ChIP-IT™

Chromatin Immunoprecipitation Kits
&
Shearing Kits

(version G1)

Catalog Nos. 53001 - 53007

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

Active Motif Europe
104 Avenue Franklin Roosevelt
B-1330 Rixensart, Belgium
UK Free Phone: 0800 169 31 47
France Free Phone: 0800 90 99 79
Germany Free Phone: 0800 181 99 10
Telephone: +32 (0)2 653 0001
Fax: +32 (0)2 653 0050

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

The manufacturer of this documentation is Active Motif, Inc.

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Overview

Chromatin Immunoprecipitation (ChIP) is a powerful tool for the study of 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 purified. The DNA is then analyzed to determine which DNA fragments were bound by the protein of interest (see Schematic on page 2).

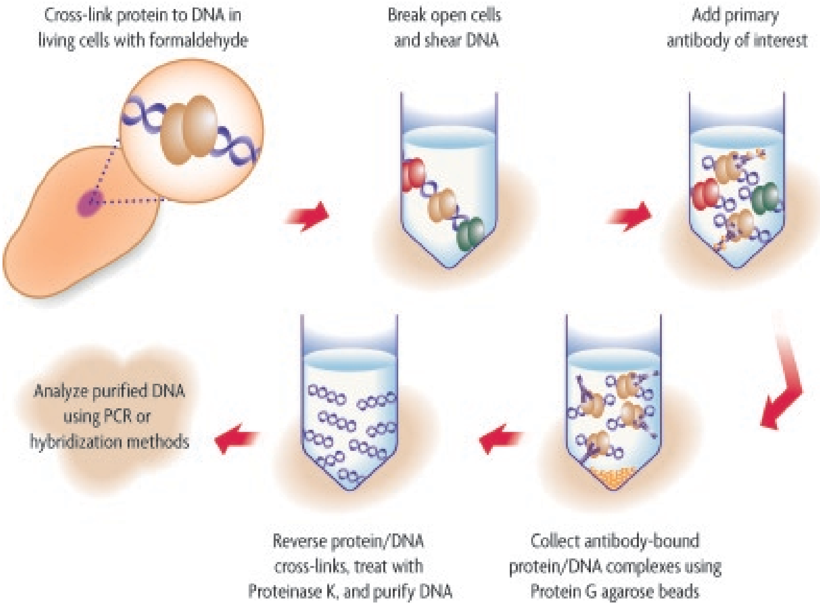
ChIP can be extremely useful in the study of transcription as it enables the identification of DNA regions that contain histone modifications or are bound by a particular transcription factor in specific cells. Furthermore, because protein/DNA interactions are fixed while in an endogenous, chromosomal context, ChIP results reflect the influence of chromosomal topology and the effects of cellular regulatory proteins[3, 4, 5].

ChIP, in particular ChIP performed with antibodies directed against transcription factors rather than abundant histone proteins, is technically demanding. This is because the method requires high-quality antibodies capable of recognizing the fixed, target-bound transcription factor of interest. In addition, specialized buffers, inhibitor cocktails and blocking reagents are required to minimize non-specific enrichment and reduce background. Result interpretation can also be difficult without appropriate controls.

Active Motif's ChIP-IT™ Kit is designed specifically for transcription factor-based ChIP and provides a complete solution for convenient, accurate monitoring of transcription factor/DNA interactions. The ChIP-IT Kit contains proven reagents and controls to ensure quick and reliable results. For users who are more familiar with ChIP, or who are using antibodies and primers that have already been shown to work well in ChIP, the ChIP-IT Kit is also offered without the positive and negative control antibodies and primers. In addition to the ChIP-IT Kits, the ChIP-IT Shearing Kit for sonication and Salmon Sperm DNA/Protein G agarose are available separately. Active Motif also offers a ChIP-IT Enzymatic and Enzymatic Shearing Kit which use an enzymatic cocktail to digest DNA into small fragments which are then suitable for use in ChIP.

product	format	catalog no.
ChIP-IT™	25 rxns	53001
ChIP-IT™ w/o controls	25 rxns	53004
ChIP-IT™ Shearing Kit	10 rxns	53002
ChIP-IT™ Enzymatic	25 rxns	53006
ChIP-IT™ Enzymatic w/o controls	25 rxns	53007
Enzymatic Shearing Kit	10 rxns	53005
Salmon Sperm DNA/Protein G agarose	25 rxns	53003

Flow Chart of Process



Schematic of chromatin immunoprecipitation.
In ChIP, protein/DNA interactions are fixed, and then the DNA is sheared and precipitated using an antibody. After reversing the cross-links, the DNA is purified and then analyzed to determine which genes were bound by the protein of interest.

Kit Performance and Benefits

The ChIP-IT Kit and ChIP-IT Enzymatic Kit provide reagents and protocols to simplify all aspects of the Chromatin Immunoprecipitation procedure. The kit can be used to: 1) Prepare chromatin, 2) Determine optimal conditions for shearing chromatin, 3) Perform ChIP reactions and 4) Analyze ChIP results by PCR. A brief discussion of the reagents provided for each of these procedures is below.

Preparation of chromatin - Sonication Shearing

The original ChIP-IT Kit 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 over cross-linking and a Protease Inhibitor Cocktail (PIC) and PMSF are included to ensure that the protein/DNA interactions are preserved during the chromatin purification and immunoprecipitation steps.

The kit provides reagents sufficient for making 5 preparations of sheared chromatin by sonication that are suitable for use in ChIP. Using this protocol, each preparation of sheared chromatin requires three 15 cm tissue culture plates of cells and yields chromatin sufficient for 16 ChIP reactions (one ChIP reaction is considered to be the incubation of one sample of chromatin with one antibody). If you wish to prepare chromatin from a smaller number of plates, simply scale the protocols down accordingly. The ChIP-IT Shearing Kit for sonication is also sold separately.

Note: An excess of chromatin isolation and shearing buffers are provided to enable optimization of shearing (see the discussion on Shearing in the Experimental Design section). The excess buffers will allow for 3 optimizations of chromatin shearing by sonication. If you want to prepare additional samples, the ChIP-IT Shearing Kit for sonication is sold separately (Catalog No. 53002).

Preparation of chromatin - Enzymatic Digestion

The Enzymatic Shearing Kit is a new kit available both separately and as part of the ChIP-IT Enzymatic Kit and contains similar components to the original ChIP-IT Kits (see Kit Contents on page 7), with the addition of a proprietary Enzymatic Shearing Cocktail and Digestion Buffer. Enzymatic shearing is easily controlled by time and temperature to yield fragments which are ideal for performing ChIP.

The Enzymatic Shearing Kit replaces the need to perform sonication shearing, thus eliminating problems due to variability in sonication power as well as complications arising from the emulsification of chromatin during sonication.

Note: The ChIP-IT Enzymatic Kits (Cat. Nos. 53006 & 53007) provide sufficient reagents to perform 5 shears. Each shear is based on using three 15 cm plates and yields chromatin sufficient for 16 ChIP reactions. If you wish to prepare chromatin from a smaller number of plates, simply scale the protocols down accordingly. An excess of shearing reagents are provided to enable optimization of shearing (see the discussion on Shearing in the Experimental Design section). The Enzymatic Shearing Kit is also sold separately (Cat. No. 53005).

DNA immunoprecipitation and purification

ChIP-IT includes all buffers, reagents and components required to perform 25 ChIP reactions. The provided Protein G agarose beads have been blocked with a mixture of Salmon Sperm DNA and BSA and are ready to use. These beads have a high binding capacity for IgG and are used in small volumes to reduce non-specific binding. The provided siliconized microcentrifuge tubes (0.65 ml) simplify wash steps and ensure a minimal loss of Protein G beads during the protocol.

The included DNA Mini-columns facilitate DNA purification by eliminating the need for phenol/chloroform extraction and minimizing tube handling, which reduces the risk of sample contamination. Columns and buffers sufficient for 30 DNA purifications are provided. Reagents required for reversing crosslinks (5 M NaCl), removing RNA (RNase) and removing DNA-bound proteins (Proteinase K) are also included.

