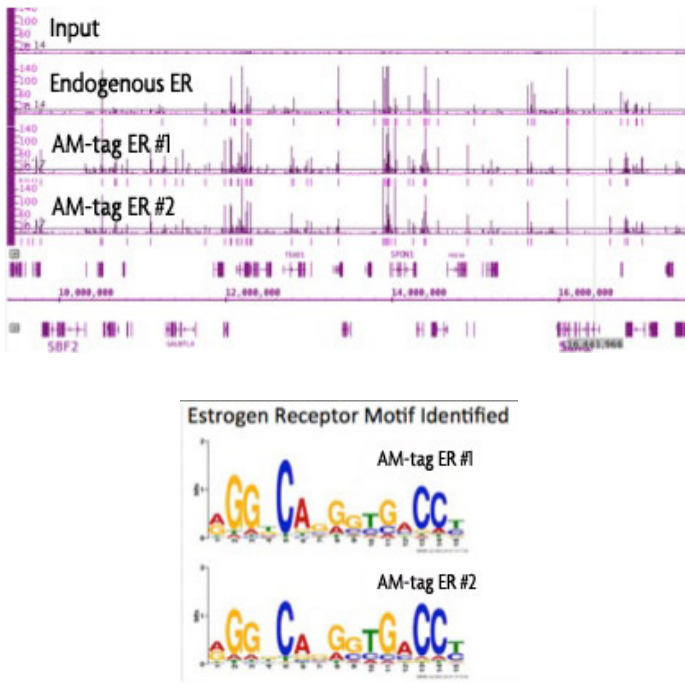


Figure 1: Tag-ChIP-IT is used to identify Estrogen Receptor binding motifs.

Estrogen Receptor (ER) cDNA was cloned into pAM_IC empty vector (Catalog No. 53023) and sequence verified. Transient transfections were performed into Ishikawa cells. Cells were induced with estradiol (E_2) and chromatin was harvested according to the instructions in the Tag-ChIP-IT Kit. The AM-Tag antibody was used to immunoprecipitate the cross-linked AM-tag-ER fusion protein. Following reversal of cross-links, enriched DNA was submitted for next generation sequencing. Data was compared to published ChIP-Seq results using an anti-ER antibody in the same cell line and induction conditions. ChIP-Seq data shows the same ER peak profile with the AM-tag immunoprecipitation as endogenous ER. The binding sites detected with the AM-tag samples were further evaluated for binding motifs. Results show that the estrogen receptor motif was identified in both Tag-ChIP-IT samples.

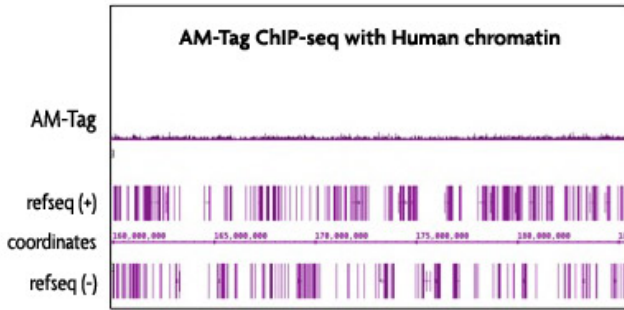


Figure 2: Specificity of AM-Tag Antibody.

ChIP-Seq was performed with the AM-Tag Antibody on chromatin prepared from Human samples. The sequencing results show an absence of DNA binding peaks which indicates there is little to no cross-reactivity of the AM-tag with human chromatin, thereby eliminating background signal in the Tag-ChIP-IT assay.

Tag-ChIP-IT Protocol Overview and Time Table

	Required Time
Cell Fixation and Lysis	1.5 hours
Chromatin Sonication	15 minutes per sample
Assessment of Chromatin Size	4.5 hours
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

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
AM-Tag Antibody (rAb)	160 µl	-20°C
RNase A (10 µg/µl)	40 µl	-20°C
Proteinase K (10 µg/µl)	200 µl	-20°C
Blocker	100 µl	-20°C
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
10X PBS	120 ml	-20°C
Fixation Buffer	2 x 1.5 ml	4°C
Protein G Agarose Beads*	500 µl	4°C
TE pH 8.0	2 x 1.5 ml	RT
Detergent	25 ml	RT
5 M NaCl	400 µl	RT
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
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. Store beads at 4°C.

