Re-ChIP-IT®

Magnetic Chromatin Re-Immunoprecipitation Kit

Catalog No. 53016

(version A4)

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Overview

Chromatin Immunoprecipitation (ChIP) is a powerful tool for studying protein/DNA interactions[1, 2]. In this method, intact cells are fixed using formaldehyde, which cross-links and preserves protein/DNA interactions. The DNA is then sheared into small, uniform fragments using either sonication or enzymatic digestion and specific protein/DNA complexes are immunoprecipitated using an antibody directed against the DNA-binding protein of interest. Following immunoprecipitation, cross-linking is reversed, the proteins are removed by treatment with Proteinase K and the DNA is recovered. The DNA is then analyzed to determine which DNA fragments were bound by the protein of interest (see Figure 1 on page 2).

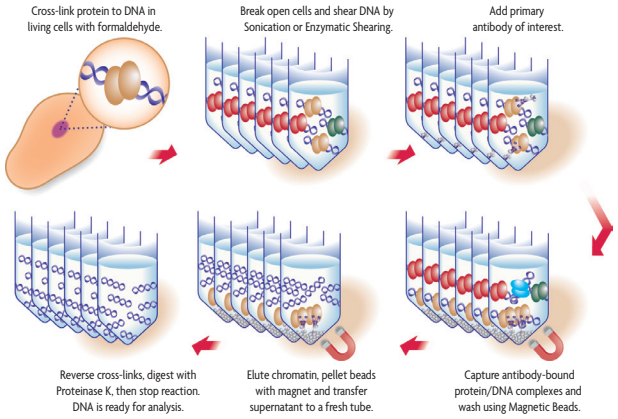
ChIP is extremely useful for studying chromatin biology and transcriptional regulation because it enables the localization of chromatin proteins, modified histones and transcription factors that are bound to specific DNA regions in specific cells. Furthermore, because the protein/DNA interactions are fixed while in an endogenous, chromosomal context, ChIP results reflect the influence of chromosomal topology and the effects of cellular regulatory proteins[3, 4, 5].

When performing chromatin immunoprecipitation (ChIP) experiments, it is often useful to prove that two different proteins or histone modifications are present at the same site in the genome. Or, you may want to determine if a protein coincides with a specific histone modification at the same regulatory element. Re-ChIP (aka Sequential ChIP, Chromatin Re-IP and ChIP Re-ChIP) is a relatively new technique that enables sequential chromatin immunoprecipitations to be performed using two different antibodies so that you can assay for the simultaneous presence of two proteins or distinct histone modifications at the same genomic region of interest[6, 7].

The Re-ChIP-IT Kit enables you to identify the simultaneous binding of two transcription factors or two histone modifications on a DNA fragment of interest. Immunoprecipitated chromatin is removed from the magnetic beads with a special buffer that prevents the majority of the first antibody from participating in the second IP reaction. The chromatin is then desalted and a second ChIP step is performed using a different antibody from the first. The cross-links of these sequentially immunoprecipitated protein-DNA complexes are then reversed and the DNA is analyzed by PCR, similar to conventional ChIP samples.

product	format	catalog no.
Re-ChIP-IT®	25 rxns	53016

Flow Chart of Process & Example



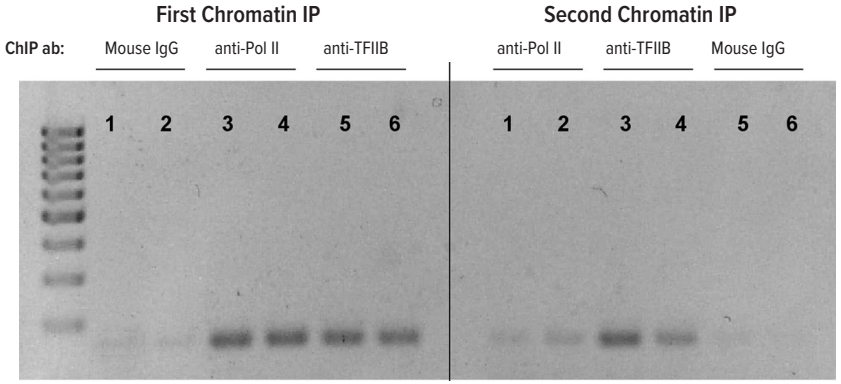


Figure 1: Sequential chromatin immunoprecipitation using Re-ChIP-IT.

In Re-ChIP-IT, sheared chromatin (prepared from formaldehyde-fixed cells) is incubated with a first antibody, and antibody-bound protein/DNA complexes are precipitated by magnetic Protein G-coated beads. The captured "First ChIP" chromatin is then eluted with a specialized buffer that prevents most of the first antibody from participating in the second ChIP reaction. A second ChIP reaction is performed with a different antibody, then the cross-links are reversed and the released DNA is analyzed by PCR to examine the co-localization of the two proteins at a specific region of interest.

In the gel above, HeLa cells were fixed for 10 minutes with 1% formaldehyde and chromatin was prepared by sonication shearing (5 pulses). ChIP was performed in duplicate on chromatin isolated from 750,000 cells. DNA was PCR analyzed using GAPDH positive control primers (36 cycles, T_m = 59°C). This figure demonstrates successful sequential chromatin IP with two different antibodies using the Re-ChIP-IT Kit. The antibodies used are listed above the duplicate lanes.

The lane numbers are the same in each panel to indicate that the DNA is from the same chromatin sample. The left panel shows the results of PCR performed on an aliquot of DNA removed from the experiment after the first ChIP step; the right panel represents PCR results on DNA from chromatin samples after both ChIP steps. For example, chromatin samples subjected to first ChIP using Mouse IgG as a negative control (lanes 1 and 2 in the left panel) were then subjected to a second ChIP with an RNA Pol II antibody (lanes 1 and 2 in the right panel). Chromatin samples in which Mouse IgG was used as either the first antibody (lanes 1 and 2) or second antibody (lanes 5 and 6) show little amplification of GAPDH DNA in either the left (first ChIP) or right panel (first and second ChIP). Chromatin samples in which the first antibody used was anti-RNA Pol II and the second antibody was anti-TFIIB (lanes 3 and 4) show good amplification of GAPDH DNA after the second ChIP (right panel) indicating the co-localization of RNA Pol II and TFIIB at the same region of the GAPDH promoter.

Re-ChIP-IT Advantages

A complete solution for rapid and efficient Re-ChIP

- Convenient magnetic bead-based protocol
- Fewer cells required than other Re-ChIP methods
- Compatible with multi-channel pipetting

The Re-ChIP-IT Kit from Active Motif contains all the reagents to perform 25 Re-ChIP reactions, but does not contain the reagents to prepare chromatin. Other Re-ChIP methods are tedious and technically challenging, requiring the covalent linkage of the primary antibodies to the immunoselection matrix. Using the Re-ChIP-IT Kit from Active Motif, we have eliminated the need to use direct antibody conjugates. We have developed a specialized buffer for elution of the first ChIP reaction that prevents the majority of the first antibody from participating in the second ChIP step, so you can be confident in your results.

The included protein-G coated magnetic beads are provided ready to use. These beads have a high binding capacity for IgG and low non-specific binding. As a result, these magnetic beads require fewer washing steps than agarose beads and it is not necessary to pre-clear the chromatin prior to ChIP. An added advantage is that the magnetic beads pellet much more quickly than standard agarose beads, which must be pelleted by centrifugation steps. In addition, magnetic stands (and the included bar magnet) are designed to pellet the beads onto the side of the tube. This makes it easier to remove buffers without disturbing the beads, so washing can be performed using multi-channel pipettors. This dramatically reduces hands-on time and ensures sample-to-sample consistency. **The provided siliconized microcentrifuge tubes (1.7 ml) simplify wash steps and ensure a minimal loss of Protein G Magnetic Beads and chromatin.**

Other steps in the ChIP protocol have also been optimized. The specialized Elution Buffer, coupled with a reagent to inactivate Proteinase K, eliminates the need for DNA purification after the ChIP is complete. This saves time, minimizes manipulations and eliminates the DNA loss that can occur during purification.

