ChIP-Bis-Seq Kit

(version A1)

Catalog No. 53048

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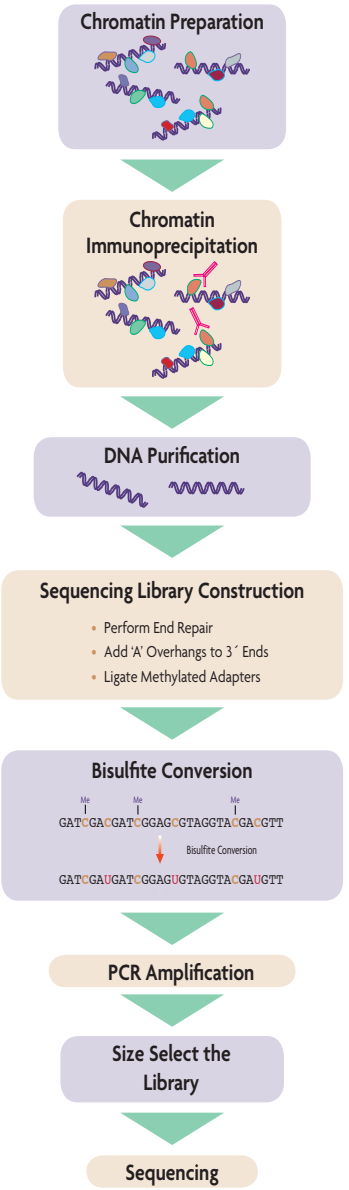
Overview

Active Motif's ChIP-Bisulfite-Sequencing (ChIP-Bis-Seq) Kit combines the target-specific selection of chromatin immunoprecipitation (ChIP) with bisulfite conversion and sequencing (Bis-Seq) to provide single-nucleotide resolution of immunoprecipitated DNA. ChIP is a powerful tool for studying protein/DNA interactions because it identifies the localization of proteins bound to specific DNA loci. While bisulfite conversion and subsequent DNA sequencing is the gold standard for DNA methylation analysis because it provides detailed information on the methylation pattern of individual DNA molecules at single-base-pair resolution. When the two techniques are used in combination, it generates multi-parametric information about individual DNA molecules enabling better understanding of the biological context and significance regarding gene regulation, gene expression, mechanisms of chromatin modification and pathway analysis.

The ChIP-Bis-Seq Kit consists of three modules. The first is Active Motif's ChIP-IT High Sensitivity Kit which is used to immunoprecipitate chromatin bound to a protein of interest in order to identify DNA binding sequences. Using our ChIP-IT High Sensitivity assay, chromatin is prepared from cells or tissues, sonicated to fragment the chromatin and up to 16 chromatin immunoprecipitation reactions are performed. The second kit module contains methylated adapters and enzymes necessary to prepare 10 next generation sequencing (NGS) libraries from the ChIP DNA. The third kit module contains reagents to perform bisulfite conversion on the adapter-ligated DNA to determine the methylation status of each DNA strand. For successful ChIP-Bis-Seq, we recommend using at least 30 µg chromatin (or 4.5 million cell equivalents) per ChIP reaction.

product	format	catalog no.
ChIP-Bis-Seq Kit	10 libraries	53048

Flow Chart of Process

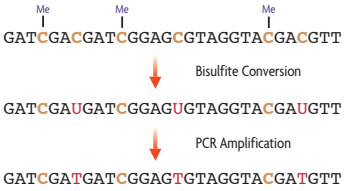


Flow chart of the ChIP-Bisulfite-Sequencing Method.

Introduction

DNA methylation is an essential epigenetic modification that plays a key role in transcriptional regulation and assures the proper regulation of gene expression and stable gene silencing in normal cells. DNA methylation is the covalent addition of a methyl group to the fifth position of the cytosine pyrimidine ring in DNA and usually occurs within CpG dinucleotides, although in some cases the DNA methylation can also be found in a non-CpG context[1, 2]. CpG dinucleotides are concentrated in large clusters called CpG islands, which are often positioned in the promoter regions of many genes and are usually unmethylated[3]. It is estimated that the human genome contains around 29,000 CpG islands and that almost 60% of all human genes are associated with CpG islands[4]. Aberrant cytosine methylation is associated with silencing of tumor suppressor genes[5] and plays a decisive role in the development of many cancers[6-14]. Alterations in the methylation status of DNA are promising candidates for highly specific and sensitive indicators of cancer diagnosis and prognosis[15-17]. Apart from carcinogenesis, DNA methylation is crucial for a variety of processes, such as genomic imprinting, X-chromosome inactivation, and suppression of repetitive elements[4, 18]. Thus profiling DNA methylation across the genome is vital to understanding the influence of epigenetics.

The bisulfite reaction was first described in the early 1970s[19] and was used by Frommer *et al.*[20], and Clark *et al.*[21] to distinguish between cytosine and 5-methylcytosine (5-mC) in DNA. It is now known that both 5-methylcytosine and 5-hydroxymethylcytosine (5-hmC) remain unchanged during conversion and therefore this method cannot be used to distinguish between the different methyl modifications[22-24]. In the bisulfite conversion reaction, DNA is first treated with sodium bisulfite to convert cytosine residues into uracil in denatured (single-stranded) DNA, under conditions whereby 5-mC and 5-hmC remain essentially non-reactive. The DNA is then amplified by PCR where the uracils are converted to thymines. Bisulfite converted DNA can be analyzed for gene- or allele-specific methylation patterns, or adapted for genome-wide DNA methylation analysis. A methylation profile of the sample can then be created by comparing the sequence of the converted DNA to untreated DNA.



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Kit Performance and Benefits

The ChIP-Bis-Seq Kit is for research use only. Not for use in diagnostic procedures.

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

ChIP Bisulfite Sequencing Advantages:

- Directly interrogate both chromatin and DNA methylation profiles on the same sample with higher resolution than either ChIP or bisulfite sequencing alone
- Evaluate allele-specific difference in DNA (imprinting, X-inactivation)
- Method works with histone and transcription factor ChIP antibodies
- Optimized ChIP reagents reduce background levels caused by non-specific binding events providing high quality DNA for analysis
- Reduce sequencing costs by focusing on specific genomic regions

Protocol Overview and Time Table

The ChIP-Bis-Seq Kit requires multiple days to complete. It is strongly advised to read the entire protocol before starting and to plan your experiments in advance.

