High Sensitivity Chromatin Preparation

(version A1)

Catalog No. 53046

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

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

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

Active Motif China
787 Kangqiao Road
Building 10, Suite 202, Pudong District
Shanghai, 201315, China
Telephone: (86)-21-20926090
Hotline: 400-018-8123

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Overview

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

Active Motif's High Sensitivity Chromatin Preparation Kit is designed to isolate formaldehyde-fixed chromatin from cultured cells or tissue samples for use in ChIP. The kit contains optimized protocols and buffers to yield high quality chromatin, including recommendations for working with difficult to lyse peripheral blood mononuclear cells (PBMCs). The High Sensitivity Chromatin Preparation Kit is ideal for optimization of chromatin preparation and sonication conditions when working with different sample types. Prepared chromatin is suitable for use with Active Motif's ChIP-IT® High Sensitivity Kit, ChIP-IT® ChIP-Seq Kit, ChIP-IT® PBMC Kit, ChIP-exo Kit, ChIP-Bisulfite-Sequencing Kit, Tag ChIP-IT® Kit and enChIP Kit.

The High Sensitivity Chromatin Preparation Kit contains reagents for 16 chromatin preparations. A chromatin preparation is defined as one 150 mm cell culture dish, 10 million PBMCs or 100 mg tissue sample. To learn about available ChIP Kits, ChIP-Seq validated antibodies, or Active Motif's EpiShear™ sonication devices, please visit our website at **www.activemotif.com/chip**.

product	format	catalog no.
High Sensitivity Chromatin Preparation	16 rxns	53046
ChIP-IT® High Sensitivity Kit	16 rxns	53040
Protein G Agarose Columns	30 rxns	53039

Kit Components and Storage

Please store each component at the temperature indicated in the table below.

Reagents	Quantity	Storage
RNase A (10 µg/µl)	40 µl	-20°C
Proteinase K (10 µg/µl)	80 µl	-20°C
10X PBS	120 ml	-20°C
100 mM PMSF	500 µl	-20°C
Protease Inhibitor Cocktail (PIC)	500 µl	-20°C
Precipitation Buffer	1.5 ml	-20°C
Carrier	35 µl	-20°C
Fixation Buffer	2 x 1.5 ml	4°C
Swelling Buffer	120 ml	4°C
Detergent	25 ml	RT
Stop Solution	20 ml	RT
Chromatin Prep Buffer	85 ml	RT
ChIP Buffer	35 ml	RT
5 M NaCl	400 µl	RT
TE pH 8.0	2 x 1.5 ml	RT
DNA Purification Elution Buffer	5 ml	RT

Additional materials required

- Dounce homogenizer with a small clearance pestle (*e.g.* Active Motif Catalog Nos. 40401 & 40415) with the tight-fitting "A" pestle). Use of a homogenizer is necessary for shearing chromatin.
- 37% formaldehyde solution with 10-15% methyl alcohol to prevent polymerization (*e.g.* Sigma Aldrich Catalog No. 252549). Do not use paraformaldehyde.
- For tissue and PBMC preparations you will need phenol, saturated (DNA Purification, Molecular Biology Grade, Amresco Catalog No. 0945)
- For tissue and PBMC preparations you will need chloroform/isoamyl alcohol (24:1) (DNA Purification, Molecular Biology Grade)
- 100% ethanol (absolute)
- 70% ethanol
- DNase-free H_2O
- Rocking platform for culture plates

- Microcentrifuge (table top centrifuge 4°C) and microcentrifuge tubes
- 250 µl PCR tubes
- Thermocycler
- 15 and 50 ml conical tubes
- Spectrophotometer for DNA quantitation
- Pipettors and tips (filter tips are recommended)
- Sonicator (*e.g.* Active Motif's EpiShear™ Sonicator with a 1/8" probe (Catalog No. 53051) with the EpiShear™ Cooled Sonication Platform (Catalog No. 53080))
- Agarose gel electrophoresis apparatus
- Razor blades (for tissue preparations)
- Hand-held homogenizer for tissue preparations (*e.g.* Biospec Products Tissue-Tearor)
- Cell scraper (rubber policeman)
- Dry ice (for PBMC cells)
- Metal spatula for cell scraping (for PBMC cells)

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Cell Growth Recommendations

When planning an experiment, calculate the number of chromatin preparations you will require and determine the number of ChIP reactions you plan to perform on each chromatin preparation. Be sure to include the appropriate positive and negative control ChIP reactions in your calculations. Also, note that if you wish to analyze the effect of particular compounds or culturing conditions on transcription factor/DNA interactions, you should prepare chromatin from control (untreated) cells as a reference sample. PBMCs (including lymphocytes and monocytes) are difficult to lyse cells and as a result will yield approximately 30-50% as compared to cell lines.

Suggested cell number minimums for the preparation of chromatin are provided below:

Cell Culture: We recommend the use of 100,000 – 15 million cells
PBMC Cells: We recommend the use of 10 – 20 million cells
Tissue Samples: We recommend the use of 100-400 mg

Cell Culture Guidelines	24-well plate	12-well plate	6-well plate	60 mm dish	100 mm dish	150 mm dish
Seeding Density	0.05 x 10^6	0.1 x 10^6	0.3 x 10^6	0.8 x 10^6	2.2 x 10^6	5.0 x 10^6
Cells at 70-80% Confluency*	0.15 x 10^6	0.3 x 10^6	0.9 x 10^6	2.4 x 10^6	6.6 x 10^6	15.0 x 10^6
Growth Medium Volume	1 ml	2 ml	3 ml	5 ml	10 ml	20 ml
Cell Fixative Solution	100 µl	200 µl	300 µl	500 µl	1 ml	2 ml
Stop Solution	55 µl	110 µl	165 µl	275 µl	550 µl	1.1 ml
PBS Wash Buffer (used per wash)	500 µl	500 µl	1 ml	2 ml	5 ml	10 ml
Chromatin Prep Buffer	500 µl	500 µl	1 ml	2 ml	5 ml	5 ml
ChIP Buffer	500 µl	500 µl	500 µl	500 µl	500 µl	500 µl

* The number of cells on a confluent plate or dish will vary with cell type. For this table, HeLa cells were used. Please adjust as needed based on your particular cell type.

