ChIP-IT® Express Enzymatic

Magnetic Chromatin Immunoprecipitation Kit & Enzymatic Shearing Kit

(version F7)

Catalog Nos. 53009 & 53035

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Overview

Chromatin Immunoprecipitation (ChIP) is a powerful tool for studying protein/DNA interactions[1, 2]. Intact cells are fixed with formaldehyde, which cross-links and preserves protein/DNA interactions. The DNA is then sheared into small, relatively uniform fragments using either sonication or enzymatic digestion, and specific protein/DNA complexes are immunoprecipitated with 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 in complex with the protein of interest (see Figure 1 on page 2).

ChIP is extremely useful for the study of chromatin biology and transcriptional regulation because it enables the localization of chromatin proteins, modified histones and transcription factors to specific DNA loci. Furthermore, because 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].

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

Active Motif's ChIP-IT® Express Enzymatic Kits contain proven ChIP reagents that provide a complete solution for convenient, accurate monitoring of protein/DNA interactions. The ChIP-IT Express Enzymatic Kits utilize protein G-coupled magnetic beads, which have greatly simplified and streamlined the ChIP protocol. A number of steps have been reduced in length or completely eliminated (see page 3 for details), making ChIP-IT Express an extremely rapid and efficient way to perform ChIP.

ChIP-IT Express Enzymatic Kits contain reagents sufficient to make 10 chromatin preparations, as well as 2 shearing optimizations, and to perform 25 ChIP reactions. If you need to shear more chromatin, the stand-alone ChIP-IT Express Shearing Kit provides just the reagents to perform and validate the shearing portion of the protocol. For additional protocols and to learn about products related to ChIP-IT Express, please visit **www.activemotif.com/chipitdocs**.

product	format	catalog no.
ChIP-IT® Express Enzymatic	25 rxns	53009
ChIP-IT® Express Enzymatic Shearing Kit	10 rxns	53035
ChIP-IT® Protein G Magnetic Beads	25 rxns	53014

Flow Chart of Process

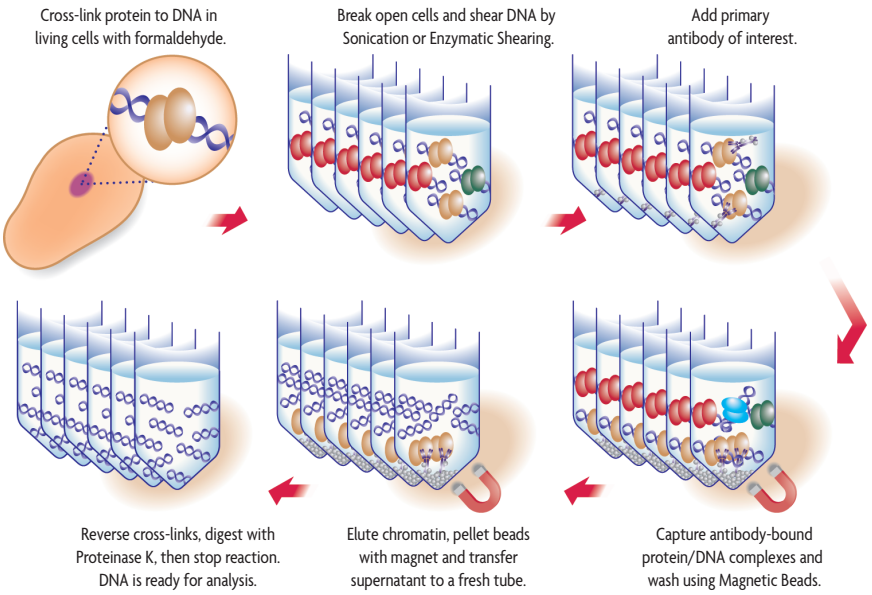

Figure 1: Improved chromatin immunoprecipitation using ChIP-IT Express.

In ChIP-IT Express, cells are treated with formaldehyde to fix protein/DNA interactions and then the fixed chromatin is sheared by either sonication or enzymatic digestion. The sheared chromatin is incubated with an antibody directed against a protein of interest, and antibody-bound protein/DNA complexes are precipitated through use of magnetic Protein G-coupled beads. The captured chromatin is then eluted, the cross-links are reversed, and the recovered DNA is analyzed by PCR to identify DNA loci associated with the protein of interest.

References

1. Solomon, M.J. *et al.* (1988) *Cell* 53(6): 937-47.
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3. Kuo, M.H. and Allis, C.D. (1999) *Methods* 19(3): 425-33.
4. Weinman, A.S. and Farnham, P.J. (2002) *Methods* 26: 37-47.
5. Caretti, G. *et al.* (2003) *J Biological Chem.* 278: 30435-30440.

ChIP-IT Express Enzymatic Advantages

Complete kits for rapid and efficient ChIP

- Shorter protocol and dramatically reduced hands-on time
- The possibility to perform numerous ChIP experiments simultaneously
- Compatible with multi-channel pipetting

The ChIP-IT Express and ChIP-IT Express Enzymatic Kits provide reagents and protocols to simplify all aspects of the chromatin immunoprecipitation procedure. The kits can be used to prepare chromatin, determine optimal conditions for shearing chromatin and perform ChIP reactions.

ChIP-IT Express Enzymatic Kits contain components to make 10 chromatin preparations as well as to perform 2 shearing optimizations, and quantities of all other components to perform 25 ChIP reactions. 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 beads and DNA.

Other steps in the ChIP protocol have also been optimized. The specialized Elution Buffer in combination with the Reverse Cross-linking Buffer saves time and eliminates the DNA loss that can occur during manipulations.

These improvements greatly reduce hands-on time and 8-, 16- or 24-ChIP experiments can easily be performed at the same time. This is not possible with traditional ChIP methods, which are time- and labor-intensive. For even higher throughput ChIP, Active Motif offers ChIP-IT Express HT (Catalog No. 53018), which enables you to perform 96 ChIPs simultaneously.

Shearing options – ChIP-IT Express (Sonication) and ChIP-IT Express Enzymatic (Enzymatic)

The **ChIP-IT Express Kit** provides reagents sufficient to prepare 10 sonication-sheared chromatin preparations as well as to perform 2 shearing optimizations. Using this protocol, each preparation of sheared chromatin requires one 15 cm tissue culture plate of cells and yields chromatin sufficient for up to 6 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 (see Appendix – Section D of this manual). If you want to prepare additional samples using sonication, the ChIP-IT Express Shearing Kit is sold separately (Catalog No. 53032).

The **ChIP-IT Express Enzymatic Kit** is similar to the ChIP-IT Express Kit, but it utilizes a proprietary Enzymatic Shearing Cocktail and Digestion Buffer to shear chromatin using enzymatic digestion instead of sonication. Because enzymatic shearing is solely time and temperature dependent, the problems associated with sonication (overheating and emulsification) are eliminated. Thus, enzymatic shearing is not only simpler, it is also easier to optimize shearing conditions and to get more reproducible shearing from prep to prep, which improves your ChIP results.

Companion Products

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

Because appropriate controls make ChIP interpretation and troubleshooting easier, Active Motif offers its **ChIP-IT Control Kits** for human, mouse and rat samples. These useful kits contain positive & negative control antibodies and species-specific positive control PCR primers. The kits are invaluable when validating antibodies for use in ChIP.

Validated **ChIP Control qPCR primer sets** are also available separately for use as positive and negative controls with many of the more common ChIP targets to confirm the success of the ChIP reactions. Use of our primer sets will save you the time and effort required to synthesize and test your own gene/species-specific control primers.

For users who need to make more sheared chromatin samples than is possible with the reagents provided in the ChIP-IT Express Kits, **ChIP-IT Express Shearing Kits** are also available separately.

If you are using an isotype of mouse monoclonal antibody that does not bind well to protein G, consider our **Bridging Antibody for Mouse IgG**; it binds with a strong affinity to both the protein G beads and the mouse primary antibody. This maximizes capture of mouse antibody-immune complexes, which improves results of ChIP and IP experiments that use mouse primary antibody.