Antibody controls

The ChIP-IT Kit includes a positive control antibody (anti-RNA pol II) and a Negative Control IgG. RNA pol II is a basal transcription factor that, through its carboxy-terminal domain (CTD), interacts with a large multi-subunit complex that contains TATA-binding protein (TBP). RNA pol II is an integral component of the transcription initiation complex. RNA pol II is known to bind to the promoter region of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene[6]. The included GAPDH primers can be used as a positive control to demonstrate the efficacy of the ChIP-IT Kit reagents and protocol. Similarly, because RNA pol II binds to a variety of active promoters, DNA immunoprecipitated using anti-RNA pol II can be analyzed with a variety of promoter-specific PCR primers to preliminarily assess the transcriptional activity of those promoters. The provided RNA pol II antibody is sufficient for 5 ChIP reactions and reacts with human, S. cerevisiae and C. elegans. Cross-reactivity with Drosophila, Hamster, Mouse, S. pombe and Arabidopsis is expected due to high sequence homology. The RNA pol II antibody is also sold separately. The Negative Control IgG is sufficient for performing 5 negative control ChIPs; experiments performed with the Negative Control IgG should not enrich for specific DNA sequences.

PCR Analysis of DNA isolated through ChIP

The ChIP-IT Kit's positive control PCR primers flank the RNA pol II site of the constitutively active glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter. The sequences are:

GAPDH For: 5´-TACTAGCGGTTTTACGGGCG-3´
GAPDH Rev: 5´-TCGAACAGGAGGAGCAGAGAGCGA-3´

The PCR product obtained with these primers is 166 bp long. PCR performed on templates that are enriched for the GAPDH promoter locus (*e.g.* DNA immunoprecipitated with anti-RNA pol II) will yield more product than PCR performed on templates that are not enriched for the GAPDH promoter locus (*e.g.* DNA immunoprecipitated with the Negative Control IgG).

ChIP-IT's negative control primers flank a region of genomic DNA between the GAPDH gene and the chromosome condensation-related SMC-associated protein (CNAP1) gene. The sequences are:

Neg For: 5´-ATGGTTGCCACTGGGGATCT-3´
Neg Rev: 5´-TGCCAAAGCCTAGGGGAAGA-3´

The PCR product generated with these primers is 174 bp long. Because the genomic DNA flanked by these primers (cytogenetic location 12 p13.3) should not be bound be transcription factors, this locus should not be enriched by ChIP. Thus, PCR with the negative control primers should give rise to equivalent amounts of product whether it is performed on DNA isolated by immunoprecipitation with anti-RNA pol II or with the Negative Control IgG (see Figure 1).

Note: The included positive and negative control primers function only with human DNA. See the Troubleshooting section in the Appendix for experiments with non-human DNA.

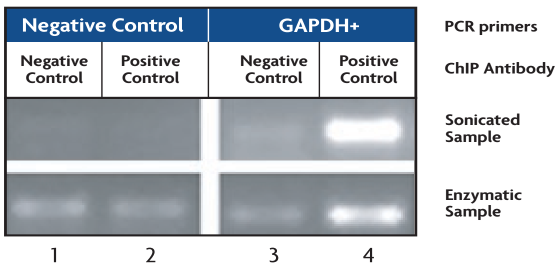


Figure 1: PCR analysis of immunoprecipitated DNA. This figure was selected to demonstrate the use of the ChIP-IT Kit's control reagents.

HeLa cells were fixed for 10 minutes with 1% formaldehyde and then chromatin was prepared and immunoprecipitated using either sonication shearing (5 pulses) or enzymatic shearing (10 minutes) using the ChIP-IT Kit's protocols. PCR analysis was performed on DNA isolated through ChIP using the Negative Control IgG (lanes 1 and 3) and the RNA pol II antibody (lanes 2 and 4). The PCR amplification was performed for 36 cycles using the kit's Negative Control (lanes 1 and 2) and GAPDH positive control PCR primers (lanes 3 and 4). Eight µl of each PCR was separated by electrophoresis on a 1% agarose gel and visualized by UV-illumination following ethidium bromide staining. See the PCR Analysis section for the composition of the PCR reactions and the cycling program.

PCR amplifications using the GAPDH primers on DNA isolated using the RNA pol II antibody generated more product than similar reactions performed on DNA isolated using the Negative Control IgG (compare lanes 3 and 4). In contrast, PCR amplifications using the Neg control primers on these same DNA templates generated PCR products of roughly equal intensity (lanes 1 and 2). Taken together, these results suggest that immunoprecipitation with anti-RNA pol II enriched for GAPDH promoter DNA, and that this enrichment was not the result of an unusually high degree of non-specific binding of the RNA pol II antibody to chromatin.

Note: As sonication and enzymatic shearing produce differing amounts of chromatin, which were not normalized for PCR, the band intensities should be compared only across each row; row-to-row comparisons do not assess the quality or quantity of the chromatin produced by the 2 methods.

Kit Components and Storage - ChIP-IT Kit

Please store each component at the temperature indicated in the table below. Do not freeze the Protein G agarose.

Reagents	ChIP Kit Quantity	Storage / Stability
10X PBS	120 ml	-20°C to room temp for 6 months
10X Glycine	33 ml	-20°C for 6 months
1X Lysis Buffer	12 ml	-20°C for 6 months
Protease Inhibitor Cocktail (PIC)	500 µl	-20°C for 6 months
PMSF	350 µl	-20°C for 6 months
Proteinase K (10 µg/µl)	80 µl	-20°C for 6 months
RNase A (10 µg/µl)	40 µl	-20°C for 6 months
5 M NaCl	200 µl	4°C for 6 months
Shearing Buffer	8 ml	-20°C for 6 months
0.5 M EDTA	60 µl	4°C for 6 months
ChIP IP Buffer	12 ml	-20°C for 6 months
Salmon Sperm DNA/Protein G agarose	5 ml	4°C for 6 months
Wash Buffer 1	40 ml	4°C for 6 months
Wash Buffer 2	10 ml	-20°C for 6 months
Wash Buffer 3	20 ml	4°C for 6 months
1% SDS	20 ml	4°C for 6 months
1 M $NaHCO_3$	125 µl	-20°C for 6 months
1 M Tris-Cl pH 6.5	60 µl	4°C for 6 months
DNA Mini-columns	30 columns	Room temp for 6 months
DNA Binding Buffer	15 ml	4°C to room temp for 6 months
DNA Wash Buffer	27 ml	4°C to room temp for 6 months
10X PCR Buffer	1.5 ml	-20°C for 6 months
Siliconized 0.65 ml tubes	30 tubes	Room temp for 6 months
DEPC H_2O	10 ml	Room temp for 6 months
Negative Control mouse IgG*	50 µl	4°C for 6 months
RNA pol II mouse mAb*	50 µl	-20°C for 6 months
GAPDH For Primer (5 pmol/µl)*	200 µl	-20°C for 6 months
GAPDH Rev Primer (5 pmol/µl)*	200 µl	-20°C for 6 months
Neg For Primer (5 pmol/µl)*	200 µl	-20°C for 6 months
Neg Rev Primer (5 pmol/µl)*	200 µl	-20°C for 6 months

*The ChIP-IT Kit w/o controls (53004) does not contain positive or negative control antibodies or primers.

Kit Components and Storage - ChIP-IT Enzymatic Kit

Please store each component at the temperature indicated in the table below. Do not freeze the Protein G agarose.