**Please refer to the descriptions below for complete details on buffer preparations

Buffer Preparation

Follow the instructions below to prepare buffers for chromatin preparation. A Quick Chart for Buffer Preparation is provided on page 7. Buffers should be scaled accordingly if working with different amounts of cells.

Complete Cell Fixation Solution

Buffer should be prepared fresh before each experiment.

For each 150 mm cell culture dish, prepare 2.5 ml of Complete Cell Fixation Solution by adding 180 µl Fixation Buffer to 1.57 ml sterile water in a 15 ml conical tube. Using appropriate precautions (*i.e.* safety glasses, gloves and lab coat), add 750 µl 37% formaldehyde to the tube and vortex to mix. Use 1/10 growth medium volume per plate. Complete cell fixation solution can be added to the growth medium in the presence or absence of serum.

For each PBMC pellet, prepare 5 ml of Complete Fixation Solution by adding 0.5 ml 10X PBS to 4.36 ml sterile water in a 15 ml conical tube. Using appropriate precautions (*i.e.* safety glasses, gloves and lab coat), add 140 µl 37% formaldehyde to the tube and vortex to mix. Use 5 ml Complete Fixation Solution per PBMC pellet.

Complete Tissue Fixation Solution

Buffer should be prepared fresh before each experiment. Prepare 10 ml of Tissue Fixation Solution for each tissue sample to be processed by adding 1 ml 10X PBS to 8.7 ml sterile water in a 15 ml conical tube. Using appropriate precautions (*i.e.* safety glasses, gloves and lab coat), add 280 µl 37% formaldehyde to the tube and vortex to mix.

Stop Solution

Is provided ready to use. Use 1/20 media volume per cell culture plate, use 250 µl per PBMC pellet and 515 µl per tissue sample.

Detergent

Is supplied ready to use.

PBS Wash Buffer

For each 150 mm cell culture dish or tissue sample, prepare 25 ml of PBS Wash Buffer. To a 50 ml conical tube add 21.25 ml sterile water, 2.5 ml 10X PBS and 1.25 ml Detergent. Mix by inverting. Place PBS Wash Buffer on ice to chill. PBS Wash Buffer can be prepared in large quantities and stored at 4°C for 6 months.

For each PBMC pellet, prepare 10 ml of PBS Wash Buffer. To a 15 ml conical tube add 8.5 ml sterile water, 1 ml 10X PBS and 0.5 ml Detergent. Just before use, add 20 µl 100 mM PMSF.

100 mM PMSF and Protease Inhibitor Cocktail (PIC)

Thaw PMSF and PIC at room temperature until fully dissolved, which takes about 30 minutes. Vortex gently and spin down briefly before use, then add to the buffers immediately before use.

Chromatin Prep Buffer

Is supplied ready to use.

Swelling Buffer

Is supplied ready to use.

ChIP Buffer

Is supplied ready to use.

Recommendations

Chromatin Shearing Tips

We suggest using a probe sonicator (*i.e.* Active Motif's EpiShear Probe Sonicator) which employs a direct sonication method to prepare chromatin for use in the ChIP. Indirect sonication systems may require longer sonication times to achieve optimal chromatin shearing. ChIP experiments usually require chromatin that has been sheared to a size of 200-1200 bp. In general, shearing efficiency is improved through the use of a small shearing volume and a V-bottom tube rather than a round-bottom tube. Also, note that shearing is inefficient if the chromatin sample becomes emulsified with air bubbles. To determine the appropriate shearing level for your sample, set up a "practice" tube containing only ChIP Buffer. Slowly increase the sonication amplitude until foaming starts to occur. Reduce the amplitude setting down slightly and mark this as the highest possible intensity to use without foaming. If a chromatin preparation becomes emulsified inadvertently, discontinue shearing and centrifuge the sample at maximum speed for 4 minutes at 4°C in a microcentrifuge to remove trapped air. Finally, to prevent overheating and denaturation of chromatin, samples should be kept on ice as much as possible during shearing, and shearing should be performed discontinuously (*i.e.* sonicate for 20 seconds, then place on ice/water for 30 seconds, sonicate again for 20 seconds, *etc.*). If possible, shear while on ice or use Active Motif's EpiShear™ Cooled Sonication Platform (Catalog No. 53080) to help regulate sample temperature.

Chromatin Quantity

It is recommended to use 10-30 µg chromatin per IP reaction. Follow the recommendations for the chromatin immunoprecipitation protocol to prepare the IP reaction. Chromatin prepared with the High Sensitivity Chromatin Preparation Kit is suitable for use in Active Motif's ChIP-IT® High Sensitivity Kit, ChIP-IT® ChIP-Seq Kit, ChIP-IT® PBMC Kit, ChIP-exo Kit, ChIP-Bisulfite-Sequencing Kit, Tag ChIP-IT® Kit and enChIP Kit.

Safety Precautions

Formaldehyde and PMSF are highly toxic chemicals. Appropriate safety precautions (*i.e.* safety glasses, gloves and lab coat) should be used. Also, formaldehyde is highly toxic by inhalation and should be used only in a ventilated hood. Finally, chromatin sonication should be performed in a biosafety hood if the chromatin is extracted from biohazardous or infectious materials.