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 blotting or 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 Appendix – Section G 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.

Active Motif's **ChIP-IT High Sensitivity Kit** is ideal for use when studying low abundance transcription factor targets, working with antibodies with sub-optimal binding affinities, or when starting ChIP with a limited number of cells. This assay provides higher sensitivity to overcome these obstacles. The ChIP-IT High Sensitivity Kit is compatible with the **ChIP-IT qPCR Analysis Kit** which can be used to simplify qPCR analysis and to enable normalization of data across multiple sample types and experiments.

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

A common experimental objective is to establish that two epigenetic marks or chromatin-associated proteins are present at the same locus in a single chromatin sample. This is done by sequential ChIP, where the sample is subjected to a second ChIP with a different antibody. Because sequential ChIP can be a complicated process, Active Motif offers its **Re-ChIP-IT Kit**, which uses the methodology developed for ChIP-IT-Express to simplify the technique of sequential ChIP.

ChIP is traditionally performed using formaldehyde-fixed tissues or cultured cell lines. In order to utilize the valuable retrospective data that is available from clinical samples, Active Motif has developed the **ChIP-IT FFPE Kit** for use with formalin-fixed paraffin-embedded (FFPE) tissue blocks and histology slides.

Kit Components and Storage
ChIP-IT Express Enzymatic Kit (Catalog No. 53009)

Please store each component at the temperature indicated in the table below. **Do not re-freeze the Protein G Magnetic Beads after you have received this kit.**

Reagents	Quantity	Storage / Stability
RNase A (10 µg/µl)	40 µl	-20°C for 6 months
5 M NaCl	200 µl	-20°C for 6 months
100 mM PMSF	475 µl	-20°C for 6 months
Proteinase K (0.5 µg/µl)	250 µl	-20°C for 6 months
Proteinase K Stop Solution	150 µl	-20°C for 6 months
Protease Inhibitor Cocktail (PIC)	2 x 100 µl	-20°C for 6 months
1X Lysis Buffer	16 ml	-20°C for 6 months
10X Glycine	33 ml	-20°C for 6 months
10X PBS	120 ml	-20°C for 6 months
Elution Buffer AM2	1.6 ml	-20°C for 6 months
Reverse Cross-linking Buffer	1.6 ml	-20°C for 6 months
ChIP Buffer 1	70 ml	-20°C for 6 months
ChIP Buffer 2	70 ml	-20°C for 6 months
Digestion Buffer	11 ml	-20°C for 6 months
Enzymatic Shearing Cocktail	6 µl	-20°C for 6 months
0.5 M EDTA	280 µl	-20°C for 6 months
Protein G Magnetic Beads*	650 µl	4°C for 6 months
Siliconized 1.7 ml microcentrifuge tubes	25	Room temperature
Bar Magnet	1	Room temperature
Mini Glue Dots	1 sheet	Room temperature

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

Kit Components and Storage
ChIP-IT Express Enzymatic Shearing Kit (Catalog No. 53035)

This component list is for the stand-alone ChIP-IT Express Enzymatic Shearing Kit, which contains only shearing reagents. The component list for the complete ChIP-IT Express Enzymatic Kit is on the preceding page. Please store each component at the temperature indicated in the table below.

Reagents	Quantity	Storage / Stability
RNase A (10 µg/µl)	40 µl	-20°C for 6 months
5 M NaCl	200 µl	-20°C for 6 months
100 mM PMSF	475 µl	-20°C for 6 months
Proteinase K (0.5 µg/µl)	250 µl	-20°C for 6 months
Proteinase K Stop Solution	150 µl	-20°C for 6 months
Protease Inhibitor Cocktail (PIC)	2 x 100 µl	-20°C for 6 months
1X Lysis Buffer	16 ml	-20°C for 6 months
10X Glycine	33 ml	-20°C for 6 months
10X PBS	120 ml	-20°C for 6 months
Digestion Buffer	11 ml	-20°C for 6 months
Enzymatic Shearing Cocktail	6 µl	-20°C for 6 months
0.5 M EDTA	280 µl	-20°C for 6 months

Additional materials required

- A ChIP-validated antibody directed against the protein of interest
- Dounce homogenizer with a small clearance pestle (*e.g.* Active Motif Catalog Nos. 40401 & 40415 with the tight-fitting pestle). Use of a homogenizer is required for enzymatic shearing and strongly recommended for shearing chromatin by sonication. Dounce homogenization greatly improves your chances for successful ChIP.
- Magnetic stand. You can assemble a magnetic stand using the provided bar magnet (see Appendix – Section E) or use commercially available stands (*e.g.* the Promega Magne-Sphere®
  Technology twelve-position Magnetic Separation Stand).
- 37% formaldehyde solution (formalin) with 10-15% methyl alcohol to prevent polymerization. We do not recommend paraformaldehyde.
- Phase contrast/tissue culture microscope and hemocytometer
- 50% Glycerol in dH_2O
- Phenol/chloroform (1:1) TE saturated pH 8 (DNA Purification, Molecular Biology Grade)
- 3 M Sodium Acetate pH 5.2 (purification of Input DNA and purification of sheared DNA prior to checking concentration by spectrophotometry or gel electrophoresis)
- 100% ethanol
- 70% ethanol
- 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 with a tube holder for 1.7 ml microcentrifuge tubes)
- Microcentrifuge (table top centrifuge 4°C) and microcentrifuge tubes
- Spectrophotometer for DNA quantitation
- Pipettors and tips (filter tips are recommended)
- Agarose gel electrophoresis apparatus
- Minimal cell culture media (growth media without serum)
- Cell scraper (rubber policeman)

Optional materials

- 8-well PCR strips (*e.g.* Thermo Fisher Part No. AB-0451)
- 1.7 ml siliconized Eppendorf tubes (*e.g.* Active Motif Catalog No. 53036)
- Chromatin IP DNA Purification Kit (*e.g.* Active Motif Catalog No. 58002)

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Points to consider:

- **Cell growth and chromatin preparation.** When planning an experiment, calculate the number of chromatin preparations you will require and determine the number of ChIP reactions you plan to perform on each chromatin preparation. Be sure to include the appropriate control ChIP reactions in your calculations. Also, note that if you wish to analyze the effect of particular compounds or culturing conditions on transcription factor/DNA interactions, you should prepare chromatin from control (untreated) cells as a reference sample.
- **Protein G-coated magnetic beads.** The supplied magnetic beads are ready to use once fully resuspended to form 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, and therefore should be resuspended just before pipetting. **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 4ºC.** The ChIP-IT Protein G Magnetic Beads are also sold separately (Catalog No. 53014).
- **Antibodies must be suitable for ChIP.** ChIP antibodies must recognize fixed, native protein that is bound to DNA and/or complexed with other proteins. Many antibodies that perform well in other applications do not perform in ChIP. Thus, ChIP performed with an antibody that has not been ChIP-validated must include appropriate controls (such as Active Motif's RNA pol II antibody, Catalog No. 39097) and a negative control IgG (mouse IgG for mouse monoclonals and rabbit IgG for rabbit polyclonals) to validate the chromatin preparation and the ChIP methodology. For your convenience, Active Motif sells ChIP-IT Control Kits for human, mouse and rat samples; these kits contain positive and negative control antibodies, appropriate positive PCR primers, PCR buffer and loading dye (see Related Products in Appendix).
- **Siliconized tubes.** Perform the ChIP reactions in the provided siliconized 1.7 ml microcentrifuge tubes or in 8-well PCR strips. (Do not use these tubes for preparing chromatin or isolating Input DNA, as you will then not have enough of them for the ChIP reactions.)

- **Bar magnet.** The provided bar magnet can be used with the provided siliconized 1.7 ml microcentrifuge tubes or 8-well PCR strips (see Appendix – Section E 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) can also be used.

