enChIP®

(version B1)

Catalog No. 53125

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TABLE OF CONTENTS **Page**

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 the identification of proteins bound to specific DNA loci. With the advent of the CRISPR/Cas9 technology, new applications are being applied to study chromatin interactions.

With enChIP®* (engineered DNA-binding molecule-mediated chromatin immunoprecipitation), the CRISPR (clustered regularly interspaced short palindromic repeats) system is used to direct a guide RNA (gRNA) to a specific genomic locus for immunoprecipitation. The gRNA is designed as the complementary sequence of the desired target locus. The gRNA is co-expressed with a deactivated (enzymatically inactive) form of the *Streptococcus pyogenes* Cas9 endonuclease (dCas9). The dCas9 protein is expressed with Active Motif's unique AM-tag sequence, which was specifically designed for use in ChIP. The AM-tag has minimal cross-reactivity with mammalian samples, thereby reducing background signal. Another benefit of the AM-tag is that the sequence is unstructured which allows the tag to protrude from the protein for maximum exposure during immunoprecipitation. Following transfection of the gRNA and AM-tagged dCas9, cells are formaldehyde fixed, lysed and the chromatin is fragmented by sonication. An antibody directed against the AM-tag is used to enrich for genomic sequences bound by the gRNA/dCas9 complex. Following immunoprecipitation, DNA can be analyzed by qPCR or NGS to identify the enriched genomic regions. enChIP provides the opportunity to investigate off-target effects of gRNA design, or to study *cis*- and *trans*-interacting chromosomal looping events.

To use Active Motif's enChIP® Kit simply clone your gRNA sequence into one of Active Motif's expression vectors. Following transfection and expression of the gRNA and AM-tagged dCas9, the enChIP Kit can be used to isolate chromatin and perform immunoprecipitation using an antibody directed against the AM-tag. The enChIP Kit contains sufficient reagents to perform 16 chromatin preparation and immunoprecipitation reactions.

product	format	catalog no.
enChIP® Kit	16 rxns	53125
pAM_gRNA Vector	10 µg	53121
pAM_dCas9 Vector	10 µg	53122
pAM_gRNA_CTCF Vector	10 µg	53123
pAM_dCas9_CTCF Vector	10 µg	53124
FuGENE® HD Transfection Reagent	0.2 ml	32042
AM-Tag polyclonal antibody	100 µg	61677
ChIP-IT qPCR Analysis Kit	10 rxns	53029

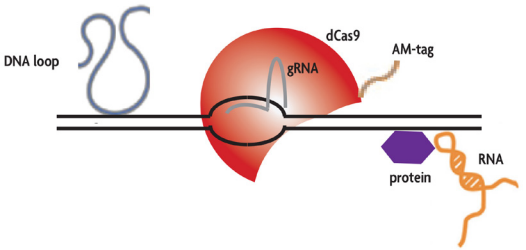
***Patent number: WO2014/125668; JP5954808**

Introduction

The CRISPR/Cas system can be traced to bacteria and archaea which use the system to target and inactivate foreign genetic material. This adaptive immunity mechanism relies on the acquisition of spacers, the assembly of the CRISPR RNAs (crRNAs) and Cas proteins to the invading nucleic acids and degradation of the foreign material by nucleases[1]. There are three main types of CRISPR/Cas systems, each with a specific Cas protein. Type II systems utilize Cas9 complexes[1]. Recently, researchers have repurposed the CRISPR/Cas9 system for genome engineering of mammalian systems[2-6].

In type II CRISPR/Cas systems, the crRNA hybridizes with a transactivating crRNA (tracrRNA) that contains additional nucleotide sequences. In the presence of Cas9, the crRNA-tracrRNA hybrid is processed to generate a spacer that is 20 nucleotides in length[7,8]. The Cas9 complex then undergoes a conformational change for DNA binding[1,4,9]. The spacer sequence directs the Cas9 complex to a complementary DNA sequence containing a protospacer adjacent motif, or PAM site (5´-NGG). Recognition of a PAM site leads to unwinding of the DNA and formation of an RNA-DNA heteroduplex[7,9,10]. Nucleases are then activated to create a double-stranded break in the target DNA.

Engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) was first described in 2013 by Toshitsugu Fujita and Hodaka Fujii[11, 12]. enChIP was designed to purify specific genomic regions using the CRISPR/Cas9 system. A single guide RNA vector containing a fusion of the crRNA-tracrRNA sequence (5´side to identify the DNA target sequence and a 3´duplex RNA structure to bind Cas9) is used to clone the target sequence of interest. When the gRNA is expressed in combination with a tagged deactivated *Streptococcus pyogenes* Cas9 (dCas9) protein, specific genomic regions can be immunoprecipitated using an antibody directed against the tag. By using a catalytically inactive form of Cas9 endonuclease, double-stranded breaks are not introduced and DNA, RNA and proteins associated with the target sequence can be recovered[11-15].