Quick Chart for Preparing Buffers

Reagents to prepare	Components	Per 150 mm dish	Per PBMC pellet	Per Tissue sample
Fixation Solution	Sterile Water	1.57 ml	4.36 ml	8.7 ml
	Fixation Buffer	180 µl	–	–
	10X PBS	–	0.5 ml	1 ml
	37% Formaldehyde	750 µl	140 µl	280 µl
	TOTAL REQUIRED	**2.5 ml**	**5 ml**	**10 ml**
Stop Solution	**TOTAL REQUIRED**	**1.1 ml**	**250 µl**	**515 µl**
PBS Wash Buffer	Sterile Water	21.25 ml	8.5 ml	21.25 ml
	10X PBS	2.5 ml	1 ml	2.5 ml
	Detergent	1.25 ml	0.5 ml	1.25 ml
	100 mM PMSF	–	20 µl	–
	TOTAL REQUIRED	**25 ml**	**10 ml**	**25 ml**
Chromatin Prep Buffer	Chromatin Prep Buffer	5 ml	–	5 ml
	Protease Inhibitor Cocktail	5 µl	–	5 µl
	100 mM PMSF	5 µl	–	5 µl
	TOTAL REQUIRED	**5 ml**	**N/A**	**5 ml**
Swelling Buffer	Swelling Buffer	–	5 ml	–
	Protease Inhibitor Cocktail	–	5 µl	–
	100 mM PMSF	–	5 µl	–
	TOTAL REQUIRED	**N/A**	**5 ml**	**N/A**
Detergent	**TOTAL REQUIRED**	**N/A**	**375 µl**	**N/A**
ChIP Buffer	ChIP Buffer	500 µl	500 µl	500 µl – 1 ml
	Protease Inhibitor Cocktail	5 µl	5 µl	5 µl – 10 µl
	100 mM PMSF	5 µl	5 µl	5 µl – 10 µl
	TOTAL REQUIRED	**510 µl**	**510 µl**	**510 µl – 1 ml**

Section A: Cell Fixation Starting with Cultured Cells

This protocol describes cell fixation and chromatin preparation from one 150 mm dish (approximately 1.5 x 10^7 cells). We recommend using 20 ml growth medium per 150 mm dish. Please refer to page 4 for information on scaling the protocol for use with other amounts of cells. The minimum cell number to be used for the preparation of chromatin is 100,000 cells.

1. Prepare 150 mm dishes for each cell line to be tested. Grow the cells to 70-80% confluency. Stimulate cells as desired to activate the pathway of interest.
2. Freshly prepare Complete Cell Fixation Solution for each 150 mm dish.
3. To fix cells, add 1/10 growth medium volume of freshly prepared Complete Cell Fixative Solution to the existing culture media for the cells (*e.g.* 20 ml growth medium would get 2 ml Complete Cell Fixation Solution). Shake gently at room temperature for 15 minutes.
4. Stop the fixation reaction by adding 1/20 media volume of Stop Solution to the existing culture media for the cells (*e.g.* 20 ml growth medium would get 1.1 ml Stop Solution). Swirl to mix and incubate at room temperature for 5 minutes.
5. Following the incubation, hold the plate at an angle and using a rubber policeman scrape cells down to collect them at the bottom edge of the plate. Use a pipette to transfer the cells to a 50 ml conical tube on ice.
6. Pellet the cells from step 5 by centrifugation for 3 minutes at 1,250 x g at 4°C.
7. Remove the supernatant and discard. Resuspend the pellet(s) in 10 ml ice-cold PBS Wash Buffer by pipetting up and down. Keep samples ice-cold for the remainder of the procedure.
8. Centrifuge for 3 minutes at 1,250 x g at 4°C. Remove the supernatant and discard. Wash the pellet(s) a second time in 10 ml ice-cold PBS Wash Buffer by pipetting up and down. Centrifuge for 3 minutes at 1,250 x g at 4°C. Remove the supernatant and discard. (Cell pellets may be stored at -80°C at this stage).
9. Resuspend each pellet(s) in 5 ml Chromatin Prep Buffer supplemented with 5 µl PIC and 5 µl 100 mM PMSF. Pipet up and down to mix.
10. Incubate on ice for 10 minutes.
11. Transfer the resuspended pellets individually to a chilled dounce homogenizer on ice. Use the tight fitting pestle (Type A) to homogenize the sample for 30 strokes. Transfer the contents to a new 15 ml conical tube and centrifuge for 3 minutes at 1,250 x g at 4°C.

    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.

12. Remove the supernatant and discard. Resuspend each pellet in 500 µl ChIP Buffer supplemented with 5 µl PIC and 5 µl 100 mM PMSF. Transfer the contents to a new 2 ml microcentrifuge tube.
13. Incubate on ice for 10 minutes. Proceed to Step B: Chromatin Sonication of Cultured Cells.

Section B. Chromatin Sonication of Cultured Cells

The section below describes the fragmentation of chromatin using sonication. Sonication results may vary depending on cell type and sonication device being used. This protocol has been validated using Active Motif's EpiShear™ Probe Sonicator in combination with an EpiShear™ Cooled Sonication Platform to maintain probe height and temperature consistency between samples. We do not recommend sonication of samples containing less than 100,000 cells and/or 350 µl volume.

The ChIP Buffer has been optimized for immunoprecipitation performance, however, due to its unique composition optimization of sonication conditions may be required. To maintain the high sensitivity of the assay, we recommend using our buffer system and altering the sonication time and/or amplitude of your sonication system to achieve the desired fragmentation (*e.g.* some systems may require as much as a three-fold increase in sonication time to improve chromatin shearing). Please pay particular attention to our protocol regarding the processing of input chromatin for agarose gel analysis prior to the chromatin immunoprecipitation reaction as many steps may differ from traditional ChIP protocols and failure to follow the outlined procedure may lead to artifacts in the gel images as shown in Figure 1 on page. 11

1. Place the 2 ml microcentrifuge tube containing the chromatin into the tube cooler or packed ice. Open cap and submerge the microtip into the liquid until the microtip is approximately 5 mm from the bottom of the tube. Sonicate according to optimized settings for the cell type being used (see Recommendations on page 6). A recommended starting range for cultured cells is: 25% amplitude, pulse for 30 seconds on and 30 seconds off for a total sonication "on" time of 10 minutes (or 20 minutes elapsed time).
2. Spin tubes at 4°C in a microcentrifuge at maximum speed for 2 minutes to pellet the cellular debris.
3. Transfer 25 µl of each chromatin preparation into a 250 µl PCR tube for analysis of shearing efficiency and chromatin quantification. This sample will be used to generate the Input DNA.
4. Aliquot the remainder of each chromatin preparation into 1.5 ml microcentrifuge tubes. We recommend making aliquots of 150 µl volume and storing at -80°C.

   Note: The size of the chromatin sonication should be verified before proceeding to the immunoprecipitation step.