	Required Time
Cell or Tissue Fixation and Lysis	1.5 hours
Chromatin Sonication	20 minutes per sample
Assessment of Chromatin Size*	4.5 hours for cell culture Overnight for tissue
Immunoprecipitation	Overnight incubation
Binding to Protein G agarose Beads	3 hours
Wash Immune Complexes	20 minutes
Reversal of Cross-links	2.5 hours
ChIP DNA Purification	15 minutes
(Optional) qPCR Analysis of ChIP enriched DNA	2 hours
Perform End Repair	45 minutes
Add 'A' Overhang to 3 ´ Ends of DNA fragments	45 minutes
Ligate Methylated Adapters to DNA Fragments	30 minutes
Bisulfite Conversion	5.5 hours
PCR Amplify Adapter-Modified Library	1 hour
Size Select the Library	1.5 hours

* The protocol varies between cell culture and tissue samples.

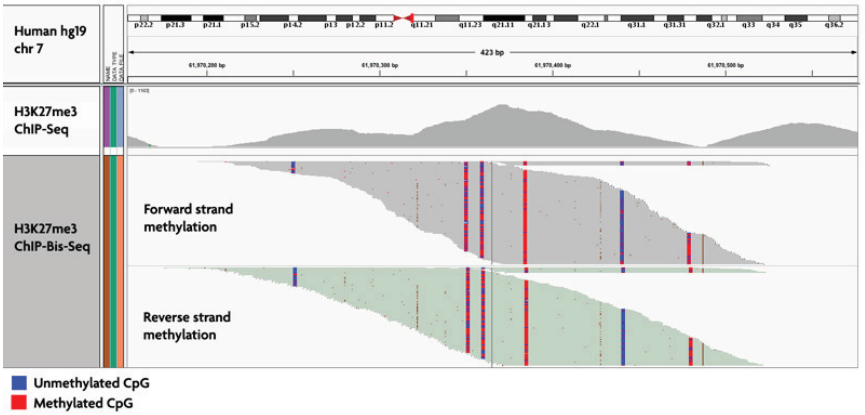

Figure 1: ChIP-Bis-Seq sequencing and DNA methylation results.

This figure shows an example of sequencing results using the ChIP-Bis-Seq Kit to analyze the DNA methylation profile of DNA that was enriched using a ChIP antibody for Histone H3K27me3 in PC9 cells. The top track shows a 423 bp region of chromosome 7 from Human Hg19 genome. The next track reveals the ChIP-seq peak profile for Histone H3K27me3. The final track shows the DNA methylation profile of forward and reverse reads as analyzed by bisulfite conversion of the ChIP-enriched DNA. The blue bars represent unmethylated CpGs, while the red bars represent CpGs containing methylation. (For a color image, please visit our website at www.activemotif.com/chipbisseq to download a color pdf of the manual).

Kit Components and Storage

Module 1: ChIP-IT High Sensitivity (16 ChIP reactions)

Please store each component at the temperature indicated in the table below. **Do not re-freeze the Protein G Agarose Beads. Once thawed, Protein G beads should be stored at 4°C.**

Reagents	Quantity	Storage
RNase A (10 µg/µl)	40 µl	-20°C
Proteinase K (10 µg/µl)	200 µl	-20°C
Blocker	100 µl	-20°C
100 mM PMSF	500 µl	-20°C
Protease Inhibitor Cocktail (PIC)	500 µl	-20°C
Precipitation Buffer	1.5 ml	-20°C
Carrier	35 µl	-20°C
10X PBS	120 ml	-20°C
Fixation Buffer	2 x 1.5 ml	4°C
Protein G Agarose Beads*	500 µl	4°C
Stop Solution	20 ml	RT
Chromatin Prep Buffer	85 ml	RT
ChIP Filtration Columns	16 ea	RT
ChIP Buffer	35 ml	RT
5 M NaCl	400 µl	RT
TE pH 8.0	2 x 1.5 ml	RT
Detergent	25 ml	RT
Wash Buffer AM1	100 ml	RT
Elution Buffer AM4	2 x 1.5 ml	RT
DNA Purification Binding Buffer	50 ml	RT
3 M Sodium Acetate	500 µl	RT
DNA Purification Wash Buffer**	10 ml	RT
DNA Purification Elution Buffer	5 ml	RT
DNA Purification Columns	16 ea	RT

* The Protein G Agarose Beads are shipped on dry ice and can be stored frozen until their first use. Once thawed, the Protein G beads **should not be re-frozen** by the customer, but stored at 4°C.
**Requires the addition of ethanol before use.

Additional materials required

- A ChIP-validated antibody directed against the protein of interest
- Dounce homogenizer with a small clearance pestle (*e.g.* Active Motif Catalog Nos. 40401 & 40415) with the tight-fitting "A" pestle). Use of a homogenizer is necessary for shearing chromatin.
- 37% formaldehyde solution with 10-15% methyl alcohol to prevent polymerization. Do not use paraformaldehyde.
- For tissue preparations you will need Phenol and chloroform/isoamyl alcohol (24:1) (DNA Purification, Molecular Biology Grade)
- 100% ethanol (absolute)
- 70% ethanol
- DNase-free H_2O
- Rocking platform for culture plates
- Apparatus to rotate tubes end-to-end at 4°C (*e.g.* a Labquake from Barnstead/Thermolyne with a tube holder for 1.5 ml microcentrifuge tubes)
- Microcentrifuge (table top centrifuge 4°C) and microcentrifuge tubes
- 250 µl PCR tubes
- Thermocycler
- 15 and 50 ml conical tubes
- Spectrophotometer for DNA quantitation
- Pipettors and tips (filter tips are recommended)
- Sonicator (*e.g.* Active Motif's EpiShear™ Sonicator with a 1/8" probe (Catalog No. 53051) with the EpiShear™ Cooled Sonication Platform (Catalog No. 53080))
- Agarose gel electrophoresis apparatus
- Razor blades (for tissue preparations)
- Hand-held homogenizer for tissue preparations (*e.g.* Biospec Products Tissue-Tearor)
- Cell scraper (rubber policeman)
- (Optional) ChIP-IT® qPCR Analysis Kit (Catalog No. 53029)
- (Optional) Gene-specific qPCR primer pairs for enrichment analysis; see Appendix Section I
- (Optional) SYBR Green qPCR master mix (Bio-Rad Catalog No. 170-8882)

Module 2: Sequencing Library Preparation (10 libraries)

