Low Cell ChIP Kit

(version A1)

Catalog No. 53086

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Overview

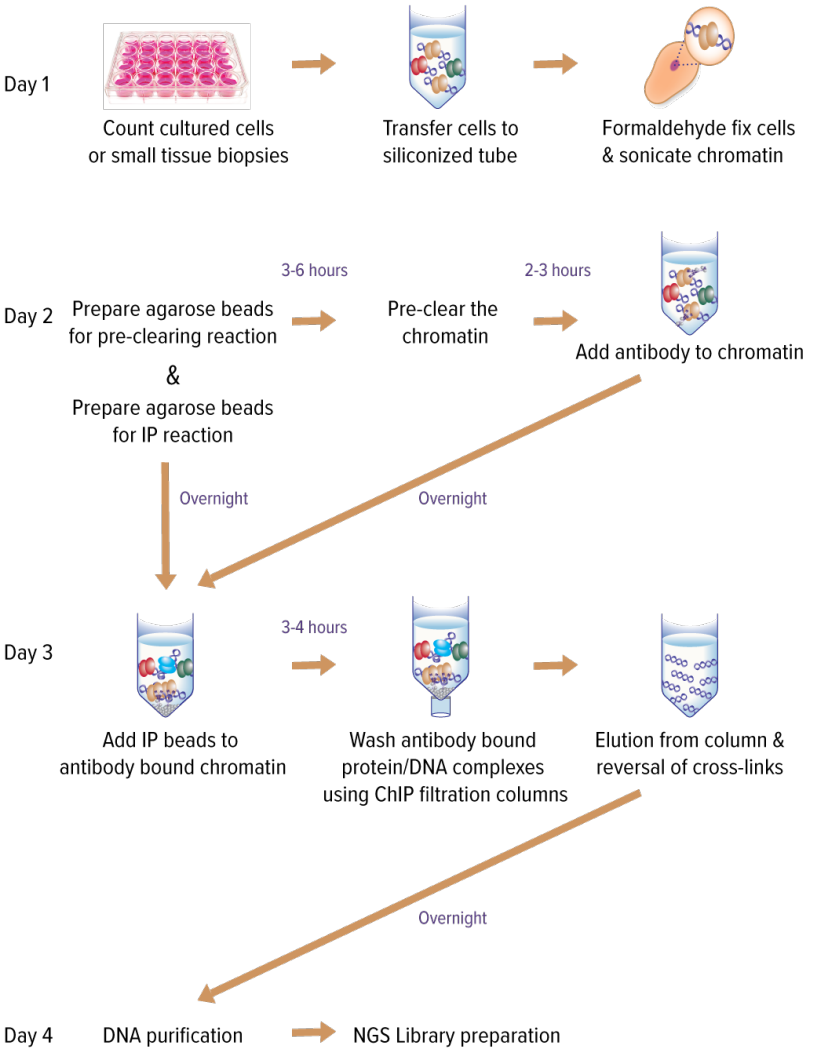
Chromatin Immunoprecipitation (ChIP) is a powerful tool for studying protein/DNA interactions, including transcription factors, co-regulatory proteins, modified histones, chromatin-modifying enzymes and polymerases because it enables identification of the localization of proteins bound to specific DNA 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 Kit.

The Low Cell ChIP Kit provides an optimized ChIP workflow to generate genome-wide binding profiles from as few as 1,000 cells, or small tissue biopsies. Low Cell ChIP 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 Low Cell ChIP Kit contains sufficient reagents to perform 16 ChIP-Seq reactions. Reagents are included for chromatin preparation and immunoprecipitation.

product	format	catalog no.
Low Cell ChIP Kit	16 rxns	53086

Flow Chart of Low Cell ChIP Process



Protocol Overview and Time Table

Protocol Steps	Required Time
Cell Collection and Fixation	30 minutes
Chromatin Sonication*	20 minutes per sample
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

Kit Performance and Benefits

Low Cell ChIP-Seq Advantages:

- Reproducible ChIP data from as little as 1,000 cells for high abundance target proteins and as little as 50,000 cells for lower abundance transcription factors
- Works with cultured cell lines, primary cells, including FACS sorted cells, and small tissue biopsies
- Works with active and repressive histone modifications and transcription factor targets