Input Preparation

5. To each 25 µl chromatin preparation from Step 3 above, add 175 µl TE pH 8.0 and 1 µl RNAse A. Cap the PCR tubes and vortex to mix
6. Incubate in a thermocycler at 37°C for 30 minutes.
7. Add 2 µl Proteinase K to each tube and vortex. Incubate tubes in a thermocycler at 55°C for 30 minutes and then increase the temperature to 80°C for 2 hours.
8. Transfer each chromatin input to a 1.5 ml microcentrifuge tube. Add 83 µl Precipitation Buffer, 2 µl Carrier and 750 µl absolute ethanol. Vortex to mix and chill at -80°C for 30 minutes to overnight.
9. Spin tubes at 4°C in a microcentrifuge at maximum speed for 15 minutes.
10. Carefully remove the supernatant taking care not to disturb the pellet. Wash the pellet with 500 µl 70% ethanol and spin at 4°C in a microcentrifuge at maximum speed for 5 minutes.
11. Carefully remove the supernatant taking care not to disturb the pellet. Remove residual ethanol with a pipet tip. Leave the tubes uncapped and air dry for 10-15 minutes.
12. When the pellets are dry, add 25 µl DNA Purification Elution Buffer to each tube. Incubate at room temperature for 10 minutes. Then vortex to ensure the pellet is completely resuspended. This solution contains your Input DNA.
13. Read the absorbance of each sample on a NanoDrop or other spectrophotometer at 260 nm to determine the DNA concentration of each chromatin preparation. Set aside 500 ng of DNA for analysis as described in Step 14. Store the remaining Input DNA at -20°C.
14. Analyze each chromatin preparation on an agarose gel by following the instructions below.
    a. Prepare 500 mM NaCl by adding 2 µl 5M NaCl to 18 µl sterile water. Vortex to mix.
    b. Transfer 500 ng of Input DNA to a 250 µl PCR tube and add 1 µl 500 mM NaCl. Adjust the final volume to 10 µl with sterile water if needed.
    c. Heat samples in a thermocycler at 100°C for 20 minutes followed by ramping the temperature down to 50°C.
    d. Remove tubes from the thermocycler and incubate at room temperature for 5 minutes.
    e. Add gel loading buffer to each sample and run on a 1.5% agarose gel. Include 100 bp and 1 kb DNA ladders to analyze chromatin size. DNA should appear as a smear anywhere between 200-1200 bp.

Note: Chromatin prepared with the High Sensitivity Chromatin Preparation Kit may look different on an agarose gel compared to chromatin prepared using traditional ChIP methods. However, this will not affect the sensitivity of the assay or increase background signal. Please follow the protocol as listed above for preparing Input DNA. Use of an alternative reverse cross-linking method or omitting the 20 minute incubation at 100°C in NaCl is not recommended as this will cause artifacts that make the DNA appear larger. As long as the chromatin falls within the recommended 200-1200 bp range, proceed with the ChIP reaction. If fragments do not fall within this range, continue to optimize sonication conditions.

15. If chromatin preparations were successful, the aliquots stored at -80°C from Section B, Step 4 can be used to perform the ChIP reactions.

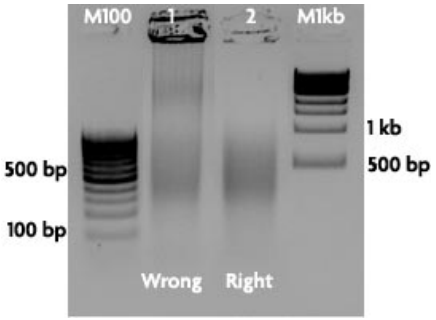

Figure 1: Validation of chromatin shearing efficiency following reversal of cross-links at 80°C for 2 hours.

Chromatin preparations of MCF-7 cells were fixed and sonicated using the EpiShear™ Probe Sonicator and EpiShear™ Cooled Sonication Platform from Active Motif. Input DNA was prepared in duplicate according to Section B, Steps 5-13 in the manual. In Sample 1, Step 14 was not performed and 500 ng of input DNA was loaded directly onto a 1.5% agarose gel without receiving the addition of NaCl and incubation at 100°C. The omission of Step 14 has caused a buffer artifact that makes the DNA appear larger on a gel. The duplicate sample, Sample 2, was processed according to the manual instructions and included the addition of NaCl and incubation at 100°C as stated in Step 14. Analysis of 500 ng of this input DNA on a 1.5% agarose gel shows the expected fragmentation between 200-1200 bp. The difference in DNA sizing on the gel between the two samples illustrates the importance of following the protocol recommendations regarding the processing of input chromatin for agarose gel analysis prior to chromatin immunoprecipitation. Omission of key steps can lead to inaccurate analysis of chromatin shearing efficiency. If the protocol steps were followed and the DNA fragments fall outside of the recommended range, sonication conditions should be further optimized.

Section C: Cell Fixation Starting with Fresh or Frozen Tissue

This protocol describes cell fixation and chromatin preparation from 100-400 mg fresh or frozen animal tissue. If performing chromatin preparation on multiple tissue samples, we recommend completing Steps 1-7 for each sample before processing the next sample.

1. For tissue fixation, transfer 10 ml Complete Tissue Fixation Solution (see Buffer Preparation on page 7) to a 60 mm petri dish. Place the dish on ice.
2. Add 100-400 mg fresh or frozen tissue sample to the petri dish and ensure that the sample is fully immersed. Cut the tissue sample into small pieces (approximately 1 mm cubes) using a razor blade.
3. Transfer the sample plus the Complete Tissue Fixation Solution to a 15 ml conical tube and rotate at room temperature for 15 minutes.
4. Stop the fixation reaction by adding 515 µl Stop Solution to the conical tube and rotate at room temperature for 5 minutes.
5. Place the conical tube on ice and homogenize the contents with a hand-held tissue homogenizer set at 30,000 rpm for 45 seconds.
6. Pellet the cells from step 5 by centrifugation for 3 minutes at 1,250 x g at 4°C.
7. Remove the supernatant and discard. Resuspend the pellet in 10 ml ice-cold PBS Wash Buffer by pipetting up and down. Keep samples ice-cold for the remainder of the procedure.
8. Centrifuge for 3 minutes at 1,250 x g at 4°C. Remove the supernatant and discard. Wash the pellet(s) a second time in 10 ml ice-cold PBS Wash Buffer by pipetting up and down. Centrifuge for 3 minutes at 1,250 x g at 4°C. Remove the supernatant and discard. (Cell pellets may be stored at -80°C at this stage).
9. Resuspend each pellet in 5 ml Chromatin Prep Buffer supplemented with 5 µl PIC and 5 µl 100 mM PMSF.
10. Incubate on ice for 10 minutes.
11. Transfer the resuspended pellet(s) individually to a chilled dounce homogenizer on ice. Use the tight fitting pestle (Type A) to homogenize the sample for 30 strokes. Once finished, transfer the contents to a new 15 ml conical tube.