With Active Motif's enChIP Kit, simply clone your 20 nucleotide target sequence into Active Motif's gRNA or dCas9 expression vectors. Following transfection and expression of the gRNA and AM-tagged dCas9, cells are formaldehyde fixed, lysed and the chromatin is fragmented by sonication. An antibody directed against the AM-tag is used to enrich for genomic sequences bound by the gRNA/dCas9 complex. Following immunoprecipitation, DNA can be analyzed by qPCR or NGS to identify the enriched genomic regions.

For researchers looking to perform genome editing experiments, determining the specificity of the gRNA binding site is critical. If the gRNA target sequence demonstrates off-target binding, then genome editing methods using active Cas9 protein could lead to unwanted downstream effects. By using the enChIP Kit containing the dCas9 protein, off-target gRNA binding sites can be identified to determine the quality of the gRNA design prior to use in genome editing experiments. Additionally, enChIP can be used to study *cis*- and *trans*- chromosomal interactions. Evaluation of the immunoprecipitated DNA sequences may reveal chromosomal looping events.

References

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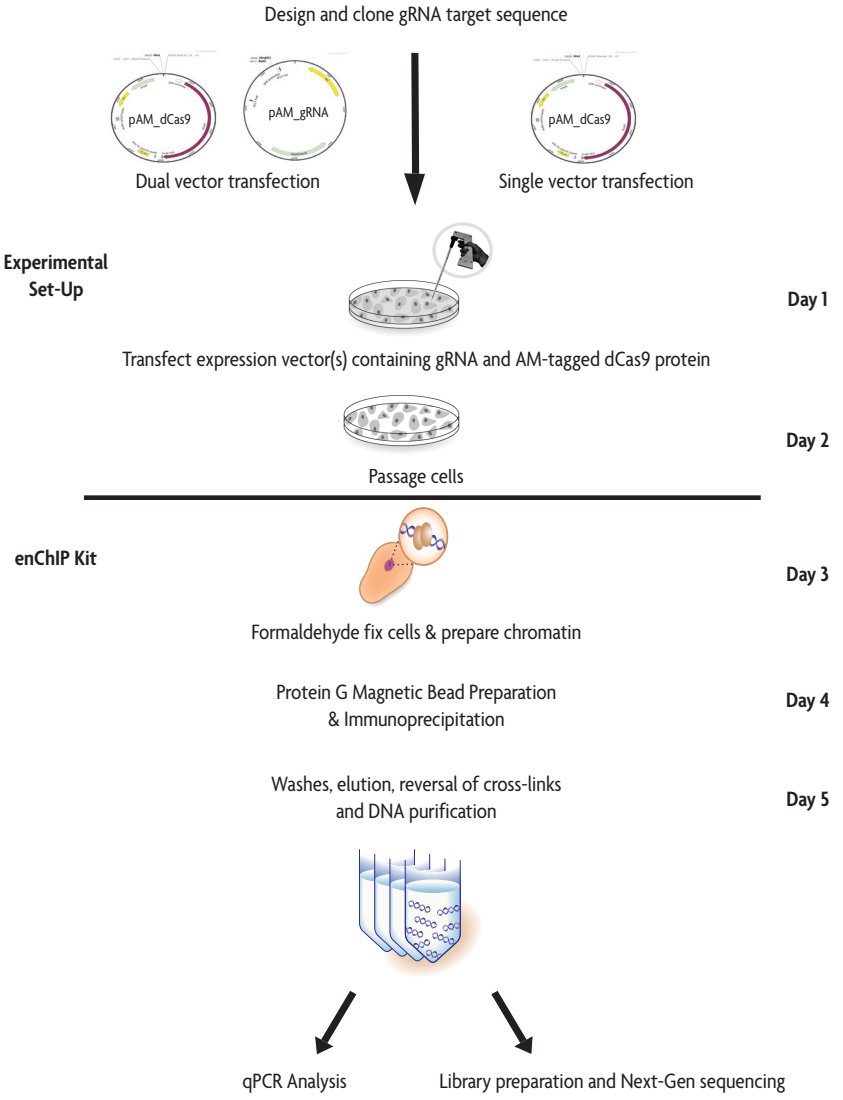
Flow Chart of Process



Flow Chart of the enChIP method.

Prior to using the enChIP Kit (Catalog No. 53125), a guide RNA (gRNA) target sequence is selected to target a specific genomic region. The gRNA sequence is then cloned into either the pAM_gRNA expression vector for dual vector transfections, or cloned into the pAM_dCas9 expression vector for single vector transfection. 24 hours post-transfection of the gRNA and AM-tagged dCas9 expression vectors, cells are passaged. Another 24 hours later cells are formaldehyde fixed and chromatin is prepared. Protein G magnetic beads are blocked and the chromatin is pre-cleared. An overnight immunoprecipitation using the AM-Tag monoclonal antibody captures the gRNA/Cas9 genomic complexes. Following washes, the DNA is eluted from the beads, the cross-links are reversed and the enriched DNA is purified. Recovered DNA can be analyzed by qPCR or Next-Generation sequencing.

