Low Cell Optimization Module

(version A2)

Catalog No. 53085

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

Revision History

Revision	Date	Description of Change
A2	Aug 2019	Additional qPCR and sequencing data was added

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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. ChIP-Seq analysis typically requires millions of cells per immunoprecipitation reaction in order to obtain meaningful information about global changes across a large population of cells. To address the needs of researchers working with limited sample material, or those wanting to study the complexities of protein-DNA interactions within a small population of cells, Active Motif has utilized its expertise in ChIP to develop the Low Cell ChIP-Seq Kit.

The Low Cell ChIP-Seq Kit provides a complete, optimized ChIP-Seq workflow to generate genome-wide binding profiles from as few as 1,000 cells, or small tissue biopsies. Low Cell ChIP-Seq not only reduces sample input requirements, but also improves signal-to-noise through the use of low background Protein G agarose beads and blockers. The included Next Gen DNA Library and Next Gen Indexing Kits* generate high complexity Illumina®-compatible sequencing libraries. The specialized enzyme formulations are optimized to work with limited input amounts and the P5 adapter contains molecular identifiers (MIDs) to distinguish PCR duplicates from fragmentation duplicates for accurate de-duplication from single read sequencing. This helps to increase the number of unique alignments from sequencing for more accurate data.

Together with Low Cell ChIP-Seq Kit reagents, the Low Cell Optimization Module can be used to examine the quantity and quality of your samples following chromatin preparation and ChIP enrichment. **This module should only be used in conjunction with the Low Cell ChIP-Seq Kit.**

The Low Cell Optimization Moduel contains sufficient reagents to perform 6 ChIP-Seq reactions. Reagents are included for qualification of DNA following chromatin preparation and ChIP enrichment -- you can choose to use these reagents with your own samples and antibodies, or you can use them with the standardized sample and antibody included in the module.

product	format	catalog no.
Low Cell Optimization Module	6 rxns	53085

*The Next Gen DNA Library and Next Gen Indexing Kits are powered by Swift Biosciences.

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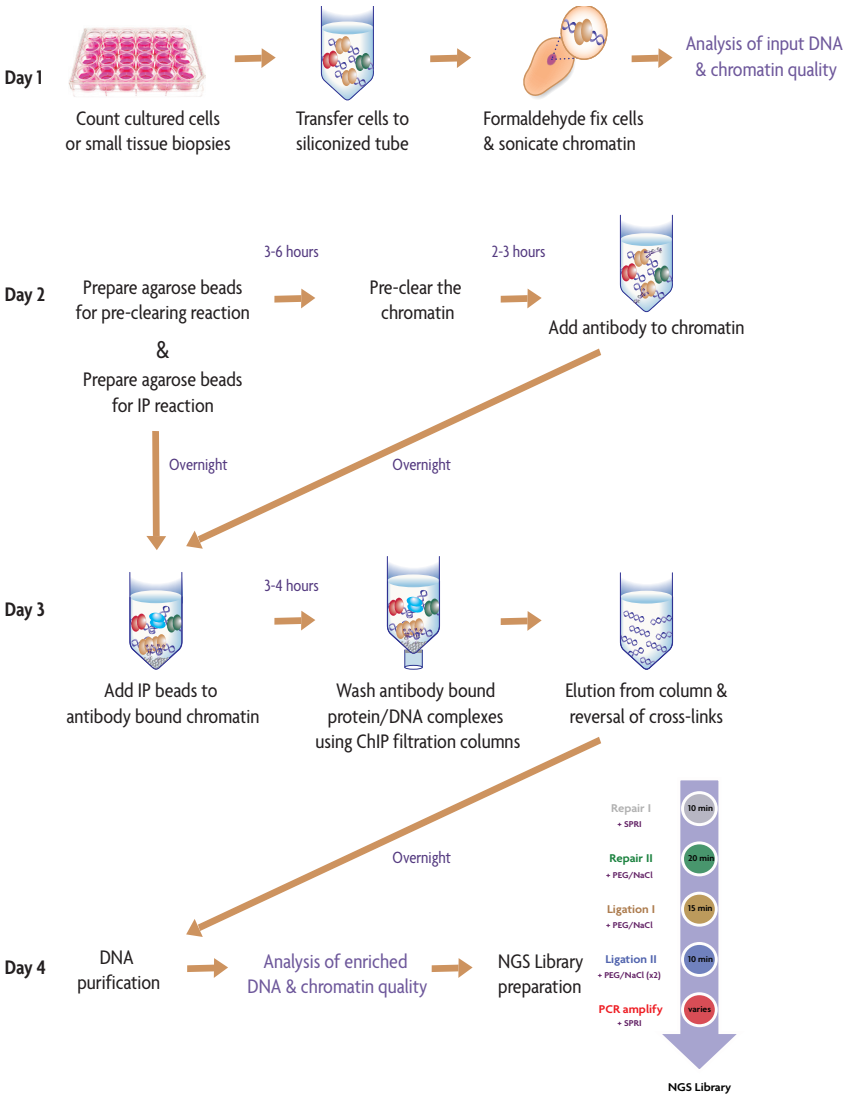
Flow Chart of Low Cell Optimization Module Process



The Low Cell Optimization Module allows for optimization and troubleshooting when using the Low Cell ChIP-Seq Kit (Catalog No. 53084). This module contains a standardized sample and antibody, as well as reagents to examine input DNA and chromatin quality (Day 1) and enriched DNA and chromatin quality (Day 4).

Protocol Overview and Time Table

Protocol Steps	Required Time
Cell Collection and Fixation	30 minutes
Chromatin Sonication*	20 minutes per sample
Analysis of Input DNA & Chromatin Quality*	Overnight incubation
Protein G Agarose Bead Preparation*	3 - 6 hours (Pre-clearing beads) to overnight (IP beads)
Pre-clearing the Chromatin*	2 - 3 hours
Immunoprecipitation	Overnight incubation
Binding to Protein G Agarose Beads	3 - 4 hours
Wash Immune Complexes & Elute DNA	45 minutes
Reversal of Cross-links	Overnight incubation
DNA Purification	1.5 hours
Analysis of Enriched DNA & Chromatin Quality	1 hour
Repair I**	10 minutes
Repair II**	20 minutes
Ligation I**	15 minutes
Ligation II**	10 minutes
PCR amplification**	20 minutes

* Some steps may be performed concurrently.

** Time indicated represents hands on time.

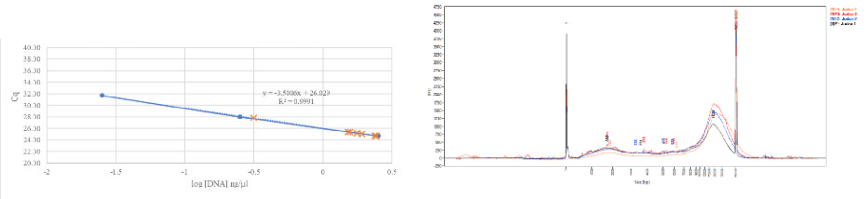


Figure 1: qPCR Analysis & Sonication Profiles of Input DNA from Fixed Jurkat Cell Pellet Samples.

The Low Cell ChIP-Seq Kit and Low Cell Optimization Module were used to analyze solubilized chromatin from 14 µl of Fixed Jurkat Cell Pellet samples (equivalent to 100,000 Jurkat cells). (Left) GAPDH-2 Cq values were plotted against DNA Standards AM1, AM2, and AM3. Samples with at least 350 ng of soluble chromatin can proceed into the ChIP enrichment step. (Right) Fragmentation profiles of solubilized chromatin were analyzed on a Fragment Analyzer. High molecular weight peaks are commonly seen at this point in the protocol, but do not affect the ChIP enrichment or sequencing data.

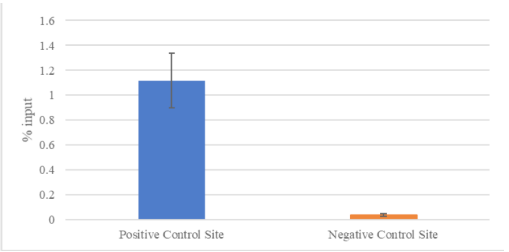


Figure 2: qPCR Analysis of H3K9ac Enriched DNA from 30 Fixed Jurkat Cell Pellet Samples.