Sequencing Library components arrive on dry ice and should be stored at -20°C. Components are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity	Storage
DNA Polymerase I Klenow (5 U/µl)	5 µl	-20°C
T4 DNA polymerase (3 U/µl)	10 µl	-20°C
T4 Polynucleotide Kinase (10 U/µl)	10 µl	-20°C
Klenow Fragment (3'-5' exo-) (5 U/µl)	10 µl	-20°C
10X Reaction Buffer AM3	50 µl	-20°C
T4 DNA Ligase (2,000 U/µl)	10 µl	-20°C
10X T4 DNA Ligase Buffer	50 µl	-20°C
2X Quick Ligation Buffer	2 x150 µl	-20°C
dNTP Mix, 10 mM each	35 µl	-20°C
1 mM dATP	100 µl	-20°C
Methylated Adapters (5 µM)	30 µl	-20°C
Glycogen (for use with Module 3)	10 µl	-20°C

Additional materials required

- Purified ChIP DNA for each sample to be tested
- QIAquick PCR Purification Kit (QIAGEN Cat. No. 28104)
- MinElute PCR Purification Kit (QIAGEN Cat. No. 28004)
- 250 µl PCR tubes
- Thermocycler

Module 3: Bisulfite Conversion (10 conversion reactions)

Bisulfite Conversion components arrive at room temperature and can be stored at 4°C prior to first use. Then, we recommend storing each component at the temperatures listed in the table below. Components are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity	Storage
Conversion Reagent	2 tubes	RT
Hydroquinone	2 tubes	RT
Buffer A	1.5 ml	RT
Buffer B	1 ml	RT
DNA Binding Buffer	15 ml	RT
DNA Wash Buffer	20 ml	RT
DNA Elution Buffer	2.5 ml	RT
ChIP-Bis-Seq purification columns	10 ea	RT

Additional materials required

- Purified adapter-ligated sample DNA
- 0.2 ml PCR tubes and caps
- 100% isopropanol
- Microcentrifuge tubes and microcentrifuge
- Thermocycler
- Hot Start *Taq* polymerase and PCR reaction buffer recommended for use with bisulfite converted DNA (*e.g.* KAPA HiFi HotStart Uracil+ Ready Mix, KAPA Biosystems Catalog No. KK2801
- PCR primers for library amplification (see recommendations in Appendix X)
- 1.5% agarose TAE gel
- Gel apparatus and power supply
- 6x Loading dye
- Molecular weight marker
- QIAquick Gel Extraction Kit (QIAGEN Cat. No. 28704)
- Razor blades (for gel extraction)
- Picogreen or equivalent fluorescent quantitation method to determine DNA concentration

Module 1 – ChIP-IT® High Sensitivity

The first step of the assay is to perform chromatin immunoprecipitation using the ChIP-IT® High Sensitivity Kit module. Please refer to pages 7-8 of the manual for the kit components and additional materials required to complete the chromatin preparation and immunoprecipitation protocols.

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.

The minimum recommended number of cells that should be used for the preparation of chromatin is 4.5 million cells per sample. It is then recommended to use 30 µg chromatin for each ChIP reaction to ensure a minimum of 10 ng DNA for downstream bisulfite conversion. The quantity of the ChIP enrichment will depend on the abundance of the protein of interest and the affinity of the ChIP antibody. For highly abundant histone proteins, less chromatin may be used. For low abundance transcription factors, it may be necessary to set up multiple ChIP reactions and combine the samples onto a single DNA purification column during the DNA purification and elution in Section F, step 3

	60 mm dish	100 mm dish	150 mm dish
Seeding Density	0.8 x 10^6	2.2 x 10^6	5.0 x 10^6
Cells at 70-80% Confluency*	2.4 x 10^6	6.6 x 10^6	15.0 x 10^6
Growth Medium Volume	5 ml	10 ml	20 ml
Cell Fixative Solution	500 µl	1 ml	2 ml
Stop Solution	275 µl	550 µl	1.1 ml
PBS Wash Buffer (used per wash)	2 ml	5 ml	10 ml
Chromatin Prep Buffer	2 ml	5 ml	5 ml
ChIP Buffer	500 µl	500 µl	500 µl

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

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

Reagent Information

Complete Cell Fixation Solution

Buffer should be prepared fresh before each experiment. For every 20 ml of cell growth medium used, 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 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 or 515 µl per 10 ml Complete Tissue Fixation Solution.

PBS Wash Buffer

Prepare 25 ml PBS Wash Buffer for every 15 cm plate or tissue sample. 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.

100 mM PMSF and Protease Inhibitor Cocktail (PIC)

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

Chromatin Prep Buffer

Is supplied ready to use.

ChIP Buffer

Is supplied ready to use.

Protein G Agarose Beads

The supplied agarose beads require washing prior to use. Follow the instructions in the manual to wash the beads for use in the ChIP reactions. There is no need to pre-block the beads or pre-clear the sample. For best results, gently shake and invert the tube to resuspend the agarose beads. The beads settle quickly, and therefore should be resuspended just before pipetting. We recommend cutting 2 mm from the end of a pipet tip prior to pipetting to prevent the tip from becoming clogged. **Protein G Agarose Beads are shipped on dry ice, but should not be re-frozen by the customer. The beads should be stored at 4°C.**

DNA Purification Wash Buffer

The DNA Purification Wash Buffer requires the addition of ethanol before use. The final concentration of ethanol should be 80%. Add 40 ml of fresh 100% ethanol to the DNA Purification Wash Buffer bottle. Invert repeatedly. The buffer can be stored at room temperature after the addition of ethanol. The ethanol only needs to be added before the first use, after that the Wash Buffer is ready for use.

3M Sodium Acetate

It is important to check the sodium acetate before use to ensure that the salts have not precipitated out of solution. Once the sodium acetate is in solution it should be stored at room temperature.

Recommendations

ChIP-validated Antibody

We recommend using 4 µg antibody per ChIP reaction in a maximum volume of 30 µl. However, this will vary according to the affinity of the antibody and the quality of the chromatin; you may need to use more of a particular antibody. ChIP antibodies must recognize fixed, native protein that is bound to DNA and/or complexed with other proteins. Many antibodies that perform well in other applications do not perform in ChIP. Thus, ChIP performed with an antibody that has not been ChIP-validated must include appropriate controls (such as Active Motif's ChIP-IT Control qPCR Kits, Catalog Nos. 53026, 53027 and 53028) to validate the chromatin preparation and the ChIP methodology. To see a list of available ChIP-validated antibodies available from Active Motif, please visit www.activemotif.com/chipabs.