- **Resuspend solutions completely.** Thaw the PMSF and the Proteinase K Stop Solution at room temperature until fully dissolved, which takes about 30 minutes. Vortex gently and spin down briefly before use.
- **Quantity of antibody.** Optimal results are typically achieved with 1-3 µg of antibody. However, this will vary according to the affinity of the antibody and the quality of the chromatin; you may need to use more of a particular antibody.
- **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

A. Cell Fixation & Shearing

This protocol describes fixation and enzymatic shearing of cells from one 15 cm plate (approximately 1.5 x 10^7 cells). (Appendix – Section D includes information on scaling the protocol for use with other amounts of cells.) This shearing protocol assumes that you have already optimized shearing conditions for your specific cell line and treatment. If you have not, do not use this protocol now. Instead, use the protocols in Appendix – Sections A-C to test multiple shearing conditions for your cell line and treatment to determine which are optimal. The optimized conditions can then be used with the protocols below.

Shearing tips: The most critical aspect of enzymatic shearing is efficient lysis of the cells so that the enzymes can access the chromatin. We strongly recommend a dounce homogenizer with a small clearance size pestle to mechanically shear the plasma membrane without disrupting the nuclei. Monitoring the cell lysis using a microscope can be very helpful. If the cells are difficult to lyse, even with the proper dounce homogenization, reduce the fixation to 5 minutes. It is important to not add the Enzymatic Cocktail until the cells are lysed.

The enzymes used in the Enzymatic Cocktail are random cleavers (sequence independent). How-

ever, they prefer open regions that are easily accessible, such as between nucleosomes. Therefore, digestion produces a specific DNA fragment size banding pattern: ~150 bp (1 nucleosome), ~300 (2 nucleosomes), ~450 bp (3 nucleosomes) *etc.*, with an ever decreasing band intensity as the molecular weights increase. This is an excellent method of producing sheared chromatin, provided you take a few extra steps to check that you have lysed the cells prior to adding the Enzymatic Shearing Cocktail. Once you have optimized the lysing conditions and the shearing time for a cell type, you can get consistent results every time.

ChIP-IT Enzymatic is also excellent for downstream applications that require very small DNA fragments (*i.e.* ChIP-Seq). Sequencing platforms require small DNA fragments (100-200 bp) so we suggest enzymatic shearing, which can produce smaller fragments more readily than sonication. Producing such small fragments with enzymatic digestion simply requires a longer incubation time (in the range of 15-30 minutes). With sonication, the size of the fragments may be limited to slightly larger sizes, which is mostly dependent on the equipment used.

Note: Several of the buffers used below require addition of PMSF and protease inhibitors (PIC). Thaw these reagents before starting the chromatin preparation (*i.e.* 30 minutes at room temperature), then add to the buffers immediately before use.

1. Grow cells to 70-80% confluency in one 15 cm plate. Stimulate cells as desired to activate the pathway of interest.
2. When cells are ready to harvest, freshly prepare the following solutions. The volumes below are calibrated to one 15 cm plate:
   a. **Fixation Solution:** Add 0.54 ml 37% formaldehyde to 20 ml minimal cell culture medium and mix thoroughly. Leave at room temperature.
   b. **1X PBS Solution**: Add 2.33 ml 10X PBS to 21 ml dH_2O, mix and place on ice.
   c. **Glycine Stop-Fix Solution:** Combine 1 ml 10X Glycine Buffer, 1 ml 10X PBS and 8 ml dH_2O. Mix well and leave at room temperature.
   d. **Cell Scraping Solution:** Add 0.6 ml 10X PBS to 5.4 ml dH_2O, mix and place on ice.
3. Pour medium off the cells and add 20 ml Fixation Solution to each plate. Incubate on a shaking platform for 10 minutes at room temperature.

   Note: In standard protocols, chromatin is fixed for 10 minutes prior to shearing. While these are typical fixation conditions, some antibody/chromatin combinations may work better with shorter fixation times.

4. Pour Fixation Solution off and wash by adding 10 ml ice-cold 1X PBS to each plate. Rock the plate for 5 seconds, then pour off the PBS.
5. Stop the fixation reaction by adding 10 ml Glycine Stop-Fix Solution to each of the plates. Swirl to cover, and then rock at room temperature for 5 minutes.
6. Wash each plate by pouring off the Glycine Stop-Fix Solution, then adding 10 ml ice-cold 1X PBS. Rock the plate for 5 seconds, then pour off the PBS.
7. Just before use, add 30 µl 100 mM PMSF to Cell Scraping Solution. Add 5 ml of this ice-cold

Cell Scraping Solution to each plate and scrape cells with a rubber policeman. Hold the plate at an angle and scrape cells down to collect them at the bottom edge of the plate. Use a 1 ml pipette to transfer the cells to a 15 ml conical tube on ice.

8. Pellet the cells from step 7 by centrifugation for 10 minutes at 2,500 rpm (720 RCF) at 4°C.
9. Remove the supernatant and discard. At this point the protocol can be continued or the pellet can be frozen. If freezing the pellet, add 1 µl 100 mM PMSF and 1 µl PIC and freeze at -80°C.

Preparation of Sheared Chromatin by Enzymatic Shearing

The section below describes the isolation and preparation of chromatin using enzymatic shearing.

1. Thaw pellet (if necessary) on ice and resuspend cells in 1 ml ice-cold Lysis Buffer supplemented with 5 µl PIC + 5 µl PMSF. Pipette gently and vortex briefly to resuspend. Incubate on ice for 30 minutes.

   During this incubation, prepare a working stock of Enzymatic Shearing Cocktail (200 U/ml) by diluting the supplied Enzymatic Shearing Cocktail (2 x 10^4 U/ml) 1:100 with 50% glycerol in dH_2O (not provided). The 200 U/ml working stock will be used in step 5 below and is stable at 4°C for 1-2 weeks.

Reagent	1-2 rxns	3-5 rxns	6-10 rxns
Stock Enzymatic Shearing Cocktail (2 x 10^4 U/ml)	0.5 µl	1 µl	2 µl
50% glycerol	49.5 µl	99 µl	198 µl

2. Transfer the cells to an ice-cold dounce homogenizer. Dounce on ice with 10 strokes to aid in nuclei release.

   Monitor Cell Lysis: To ensure cell lysis, take 10 µl of the cell lysate from the dounce and look at it under a phase contrast microscope using a hemocytometer to verify that the nuclei have been released. It is often helpful to look at the cells before and after the lysis step as this makes it easier to identify the nuclei versus whole cells. Intact cells should have a dark central region (nucleus) surrounded by a halo of less dense cytoplasm. In lysed cells, the nuclei will appear as dots surrounded by asymmetric debris. If the cells are not lysed, then dounce on ice with an additional 10 strokes, or until the cells are lysed.
3. Transfer cells to a 1.7 ml microcentrifuge tube and centrifuge for 10 minutes at 5,000 rpm (2,400 RCF) in a 4°C microcentrifuge to pellet the nuclei.
4. Carefully remove the supernatant and discard. Resuspend the nuclei pellet in 350 µl Digestion Buffer (supplemented with 1.75 µl PIC and 1.75 µl PMSF) and incubate for 5 minutes at 37°C.
5. Add 17 µl of the working stock of Enzymatic Shearing Cocktail (200 U/ml) to the pre-warmed nuclei and vortex to mix.
6. Incubate the tube at 37°C for the amount of time that you determined to be optimal for your cell line (previously determined in Appendix – Sections A-C). Vortex the tube briefly approximately every 2 minutes during the incubation to increase the shearing efficiency.

7. Stop the reaction by adding 7 µl ice-cold 0.5 M EDTA to each tube; chill on ice 10 minutes.

8. Centrifuge the sheared chromatin samples for 10 minutes at 15,000 rpm (18,000 RCF) in a 4°C microcentrifuge. Carefully transfer supernatant to a fresh 1.7 ml microcentrifuge tube. This is the sheared chromatin. It can be used right away or stored at -80°C. Before freezing, remove 50 µl for use in assessing the efficiency of your DNA shearing and determining the DNA concentration. The remaining chromatin (approximately 350 µl) is sufficient for up to 6 ChIP reactions and should be aliquoted before freezing to minimize freeze-thaw cycles.