Reagents	ChIP Kit Quantity	Storage / Stability
10X PBS	120 ml	-20°C to room temp for 6 months
10X Glycine	33 ml	-20°C for 6 months
1X Lysis Buffer	12 ml	-20°C for 6 months
Protease Inhibitor Cocktail (PIC)	500 µl	-20°C for 6 months
PMSF	350 µl	-20°C for 6 months
Proteinase K (10 µg/µl)	80 µl	-20°C for 6 months
RNase A (10 µg/µl)	40 µl	-20°C for 6 months
5 M NaCl	200 µl	4°C for 6 months
Enzymatic Shearing Cocktail	6 µl	-20°C for 6 months
Digestion Buffer	11 ml	4°C for 6 months
0.5 M EDTA	280 µl	4°C for 6 months
ChIP IP Buffer	12 ml	-20°C for 6 months
Salmon Sperm DNA/Protein G agarose	5 ml	4°C for 6 months
Wash Buffer 1	40 ml	4°C for 6 months
Wash Buffer 2	10 ml	-20°C for 6 months
Wash Buffer 3	20 ml	4°C for 6 months
1% SDS	20 ml	4°C for 6 months
1 M $NaHCO_3$	125 µl	-20°C for 6 months
1 M Tris-Cl pH 6.5	60 µl	4°C for 6 months
DNA Mini-columns	30 columns	Room temp for 6 months
DNA Binding Buffer	15 ml	4°C to room temp for 6 months
DNA Wash Buffer	27 ml	4°C to room temp for 6 months
10X PCR Buffer	1.5 ml	-20°C for 6 months
Siliconized 0.65 ml tubes	30 tubes	Room temp for 6 months
DEPC H_2O	10 ml	Room temp for 6 months
Negative Control mouse IgG*	50 µl	4°C for 6 months
RNA pol II mouse mAb*	50 µl	-20°C for 6 months
GAPDH For Primer (5 pmol/µl)*	200 µl	-20°C for 6 months
GAPDH Rev Primer (5 pmol/µl)*	200 µl	-20°C for 6 months
Neg For Primer (5 pmol/µl)*	200 µl	-20°C for 6 months
Neg Rev Primer (5 pmol/µl)*	200 µl	-20°C for 6 months

*The ChIP-IT Enzymatic Kit w/o controls (53007) does not contain positive or negative control antibodies or primers.

Kit Components and Storage - Shearing Kits

Kit components will arrive at 4°C. Store each component at the temperature indicated in the table below.

	Quantity		
Reagents	**Shearing Kit**	**Enzymatic Shearing Kit**	**Storage / Stability**
10X PBS	120 ml	120 ml	4°C for 6 months
10X Glycine	33 ml	33 ml	-20°C for 6 months
1X Lysis Buffer	12 ml	12 ml	-20°C for 6 months
Protease Inhibitor Cocktail (PIC)	2 x 100 µl	2 x 100 µl	-20°C for 6 months
PMSF	500 µl	500 µl	-20°C for 6 months
Proteinase K (10 µg/µl)	80 µl	80 µl	-20°C for 6 months
RNase A (10 µg/µl)	40 µl	40 µl	-20°C for 6 months
5 M NaCl	200 µl	200 µl	4°C for 6 months
Shearing Buffer	11 ml	-	-20°C for 6 months
Digestion Buffer	-	11 ml	4°C for 6 months
Enzymatic Shearing Cocktail	-	6 µl (2 x 10^4 U/ml)	-20°C for 6 months
0.5 M EDTA	-	280 µl	4°C for 6 months

Additional materials required

- 37% Formaldehyde (Fixation)
- 50% Glycerol in dH_2O (Enzymatic shearing)
- 3 M Sodium Acetate pH 5.2 (DNA precipitation)
- Phenol/chloroform (DNA extraction - optional)
- 100% ethanol (DNA precipitation - optional)
- 70% ethanol (DNA precipitation - optional)
- Rocking platform for culture plates
- Apparatus to rotate tubes end-to-end at 4°C (*e.g.* a Labquake from Barnstadt/Thermolyne)
- Microcentrifuge and microcentrifuge tubes
- Spectrophotometer
- Pipettors and tips (filter tips are recommended)
- Sonicator (Recommend Sonics Vibracell VC 130 with 3 mm stepped microtip)
- Thermocycler and thermocycling tubes
- *Taq* polymerase
- dNTP mixture (5 mM each dNTP)
- 2 ml Dounce homogenizer w/ small clearance pestle
- Agarose gel electrophoresis apparatus
- Minimal cell culture media
- Cell Scraper (rubber policeman)
- 10 ml pipette and aspirator and 100 ml graduated cylinder

ChIP Experimental Design

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Points to consider:

1. **Cell growth and chromatin preparation.** The ChIP-IT Kit includes excess shearing reagents for three optimizations and for five preparations of chromatin for ChIP. Each chromatin preparation requires cells grown in three 15 cm tissue culture plates (approximately 4.5 x 10^7 cells) and yields enough material to perform 16 ChIP reactions. The kit contains Protein G agarose sufficient to perform a total of 25 ChIP reactions. 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. 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 an uninduced reference sample. See the Troubleshooting section in the Appendix for comments regarding chromatin yield and the amount of chromatin used for each ChIP reaction.

2. **Shearing by Sonication.** 200-1500 bp sheared chromatin is required for performing ChIP experiments. Optimize shearing conditions using chromatin material that is not planned for use in ChIP. Once shearing conditions are optimized, then prepare chromatin for ChIP. 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 very 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 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 (*e.g.* sonicate for 15 seconds, then place on ice for 30 seconds, sonicate again for 15 seconds, *etc.*).

4. **Shearing by Enzymatic Digestion.** The most common method for preparing sheared DNA for ChIP is by sonication. However, during the sonication shearing process complications arising from emulsification and overheating can occur. To address the need for a more robust and user-friendly shearing protocol, Active Motif has introduced an Enzymatic Shearing Kit and ChIP-IT Enzymatic Kit which uses a proprietary Enzymatic Shearing Cocktail to quickly and easily digest DNA to 200-1500 bp fragments. Enzymatic activity can be easily controlled by time and temperature.

5. **Salmon Sperm DNA/Protein G agarose**. The protein G agarose beads are ready to use following complete resuspension to a homogeneous slurry. For best results, gently shake and roll the tube. The beads settle quickly, thus resuspend just before pipetting. The beads can clog the small opening of some 200 µl pipette tips. This can be avoided by cutting the pipette tip to make a wider opening.

6. **Antibodies to be used must be suitable for ChIP.** 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 (such as the included RNA pol II antibody) to demonstrate that the antibody and the prepared chromatin are appropriate for ChIP.

7. **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, PCR analysis 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 to ensure consistent PCR amplification results.

   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 150-250 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. See Figure 1 on page 5 and Section K. PCR Analysis for further discussion.

8. **PCR primers.** PCR primers should efficiently and specifically amplify the desired target. This should be proven on a relevant template, such as DNA that has been taken through a "mock" ChIP (*e.g.* chromatin that has been sheared, treated to remove the cross-links, treated with Proteinase K and purified by phenol/chloroform and precipitation or through use of the DNA purification columns).

9. **Negative control primers.** Because ChIP reactions are unavoidably contaminated with a small amount of non-specifically captured DNA, all PCR primer pairs will produce a PCR product when used on DNA obtained through ChIP, provided that the PCR is performed for enough cycles. The provided negative control primers take advantage of this fact and are used to demonstrate the relative amount of contaminating DNA in different DNA samples. (See Figure 1 on page 5 for further discussion.)

10. **Buffer precipitation.** The 1 M NaHCO3, 1% SDS and DNA Binding Buffer may precipitate during shipping on dry ice, or during storage at 4°C or -20°C. If this occurs, resuspend completely before use by warming to room temperature and shaking or vortexing.

11. **Stopping points in the protocol.** The included ChIP protocols divide the process into four workdays. After you are familiar with the process, you can choose to adjust the stopping points to complete a ChIP experiment in two or three days.

12. **Safety precautions.** Formaldehyde, and PMSF are highly toxic chemicals. Appropriate safety precautions (*i.e.* safety glasses, gloves and labcoat) should be used. In addition, formaldehyde is highly toxic by inhalation and should be used only in a ventilated hood.

A. Optimization of Chromatin Shearing - Sonication

Please read the Experimental Design section that begins on page 9 before starting. The section below is written strictly for the optimization of shearing conditions. It is expected that the resulting sheared DNA will be used only to analyze shearing efficiency, not for ChIP. For this reason, protease inhibitors and PMSF are not included in the buffers. The cells used should be of the same type that will be used in the ChIP experiments and can be grown in standard culturing conditions. The protocol below was developed using a Sonics Vibracell VC 130 sonicator with a 3 mm stepped microtip at 25% power and in a volume of approximately 300 µl.