    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.
12. Centrifuge for 3 minutes at 1,250 x g at 4°C.

13. Remove the supernatant and discard. Resuspend each pellet in 500 µl - 1 ml ChIP Buffer supplemented with PIC and 100 mM PMSF. (For 500 µl add 5 µl PIC and 5 µl PMSF. For 1 ml add 10 µl PIC and 10 µl PMSF.) Transfer the contents to a new 2 ml microcentrifuge tube.

14. Incubate on ice for 10 minutes. Proceed to Section D: Chromatin Sonication of Tissue.

Section D. Chromatin Sonication of Tissue

The section below describes the fragmentation of chromatin using sonication. Due to the increased concentration of protein and cellular debris present in animal tissue, we recommend following this protocol for the preparation of chromatin and input DNA from tissue. Sonication results may vary depending on tissue type and sonication device being used. This protocol has been validated using Active Motifs EpiShear™ Probe Sonicator in combination with the EpiShear™ Cooled Sonication Platform to maintain probe height and temperature consistency between samples. We do not recommend sonication of samples containing less than 50 mg tissue and/or 350 µl volume.

The ChIP has been optimized for immunoprecipitation performance, however, due to its unique composition optimization of sonication conditions may be required. To maintain the high sensitivity of the assay, we recommend using our buffer system and altering the sonication time and/or amplitude of your sonication system to achieve the desired fragmentation (*e.g.* some systems may require as much as a three-fold increase in sonication time to improve chromatin shearing). Please pay particular attention to our protocol regarding the processing of input chromatin for agarose gel analysis prior to the chromatin immunoprecipitation reaction as many steps may differ from traditional ChIP protocols and failure to follow the outlined procedure may lead to artifacts in the gel images as shown in Figure 2 on page 15.

1. Place the 2 ml microcentrifuge tube containing the chromatin into the tube cooler or packed ice. Open cap and submerge the microtip into the liquid until the microtip is approximately 5 mm from the bottom of the tube. Sonicate according to optimized settings for the tissue type being used (see Recommendations on page 6). A recommended starting range for tissue samples is: 25% amplitude, pulse for 30 seconds on and 30 seconds off for a total sonication "on" time of 10 minutes (or 20 minutes elapsed time).

2. Spin tubes at 4°C in a microcentrifuge at maximum speed for 2 minutes to pellet the cellular debris.

3. Transfer 25 µl of each chromatin preparation into a 250 µl PCR tube for analysis of shearing efficiency and chromatin quantification. This sample will be used to generate the Input DNA.

4. Aliquot the remainder of each chromatin preparation into 1.5 ml microcentrifuge tubes. We recommend making aliquots of 150 µl volume and storing at -80°C.

   Note: The size of the chromatin sonication should be verified before proceeding to the immunoprecipitation step.

Input Preparation

5. To each 25 µl chromatin preparation from Step 3 above, add 175 µl TE pH 8.0 and 2 µl RNAse A. Cap the PCR tubes and vortex to mix.
6. Incubate in a thermocycler at 37°C for 1 hour.
7. Add 5 µl Proteinase K to each tube, vortex and incubate in a thermocycler at 37°C for 3 hours.
8. Add 10 µl 5 M NaCl, vortex and incubate at 65°C for 6-16 hours to reverse cross-links.
9. Remove tubes from the thermocycler and add 250 µl phenol and 125 µl chloroform:isoamyl alcohol (24:1). Vortex vigorously and spin tubes in a room temperature microcentrifuge at maximum speed for 2 minutes.
10. Transfer each upper aqueous layer to a new 1.5 ml microcentrifuge tube and add 250 µl chloroform:isoamyl alcohol (24:1). Vortex vigorously and spin tubes in a room temperature microcentrifuge at maximum speed for 2 minutes.
11. Transfer the upper aqueous layer to a new 1.5 ml microcentrifuge tube. Add 83 µl Precipitation Buffer, 2 µl Carrier and 900 µl absolute ethanol. Vortex to mix and chill at -80°C for 30 minutes to overnight.
12. Spin at 4°C in a microcentrifuge at maximum speed for 15 minutes.
13. Carefully remove the supernatant taking care not to disturb the pellet. Wash the pellet with 500 µl 70% ethanol and spin at 4°C in a microcentrifuge at maximum speed for 5 minutes.
14. Carefully remove the supernatant taking care not to disturb the pellet. Remove residual ethanol with a pipet tip. Leave the tubes uncapped and air dry for 10-15 minutes.
15. When the pellets are dry, add 25 µl DNA Purification Elution Buffer to each tube. Incubate at room temperature for 10 minutes. Then vortex to ensure the pellet is completely resuspended. This solution contains your Input DNA.
16. Read the absorbance of each sample on a NanoDrop or other spectrophotometer at 260 nm to determine the DNA concentration of each chromatin preparation. Set aside 500 ng of DNA for analysis as described in Step 17. Store the remaining Input DNA at -20°C.
17. Analyze each chromatin preparation on an agarose gel by following the instructions below.
    a. Prepare 500 mM NaCl by adding 2 µl 5M NaCl to 18 µl sterile water. Vortex to mix.
    b. Transfer 500 ng of Input DNA to a 250 µl PCR tube and add 1 µl 500 mM NaCl. Adjust the final volume to between 10 µl with sterile water if needed.
    c. Heat samples in a thermocycler at 100°C for 20 minutes followed by ramping the temperature down to 50°C.
    d. Remove tubes from the thermocycler and incubate at room temperature for 5 minutes.
    e. Add gel loading buffer to each sample and run on a 1.5% agarose gel. Include 100 bp and 1 kb DNA ladders to analyze chromatin size. DNA should appear as a smear anywhere between 200-1200 bp.