Kit Performance and Benefits

enChIP Kit Applications:

- Identify off-target binding events of the gRNA
- Evaluate cis- and trans-interacting chromosomal looping
- Works well with open chromatin regions to detect promoter, enhancer and insulator elements. enChIP is not optimized for use on repressive regions.

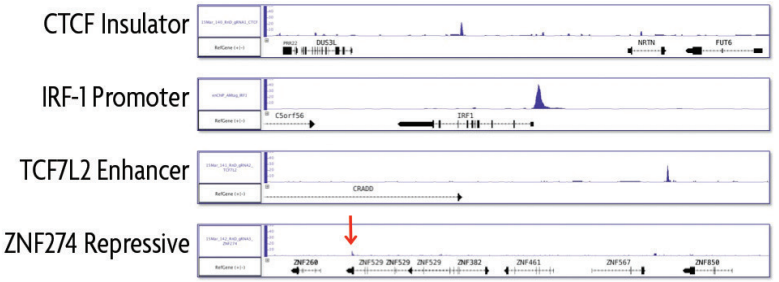


Figure 1: Comparison of enChIP performance to evaluate different genomic loci.

To evaluate different genomic loci, gRNAs were designed to target known insulator, promoter, enhancer and repressive regions of the genome. Experiments were run according to the instructions in the enChIP Kit. ChIP-seq results show highly specific enrichment of the insulator, promoter and enhancer regions. For the repressive genomic region, only a small ChIP-seq peak was observed indicating that the enChIP method is not optimized for repressive regions.

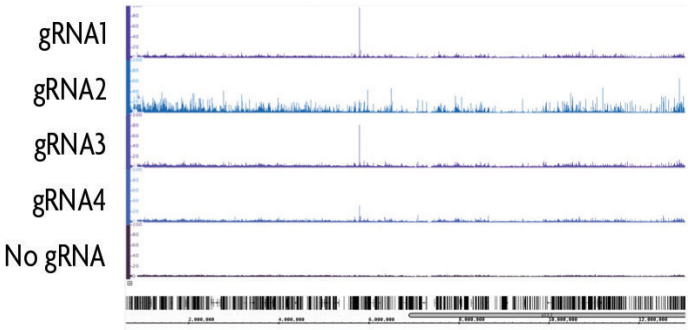


Figure 2: Determining gRNA target sequence specificity.

Four different gRNAs were designed using the CRISPRdirect program to target a 200 bp region surrounding a CTCF binding site on chromosome 19. Following transfection of the gRNA and dCas9 into HEK293T cells, chromatin was prepared and immunoprecipitated according to the instructions in the enChIP Kit. enChIP-seq results show the differences in gRNA specificity. gRNA 2 has a high degree of background as compared to gRNA 4 despite having only a single bp difference in the target sequence. This confirms the value of performing enChIP to identify off-target binding events of the gRNA prior to use in genome engineering experiments.

Kit Components and Storage

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

Reagents	Quantity	Storage
AM-Tag monoclonal Ab (1 mg/ml)	80 µg	-20°C
RNase A (10 µg/µl)	40 µl	-20°C
Proteinase K (10 µg/µl)	80 µl	-20°C
100 mM PMSF	500 µl	-20°C
Protease Inhibitor Cocktail (PIC)	500 µl	-20°C
Precipitation Buffer	1.5 ml	-20°C
Carrier	35 µl	-20°C
10X PBS	120 ml	-20°C
Anti-mouse IgG (1 mg/ml)	60 µg	4°C
Fixation Buffer	2 x 1.5 ml	4°C
Protein G Magnetic Beads*	1.5 ml	4°C
Detergent	25 ml	RT
Stop Solution	20 ml	RT
Chromatin Prep Buffer	85 ml	RT
ChIP Buffer	35 ml	RT
Equilibration Buffer	2 x 120 ml	RT
Blocking Buffer AM4	15 ml	RT
LS Buffer	35 ml	RT
Wash Buffer AM1	100 ml	RT
LiCl Buffer	35 ml	RT
Elution Buffer AM4	2 x 1.5 ml	RT
TE	50 ml	RT
DNA Purification Binding Buffer	50 ml	RT
3 M Sodium Acetate	500 µl	RT
DNA Purification Wash Buffer**	10 ml	RT
DNA Purification Elution Buffer	5 ml	RT
DNA Purification Columns	16 ea	RT

* The Protein G Magnetic 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 Magnetic Beads should be stored at 4°C.