Chromatin Shearing Tips

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 30 µg chromatin per IP reaction (4.5 million cell equivalents). However, if chromatin is in limited supply and the target protein is a highly abundant histone, lower chromatin amounts (10 µg or 1.5 million cell equivalents) may be used due to the high quality of the DNA obtained from the ChIP-IT Bisulfite Sequencing 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.

Section A: Cell Fixation Starting with Cultured Cells

This protocol describes cell fixation and chromatin preparation from one 15 cm plate (approximately 1.5 x 10^7 cells). We recommend using 20 ml growth medium per 15 cm plate. Please refer to page 11 for information on scaling the protocol for use with other amounts of cells.

1. Prepare 15 cm plates 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 15 cm plate. The volumes listed in the protocol below are enough to process one 15 cm plate. Please refer to the chart on page 8 to scale the solution volumes.
3. To fix cells, add 1/10 growth medium volume of freshly prepared Complete Cell Fixative Solution to the existing culture media for the cells (*e.g.* 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.
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.

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. 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 (which is equivalent to 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 Section B, 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 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 on an agarose gel. Store the remaining Input DNA at -20°C.

14. Analyze each chromatin preparation on an agarose gel.

    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 a 100 bp DNA ladder to analyze chromatin size. DNA should appear as a smear anywhere between 200-1200 bp.

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

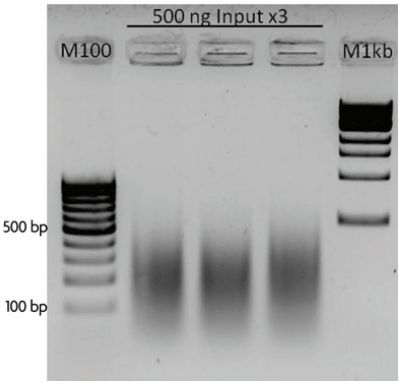

Figure 1: Validation of chromatin shearing efficiency.

Three chromatin preparations of MCF-7 cells were fixed and sonicated using the EpiShear™ Probe Sonicator and EpiShear™ Cooled Sonication Platform from Active Motif. The chromatin was prepared according to the instructions in the manual and 500 ng each was run on a 1.5% agarose gel. The three samples show a DNA smear concentrated around 200-500 bp.

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 9) 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.
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.

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. 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 (which is equivalent to 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 Section D, 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 on an agarose gel. Store the remaining Input DNA at -20°C.
17. Analyze each chromatin preparation on an agarose gel.
    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 a 100 bp DNA ladder to analyze chromatin size. DNA should appear as a smear anywhere between 200-1200 bp. (See Figure 1 on page X as an example)
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 in Section E.

Section E. Immunoprecipitation

Successful chromatin immunoprecipitation depends on the quality of the ChIP antibody and the abundance of the target protein. We suggest using 30 µg chromatin (4.5 x 10^6 cell equivalents) per immunoprecipitation reaction to ensure good ChIP DNA recovery and successful library preparations, although 10 µg may be used for highly abundant histone target proteins.

1. Thaw sonicated chromatin on ice. Spin chromatin at 4°C in a microcentrifuge at maximum speed for 2 minutes.
2. Set up the ChIP reactions by adding the components in the order shown in Table 1 below to 1.5 ml microcentrifuge tubes. Be sure to use the DNA concentration that was determined for your sonicated chromatin sample to calculate the volume to use.
3. In a 1.5 ml microcentrifuge tube prepare the antibodies to be used in the ChIP reactions. Use a separate tube for each antibody. To the tube add 5 µl Blocker and 4 µg ChIP antibody. (Antibody volume should not exceed 30 µl per reaction). Incubate Antibody/Blocker mix for 1 minute at room temperature and then add to the ChIP reactions.

Table 1

Reagent	1 reaction
Sheared Chromatin (10 - 30 µg)	X µl
ChIP Buffer	adjust up to 200 µl
Protease Inhibitor Cocktail (PIC)	5 µl
Antibody/Blocker mix (from Step 3 above)	not to exceed 35 µl
Maximum Volume Allowed	**240 µl**

4. Cap tubes and incubate on an end-to-end rotator overnight at 4°C.
5. The Protein G agarose beads require washing before use. Transfer 30 µl Protein G agarose beads for each IP reaction to a 1.5 ml microcentrifuge tube. Add an equal volume of TE, pH 8.0 and invert to mix. Spin at 1250 x g in a microcentrifuge for 1 minute. Remove the supernatant equivalent to the volume of TE added to the agarose beads.

   Note: Before pipetting the Protein G agarose beads, they should be fully resuspended by inverting the tube. When pipetting the beads, cut 2 mm from the end of a pipet tip to prevent the tip from becoming clogged.

6. Wash the beads a second time with the same volume of TE, pH 8.0. Invert to mix. Spin at 1250 x g for 1 minute in a microcentrifuge. Remove the supernatant equivalent to the volume of TE added to the agarose beads. The beads are now ready to use.
7. Spin the ChIP reactions at 1250 x g for 1 minute to collect liquid from the inside of the cap.
8. Using a cut pipet tip, add 30 µl washed Protein G agarose beads to each immunoprecipitation

reaction. Cap tubes and incubate on an end-to-end rotator at 4°C for 3 hours.

9. Label a ChIP Filtration Column for each ChIP reaction. Remove the tab from the bottom of the column and place in an empty 1 ml pipet tip box as a holder (see Figure 2 below).
10. Remove ChIP reactions from rotator and spin at 1250 x g for 1 minute to collect liquid from inside of the cap.
11. Add 600 µl ChIP Buffer to each ChIP reaction then transfer the entire reaction (including the protein G agarose beads) to its labeled column. Allow flow-through to occur by gravity.
12. During the gravity flow, transfer 100 µl per ChIP reaction of Elution Buffer AM4 to a 1.5 ml microcentrifuge tube and allow to pre-warm at 37°C during the wash steps.
13. Wash each column with 900 µl Wash Buffer AM1. Let stand for 3 min.
14. Repeat Step 13 four more times for a total of five washes.
15. Transfer columns to a new 1.5 ml microcentrifuge tube and spin in a room temperature microcentrifuge at 1250 x g for 3 minutes to remove residual Wash Buffer.
16. Following the spin, transfer the ChIP Filtration Columns to new 1.5 ml microcentrifuge tubes. Add 50 µl 37°C Elution Buffer AM4 to each column. Incubate at room temperature for 5 minutes. Spin in a room temperature microcentrifuge at 1250 x g for 3 minutes.
17. With columns remaining in the same microcentrifuge tube, add another 50 µl 37°C Elution Buffer AM4 to each column. Incubate at room temperature for 5 minutes and spin in a room temperature microcentrifuge at 1250 x g for 3 minutes.
18. Discard the ChIP Filtration Columns. The flow-through (~100 µl volume) contains the ChIP DNA. Proceed to Section F: Reversal of Cross-links and DNA Purification.