Note: Three ~350 µl samples of chromatin are prepared to optimize shearing, but only 50 µl of each are analyzed. If PIC and PMSF are included in the buffers during optimization, one or more of the remaining chromatin samples may be able to be used in ChIP, if the tested shearing conditions were successful. (However, adding PIC and PMSF to the buffers during optimization will reduce the number of shearing reactions that can be performed once you have established optimal conditions.) If you wish to be able to use the unused chromatin, include PIC and PMSF as directed in Section B, and freeze the unused chromatin during shearing optimization. The saved chromatin sample(s) that were sheared using optimal conditions can then be thawed and used for at least 6 ChIP reactions.

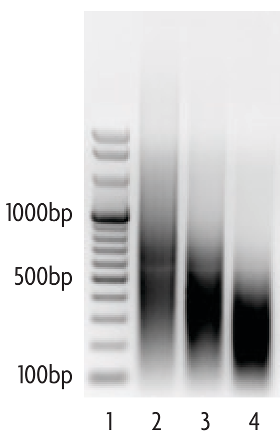


Figure 2: Analysis of DNA sheared using the ChIP-IT Kit. HeLa cells were fixed for 10 minutes with 1% formaldehyde and then chromatin was prepared using the ChIP-IT Kit. Chromatin was sheared with 5, 10 and 20 pulses at 25% power using a Sonics Vibracell VC 130 sonicator with a 3 mm stepped microtip in a volume of approximately 300 µl. Each pulse consisted of a 20 second sonication followed by a 30 second rest on ice. The sheared and unsheared chromatin samples were subjected to cross-link reversal, treated with Proteinase K, phenol/chloroform extracted and precipitated as described. Samples were separated by electrophoresis through a 1% agarose gel.

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

1. Grow cells to 70-80% confluency on three 15 cm plates (approximately 4.5 x 107 cells).
2. Follow steps 2-12 that begin on page 13 of the protocol in Section B. Cell Fixation, Chromatin Isolation and Sonication Shearing to prepare 3 aliquots (approximately 350 µl each) of fixed chromatin.

3. Shear the three aliquots of fixed chromatin at 25% power using three different conditions:
    a. Five pulses of 20 seconds each, with a 30 second rest on ice between each pulse.
    b. Ten pulses of 20 seconds each, with a 30 second rest on ice between each pulse.
    c. Twenty pulses of 20 seconds each, with a 30 second rest on ice between each pulse.
4. Centrifuge the three sheared chromatin samples at 10,000 to 15,000 rpm in a 4°C microcentrifuge for 12 minutes. Transfer supernatant to a fresh tube. This sheared chromatin can be stored at -80°C. Alternatively, continue with the steps below to reverse cross-links and purify the chromatin.
5. Transfer 50 µl of each sheared chromatin to a fresh microcentrifuge tube.
6. To reverse cross-links, add 150 µl dH_2O, 8 µl 5 M NaCl and 1 µl RNase to each of the sheared DNA samples. Vortex and then incubate for 4 hours at 65°C; samples can be left overnight.
7. After the 4 hour incubation, centrifuge the tubes briefly to collect condensed material and then allow the samples to return to room temperature. Add 2 µl of the Proteinase K solution to each tube and incubate at 42°C for 1.5 hours. This DNA can now be run on a 1% or 2% agarose gel to determine the size range of the sheared DNA.

    Note: If you intend to use a spectrophotometer to determine the DNA concentration, the DNA should first be 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 -20°C for at least 1 hour.
    c. Centrifuge at maximum speed for 10 minutes in 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 50 µl dH_2O and determine the OD at 260 nm to determine the DNA concentration (1.0 A_{260} unit = 50 µg/ml).
8. It is recommended to load each shearing sample on the gel in two different amounts to avoid over- or under-loading. (For example, if loading non-precipitated samples, load 2 and 10 µl of each sample; if loading precipitated samples, load 1 and 5 µl of each sample.)
9. Shearing should result in a smear similar to that shown in lanes 2 or 3 of Figure 2.

B. Cell Fixation, Chromatin Isolation and Sonication Shearing (Day 1)

This section describes preparation of chromatin from three 15 cm plates (this corresponds to approximately 4.5 x 10^7 cells). Note: Several of the buffers used below require the addition of PMSF and/or protease inhibitors (PIC). Thaw these reagents before starting the protocol and add to the buffers immediately before use.

1. Grow cells to 70-80% confluency on three 15 cm plates. Treat all three plates equally to influence the pathway of interest.
2. When cells are ready to harvest prepare fresh Fixation Solution, ice-cold 1X PBS, Glycine Stop-Fix solution and Cell Scraping solution as follows:
   a. Fixation Solution: Add 1.62 ml of 37% formaldehyde to 60 ml minimal cell culture medium and mix thoroughly. Leave at room temperature.
   b. 1X PBS: Add 7 ml 10X PBS to 63 ml dH_2O, mix and place on ice.
   c. Glycine Stop-Fix Solution: Combine 3 ml 10X Glycine Buffer, 3 ml 10X PBS and 24 ml dH_2O. Mix well and leave at room temperature.
   d. Cell Scraping Solution: Add 600 µl 10X PBS to 5.4 ml dH_2O, mix and place on ice. Just before use (in step 7 below) add 30 µl 100 mM PMSF.
3. Pour medium off the three plates and add 20 ml Fixation Solution to each plate. Incubate on a shaking platform for 10 minutes at room temperature.
4. Pour Fixation Solution off the plates and wash by adding 10 ml ice-cold PBS to each plate, rocking the plate for 5 seconds and then pouring off the PBS.
5. Stop the fixation reaction by adding 10 ml Glycine Stop-Fix solution to each of the plates, swirling to cover and then rocking at room temperature for 5 minutes.
6. Wash each plate by pouring off the Glycine Stop-Fix solution and adding 10 ml ice-cold PBS, rocking the plate for 5 seconds, and then pouring off the PBS.
7. Add 2 ml ice cold Cell Scraping Solution to each of the plates and scrape cells with a rubber policeman. Hold the plate at an angle and scrape the 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. Do the same for the other two plates and pool cells from the three plates in one 15 ml conical tube.
8. Pellet the pooled cells by centrifugation for 10 minutes at 2500 rpm (720 RCF) at 4°C.
9. Remove the supernatant. 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. When you are ready, continue with step 10.
10. Thaw pellet (if necessary) and resuspend cells in 1 ml ice-cold Lysis Buffer (supplemented with 5 µl PIC + 5 µl PMSF). Incubate on ice for 30 minutes.
11. Transfer the cells to an ice-cold dounce homogenizer. Gently dounce on ice with 10 strokes to aid in nuclei release. Transfer cells to a 15 ml conical tube and centrifuge at 5000 rpm (approximately RCF 2400) for 10 minutes at 4°C to pellet the nuclei.

12. Carefully remove the supernatant. Resuspend the nuclei pellet in 1.0 ml Shearing Buffer (supplemented with 5 µl PIC) and aliquot into three 1.7 ml microcentrifuge tubes. Each aliquot should be approximately 350 µl.
13. Shear the three aliquots of DNA using your optimized conditions (from page 11).
14. Centrifuge the sheared DNA samples at 10,000 to 15,000 rpm in a 4°C microcentrifuge for 12 minutes. Pool the supernatants by transferring each to the same fresh tube. This contains the sheared chromatin. It can be used right away or aliquoted and stored at -80°C. Remove

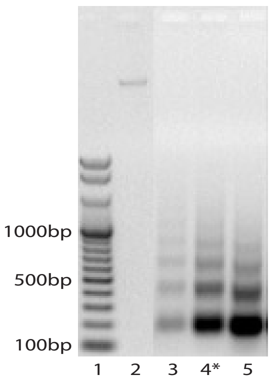


Figure 3: Analysis of DNA sheared using the Enzymatic Shearing Kit. HeLa cells were fixed for 10 minutes with 1% formaldehyde and then chromatin was prepared using the ChIP-IT Kit protocol. Chromatin was sheared with the Enzymatic Shearing Cocktail for 5, 10 & 15 minutes and the reaction was stopped 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.