The Low Cell ChIP-Seq Kit and Low Cell Optimization Module were used to analyze enriched chromatin from Fixed Jurkat Cell Pellet samples. ACTB-2 (postive control site) and Hum Neg primers (negative control site) were used to analyze enrichment using a H3K9ac antibody. Average fold enrichment = 28-fold; n=30; $p=5.66x10^{-5}$

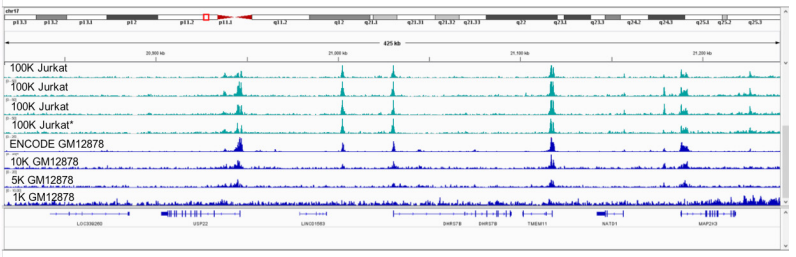


Figure 3: Sequencing Analysis of H3K9ac Enriched DNA from Fixed Jurkat Cell Pellet Samples.

The Low Cell ChIP-Seq Kit and Low Cell Optimization Module were used to analyze enriched chromatin from (100,000 cell) Fixed Jurkat Cell Pellet samples (top 4 tracks, green). A section of chromosome 17 is shown here. The bottom Jurkat track (marked with *) exhibited much lower fold-enrichemnt of positive control regions, as analyzed by qPCR. As a result, slightly degraded peaks can be observed in this sample. For comparison, H3K9ac enrichment of GM12878 cells is also shown (blue). The top track is publicly available ENCODE data, the lower tracks demonstrate peak degradation when using just 10,000, 5,000 or 1,000 cells.

Kit Components and Storage

The Low Cell Optimization Module is for research use only. Not for use in diagnostic procedures. We recommend storing each component at the temperatures indicated in the tables below.

Reagents for Low Cell Optimization Module

Reagents	Quantity	Storage
Fixed Jurkat Cell Pellet	6 x 14 µl	-80°C
RNase A	25 µl	-20°C
DNA Standard AM1	20 µl	-20°C
DNA Standard AM2	20 µl	-20°C
DNA Standard AM3	20 µl	-20°C
Human Pos Control Primer Set, GAPDH-2	400 µl	-20°C
Human Neg Control Primer Set 1	400 µl	-20°C
Carrier	35 µl	-20°C
Human Positive Control Primer Set, ACTB-2	400 µl	-20°C
AbFlex® Histone H3K9ac Antibody	100 µg	-20°C

Please note: A subset of Low Cell ChIP-Seq reagents will also be used in steps specific to the Low Cell Optimization Module.

Additional materials required

- Instrument for visualizing fragmentation profile (e.g. Fragment Analyzer)
- SYBR Green qPCR master mix (e.g. Bio-Rad Cat # 170-8882)
- A ChIP-Seq-validated antibody directed against the protein of interest
- 37% formaldehyde solution with 10-15% methyl alcohol to prevent polymerization (*e.g.* Sigma Aldrich Catalog No. 252549). Alternatively, methanol-free formaldehyde may be used in order to ensure compatibility with specific sonciation instruments (e.g. 16% Methanol-Free Formaldehyde, Thermo Fisher Cat # 28906). Do not use paraformaldehyde.
- Phenol, saturated (DNA Purification, Molecular Biology Grade, Amresco Catalog No. 0945)
- Chloroform/isoamyl alcohol (24:1) (DNA Purification, Molecular Biology Grade)
- 100% ethanol (absolute)
- 70% ethanol
- DNase-free H_2O
- 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
- Thermal cycler
- 15 ml conical tubes
- 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))
- (Optional) Enzymes and apparatus to create single cell suspension from tissue samples (*e.g.* Disposable Pellet Mixer, VWR 47747-370 or Mini Mortar and Pestle, Bel-Art H37260-0100)
- Magnetic SPRI beads for clean-up steps (*e.g.* Beckman Coulter, B23317, B23318, & B23319)
- Magnetic rack or bar magnet
- qPCR-based library quantification kit (*e.g.* Kapa Biosystems, Cat. KR0405)
- Multichannel pipette and aerosol-resistant pipette tips from 2- 1000 µl
- Microcentrifuge & 1.5 ml low retention microcentrifuge tubes
- 0.2 ml PCR tubes
- Nuclease-free wate
- Real time PCR instrument

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

The Low Cell ChIP-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. For ease-of-use the manual is divided into Protocols to represent the different steps of the workflow: Low Cell ChIP-Seq and NGS Library Preparation.

Steps that are specific to the Low Cell Optimization Module, and are not performed in the standard Low Cell ChIP-Seq workflow, are indicated in bold throughout the manual.

Protocol 1 – Low Cell ChIP-Seq

This section describes the experimental set-up and buffer preparation steps needed for the low cell chromatin preparation and immunoprecipitation portion of the protocol.

Cell Growth Recommendations

When planning an experiment, calculate the number of ChIP reactions you plan to perform. 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 condition on transcription factor/DNA interactions, you should prepare chromatin from control (untreated) cells as a reference sample.

Below are guidelines for cell culturing conditions. If multiple ChIP reactions will be performed using the same cell type, cells can be cultured in a larger volume and will be transferred to individual tubes for chromatin preparation and immunoprecipitation reactions.

	96-well plate	24-well plate	12-well plate	6-well plate	T-25 flask
Seeding Density	0.02 x 10^6	0.05 x 10^6	0.1 x 10^6	0.3 x 10^6	0.7 x 10^6
Cells at 70-80% Confluency*	0.06 x 10^6	0.15 x 10^6	0.3 x 10^6	0.9 x 10^6	2.1 x 10^6
Growth Medium volume to culture cells	100 µl	1 ml	2 ml	3 ml	5 ml
Trypsin	50 µl	500 µl	1 ml	2 ml	3 ml
Growth medium	50 µl	500 µl	1 ml	2 ml	3 ml
1 X PBS (volume per wash)	100 µl	1 ml	2 ml	3 ml	5 ml

Table 1: Cell culture recommendations.

*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.

Recommendations about the number of cells to use per ChIP reaction are provided in the tables below and are based on the relative abundance of the target protein. ChIP efficiency will also be influenced by the quality of the ChIP-Seq antibody used. Optimization may be required.

Target protein	Examples	Minimum Cell Number
Robust histone	H3K4me1, H3K4me3, H3K27me3	1,000 cells
Low abundance histone	H3K27ac, H3K9ac, H3K9me3	5,000 cells
High abundance protein	CTCF, BRD4	10,000 cells
Low abundance protein	Transcription factors	50,000 cells

Buffer Preparation

Complete Cell Fixation Solution

Buffer should be prepared fresh before each experiment. In a 1.5 ml microcentrifuge tube add the appropriate volume of sterile water and fixation buffer (see table below). Using appropriate precautions (*i.e.* safety glasses, gloves and lab coat), add 37% or 16% formaldehyde to the tube and vortex to mix. Use 5 µl Complete cell fixation solution per 50 µl cell pellet. Discard unused solution.

	37% Methanol Buffered Formaldehyde	16% Methanol Free Formaldehyde
Sterile Water	62.8 µl	23.4 µl
Fixation Buffer	7.2 µl	7.2 µl
Formaldehyde	30 µl	69.4 µl
Total Volume	100 µl	100 µl

Complete Tissue Fixation Solution

Buffer should be prepared fresh before each experiment. In a 1.5 ml microcentrifuge tube add 1 ml 1X PBS. Using appropriate precautions (*i.e.* safety glasses, gloves and lab coat), add 37% or 16% formaldehyde to the tube and vortex to mix. Use 1 ml Complete tissue fixation solution per tissue sample. Discard unused solution.