Note: Chromatin prepared with the High Sensitivity Chromatin Preparation Kit may look different on an agarose gel than chromatin prepared using traditional ChIP

methods. However, this will not affect the sensitivity of the assay or increase background signal. Please follow the protocol as listed above for preparing Input DNA. Use of an alternative reverse cross-linking method or omitting the 20 minute incubation at 100°C in NaCl is not recommended as this will cause artifacts that make the DNA appear larger. As long as the chromatin falls within the recommended 200-1200 bp range, proceed with the ChIP reaction. If fragments do not fall within this range sonication conditions should be further optimized.

18. If chromatin preparations were successful, the aliquots stored at -80°C from Section D, Step 4 can be used to perform the ChIP reactions.

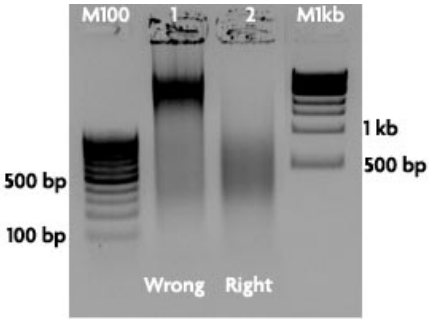

Figure 2: Validation of chromatin shearing efficiency following reversal of cross-links overnight at 65°C.

Chromatin preparations were fixed and sonicated using the EpiShear™ Probe Sonicator and EpiShear™ Cooled Sonication Platform from Active Motif. Input DNA was prepared in duplicate according to Section D, Steps 5-16 in the manual. In Sample 1, Step 17 was not performed and 500 ng of input DNA was loaded directly onto a 1.5% agarose gel without receiving the addition of NaCl and incubation at 100°C. The omission of Step 17 has caused a buffer artifact that makes the DNA appear larger on a gel. The duplicate sample, Sample 2, was processed according to the manual instructions and included the addition of NaCl and incubation at 100°C as stated in Step 17. Analysis of 500 ng of this input DNA on a 1.5% agarose gel shows the expected fragmentation between 200-1200 bp. The difference in DNA sizing on the gel between the two samples illustrates the importance of following the protocol recommendations regarding the processing of input chromatin for agarose gel analysis prior to chromatin immunoprecipitation. Omission of key steps can lead to inaccurate analysis of chromatin shearing efficiency. If the protocol steps were followed and the DNA fragments fall outside of the recommended range, sonication conditions should be further optimized.

Section E: Cell Fixation Starting with PBMCs

PBMCs (including lymphocytes and monocytes) are difficult to lyse cells and as a result will yield approximately 30-50% as compared to cell lines. We recommend using a minimum of 10 million cells for the preparation of chromatin.

1. Collect your PBMCs, T cells, B cells, NK cells or moncytes according to your preferred protocol. Transfer the appropriate number of cells required for each chromatin preparation into a conical tube and centrifuge at 1,250 x g at 4°C to collect the cells. If there are cells that are floating or are stuck to the sides of the tube following the spin, use a metal spatula to scrape the cells down into solution and repeat the centrifugation at 1,250 x g at 4°C for 5 minutes. Continue to repeat the scraping and spin as many times as needed to pellet all the cells. Remove and discard supernatant.

   Note: PBMCs may stick to certain plastics. Therefore, it is recommended to use a metal spatula for cell scraping to avoid losing any sample material.

2. Flash freeze the cell pellet(s) by imersing conical tube into dry ice. Incubate on dry ice for 10 minutes. Cell pellets may be stored at -80°C at this stage, or proceed with the next step.
3. Prepare fresh Fixation Solution. To fix cells, add 5 ml of freshly prepared Fixative Solution to the cell pellet and resuspend cells by pipetting up and down. Incubate at room temperature for 15 minutes on a roller.
4. Stop the fixation reaction by adding 250 µl Stop Solution to the existing fixation solution. Swirl to mix and incubate at room temperature for 5 minutes on a roller.
5. Following the incubation, add 250 µl Detergent to each conical tube. Invert to mix. Pellet the cells by centrifugation for 5 minutes at 1,250 x g at 4°C.
6. If there are cells that are floating or are stuck to the sides of the tube following the spin, use a metal spatula to scrape the cells down into solution and repeat the centrifugation at 1,250 x g at 4°C for 5 minutes. Continue to repeat the scraping and spin as many times as needed to pellet all the cells.
7. Remove the supernatant and discard. Resuspend the pellet(s) in 5 ml ice-cold PBS Wash Buffer by pipetting up and down. Keep samples ice-cold for the remainder of the procedure.
8. Centrifuge for 5 minutes at 3,200 x g at 4°C. The cell pellet will tend to be loose, so carefully remove the supernatant and discard taking care to avoid disturbing the pellet. Wash the pellet(s) a second time in 5 ml ice-cold PBS Wash Buffer by pipetting up and down. Centrifuge for 5 minutes at 3,200 x g at 4°C. Carefully remove the supernatant and discard.
9. Flash freeze the cell pellet(s) by imersing conical tube into dry ice. The freeze/thaw will to help facilitate cell lysis. Incubate on dry ice for 10 minutes. Cell pellets may be stored at -80°C at this stage, or proceed with the next step.
10. Resuspend each pellet(s) in 5 ml ice-cold Swelling Buffer supplemented with 5 µl PIC and 5 µl 100 mM PMSF. Pipet up and down to mix.

11. Incubate on ice for 30 minutes.
12. Add 125 µl Detergent to each cell pellet. Vortex for 30 sec on highest setting to lyse the cells.

    Note: We do not recommend the use of a dounce homogenizer to assist in cell lysis as the transfer process to and from the dounce increases sample loss. If cells continue to have difficulty lysing, we suggest additional sonication.