   Note: Use the 50 µl sample removed above in the DNA Clean Up protocol in Appendix – Section C to check the DNA concentration and confirm that the chromatin has been sheared. You will need the concentration to set up you ChIP reactions.

Protocols – Chromatin Immunoprecipitation

B. Immunoprecipitation

1. Thaw chromatin (if necessary) on ice. Transfer 10 µl to a microcentrifuge tube; this tube is the "Input DNA" that will be processed in Step D6 and then be used as a control in PCR analysis. Store this sample at 4ºC if it will be used within 6 hours; otherwise, store it at -20ºC.

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. Be sure to use the DNA concentration that was determined for your sheared chromatin sample to calculate the volume to use. 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 (7-25 µg)*	20-60 µl	61-150 µl
Protease Inhibitor Cocktail (PIC)	1 µl	2 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 with 1-50 µg of chromatin.

3. Cap tubes and incubate on an end-to-end rotator for 4 hours at 4°C. In some cases, sensitivity may be improved if the incubation is performed overnight.

4. Spin tube briefly to collect liquid from the inside of the cap.
5. Place tube on magnetic stand to pellet beads on the tube side.
6. Carefully remove and discard supernatant.

C. Wash Magnetic Beads

Note: Do not allow the beads to "dry out". Allow no more than 1 minute to elapse between removing buffer and then adding the next wash or the elution buffer. For suggestions regarding bead washing methods, see Appendix – Section E.

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.

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

1. Resuspend the 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 the eluted chromatin and mix by pipetting up and down. Place tubes in magnetic stand; allow beads to pellet to the sides of the tubes.
5. Transfer the supernatant, which contains the chromatin, to a fresh tube.
6. It is now time to process the "Input DNA" sample: take the 10 µl Input DNA aliquot (that was set aside in Step B1 above) and thaw on ice, if needed. Add 88 µl ChIP Buffer 2 and 2 µl 5M NaCl to the Input DNA sample only, so that its final volume is 100 µl.
7. Incubate the ChIP and Input DNA samples at 95°C for 15 minutes in a thermocycler.

   Note: If you are using larger, thicker-walled microcentrifuge tubes, perform a 2.5 hour incubation at 65°C. More proteinaceous samples may need a longer incubation time. The sample may be stored at -20°C at this point.

8. Return tubes to room temperature, spin the tubes briefly if liquid has collected on the inside of the caps, then add 2 µl Proteinase K.
9. Cap the tubes, mix well and incubate at 37°C for 1 hour. During this incubation, place the Proteinase K Stop Solution at room temperature for 30 minutes to 1 hour.

10. Return the tubes to room temperature and add 2 µl Proteinase K Stop Solution. Briefly centrifuge the tubes to collect liquid from the caps. The DNA can be used immediately in PCR or stored at -20°C.

Protocols – PCR Analysis

E. End Point PCR

The protocol below is a guideline for optimizing PCR analysis of DNA collected by ChIP. In order to obtain reliable comparisons of the DNA amounts collected in different ChIP reactions, end point PCR must be stopped during the linear phase of amplification. As this window of amplification differs between samples and PCR primer sets, the correct number of PCR cycles must be determined empirically. Real-time PCR analysis simplifies these considerations, as it is possible to determine the Ct value of each sample, representing the cycle number at which linear amplification begins. Due to these advantages, we advise that you use real-time PCR whenever possible.

PCR should be performed on four DNA templates: DNA from ChIP performed using positive and negative control antibodies (such as the positive control RNA pol II antibody and Negative Control IgG supplied in Active Motif's ChIP-IT Control Kits), the Input DNA sample and DNA from ChIP with the test antibody. A water-only control should also be performed to ensure the PCR reagents are not contaminated.

Design of the primers

Analyze your potential primer pairs using an *in silico* PCR program (*i.e.* the UCSC Genome Browser at http://genome.cse.ucsc.edu/cgi-bin/hgPcr) to ensure that the primers selected will produce a single amplicon from the genomic DNA of the species from which the DNA is being amplified. Ideally, the amplicons should be 150-400 bp in length for standard PCR. Use of PCR design programs can be helpful in selecting good primer pairs.

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.

Setting up and performing PCR

In the example below, PCR reactions are set up using 2 different PCR cocktails, which contain positive & negative control PCR primer sets. If you are using a positive control antibody from one of Active Motif's ChIP-IT Control Kits, use only positive control PCR primers with that antibody; our positive control antibodies were chosen because they bind to many regions in the genome, making it impractical to design "negative control" primers for our positive control antibodies. For analysis of ChIP performed with other antibodies, it may be possible, depending on the antibody, to include both positive and negative PCR primer sets that are appropriate for that antibody.

1. Program the thermocycler. The program should start with an initial melt step at 94°C for 3 minutes, then 30-36 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. 36 cycles is a good starting point; you may need to optimize the number of cycles for your system.
2. **IMPORTANT:** Dilute the Input DNA sample 1:10 by adding 20 µl Input DNA to 180 µl dH_2O.

3. Use the table below to label PCR tubes and add the PCR templates and water-only control, keeping the tubes on ice. Add the PCR cocktails you will make in Step 4:

Reaction No.	PCR Template (5 µl each)	PCR cocktail (20 µl each)
1	ChIP DNA – Positive control antibody	Positive PCR cocktail
2	ChIP DNA – Negative control IgG	Positive PCR cocktail
3	Input DNA (diluted 1:10)	Positive PCR cocktail
4	ChIP DNA – Test antibody	Positive PCR cocktail
5	H_2O (no DNA control)	Positive PCR cocktail
6	ChIP DNA – Positive control antibody	Negative PCR cocktail
7	ChIP DNA – Negative control IgG	Negative PCR cocktail
8	Input DNA (diluted 1:10)	Negative PCR cocktail
9	ChIP DNA – Test antibody	Negative PCR cocktail
10	H_2O (control)	Negative PCR cocktail

4. Set up the Positive PCR cocktail and the Negative PCR cocktail on ice according to the tables below. 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, in Active Motif's ChIP-IT Control Kits only positive control PCR primers are provided with the positive control antibody and Negative control IgG. (These are provided as a mixture of forward and reverse primers, so use 4 µl). However, for your test antibody we recommend to design and test both positive and negative PCR primer sets, if possible.

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

5. Add 20 µl of the appropriate PCR cocktail to each of the 5 µl PCR templates (on ice) prepared in Step 3, for a total volume of 25 µl. Mix each reaction by pipetting up and down. 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 1. After the cycles are complete, remove the tubes and place on ice.
7. These PCR reactions can be immediately analyzed as described below, or stored at -20°C.

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 human GAPDH positive control primers are 166 bp; those from the mouse EF1α primers are 233 bp, while the rat actin primers produce 223 bp PCR products. Use either a 50 or 100 bp ladder as the migration standard. Run the gel until the PCR products are well separated from the primers and primer dimers. Stain gel and analyze.

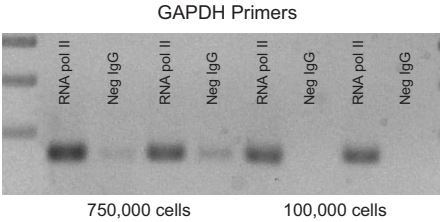


Figure 2: PCR of chromatin immunoprecipitation performed on 100,000 cells using ChIP-IT Express.

HeLa cells were fixed for 10 minutes with 1% formaldehyde and then chromatin was prepared by sonication shearing (5 pulses). ChIP was performed in duplicate on chromatin isolated from 100,000 and 750,000 cells using a Negative Control IgG and an RNA pol II antibody. The DNA isolated through these ChIP reactions was then analyzed by 36 cycles of PCR using GAPDH positive control primers. (These antibodies and primers are available as the ChIP-IT Control Kit – Human. Kits for mouse and rat are also available; see Related Products.) Ten µl of each PCR was separated on a 1% agarose gel and visualized by UV-illumination following ethidium bromide staining. PCR using the GAPDH primers on DNA isolated with the RNA pol II antibody reproducibly generated more product than similar reactions performed on DNA isolated using the Negative Control IgG. These results demonstrate that ChIP performed with RNA pol II antibody greatly enriched for GAPDH promoter DNA, while ChIP performed with negative IgG did not.