Re-ChIP-IT Kit & Related Products

The Re-ChIP-IT Kit provides reagents and protocols to perform 25 sequential chromatin IP experiments in a convenient magnetic bead-based format. The Re-ChIP-IT Kit can be used in conjunction with Active Motif's Chromatin Shearing Kits, which are used to prepare chromatin after first determining the optimal conditions for shearing chromatin from a particular cell line. Although not included in this kit, a brief discussion of the reagents provided in the shearing kits is included below for your reference.

Preparation of chromatin – Sonication Shearing

The ChIP-IT Express Shearing Kit (Catalog No. 53032) includes all buffers (excluding formaldehyde) required for cell fixation, nuclei purification and chromatin shearing by sonication. A Glycine "Stop-Fix" Buffer is included to prevent excessive cross-linking and Protease Inhibitor Cocktail (PIC) and PMSF are included to ensure that the protein/DNA interactions are preserved during the chromatin purification and immunoprecipitation steps.

Preparation of chromatin – Enzymatic Digestion

The ChIP-IT Express Enzymatic Shearing Kit (Catalog No. 53035) is similar to the ChIP-IT Express Shearing Kit, except that it uses a proprietary Enzymatic Shearing Cocktail and Digestion Buffer to enable enzymatic shearing, rather than sonication shearing. Enzymatic shearing is easily controlled by time and temperature to yield fragments that are ideal for performing ChIP. The Enzymatic Shearing Kit replaces sonication shearing, thus eliminating problems due to variability in sonication power as well as complications arising from the emulsification of chromatin during sonication.

Related Products

Please visit our website for complete information on the items below.

The ChIP-IT Express Kit provides reagents sufficient to prepare 15 sonication-sheared chromatin preparations and then perform 25 ChIPs. Each preparation of sheared chromatin requires a single 15 cm tissue culture plate of cells and yields chromatin sufficient for up to 14 ChIP-IT Express reactions (one ChIP reaction is considered to be the incubation of one sample of chromatin with one antibody). However, the chromatin preparation protocols can be scaled up or down depending on how many cells you would like to work with.

The ChIP-IT Express Enzymatic Kit is similar to ChIP-IT Express, but it uses a proprietary Enzymatic Shearing Cocktail and Digestion Buffer instead of sonication shearing. Enzymatic shearing is easily controlled by time and temperature to yield fragments ideal for use in ChIP, and it eliminates problems due to variability in sonication power as well as complications arising from emulsification of the chromatin during sonication. The ChIP-IT Express Enzymatic Kit provides sufficient reagents to perform 15 shearing reactions and then perform 25 ChIPs.

The **ChIP-IT Express HT Kit** was designed for users who have many ChIPs to perform. It enables true high-throughput ChIP by providing you with the reagents and protocols that adapts the magnetic bead-based ChIP-IT Express Kit method to a format that makes possible 96-well ChIP.

Because appropriate controls make ChIP interpretation and troubleshooting easier, Active Motif sells **ChIP-IT Control Kits** for human, mouse and rat samples. These useful kits contain species-specific positive & negative control antibodies, appropriate positive control PCR primers, PCR buffer and loading dye.

Active Motif's **Ready-to-ChIP Chromatin** is another useful reagent that will save you time. This High-quality chromatin from HeLa, Hep G2, K-562 and NIH/3T3 cells has been sheared by sonication and is ready for use in ChIP.

The **EpiShear™ Sonicator** is a compact 120-watt unit that is ideal for preparing sheared chromatin from small or large sample sizes for use in ChIP, as well as for cell disruption, DNA/RNA shearing and other homogenization applications.

For users who need to make more chromatin than is possible with the reagents included in the ChIP-IT Kits, the **ChIP-IT Protein G Magnetic Beads** and the **ChIP-IT Express Shearing Kits** are also available separately.

Finally, one difficult aspect of ChIP is finding an antibody that recognizes the target protein when it is bound to DNA and fixed by formaldehyde. Antibodies that perform well in Western, Supershift and other applications may not work well in ChIP. For this reason, Active Motif offers an ever-increasing number of **ChIP-validated antibodies** that have been verified to work in ChIP. See Related Products in the Appendix of this manual or go to **www.activemotif.com/chipabs** to generate an up-to-date list of antibodies that will help make your ChIP successful.

Kit Components and Storage

Please store each component at the temperature indicated in the table below. Do not re-freeze the Protein G Magnetic Beads or the LSV Protein G Magnetic Beads.

Reagents	Quantity	Storage / Stability
Buffered NaCl	100 µl	-20°C for 6 months
5 M NaCl	400 µl	-20°C for 6 months
Proteinase K (0.5 µg/µl)	100 µl	-20°C for 6 months
Proteinase K Stop Solution	100 µl	-20°C for 6 months
Protease Inhibitor Cocktail (PIC)	100 µl	-20°C for 6 months
Re-ChIP-IT Elution Buffer	100 µl	-20°C for 6 months
10X PCR Buffer	1.5 ml	-20°C for 6 months
10X PCR Loading Dye	1.5 ml	-20°C for 6 months
ChIP Buffer 1	75 ml	-20°C for 6 months
ChIP Buffer 2	75 ml	-20°C for 6 months
Elution Buffer AM2	1.5 ml	-20°C for 6 months
Reverse Cross-linking Buffer	1.5 ml	-20°C for 6 months
Protein G Magnetic Beads*	700 µl	4°C for 6 months
LSV Protein G Magnetic Beads*	700 µl	4°C for 6 months
Re-ChIP Purification Columns	25	4°C for 6 months
Bar Magnet	1	Room temperature
15 ml Conical Tube	1	Room temperature
Mini Glue Dots	2 sheets	Room Temperature
DEPC-treated Water	10 ml	Room temperature
Siliconized 1.7 ml microfuge tubes	50	Room Temperature
0.2 ml PCR stripwell tubes	96	Room Temperature

* The Protein G Magnetic Beads and LSV Protein G Magnetic Beads are shipped on dry ice, but **should not be re-frozen** by the customer. Upon receipt of this kit, store the beads at 4ºC.

Additional materials required

- A ChIP-validated antibody directed against the protein of interest
- 37% Formaldehyde (Fixation)
- Phenol/chloroform (Purification of sheared DNA prior to checking concentration by spectrophotometry or gel electrophoresis)
- 3 M Sodium Acetate pH 5.2 (Purification of sheared DNA prior to checking concentration by spectrophotometry or gel electrophoresis)
- 100% ethanol (Purification of sheared DNA prior to checking concentration by spectrophotometry or gel electrophoresis)
- 70% ethanol (Purification of sheared DNA prior to checking concentration by spectrophotometry or gel electrophoresis)
- DNase-free H_2O (Purification of Input DNA)
- Rocking platform for culture plates
- Apparatus to rotate tubes end-to-end at 4°C (*e.g.* a Labquake from Barnstead/Thermolyne)
- Microcentrifuge and microcentrifuge tubes
- Microplate centrifuge
- Spectrophotometer
- Pipettors and tips (a multi-channel pipettor and filter tips are recommended)
- Reagent reservoir (*e.g.* Thermo Fisher MatrixTech 25 ml Reagent Reservoir part no. 8094)
- Agarose gel electrophoresis apparatus
- Minimal cell culture media
- Cell scraper (rubber policeman)
- 10 ml pipette, aspirator and 100 ml graduated cylinder

Optional materials

- Chromatin IP DNA Purification Kit (*e.g.* Active Motif Catalog No. 58002)

Re-ChIP-IT Experimental Design

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Choosing Antibodies and Performing Control Reactions