13. Centrifuge for 10 minutes at 3,200 x g at 4°C. Remove and discard the supernatant.
14. Resuspend each pellet in 500 µl ChIP Buffer supplemented with 5 µl PIC and 5 µl 100 mM PMSF. Transfer the contents to a new 2 ml microcentrifuge tube.
15. Incubate on ice for 10 minutes. Proceed to Step F: Chromatin Sonication of PBMCs.

Section F. Chromatin Sonication of PBMCs

The section below describes the fragmentation of chromatin using sonication. Sonication results may vary depending on cell type and sonication device being used. This protocol has been validated using Active Motif's EpiShear™ Probe Sonicator in combination with an EpiShear™ Cooled Sonication Platform to maintain probe height and temperature consistency between samples. We do not recommend sonication of samples containing less than 10 million cells or 350 µl volume.

The ChIP Buffer has been optimized for immunoprecipitation performance, however, due to its unique composition optimization of sonication conditions may be required. To maintain the high sensitivity of the assay, we recommend using our buffer system and altering the sonication time and/or amplitude of your sonication system to achieve the desired fragmentation (*e.g.* some systems may require as much as a three-fold increase in sonication time to improve chromatin shearing). Please pay particular attention to our protocol regarding the processing of input chromatin for agarose gel analysis prior to the chromatin immunoprecipitation reaction as many steps may differ from traditional ChIP protocols and failure to follow the outlined procedure may lead to artifacts in the gel images as shown in Figure 3 on page 19.

1. Place the 2 ml microcentrifuge tube containing the chromatin into the tube cooler or packed ice. Open cap and submerge the microtip into the liquid until the microtip is approximately 5 mm from the bottom of the tube. Sonicate according to optimized settings for the cell type being used (see Recommendations on page 6). A recommended starting range for PBMCs is: 4 rounds of sonication at 42% amplitude, pulse for 30 seconds on and 30 seconds off for a total sonication "on" time of 5 minutes per round. (Total sonication "on" time is 20 minutes and total elapsed time is 40 minutes per sample.)
2. Spin tubes at 4°C in a microcentrifuge at maximum speed for 5 minutes to pellet the cellular debris.
3. Transfer 25 µl of each chromatin preparation into a 250 µl PCR tube for analysis of shearing efficiency and chromatin quantification. This sample will be used to generate the Input DNA.
4. Aliquot the remainder of each chromatin preparation into 1.5 ml microcentrifuge tubes. We recommend making aliquots of 250 µl volume.

5. Flash freeze the chromatin by imersing tubes into dry ice for 10 minutes and store at -80°C.

   Note: The size of the chromatin sonication should be verified before proceeding to the immunoprecipitation step.

Input Preparation

6. To each 25 µl chromatin preparation from Step 5 above, add 175 µl TE pH 8.0 and 2 µl RNAse A. Cap the PCR tubes and vortex to mix.
7. Incubate in a thermocycler at 37°C for 1 hour.
8. Add 5 µl Proteinase K to each tube, vortex and incubate in a thermocycler at 37°C for 3 hours.
9. Add 10 µl 5 M NaCl, vortex and incubate at 65°C for 6-16 hours to reverse cross-links.
10. Remove tubes from the thermocycler and add 250 µl phenol and 125 µl chloroform:isoamyl alcohol (24:1). Vortex vigorously and spin tubes in a room temperature microcentrifuge at maximum speed for 2 minutes.
11. Transfer each upper aqueous layer to a new 1.5 ml microcentrifuge tube and add 250 µl chloroform:isoamyl alcohol (24:1). Vortex vigorously and spin tubes in a room temperature microcentrifuge at maximum speed for 2 minutes.
12. Transfer the upper aqueous layer to a new 1.5 ml microcentrifuge tube. Add 83 µl Precipitation Buffer, 2 µl Carrier and 900 µl absolute ethanol. Vortex to mix and chill at -80°C for 30 minutes to overnight.
13. Spin at 4°C in a microcentrifuge at maximum speed for 15 minutes.
14. Carefully remove the supernatant taking care not to disturb the pellet. Wash the pellet with 500 µl 70% ethanol and spin at 4°C in a microcentrifuge at maximum speed for 5 minutes.
15. Carefully remove the supernatant taking care not to disturb the pellet. Remove residual ethanol with a pipet tip. Leave the tubes uncapped and air dry for 10-15 minutes.
16. When the pellets are dry, add 25 µl DNA Purification Elution Buffer to each tube. Incubate at room temperature for 10 minutes. Then vortex to ensure the pellet is completely resuspended. This solution contains your Input DNA.
17. Read the absorbance of each sample on a NanoDrop or other spectrophotometer at 260 nm to determine the DNA concentration of each chromatin preparation. Set aside 500 ng of DNA for analysis as described in Step 18. Store the remaining Input DNA at -20°C.
18. Analyze each chromatin preparation on an agarose gel by following the instructions below.
    a. Prepare 500 mM NaCl by adding 2 µl 5M NaCl to 18 µl sterile water. Vortex to mix.
    b. Transfer 500 ng of Input DNA to a 250 µl PCR tube and add 1 µl 500 mM NaCl. Adjust the final volume to between 10 µl with sterile water if needed.
    c. Heat samples in a thermocycler at 100°C for 20 minutes followed by ramping the temperature down to 50°C.
    d. Remove tubes from the thermocycler and incubate at room temperature for 5 minutes.

e. Add gel loading buffer to each sample and run on a 1.5% agarose gel. Include 100 bp and 1 kb DNA ladders to analyze chromatin size. DNA should appear as a smear anywhere between 200-1200 bp.

19. If chromatin preparations were successful, the aliquots stored at -80°C from Section F, Step 5 can be used to perform the ChIP reactions.

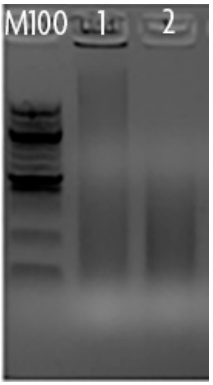

Figure 3: Validation of chromatin shearing efficiency following reversal of cross-links overnight at 65˚C.