	37% Methanol Buffered Formaldehyde	16% Methanol Free Formaldehyde
1X PBS	1 ml	1 ml
Formaldehyde	28 µl	67 µl
Total Volume	1.028 ml	1.067 ml

Stop Solution

Is provided ready to use.

1X PBS Buffer

Prepare a 1X PBS solution by adding 1 ml 10X PBS to 9 ml sterile water. Mix by inverting and place on ice to chill. 1X PBS 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.

ChIP Buffer

Is supplied ready to use.

Protein G Agarose Beads

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 and can be stored frozen until their first use. Once thawed, beads should not be re-frozen by the customer. Protein G Agarose Beads should be stored at 4°C.

Recommendations

ChIP-Seq-validated Antibody

We recommend using 2-4 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. Many antibodies that perform well in other applications do not perform in ChIP. Thus, Low Cell ChIP-Seq performed with an antibody that has not been ChIP-Seq-validated must include appropriate controls. 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 to help regulate sample temperature.

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.

Reduce Contamination

To reduce the risk of DNA and library contamination, particularly with ultra-low input samples, we suggest to physically separate the laboratory space, equipment and supplies where pre-PCR and post-PCR processes are performed. We also suggest to clean lab areas using 0.5% Sodium Hypochlorite (10% bleach). The use of specialty barrier pipette tips also helps to avoid exposure to potential contaminants.

Protocols

Section A: Cell Fixation Starting with Cultured Cells

This protocol describes cell fixation and chromatin preparation from cultured cells.

1. Use the table on page 7 as a guideline to culture each cell line to be tested. Grow the cells to 70-80% confluency. Stimulate cells as desired to activate the pathway of interest.
2. Aspirate growth medium and wash the cells twice with the recommended volume of 1X PBS.
3. Detach cells from plate using trypsin. Transfer cells to a 15 ml conical tube or 1.5 ml microcentrifuge tube based on the volume.
4. Add an equal volume of growth medium to the tube. Pipet to mix.
5. Perform a cell count and determine cell viability. Record the number of viable cells.

   # viable cells: _____
6. Transfer the desired number of cells for each ChIP reaction into separate 1.7 ml siliconized microcentrifuge tubes (provided in the Low Cell ChIP-Seq Kit). The use of siliconized tubes is recommended to avoid sample loss due that can result from material sticking to the plastic walls of microcentrifuge tubes. Below are the minimum number of cells that should be used for each ChIP based on the abundance of the target protein.

Target protein	Examples	Minimum Cell Number
Robust histone	H3K4me1, H3K4me3, H3K27me3	1,000 cells
Low abundance histone	H3K27ac, H3K9ac, H3K9me3	5,000 cells
High abundance protein	CTCF, BRD4	10,000 cells
Low abundance protein	Transcription factors	50,000 cells

Note: In working with low cell numbers, it is important to minimize the transfer of cellular material and/or pipetting whenever possible to avoid sample loss.

7. If the total volume of the cell suspension is less than 50 µl, add ice-cold 1X PBS to adjust to a total volume of 50 µl. If the total volume of the cell suspension is larger than 50 µl, pellet the cells by centrifugation in a microcentrifuge at 1250 x g for 5 minutes at 4°C. Carefully remove

all but 10 µl of supernatant from the tube to avoid disturbing the pellet. The pellet may not be visible. Add 40 µl ice-cold 1X PBS to the tube for a total volume of 50 µl.

8. Freshly prepare Complete Cell Fixation Solution as described on page 8.
9. To fix cells, add 5 µl Complete Cell Fixation Solution to the 50 µl cell suspension. Do not pipette sample. Flick the tube gently to mix and incubate at room temperature for 10 minutes.
10. Stop the fixation reaction by adding 2.8 µl Stop Solution. Do not pipette sample. Flick the tube gently to mix and incubate at room temperature for 5 minutes.

11.a. If utilizing Fixed Jurkat Cell Pellet samples, add 2 µl PIC, 2 µl PMSF, and 184.4 µl ChIP Buffer to each tube.

11.b. For your samples, add 2 µl PIC, 2 µl PMSF, and 138.2 µl ChIP Buffer to each tube.

12. Transfer the cells to a 2 ml V-bottom tube for sonication. Repeat for each sample. **Note:** If not using a probe sonicator, please follow volume guidelines compatible with your sonication instrument.
13. Proceed to Section C: Chromatin Sonication.

Section B. Cell Fixation Starting with Small Tissue Biopsies

This protocol provides guidelines for working with small tissue biopsies. Optimization may be required based on the tissue type used. Keep tissue samples on dry ice until fixed.

1. Prepare Complete Tissue Fixation Solution as described on page 8.
2. Record the weight of an empty 1.7 ml siliconized microcentrifuge tube (provided).

   Tube weight _____ mg
3. Place a small tissue sample in the 1.7 ml siliconized tube and record the total weight.

   Tube + tissue weight _____mg
4. Subtract the tube weight from the total weight to determine the weight of the tissue.

   Tissue weight _____ mg
5. Calculate the expected cell number based on the conversion of 5,000 cells per mg of tissue.

   # cells: _____
6. Add 1 ml Complete Tissue Fixation Solution to the tissue.
7. Incubate for 15 minutes at room temperature with agitation.
8. Add 50 µl Stop Solution. Incubate for 5 minutes at room temperature with agitation.
9. Use your preferred method to generate a single cell suspension. Optimization may be required depending on the tissue type used. If possible, try to work within the siliconized tube to avoid sample loss. Count cells with a hemacytometer. # viable cells _____

   Note: In working with low cell numbers, it is important to minimize the transfer of cellular material and/or pipetting whenever possible to avoid sample loss.

10. Once cells are in a single cell suspension, centrifuge at 1,250 x g for 5 minutes at 4°C.
11. Carefully remove and discard supernatant, taking care to avoid the cell pellet.
12. Resuspend cell pellet in 200 µl ChIP Buffer supplemented with 2 µl PIC and 2 µl PMSF. Using the same pipette tip, transfer the cells to a 2 ml V-bottom tube for sonication.
13. Proceed to Section C: Chromatin Sonication.

Section C. Chromatin Sonication

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.

1. Place the 2 ml microcentrifuge tube containing the cells into the tube cooler or packed ice. Open cap and submerge the microtip into the liquid until the microtip is approximately 2 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 (or 20 minutes elapsed time).
2. Spin tubes at 4°C in a microcentrifuge at maximum speed (18,000 x g) for 2 minutes to pellet the cellular debris.
3. Transfer 200 µl of soluble chromatin to a new 1.7 ml siliconized microcentrifuge tube. Place on ice and Proceed to Section F: Protein G Agarose Bead Preparation if pre-clearing of the chromatin will be performed the same day. Alternatively, chromatin can be stored at -80°C.
4. **To analyze the quantity and shearing profile of samples, transfer 20 µl (or more, see Section D, Step 18) of chromatin from samples or Fixed Jurkat Cell Pellet sample to a new PCR tube. Add enough TE, pH 8.0 to bring to a total volume of 100 µl. Proceed to Section D: Input Preparation**

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Section D. Input Preparation

1. **To each 100 µl Input preparation from Section C, Step 4, add 2 µl RNase A. Cap the PCR tubes and vortex to mix.**
2. **Incubate in a thermocycler at 37°C for 30 minutes.**
3. **Add 2 µl Proteinase K and 5 µl 5 M NaCl. Cap the PCR tubes and vortex to mix.**
4. **Incubate in a thermocycler at 65°C overnight.**
5. **Following reversal of cross-links, transfer DNA to a new 1.5 ml microcentrifuge tube. Add 125 µl phenol and 64 µl chloroform/isoamyl alcohol (24:1). Shake vigorously for 15 seconds to mix (do not vortex). Incubate at room temperature for 5 minutes.**
6. **Centrifuge in a microcentrifuge at maximum speed for 2 minutes. Transfer the aqueous**

layer (top layer) to a new 1.5 ml microcentrifuge tube. A hazy white layer at interface may still be present.