**Requires the addition of ethanol before use.

Additional materials required

- Stable cells or transiently transfected cell line expressing the AM-tag fusion protein construct. See Appendix, Sections G–H for details on vector design and transfection.
- 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. Do not use paraformaldehyde.
- 100% ethanol (absolute)
- 70% ethanol
- DNase-free H₂O
- 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
- 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)

Protocols – Experimental Set Up

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Chromatin Preparation

Tag-ChIP-IT requires 20-30 μg chromatin isolated from stable cells or transiently transfected cells expressing the AM-tag fusion protein. For details on preparing cells for use in Tag-ChIP-IT, please refer to Appendix Sections G–H on page 20.

Chromatin should be prepared 24-72 hours post-transfection, or when the cells reach 70-80% confluency. Refer to the table below to scale buffer volumes for chromatin preparation.

	60 mm dish	100 mm dish	150 mm dish
Seeding Density	0.8×10^6	2.2×10^6	5.0×10^6
Cells at 70-80% Confluency*	2.4×10^6	6.6×10^6	15.0×10^6
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

Buffer Preparation

Complete Cell Fixation Solution

Buffer should be prepared fresh before each experiment. For every 10 ml of cell growth medium used, prepare 1.25 ml of Complete Cell Fixation Solution by adding 90 μl Fixation Buffer to 785 μl sterile water in a 15 ml conical tube. Using appropriate precautions (*i.e.* safety glasses, gloves and lab coat), add 375 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.

Stop Solution

Is provided ready to use. Use 1/20 media volume per cell culture plate.

PBS Wash Buffer

Prepare 12 ml PBS Wash Buffer for every 100 mm dish. To a 15 ml conical tube add 10.2 ml sterile water, 1.2 ml 10X PBS and 0.6 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.

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 4°C.**

AM-Tag Antibody (rAb)

We recommend using 10 µg AM-Tag antibody per ChIP reaction. The AM-Tag antibody is provided at a concentration of 1 µg/µl, so you will need to use 10 µl per immunoprecipitation.

Recommendations

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 Tag-ChIP-IT 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 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 20-30 µg chromatin per IP reaction. If ChIP-Seq will be performed following enrichment, please read the recommendations listed in Section F on page 18 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.

Protocols – Preparation of Sheared Chromatin

Section A: Cell Fixation Starting with Cultured Cells

This protocol describes cell fixation and chromatin preparation from one 100 mm dish (approximately 6.6×10^6 cells) containing stable cells or transiently transfected cells expressing the AM-tag fusion protein. We recommend using 10 ml growth medium per 100 mm dish. Please refer to page 7 for information on scaling the protocol for use with other amounts of cells.

1. Follow the recommendations outlined in Appendix Sections G-H for the cloning and transfection of your protein of interest expressing the AM-tag. We recommend using untransfected cells to provide an estimate of background. Prepare chromatin 24-72 hours post-transfection, or when the cells reach 70-80% confluency.
2. Freshly prepare Complete Cell Fixation Solution for each 100 mm dish. The volumes listed below are enough to process one 100 mm dish. 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. 10 ml growth medium would get 1 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. 10 ml growth medium would get 550 μ l 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 5 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 5 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 μ l PIC and 5 μ l 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 μ l 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 μ l ChIP Buffer supplemented with 5 μ l PIC and 5 μ l 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 μ l volume.

The ChIP 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 μ l of each chromatin preparation into a 250 μ l 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 μ l 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 μ l chromatin preparation from Step 3 above, add 175 μ l TE pH 8.0 and 1 μ l RNase A. Cap the PCR tubes and vortex to mix
6. Incubate in a thermocycler at 37°C for 30 minutes.
7. Add 2 μ l 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 μ l Precipitation Buffer, 2 μ l Carrier and 750 μ l 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 μ l 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 μ l 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 μ l 5M NaCl to 18 μ l sterile water. Vortex to mix.
 - b. Transfer 500 ng of Input DNA to a 250 μ l PCR tube and add 1 μ l 500 mM NaCl. Adjust the final volume to 10 μ l 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 Tag-ChIP-IT 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.

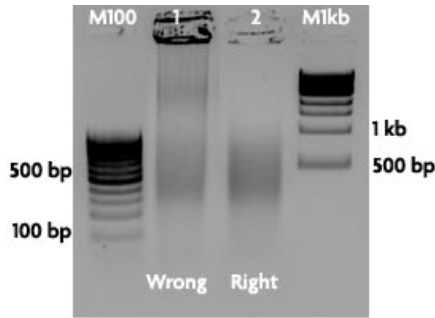


Figure 3: Validation of chromatin shearing efficiency following reversal of cross-links at 80°C for 2 hours.