**Requires the addition of ethanol before use.

Additional materials required for enChIP Kit

- Transfected cultured cells containing a sequence verified gRNA target in combination with the pAM_dCas9 expression vector and a no gRNA control transfection. Refer to Experimental Set-Up on pages 8-13 to design, clone and transfect cells.
- Dounce homogenizer with a small clearance pestle (*e.g.* Active Motif Catalog Nos. 40401 & 40415) with the tight-fitting "A" pestle). Use of a homogenizer is necessary for shearing chromatin.
- 37% formaldehyde solution with 10-15% methyl alcohol to prevent polymerization (*e.g.* Sigma Aldrich Catalog No. 252549). Do not use paraformaldehyde.
- 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))
- Pipettors and tips (filter tips are recommended)
- Rocking platform for culture plates
- Cell scraper (rubber policeman)
- 15 and 50 ml conical tubes
- 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
- 0.2 ml PCR tubes
- Thermocycler
- Spectrophotometer for DNA quantitation
- Agarose gel electrophoresis apparatus
- (Optional) ChIP-IT® qPCR Analysis Kit (Catalog No. 53029)
- (Optional) Gene-specific qPCR primer pairs for enrichment analysis
- (Optional) SYBR Green qPCR master mix (Bio-Rad Catalog No. 170-8882)

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Design of gRNA

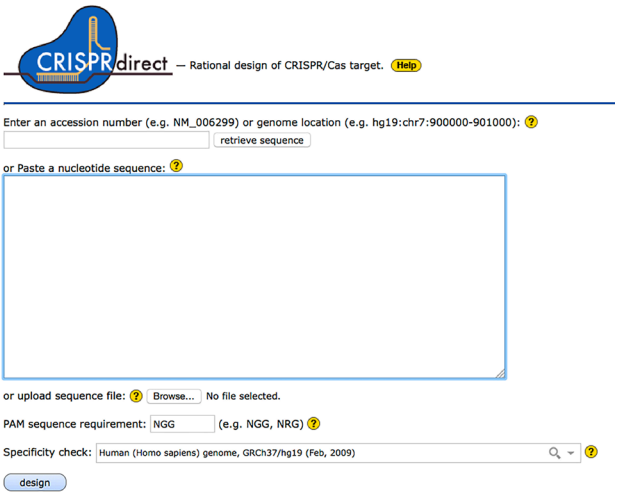
In the enChIP Kit, a guide RNA (gRNA) is designed to target a specific genomic locus. Online software programs are available to assist in the design of a 23 bp gRNA in the form of 5´-N_{20}NGG-3´, where the NGG represents the Protospacer Adjacent Motif (PAM) sequence immediately following the target sequence that is required for successful binding of Cas9 protein. The identified gRNA sites may reside on the + or - strand. We recommend designing multiple gRNAs to a specific locus and then experimentally testing each gRNA for specificity using the enChIP Kit. If possible, try to avoid target sequences that overlap with known recognition sites for DNA-binding proteins as this may block access of the gRNA to its target location.

1. Determine the genomic region of interest for gRNA targeting. Retrieve the DNA sequence of approximately 500 bp surrounding this target locus. This sequence will be analyzed using online software programs and gRNA selection tools to identify the best gRNA sequences.
2. There are several online programs available to assist with gRNA design. A few are listed below. Select a program and follow the software provider's instructions for use.

   CRISPRdirect - http://crispr.dbcls.jp

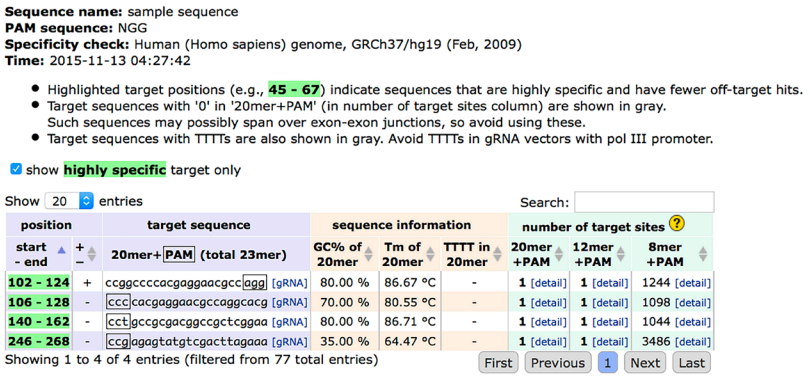
   Michael Boutros Lab - http://www.e-crisp.org/E-CRISP/designcrispr.html

   Feng Zhang Lab - http://crispr.mit.edu
3. Below is an example of gRNA design using CRISPRdirect. Software instructions may change over time, so please follow the recommendations of the software provider for use.
   a. Paste the genomic coordinates or genomic sequence from Step 1 above into the CRISPRdirect site.



b. Check the box for "show highly-specific targets only".

c. The output box will show potential gRNA sequencing for the genomic target.



Sequence name: sample sequence
PAM sequence: NGG
Specificity check: Human (Homo sapiens) genome, GRCh37/hg19 (Feb, 2009)
Time: 2015-11-13 04:27:42

- Highlighted target positions (e.g., **45 - 67**) indicate sequences that are highly specific and have fewer off-target hits.
- Target sequences with '0' in '20mer+PAM' (in number of target sites column) are shown in gray. Such sequences may possibly span over exon-exon junctions, so avoid using these.
- Target sequences with TTTTs are also shown in gray. Avoid TTTTs in gRNA vectors with pol III promoter.