- **Save aliquots for analysis.** Re-ChIP is performed by performing sequential ChIP reactions using two different antibodies that are directed against proteins expected to be at or near the same genomic locus in the prepared chromatin. ChIP is performed with a 1st antibody and the eluted chromatin is then used in a 2nd ChIP reaction with a 2nd antibody. You will save an aliquot of Input DNA before performing the 1st ChIP, as well as small aliquots of each 1st ChIP reaction. These will be used as controls in PCR analysis alongside the 2nd ChIP samples so that you can confirm the locus was present to begin with in the sheared chromatin and in the eluted chromatin after the 1st ChIP.
- **Use ChIP-validated antibodies and chromatin.** ChIP antibodies must recognize fixed 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 unproven antibody must include appropriate controls; additional controls may be needed for Re-ChIP. To avoid the need to perform a large number of control reactions, it is preferable to use antibodies that have already been successfully used in regular ChIP, ideally with the same chromatin preparation that will be used in Re-ChIP. If you cannot use the same chromatin preparation, use chromatin that was prepared the same way, from the same cell type and stimulation, as the chromatin that was used to ChIP-validate the antibodies.
- **Control reactions in the 1st ChIP.** If the 1st ChIP is not successful, then the 2nd ChIP cannot be successful. Therefore, if you cannot use a 1st antibody that has been ChIP-validated with the type of chromatin being used in the 1st ChIP, you should perform positive and negative control 1st ChIP reactions. This way, if the 2nd ChIP is not successful, you will at least know if the 1st ChIP was successful with the positive control. Because the 1st ChIP is performed on a non-eluted chromatin preparation, the positive control antibody can be directed against any protein expected to be present in the prepared chromatin. For your convenience, Active Motif sells ChIP-IT Control Kits for human, mouse and rat samples that contain positive and negative control antibodies (that are suitable for use in all 1st ChIPs and some 2nd ChIPs), appropriate PCR primers, PCR buffer and loading dye (see Related Products in Appendix).
- **"No antibody" control in the 1st ChIP.** During the 1st ChIP, the majority of the 1st antibody remains bound to the protein G magnetic beads while the chromatin is eluted. However, a small amount of 1st antibody may elute with the chromatin. The Re-ChIP-IT Elution Buffer was designed to prevent any of the small amount of eluted 1st antibody from interacting with protein G during the 2nd ChIP. So, any 1st antibody carried over should not be able to cause immunoprecipitation during the 2nd ChIP. Even so, it is strongly advised to perform a "no antibody" control during the 2nd ChIP. Adding no antibody when performing the 2nd ChIP should result in no immunoprecipitation, and little or no PCR band. This will validate that successful 2nd ChIP is caused only by the 2nd antibody, and not by any carried over 1st antibody. Thus, you should perform each 1st ChIP in duplicate, so that the eluted chromatin samples can be used in 2nd ChIP with the 2nd antibody and with a "no antibody" control.
- **Control reactions in the 2nd ChIP.** During the 2nd ChIP, a 2nd antibody is used to perform

ChIP on the chromatin that was eluted by the 1st ChIP reaction. Again, if possible, use a 2nd antibody that already has been shown to perform in ChIP. Choosing appropriate control reactions for the 2nd ChIP is not as straight forward as with the 1st ChIP because the eluted chromatin used for the 2nd ChIP is already a small sub-set of the original chromatin. Thus, any antibody to be used as a control in the 2nd ChIP must be directed against a protein that is in a protein complex, or near a locus, that will have been immunoprecipitated in the 1st antibody. For example, Active Motif's positive control RNA Pol II antibody is an appropriate control in a 2nd ChIP only if you know that RNA Pol II will have been brought down with the chromatin eluted by the 1st antibody during the 1st ChIP.

- **Use of the 1st antibody as a control in the 2nd ChIP.** The 1st antibody is sometimes used as a positive control in the 2nd ChIP. Because the chromatin used in the 2nd ChIP was eluted by the 1st antibody, the chromatin can be brought down again by the same antibody if it is freshly added for the 2nd ChIP. This confirms that the 2nd ChIP was successful, at least with the control. However, it is best to not use the same antibody in the 1st and 2nd ChIPs if it is a monoclonal. See Troubleshooting in the Appendix for an explanation.

Sample Re-ChIP Experiment

Below is a table showing the 1st and 2nd ChIP reactions that should be set up when performing a typical Re-ChIP experiment, as well as the PCR analysis that will be performed after Re-ChIP. In this experiment, NFκB p65 antibody will be used for the 1st antibody, while an NFκB p50 antibody will be the 2nd antibody. Both of these antibodies had been ChIP-validated previously.

	Rxn 1	Rxn 2	Rxn 3 (optional)	Rxn 4 (optional)	Rxn 5 (optional)
1st ChIP	NFκB p65	NFκB p65	NFκB p65	RNA Pol II	Negative control IgG
2nd ChIP	NFκB p50	No antibody	NFκB p65	RNA Pol II	Negative control IgG
PCR	Input DNA, 1st ChIP NFκB p65, 1st ChIP RNA Pol II*, 1st ChIP Negative control IgG*, 2nd ChIP NFκB p50, 2nd ChIP No antibody, 2nd ChIP NFκB p65*, 2nd ChIP RNA Pol II*, 2nd ChIP Negative control IgG*. * If the control was performed.				

Reaction 1 is the Re-ChIP to test if p65 and p50 co-occur at the locus of interest.

Reaction 2 is a "no antibody" control that will confirm that any PCR band caused by the 2nd ChIP with p50 (Rxn 1) is caused by p50, and is not caused by carry-over of the p65 from the 1st ChIP.

Rxns 3 and 4 are possible positive controls for 1st and 2nd ChIPs. Use a ChIP-validated antibody as a control (Rxn 4) if your test antibodies are not ChIP-validated. You should perform controls even when using ChIP-validated antibodies controls to confirm ChIP worked. This prevents mistakenly concluding that your proteins don't co-occur. For example, if you were to see a p65 PCR band but no p50 PCR band for Rxn 1, either p65 and p50 did not co-occur OR the 2nd ChIP simply did not work. Only by performing adequate controls can you rule out failure of the ChIP procedures.

Rxn 5 uses a Negative control IgG to demonstrate that ChIPs 1 and 2 are specific. If you are using ChIP-validated antibodies that you have shown are specific, this is optional.

Other Points to Consider

- **Cell growth and chromatin preparation.** This kit contains sufficient reagents to perform a total of 25 Re-ChIP reactions. Chromatin can be prepared for use in ChIP through sonication by using Active Motif's ChIP-IT Express Shearing Kit (Catalog No. 53032) or by enzymatic digestion with the ChIP-IT Express Enzymatic Shearing Kit (Catalog No. 53035). Re-ChIP-IT is also compatible for use with Active Motif's Ready-to-ChIP Chromatin. Before starting an experiment, calculate the number of chromatin preparations you 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 (see the points above). 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. See Troubleshooting in the Appendix for comments regarding chromatin yield and the amount of chromatin used for each ChIP reaction.
- **Formaldehyde fixation (1% solution).** In standard protocols, chromatin is fixed for 10 minutes prior to shearing by sonication or enzymatic digestion. While these are typical fixation conditions, some antibody/chromatin combinations may work better with shorter fixation. Fixing for two minutes with 1% formaldehyde has been very effective in some cases. When optimizing ChIP experiments, it can be useful to test reducing the time of fixation.
- **Shearing by Sonication.** Chromatin sheared to a size of 200-1500 bp is usually used for ChIP experiments. 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. This can be avoided by using lower shearing power and by turning the power on gradually. If a shearing reaction is allowed to emulsify, discontinue shearing and centrifuge the sample at 4°C for 4 minutes at 8000 rpm 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 15 seconds, then place on ice for 30 seconds, sonicate again for 15 seconds, *etc.*).
- **Shearing by Enzymatic Digestion.** While sonication is the most common method used to shear chromatin for ChIP, in some cases (*e.g.* when the sample is limited, when nucleosome ChIP is to be performed, when a sonicator is not available, or for high-throughput applications) enzymatic shearing is an excellent alternative. To provide a robust and user-friendly enzymatic shearing method, Active Motif offers the ChIP-IT Express Enzymatic Kit, which uses a proprietary Enzymatic Shearing Cocktail to quickly digest the chromatin DNA to 200-1500 bp fragments. Enzymatic activity can be easily controlled by time and temperature.
- **Protein G-coated magnetic beads.** The supplied magnetic beads are ready to use following complete resuspension to a homogeneous slurry. There is no need to pre-block the beads or pre-clear the sample. For best results, gently shake and roll the tube. The beads settle quickly, so should be resuspended just before pipetting. **Protein G Magnetic Beads and LSV Protein G Magnetic Beads are shipped on dry ice, but should not be re-frozen by the customer. Upon receipt, the beads should be stored at 4°C**.