Chromatin preparations of MCF-7 cells were fixed and sonicated using the EpiShear™ Probe Sonicator and EpiShear™ Cooled Sonication Platform from Active Motif. 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.

Protocols – Chromatin Immunoprecipitation

Section C. Immunoprecipitation

Successful chromatin immunoprecipitation depends on the efficiency of the transfection, the expression levels of the AM-tag fusion protein of interest and the ability of the tagged protein to compete against endogenous protein for chromatin binding sites. It is recommended to optimize the transfection and expression of tagged protein prior to performing the immunoprecipitation.

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 20-30 µg chromatin per ChIP reaction.
3. In a 1.5 ml microcentrifuge tube prepare the AM-Tag Antibody. To the tube add 5 µl Blocker and 10 µg AM-Tag antibody. Incubate Antibody/Blocker mix for 1 minute at room temperature and then add to the ChIP reactions.

Table 1

Reagent	1 reaction
Sheared Chromatin (20 - 30 µg)	X µl
ChIP Buffer	adjust up to 200 µl
Protease Inhibitor Cocktail (PIC)	5 µl
Antibody/Blocker mix (from Step 3)	15 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 μ l 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 4 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 μ l 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 μ l 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 μ l 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 μ l 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 μ l 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 μ l volume) contains the ChIP DNA. Proceed to Section F: DNA purification.



Figure 4: 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 D. Reversal of Cross-links and DNA Purification

1. Transfer each eluted ChIP DNA to a 250 μ l PCR tube and add 2 μ l 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 μ l) DNA Purification Binding Buffer to each tube and vortex to mix. Adjust the pH with 5 μ l 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 24 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 μ l 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 μ l of DNA Purification Elution Buffer (AM #103498) to the center of the column matrix and incubate for 5 minutes at room temperature. Spin at 14,000 rpm for 1 minute in a microcentrifuge. Add an additional 100 μ l DNA Purification Elution Buffer to the column and incubate for 5 minutes at room temperature. Spin at 14,000 rpm for 1 minute in a microcentrifuge. Total elution volume is 200 μ l.
 - b. **For ChIP-Seq or ChIP-chip analysis:** add 36 μ l of 37°C DNA Purification Elution Buffer (AM #103498) to the center of the column matrix and incubate for 5 minutes 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.

Protocols – ChIP DNA Analysis

Section E. 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.

Section F. ChIP-Seq

Genome-wide analysis with Next Generation sequencing 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. Please select an appropriate library preparation method compatible with the sequencing apparatus 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 protein
- Library generation usually requires 10 ng of ChIP enriched DNA. However, due to the low background level with the Tag-ChIP-IT Kit 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 factors
- 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) for library generation.

B. ChIP-Seq Requirements

It is commonly recommended that library generation of ChIP DNA requires 10 ng of ChIP enriched DNA. However, 10 ng is an unrealistic number for a sequence specific DNA binding transcription factor. Using the Tag-ChIP-IT Kit, we have 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 E on page 17 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 5). If the qPCR enrichments are satisfactory then the remaining 30 µl of ChIP DNA can be used for library generation.

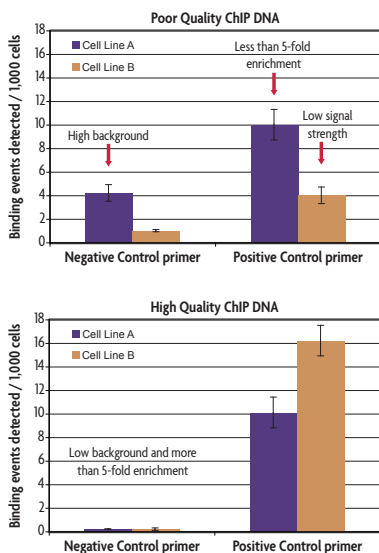


Figure 5: Comparison of qPCR results showing good versus poor enrichment over negative control primers.

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 TAG-ChIP-IT Kit has been validated for ChIP-Seq using Active Motif's Next Gen DNA Library and Next Gen Indexing Kits (Catalog Nos. 53216 & 53264).

Appendix

Section G: Prepare the AM-tag Fusion Protein Construct

Active Motif's Tag-ChIP-IT Kit requires stable cells or transiently transfected cells expressing the AM-tag. The following outlines the design and preparation of the AM-tag vector.