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 were optimal and used successfully in ChIP.

25 µl for use in checking the DNA shearing efficiency and DNA concentration. Aliquot the remainder into four equal aliquots (usually about 220 µl each). Each aliquot can then be used for 4 ChIP reactions (*i.e.* each aliquot can be tested with four different antibodies).

Note: If you wish to use the reserved 25 µl sample to determine DNA concentration and shearing efficiency, add 25 µl dH_2O and follow the protocol for DNA clean up described in steps 4-7 of Section A. Optimization of Shearing on page 11.

A1. Optimization of Chromatin Shearing - Enzymatic

Please read the Experimental Design section that begins on page 9 before starting. The section below is written strictly for the optimization of enzymatic shearing conditions. It is expected that the resulting sheared DNA will be used only to analyze shearing efficiency, not for ChIP. For this reason, protease inhibitors and PMSF are not included in the buffers. The cells used should be of the same type that will be used in the ChIP experiments and can be grown in standard culturing conditions.

Note: 1 ml of chromatin is prepared to optimize shearing, but only 200 µl is analyzed. If PIC and PMSF are included during optimization, the 800 µl of unused chromatin can be used in ChIP. (However, adding PIC and PMSF to the buffers during optimization reduces the number of shearing reactions that can be performed once you have established optimal conditions.) If you wish to be able to use the unused chromatin, include PIC and PMSF as directed in Section B, and freeze the unused chromatin during shearing optimizing. The saved chromatin can then be thawed and enzymatically sheared using the optimal conditions identified. This chromatin should be sufficient for at least 12 ChIP reactions.

1. Grow cells to 70-80% confluency on three 15 cm plates.
2. Follow steps 2-12 from page 16 of the protocol in Section B1. Cell Fixation, Chromatin Isolation and Enzymatic Shearing.
3. Transfer 50 µl of the chromatin in the Digestion Buffer to 4 fresh microcentrifuge tubes and pre-warm the chromatin at 37°C for 5 minutes.
4. Prepare a working Enzymatic Shearing Cocktail solution by diluting the supplied mixture (2 x 10^4 U/ml) to 1:100 with 50% glycerol in dH_2O (not provided) to make a final concentration of 200 U/ml. (The 200 U/ml stock is stable at 4°C for 1-2 weeks.)

Reagent	µl for 5 rxns
Stock Enzymatic Cocktail (2 x 10^4 U/ml)	0.125 µl
50% glycerol	12.375 µl

5. To optimize shearing conditions using Enzymatic Digestion, set up 4 reactions as indicated below. Vortex the tubes on a low setting to mix components.
   a. 50 µl chromatin plus 2.5 µl dH_20 (No Enzyme) - incubate for 10 minutes at 37°C
   b. 50 µl chromatin plus 2.5 µl Enzyme - incubate for 5 minutes at 37°C
   c. 50 µl chromatin plus 2.5 µl Enzyme - incubate for 10 minutes at 37°C
   d. 50 µl chromatin plus 2.5 µl Enzyme - incubate for 15 minutes at 37°C
6. Stop each reaction by adding 1 µl ice-cold EDTA to each tube, chill on ice for 10 minutes.
7. Centrifuge the sheared DNA samples at 10,000 to 15,000 rpm in a 4°C microcentrifuge for 10 minutes. Collect the supernatant. This sheared chromatin can be stored at -80°C. Alternatively, continue with the steps below to reverse cross-links and purify the chromatin.
8. Transfer 50 µl of each sheared chromatin fraction to a fresh microcentrifuge tube.
9. To reverse cross-links, add 150 µl dH_2O, 8 µl 5 M NaCl and 1 µl RNase to each of the sheared DNA samples. Vortex and then incubate for 4 hours at 65°C. (This incubation can be performed overnight if desired.)
10. After the 4 hour incubation, centrifuge the tubes briefly to collect condensed material and then allow the samples to return to room temperature. Add 2 µl of the Proteinase K solution to each tube and incubate at 42°C for 1.5 hours. This DNA can now be run on a 1% or 2% agarose gel to determine the size range of the sheared DNA.

    Note: If you intend to use a spectrophotometer to determine the DNA concentration, the DNA should first be 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 -20°C for at least 1 hour.

c. Centrifuge at maximum speed for 10 minutes in 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 load 10 µl of each sheared sample to a 1-2% agarose gel and 12 µl of a 100 bp ladder on one side. Run the gel at 100V for approximately 45 minutes to 1 hour until the loading dye reaches 3/4 of the way to the end of the gel rack.

11. Shearing should result in a 200-1000 bp ladder like smear similar to that shown in Lane 4 of Figure 3.

B1. Cell Fixation, Chromatin Isolation and Enzymatic Shearing (Day 1)

This section describes preparation of chromatin from three 15 cm plates (this corresponds to approximately 4.5×10^7 cells). Note: Several of the buffers used below require the addition of PMSF and/or protease inhibitors (PIC). Thaw these reagents before starting the protocol and add to the buffers immediately before use.

1. Grow cells to 70-80% confluency on three 15 cm plates. Treat all three plates equally to influence the pathway of interest.
2. When cells are ready to harvest prepare fresh Fixation Solution, ice-cold 1X PBS, Glycine Stop-Fix solution and Cell Scraping solution as follows:

   a. Fixation Solution: Add 1.62 ml of 37% formaldehyde to 60 ml minimal cell culture medium and mix thoroughly. Leave at room temperature.

   b. 1X PBS: Add 7 ml 10X PBS to 63 ml dH_2O, mix and place on ice.

   c. Glycine Stop-Fix Solution: Combine 3 ml 10X Glycine Buffer, 3 ml 10X PBS and 24 ml dH_2O. Mix well and leave at room temperature.

   d. Cell Scraping Solution: Add 600 µl 10X PBS to 5.4 ml dH_2O, mix and place on ice. Just before use (in step 7 below) add 30 µl 100 mM PMSF.
3. Pour medium off the three plates and add 20 ml Fixation Solution to each plate. Incubate on a shaking platform for 10 minutes at room temperature.
4. Pour Fixation Solution off the plates and wash by adding 10 ml ice-cold PBS to each plate, rocking the plate for 5 seconds and then pouring off the PBS.
5. Stop the fixation reaction by adding 10 ml Glycine Stop-Fix solution to each of the plates, swirling to cover and then rocking at room temperature for 5 minutes.
6. Wash each plate by pouring off the Glycine Stop-Fix solution and adding 10 ml ice-cold PBS,

rocking the plate for 5 seconds, and then pouring off the PBS.

7. Add 2 ml ice cold Cell Scraping Solution to each of the plates and scrape cells with a rubber policeman. Hold the plate at an angle and scrape the 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. Do the same for the other two plates and pool cells from the three plates in one 15 ml conical tube.
8. Pellet the pooled cells by centrifugation for 10 minutes at 2500 rpm (720 RCF) at 4°C.
9. Remove the supernatant. 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. When you are ready, continue with step 10.
10. Thaw pellet (if necessary) and resuspend cells in 1 ml ice-cold Lysis Buffer (supplemented with 5 µl PIC + 5 µl PMSF). Incubate on ice for 30 minutes.
11. Transfer the cells to an ice-cold dounce homogenizer. Gently dounce on ice with 10 strokes to aid in nuclei release. Transfer cells to a 15 ml conical tube and centrifuge at 5000 rpm (approximately RCF 2400) for 10 minutes at 4°C to pellet the nuclei.
12. Carefully remove the supernatant. Resuspend the nuclei pellet in 1.0 ml Digestion Buffer (supplemented with 5 µl PIC + 5 µl PMSF) and pre-warm this solution at 37°C for 5 minutes.
13. Prepare a working Enzymatic Shearing Cocktail solution by diluting 1:100 of the supplied mixture (2 x 10^4 U/ml) with 50% glycerol in dH_2O (not provided) to make a final stock at 200 U/ml.