7. Add 125 µl chloroform/isoamyl alcohol (24:1) to the aqueous layer . Shake vigorously for 15 seconds to mix (do not vortex). Incubate at room temperature for 5 minutes.
8. During the incubation, set up new 1.5 ml microcentrifuge tubes for each ChIP reaction and add 2 µl Carrier to each tube.
9. Centrifuge the DNA purifications in a microcentrifuge at maximum speed for 2 minutes. Transfer the aqueous layer (top layer) to the microcentrifuge tube containing Carrier.
10. Add 300 µl 100% ethanol to the DNA solution and briefly vortex to mix.
11. Place samples at -80°C for 2 hours to precipitate the DNA.
12. Centrifuge in a microcentrifuge at maximum speed for 15 minutes at 4°C. Mark the tube where you expect the pellet to form as it may not be visible.
13. Carefully remove the supernatant taking care not to disturb the location of the DNA pellet.
14. Add 500 µl 70% ethanol to each tube and invert to mix.
15. Centrifuge in a microcentrifuge at maximum speed for 5 minutes at 4°C. Mark the tube where you expect the pellet to form as it may not be visible.
16. Remove 400 µl supernatant and centrifuge a second time at maximum speed for 2 minutes at 4°C. Carefully remove as much residual ethanol as possible with a P200 pipet taking care not to disturb the pellet.
17. Air-dry the pellet for 10-15 minutes (until residual ethanol has evaporated). Resuspend the pellet in 20 µl Low-EDTA TE.
18. Remove 5 µl to use for visualization of the shearing profile on a Fragment Analyzer or Bioanalyzer. If you find your sample is lower than the limit of detection for visualization, consider reserving more of your sample for this step.
19. To make a 1:10 dilution, add 135 µl of Low EDTA TE to the remaining 15 µl of sample for a total volume of 150 µl. Reserve 20 µl of the 150 µl for use in Section K: Analysis of Enriched DNA and Chromatin Quality.
20. With the remaining 130 µl, proceed to Section E: Analysis of Input DNA and Chromatin Quality.

Section E. Analysis of Input DNA and Chromatin Quality

1. Set up a qPCR reaction to quantify the chromatin input and to evaluate the quality of the chromatin. Use the provided DNA Standards and Human Positive Control GAPDH-2 PCR Primer Set (Cat # 71006) to analyze the Input DNA. Below is an example qPCR reaction. Please follow the specific instructions for your qPCR instrument. We recommend using a commercially available SYBR Green qPCR master mix (e.g. Bio-Rad Cat # 170-8882) and preparing triplicate reactions.

Note: Quantification by Qubit is not recommended due to the low amount of input. qPCR is the only recommended method for quantification at this step.

Reagent	10 µl PCR reactions
2X SYBR Green master mix	5 µl
GAPDH-2 Primer mix (2.5 µM each primer)*	2.5 µl
DNA sample (DNA Std or Input)	2.5 µl
Total volume	10 µl

2. **Place the PCR tubes in a real time PCR instrument. Using the software for your qPCR machine, assign a value of 2.5 ng/µl to DNA Standard AM1, a value of 0.25 ng/µl to DNA Standard AM2 and a value of 0.025 ng/µl to DNA Standard AM3. These values will be used to quantify your sample chromatin Input.**
3. **Place tubes in a real time PCR instrument and program as below:**

   95°C for 2 minutes
   (95°C for 15 seconds, 58°C for 20 seconds, 72°C for 20 seconds) for 40 cycles
4. **Include and inspect the melt curve based on the protocols recommended by the qPCR instrument manufacturer to ensure that primer pairs amplify only a single product.**
5. **Your qPCR instrument will assign values (in ng/µl) to each qPCR reaction based on the values assigned to DNA Standards AM1, AM2 and AM3. If your machine does not average your triplicate reactions automatically, you will need to calculate these averages.**
6. **For each qPCR reaction you will have used a percentage of your total chromatin. In order to calculate the total amount of chromatin, first multiply the calculated concentration by 10, to account for the 1:10 dilution. Next, by multiply the recovered volume of the soluble fraction (for example, 180 µl is the recovered volume if you transferred 20 µl at Section C, Step 4).**
7. **Generate a graph to evaluate the total chromatin yield (ng) for each chromatin preparation.**
   a. **We recommend at least 350 ng of chromatin is available in the recovered volume of the soluble fraction of the Fixed Jurkat Cell Pellet samples (the aliquots stored at -80°C from Section C, Step 3) to proceed.**

Section F. Protein G Agarose Bead Preparation

1. Set up two 1.5 ml microcentrifuge tubes to prepare the Protein G Agarose beads for the IP and pre-clearing. We recommend cutting 2 mm from the end of a pipet tip prior to pipetting Protein G agarose beads to prevent the tip from becoming clogged. If more than 12 IP reactions will be performed, we suggest setting up multiple tubes to ensure sufficient room in the tube for the addition of all the required components. Volumes to add will be based on the number of IP reactions performed. Include negative and positive control IPs into the cal-

culation for the number of IP reactions. Volumes shown below include excess for pipetting.

Reagents	IP Reactions 4 rxns	8 rxns	12 rxns	Pre-clearing Reactions 4 rxns	8 rxns	12 rxns
Protein G Agarose	140 µl	270 µl	400 µl	140 µl	270 µl	400 µl

* We do not recommend more than 12 IP reactions in a single tube. If more than 12 IPs are to be performed in a single experiment, set up multiple tubes.

2. Centrifuge the tubes at 1250 x g for 3 minutes. Carefully remove and discard supernatant, taking care to avoid the beads.
3. Add the calculated volume of TE, pH 8.0 to the each tube of beads:

Reagents	IP Reactions 4 rxns	8 rxns	12 rxns	Pre-clearing Reactions 4 rxns	8 rxns	12 rxns
TE, pH 8.0	460 µl	900 µl	1330 µl	460 µl	900 µl	1330 µl

4. Centrifuge the tubes at 1250 x g for 3 minutes. Carefully remove and discard supernatant, taking care to avoid the beads.
5. Set up bead blocking reactions according to the table below:

Reagents	IP Reactions 4 rxns	8 rxns	12 rxns	Pre-clearing Reactions 4 rxns	8 rxns	12 rxns
TE, pH 8.0	126 µl	243 µl	360 µl	140 µl	270 µl	400 µl
Blocking Reagent AM1	14 µl	27 µl	40 µl	14 µl	27 µl	40 µl
BSA	14 µl	27 µl	40 µl	14 µl	27 µl	40 µl
Blocker	14 µl	27 µl	40 µl	–	–	–
Incubate at 4°C with rotation	Overnight			3 - 6 hours		

6. Cap tubes and incubate on an end-to-end rotator at 4°C. Follow the recommendations in the chart above for the incubation time.
7. Following the incubation, proceed to Section G: Pre-clearing the Chromatin.

Section G. Pre-clearing the Chromatin

1. If chromatin from Section C, Step 3 was stored at -80°C, place on ice to thaw.
2. **If 20 µl of chromatin was removed from the sample for input preparation and analysis (Sections D and E), add 20 µl of ChIP buffer to each sample to bring the total volume to 200 µl.**
3. Remove the pre-clearing reactions from the rotator and quick spin to collect contents to the bottom of the tube. Allow the IP beads to continue to incubate on the rotator.
4. Centrifuge the tubes at 1250 x g for 3 minutes. Carefully remove and discard supernatant,

taking care to avoid the beads.

5. Add the calculated volume of ChIP Buffer to the pre-clearing reaction. Mix by inverting.

	IP Reactions			Pre-clearing Reactions		
Reagents	**4 rxns**	**8 rxns**	**12 rxns**	**4 rxns**	**8 rxns**	**12 rxns**
ChIP Buffer	–	–	–	460 µl	900 µl	1330 µl

6. Centrifuge the tubes at 1250 x g for 3 minutes. Carefully remove and discard supernatant, taking care to avoid the beads.
7. Perform a second wash with ChIP Buffer using the volumes below. Mix by inverting.