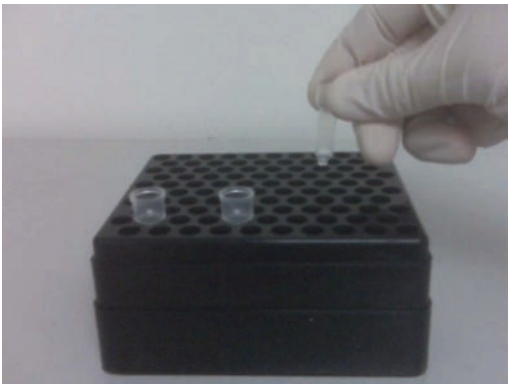

Figure 2: Using the ChIP Filtration Columns.
Remove the tab from the bottom of the ChIP Filtration Columns and place columns in an empty 1 ml pipet tip box to perform the wash steps.

Section F. Reversal of Cross-links and DNA Purification

1. Transfer each eluted ChIP DNA to a 250 µl PCR tube and add 2 µl Proteinase K. Vortex to mix and heat in a thermocycler at 55°C for 30 minutes and then increase the temperature to 80°C for 2 hours.
2. Transfer the DNA to a 1.5 ml microcentrifuge tube and add 5 volumes (500 µl) DNA Purification Binding Buffer to each tube and vortex to mix. Adjust the pH with 5 µl 3M Sodium Acetate. The sample should be bright yellow in color to indicate a proper pH. If your sample is not bright yellow, please refer to the Troubleshooting guide in the Appendix on page 31 for details to adjust pH prior to loading the sample into the purification column.
3. For each sample, place a DNA purification column (AM #103928) in the collection tube and add each pH adjusted sample to its own column. Close the cap on each column, place them with the collection tubes in a microcentrifuge and spin them at 14,000 rpm for 1 minute.
4. Remove the column from the collection tube, then remove and discard the flow through from the collection tube. Return the column to the collection tube.
5. Prepare DNA Purification Wash Buffer (AM #103497) before the first use. Follow the instruction on page 10 for the addition of ethanol prior to using the solution. Add 750 µl DNA Purification Wash Buffer to each column and cap the column.
6. Spin at 14,000 rpm for 1 minute in a microcentrifuge.
7. Remove the column from the collection tube, then remove and discard the flow through from the collection tube. Return the column to the collection tube.
8. With the column cap open, spin at 14,000 rpm for 2 minutes in a microcentrifuge to remove any residual Wash Buffer from the column.
9. Transfer the column to a clean microcentrifuge tube. Pre-warm the required elution buffer volume at 37°C for 5 minutes prior to use.
10. Add 36 µl of 37°C DNA Purification Elution Buffer (AM #103498) to the center of the column matrix and incubate for 1 minute at room temperature. Spin at 14,000 rpm for 1 minute in a microcentrifuge.
11. Discard column. Purified DNA may be stored at -20°C for future use.

Optional: Validation of ChIP-enriched DNA

The quality of the ChIP-enriched DNA can be evaluated using Active Motif's ChIP-IT™ qPCR Analysis Kit (Catalog No. 53029). Gene-specific positive and negative control primer sets should be included in the analysis to determine the fold enrichment. Dilute 6 µl ChIP DNA in 94 µl DNA Purification Elution Buffer and use 5 µl per qPCR reaction according the instructions provided in the ChIP-IT qPCR Analysis Kit.

Module 2 – Sequencing Library Preparation

This module is designed to add methylated adapters to the ends of the ChIP-enriched DNA to prepare a sequencing library. These adapters contain methylated cytosine residues throughout the adapter sequence. This prevents conversion of the cytosines during the subsequent bisulfite conversion treatment in Module 3, thereby preserving the original adapter sequence for library amplification. Please refer to pages 9 of the manual for the kit components and additional materials required to complete the sequencing library preparation.

Reagent Information

Quick spin the vials before opening.

Enzymes used for library construction are temperature sensitive and should be kept on ice during the entire process and returned to -20°C storage as quickly as possible.

Section G: Perform End Repair

This process is to convert the overhangs into phosphorylated blunt ends.

1. Prepare a fresh 1:5 dilution of the DNA Polymerase I Klenow just before use. Add 0.5 µl DNA Polymerase I Klenow (5 U/µl) to 2 µl sterile dH_2O. Pipet up and down to mix. Discard any unused enzyme dilution.
2. Using 200 µl PCR tubes, prepare end repair reactions on the purified ChIP DNA.

Reagents	**One rxn**
ChIP enriched DNA (from Section F)	30 µl
dH_2O	Up to 40 µl
10X T4 DNA Ligase Buffer	5 µl
dNTP Mix, 10 mM each	2 µl
T4 DNA Polymerase (3 U/µl)	1 µl
Diluted DNA Polymerase I Klenow (5 U/µl) from Step K.1	1 µl
T4 Polynucleotide Kinase (10 U/µl)	1 µl
Total Volume	**50 µl**

3. Incubate the reactions in a thermal cycler for 30 minutes at 20°C.
4. Purify the reactions using QIAquick PCR Purification Kit (QIAGEN Cat. No. 28104) following the suppliers protocol. Elute the DNA using 36 µl Buffer EB.

Section H: Add 'A' Overhang to 3´ Ends of DNA Fragments

This step is designed to add an 'A' base to the 3´ end of the blunt phosphorylated DNA fragments to prepare the DNA fragments for ligation to methylated adapters.

1. Prepare reactions to add an 'A' overhang to the 3´ ends of the DNA fragments.

Reagents	**One rxn**
DNA from Step G.4	34 µl
10X Reaction Buffer AM3	5 µl
1 mM dATP	10 µl
Klenow Fragment (3´-5´ exo-) (5 U/µl)	1 µl
Total Volume	**50 µl**

2. Incubate the reactions in a thermal cycler for 30 minutes at 37°C.
3. Purify the reactions using the MinElute PCR Purification Kit (QIAGEN Cat. No. 28004) following the suppliers protocol. Elute the DNA using 11 µl Buffer EB. Perform a second elution using 11 µl Buffer EB for a total elution volume of 23 µl.