Low Cell ChIP-Seq Kit

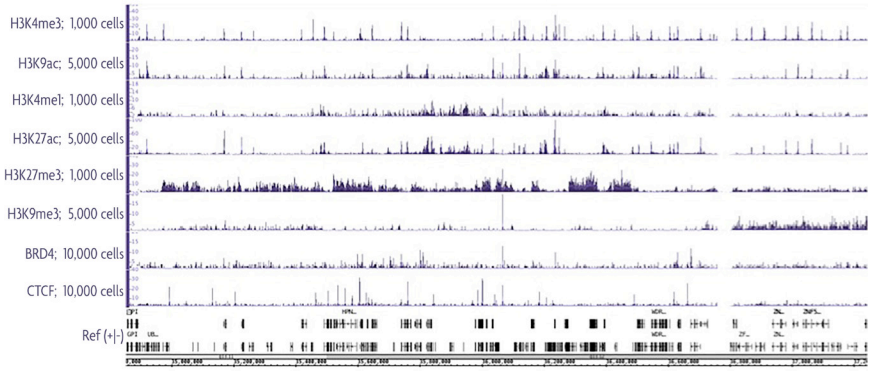


Figure 1: Sensitivity of Low Cell ChIP-Seq Kit across different target proteins and input amounts.

The Low Cell ChIP-Seq Kit was used to detect both robust and low abundance target proteins and both active and repressive histone modifications using the input cell number listed in the y-axis. Nice peaks are observed above background from as little as 1,000 cells for histone marks H3K4me3, H3K4me1 and H3K27me3. Lower abundance proteins BRD4 and insulator protein CTCF show nice ChIP-Seq signal from only 10,000 cells. Results shown represent a collection of individual Low Cell ChIP-Seq reactions from several cell lines. Data has been reproducibly generated across multiple experiments.

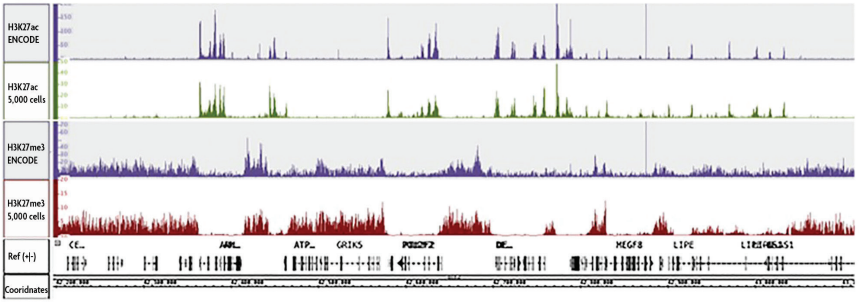


Figure 2: Comparison of Low Cell ChIP-Seq data with ENCODE validated data sets.

The Low Cell ChIP-Seq Kit was used to detect histone modifications associated with either active gene expression (H3K27ac) or repressive chromatin (H3K27me3). Data from Low Cell ChIP-Seq was generated using 5,000 cells per IP reaction, while the ENCODE data sets were generated from 20 million cells per IP reaction. Results show that Active Motif's Low Cell ChIP-Seq Kit is capable of generating high quality ChIP-Seq data that contains the same peak profiles when compared to published data sets.

The quality of the Low Cell ChIP-Seq depends not only on cell type and abundance of the target protein, but it is also influenced by the quality of the ChIP-Seq antibody. Below is a list of some of the validated antibodies from Active Motif for use with Low Cell ChIP-Seq. Please refer to our website for an up-to-date list of validated antibodies www.activemotif.com/chip-lowcell.

Protein Target	Active Motif Catalog Number	Cell Type Tested
H3K4me3	39159	GM12878; MDA-MB-468
H3K9ac	39918	GM12878
H3K9me3	39161	GM12878
H3K27ac	39133	HepG2
H3K27me3	39155	MDA-MB-468
CTCF	61311	MDA-MB-468

Kit Components and Storage

The Low Cell ChIP-Seq Kit is for research use only. Not for use in diagnostic procedures. The kit contains multiple modules to help organize your experiments. Store each component at the temperatures indicated in the tables below.

Reagents for Low Cell ChIP

Reagents	Quantity	Storage
Proteinase K (10 µg/µL)	80 µL	-20°C
10X PBS	20 mL	-20°C
100 mM PMSF	150 µL	-20°C
Protease Inhibitor Cocktail (PIC)	100 µL	-20°C
Carrier	35 µL	-20°C
Blocker	100 µL	-20°C
Blocking Reagent AM1	115 µL	-20°C
BSA (10 mg/mL)	115 µL	-20°C
Fixation Buffer	150 µL	4°C
Protein G Agarose Beads*	1.2 mL	4°C
TE pH 8.0	35 mL	RT
5 M NaCl	400 µL	RT
Stop Solution	1 mL	RT
ChIP Filtration Columns	16 ea	RT
ChIP Buffer	45 mL	RT
Wash Buffer AM1	30 mL	RT
LiCl Buffer	40 mL	RT
Elution Buffer AM4	1.6 mL	RT
1.7 mL siliconized tubes	32 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. Protein G Agarose Beads should be stored at 4ºC.