F. Real-time PCR

Following the final elution, cross-link reversal and proteinase K digestion of the immunoprecipitated chromatin, the samples should be subjected to a DNA clean-up step prior to certain downstream applications. For real-time PCR, we suggest using the Active Motif Chromatin IP DNA Purification Kit (Catalog No. 58002) prior to amplification. These columns yield 50 µl; 2 µl will be used for each PCR, giving you enough DNA for 25 PCR reactions. We suggest that you use a commercially available SYBR Green PCR Kit and a real-time thermocycler.

A. Design of the primers

- Analyze your potential primer pairs using an *in silico* PCR program (*i.e.* the UCSC Genome Browser at http://genome.cse.ucsc.edu/cgi-bin/hgPcr). Please note that primer sets that have worked in end point PCR may not work in real-time PCR.
- 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 50-150 bp in length for real-time PCR.
- G/C stretches at the 3´ ends of primers should be avoided.
- The difference in melting temperature between the forward and reverse primers should not exceed 3°C.

B. Generation of a standard curve to accurately determine fold enrichment

Note: This step is not essential, but many downstream calculations will rely on a standard curve.

1. To test the efficiency of your primers, produce a standard curve by performing qPCR with your primer set on known DNA quantities of Input DNA in triplicate. Run three to five samples that are 10-fold dilutions, *e.g.* 0.005 ng, 0.05 ng, 0.5 ng, 5 ng and 50 ng.
2. Run the ChIP and IgG samples along with the dilution series of the Input DNA standards. Every primer set will have a different amplification profile, so the Ct values can then be plotted to create a linear regression plot. This may not be necessary as most real-time thermocyclers will create a standard curve automatically.
3. Ct = Threshold Cycle (cycle number where the signal exceeds the background threshold level).
4. Plot Ct vs. DNA quantity (log scale) of the dilutions to produce the standard curve (Figure 3).

Two methods for calculating the fold enrichment using the slope of the standard curve. The first method is sufficient for most circumstances, but an optional second method is presented as well.

Method 1:

1. This first method entails (a) solving for the DNA quantity of the ChIP and IgG samples, then (b) calculating the Fold Enrichment of the ChIP sample relative to the IgG sample:

   X = DNA quantity
   Y = Ct
   M = slope of the standard curve line.
   B = the Ct value where X = 1, (*e.g.* 27.46 cycles in Figure 3)
   ChIP Ct = 22.77 cycles (from Figure 3)
   IgG Ct = 30.22 cycles (from Figure 3)

   (a) $Y = M(\log X) + B$ or
   $\log(X) = (Y - B) \div M$

   (b) Fold Enrichment = ChIP DNA quantity ÷ IgG DNA quantity

   Using the data from Figure 3, calculations are performed as follows:
   Solve for X using the known values of the slope, y-intercept, ChIP Ct and IgG Ct:

   (a) $\log(X) = (Y - B) \div M$
   i.e.: log(DNA quantity) = (Ct – y-int) ÷ slope

   For the ChIP sample, log(X) = (22.77 – 27.463) ÷ -3.508, so X = 21.767 ng
   For the IgG sample, log(X) = (30.22 – 27.463) ÷ -3.508, so X = 0.1637 ng

   (b) Solve the Fold Enrichment using the calculated DNA quantities:
   21.767 ng ÷ 0.1637 ng = 133 fold enrichment

Method 2:

2. This second method calculates enrichment as a ratio of the amplification efficiency of the ChIP sample over that of the IgG.

   The efficiency of the primers can be calculated using the slope of the standard curve and the following formula:

   % Efficiency = $[10^{(-1/\text{slope})} - 1] \times 100\%$

   In Figure 3, the slope = -3.508
   % Efficiency = $[10^{(-1/\text{slope})} - 1] \times 100\% = [10^{(-1/-3.508)} - 1] \times 100\%$
   % Efficiency = [1.928 - 1] x 100% = 0.928 x 100%
   % Efficiency = 92.8%

   The ideal efficiency is 100% ±10% (*i.e.* 90-110%, where -3.32 = 100% efficiency). In this example, the primer set falls within the acceptable range. Therefore, the amplification efficiency of the primer can be used to determine the amplification efficiency of the ChIP sample and the IgG sample.

   AE = amplification efficiency (is a factor of the primer efficiency) = $10^{(-1/\text{slope})}$
   Fd = dilution factor (*i.e.* 1)

   (a) % ChIP = $AE^{(\text{Input Ct - ChIP Ct})} * (Fd)(100)$
   (b) % IgG = $AE^{(\text{Input Ct - IgG Ct})} * (Fd)(100)$
   (c) Fold Enrichment = % ChIP ÷ % IgG

   Using the data from Figure 3, calculations are performed as follows:

Slope = -3.508 (from Figure 3)

AE = $10^{(-1/\text{slope})}$ = $10^{(-1/-3.508)}$ = 1.928

Input Ct = 21.36 cycles (use the value of the Input DNA standard closest to the ChIP sample, which is the 50 ng Input DNA sample in Figure 3)

ChIP Ct = 22.77 cycles (from Figure 3)

IgG Ct = 30.22 cycles (from Figure 3)

(a) % ChIP = $(1.928^{(21.36 - 22.77)})(1)(100)$

% ChIP = $(1.928^{(-1.41)})(1)(100)$

% ChIP = 39.62%

(b) % IgG = $(1.928^{(21.36 - 30.22)})(1)(100)$ = 0.3%

% IgG = (1.928(-8.86))(1)(100)

% IgG = 0.3%

(c) Solve the Fold Enrichment using the calculated amplification efficiencies:

39.62% ÷ 0.3% = 132 fold enrichment

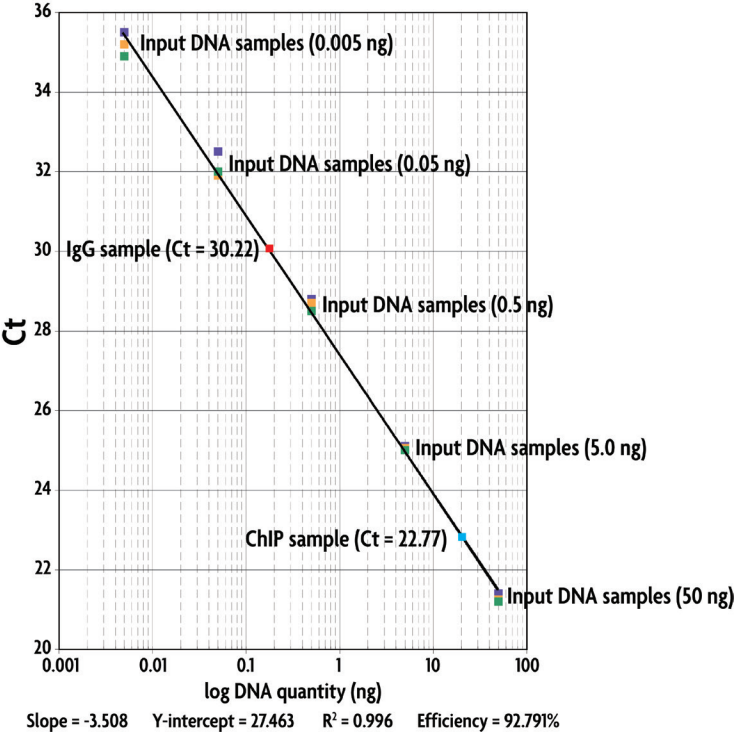


Figure 3: Standard curve produced from ten-fold dilutions of Input DNA.

Five 10-fold dilutions of Input DNA were qPCR amplified in triplicate along with the ChIP and IgG samples using GADPH primers. The values for each amount of Input DNA were plotted and used to produce the standard curve. Its slope and y-intercept values were used with the Ct values of the samples to calculate the fold enrichment.