Section I: Ligate Methylated Adapters to DNA Fragments

This protocol is for ligation of methylated adapters to the ends of the DNA fragments.

1. Methylated adapters are provided ready for use. These adapters are compatible with Illumina sequencing platforms. Please see Appendix Section O for adapter sequence information.
2. Set up adapter ligation reactions as follows:

Reagents	**One rxn**
DNA from Step H.3	21 µl
2X Quick Ligation Buffer	25 µl
5 µM Methylated Adapter Mix	3 µl
T4 DNA Ligase (2,000 U/µl)	1 µl
Total Volume	**50 µl**

3. Incubate for 20 minutes at room temperature (approximately 24°C).
4. Purify the reactions using MinElute PCR Purification Kit (QIAGEN Cat. No. 28004) according to the suppliers protocol. Elute the DNA using 11 µl Buffer EB.
5. Proceed to Module 3 to perform the bisulfite conversion reaction.

Module 3 – Bisulfite Conversion

In this module, the adapter-ligated ChIP-enriched DNA will undergo bisulfite conversion to determine the methylation status of the enriched DNA at single-base-pair resolution. Please refer to pages 10 of the manual for the kit components and additional materials required to complete the bisulfite conversion. This protocol calls for the use of Glycogen as a carrier, this component was shipped with the -20°C storage items in Module 2.

Reagent Information

Preparation of Conversion Buffer

Two tubes of Conversion Reagent are provided in the Bisulfite Conversion Kit. Each vial is sufficient for performing 10 bisulfite conversions. Prepare the Conversion Buffer by resuspending one of the Conversion Reagent tubes with 875 µl dH_2O, and 350 µl Buffer A. Mix at room temperature for 10 minutes with intermittent vortexing.

NOTE: This is a saturated solution and it is normal for the Conversion Reagent to not dissolve completely. The Conversion Buffer is stable for one week at -20°C. Conversion Reagent is sensitive to air and moisture. Keep cap on tight and re-cap immediately after use.

Preparation of Hydroquinone

Hydroquinone is an alkalizing agent and prevents DNA from strand breakage because of depurination. Each tube provided can be used for 10 conversion reactions. Resuspend one tube with 100 µl dH_2O. Keep mixture in dark and discard after use.

Buffer B

Prepare a 3-fold dilution of Buffer B by adding 10 µl Buffer B to 20 µl dH_2O. Store at RT.

Preparation of Desulfonation Buffer

For each reaction, combine 22 µl Buffer B with 88 µl dH_2O and 110 µl 100% isopropanol. If this reagent will not be used immediately, it can be stored for up to one week at -20°C.

DNA Binding Buffer / DNA Wash Buffer / DNA Elution Buffer

Are supplied ready to use.

Glycogen (20 mg/ml)

This item was shipped with the Module 2 components and should be stored at -20°C. Prepare a 1 mg/ml working solution of glycogen by adding 2 µl Glycogen stock to 38 µl dH_2O. The diluted stock may be stored at -20°C for future use.

Section J: Conversion Reaction

1. Prepare Conversion Buffer and Hydroquinone as described in the Buffer Preparation Section on page 26.
2. Set up a conversion reaction for each sample by adding the reagents in the order listed below into PCR tubes. Mix well by pipetting. If using less than 10 µl of DNA, adjust the volume with water to a final volume of 13 µl.

Reagent	Quantity
DNA from Step I.6	10 µl
Diluted Buffer B	3 µl
Total Volume	**13 µl**

3. Place tubes in the thermocycler and incubate at 90°C for 2 minutes. Transfer samples immediately to ice.
4. Add Hydroquinone and Conversion Buffer to the PCR tubes while still on ice to a final volume of 140 µl. Keep hydroquinone away from light.

Reagent	Quantity
Denatured DNA	13 µl
Hydroquinone	7 µl
Conversion Buffer	120 µl
Total Volume	**140 µl**

5. Place tubes in the thermocycler and set the following program. To prevent evaporation of samples, use the heated lid or overlay reactions with mineral oil.

   94°C for 3 minutes
   50°C for 5 hours
   Hold at 4°C

6. Remove tubes and continue with Step K below. The protocol can also be stopped here and DNA samples can be kept at 4°C in the dark for up to 5 days.

Section K: On-column Desulfonation and DNA Purification

1. Prepare Desulfonation Buffer as described in the Buffer Preparation section on page 26.
2. For each conversion reaction, aliquot 500 µl DNA Binding Buffer into a 1.5 ml microcentrifuge tube. Add the entire conversion reaction mixture to the DNA Binding Buffer in the tube. Add 1 µl Glycogen (1 mg/ml) to each tube and vortex to mix.
3. Place the desired number of DNA purification columns into collection tubes.
4. Pipet each DNA Binding Buffer/conversion reaction mixture into one of the DNA Purification

columns and spin at 10,000 rpm for 30 seconds in a microcentrifuge. Empty the collection tube and place the column back on the collection tube.

5. Add 200 µl DNA Wash Buffer to each column and spin at 10,000 rpm for 30 seconds in a microcentrifuge.
6. Add 200 µl Desulfonation Buffer to each column and incubate 20 minutes at room temperature. Spin at 10,000 rpm for 30 seconds in a microcentrifuge. Empty the collection tube and place the column back on the collection tube.
7. Add 200 µl DNA Wash Buffer to each column and spin at 10,000 rpm for 30 seconds in a microcentrifuge. Empty the collection tube and place the column back on the collection tube.
8. To remove any residual wash buffer, spin at 10,000 rpm for 30 seconds in a microcentrifuge. Remove the column and place in a new 1.5 ml microcentrifuge tube.
9. Add 24 µl DNA Elution Buffer directly to the filter of the column.
10. Incubate 3 minutes at room temperature. Spin at 10,000 rpm for 30 seconds in a microcentrifuge. The eluate will contain the converted DNA.
11. The eluted DNA is now ready for PCR amplification.

Section L: Enrich the Adapter-Modified DNA Fragments by PCR

This step is designed to PCR amplify the bisulfite converted DNA. We recommend using a DNA polymerase that is designed to amplify bisulfite converted DNA (*e.g.* KAPA HiFi HotStart Uracil+ Ready Mix, KAPA Biosystems Catalog No. KK2801). PCR primers should be compatible with Illumina sequencing platforms. Primers for single, paired-end or multiplex sequencing may be used. For information on primer sequences, please refer to Appendix Section O.