	IP Reactions			Pre-clearing Reactions		
Reagents	**4 rxns**	**8 rxns**	**12 rxns**	**4 rxns**	**8 rxns**	**12 rxns**
ChIP Buffer	–	–	–	160 µl	315 µl	465 µl

8. Cut off 2 mm from the end of pipet tip and transfer 50 µl of the pre-cleared Protein G agarose bead slurry to the 1.7 ml siliconized tube containing the sonicated chromatin from Section C, Step 3.
9. Add 5 µl Protease Inhibitor Cocktail and 5 µl PMSF to each tube.
10. If using less than 200 µl chromatin, adjust the final volume of the reaction to a final volume of 260 µl using ChIP Buffer.
11. Cap tubes and incubate on an end-to-end rotator for 2-3 hours at 4°C.

Section H. Immunoprecipitation

1. Prepare separate, labeled 1.5 ml microcentrifuge tubes for each ChIP reaction, even if the same antibody will be used for more than one sample. Prepare the antibody mixture according to the table below and place on ice. **If using the AbFlex Histone H3K9ac Antibody contained in the Low Cell Optimization Module, use 3 µg of antibody for each ChIP reaction.**

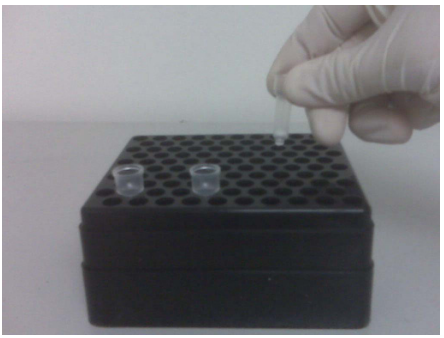
Reagent	**Antibody Mixture**
Antibody	2-4 µg
1X PBS	Up to 30 µl final volume

2. Centrifuge the tubes containing the pre-clearing reactions (Section G, Step 11) in a microcentrifuge at 1250 x g for 3 minutes. Carefully transfer the supernatant, avoiding the bead pellet, to the microcentrifuge tube containing the appropriate antibody mixture.
3. Cap tubes and incubate on an end-to-end rotator overnight at 4°C.

4. The next day, remove the IP reactions (from Section F, Step 6) from the rotator and quick spin to collect contents to the bottom of the tube.
5. Add 50 µl of the IP Reaction bead slurry to each immunoprecipitation reaction. We recommend cutting off 2 mm from the end of a pipet tip to make it easier to pipet the bead slurry.
6. Cap tubes and incubate on an end-to-end rotator for 3-4 hours at 4°C.

Section I. Washing & Elution of IP reactions

1. 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.



2. Remove the immunoprecipitation reactions from the rotator and quick spin to collect contents to the bottom of the tube.
3. Add 600 µl ChIP Buffer to each immunoprecipitation reaction to wash any remaining beads off the sides of the tube and transfer the entire volume (including the Protein G agarose beads) to its labeled column. Allow flow-through to occur by gravity.
4. During the gravity flow, transfer 100 µl Elution Buffer AM4 per ChIP reaction to a new 1.5 ml microcentrifuge tube. Pre-warm at 37°C. (DO NOT add to columns until Step 10 below.)
5. Wash each column with 900 µl ChIP Buffer. Allow a minute for the buffer to flow through the column. Wash a second time with an additional 900 µl ChIP Buffer.
6. Wash each column with 900 µl Wash Buffer AM1. Incubate for 3 minutes on the column. Allow the buffer to flow through the column. Wash a second time with an additional 900 µl Wash Buffer AM1.
7. Wash each column with 900 µl LiCl Buffer. Incubate for 3 minutes on the column. Allow the buffer to flow through the column. Wash a second time with an additional 900 µl LiCl Buffer.
8. Wash each column with 900 µl TE Buffer pH 8.0. Allow a minute for the buffer to flow through the column. Wash a second time with an additional 900 µl TE Buffer pH 8.0.
9. Transfer columns to a new 1.5 ml microcentrifuge tube. Centrifuge at 1250 x g for 3 minutes at room temperature to remove residual wash buffer.
10. Label new 1.5 ml microcentrifuge tubes. Transfer the ChIP Filtration Columns to the labeled tubes. Add 50 µl pre-warmed (37°C) Elution Buffer AM4 to each column. Incubate at room temperature for 5 minutes. Centrifuge at 1250 x g for 3 minutes at room temperature to col-

lect ChIP DNA.

11. Keeping the columns in the same microcentrifuge tube, add an additional 50 µl pre-warmed (37°C) Elution Buffer AM4 to each column. Incubate at room temperature for 5 minutes. Centrifuge at 1250 x g for 3 minutes at room temperature to collect ChIP DNA
12. Discard the ChIP Filtration columns. The flow through contains the ChIP DNA.

Section J. Reversal of Cross-links and DNA Purification

1. Transfer each eluted ChIP DNA to a 250 µl PCR tube. Add 2 µl Proteinase K and 5 µl 5 M NaCl. Vortex to mix. Heat in a thermocycler at 65°C overnight.
2. Following reversal of cross-links, transfer DNA to a new 1.5 ml microcentrifuge tube. Add 125 µl phenol and 64 µl chloroform/isoamyl alcohol (24:1). Shake vigorously for 15 seconds to mix (do not vortex). Incubate at room temperature for 5 minutes.
3. Centrifuge in a microcentrifuge at maximum speed for 2 minutes. Transfer the aqueous layer (top layer) to a new 1.5 ml microcentrifuge tube. A hazy white layer at interface may still be present.
4. Add 125 µl chloroform/isoamyl alcohol (24:1) to the aqueous layer in the new tube. Shake vigorously for 15 seconds to mix (do not vortex). Incubate at room temperature for 5 minutes.
5. During the incubation, set up new 1.5 ml microcentrifuge tubes for each ChIP reaction and add 2 µl Carrier to each tube.
6. Centrifuge the DNA purifications in a microcentrifuge at maximum speed (> 18,000 x g) for 2 minutes. Transfer the aqueous layer (top layer) to microcentrifuge tube containing Carrier.
7. Add 300 µl 100% ethanol to the DNA solution and briefly vortex to mix.
8. Place samples at -80°C for 2 hours to precipitate the DNA.
9. Centrifuge in a microcentrifuge at maximum speed (> 18,000 x g) for 15 minutes at 4°C. Mark the tube where you expect the pellet to form as it may not be visible.
10. Carefully remove the supernatant with a pipet. Avoid disturbing the location of the DNA pellet.
11. Add 500 µl 70% ethanol to each tube and invert to mix.
12. Centrifuge in a microcentrifuge at maximum speed (> 18,000 x g) for 5 minutes at 4°C. Mark the tube where you expect the pellet to form as it may not be visible.
13. Remove 400 µl supernatant and centrifuge a second time at maximum speed (> 18,000 x g) for 2 minutes at 4°C. Carefully remove as much residual ethanol as possible with a P200 pipet taking care not to disturb the pellet.
14. Air-dry the pellet for 10-15 minutes (until residual ethanol has evaporated).
15. **To perform post-IP analysis of enriched chromatin, resuspend the pellet in 20 µl Low-EDTA TE Buffer. Remove 2 µl for analysis in Section K: Analysis of Enriched DNA and Chromatin Quality. The remaining 18 µl will be diluted with Low-EDTA TE to a total volume of**

40 µl and used in Section L: Repair I to begin NGS library preparation. Alternatively, ChIP DNA may be stored at -20°C.

16. To proceed without performing post-IP analysis, resuspend the pellet in 40 µl Low-EDTA TE Buffer. Proceed to Section L: Repair I to begin NGS library preparation. Alternatively, ChIP DNA may be stored at -20°C.

Section K. Analysis of Enriched DNA and Chromatin Quality

1. **Add 18 µl of Low EDTA TE to each 2 µl of sample chromatin from Section J, Step 15, for a total volume of 20 µl. These are post-IP enriched samples.**
2. **Use the 20 µl reserves from Section D, Step 19 as pre-IP input samples.**
3. **qPCR will be used to determine % input relative quantification by directly comparing pre-IP input samples to their post-IP enriched sample counterparts. This method accounts for within sample variability and allows you to assess the quality of your ChIP DNA prior to NGS library preparation.**

   Using Human Pos Control Primer Set, ACTB-2 (positive control region for H3K9ac, Cat # 71005) and Human Neg Control Primer Set 1 (negative control region for H3K9ac, Cat # 71001) on the pre-IP input samples and the post-IP enriched samples, perform qPCR.