- **Perform Re-ChIP-IT in the provided siliconized 1.7 ml microfuge tubes or in 8-well PCR strips.** The provided bar magnet can be used with either of these formats (see Appendix – Section B for detailed instructions). Commercially available side-pulling magnetic stands (*e.g.* Promega MagneSphere® Technology twelve-position Magnetic Separation Stand – 1.7 ml microcentrifuge tube format, or the Ambion 6 Tube Magnetic Stand) can also be used with the standard 1.5 or 1.7 ml microcentrifuge tubes.

First-time users of magnetic beads in the PCR tube format should familiarize themselves with the manipulations before performing a ChIP (see Appendix – Section B). Some commercially available magnets for 96-well plates are not ideal for use with 8-well PCR strips.

- **PCR analysis of immunoprecipitated DNA.** A successful ChIP results in an enrichment of chromatin fragments that are bound by the protein of interest, not complete purification. Thus, DNA isolated by ChIP is unavoidably contaminated with non-specifically captured DNA. For this reason, Real-Time Quantitative PCR analysis is preferred. If this method is not available, PCR of ChIP DNA should be performed such that cycling is stopped while the reaction is still in the linear stage of amplification. Hot-start PCR methods are recommended.

  If you intend to analyze the binding of a known protein to a known binding site, design the PCR primers so that they flank the binding site and generate a 100-250 bp amplicon. Alternatively, if you hope to identify a protein binding site within a region of DNA, it may be best to design several primer pairs so that the DNA region in question can be systematically analyzed. In this case, design a series of primer pairs that can be used to generate amplicons that overlap one another and span the region of interest. To facilitate this, the amplicons can be 250-400 bp in length. After these primer pairs have been used to roughly localize the binding site, design a more focused set of primers. Use of PCR design programs can be helpful in selecting good primer pairs. Also, as PCR analysis is extremely sensitive, precautions against contamination should be taken throughout the entire ChIP protocol.

- **PCR primers.** PCR primers should efficiently and specifically amplify the desired target. This should be proven on a relevant template, such as genomic or Input DNA. In addition, negative control primers can be useful to control for DNA shearing efficiency and to map putative protein binding sites. Ideally, PCR primers for ChIP should be 24 nt in length, have a GC content of 50% and a T_m of 60°C. See Troubleshooting for discussion.

- **Siliconized tubes.** The siliconized 1.7 ml tubes are provided only for performing the ChIP reactions. Do not use them for preparation of chromatin or for isolation of Input DNA. Low retention PCR tube strips are also included for this purpose, if you choose.

- **Resuspend solutions completely.** Thaw the Proteinase K Stop Solution at room temperature until fully dissolved. Vortex gently and spin down briefly before use.

- **Maximum volume of chromatin.** Chromatin shearing (sonication) buffers usually contain detergents (*e.g.* 0.1% SDS and 0.5% sodium deoxycholate is typical). If you plan to use more than 60 µl sonicated chromatin in a ChIP reaction, use the 200 µl reaction volume in Table 1. This will ensure that the detergent in the shearing buffer does not interfere with antibody activity. This is not a concern with chromatin prepared by the Active Motif ChIP-IT Express Enzymatic Shearing Kit because the relevant buffers contain little or no detergent.
- **Quantity of antibody.** Best results are typically obtained by use of 1-3 µg antibody. However, this will vary according to the activity of the antibody and the quality of the chromatin, and you may need to use more of a particular antibody.
- **Stopping points in the protocol.** Convenient stopping points are mentioned in the Troubleshooting section in Appendix – Section C.
- **Safety precautions.** Formaldehyde is highly toxic. 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 shearing should be performed in a hood if the cells might contain something that could infect human cells.
- **Washing of magnetic beads.** See also Appendix – Section B. In the protocols below, bead washing is performed as follows:
    a. Place tubes in the appropriate magnetic stand and allow beads to pellet on tube side.
    b. Carefully remove the supernatant.
    c. Add the appropriate wash buffer and resuspend pellet completely by pipetting up and down 2-3 times. Ensure the beads are not clinging to the pipette tips after pipetting. You may need to move the tubes away from the magnetic field before resuspending.
    d. Repeat steps a-c for the desired number of washes.

A. First Chromatin Immunoprecipitation

1. Thaw chromatin (if necessary). Transfer 10 µl to a microcentrifuge tube; this tube is the "Input DNA" that will be processed in Step G6, then be used as a control in PCR analysis. Store this reserved chromatin at -20°C.
2. Set up the ChIP reactions by adding the components shown in Table 1 below to the provided siliconized 1.7 ml microcentrifuge tubes or to PCR tubes. Before pipetting the magnetic beads, they should be fully resuspended by inverting and/or vortexing the bottle. **The antibody should be the final component added to the reaction.**

Table 1

Reagent	One reaction (if using less than 60 µl of chromatin)	One reaction (if using more than 60 µl of chromatin)
Protein G Magnetic Beads	25 µl	25 µl
ChIP Buffer 1	10 µl	20 µl
Sheared Chromatin (~7 µg)*	20-60 µl	61-100 µl
Protease Inhibitor Cocktail (PIC)	1 µl	1 µl
dH_2O	Add enough so that the final reaction volume will be 100 µl	Add enough so that the final reaction volume will be 200 µl
Antibody (added last)	1-3 µg	1-3 µg
Total Volume	**100 µl**	**200 µl**

***Note:** Depending on the application, ChIP can be performed using anywhere from 1-50 µg of chromatin. An important factor is the volume of the chromatin being added, especially if the chromatin was prepared using sonication, as the detergents used during sonication will impact ChIP. Use the 200 µl ChIP reaction volume in the right column (above) if the volume of chromatin will be greater than 60 µl. See the Appendix for discussions on the amount of chromatin to use and for methods to quantify DNA in chromatin.

3. Cap tubes and mix thoroughly.
4. Incubate on an end-to-end rotator for 4 hours at 4°C (*e.g.* a Labquake from Barnstead/Thermolyne with a tube holder for 1.7 ml microcentrifuge tubes). In some cases, sensitivity may be improved if the incubation is performed overnight.
5. Spin tubes briefly to collect liquid from the inside of the caps.
6. Place tubes on magnetic stand to pellet beads on the side of the tubes.
7. Carefully remove and discard supernatant.

B. Wash Magnetic Beads

For suggestions regarding bead washing methods, see the Experimental Design section that begins on page 8, as well as the information in Appendix – Section B.

For 1.7 ml microcentrifuge tubes:

1. Wash beads one time with 800 µl ChIP Buffer 1.
2. Wash beads two times with 800 µl ChIP Buffer 2.
3. After the final wash, remove as much supernatant as possible without disturbing the beads. Use a 200 µl pipetteman if necessary.

For 8-well PCR strips:

1. Wash beads three times with 200 µl ChIP Buffer 1.
2. Wash beads two times with 200 µl ChIP Buffer 2. After the final wash, remove as much supernatant as possible without disturbing the beads.

C. Elute Chromatin from First ChIP Reaction

1. Prepare buffer for the first elution by diluting the Re-ChIP-IT Elution Buffer 1:100 in DEPC water. Prepare only as much buffer as you need for your required number of Re-ChIP samples as this buffer must be used freshly and cannot be stored.
2. Resuspend each sample of washed beads from Step B with 100 µl of the diluted Re-ChIP-IT Elution Buffer.
3. Incubate 30 minutes at room temperature on an end-to-end rotator.

D. Desalt Chromatin from First ChIP Reaction

1. During the elution step above, prepare the desalting columns as follows:
    a. Remove the bottom closures of the columns and loosen the caps.
    b. Place each of the columns in a fresh 1.7 ml microfuge tube (do not use the siliconized tubes provided with the kit).
    c. Spin the tubes in a microfuge at 1,500 x *g* for 1 minute to remove the storage solution.
    d. With an indelible maker, place a mark on the side of each column where the desalting resin is sloped higher on the tube.
    e. Place each column in a siliconized 1.7 ml microcentrifuge tube and remove the caps from each column.
2. Remove the tubes containing the eluted chromatin from the first ChIP reaction (Step C) from the rotator, briefly spin to collect liquid from the inside of the caps, then place the tubes on a magnetic stand to pellet the beads on the side of the tubes.