Reagent.	µl for 1 rxn	µl for 5 rxns	µl for 10 rxn
Stock Enzyme	0.5	2.5	5
50% glycerol	49.5	247.5	495

14. Add 50 µl of the working stock of Enzymatic Shearing Cocktail (200 U/ml) to the pre-warmed nuclei and vortex to mix. Next, incubate the tube at 37°C for the optimized time (from page 14). Flick or vortex the tube periodically during the incubation to ensure the chromatin is evenly sheared.
15. Stop the reaction by adding 20 µl ice-cold EDTA to each tube, chill on ice for 10 minutes.
16. Centrifuge the sheared DNA samples at 10,000 to 15,000 rpm in a 4°C microcentrifuge for 10 minutes. Collect the supernatant. This contains the sheared chromatin. It can be used right away or aliquoted and stored at -80°C. Remove 25 µl for use in checking the DNA shearing efficiency and DNA concentration. Aliquot the remainder into four equal aliquots (usually about 250 µl each). Each aliquot can then be used for 4 ChIP reactions (*i.e.* each aliquot can be tested with four different antibodies).

    Note: If you wish to use the reserved 25 µl sample to determine DNA concentration and shearing efficiency, add 25 µl dH_2O and follow the protocol for DNA clean up described in steps 8-11 of Section A1. Optimization of Shearing on page 15.

C. Pre-clearing of Chromatin (Day 2)

Chromatin is pre-cleared with Protein G beads to reduce non-specific background. Normally, each chromatin preparation will be used for several ChIPs (*e.g.* a negative control ChIP, a positive control ChIP and ChIP with antibody of interest). In these cases, the chromatin for the ChIPs can be pre-cleared in the same tube (see example below). The volumes below assume that the chromatin was prepared as described in the preceding sections.

Reagent	µl for one ChIP rxn	µl for 3 ChIP rxns
Chromatin	50 µl	150 µl
Resuspended Protein G beads	100 µl	300 µl
ChIP IP Buffer	59 µl	177 µl
PIC	1 µl	3 µl
Total Volume	210 µl	630 µl

1. Use above table to calculate amount of chromatin, resuspended Protein G beads, ChIP IP Buffer and PIC required for pre-clearing reactions. Combine reagents in a 1.7 ml microcentrifuge tube.
2. Rotate the tube at 4°C for 1 to 2 hours.
3. Place tube in a microcentrifuge for 2 minutes at 4,000 rpm.
4. After centrifugation is complete, place tube on ice for 2 minutes to let the beads settle.
5. Transfer the supernatant (chromatin) to a fresh tube. Do not disturb the beads.
6. Repeat steps 3 through 5 to ensure that all beads are removed from the chromatin.

D. Addition of Antibodies to Pre-cleared Chromatin

Note: Some volume (~15%) is lost during pre-clearing. Assume that 170 µl of pre-cleared chromatin is available for each ChIP reaction.

1. Transfer 10 µl of the pre-cleared chromatin to a microcentrifuge tube and store at -20°C. This sample is the "Input DNA" and will be used in PCR analysis. It will be treated to remove cross-links at a later stage (see Section H. Reverse Cross-links and Remove RNA).
2. Perform the antibody incubations in the provided 0.65 ml siliconized tubes. To begin, label the appropriate number of tubes.
3. Add 170 µl of the pre-cleared chromatin from Step C to each of the labeled 0.65 ml tubes.
4. Add the appropriate antibody to each of the labeled tubes. We recommend using between 1 and 3 µg of antibody for each ChIP reaction. The kit's Negative Control IgG and RNA pol II antibody should be used at 10 µl (2 µg) per ChIP reaction.
5. Incubate the tubes 4 hours to overnight on a rotator at 4°C (sensitivity may be improved by overnight incubation).

E. Addition of Protein G to Antibody/Chromatin Mixture (Day 3)

1. Resuspend the stock of Protein G beads fully by inverting the bottle several times.
2. Aliquot a fresh 100 µl aliquot of the fully resuspended beads into each of the antibody/ chromatin incubations performed in the preceding step.
3. Incubate the tubes on a rotator for 1.5 hours at 4°C.
4. During this incubation, prepare the ChIP IP and Wash Buffers as described below. Each ChIP reaction will be washed once with ChIP IP Buffer + PIC, four times with Wash Buffer 1 + PIC, once with Wash Buffer 2 + PIC and twice with Wash Buffer 3. The quantities listed below are sufficient for one ChIP reaction. (See Troubleshooting Section for additional comments on washing conditions)

   ChIP IP Buffer + PIC: Add 2 µl Protease Inhibitor Cocktail (PIC) to 400 µl ChIP IP Buffer, mix and place on ice.

   Wash Buffer 1 + PIC: Add 1.6 µl PIC to 1.6 ml Wash Buffer 1, mix and place on ice.

   Wash Buffer 2 + PIC: Add 0.4 µl PIC to 400 µl Wash Buffer 2, mix and place on ice.

   Wash Buffer 3: Place on ice (supplied ready-to-use).

F. Washing ChIP Reactions

1. Following incubation of the beads with the antibody/chromatin mixture, pellet the beads by centrifuging each ChIP reaction for 2 minutes at 4000 rpm. Place tubes in a rack and allow 30 seconds for the beads to fully settle.

   Note: All subsequent bead pelleting steps should be performed in this manner.

2. Remove the supernatant. Use a 200 µl pipette to withdraw 200 µl twice (discard supernatants). Avoid disturbing the beads.
3. Add 400 µl ChIP IP Buffer + PIC to each tube of beads and cap the tubes. Vortex the tubes for 10 seconds on a low setting and then incubate on rotator for 1-3 minutes. Pellet beads and remove supernatant.
4. Add 400 µl Wash Buffer 1 and cap tubes. Resuspend beads and vortex the tubes for 10 seconds on a low setting and then incubate on rotator for 1-3 minutes. Pellet beads and remove supernatant.
5. Repeat Step 4 three times.
6. Add 400 µl Wash Buffer 2 + PIC and cap tubes. Vortex the tubes for 10 seconds on a low setting and then incubate on rotator for 1-3 minutes. Pellet beads and remove supernatant.
7. Add 400 µl Wash Buffer 3 and cap tubes. Vortex the tubes for 10 seconds on a low setting and then incubate on rotator for 1-3 minutes. Pellet beads and remove supernatant.
8. Repeat Step 7 once. This is the final wash. Remove as much buffer as possible without disturbing the beads.

G. DNA Elution from Protein G

In this section, immunoprecipitated DNA will be collected from the washed Protein G beads using two elutions with 50 µl ChIP Elution Buffer (the ChIP Elution Buffer is prepared below in step 1).

1. Freshly prepare 105 µl of ChIP Elution Buffer for each ChIP reaction by adding 5 µl 1M $NaHCO_3$ to 100 µl 1% SDS and mixing thoroughly.
2. Add 50 µl ChIP Elution Buffer to each of the washed Protein G bead pellets in the 0.65 ml tubes. Cap tubes, vortex briefly and incubate for 15 minutes at room temperature with gentle rotation.
3. Centrifuge tubes for 2 minutes at 4000 rpm to pellet beads. Remove tubes from centrifuge, place in tube holder in vertical position and wait several seconds for the beads to settle completely.
4. Use a 200 µl pipette to transfer each supernatant to an appropriately labeled 1.5 or 1.7 ml microcentrifuge tube.
5. Repeat steps 2 to 4 and pool the appropriate elutions.

H. Reverse Cross-links and Remove RNA

Note: The reserved Input DNA (from Step 1 of Section D) must also be taken through the following steps. Remove the reserved Input DNA from the freezer and add 90 µl dH_2O to bring the volume to 100 µl and treat this sample along with the ChIP elutions below.

1. Add 4 µl 5 M NaCl and 1 µl RNase A to each ChIP elution and to the sample of Input DNA.
2. Vortex to mix completely and centrifuge the tubes briefly to remove liquid from the sides of the tubes. Place tubes in a 65°C incubator or water bath for 4 hours to overnight. (The experiment can be stopped here and tubes stored at -20°C until use.)