Additional materials required

- 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). 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)

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. Please refer to the kit contents and additional materials required section of the manual (pages 6-7) to identify the components that are needed for each protocol.

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 table on page 11 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 62.8 µL sterile water and 7.2 µL fixation buffer. Using appropriate precautions (*i.e.* safety glasses, gloves and lab coat), add 30 µL 37% formaldehyde to the tube and vortex to mix. Use 5 µL Complete cell fixation solution per 50 µL cell pellet. Discard unused solution.

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 28 µL 37% formaldehyde to the tube and vortex to mix. Use 1 mL Complete tissue fixation solution per tissue sample. Discard unused solution.

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 4ºC 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 4ºC.**

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. 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, 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

We suggest using a probe sonicator (*i.e.* Active Motif's EpiShear Probe Sonicator) which employs a direct sonication method to prepare chromatin for use in the Low Cell ChIP-Seq Kit. Indirect sonication systems may require longer sonication times to achieve optimal chromatin shearing. ChIP experiments usually require chromatin that has been sheared to a size of 200-1200 bp. In general, shearing efficiency is improved through the use of a small shearing volume and a V-bottom tube rather than a round-bottom tube. Also, note that shearing is inefficient if the chromatin sample becomes emulsified with air bubbles. To determine the appropriate shearing level for your sample, set up a "practice" tube containing only ChIP Buffer. Slowly increase the sonication amplitude until foaming starts to occur. Reduce the amplitude setting down slightly and mark this as the highest possible intensity to use without foaming. If a chromatin preparation becomes emulsified inadvertently, discontinue shearing and centrifuge the sample at maximum speed for 4 minutes at 4ºC 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.

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.

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 10 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 11.
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. Add 2 µL PIC and 2 µL PMSF to each tube. Then, add 138.2 µL ChIP Buffer and using the

same pipette tip, transfer the cells to a 2 mL V-bottom tube for sonication. Repeat for each sample. The final cell suspension volume should be 200 µL.

12. 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 11.
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 D: 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.

Section D. 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 calculation for the number of IP reactions. Volumes shown below include excess for pipetting.

	IP Reactions			Pre-clearing Reactions		
Reagents	**4 rxns**	**8 rxns**	**12 rxns**	**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:

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

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:

	IP Reactions			Pre-clearing Reactions		
Reagents	**4 rxns**	**8 rxns**	**12 rxns**	**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 E with the pre-clearing bead reactions.

Section E. Pre-clearing the Chromatin

1. If chromatin from Section C, Step 3 was stored at -80°C, place on ice to thaw.
2. 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.
3. Centrifuge the tubes at 1250 x g for 3 minutes. Carefully remove and discard supernatant, taking care to avoid the beads.
4. 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 µL	–	–	–	460 µL	900 µL	1330

5. Centrifuge the tubes at 1250 x g for 3 minutes. Carefully remove and discard supernatant, taking care to avoid the beads.
6. 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

7. 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.
8. Add 5 µL Protease Inhibitor Cocktail and 5 µL PMSF to each tube.
9. If using less than 200 µL chromatin, adjust the final volume of the reaction to a final volume of 260 µL using ChIP Buffer.
10. Cap tubes and incubate on an end-to-end rotator for 2-3 hours at 4°C.

Section F. Immunoprecipitation

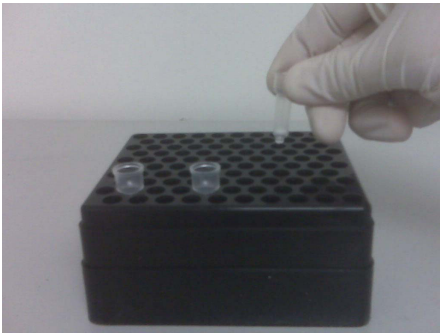
1. Prepare separate, labeled 1.5 mL microcentrifuge tubes for each ChIP reaction, even if the same antibody will be used for more than one sample. Prepare the antibody mixture according to the table below and place on ice.

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 E, Step 10) in a microcentrifuge at 3500 rpm 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 D, Step 5) 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 G. 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 collect 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 H. 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 30 minutes 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). Resuspend the pellet in 40 µL **Low-EDTA TE Buffer**. Proceed to NGS library preparation. The Low Cell ChIP DNA fragments are double-stranded and should work with any commercial DNA library preparation kit designed for sub-nanogram levels of DNA. Please contact our technical support for guidance. Alternatively, ChIP DNA may be stored at -20°C.

Section I. 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.
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.

Problem/question	Recommendation
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

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