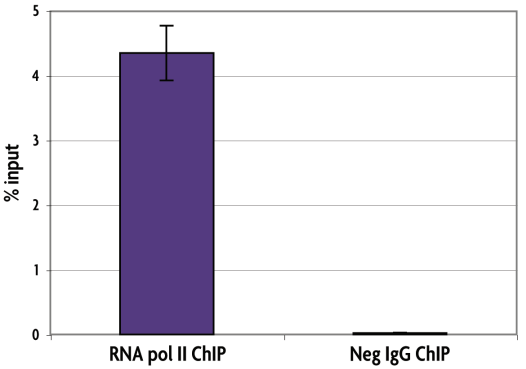


Figure 4: ChIP enrichment of GAPDH promoter DNA using RNA pol II antibody and Negative Control IgG.

HeLa cells were fixed for 10 minutes with 1% formaldehyde and then chromatin was prepared by sonication shearing (5 pulses). ChIP was performed using ChIP-IT Express on chromatin isolated from 750,000 cells using a Negative Control IgG and an RNA pol II antibody (Catalog No. 39097). Real-time PCR was performed on DNA purified from each of the ChIP reactions using a primer pair specific for the GAPDH gene. (These antibodies and primer set are available together in the ChIP-IT Control Kit – Human (Catalog No. 53010)). These results demonstrate that ChIP performed with RNA pol II antibody greatly enriched for GAPDH promoter DNA, while ChIP performed with negative IgG did not.

Appendix

Optimizing the Shearing Conditions

Chromatin shearing conditions can vary significantly depending on the cell type and, occasionally the cell culture & cell stimulation conditions. However, after shearing has been optimized for a given cell type, those conditions usually give consistent results with that cell type. For this reason, we recommend use of the following protocols to determine the optimal shearing conditions the first time you make chromatin from a cell line. You can then use the optimized conditions to prepare chromatin from this cell type and stimulation conditions.

Section A. Cell Fixation to Optimize Shearing Conditions

Please read Experimental Design before starting. Note that although ~350 µl of chromatin is prepared, only 200 µl is used to analyze shearing efficiency. If PIC and PMSF are included in the fixation and digestion buffers as directed, the unused ~150 µl of chromatin can be stored frozen while optimal shearing conditions are identified. This chromatin can then be thawed, sheared according to the optimal conditions, and used in ChIP. Use of PIC and PMSF during optimization will, however, reduce the number of shearing and ChIP reactions that can be performed once you have established optimal shearing conditions and are ready to perform ChIP.

Note: If you wish to use the unused chromatin in ChIP, thaw the PIC and PMSF before starting the protocol and add to the buffers immediately before use. Freeze the unused chromatin during shearing analysis. If you are performing optimization simply to identify shearing parameters, do not add PIC and PMSF to the buffers. This will conserve these reagents so that you can use them to prepare additional samples of sheared chromatin using your optimized conditions, and to perform additional ChIP reactions.

1. Grow cells to 70-80% confluency in one 15 cm plate. Stimulate cells as desired to activate the pathway of interest.
2. When cells are ready to harvest, freshly prepare the following solutions. The volumes below are calibrated to one 15 cm plate (approximately 1.5 x 10^7 cells):
   a. **Fixation Solution:** Add 0.54 ml 37% formaldehyde to 20 ml minimal cell culture medium and mix thoroughly. Leave at room temperature.
   b. **1X PBS Solution**: Add 2.33 ml 10X PBS to 21 ml dH_2O, mix and place on ice.
   c. **Glycine Stop-Fix Solution:** Combine 1 ml 10X Glycine Buffer, 1 ml 10X PBS and 8 ml dH_2O. Mix well and leave at room temperature.
   d. **Cell Scraping Solution:** Add 0.6 ml 10X PBS to 5.4 ml dH_2O, mix and place on ice.
3. Pour medium off the cells and add 20 ml Fixation Solution to each plate. Incubate on a shaking platform for 10 minutes at room temperature.

Note: In standard protocols, chromatin is fixed for 10 minutes prior to shearing. While these are typical fixation conditions, some antibody/chromatin combinations may work better with shorter fixation times.

4. Pour Fixation Solution off and wash by adding 10 ml ice-cold 1X PBS to each plate. Rock the plate for 5 seconds, then pour off the PBS.
5. Stop the fixation reaction by adding 10 ml Glycine Stop-Fix Solution to each of the plates. Swirl to cover, and then rock at room temperature for 5 minutes.
6. Wash each plate by pouring off the Glycine Stop-Fix Solution, then adding 10 ml ice-cold 1X PBS. Rock the plate for 5 seconds, then pour off the PBS.
7. Just before use, add 30 µl 100 mM PMSF to Cell Scraping Solution. Add 5 ml of this ice-cold Cell Scraping Solution to each plate and scrape cells with a rubber policeman. Hold the plate at an angle and scrape cells down to collect them at the bottom edge of the plate. Use a 1 ml pipette to transfer the cells to a 15 ml conical tube on ice.
8. Pellet the cells from step 7 by centrifugation for 10 minutes at 2,500 rpm (720 RCF) at 4°C.
9. Remove the supernatant and discard. At this point the protocol can be continued or the pellet can be frozen. If freezing the pellet, add 1 µl 100 mM PMSF and 1 µl PIC and freeze at -80°C.

Section B. Optimization of Enzymatic Shearing

Please read **Shearing tips** on page 9 before beginning. Note that although ~350 µl of chromatin is prepared, only 200 µl is used to analyze shearing efficiency.

1. Thaw pellet (if necessary) on ice and resuspend cells in 1 ml ice-cold Lysis Buffer supplemented with 5 µl PIC + 5 µl PMSF. Pipette gently and vortex briefly to resuspend. Incubate on ice for 30 minutes.

   During this incubation, prepare a working stock of Enzymatic Shearing Cocktail (200 U/ml) by diluting the supplied Enzymatic Shearing Cocktail (2 x 10^4 U/ml) 1:100 with 50% glycerol in dH_2O (not provided). The 200 U/ml working stock will be used in step 5 below and is stable at 4°C for 1-2 weeks.

Reagent	5 rxns
Stock Enzymatic Shearing Cocktail (2 x 10^4 U/ml)	0.5 µl
50% glycerol	49.5 µl

2. Transfer the cells to an ice-cold dounce homogenizer. Dounce on ice with 10 strokes to aid in nuclei release.

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

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

3. Transfer cells to a 1.7 ml microcentrifuge tube and centrifuge for 10 minutes at 5,000 rpm (2,400 RCF) in a 4°C microcentrifuge to pellet the nuclei.
4. Carefully remove the supernatant and discard. Resuspend the nuclei pellet in 350 µl Digestion Buffer (supplemented with 1.75 µl PIC and 1.75 µl PMSF) and incubate this solution for 5 minutes at 37°C.
5. Transfer 50 µl of the chromatin in Digestion Buffer to each of 4 fresh microcentrifuge tubes and incubate the tubes at 37°C for 2 minutes. Freeze the remaining ~150 µl chromatin at -80°C. (After you have determined which condition provides optimal shearing, this chromatin can be sheared as in step 6 using 7.5 µl working stock Enzyme.)
6. To optimize shearing conditions using Enzymatic Digestion, set up 4 reactions as indicated below. Vortex the tubes on a low setting to mix components. Vortex the tube briefly approximately every 2 minutes during the incubation to increase the shearing efficiency.
    a. 50 µl chromatin plus 2.5 µl dH_2O (No Enzyme) – incubate for 10 minutes at 37°C
    b. 50 µl chromatin plus 2.5 µl working stock Enzyme – incubate for 5 minutes at 37°C
    c. 50 µl chromatin plus 2.5 µl working stock Enzyme – incubate for 10 minutes at 37°C
    d. 50 µl chromatin plus 2.5 µl working stock Enzyme – incubate for 15 minutes at 37°C
7. Stop the reactions by adding 1 µl ice-cold 0.5 M EDTA to each tube. Chill on ice 10 minutes.
8. Centrifuge the sheared chromatin samples for 10 minutes at 15,000 rpm (18,000 RCF) in a 4°C microcentrifuge. Collect the supernatant. This sheared chromatin can be stored at -80°C. Or, continue immediately with Section C below to reverse cross-links and purify the chromatin prior to gel analysis.