☑ show **highly specific** target only

Show 20 entries Search:

position		target sequence	sequence information			number of target sites		
start - end	+ –	20mer+PAM (total 23mer)	GC% of 20mer	Tm of 20mer	TTTT in 20mer	20mer +PAM	12mer +PAM	8mer +PAM
102 - 124	+	ccggccccacgaggaacgccagg [gRNA]	80.00 %	86.67 °C	-	1 [detail]	1 [detail]	1244 [detail]
106 - 128	-	cccgcacgaggaacgccaggcacg [gRNA]	70.00 %	80.55 °C	-	1 [detail]	1 [detail]	1098 [detail]
140 - 162	-	cctgccgcgacggccgctcggaa [gRNA]	80.00 %	86.71 °C	-	1 [detail]	1 [detail]	1044 [detail]
246 - 268	-	ccgagagtatgtcgacttagaaa [gRNA]	35.00 %	64.47 °C	-	1 [detail]	1 [detail]	3486 [detail]

Showing 1 to 4 of 4 entries (filtered from 77 total entries) First Previous 1 Next Last

d. Click the "gRNA" link to the right of the sequence and copy the **20 bp non-PAM** sequence.

e. Select the transfection method (dual vector or single vector) desired to perform your experiment. Based on this decision, paste the 20 bp non-PAM sequence from step 3d above into the correct gRNA insert template. gRNA templates can be found on page 10 and downloadable versions are available online at www.activemotif.com/enchip. Please note that each cloning vector has unique overlapping ends and gRNA inserts are not interchangeable between the different vectors.

Dual Vector Transfection: The gRNA is cloned into a vector designed specifically to express the gRNA. The dCas9 protein is expressed in a separate expression vector. This option requires co-transfection of two vectors (gRNA vector + dCas9 vector) into your cell line of interest. Perform cloning using the pAM_gRNA vector (Catalog No. 53121).

Single Vector Transfection: The gRNA sequence of interest is cloned directly into the dCas9 expression vector. Both the gRNA and the dCas9 protein will be expressed from the single vector. This option requires only a single transfection, but the vector size is extremely large (>10 kb) and may prove challenging to transfect in some cell lines. Perform cloning using the pAM_dCas9 vector (Catalog No. 53122).

Using existing gRNA vectors: For researchers who already have their gRNA of interest cloned into an expression vector, the dual vector transfection system should be used. This involves co-transfection of the researchers' gRNA expression vector with Active Motif's pAM_dCas9 vector (Catalog No. 53122). No additional cloning is required. Proceed to the next section for Transfection of gRNA and dCas9 expression plasmids.

Dual Vector Transfection – Perform cloning with pAM_gRNA vector (Cat. No. 53121)

*GGCGGCCGTTACTAG*TGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCG
ACTGGATCCGGTACCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTT
CATATTTGCATATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGAC
TGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCT
TGGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACC
GTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAA
ACACCGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTA
AAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCT
TTTTTTT*AGCTTGATGCATAGC*

Green:	20 bp of the selected target sequence
Yellow:	U6 promoter
Blue:	guide RNA scaffold
Red:	termination signal
Copper:	Overlapping ends for subcloning into pAM_gRNA expression vector

Single Vector Transfection – Perform cloning with pAM_dCas9 vector (Cat. No. 53122)

*GCTTGACCGACAATT*TGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGA
CTGGATCCGGTACCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTC
ATATTTGCATATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACT
GTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTT
GGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCG
TAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAA
CACCGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAA
AATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTT
TTTTTT*AATTGCATGAAGAAT*

Green:	20 bp of the selected target sequence
Yellow:	U6 promoter
Blue:	guide RNA scaffold
Red:	termination signal
Copper:	Overlapping ends for subcloning into pAM_dCas9 expression vector

Figure 3: gRNA insert templates should be used for the design of gBlocks® Gene Fragments for cloning into the appropriate expression vector for either single or dual vector transfections. Color images for the gRNA insert templates can be found online at www.activemotif.com/enchip.

4. Synthesize gBlocks® Gene Fragments (Integrated DNA Technologies, https://www.idtdna.com/site/order/gblockentry) representing the entire 453 bp sequence to be cloned into the gRNA expression plasmid. The gBlocks sequence should contain the overlapping ends needed for subcloning into the expression vector of choice, the 20 bp target sequence, the guide RNA scaffold and the termination signal.
5. Linearize the appropriate expression plasmid using restriction enzyme digestion.
   a. **pAM_gRNA vector:** Linearize the pAM_gRNA vector with a double digest using restriction enzymes SpeI and HindIII. Gel purify the 3891 bp digested backbone.
   b. **pAM_dCas9 vector:** Linearize the pAM_dCas9 vector with a single digest using restriction enzyme MfeI.

6. Clone each gBlocks gRNA insert into Active Motif's expression plasmid using directional cloning methods such as InFusion® (Clontech) or Gibson Assembly® (New England Biolabs). Follow the manufacturer's recommendations for cloning.

    a. For InFusion cloning, we recommend a 1:3 molar ratio of vector : insert. For example:
    Use 150 ng pAM_gRNA vector with 50 ng gRNA insert.
    Use 150 ng pAM_dCas9 vector with 22 ng gRNA insert.