1. Using Active Motif's pAM_IC Empty Vector (Catalog No. 53023), design a fusion protein construct containing the AM-tag in-frame with the C-terminus of the protein of interest. We recommend the use of directional cloning methods such as InFusion® (Clontech) or Gibson Assembly® (New England Biolabs). Follow the manufacturer's recommendations for cloning.

Alternatively, the AM-tag sequence can be cloned into the expression vector of choice for your protein of interest. Ensure that the open reading frame of interest begins with the ATG start codon, that the AM-tag remains in-frame with the C-terminus and that a stop codon is present. We do not recommend the use of the AM-tag at the amino-terminus of the protein as this may significantly reduce expression efficiency. If you require a N-terminal tag, please contact Active Motif's technical support.

AM-tag: 5' – TGCCAAGATCCTCAACGCAAAGGCAACGTGATACTCTCTCAGGCTTACGGGTGC-CAAGATCCTCAACGCAAAGGCAACGTGATACTCTCTCAGGCTTAC – 3'

2. Perform transformations of the AM-tag fusion protein construct into competent *E.coli*. We recommend the use of Active Motif's Rapid Trans TAMI Competent cells (Catalog No. 11096). Use the appropriate antibiotic selection for the cloning vector chosen (*e.g.* pAM_IC contains the ampicillin resistance gene).
3. Select 2-4 colonies for each construct and isolate the plasmid DNA. Sequence validate each plasmid to confirm the correct open reading frame sequence. We suggest preparing a glycerol stock for each candidate construct.
5. Determine the amount of DNA needed for transfection. If necessary, prepare additional DNA. Ensure that the DNA is free of nucleases or other contaminants prior to transfection. Quantify the DNA.

Section H: Transfection of Cells with AM-tag Construct

Following sequence validation of the AM-tag cloning vector, cells can be transiently transfected or selected to make stable cells expressing the AM-tagged protein of interest. Chromatin will be prepared from these cells for analysis in the Tag-ChIP-IT Kit (Catalog No. 53022). Successful chromatin immunoprecipitation depends on the efficiency of the transfection and the expression levels of the AM-tag fusion protein of interest. It is recommended to optimize the transfection and expression of tagged protein prior to performing the chromatin immunoprecipitation.

Once transfection conditions have been optimized, 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 control ChIP reactions in your calculations. We recommend using untransfected cells (or a non-expressing cell line) as an estimate of the

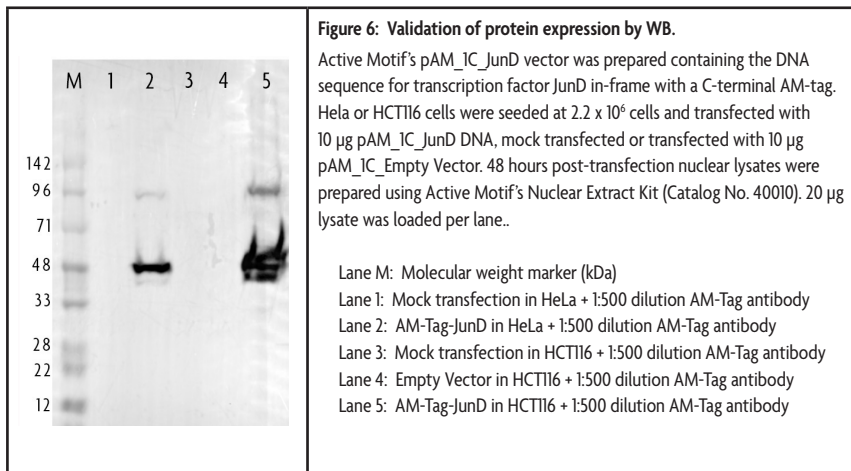
background. It is important to transfect enough cells to yield the necessary 20-30 µg of chromatin per immunoprecipitation reaction.

We recommend using FuGENE® HD Transfection Reagent (Catalog No. 32042) with the pAM_IC vector at a ratio of 3:1 (µl FuGENE : µg DNA vector). Optimization may be required for each cell line and construct tested. Below are some general guidelines.

1. In a 100 mm dish, seed 2.2×10^6 cells per plate in the appropriate growth medium. Incubate in a humidified incubator for 24 hours.