3. Quickly transfer the chromatin-containing supernatant to the center of the compact resin bed in a prepared desalting column. Place column in the microcentrifuge tube with the mark facing outward.
4. Centrifuge at 1,500 x *g* for two minutes to collect the desalted chromatin.
5. Put the microcentrifuge tubes containing the collected chromatin on ice. Remove 10 µl from each of these chromatin samples and put in new PCR tubes to be used as controls for the first ChIP reaction. Cap and store these PCR tubes at -20°C for further application. The remaining chromatin can be used immediately in the second ChIP reaction in Step E or stored at -80°C for several days.

E. Second Chromatin Immunoprecipitation

1. Set up the Re-ChIP reactions by adding the components shown in Table 2 (below) to the provided PCR tubes. Before pipetting the magnetic beads, they should be fully resuspended by inverting and/or vortexing the bottle. **The second antibody should be the final component added to the reaction.**

Table 2

Reagent	One reaction
LSV Protein G Magnetic Beads	25 µl
ChIP Buffer 1	10 µl
Desalted Chromatin from Step D (above)	90 µl
Protease Inhibitor Cocktail (PIC)	1 µl
dH_2O	Add enough so that the final reaction volume will be 140 µl
Second Antibody (added last)	1-3 µg
Total Volume	**140 µl**

2. Cap the tubes and mix thoroughly.
3. Incubate overnight at 4°C on an end-to-end rotator (incubation may be as short as 4 hours).
4. Spin tubes briefly to collect liquid from the inside of the cap.
5. Place tubes on magnetic stand to pellet beads on the side of the tubes.
6. Carefully remove and discard supernatant.

F. Wash Magnetic Beads

For 8-well PCR strips:

1. Wash beads three times with 200 µl ChIP Buffer 1.
2. Wash beads two times with 200 µl ChIP Buffer 2. After the final wash, remove as much supernatant as possible without disturbing the beads.

G. Elute Chromatin, Reverse Cross-links and Treat with Proteinase K

1. Resuspend washed beads with 50 µl Elution Buffer AM2.
2. Incubate 15 minutes at room temperature on an end-to-end rotator.
3. Briefly spin tubes to collect liquid from caps.
4. Add 50 µl of the Reverse Cross-linking Buffer to eluted chromatin and immediately place tubes in magnetic stand; allow beads to pellet to sides of tubes.
5. Quickly transfer the supernatant, which contains the chromatin, to a fresh PCR tube.
6. "Input DNA" sample: take the 10 µl Input DNA aliquot (that was reserved in Step A1 above) from the -20°C freezer. Add 88 µl ChIP Buffer 2 and 2 µl 5 M NaCl to the Input DNA sample only, so that its final volume is 100 µl.
7. Remove the PCR tubes containing 10 µl of the first chromatin IP from the -20°C (from Step D5 on page 15). Add 2 µl of Buffered NaCl.
8. Incubate all samples (Input DNA, 1st ChIP 10 µl samples and 2nd ChIP elution samples) at 94°C for 15 minutes in a thermocycler.

   Note: If you are using larger microcentrifuge tubes, it may be easier to perform a 2.5 hour incubation at 65°C.

9. Return tubes to room temperature and spin briefly if liquid has collected on the inside of the caps. Add 2 µl Proteinase K to the "Input DNA" and to the samples from the 2nd ChIP. For samples from the 1st ChIP (10 µl samples), dilute the stock Proteinase K solution 1:5 in DEPC-treated water (*e.g.* for 25 samples, add 6 µl of Proteinase K to 24 µl DEPC water), then add 1 µl of this 1:5-diluted Proteinase K to each tube.
10. Cap tubes, mix well and incubate at 37°C for 1 hour. During this incubation, heat the Proteinase K Stop Solution at 37°C for use in the next step.
11. Return the tubes to room temperature and add 2 µl Proteinase K Stop Solution to the "Input DNA" and to the samples from the 2nd ChIP. For samples from the 1st ChIP (10 µl samples), dilute the Proteinase K Stop Solution 1:5 in DEPC-treated water (*e.g.* for 25 samples, add 6 µl Proteinase K Stop Solution to 24 µl DEPC water), then add 1 µl of this 1:5-diluted Proteinase K Stop Solution to each tube. **Make the dilution for the required number of samples only and use immediately; the diluted Proteinase K Stop Solution cannot be stored.**
12. Briefly centrifuge the tubes to collect liquid from the caps. DNA can be immediately used in PCR or stored at -20°C.

H. PCR Analysis

The protocol below is a guideline for optimizing PCR analysis of DNA collected through Re-ChIP. Accurate PCR analysis of ChIP DNA requires that the PCR be stopped during linear amplification. The appropriate number of PCR cycles must be determined empirically.

PCR will be performed on a number of different DNA templates, depending on which controls you have performed. At the minimum, you will set up PCR for the Input DNA (reserved in Step A1), DNA from the 1st ChIP with a test antibody (reserved in Step D5), DNA from the 2nd ChIP with a 2nd test antibody (eluted in Step G5) and DNA from the 2nd ChIP with no antibody (eluted in Step G5). A water-only control is also performed to ensure the PCR reagents are not contaminated. You might also perform PCR on 1st and 2nd ChIPs using positive and negative control antibodies.

In the example below, PCR reactions are set up using 2 different PCR cocktails, which contain positive & negative PCR primer sets. If you are using a positive control antibody from one of Active Motif's ChIP-IT Control Kits, only positive control PCR primers are used because the positive control antibodies included in those kits can bind many regions along a chromosome, making it difficult to design "negative control" primers that function with all cell types and shearing conditions. For PCR analysis of ChIP performed with other antibodies, we recommend that you use both positive and negative PCR primer sets that are appropriate for your antibody. See the Experimental Design section that begins on page 8 and Troubleshooting in the Appendix for details.

Note: PCR is extremely sensitive and all precautions should be taken to guard against contamination. Gloves should be worn and filter-tip pipettes should be used.

1. Program the thermocycler. The program should start with a initial melt step at 94°C for 3 minutes, then 36 cycles of [94°C for 20 seconds, 59°C for 30 seconds and 72°C for 30 seconds], then a hold cycle at 10°C. The total volume of each PCR will be 25 µl. You may need to optimize the number of cycles for your specific system.
2. Dilute the Input DNA 1:10 by adding 20 µl Input DNA to 180 µl dH_2O.
3. Use the table on the following page to label PCR tubes and add the PCR templates and water-only control, keeping the tubes on ice. Add the PCR cocktails to be made in Step H4:

Reaction No.	PCR Template (5 µl each)	PCR cocktail (20 µl each)
1	Input DNA (diluted 1:10)	Positive PCR cocktail
2	1st ChIP DNA – Test antibody	Positive PCR cocktail
3	2nd ChIP DNA – Test antibody	Positive PCR cocktail
4	2nd ChIP DNA – No antibody	Positive PCR cocktail
5	H_2O (no DNA control)	Positive PCR cocktail
6*	1st ChIP DNA – Positive control antibody	Positive PCR cocktail
7*	2nd ChIP DNA – Positive control antibody	Positive PCR cocktail
8*	1st ChIP DNA – Negative control IgG	Positive PCR cocktail
9*	2nd ChIP DNA – Negative control IgG	Positive PCR cocktail
10	Input DNA (diluted 1:10)	Negative PCR cocktail
11	1st ChIP DNA – Test antibody	Negative PCR cocktail
12	2nd ChIP DNA – Test antibody	Negative PCR cocktail
13	2nd ChIP DNA – No antibody	Negative PCR cocktail
14	H_2O (no DNA control)	Negative PCR cocktail
15*	1st ChIP DNA – Positive control antibody	Negative PCR cocktail
16*	2nd ChIP DNA – Positive control antibody	Negative PCR cocktail
17*	1st ChIP DNA – Negative control IgG	Negative PCR cocktail
18*	2nd ChIP DNA – Negative control IgG	Negative PCR cocktail

* Optional controls.