7. Perform transformations of each gRNA construct into competent *E.coli*. We recommend the use of Active Motif's RapidTrans TAM1 Competent cells. (Catalog No. 11096). Plate transformation reactions onto LB agar plates containing the appropriate antibiotic selection for the cloning vector used. Grow overnight at 37°C.

    pAM_gRNA vector: Kanamycin selection is required
    pAM_dCas9 vector: Ampicillin selection is recommended

8. Select 2-4 colonies for each gRNA construct and isolate the plasmid DNA. The DNA should be free of nucleases or other contaminants prior to transfection. Sequence validate each plasmid to confirm the correct sequence. We suggest preparing a glycerol stock for each candidate construct.

9. Proceed to the next section for Transfection of gRNA and dCas9 expression plasmids.

Transfection of gRNA and dCas9 expression plasmids

Following sequence validation of the expression constructs, cells can be transiently transfected with the gRNA and pAM_dCas9 expression plasmids. The following protocol provides recommendations for transfection using FuGENE® HD Transfection Reagent (Catalog No. 32042). Optimization may be required for each cell line and expression construct tested.

To determine the efficiency of the transfection, set up a duplicate transfection for each cell type used. Cell lysates can be prepared from the duplicate transfection reaction using Active Motif's Nuclear Extract Kit (Catalog No. 40010) for analysis by Western blot. Use the AM-Tag polyclonal antibody (Catalog No. 61677) at a 1:250 - 1:1,000 dilution for detection of the AM-tagged dCas9 protein. If the tagged protein is not detected, continue to optimize transfection conditions.

Calculate the number of transfections you will perform with each gRNA and cell line. Small scale reactions provide enough chromatin to perform one enChIP reaction. Large scale reactions provide enough chromatin to perform 5 enChIP reactions. We recommend running a no gRNA negative control reaction for each cell line tested. Active Motif also offers positive control vectors for both dual or single transfection. The positive control gRNA targets a locus corresponding to a CTCF binding site on chromosome 19 (5,804,115 – 5,804,290). These vectors are available as pAM_gRNA_CTCF vector (Catalog No. 53123) and pAM_dCas9_CTCF vector (Catalog No. 53124).

1. Seed cells in either a 6-well plate (small scale) or 10cm dish (large scale) using the appropriate growth medium. Incubate in a humidified incubator for 24 hours. Cells should be 70-80% confluent at the time of transfection.

	Small Scale Cell Culture (1 enChIP rxn)		Large Scale Cell Culture (5 enChIP rxns)	
	Single Vector	Dual Vector	Single Vector	Dual Vector
Cell culture plate	6-well plate	6-well plate	10 cm dish	10 cm dish
Cell seeding density*	5 x 10^5 cells	5 x 10^5 cells	3 x 10^6 cells	3 x 10^6 cells
Growth medium	2 ml	2 ml	10 ml	10 ml

*These conditions were established for cells with doubling times of 14-18 hours. Cell seeding densities may need to be optimized to ensure that cells are ~80% confluent at the time of transfection.

2. Prepare a separate microcentrifuge tube for each transfection reaction. To each tube add the recommended amount of DNA and Opti-MEM according to the table below.

		Small Scale Cell Culture		Large Scale Cell Culture	
		Single Vector	Dual Vector	Single Vector	Dual Vector
Test gRNA or Positive control CTCF gRNA	**gRNA vector**	–	1 µg	–	2 µg
	dCas9 vector	–	1 µg	–	2 µg
	(dCas9 + gRNA) vector	1 µg	–	2 µg	–
	Opti-MEM	Up to 275 µl	Up to 275 µl	Up to 550 µl	Up to 550 µl
No gRNA negative control	**gRNA vector**	–	–	–	–
	dCas9 vector	1 µg	1 µg	2 µg	2 µg
	(dCas9 + gRNA) vector	–	–	–	–
	Opti-MEM	Up to 275 µl	Up to 275 µl	Up to 550 µl	Up to 550 µl

3. Add FuGENE HD Transfection reagent drop wise directly to the DNA/media mixture. Do not allow FuGENE to come directly in contact with the plastic from the tube. Mix the solution by pipetting up and down and incubate at room temperature for 30 minutes.

   Small scale cell culture: Add 6 µl FuGENE to each transfection reaction.
   Large scale cell culture: Add 12 µl FuGENE to each transfection reaction.

4. Add the entire DNA/media/FuGENE mixture drop wise to each cell culture plate. Incubate on a shaking platform at 100 rpm for 2 minutes to evenly distribute the transfection mixture.

5. Return plate to humidified incubator for 24 hours.

6. 24 hours post-transfection, passage cells to a larger dish. Add additional complete growth medium to the cells.

   Small scale cell culture: Transfer cells in each well of a 6-well plate to a 10 cm dish. Culture cells in 10 ml complete growth medium.

Large scale cell culture: Transfer cells from a 10 cm dish to a 15 cm dish. Culture cells in 20 ml complete growth medium.