   Note: The SDS in the samples may precipitate if they are stored on ice or at -20°C. Warm the tubes to room temperature and vortex to resuspend the SDS.

I. Treat with Proteinase K (Day 4)

1. Remove tubes from 65°C incubator and centrifuge for 1 minute to collect liquid from the sides of the tubes.
2. Add the following three components to each tube: 2 µl 0.5 M EDTA, 2 µl 1 M Tris-Cl pH 6.5 and 2 µl Proteinase K solution.
3. Vortex to mix, centrifuge briefly to collect liquid from the sides of the tubes and incubate at 42°C for 1.5 to 2 hours to digest the proteins. During this incubation, prepare the reagents that will be needed for DNA purification in the next section.

J. Purify Eluted DNA

1. Label and place the required number of DNA purification mini-columns in their provided collection tubes in a rack. The DNA will be eluted from the columns into microcentrifuge tubes (1.5 ml or 1.7 ml). Label and set aside the appropriate number of tubes. (We recommend labeling the sides of the tubes as the caps often break off during centrifugation.)
2. Remove the Proteinase K-treated samples from the 42°C incubator and centrifuge briefly to collect the liquid condensed on the sides of the tubes.
3. Add 500 µl of DNA Binding Buffer to each DNA sample and vortex to mix completely. A cloudy solution may form upon this addition. This does not effect results. Transfer each sample into a labeled DNA purification mini-column and centrifuge for 30 seconds at 10,000 to 15,000 rpm.
4. Remove the mini-column from the collection tube, discard the flow-through and replace the mini-column in the tube.
5. Add 600 µl of DNA Wash Buffer to each mini-column.
6. Centrifuge for 30 seconds at 10,000 to 15,000 rpm.
7. Remove the mini-column from the collection tube, discard the flow-through and replace the mini-column in the tube.
8. Add 300 µl of DNA Wash Buffer to each mini-column.
9. Centrifuge for 2 minutes at 10,000 to 15,000 rpm.
10. Place each dry mini-column into the appropriately labeled 1.5 ml or 1.7 ml tube prepared in Step 1. Add 50 µl DEPC H_2O directly to the resin at the bottom of each mini-column. Incubate for 3 minutes at room temperature.
11. Spin for 1 minute at 10,000 to 15,000 rpm.
12. Remove the mini-columns and collection tubes from the centrifuge and place in a tube rack. Repeat the DNA elution by adding 50 µl DEPC H_2O directly to the resin, incubating 3 minutes at room temperature and then centrifuge for 1 minute at 10,000 to 15,000 rpm.
13. The eluted DNA can be used immediately in PCR or stored at -20°C.

K. PCR Analysis

The protocol below is a guideline for optimizing the PCR analysis of DNA collected through ChIP. Accurate PCR analysis of ChIP DNA requires that the PCR be stopped during the linear amplification phase. The appropriate number of PCR cycles must be determined empirically. In the example below, PCR will be performed on three DNA templates: DNA isolated through RNA pol II ChIP, DNA isolated through the Negative Control IgG ChIP and the Input DNA. A control reaction with no template will also be performed to ensure that the PCR reagents are not contaminated. Two sets of PCR primers will be used: The GAPDH primer pair and the Negative (Neg) control primer pair. Each reaction will be cycled 36 times. For PCR analysis using other PCR primers and templates, PCR conditions may need additional optimization, we provide an excess of reagents to perform this optimization.

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. During the hold cycle, remove the tubes and place on ice (see step 6 below). The total volume of each PCR will be 25 µl.
2. Dilute the eluted Input DNA 1:10 by adding 20 µl eluted Input DNA to 180 µl dH_2O.
3. Label PCR tubes and add the PCR templates and cocktails as shown in the table below.

Reaction No.	PCR Template	PCR cocktail	No. of PCR cycles
1	5 µl RNA pol II ChIP DNA	20 µl GAPDH	36
2	5 µl Neg control ChIP DNA	20 µl GAPDH	36
3	5 µl 1:10 diluted Input DNA	20 µl GAPDH	36
4	5 µl DEPC H_2O (control)	20 µl GAPDH	36
5	5 µl RNA pol II ChIP DNA	20 µl Negative control	36
6	5 µl Neg control ChIP DNA	20 µl Negative control	36
7	5 µl 1:10 diluted Input DNA	20 µl Negative control	36
8	5 µl DEPC H_2O (control)	20 µl Negative control	36

4. Set up the GAPDH PCR cocktail and the Negative control PCR cocktail on ice according to the tables below. Add the dH_2O first and the *Taq* polymerase last. Mix thoroughly by vortexing and keep on ice. This ensures that the reaction mixture is inactive until the cycling is started.

GAPDH PCR cocktail:

Reagent	One reaction	4 reactions
DEPC H_2O	12.3 µl	61.5 µl
GAPDH For primer (5 pmol/µl)	2.0 µl	10 µl
GAPDH Rev 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 control PCR cocktail:

Reagent	One reaction	4 reactions
DEPC H_2O	12.3 µl	61.5 µl
Neg For primer (5 pmol/µl)	2.0 µl	10 µl
Neg Rev 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 Kit. 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 2. 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 36 cycles, remove tubes 1-8 and place on ice.
7. These PCR reactions can be immediately analyzed as described below or stored at -20°C.

L. Analysis of PCR Products

1. Add 8 µl of each PCR product to 2 µl 6X DNA loading dye and separate on 3% agarose gel. Save remaining PCR product in case additional gels must be run. Use gels with 2.5 mm-wide combs.
2. PCR products obtained with the GAPDH and Neg primers are 166 and 174 bp, respectively. 50 bp or 100 bp ladder should be used as a migration standard. Run gel until PCR amplification products are well separated from PCR primers and primer dimers (see Figure 1). Stain gel and analyze.

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Appendix

Section A. Troubleshooting Guide

Problem/question	Possible Cause	Recommendation
The PCR products are the correct size but are very light.	Not enough PCR product is loaded, agarose gel wells are too wide or not enough PCR cycles were performed.	Load more PCR product, 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 performed for maximum product yield.
Can I perform more than 5 chromatin preparations?		Yes. However, to conserve chromatin preparation reagents, start with cells from one or two 15 cm plates and scale down the reagent volumes used in the chromatin preparation accordingly. (One plate corresponds to approx. 1.5×10^7 cells.)
How much DNA is used in a typical ChIP reaction?		ChIP reactions are routinely performed with 12 µg of DNA. Accurate quantification of DNA contained within a chromatin sample requires cross-link reversal and DNA clean-up through phenol/chloroform extraction and precipitation. DNA in a chromatin sample can be estimated as: # number of cells harvested x 6 pg DNA/cell x estimated % recovery (we assume 75% yield). Each shear produces enough chromatin to perform 16 ChIPs.
There are precipitates in the solution		The 1 M $NaHCO_3$, the 1% SDS and the DNA Binding Buffer may precipitate during shipping on dry ice or during storage at 4°C or -20°C. If this occurs, resuspend completely before use by warming to room temperature and shaking or vortexing. The precipitate will not effect ChIP results.
20 pulses of sonication does not result in adequately sheared DNA		Sonicate for more pulses and sonicate for 30 seconds per pulse rather than 20 seconds. Take care to keep chromatin well-chilled on ice during these extended sonication pulses. It is also possible that the chromatin has been "overfixed" and is resistant to shearing. In these cases, it may be best to prepare new chromatin. If you continue to experience difficulties with sonication shearing we suggest using our new Enzymatic Shearing Kit as detailed in the manual.
PCR using the negative control (Neg) PCR primers on DNA obtained through RNA pol II ChIP generates more PCR product than PCR using the GAPDH primers on the same DNA.	The negative control PCR primers are more efficient in PCR than the GAPDH primers and a robust product can be generated in a relatively few cycles.	This is not a problem because the Neg control primers are used to indicate the **relative** amount of non-specifically precipitated (contaminating) DNA in various ChIP DNA samples. Generally, because all PCR primers vary in their amplification efficiency, one needs to be careful when drawing conclusions based on the intensity of PCR products generated with different primer pairs. See Figure 1 on page 5 and the sections "Kit performance and Benefits" and ChIP Experimental Design" for more discussion.
Can I modify the Wash Steps in Part F?		Yes. The wash steps using Wash Buffer 1 and Wash Buffer 3 may be reduced, if desired. However, higher background may result from reduced washing. In addition, you may use 1.7 ml eppendorf tubes rather then the provided 0.65 ml siliconized tubes. In this case, the 0.4 ml Wash Buffer 1 or Wash Buffer 3 steps can be combined to make 0.8 ml washes.
Can ChIP-IT Kit reagents be used on chromatin prepared from non-human cells?		Yes. The RNA pol II antibody is suitable for use with human, S. cerevisiae and C. elegans. Cross-reactivity with Drosophila, Hamster, Mouse, S. pombe and Arabidopsis is expected due to homology. However, PCR primers included in the kit function only on human DNA. Please call technical service for information on GAPDH primer sequences for other species.