Section C. DNA Clean Up to Assess Shearing Efficiency and DNA Concentration

1. If necessary, thaw the 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 65°C in a water bath or a thermocycler for 4 hours to overnight 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 10 µl Proteinase K to each sample and incubate at 42°C for 1.5 hours.

Note: If you intend to use a spectrophotometer to determine the DNA concentration, the DNA must first be cleaned up. Column purification is not recommended as the high protein content may clog the column. Therefore, the DNA should be phenol/chloroform extracted and precipitated, which is performed as follows:

a. Add 200 µl 1:1 phenol/chloroform TE saturated pH 8 to the sample, vortex to mix completely and centrifuge for 5 minutes at maximum speed in a microcentrifuge.

b. Transfer the aqueous phase 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% ice cold ethanol without disturbing the pellet and spin for 5 minutes at maximum speed in a 4°C microcentrifuge.

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 absorbance at 260 nm to determine the DNA concentration (1.0 A_{260} unit = 50 µg/ml).

The DNA concentration of the aliquot can be used to back calculate the concentration of the sheared chromatin sample. ***Optional, but recommended:*** If you are performing ChIP on multiple chromatin samples, such as non-treated and treated samples, use the DNA concentrations so that the initial quantity of DNA is equal for all ChIP reactions (7-25 µg is recommended). This ensures that equal amounts of chromatin are used per IP, and that the relative differences between the treatment groups are comparable.

6. We recommended loading two different quantities of each sheared sample on the gel to ensure one falls within an acceptable range. 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.

7. Optimal enzymatic shearing should result in a 200-1500 bp banded pattern similar to that shown in lane 4 of Figure 5 below.

8. If PIC and PMSF were used during the optimization experiment, the remaining ~150 µl of chromatin that was frozen in Step B5 above can be. sheared according to the optimal conditions. The digest volumes should be proportionate to the digest used in step 6 of Section B above. For example, ~150 µl unsheared chromatin will require 7.5 µl working stock Enzyme. Incubate at 37°C for the time that yielded suitable chromatin. Stop the reaction with 3 µl ice-cold 0.5 M EDTA. Chill on ice 10 minutes. Centrifuge the sample for 10 minutes at 15,000 rpm (18,000 RCF) in a 4°C microcentrifuge. Collect the supernatant. This sheared chromatin can be stored at -80°C for up to a year or used immediately in ChIP reactions.

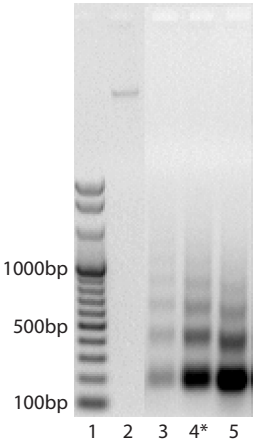


Figure 5: Gel analysis of enzymatic shearing (ChIP-IT Express Enzymatic)
HeLa cells were fixed for 10 minutes with 1% formaldehyde and then chromatin was prepared using the ChIP-IT Express Enzymatic Kit protocol. Chromatin was sheared with the Enzymatic Shearing Cocktail for 5, 10 & 15 minutes and the 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: In this experiment, the DNA treated for 10 minutes was optimal, and was then used successfully in ChIP.

Section D. Scale Up/Down of Chromatin Preparation

Our standard chromatin preparation protocols use cells grown in one 15 cm tissue culture plate (approximately 1.5 x 10^7 cells) and yield enough material to perform up to 6 ChIP reactions. Depending on your experiments, you may wish to work with different volumes of cells. The comments and table below are designed to help adapt our protocols for using different cell numbers.

- It is not recommended to use a sample with less than 500 µl or more than 2 ml of the Lysis Buffer for the cell lysis and dounce homogenization steps.
- If you intend to compare ChIP results from various samples, treat the samples equally. For example, grow induced and uninduced cells in the same size plate and to the same density, then use equal volumes and shearing conditions. This will help ensure that the chromatin preparations are equivalent in terms of cell numbers (genome equivalents), DNA shearing efficiency, *etc*. Quantify the DNA in the chromatin preparations (by following the DNA Clean Up protocol above) and then use equal mass quantities of chromatin in each ChIP assay.
- Each human diploid cell contains 6.6 picograms of DNA. This can be used to estimate DNA in a chromatin preparation if the number of cells in the starting material is known. We estimate DNA recovery of chromatin shearing to be about 60-70%, depending on the cell/tissue type.

	1 well of a 24-well plate	10 cm plate	15 cm plate	3 x 15 cm plates
Number of Cells (tissue weight)	130,000 (–)	0.66 x 10^7 (–)	1.5x 10^7 (~75 mg)	4.5x 10^7 (~225 mg)
Fixation Solution	2 ml	10 ml	20 ml	60 ml (20 ml/plate)
Glycine Stop-Fix	1 ml	5 ml	10 ml	30 ml (10 ml/plate)
1X PBS	2 x 1 ml	2 x 5 ml	2 x 10 ml	2 x 30 ml (2 x 10 ml/ plate)

	1 well of a 24-well plate	10 cm plate	15 cm plate	3 x 15 cm plates
Cell Scraping Solution + PMSF	500 µl + 2.5 µl PMSF	1 ml + 5 µl PMSF	5 ml + 30 µl PMSF	15 ml + 90 µl PMSF (5 ml + 30 µl/plate) Pool the 3 plates
Lysis Buffer + PIC + PMSF	200 µl + 1 µl PIC + 1 µl PMSF	500 µl + 2.5 µl PIC + 2.5 µl PMSF	1 ml + 5 µl PIC + 5 µl PMSF	3 ml + 15 µl PIC + 15 µl PMSF
Digestion Buffer + PIC + PMSF	50 µl + 0.25 µl PIC + 0.25 µl PMSF	175 µl + 0.875 µl PIC + 0.875 µl PMSF	350 µl + 1.75 µl PIC + 1.75 µl PMSF	1000 µl + 5 µl PIC + 5 µl PMSF
Enzymatic Shearing Cocktail, diluted	2.5 µl	8 µl	17 µl	50 µl
0.5 M EDTA	1 µl	3.5 µl	7 µl	20 µl

Section E. Use of Magnetic Beads and Included Bar Magnet

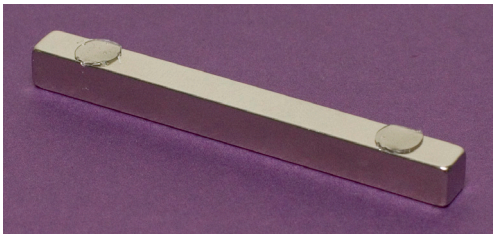
- The magnet should be stored in the provided tube.
- Be careful when working near metal objects or surfaces. A free magnet will jump great distances onto nearby metal surfaces with surprising speed. This can break the magnet.
- Use the provided Mini Glue Dots to attach the bar magnet to an empty pipette tip box to create an effective magnetic 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 break if you attempt to pull one end or pry it 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 for use with standard 96-well PCR cyclers are recommended (*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.

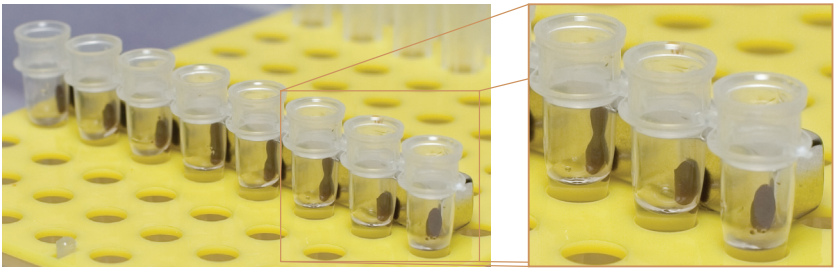


3. Remove the covering tape from the exposed side of the glue dots. Fix the magnet to the tip box so that it is against the PCR tubes. The magnetic stand is now ready for use.