7. Return plate to humidified incubator for 24 hours.
8. Transfected cells are now ready to be processed for chromatin fixation. Alternatively, if duplicate reactions were performed cell lysates can be prepared using Active Motif's Nuclear Extraction Kit (Catalog No. 40010) for Western blot analysis of transfection efficiency using the AM-Tag polyclonal antibody (Catalog No. 61677) to recognize the tagged dCas9 protein.

Buffer Preparation & Recommendations

Prepare the following buffers for use in chromatin preparation and immunoprecipitation reactions. All other buffers and reagents are supplied ready for use.

Complete Cell Fixation Solution

Buffer should be prepared fresh before each experiment. For every 20 ml of cell growth medium used, prepare 2.5 ml of Complete Cell Fixation Solution by adding 180 µl Fixation Buffer to 1.57 ml sterile water in a 15 ml conical tube. Using appropriate precautions (*i.e.* safety glasses, gloves, lab coat and working in a ventilated hood), add 750 µl 37% formaldehyde to the tube and vortex to mix. Use 1/10 growth medium volume per plate. Complete cell fixation solution can be added to the growth medium in the presence or absence of serum.

Stop Solution

Is provided ready to use. Use 1/20 media volume per cell culture plate.

PBS Wash Buffer

Prepare 25 ml PBS Wash Buffer for every 15 cm plate. To a 50 ml conical tube add 21.25 ml sterile water, 2.5 ml 10X PBS and 1.25 ml Detergent. Mix by inverting. Place PBS Wash Buffer on ice to chill. PBS Wash Buffer can be prepared in large quantities and stored at 4°C for 6 months.

100 mM PMSF and Protease Inhibitor Cocktail (PIC)

Thaw the PMSF and the PIC at room temperature until fully dissolved, which takes about 30 minutes. Vortex gently and spin down briefly before use, then add to the buffers immediately before use. Use appropriate safety precautions (*i.e.* safety glasses, gloves and lab coat) when working with PMSF as it is highly toxic.

Protein G Magnetic Beads

The supplied magnetic beads require processing prior to use. Follow the instructions in the manual to prepare the beads for use in the enChIP reactions. For best results, gently shake and invert the tube to resuspend the magnetic beads. The beads settle quickly, and therefore should be resuspended just before pipetting. **Protein G Magnetic 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 Magnetic Beads should be stored at 4°C.**

ChIP Buffer, LS Buffer, Wash Buffer AM1, LiCl Buffer and TE

These buffers are provided at room temperature. We recommend using chilled buffers for the immunoprecipitation set-up and wash steps. Place these buffers on ice prior to use.

AM-Tag Monoclonal Antibody

We recommend using 5 µg AM-Tag monoclonal antibody per enChIP reaction. The AM-Tag monoclonal antibody is provided at a concentration of 1 µg/µl, so you will need to use 5 µl per enChIP.

Anti-Mouse IgG

The anti-mouse IgG is provided at a concentration of 1 µg/µl, so you will need to use 3.5 µl per chromatin pre-clearing reaction.

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 enChIP Kit. Indirect sonication systems may require longer sonication times to achieve optimal chromatin shearing. enChIP experiments usually require chromatin that has been sheared to a size of 200-2000 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. Then, resuspend any pelleted material and transfer chromatin to a new tube before continuing sonication. 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.

DNA Purification Wash Buffer

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

3M Sodium Acetate

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

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Section A: Cell Fixation of Cultured Cells

This protocol describes cell fixation and chromatin preparation using the transfected cell lines.

	10 cm dish	15 cm dish
Cell Fixative Solution	1 ml	2 ml
Stop Solution	550 µl	1.1 ml
PBS Wash Buffer	2 x 5 ml	2 x 10 ml
Chromatin Prep Buffer	5 ml	5 ml
ChIP Buffer	Up to 500 µl	Up to 1 ml

1. Prepare Complete Cell Fixation Buffer as described on page 13. To fix cells, add 1/10 growth medium volume of freshly prepared Complete Cell Fixative Solution to the existing culture media for the cells (*e.g.* 20 ml growth medium would get 2 ml Complete Cell Fixation Solution). The cell fixation solution can be added to the growth medium in the presence or absence of serum.
2. Shake gently at room temperature for 10 minutes.
3. Stop the fixation reaction by adding 1/20 media volume of Stop Solution to the existing culture media for the cells (*e.g.* 20 ml growth medium would get 1.1 ml Stop Solution).
4. Swirl to mix and incubate at room temperature for 5 minutes.
5. Following the incubation, hold the plate at an angle and using a rubber policeman scrape cells down to collect them at the bottom edge of the plate. Use a pipette to transfer the cells to a 50 ml conical tube on ice.
6. Pellet the cells from step 5 by centrifugation for 3 minutes at 1,250 x g at 4°C.
7. Remove the supernatant and discard. Resuspend the pellet(s) in 5-10 ml ice-cold PBS Wash Buffer by pipetting up and down. Keep samples ice-cold for the remainder of the procedure.
8. Centrifuge for 3 minutes at 1,250 x g at 4°C. Remove the supernatant and discard. Wash the pellet(s) a second time in 5-10 ml ice-cold PBS Wash Buffer by pipetting up and down. Centrifuge for 3 minutes at 1,250 x g at 4°C. Remove the supernatant and discard. (Cell pellets may be stored at -80°C at this stage).
9. Resuspend each pellet(s) in 5 ml Chromatin Prep Buffer supplemented with 5 µl PIC and 5 µl 100 mM PMSF. Pipet up and down to mix.
10. Incubate on ice for 10 minutes.
11. Transfer the resuspended pellets individually to a chilled dounce homogenizer on ice. Use the tight fitting pestle (Type A) to homogenize the sample for 30 strokes. Transfer the contents to a new 15 ml conical tube and centrifuge for 3 minutes at 1,250 x g at 4°C.