Chromatin preparations of PBMC cells were fixed and sonicated using the EpiShear™ Probe Sonicator and EpiShear™ Cooled Sonication Platform from Active Motif. Input DNA was prepared in duplicate according to Section F, Steps 6-17 in the manual. In Sample 1, Step 18 was not performed and 500 ng of input DNA was loaded directly onto a 1.5% agarose gel without receiving the addition of NaCl and incubation at 100°C. The omission of Step 18 has caused a buffer artifact that makes the DNA appear larger on a gel. The duplicate sample, Sample 2, was processed according to the manual instructions and included the addition of NaCl and incubation at 100°C as stated in Step 18. Analysis of 500 ng of this input DNA on a 1.5% agarose gel shows the expected fragmentation between 200-1200 bp. The difference in DNA sizing on the gel between the two samples illustrates the importance of following the protocol recommendations regarding the processing of input chromatin for agarose gel analysis prior to chromatin immunoprecipitation. Omission of key steps can lead to inaccurate analysis of chromatin shearing efficiency. If the protocol steps were followed and the DNA fragments fall outside of the recommended range, sonication conditions should be further optimized.

Section G. 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 Input DNA clean up, -20°C.
After sonication shearing and centrifugation, a viscous or cloudy layer is visible in the chromatin.	Depending upon the cell type, lipid or glycogen layers may be seen after centrifugation. For example, fatty tissues may have a lipid layer. Avoid such layers when you remove the supernatant. However, if the whole supernatant is cloudy, it should not interfere with the IP reaction.
Poor yield of sheared chromatin.	Insufficient cell numbers were used. Repeat chromatin preparation using a larger number of cells.
	Nuclei not released. It is highly recommended to perform dounce homogenization, even when using sonication. Use a dounce homogenizer with a small clearance pestle (Active Motif Catalog Nos. 40401 & 40415). Monitor cell lysis under a microscope. Generally, the more cells that are lysed, the higher the sheared chromatin yield.
	Sonication samples were emulsified. Avoid emulsification by turning up the power of the sonicator gradually. If a chromatin preparation becomes emulsified inadvertently, discontinue shearing and centrifuge the sample for 4 minutes at 8,000 rpm in a 4°C microcentrifuge to remove trapped air.
	Use fresh formaldehyde when preparing Complete Cell Fixation Solution and Complete Tissue Fixation Solution.
	Buffers were not scaled proportionally to the size of the sample. Use the chart in Cell Growth Recommendations to scale up or down chromatin preparation.
Shearing efficiency is not clear from gel analysis or is inefficient.	Material is stuck in the wells, and smears or streaks are seen from the top to bottom of the lane. The sheared chromatin needs to have the cross-links reversed, protein removed (Proteinase K) and RNA removed (RNase), followed by DNA purification.
	High molecular weight products. Decrease the size of the fragments by re-sonicating the sample. If an alternative reverse cross-linking method was used, or the 20 minute incubation at 100°C in NaCl was omitted prior to running the agarose gel for analysis, please repeat the input chromatin preparation and follow the manual instructions.
	Chromatin not sheared enough. Shearing should produce DNA fragments that are small enough to exclude background from neighboring chromosomal sequences, but still large enough that there is a good possibility your amplicon remains intact. We recommend 200-1200 bp fragments. If necessary, optimize sonication settings. Conditions provided are recommended starting points. Increase amplitude of sonication to 42% with cultured cells in a 500 µl volume. Increase amplitude of sonication to 63% with tissue samples in a 1 ml volume. Check the fragment size on a gel to assess your shearing efficiency.

Section H. Related Products

ChIP-IT® Kits	Format	Catalog No.
ChIP-IT® Express	25 rxns	53008
ChIP-IT® Express Enzymatic	25 rxns	53009
ChIP-IT® Express Shearing Kit	10 rxns	53032
ChIP-IT® Express Enzymatic Shearing Kit	10 rxns	53035
ChIP-IT® High Sensitivity	16 rxns	53040
High Sensitivity Chromatin Preparation Kit	16 rxns	53046
ChIP-IT® qPCR Analysis Kit	10 rxns	53029
ChIP-IT® ChIP-Seq	10 libraries	53041
ChIP-Bis-Seq	10 libraries	53048
ChIP-IT® FFPE	16 rxns	53045
ChIP-IT® FFPE Chromatin Preparation Kit	5 rxns	53030
ChIP-exo	12 rxns	53043
enChIP	16 rxns	53125
Tag ChIP-IT®	16 rxns	53022
ChIP-IT® Express HT	96 rxns	53018
Re-ChIP-IT®	25 rxns	53016
RNA ChIP-IT®	25 rxns	53024
Chromatin IP DNA Purification Kit	50 rxns	58002
EpiShear™ Probe Sonicator	110 V	53051
EpiShear™ Cooled Sonication Platform, 1.5 ml	1 platform	53080
ChIP-IT® Protein G Magnetic Beads	25 rxns	53014
Protein G Agarose Columns	30 rxns	53039
Siliconized Tubes, 1.7 ml	25 tubes	53036
ChIP-IT® Control qPCR Kit – Human	5 rxns	53026
ChIP-IT® Control qPCR Kit – Mouse	5 rxns	53027
ChIP-IT® Control qPCR Kit – Rat	5 rxns	53028
ChIP-IT® Control Kit – Human	5 rxns	53010
ChIP-IT® Control Kit – Mouse	5 rxns	53011
ChIP-IT® Control Kit – Rat	5 rxns	53012
Ready-to-ChIP HeLa Chromatin	10 rxns	53015
Ready-to-ChIP Hep G2 Chromatin	10 rxns	53019
Ready-to-ChIP K-562 Chromatin	10 rxns	53020
Ready-to-ChIP NIH/3T3 Chromatin	10 rxns	53021
Bridging Antibody for Mouse IgG	500 µg	53017
Dounce Homogenizer	1 ml	40401
Dounce Homogenizer	15 ml	40415

ChIP-validated Antibodies

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

Technical Services

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

Active Motif North America

1914 Palomar Oaks Way, Suite 150
Carlsbad, CA 92008, USA
E-mail: tech_service@activemotif.com

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

Active Motif Europe

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

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

Active Motif Japan

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

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

Active Motif China

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

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

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