Section B. Related Products

ChIP-IT™ Kits	Format	Catalog No.
ChIP-IT™ Express	25 rxns	53008
ChIP-IT™ Express Enzymatic	25 rxns	53009
ChIP-IT™ Protein G Magnetic Beads	25 rxns	53014
ChIP-IT™	25 rxns	53001
ChIP-IT™ w/o controls	25 rxns	53004
ChIP-IT™ Shearing Kit	10 rxns	53002
ChIP-IT™ Enzymatic	25 rxns	53006
ChIP-IT™ Enzymatic w/o controls	25 rxns	53007
Enzymatic Shearing Kit	10 rxns	53005
Salmon Sperm DNA/Protein G agarose	25 rxns	53003
ChIP-IT™ Control Kit – Human	5 rxns	53010
ChIP-IT™ Control Kit – Mouse	5 rxns	53011
ChIP-IT™ Control Kit – Rat	5 rxns	53012
Ready-to-ChIP HeLa Chromatin	10 rxns	53015

Chromatin Assembly	Format	Catalog No.
Chromatin Assembly Kit	10 rxns	53500

Histone Acetyltransferase Activity	Format	Catalog No.
HAT Assay Kit (Fluorescent)	1 x 96 rxns	56100

Co-Immunoprecipitation	Format	Catalog No.
Nuclear Complex Co-IP Kit	50 rxns	54001

ChIP-validated Antibodies	Application	Format	Catalog No.
AP-2 pAb	ChIP, SS	17 rxns	39304
c-Jun pAb	ChIP, SS IF	100 µg	39309
C/EBPα pAb	ChIP, WB, SS, IF	100 µg	39306
DNMT1 mAb	ChIP, IP, WB	100 µg	39204
DNMT3B mAb	ChIP, IF, WB,	100 µg	39206
E2F-1 pAb	ChIP, SS	17 rxns	39313
E2F-6 mAb	ChIP, WB	100 µl	39509
GATA-1 pAB	ChIP, WB	100 µl	39025
HBP-1 mAb	ChIP, IF WB	100 µl	39511
HDAC3 pAb	ChIP, WB	100 µg	40968
HDAC4 pAb	ChIP, WB	100 µg	40969
HDAC5 pAb	ChIP, WB	100 µg	40970
HDAC6 pAb	ChIP, WB	100 µg	40971
IRF-3 pAb	ChIP, WB	100 µl	39033
Jun B pAb (Crude serum)	ChIP, SS	17 rxns	39326
Jun D pAb (Crude serum)	ChIP, SS	100 µl	39328
p53 pAb	ChIP, SS	17 rxns	39334
Pax-5 pAb	ChIP, SS	17 rxns	39336
PPARγ pAb	ChIP, SS	17 rxns	39338
RNA pol II mouse mAb	ChIP, WB	200 µl	39097
SP1 pAb	ChIP, WB	100 µl	39058
TRF2 pAb	ChIP, WB, IP	100 µg	39223

Application Key: ChIP = Chromatin Immunoprecipitation; **WB** = Western blot; **IF** = Immunofluorescence; **IP** = Immunoprecipitation; **SS** = Supershift; For our complete line of antibodies visit www.activemotif.com

Transcription Factor ELISAs	Format	Catalog No.
TransAM™ AML-1/Runx1	1 x 96-well plate	47396
TransAM™ AML-3/Runx2	1 x 96-well plate	44496
TransAM™ AP-1 Family	2 x 96-well plates	44296
TransAM™ AP-1 c-Fos	1 x 96-well plate	44096
TransAM™ AP-1 c-Jun	1 x 96-well plate	46096
TransAM™ AP-1 FosB	1 x 96-well plate	45096
TransAM™ AP-1 JunD	1 x 96-well plate	43496
TransAM™ ATF-2	1 x 96-well plate	42396
TransAM™ c-Myc	1 x 96-well plate	43396
TransAM™ C/EBP α/β	1 x 96-well plate	44196
TransAM™ CREB	1 x 96-well plate	42096
TransAM™ pCREB	1 x 96-well plate	43096
TransAM™ Elk-1	1 x 96-well plate	44396
TransAM™ ER	1 x 96-well plate	41396
TransAM™ FKHR (FOXO1/4)	1 x 96-well plate	46396
TransAM™ GATA Family	2 x 96-well plates	48296
TransAM™ GATA-4	1 x 96-well plate	46496
TransAM™ GR	1 x 96-well plate	45496
TransAM™ HIF-1	1 x 96-well plate	47096
TransAM™ HNF Family	2 x 96-well plates	46296
TransAM™ HNF-1	1 x 96-well plate	46196
TransAM™ MAPK Family	2 x 96-well plates	47296
TransAM™ MEF2	1 x 96-well plate	43196
TransAM™ MyoD	1 x 96-well plate	47196
TransAM™ NF-YA	1 x 96-well plate	40396
TransAM™ NFATc1	1 x 96-well plate	40296
TransAM™ NFκB Family	2 x 96-well plates	43296
TransAM™ Flexi NFκB Family	2 x 96-well plates	43298
TransAM™ NFκB p50	1 x 96-well plate	41096
TransAM™ Flexi NFκB p50	1 x 96-well plate	41098
TransAM™ NFκB p50 Chemi	1 x 96-well plate	41097
TransAM™ NFκB p52	1 x 96-well plate	48196
TransAM™ NFκB p52 Chemi	1 x 96-well plate	48197
TransAM™ NFκB p65	1 x 96-well plate	40096
TransAM™ Flexi NFκB p65	1 x 96-well plate	40098
TransAM™ NFκB p65 Chemi	1 x 96-well plate	40097
TransAM™ Oct-4	1 x 96-well plate	41196
TransAM™ p53	1 x 96-well plate	42496
TransAM™ PPARγ	1 x 96-well plate	40196
TransAM™ Sp1	1 x 96-well plate	41296
TransAM™ Sp1/Sp3	1 x 96-well plate	40496
TransAM™ STAT Family	2 x 96-well plates	42296
TransAM™ STAT3	1 x 96-well plate	45196

DNA Methylation	Format	Catalog No.
MethylDetector™	50 rxns	55001
MethylCollector™	25 rxns	55002
Fully Methylated Jurkat DNA	10 µg	55003

SUMOylation	Format	Catalog No.
SUMOlink™ SUMO-1 Kit	20 rxns	40120
SUMOlink™ SUMO-2/3 Kit	20 rxns	40220

Technical Services

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

Active Motif North America
1914 Palomar Oaks Way, Suite 150
Carlsbad, CA 92008
USA

Toll Free:	877 222 9543
Telephone:	760 431 1263
Fax:	760 431 1351
E-mail:	tech_service@activemotif.com

Active Motif Europe
104 Avenue Franklin Roosevelt
B-1330 Rixensart, Belgium

UK Free Phone:	0800 169 31 47
France Free Phone:	0800 90 99 79
Germany Free Phone:	0800 181 99 10
Telephone:	+32 (0)2 653 0001
Fax:	+32 (0)2 653 0050
E-mail:	eurotech@activemotif.com

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

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

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