4. Set up the Positive PCR cocktail and the Negative PCR cocktail on ice according to the tables on the following page. Add the dH_2O first and the *Taq* polymerase last. Mix thoroughly and keep on ice. This ensures that the reaction mixture is inactive until the cycling is started. As discussed above, if you are using the positive control antibody and Negative control IgG from one of the ChIP-IT Control Kits, only positive control PCR primers are provided (as a mixture of forward and reverse primers). However, for your own test antibodies, and if you are supplying your own positive and negative control antibodies, we recommend that you design and test these using both the positive and negative PCR primer sets.

Positive PCR cocktail:

Reagent	1 reaction	5 reactions
DEPC H_2O	12.3 µl	61.5 µl
Positive Forward primer (5 pmol/µl)	2.0 µl	10 µl
Positive Reverse primer (5 pmol/µl)	2.0 µl	10 µl
dNTP mixture (5 mM each dNTP)	1.0 µl	5.0 µl
10X PCR Buffer	2.5 µl	12.5 µl
Taq (5 U/µl)	0.2 µl	1.0 µl
Total Volume (Not including DNA template)	20 µl	100 µl

Negative PCR cocktail:

Reagent	1 reaction	5 reactions
DEPC H_2O	12.3 µl	61.5 µl
Negative Forward primer (5 pmol/µl)	2.0 µl	10 µl
Negative Reverse primer (5 pmol/µl)	2.0 µl	10 µl
dNTP mixture (5 mM each dNTP)	1.0 µl	5.0 µl
10X PCR Buffer	2.5 µl	12.5 µl
Taq (5 U/µl)	0.2 µl	1.0 µl
Total Volume (Not including DNA template)	20 µl	100 µl

Note: We recommend that PCR analysis be performed using the 10X PCR Buffer included with the ChIP-IT Control Kits. The composition of the 10X PCR Buffer is 750 mM Tris-Cl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20 and 25 mM $MgCl_2$.

5. Add the appropriate PCR cocktail to each of the PCR tubes (on ice) prepared in Step H3. Cap PCR tubes carefully and ensure that each reaction mixture is in the bottom of the tube.
6. Place PCR tubes in thermocycler and start the PCR program described in Step H1. After 36 cycles, remove tubes 1-10 and place on ice.
7. These PCR reactions can be immediately analyzed as described below or stored at -20°C.

I. Analysis of PCR Products

1. Run ~8 µl of each PCR product on a 3% agarose gel. Save remaining PCR product in case additional gels must be run. Use gel combs with 2.5 mm-wide wells.
2. PCR products obtained with the GAPDH positive control primers are 166 bp. Use either a 50 or 100 bp ladder as the migration standard. Run the gel until PCR amplification products are well separated from PCR primers and primer dimers. Stain gel and analyze.

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Appendix

Section A. DNA Clean Up

Active Motif sells two kits for preparing chromatin for use in ChIP, the ChIP-IT Express Shearing Kit (Cat. No. 53032) and the ChIP-IT Express Enzymatic Shearing Kit (Cat. No. 53035), as well as Ready-to-ChIP Chromatin prepared from HeLa cells (Cat. No. 53015), Hep G2 cells (Cat. No. 53019), K-562 cells (Cat. No. 53020) and NIH/3T3 cells (Cat. No. 53021).

The protocol below provides instructions to check and quantify your sheared chromatin prior to using it in ChIP and Re-ChIP experiments.

1. If necessary, thaw 50 µl aliquots of each sheared chromatin sample.
2. Add 150 µl dH_2O, then 10 µl 5 M NaCl to each tube.
3. Heat all samples at 95°C in a water bath or a thermocycler for 15 minutes to reverse the cross-links, taking care to prevent the lids from popping open if you use a water bath.
4. Add 1 µl RNase A to each sample and incubate at 37°C for 15 minutes.
5. Add 1 µl Proteinase K (0.5 µg/µl) to each sample and incubate at 67°C for 15 minutes.

   Note: If you intend to use a spectrophotometer to determine the DNA concentration, the DNA should first be column purified or phenol/chloroform extracted and precipitated. This can be performed as follows:

   a. Add 200 µl phenol/chloroform to the sample, vortex to mix completely and centrifuge for 5 minutes at maximum speed in a microcentrifuge.

   b. Transfer supernatant to a fresh microcentrifuge tube, then add 20 µl 3 M Sodium Acetate pH 5.2 and 500 µl 100% ethanol. Vortex to mix completely and place at -80°C for at least 1 hour. Alternatively, the sample can be left at -20°C overnight.

   c. Centrifuge at maximum speed for 10 minutes in a microcentrifuge at 4°C.

   d. Carefully remove and discard supernatant. Do not disturb the pellet.

   e. Add 500 µl 70% ethanol to the pellet and spin 5 minutes at 4°C in microcentrifuge at maximum speed.

   f. Carefully remove and discard supernatant. Do not disturb pellet. Allow pellet to air-dry.

   g. Resuspend pellet in 30 µl dH_2O and use a spectrophotometer to measure the OD at 260 nm to determine the DNA concentration (1.0 A_{260} unit = 50 µg/ml).

      Alternatively, purify the DNA with the Chromatin IP DNA Purification Kit (Active Motif Catalog No. 58002). Elute in 10 µl dH_20 and measure the concentration using the NanoDrop from Thermo.

6. It is recommended to load each shearing sample on the gel in two different amounts to avoid over- or under-loading. Add 4 µl of a 6X Loading Buffer to 16 µl of sample, then load 5 µl & 10 µl of each sample on a 1% TAE agarose gel. Run the gel at 100V for 45 minutes to 1 hour until the loading dye reaches 3/4 of the way to the end of the gel rack.
7. Optimal sonication shearing should result in a 200-1500 bp smear similar to that shown in

lanes 2 or 3 of Figure 2 below. Optimal enzymatic shearing should produce a 200-1500 bp ladder-like smear similar to Lane 4 of Figure 3 below:

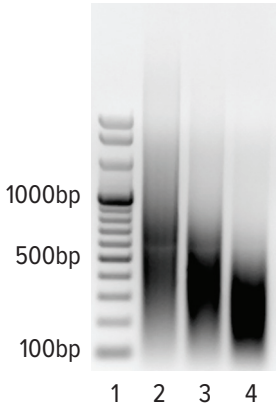


Figure 2: Gel analysis of sonication shearing (ChIP-IT Express).

HeLa cells were fixed for 10 minutes with 1% formaldehyde and then chromatin was prepared using the ChIP-IT Express Kit. Three samples of approximately 300 µl each were sheared with 5, 10 and 20 pulses at 25% amplitude using the Active Motif EpiShear™ Sonicator with a 1/8" probe. 300 µl. Each pulse consisted of a 20-second sonication followed by a 30-second rest on ice to prevent heat build up. The sheared and unsheared chromatin samples were subjected to cross-link reversal, treated with Proteinase K and RNase A, then phenol/chloroform extracted and precipitated as described. Samples were separated by electrophoresis through a 1% agarose gel. Optimally sheared chromatin will yield a smear between 200-1500 bp.

Lane 1: 100 to 1000 bp ladder.
Lane 2: HeLa DNA sheared for 5 pulses (optimal).
Lane 3: HeLa DNA sheared for 10 pulses (optimal).
Lane 4: HeLa DNA sheared for 20 pulses (over-sheared).
Note: From this experiment, the DNA sonicated for both 5 and 10 pulses are suitable for use in ChIP.

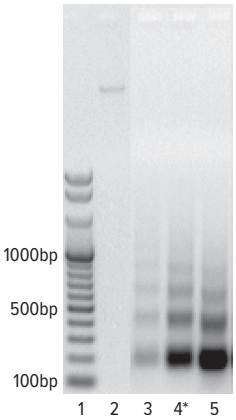


Figure 3: Gel analysis of enzymatic shearing (ChIP-IT Express Enzymatic).

HeLa cells were fixed for 10 minutes with 1% formaldehyde and then chromatin was prepared using the ChIP-IT Express Enzymatic Kit protocol. Chromatin was sheared with the Enzymatic Shearing Cocktail for 5, 10 & 15 minutes and the reaction was stopped with the addition of cold EDTA. The sheared and unsheared chromatin samples were subjected to cross-link reversal, treated with Proteinase K, phenol/chloroform extracted and precipitated as described in the protocol. Samples were separated by electrophoresis through a 1% agarose gel. Optimally sheared chromatin will yield a bands between 200-1500 bp.