(Optional) Prepare transfection reactions to verify expression of the AM-tagged fusion protein. We suggest performing a time course experiment to evaluate tagged-protein expression levels at 24, 48 and 72 hours post-transfection to identify the optimal harvest time point for the protein of interest. Cell lysates can be prepared from these transfection reactions for analysis by Western blot. Use the AbFlex™ AM-Tag antibody (Catalog No. 91111) at a 1:500 dilution for detection of the tagged protein as compared to untransfected cells (Figure 6).

2. Prepare a separate microcentrifuge tube for each transfection reaction. Add 10 µg AM-tag construct to Opti-MEM media in a final volume of 550 µl.
3. Add 30 µl FuGENE HD Transfection Reagent drop wise directly to the media/DNA mixture. Do not allow FuGENE to come directly into contact with the plastic from the tube. Mix the solution by pipetting up and down and incubate at room temperature for 15-30 minutes.
4. Add 580 µl of the media/DNA/FuGENE mixture drop wise to each 100 mm dish. Gently swirl dish or incubate on a shaker at 100 rpm for 2 minutes to evenly distribute transfection mixture.
5. Return dish to humidified incubator for 24-72 hours before proceeding with the chromatin preparation. If desired, treat with compounds.
6. Follow the protocol on page 7 of the manual to prepare chromatin for use in Tag ChIP-IT.



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/> or the UCSC Genome Browser at <http://genome.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

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) 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 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) 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 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 μ l and used 5 μ l 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 \times 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 μ g 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: <ol style="list-style-type: none"> 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. 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
	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 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.
	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
<p>Poor or no enrichment with target antibody.</p>	<p>Too little chromatin. Generally, we recommend using 20 - 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.</p>
	<p>Inefficient transfection Optimize transfection conditions for your expression construct of choice.</p>
	<p>Low protein expression levels. Confirm expression of AM-tagged protein by Western blot with AM-Tag antibody (Catalog No. 61677). If necessary, perform a time course experiment to determine the optimal time post-transfection to harvest chromatin.</p>
	<p>Perform a control transfection using Active Motif's pAM_IC_JunD vector (Catalog No. 53044) under the conditions described on the certificate of analysis. This control transfection and chromatin preparation can be used to validate the Tag-ChIP-IT technology. Optimization may be needed for each expression construct and cell line tested.</p>
	<p>Problems with PCR. Confirm the amplified sequence for the positive control primer set is bound by the antibody target. Identify other binding sites.</p>
<p>No PCR products for the ChIP'd samples (but the Input DNA yields the correct PCR product)</p>	<p>Increase the amount of chromatin used in the ChIP reaction, the amount of antibody used, or both.</p>
<p>No PCR products with real-time PCR</p>	<p>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.</p>
	<p>No ethanol in DNA Purification Wash Buffer. Make sure that ethanol has been added to the DNA Purification Wash Buffer prior to first use.</p>

Section K. Related Products

ChIP-IT® Kits	Format	Catalog No.
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® High Sensitivity	16 rxns	53040
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
UnMethylCollector™	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
E-mail: tech_service@activemotif.com

Toll Free: 877 222 9543
Direct: 760 431 1263
Fax: 760 431 1351

Active Motif Europe

Avenue Reine Astrid, 92
B-1310 La Hulpe, Belgium
E-mail: eurotech@activemotif.com
Direct: +32 (0)2 653 0001

Germany Free Phone: 0800 181 99 10
France Free Phone: 0800 90 99 79
UK Free Phone: 0800 169 31 47
Fax: +32 (0)2 653 0050

Active Motif Japan

Azuma Bldg, 7th Floor
2-21 Ageba-Cho, Shinjuku-Ku
Tokyo, 162-0824, Japan

Direct: +81 3 5225 3638
Fax: +81 3 5261 8733
E-mail: japantech@activemotif.com

Active Motif China

787 Kangqiao Road
Building 10, Suite 202
Pudong District
Shanghai, 201315, China

Direct: (86)-21-20926090
Hotline: 400-018-8123
E-mail: techchina@activemotif.com

Visit Active Motif on the worldwide web at <http://www.activemotif.com>

At this site:

- Read about who we are, where we are, and what we do
- Review data supporting our products and the latest updates
- Enter your name into our mailing list to receive our catalog, *MotifVations* newsletter and notification of our upcoming products
- Share your ideas and results with us
- View our job opportunities

Don't forget to bookmark our site for easy reference! The minimum recommended number of cells that should be used for the preparation of chromatin is 100,000 cells.