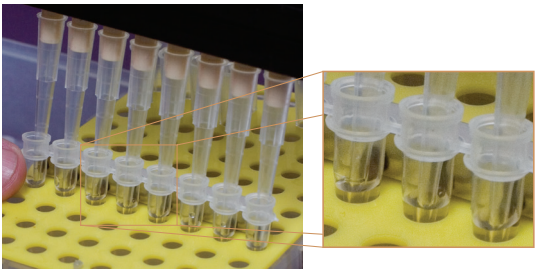
   Note: Familiarize yourself with using the magnetic stand before performing with PCR tubes for the first time. Add 5 µl of magnetic beads to 100 µl ChIP Buffer 1 in one tube of an 8-well strip of PCR tubes. Use this tube with the assembled bar magnet stand to become familiar with use of the beads and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

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 insides of 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, 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 F. 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 cross-link reversal, -20°C. 4. After DNA clean up, -20°C.
Poor yield of sheared chromatin.	Nuclei not released. It is critical to perform dounce homogenization when using enzymatic shearing (and is highly recommended when using sonication shearing). Use a dounce homogenizer with a small clearance pestle (see reference in Optional materials on page 7). Monitor cell lysis under a microscope as described in the procedures. Generally, the more cells that are lysed, the higher the sheared chromatin yield.
	Decrease the fixation time. Over-fixed cells are often very resistant to lysis and shearing. 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 to 5 minutes.
	Use fresh formaldehyde when preparing Fixation Solution.
	Buffers were not scaled proportionally to the size of the sample. Use the chart in Appendix – Section D 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 using phenol/chloroform. Follow the DNA Clean Up protocol in Appendix – Section C. DNA purification columns are not recommended, as the high protein content may clog the columns.
	For samples with a lot of chromatin, you may need to reverse cross-links for a longer period (4 hours to overnight at 65°C).
	Lost DNA during the purification step. Phenol should be saturated with TE pH 8. Lower pH solutions will degrade the DNA. Column purification is not recommended due to the high protein content of the sample, which may clog the column.
	High molecular weight products. You must repeat the chromatin preparation using longer digestion times. Be sure to vortex the sample every 2 minutes during the digestion.
The ladder-like banding pattern is not seen on the gel	A single ~100-200 bp band is present, and its appearance is not altered by the different digestion times. This usually indicates that the cells were not completely lysed, so the enzymes did not have access to chromatin. Follow the instructions for douncing the sample; increasing the digestion time will not solve this problem.
	No DNA. This is normal for the undigested DNA lane. The DNA is present, but in such a wide range of sizes it is not visible on the gel. No DNA in a digested sample lane indicates that the sample was too small (not enough chromatin to detect by gel) or the DNA was lost during DNA purification.
	Low molecular weight smear. The digestion time was too long or the Enzymatic Shearing Cocktail was not properly diluted.
	Smears/streaks from the wells. The cross-links were not completely reversed. The protein and DNA are still cross-linked and do not migrate in the gel properly and results in much of the DNA being stuck in the well or being lost during DNA purification.
	Large band/smear at bottom of gel. The RNA was not removed using the provided RNase.
	No specific bands, but a smear that decreases in size in lanes with longer digestion time. This indicates degraded DNA. Process the samples (particularly tissue) more quickly, be sure to keep on ice as directed and to add the appropriate proteinase inhibitors.
Cells will not lyse.	A few cell types are resistant to lysis and enzymatic shearing. Switch to sonication shearing.

Problem/question	Recommendation
Performing ChIP with a large volume of chromatin.	This is not recommended. 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. Do not perform a single scaled-up reaction, as the capture efficiency is lower.
Poor or no enrichment with target antibody.	Too little chromatin. Generally, we recommend using 7-10 µg of chromatin for a "regular" ChIP (with highly abundant, DNA-associated targets such as histones). For moderately abundant transcription factors, 7-25 µg of chromatin is recommended; for very low abundance transcription factors, a maximum of 50 µg chromatin is recommended for each IP reaction. For small-scale ChIP, chromatin quantities have been reported as low as 1 µg, but this is largely dependant upon transcription factor abundance and the affinity strength of the antibody, and may need to be adjusted accordingly. Be sure to quantitate the concentration of the sheared chromatin sample(s) being ChIP'd to ensure both that adequate chromatin is used per sample, and that equal mass quantities of chromatin are used in each ChIP.
	Antibody is not ChIP validated. The antibody does not efficiently recognize fixed proteins, either because the epitope is destroyed by fixation or because the epitope is masked by other proteins in a larger complex. To assist in ChIP validating an antibody, it is very useful to use a positive control antibody such as RNA Pol II and a negative IgG from the same species, and primers that have been proven to work in the type of PCR being used.
	Low-affinity antibody. Increase the incubation time of the ChIP reaction to overnight at 4°C on an end-to-end rotator.
	Antibody affinity to protein G is weak. Individual monoclonals have variable binding affinities to protein G, which are pH dependent; the optimal pH may vary for each Ig, For monoclonals with low to medium antibody affinity, capture efficiency by protein G can be dramatically improved through use of our Bridging Antibody (Cat. No. 53017). This antibody is a rabbit anti-mouse pAb that recognizes all subclasses of mouse immunoglobulins. If your mouse IgG has a weak/medium affinity to protein A or G, the Bridging Antibody will increase antibody capture by the beads without significantly increasing background.
	Problems with PCR. DNA to be used in real-time PCR must be purified prior to amplification.
	Primer issues. Confirm the species specificity of your primers. You may need to redesign your primers. Primers that work in end point PCR do not always work in real-time PCR.
The PCR products are the correct size, but are very faint.	Load more PCR product, and/or use smaller wells for the agarose gel. It should be noted that because the PCR reactions are stopped in log phase of amplification, the yield of PCR product may be lower than in typical PCR amplifications, which are performed for maximum product yield. You can also perform more PCR cycles. **Note:** a primer dimer band may be visible underneath the correct size PCR product.
No PCR bands with Input DNA samples (but the ChIP'd samples have the correct PCR product).	Perform the 1:10 dilution on the Input DNA prior to PCR, as indicated in the manual.
	Proteinase K may not have been completely inhibited. Warm the Proteinase K Stop Solution to room temperature for 30 minutes and then vortex briefly to get the material in solution, then add the recommended 2 µl and perform the recommended incubation.
	Purification of the Input DNA with a DNA purification column prior to PCR will effectively address either of the above issues.
No PCR products for the ChIP'd samples (but the Input DNA yields the correct PCR product)	Increase the amount of chromatin used in the ChIP reaction, the amount of antibody used, or both.
	Use a different antibody.
No PCR products with real-time PCR	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.

Problem/question	Recommendation
High background.	Chromatin not sheared enough. Shearing should produce DNA fragments that are small enough to exclude background from neighboring chromosomal sequences, but still large enough that there is a good possibility your amplicon remains intact. We recommend 200-1500 bp fragments. If the DNA fragments are too large, the background is increased. Consider increasing the time of the enzymatic digestion. Check the fragment size on a gel to assess your shearing efficiency.
	Antibody issue. Too much antibody relative to the amount of chromatin in the ChIP reaction. Excess antibody will result in more non-specific binding, which will be detected as increased background
	Too much template DNA. Reduce the amount of DNA in the PCR reaction.
	Increase washes. In most cases, the washing protocol in this manual is appropriate. However, if 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 is provided for two "extra" washes per sample. Sufficient ChIP Buffer 2 is provided for one additional wash. 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.
	Block the magnetic beads. The magnetic 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 in other applications), you may add blocking reagents to the ChIP reaction. Blocking reagents: 2.5 µg/µl BSA (*e.g.* Sigma Cat. No. 4503) and either 1.25 µg/µl tRNA (*e.g.* Sigma Cat. No. R3629) or 2.5 µg/µl Salmon sperm DNA (*e.g.* Sigma Cat. No. A-7888) (final concentrations shown for each) can be added directly to the ChIP reaction. These amounts are a good starting point, and can be increased as needed.

Technical Services

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

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