Please note: Positive and negative control regions are specific to the antibody used for enrichment. To learn more about the best control regions for your antibody, please visit www.activemotif.com/chipprimers.

Below is a portion of an example plate depicting qPCR reactions for pre-IP inputs and post-IP enrichments from two Jurkat H3K9ac-enriched samples:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1-Input ACTB-2	S1-Input ACTB-2	S1-Input ACTB-2	S2-Input ACTB-2	S2-Input ACTB-2	S2-Input ACTB-2	-	-	-	BL ACTB-2	BL ACTB-2	BL ACTB-2
B	-	-	-	-	-	-	-	-	-	-	-	-
C	S1-IP ACTB-2	S1-IP ACTB-2	S1-IP ACTB-2	S2-IP ACTB-2	S2-IP ACTB-2	S2-IP ACTB-2	-	-	-	-	-	-
D	-	-	-	-	-	-	-	-	-	-	-	-
E	-	-	-	-	-	-	-	-	-	-	-	-
F	S1-IP HNeg	S1-IP HNeg	S1-IP HNeg	S2-IP HNeg	S2-IP HNeg	S2-IP HNeg	-	-	-	-	-	-
G	-	-	-	-	-	-	-	-	-	-	-	-
H	S1-Input HNeg	S1-Input HNeg	S1-Input HNeg	S2-Input HNeg	S2-Input HNeg	S2-Input HNeg	-	-	-	BL HNeg	BL HNeg	BL HNeg

2. Set up qPCR reactions as follows:

Reagent	10 µl PCR reactions
2X SYBR Green qPCR master mix	5 µl
PCR primer pair (2.5 µM each)	2.5 µl
DNA sample (ChIP or Input)	2.5 µl
Total volume	10 µl

3. Place the PCR plate in a real time PCR instrument and follow the amplification conditions listed below. The provided primers have been optimized for use with Bio-Rad SYBR Green qPCR Master Mix (Catalog No. 170-8882). Other master mixes could affect PCR performance and melt curves. If necessary, optimize conditions based on the SYBR Green master mix reagent and PCR instrument used.

   95°C for 2 minutes
   (95°C for 15 seconds, 58°C for 20 seconds, 72°C for 20 seconds) for 40 cycles

4. Include and inspect the melt curve based on the protocols recommended by the qPCR instrument manufacturer to ensure that primer pairs amplify only a single product.

5. Calculate average Cq values for sample inputs and IPs, and for ACTB-2 and Human Negative Control Primers. The following example calculations demonstrate how to perform cycle adjustments, calculate cycle differences between input and IP samples, convert cycle numbers to a percentage of input, and finally determine the fold enrichment of your IP.

   Cq values are expected to be sample- and antibody-specific. However, the following pattern is indicative of a specific and successful enrichment: Input sample Cq values are relatively low (ex: mid-20s) and roughly equivalent for ACTB-2 and Hum Neg. IP sample ACTB-2 Cq values are higher (ex: 30) than input sample Cq values, but lower than IP sample Hum Neg values (ex: mid-30s or higher). In fact, it is not unusual for IP sample Hum Neg samples to exhibit no amplification at all.

Sample	Avg Cq	Adj Input*	Delta Cq	% Input**	Enrichment
S1-Input (ACTB-2)	26.70	26.70-3.32= 23.38			
S1-Input (Hum Neg)	26.49	26.49-3.32= 23.17			
S1-IP (ACTB-2)	30.43		30.43-23.38= 7.05	$2^{-7.05}$=0.75%	0.75/0.01= 75-fold
S1-IP (Hum Neg)	36.31		36.31-23.17= 13.14	$2^{-13.14}$=0.01%	

*** During Section C, Step 4, we used just 10% of sonicated chromatin for our input prep. To account for the other 90% of the chromatin, we adjust the Cq cycle number by log(0.1, 2)=3.32 cycles.**

****A base of 2 in the input calculation assumes perfect primer efficiency (product doubles with each PCR cycle).**

Protocol 2 – NGS Library Preparation

This protocol is designed to add Illumina-compatible adapters to the ends of the ChIP-enriched DNA to prepare a sequencing library. These adapters contain molecular identifiers (MIDs) that can be used to remove PCR duplicates through bioinformatic analysis. Both the Next Gen DNA Library Kit and Next Gen Indexing Kit (included in the Low Cell ChIP-Seq Kit) will be required to complete this section of the protocol.

Reagent Preparation

Enzyme Reagents

To maximize efficient use of enzyme reagents, remove enzymes from -20°C storage and place on ice, NOT in a cryocooler, for at least 10 minutes to allow reagents to reach 4°C prior to pipetting. After thawing reagents, invert to mix well (do not vortex enzymes). Spin tubes in a microcentrifuge to collect contents prior to opening. Attempting to pipette enzymes at -20°C, or failure to spin enzyme contents prior to opening may result in a shortage of enzyme reagents.

80% Ethanol Solution

Before starting the assay, prepare a fresh 80% ethanol solution using 200-proof/absolute ethanol and nuclease-free water. Calculate the amount of 80% ethanol solution to prepare based on needing approximately 2.0 ml per sample. Discard after use.

SPRIselect beads (not included)

SPRIselect beads are used during the NGS library preparation. Section N through Section R are per-

formed on the bead. DNA is eluted from the bead in Section S followed by library amplification in Section T. Fresh beads are added to clean up the library in Section U using the two-sided SPRI cleanup approach. Follow the recommendations in each step of the protocol for the appropriate reagents and volumes required.

Recommendations

Assemble reagent master mixes on ice and scale volumes as needed, using a 5% excess volume to compensate for pipetting loss. We suggest preparing reagent mixes in advance to ensure the SPRIselect beads do not dry out. Reaction master mixes for all the library preparation steps may be prepared at the start of the protocol ONLY if the entire library preparation will be performed immediately. Do not store master mixes. Always add reagents in the specified order.

Section L: Repair I

1. Transfer the 40 µl ChIP DNA to a 0.2 ml PCR tube.
2. Place a new 0.2 ml PCR tube on ice and prepare the Repair I reaction mix by adding reagents in the order listed below. Multi-reaction volumes shown below include excess for pipetting.

Reagent	One rxn	4 rxns
Low EDTA TE	13 µl	54.6 µl
Buffer W1	6 µl	25.2 µl
Enzyme W2	1 µl	4.2 µl
Total Volume	**20 µl**	**84 µl**

3. Add 20 µl of the pre-mixed Repair I reaction mix to each PCR tube containing the 40 µl DNA sample. Mix by pipetting. Place in the thermal cycler and run the Repair I thermal cycler program as described below.

Repair I Thermal Cycler Program	37°C for 10 minutes, lid heating OFF* *Alternatively, the thermal cycler lid may be left open.

Section M: Post-Repair I SPRI

1. Clean up the Repair I reaction using SPRIselect beads and freshly prepared 80% ethanol. See Appendix, Section W for instructions.

Sample Volume	Bead Volume	PEG NaCl Volume
60 µl	84 µl (ratio: 1.4)	N/A

Section N: Repair II

1. Place a new 0.65 ml tube on ice and prepare the Repair II reaction mix by adding reagents in the order listed below.

Reagent	One rxn	4 rxns
Low EDTA TE	30 µl	126 µl
Buffer G1	5 µl	21 µl
Reagent G2	13 µl	54.6 µl
Enzyme G3	1 µl	4.2 µl
Enzyme G4	1 µl	4.2 µl
Total Volume	**50 µl**	**210 µl**

2. Add 50 µl of the pre-mixed Repair II reaction mix to the beads for each sample and resuspend by pipetting.
3. Place in the thermal cycler and run the Repair II thermal cycler program as described below.

Repair II Thermal Cycler Program	20°C for 20 minutes, lid heating OFF* *Alternatively, the thermal cycler lid may be left open.

Section O: Post-Repair II SPRI

1. Clean up the Repair II reaction using PEG NaCl solution and freshly prepared 80% ethanol. See Appendix, Section W for instructions.