1. Using a 200 µl PCR tube, prepare the following reactions. Add components in the order listed in the table below.

Reagents	**One rxn**
Adapter Ligated DNA	23 µl
PCR primer mix (10 µM each)	2 µl
2X High Fidelity Uracil+ Mix	25 µl
Total Volume	**50 µl**

2. Amplify the reactions in a thermal cycler according to the recommendations for the DNA polymerase being used. For example with the KAPA HiFi HotStart Uracil+:

   98°C for 45 seconds
   (98°C for 15 seconds, 65°C for 30 seconds and 72°C for 30 seconds) for 14 cycles
   72°C for 1 minute
   Hold at 4°C

Section M: Size Select the Library

This process removes excess adapters and selects a size range of DNA templates.

1. Prepare a large comb 1.5.% agarose TAE gel with 400 ng/ml ethidium bromide or equivalent.

   Note: Ethidium bromide is a mutagen. Use appropriate precaution when handling.

2. Add 2.5 µl loading buffer to each reaction.
3. Run the entire adapter ligated DNA from Step L.2 and 500 ng of 100 bp DNA ladder on a 1.5% agarose gel for size selection of the DNA library. To avoid potential cross contamination of adjacent wells, leave at least one empty lane between DNA ladder, sample and Input wells.
4. Run the gel at 120V until the tracking dye is 2/3 down the length of the gel.
5. Visualize the DNA using a Dark Reader transilluminator to avoid exposure to UV light. If using a UV light, work quickly when excising the band to minimize direct exposure to UV. Prolonged exposure to UV light can damage DNA.
6. Using a clean razor blade, excise a gel slice in the 200-300 bp range. We recommend to photograph the gel before and after the band is excised.
7. Use a QIAquick Gel Extraction Kit (QIAGEN Cat. No. 28704) to purify the DNA from the agarose. Make the following protocol modifications to the QIAquick Gel Extraction Kit protocol:
   a. QIAquick columns can handle a maximum of 400 mg agarose. If using a larger quantity of agarose, you will need to split your sample across multiple columns.
   b. Incubate the gel slice in 3 volumes Buffer QG at **37°C for 30 minutes** instead of the suggested 50°C for 10 minutes. Buffer volumes and incubation times are dependent upon the thickness of the gel and may need to be adjusted based on your sample. If necessary, add additional Buffer QG and increase the duration of the 37°C incubation.
   c. Add the recommended extra 0.5 ml Buffer QG to the QIAquick column.
   d. During the wash steps, incubate 2-5 minutes in Buffer PE before centrifugation.
   e. Elute the DNA in 38 µl Buffer EB.
8. Determine the final concentration of the library using Picogreen or equivalent fluorescent quantitation method. Libraries are now ready for sequencing.

Section N: Analyzing the Sequencing Results

Analysis of bisulfite converted DNA can be difficult given the lack of complexity of the DNA bases following conversion. As a result, shorter read length may be more difficult to accurately map. It is advised to generate longer reads and use a software program designed for analysis of genome-wide bisulfite converted DNA. Several programs exist for bisulfite-sequencing analysis, such as the freeware program GBSA (see reference http://www.ncbi.nlm.nih.gov/pubmed/23268441), or Bis-SNP (see reference http://epigenome.usc.edu/publicationdata/bissnp2011/).

1. If the CpG dinucleotide was methylated in the original DNA, the sequencing read will show

a CG at that location. If the CpG dinucleotide was unmethylated in the original DNA, the bisulfite conversion will have converted the cytosine to uracil and the sequencing read will show a TG at that location.

2. Compare the bisulfite converted sequence data against the unconverted DNA sequence to determine the percentage of methylation.

Appendix

Section O. Adapter and Primer Selection

The ChIP-Bis-Seq Kit includes methylated adpaters for use in generating Next Generation Sequencing libraries. Methylated adpaters are provided as a 5 µM mix and should be used according to the recommendations in the manual. Information regarding the sequence of the methylated adapters and corresponding primer sequences for use in the PCR amplification of the adapter-ligated DNA can be found on the Illumina website at **http://support.illumina.com/downloads/illumina-customer-sequence-letter.html**. Download the customer sequence letter and go to the section for *Oligonucleotide sequences for Genomic DNA*.

Since methylated adapters were used, the Oligonucleotide sequence for Genomic DNA adapters is retained following bisulfite conversion. Select a corresponding PCR primer sequence for use in single, paired-end or multiplex analysis to fit your sequencing needs. Due to the lack of complexity with bisulfite converted DNA, longer sequencing reads (>75 bp) will improve the ability to map the sequence back to the reference genome.

Appendix

Section P. Troubleshooting Guide

Problem/question	Recommendation
At what points in the protocol can I stop the ChIP protocol?	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 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.
	Sonicated 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.	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.
Performing ChIP with a large volume of chromatin.	This is not recommended. It is better to set up several small ChIP reactions (240 µl each) and pool the samples at the end, rather than trying to ChIP a single large sample. Do not perform a single scaled-up reaction, as the capture efficiency is lower.
ChIP DNA does not turn bright yellow following the addition of 3 M sodium acetate	If the color is light orange or violet, this indicates the pH is too high. Add more 3 M sodium acetate 5 µl at a time, mixing after addition until the color is bright yellow. This step is crucial to the success of DNA binding and purification. For a full color image please see the manual for Active Motif's Chromatin IP DNA Purification Kit Catalog No. 58002 available online at our website www.activemotif.com.
High background.	Chromatin not sheared enough. Shearing should produce DNA fragments that are small enough to exclude background from neighboring chromosomal sequences, but still large enough that there is a good possibility your amplicon remains intact. We recommend 200-1200 bp fragments. If the DNA fragments are too large, the background is increased. Consider increasing the number of pulses for sonication. Check the fragment size on a gel to assess your shearing efficiency.
	Antibody issue. Too much antibody relative to the amount of chromatin in the ChIP reaction. Excess antibody will result in more non-specific binding, which will be detected as increased background.