Lane 1: 100 to 1000 bp ladder.
Lane 2: Unsheared HeLa DNA.
Lane 3: HeLa DNA treated for 5 minutes (under-digested).
Lane 4: HeLa DNA treated for 10 minutes (optimized digestion).
Lane 5: HeLa DNA treated for 15 minutes (over-digested).
***Note:** From this experiment, the DNA treated for 10 minutes was optimal and used successfully in ChIP.

Section B. Use of Magnetic Beads and Included Bar Magnet

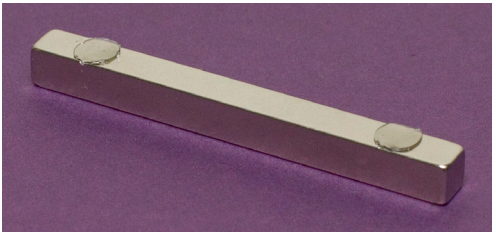
- The magnet should be stored in the provided tube.
- Be careful when working near metal objects or surfaces. A free magnet will jump great distances onto nearby metal surfaces with surprising speed. This can break the magnet.
- Use the provided Mini Glue Dots to attach the bar magnet to an empty pipette tip box to create an effective magnet stand for use with either PCR strips or microcentrifuge tubes.
- If the magnet becomes attached to a flat metal surface, it should be removed by sliding it off the edge of the surface. The magnet may be broken if you attempt to pull one end away from the metal.

Caution: The included neodymium bar magnet is extremely powerful and is easily broken if handled incorrectly.

Creating a magnetic stand for 8-well PCR strips:

Note: 8-well strip tubes are provided with the kit, but if more are needed, use strips that will work with a standard 96-well PCR cycler (*e.g.* Thermo Fisher AB-0451).

1. Place a strip of PCR tubes in the wells of an empty tip box (200 µl tips) and place the magnet directly against the tubes. This is the way the magnet will be positioned when the glue dots are used to affix it to the box.
2. Remove the covering tape from one side of two glue dots and attach the glue dots on the bar magnet (the uncovered face of the dot is placed on the magnet) as shown below.

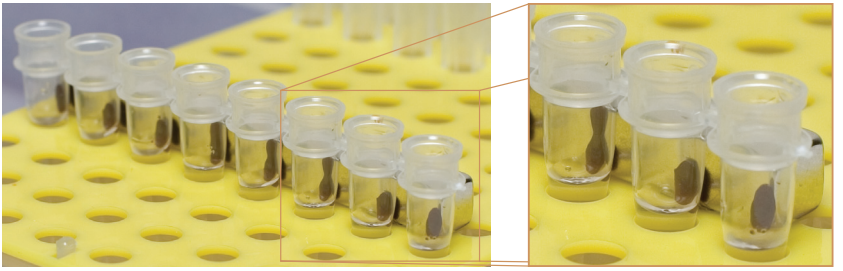


3. Remove the covering tape from the exposed side of the glue dots. Fix the magnet to the tip box so that it is against the PCR tubes. The magnetic stand is now ready for use.

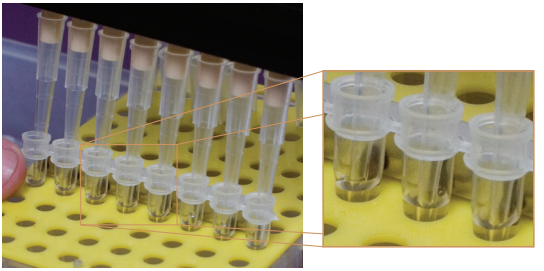
Note: Familiarize yourself with using the magnetic stand before performing with PCR tubes for the first time. Add 5 µl of magnetic beads to 100 µl ChIP Buffer 1 in one tube of an 8-well strip of PCR tubes. Use this tube with the assembled bar magnet stand to become familiar with use of the beads and magnet. It is difficult to re-suspend the beads if the tubes are directly adjacent to the magnet, so it is usually best to move the tubes away from the magnet for resuspension.

Washing should be performed as follows:

a. Place the tubes in the rack against the magnet and allow the beads to be pinned to the side of the tube, as shown below.



b. Remove supernatant with a 200 µl pipetteman or a 200 µl eight-channel pipetteman.



c. Move the tube strip into a row that is not adjacent to the magnet.

d. Add wash buffer and pipet up and down to fully re-suspend the beads. Ensure that a minimal amount of beads cling to the tips when the re-suspension is complete.

e. Repeat steps a-d until desired washing steps are complete.

Centrifugation of 8-well PCR strip tubes:

When working with 8-well PCR strip tubes, it may be desirable to centrifuge the tubes to collect the liquid and beads from the inside the caps. This is easily accomplished in a centrifuge fitted with adaptors for spinning microtiter plates. Place a standard 96-well plate in the adaptor to hold the tubes in place. Be sure to balance the rotor (*i.e.* place a microtiter plate and tubes of appropriate mass in the rotor's opposing 96-well plate adaptor). Spin the plates briefly to let the rotor reach a speed of 1000 x *g* before allowing the rotor to stop.

Creating a magnetic stand for 1.7 ml microcentrifuge tubes:

1. Remove the covering tape from one side of two glue dots.
2. Place two 1.7 ml microcentrifuge tubes in the wells of an empty tip box (1000 µl) and place the magnet directly against the tubes. This is the way the magnet will be positioned when the glue dots are used to affix it to the box.
3. Attach the glue dots on the bar magnet (the uncovered face of the dot is placed on the magnet) as shown above.
4. Remove the covering tape from the exposed side of the glue dot. Fix the magnet to the tip box so that it is against the tubes. The magnetic stand is now ready for use.

Note: 1.7 ml microcentrifuge tubes are held less securely in this assembled tube stand than in a typical commercial magnetic stand. This is not a problem if the below washing protocol is followed. Work with 1 tube at a time, and keep the tubes in the standard tube rack unless you are holding the tube next to the magnet.

Washing is best performed one tube at a time, and should be performed as follows:

1. Place the tube in a standard 1.7 ml microcentrifuge tube rack and open the cap.
2. Place the opened tube in the assembled magnetic stand. The beads will pellet more rapidly if the bottom of the tube is held against the magnet, as shown below, and then slowly lowered into the well. This will pellet the beads up onto the side of the tube.

3. Allow the beads to pellet completely and remove supernatant with a 1000 µl pipetteman. You can either leave the tube in the rack or pull it out when you remove the buffer. The beads will remain on the side of the tube, even when not next to the magnet.
4. Return the tube to the standard microcentrifuge tube rack, add 800 µl wash buffer and fully resuspend the beads by pipetting up and down.
5. Repeat steps 2-4 until desired washing steps are complete. After the final wash has been removed, the last traces of wash buffer should be removed with a 200 µl pipetteman.

Section C. 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 the 1st ChIP elution and desalting, -80°C. 4. After the 2nd ChIP elution, -20°C. 5. After the cross-link reversal, -20°C.
How much sheared chromatin should I use for a Re-ChIP reaction?	Best results are obtained when the amount of chromatin used in ChIP is from 1.5-3 x 10^6 cells. If it is assumed that human diploid cells contain 6.6 picograms of DNA and recovery after cell fixation and shearing is 70%, this is between 7 and 13.9 µg DNA. The minimum amount of sheared chromatin that can be used for Re-ChIP is 1 µg; the maximum is 50 µg.
Preparing a Re-ChIP reaction with a large volume.	It is better to set up several small ChIP reactions (200 µl each) and pool the samples at the end, rather than trying to ChIP a single large sample.