Sample Volume	Bead Volume	PEG NaCl Volume
50 µl	N/A	60 µl (ratio: 1.2)

Section P: Ligation I

1. Place a new 0.2 ml PCR tube on ice and prepare the Ligation I reaction mix by adding reagents in the order listed below.

Reagent	One rxn	4 rxns
Low EDTA TE	20 µl	84 µl
Buffer Y1	3 µl	12.6 µl
Enzyme Y3	2 µl	8.4 µl
Total Volume	**25 µl**	**105 µl**

2. Add 25 µl of the pre-mixed Ligation I reaction mix to the beads for each sample.
3. Add 5 µl of the desired Reagent Y2 Index to each sample for a total volume of 30 µl. Resuspend by pipetting. Record the identity of the index used for each sample to ensure proper de-multiplexing after sequencing.

   Note: Reagent Y2 Index samples are part of Active Motif's Next Gen Index Kit, Catalog No. 53264. Index primer sequences can be found in the Appendix Section U. All 16 unique indices provided are compatible within the same sequencing reaction.

4. Place in the thermal cycler and run the Ligation I thermal cycler program as described below.

Ligation I Thermal Cycler Program	25°C for 15 minutes, lid heating OFF* *Alternatively, the thermal cycler lid may be left open.

Section Q: Post-Ligation I SPRI

1. Clean up the Ligation I reaction using PEG NaCl solution and freshly prepared 80% ethanol. See Appendix, Section W for instructions.

Sample Volume	Bead Volume	PEG NaCl Volume
30 µl	N/A	25.5 µl (ratio: 0.85)

Section R: Ligation II

1. Place a new 0.65 ml tube on ice and prepare the Ligation II reaction mix by adding reagents in the order listed below.

Reagent	One rxn	4 rxns
Low EDTA TE	30 µl	126 µl
Buffer B1	5 µl	21 µl
Reagent B2-MID	2 µl	8.4 µl
Reagent B3	9 µl	37.8 µl
Enzyme B4	1 µl	4.2 µl
Enzyme B5	2 µl	8.4 µl
Enzyme B6	1 µl	4.2 µl
Total Volume	**50 µl**	**210 µl**

2. Add 50 µl of the pre-mixed Ligation II reaction mix to the beads for each sample and resuspend by pipetting.
3. Place in the thermal cycler and run the Ligation II thermal cycler program as described below.

Ligation II Thermal Cycler Program	40°C for 10 minutes, lid heating OFF* 25°C Hold *Alternatively, the thermal cycler lid may be left open.

Section S: Post-Ligation II SPRI

1. Clean up the Ligation II reaction using PEG NaCl solution and freshly prepared 80% ethanol. See Appendix, Section W for instructions.

Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
50 µl	N/A	42.5 µl (ratio: 0.85)	20 µl

2. At the end of the first Ligation II SPRI clean up, resuspend the beads in 20 µl Low EDTA TE buffer. Mix well by pipetting up and down until homogenous.
3. Place beads on a magnetic rack until the solution clears and a pellet is formed. Transfer supernatant to a new 0.2 ml PCR tube.

Section T: Library Amplification

1. Place a new 0.2 ml PCR tube on ice and prepare the Library PCR reaction mix by adding reagents in the order listed below.

Reagent	One rxn	4 rxns
Low EDTA TE	10 µl	42 µl
Reagent R1	5 µl	21 µl
Reagent R2	4 µl	16.8 µl
Buffer R3	10 µl	42 µl
Enzyme R4	1 µl	4.2 µl
Total Volume	**30 µl**	**126 µl**

2. Add 30 µl of the pre-mixed Library PCR reaction mix to the 20 µl eluted library and mix by pipetting. The total volume of the PCR reaction is 50 µl.
3. Place in the thermal cycler and run the Library PCR thermal cycler program as described below.

Library PCR Thermal Cycler Program	98°C for 30 seconds	
	98°C for 10 seconds 60°C for 30 seconds 68°C for 60 seconds	x 14 PCR cycles
	4°C Hold	
Proceed immediately to Post-PCR SPRI		

Section U: Post-Library PCR SPRI

It is recommended to use a two-sided SPRI cleanup approach to eliminate small and large fragments for a more uniform library selection.

1. Clean up the Library PCR reaction using SPRIselect beads and freshly prepared 80% ethanol. See Appendix page 28, Post-Library SPRI (two-sided SPRI cleanup) for detailed instructions.

Sample Volume	Bead Volume	PEG NaCl Volume
50 µl	32.5 µl (ratio: 0.65)	N/A

Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
50 µl	10 µl (ratio: 0.2)	N/A	20 µl

2. Resuspend the beads in 20 µl Low EDTA TE buffer. Mix well by pipetting up and down until homogenous.
3. Place beads on a magnetic rack until the solution clears and a pellet is formed. Transfer supernatant to a new tube.
4. Use a library quantification kit for Next-generation sequencing to quantify the library (*e.g.* Kapa Biosystems, Catalog No.KR0405). PCR amplified libraries can also be analyzed with a Bioanalyzer to assess size distribution. Please note the sensitivity limits specified by Agilent for the Bioanalyzer to ensure proper analysis of library size.
5. Libraries can be stored at 4°C for short-term storage or -20°C for long-term storage. Libraries are now ready for sequencing on the Illumina platforms.

Section V: Sequencing Guidelines

1. Guidelines for the run set-up and sample sheet preparation for use with Illumina MiSeq, HiSeq2500 and NextSeq 500 platforms can be found at **www.activemotif.com/nextgen**. Select the Documents tab to download a copy of the Sequencing Guidelines. The Sequencing Guidelines will also include instructions for the use of MIDs, including recommendations for data analysis.

Section W: SPRIselect Clean-up Protocol

Please use the following protocol for the post-repair and post-ligation SPRI steps, substituting the correct **Bead Volume**, **PEG NaCl Volume** and **Elution Volume** based on the table provided for each section. For post-library PCR SPRI, please follow the two-sided SPRI cleanup approach listed on page 28.

1. Ensure the SPRI beads are at room temperature and briefly vortex beads to homogenize the suspension before use.
2. Add the specified Bead Volume, or PEG NaCl Volume to each sample. Mix by vortexing. Quick spin the samples in a tabletop microcentrifuge.
3. Incubate the samples for 5 minutes at room temperature.
4. Place the sample on a magnetic rack until the solution clears and a pellet is formed (~5 min).
5. Remove and discard the supernatant without disturbing the pellet (<5 µl may be left behind).
6. Add 180 µl of freshly prepared 80% ethanol solution to the pellet while it is still on the magnet. Use care not to disturb the pellet. Incubate for 30 seconds, and then carefully remove the ethanol solution.
7. Repeat step 6 once more for a second wash with the 80% ethanol solution.
8. Quick spin the samples in a tabletop microcentrifuge and place back onto the magnet. Remove any residual ethanol solution from the bottom of the tube.
9. Air dry the pellet for no more than 5 minutes. Watch the pellet to avoid cracking or overdrying, which could result in reduced DNA recovery.
10. Add the specified volume of each reaction mix (Repair II, Ligation I and Ligation II) or elution volume (Post-Ligation II and Post-Library PCR) or Low EDTA TE buffer and resuspend the pellet. Mix well by pipetting up and down until homogenous.

Post-Repair I SPRI

Sample Volume	Bead Volume	PEG NaCl Volume
60 µl	84 µl (ratio 1.4)	N/A

Post-Repair II SPRI

Sample Volume	Bead Volume	PEG NaCl Volume
50 µl	N/A	60 µl (ratio: 1.2)

Post-Ligation I SPRI

Sample Volume	Bead Volume	PEG NaCl Volume
30 µl	N/A	25.5 µl (ratio: 0.85)

Post-Ligation II SPRI

Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
50 µl	N/A	42.5 µl (ratio: 0.85)	20 µl

Post-Library PCR SPRI (two-sided SPRI cleanup)

Sample Volume	Bead Volume	PEG NaCl Volume
50 µl	32.5 µl (ratio: 0.65)	N/A

1. Mix by vortexing. Quick spin the samples in a tabletop microcentrifuge.
2. Incubate the samples for 5 minutes at room temperature.
3. Place the sample on a magnetic rack until the solution clears and a pellet is formed.
4. Transfer the supernatant (approximately 50 µl) to a new tube and discard the beads. Add 10 µl fresh SPRI beads. (ratio: 0.2) to the supernatant in the new tube.

Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
50 µl	10 µl (ratio: 0.2)	N/A	20 µl

5. Mix by vortexing. Quick spin the samples in a tabletop microcentrifuge.
6. Incubate the samples for 5 minutes at room temperature.
7. Place the sample on a magnetic rack until the solution clears and a pellet is formed.
8. Remove and discard the supernatant without disturbing the pellet (leave < 5 µl in tube).
9. Add 180 µl of freshly prepared 80% ethanol solution to the pellet while it is still on the magnet. Use care not to disturb the pellet. Incubate for 30 seconds, and then carefully remove the ethanol solution.
10. Repeat step 9 once more for a second wash with the 80% ethanol solution.
11. Quick spin the samples in a tabletop microcentrifuge and place back onto the magnet. Remove any residual ethanol solution from the bottom of the tube.
12. Air dry the pellet for no more than 5 minutes. Watch the pellet to avoid cracking or over-drying, which could result in reduced DNA recovery.
13. Proceed to Section U, Step 2 on page 26 for elution and quantification.

Section X: Next Gen DNA Library & Next Gen Indexing Kits

The Next Gen DNA Library Kit is designed to generate high complexity DNA libraries for Next generation sequencing (NGS). The Next Gen DNA Library Kit offers the advantage of including molecular identifiers (MIDs) during library generation to enable removal of true PCR duplicates from the sequencing analysis. This helps to distinguish PCR duplicates from fragmentation duplicates, thereby, increasing the number of unique alignments for more accurate data analysis.

The Next Gen DNA Library Kit works by adding standard low throughput (LT) P7 adapters containing a single index for multiplexed sequencing (index 1 position) during Ligation I. Following a PEG NaCl clean-up, a high throughput (HT) P5 adapter containing the MID is incorporated during Ligation II. The MID is a 9 base random N sequence at the [i5] (index 2 position) of the P5 adapter. Addition of the MID is strand-specific with each dsDNA insert receiving two MIDs (X and Y). The two MIDs cluster and sequence independently. Bioinformatically, PCR duplicates can be removed from the data set, while fragmentation duplicates are preserved.

During Ligation I in the protocol, you must use a unique indexed adapter "Reagent Y2 Index" to label each library. If no multiplex sequencing is being performed, all libraries may be labeled with the same Index. Libraries made with unique Index adapters may be multiplexed during cluster generation and co-sequenced on the same Illumina flow cell.

Below are the unique sequences for the 16 Reagent Y2 Index adapters provided in the Next Gen Indexing Kit (Active Motif, Catalog No. 53264). Use 5 µl "Reagent Y2 Index" in the Ligation I protocol on page 23. Make sure to note which indexed adapter was used with each sample. Do not use the same index adapter on two different samples you plan to multiplex together.

Indexed Adapters	Sequence*
Reagent Y2 Index 1	ATCACG(A)
Reagent Y2 Index 2	CGATGT(A)
Reagent Y2 Index 3	TTAGGC(A)
Reagent Y2 Index 4	TGACCA(A)
Reagent Y2 Index 5	ACAGTG(A)
Reagent Y2 Index 6	GCCAAT(A)
Reagent Y2 Index 7	CAGATC(A)
Reagent Y2 Index 8	ACTTGA(A)
Reagent Y2 Index 9	GATCAG(A)
Reagent Y2 Index 12	CTTGTA(A)
Reagent Y2 Index 13	AGTCAA(C)
Reagent Y2 Index 14	AGTTCC(G)
Reagent Y2 Index 15	ATGTCA(G)
Reagent Y2 Index 16	CCGTCC(C)
Reagent Y2 Index 18	GTCCGC(A)
Reagent Y2 Index 19	GTGAAA(C)

* The base pair in parentheses is read during a seventh cycle, but is not considered part of the index sequence.

Section Y. 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 chromatin shearing, -80°C. 2. During overnight incubation of pre-cleared chromatin and antibody, 4°C. 3. During overnight reversal of cross-links, 65°C 4. After DNA clean up, -20°C. 5. After NGS library preparation, 4°C short-term or -20°C for long-term storage.
What if my tissue is too large to fit into the 1.7 ml siliconized tube?	If tissue is larger than 1 mm x 1 mm, place tissue in a 10 cm petri dish and add 10 ml ice-cold 1X PBS. Add 1 ml Complete Fixation Solution and use a razor blade to chop tissue into smaller 1 mm x 1 mm pieces. Transfer tissue with the entire volume of PBS and fixation solution to a 15 ml conical tube. Incubate for 15 minutes at room temperature with agitation. Add 550 µl Stop Solution to quench fixation. Proceed with Section B, Step 9 to prepare a single cell suspension.
What if I want to prepare a different number of IP reactions other than those listed in the manual?	The IP and Pre-Clearing reaction volumes are provided as a guideline and include excess for pipetting. If different numbers (other than 4, 8 or 12) reactions are to be performed adjust the volume of reagents added using the ratios listed as a guideline. Ensure there is some excess for pipetting, but do not include a large excess or you may run short on reagents.
A precipitation is observed during Protein G Agarose bead preparation	A small precipitation or cloudiness may be observed in the IP reaction tubes following the overnight incubation. We suggest heating the samples at 37°C for 5 minutes to dissolve the precipitation and proceed with the protocol.
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. Chromatin amounts will be too small to visualize on a gel or BioAnalyzer and still have enough material for the IP. To investigate shearing issues, you may need to prepare extra chromatin material.
	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. We recommend using 2-4 µg of antibody per IP.
Poor or no enrichment with target antibody.	Insufficient cell numbers were used. Repeat chromatin preparation using a larger number of cells. Follow the recommendations for input cell numbers based on the robustness of the target protein.
	Antibody is not ChIP-Seq 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. Active Motif provides a list of Low Cell ChIP-seq validated antibodies at www.activemotif.com/chip-lowcell.
	Low-affinity antibody. Efficiency of the Low Cell ChIP-Seq not only relies on the abundance of the target protein, but the quality of the ChIP antibody. Antibodies with low binding affinities may require the use of more cells. Alternatively, use a different antibody.
	Use fresh formaldehyde, buffered with 10-15% methanol for fixation solutions.
Library migrates unexpectedly on Bioanalyzer	Over-amplification of library leads to the formation of heteroduplex structures that migrate abnormally. A sharp band on the Bioanalyzer betwee 125-200 bp is an indication that adapter dimers are present and were not sufficiently removed using SPRI. Lower the SPRI ratio and ensure that two washes were performed with 80% ethanol. Quantify library by qPCR as other quantification methods will not accurately detect heteroduplex library molecules. Perform the minimum number of PCR cycles necessary to avoid over-amplification

Problem/question	Recommendation
Incomplete resuspension of beads after ethanol wash during SPRI steps	Over-drying of beads. Continue pipetting the liquid over the beads to break up clumps for complete resuspension. We recommend preparing reagent mixes in advance so that the SPRI beads do not dry out between steps.
Shortage of enzyme reagents	Pipetting enzymes at -20°C instead of placing enzymes at 0-4°C before use. Allow enzyme reagents to equilibrate to 0-4°C for 10 minutes prior to pipetting. Place enzymes on ice prior to use, do not use a cryocooler.
Retention of liquid in pipette tip	Viscous reagents may stick to pipette tip, especially for non-low retention tips. Pipette up and down several times to ensure all liquid and/or beads are released from the pipette tip

Section Z. 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
ChIP-IT High Sensitivity® Chromatin Preparation Kit	16 rxns	53046
ChIP-IT® qPCR Analysis Kit	10 rxns	53029
ChIP-IT® ChIP-Seq	10 libraries	53041
ChIP-IT® FFPE II	16 rxns	53047
ChIP-IT® FFPE Chromatin Preparation II Kit	5 rxns	53031
ChIP-IT® Express HT	96 rxns	53018
Low Cell ChIP-Seq	16 rxns	53084
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 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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