Problem/question	Recommendation
Poor or no enrichment with target antibody.	Too little chromatin. Generally, we recommend using 30 µg of chromatin per ChIP reaction for use downstream in bisulfite conversion. ChIP reactions should not exceed 50 µg per IP reaction. Be sure to quantitate the concentration of the sheared chromatin sample(s) being ChIP'd to ensure both that adequate chromatin is used per sample, and that equal mass quantities of chromatin are used in each ChIP.
	Antibody is not ChIP validated. The antibody does not efficiently recognize fixed proteins, either because the epitope is destroyed by fixation or because the epitope is masked by other proteins in a larger complex. To assist in ChIP validating an antibody, it is very useful to use a positive control antibody such as Histone H3K4me3 (Catalog No. 39915) and a negative IgG from the same species, and primers that have been proven to work in the type of PCR being used. Active Motif offers species-specific ChIP-IT Control qPCR Kits for antibody validation (Catalog Nos. 53026, 53027 & 53028).
	Low-affinity antibody. Use a different antibody.
	Antibody affinity to protein G is weak. Individual monoclonals have variable binding affinities to protein G, which are pH dependent; the optimal pH may vary for each IgG, For those with low to medium affinity, capture efficiency by protein G can be dramatically improved through use of our Bridging Antibody (Catalog No. 53017). This antibody is a rabbit anti-mouse pAb that recognizes all subclasses of mouse immunoglobulins. If your IgG has a weak/medium affinity to protein A or G, the Bridging Antibody will increase antibody capture by the beads without increasing background.
	Problems with PCR. Confirm the amplified sequence for the positive control primer set is bound by the antibody target. Identify other binding sites.
No PCR products for the ChIP'd samples (but the Input DNA yields the correct PCR product)	Increase the amount of chromatin used in the ChIP reaction, the amount of antibody used, or both.
	Use a different antibody.
No ChIP PCR products with real-time PCR	Confirm the species specificity and efficiency of your primers. You may need to redesign your primers. Primers that work in end point PCR do not always work in qPCR.
	No ethanol in DNA Purification Wash Buffer. Make sure that ethanol has been added to the DNA Purification Wash Buffer prior to first use.
Conversion time	For DNA sequences that are GC-rich and/or contain extensive secondary structures we suggest a 9 hour conversion at 50°C. Longer conversion times, up to 16 hours, may be used if noticing incomplete conversion, however, this may result in degradation of the DNA. If noticing a loss of DNA after the conversion reaction, the conversion time can be reduced to 3 hours at 50°C
Poor PCR amplification	We recommend performing a Hot Start PCR reaction using a polymerase that has been validated to work with bisulfite converted DNA. Follow the recommendations of the manufacturer for the preparation of the PCR reaction.
Poor sample recovery	When converting a small amount of DNA, add 1 µg Glycogen (20 ng/µl) or tRNA (10 µg/µl) as a carrier to the 500 µl DNA Binding Buffer for each reaction during the on-column desulfonation and DNA purification step to minimize sample loss.
	Quantification of the sequencing library should be performed following PCR amplification. We recommend using a fluorescent quantitation method, such as Picogreen,,to determine the DNA quantity since the yield recovered may be below the detectable range of a Nanodrop instrument.
Incomplete conversion	Conversion reagent is sensitive to air and moisture. If conversion reagent was not properly sealed and stored, repeat conversion reaction using a new tube of conversion reagent.

Problem/question	Recommendation
Can I use columns to purify my enriched adapter-modified DNA rather than AMPure® XP beads?	We recommend the use of Agencourt® AMPure® XP Beads (Beckman Coulter Cat No. A63880) for purification of the adapter-modified DNA rather than column purification. With column purification we noticed a higher degree of free adapter in the flow through. The AMPure bead purification provides better purity of the library, but optimization may be required to determine the optimal amount of beads to use per reaction.

Section Q. Related Products

DNA Methylation	Format	Catalog No.
Bisulfite Conversion Kit	50 rxns	55016
MethylCollector™ Ultra	30 rxns	55005
HypoMethylCollector™	30 rxns	55004
MeDIP	10 rxns	55009
Hydroxymethyl Collector™	25 rxns	55013
hMeDIP	10 rxns	55010
PvuRts1I restriction enzyme	50 units	55011
β-Glucosyltransferase enzyme	500 units	55012
Recombinant TET1 protein, active	25 µg	31363
DNMT Activity / Inhibition Assay	1 x 96 rxns	55006
Methylated DNA Standard Kit	3 x 2.5 µg	55008
5-Carboxylcytosine DNA Standard	0.5 µg	55014
Fully Methylated Jurkat DNA	10 µg	55003
Jurkat genomic DNA	10 µg	55007

Antibodies	Application	Format	Catalog No.
3-Methylcytosine rabbit pAb	DB	100 µg	61111
5-Carboxylcytosine rabbit pAb	DB, IF	100 µl	61225
5-Formylcytosine rabbit pAb	DB, IF	100 µl	61223
5-Hydroxymethylcytidine mouse mAb	DB, MeDIP	100 µg	39999
5-Hydroxymethylcytidine rabbit pAb	DB, IF, IHC, MeDIP	100 µl	39769
5-Methylcytosine mouse mAb	DB, FACS, IHC, IP, MeDIP	50 µg	39649
5-Methylcytosine rabbit pAb	DB, IP, MeDIP	100 µg	61255
CGBP rabbit pAb	WB	200 µl	39203
DNMT1 mouse mAb	ChIP, IHC, IP, WB	100 µg	39204
DNMT2 rabbit pAb	WB	100 µg	39205
DNMT3A mouse mAb	ChIP, IF, IHC, WB	100 µg	39206
DNMT3B mouse mAb	ChIP, IF, IP, WB	100 µg	39207
DNMT3L rabbit pAb	WB	100 µl	39907
Kaiso mouse mAb	WB	200 µg	39365
MBD1 mouse mAb	WB	100 µg	39215
MBD2 rabbit pAb	WB	200 µl	39547
MBD3 mouse mAb	WB	100 µg	39216
MBD4 rabbit pAb	WB	100 µg	39217
MeCP2 rabbit pAb	WB	100 µg	39218
MeCP2 mouse mAb	ChIP, IF, IHC, IP, WB	100 µg	61291
Tet1 rabbit pAb	ChIP, WB	100 µl	61443
Tet2 mouse mAb	IP, WB	100 µg	61389
Tet3 rabbit pAb	WB	100 µl	61395
Ubiquitin mouse mAb	IF, IHC, IP, WB	100 µg	39741
Uhrf1 rabbit pAb	WB	200 µl	39625

For more information, please visit our website at **www.activemotif.com**.

Technical Services

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