Problem/question	Recommendation
Poor enrichment with ChIP antibody.	In some cases, use of an antibody in ChIP results in lower-than-expected enrichment of a target of interest. This is often because 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. In this case, use more antibody when performing the ChIP. Alternatively, try to find an antibody that has been proven to work in ChIP, or that is known to recognize an epitope distinct from the one recognized by the unsatisfactory antibody.
There is no difference in band intensity between negative control and positive control.	See the recommendation below regarding increasing washing stringency.
	Decrease the number of PCR cycles (30, 32, 34 PCR cycles). The exponential phase of amplification occurs in PCR cycles where reaction components are still in excess and PCR products are accumulating at a constant rate. During this phase, each copy of DNA is being actively amplified, making it a better measure than endpoint PCR. In endpoint PCR, reagents such as nucleotides or primers may become exhausted. This can result in inefficient amplification, which can cause inaccurate quantification of the gene of interest. Thus, high background due to endpoint PCR can be decreased if the number of PCR cycles are reduced, so the results reflect exponential PCR. Real-time PCR can also be used in such cases.
	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 200-1500 bp fragments. If the DNA fragments are too large, you the background is increased. So, consider increasing the time of the enzymatic digestion, or the time and/or number of pulses for sonication.
	Confirm species specificity of your primers. You may need to redesign your primers.
	See the recommendation below regarding blocking the magnetic beads.
Strong PCR signal when using target PCR primers to amplify ChIP DNA that was isolated with a negative control (non-target) antibody.	In most cases, the washing procedure in the enclosed protocol is appropriate. However, when the background is high you can increase washing stringency in several ways: 1) After adding ChIP Buffer 1 and/or ChIP Buffer 2 during the wash steps, gently agitate the samples for several minutes before removing the buffer. 2) Perform additional washes. Sufficient ChIP Buffer 1 and ChIP Buffer 2 are provided for one “extra” wash of each per sample. 3) Add two washes using a high-salt buffer (20 mM Tris-Cl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 500 mM NaCl, pH 7.4), which is not provided. These additional washes should be performed after the washes with ChIP Buffer 1. Then, proceed with the ChIP Buffer 2 washes, as outlined in the protocol.
	Confirm the species specificity of your primers. You may need to redesign your primers.
	See the next recommendation regarding blocking the magnetic beads.
Is blocking of the magnetic beads ever required?	The beads provided are ready to use for most ChIPs. However, for applications highly sensitive to non-specific binding (such as when cloning ChIP DNA or using antibodies that require extra blocking), you may add blocking reagents to the ChIP reaction. In these cases, a combination of BSA (*e.g.* Sigma Cat. No. 4503) and either tRNA (*e.g.* Sigma Cat. No. R3629) or salmon sperm DNA (*e.g.* Sigma Cat. No. A-7888) can be added directly to the ChIP reaction. 2.5 µg/µl BSA and 1.25 µg/µl tRNA or 2.5 µg/µl Salmon sperm DNA (final concentrations) can be used as a starting point and more or less can be added as desired.
The gel for optimizing chromatin shearing has bands stuck in the wells, and smears from the top to bottom of the lane.	The sheared chromatin needs to have the cross-links reversed, protein removed (Proteinase K) and the RNA removed (RNase). Follow the DNA clean up protocol in Section A of the Appendix.
The PCR products are the correct size, but are very light.	Load more PCR product, and/or use smaller wells for the agarose gel. It should be noted that because the PCRs should be stopped while the reactions are in the linear phase of amplification, the yield of PCR product will be lower than in typical PCR amplifications, which are performed for maximum product yield. You can also perform more PCR cycles.

Problem/question	Recommendation
No PCR bands for Input DNA or ChIP'd samples.	In the presence of 0.8 mM total dNTP concentration, perform a $MgCl_2$ titration series in 0.5 mM increments over a range of 1-4 mM. This will identify the magnesium ion concentration that produces the highest yield of a specific PCR product. When using *Taq* DNA Polymerase, too little free magnesium ion results in little or no PCR product, while excess free magnesium ion can cause unwanted products and promote misincorporation.
	Confirm species specificity of your primers. You may need to redesign your primers.
No PCR products with real-time PCR	The DNA should be purified before performing real-time PCR. We recommend Active Motif's Chromatin IP DNA Purification Kit (Catalog No. 58002) prior to amplification. Its columns yield 50 µl; 2 µl is used for each PCR, providing enough DNA for 25 PCR reactions.
Low yield when using enzymatic shearing.	It is critical to perform dounce homogenization when using enzymatic shearing, and is highly recommended when using sonication shearing. (Our recommended dounce is the Kimble-Kontes Cat. No. 885302-002, 2 ml, with the B pestle.) To ensure cell lysis, look at 10 µl of the sample before and after homogenization under a phase contrast microscope using a hemocytometer. Check that the nuclei have been released: Intact cells have a dark central region (nucleus) surrounded by a halo of the less dense cytoplasm. Comparing cells before and after lysis makes it easier to determine the extent of lysis, as this can be difficult looking only at homogenized cells. If the cells are not lysed, perform an additional 10 strokes of the dounce homogenizer until the cells are lysed.
	Cross-linking for longer periods of time tends to cause cells to form into a giant cross-linked aggregate that is not sheared efficiently. Decrease the incubation time of the formaldehyde fixation step.
Low yield when using sonication shearing.	It is highly recommended to perform dounce homogenization, even when using sonication shearing, as this will increase your yield and improve your ChIP.
	Cross-linking for longer periods of time tends to cause cells to form into a giant cross-linked aggregate that is not sheared efficiently. Decrease the incubation time of the formaldehyde fixation step.
Low or no signal for 2nd ChIP when using the same antibody in both the 1st and 2nd ChIP reactions.	When using monoclonal antibodies, it is not recommended to use the same antibody for the 1st and 2nd ChIP reactions, which is sometimes done as a control for the 2nd ChIP. Small amounts of antibody may be present in the chromatin eluted from the 1st ChIP. While the elution conditions prevent any eluted antibody from being precipitated in the 2nd ChIP, eluted antibody can still bind its target protein. Because the eluted antibody and the 2nd antibody are the same, they both compete for the for the same binding site(s). Thus, the total number of epitopes available for binding by the 2nd antibody is reduced by binding of the eluted antibody. This effect is more pronounced for monoclonal antibodies because both antibodies are competing for a single, identical epitope on the target protein. As only the 2nd antibody can be precipitated, this may cause the PCR signal to be weak (or absent) in the 2nd ChIP when the same monoclonal antibody is used for both the 1st and 2nd ChIP reactions.
Low signal for 2nd ChIP when using ChIP-validated 1st and 2nd antibodies that should bring down interacting proteins.	Some antibody combinations seem to work better when used in one sequence than in the other. That is, in some cases the order that the antibodies are used can impact the strength of the signal seen by PCR of the 2nd ChIP. For this reason, if you get poor or unexpected results for the 2nd ChIP, you may wish to repeat the experiment using the 1st and 2nd antibodies in the opposite order. For example, in lanes 3 & 4 of Figure 1 on page 2 the RNA Pol II antibody was used in the 1st ChIP and TFIIB antibody was used in the 2nd ChIP. To test if the order these antibodies are used in Re-ChIP changes the results, you would perform a 1st ChIP with TFIIB antibody and a 2nd ChIP with RNA Pol II antibody.

Problem/question	Recommendation
How do I design PCR primers to analyze shearing efficiency and to map putative DNA-binding sites?	Negative control PCR primers can be used to demonstrate that chromatin was sufficiently sheared. For example, negative control primers can be designed to amplify a DNA fragment that is 2 kb away from the "Target DNA" (the region bound by the protein of interest). Following ChIP reactions (performed with antibody against the protein of interest and with a negative IgG), PCR is performed with the negative control primers and with primers that amplify the Target DNA. The PCR should show that the anti-protein-of-interest ChIP enriches for Target DNA, but not for the negative control DNA. This result would support the conclusion that the enrichment was due to protein binding to (or near) the putative Target, and not due to binding elsewhere on a very large (poorly sheared) chromatin fragment. Similarly, appropriately designed PCR primers can be used to roughly map the DNA Target of the protein of interest. For example, primers can be designed to amplify short (approximately 100 bp) DNA fragments that are progressively closer to the putative Target DNA (*e.g.* within 1.5 kb, 1 kb, 500 bp, 250 bp). This type of analysis can help confirm the exact binding site of the protein of interest. For such higher-resolution mapping, the chromatin must be extensively sheared (DNA fragment size should be less than 500 bp).

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

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