    Monitor Cell Lysis: To ensure cell lysis, take 10 µl of the cell lysate from the dounce and look at it under a phase contrast microscope using a hemocytometer to verify that the nuclei

have been released. It is often helpful to look at the cells before and after the lysis step as this makes it easier to identify the nuclei versus whole cells. Intact cells should have a dark central region (nucleus) surrounded by a halo of less dense cytoplasm. In lysed cells, the nuclei will appear as dots surrounded by asymmetric debris. If the cells are not lysed, then dounce on ice with an additional 10 strokes, or until the cells are lysed.

12. Prepare Complete ChIP Buffer by adding 10 µl PIC and 10 µl PMSF per 1 ml ChIP Buffer.
13. Remove the supernatant and discard. Resuspend each nuclear pellet in Complete ChIP Buffer.
    Small scale cell culture: Resuspend each pellet to reach a final volume of 500 µl.
    Large scale cell culture: Resuspend each pellet to reach a final volume of 1 ml
14. Transfer the contents to a new 2 ml microcentrifuge tube.
15. Incubate on ice for 10 minutes. Proceed to Step B: Chromatin Sonication of Cultured Cells.

Section B. Chromatin Sonication of Cultured Cells

The section below describes the fragmentation of chromatin using sonication. Sonication results may vary depending on cell type and sonication device being used. This protocol has been validated using Active Motif's EpiShear™ Probe Sonicator in combination with an EpiShear™ Cooled Sonication Platform to maintain probe height and temperature consistency between samples.

The ChIP Buffer has been optimized for immunoprecipitation performance, however, due to its unique composition optimization of sonication conditions may be required. To maintain the high sensitivity of the assay, we recommend using our buffer system and altering the sonication time and/or amplitude of your sonication system to achieve the desired fragmentation (*e.g.* some systems may require as much as a three-fold increase in sonication time to improve chromatin shearing). We recommend checking shearing efficiency prior to performing the immunoprecipitation reactions. Input DNA can be prepared and run on an agarose gel to confirm shearing efficiency. The chromatin should appear as a smear between 200-2000 bp.

1. Place the 2 ml microcentrifuge tube containing the chromatin into the tube cooler or packed ice. Open cap and submerge the microtip into the liquid until the microtip is approximately 5 mm from the bottom of the tube. Sonicate according to optimized settings for the cell type being used (see Recommendations on page 14). Suggestions for sonication conditions are provided below based on the chromatin volume:

   For 500 µl chromatin volumes: 42% amplitude, pulse for 30 seconds on and 30 seconds off for a total sonication "on" time of 10 minutes (or 20 minutes elapsed time).
   For 1 ml chromatin volumes: 61% amplitude, pulse for 30 seconds on and 30 seconds off for a total sonication "on" time of 10 minutes (or 20 minutes elapsed time).
2. Spin tubes at 4°C in a microcentrifuge at maximum speed for 2 minutes to pellet the cellular debris.
3. Transfer 25 µl of each chromatin preparation into a 250 µl PCR tube for analysis of shearing efficiency and chromatin quantification. This is the Input DNA.

4. The remaining chromatin should be stored at -80°C.
   For 500 µl chromatin volumes: The entire amount will be used in the enChIP reaction. There is no need to aliquot.
   For 1 ml chromatin volumes: Use 200 µl per enChIP reaction. Prepare 200 µl aliquots into 1.5 ml microcentrifuge tubes.

Input Preparation

5. To each 25 µl chromatin preparation from Step 3 above, add 175 µl TE and 1 µl RNAse A. Cap the PCR tubes and vortex to mix
6. Incubate in a thermocycler at 37°C for 30 minutes.
7. Add 2 µl Proteinase K to each tube and vortex. Incubate tubes in a thermocycler at 55°C for 30 minutes and then increase the temperature to 80°C for 2 hours.
8. Transfer each chromatin input to a 1.5 ml microcentrifuge tube. Add 83 µl Precipitation Buffer, 2 µl Carrier and 750 µl absolute ethanol. Vortex to mix and chill at -80°C for 30 minutes to overnight.
9. Spin tubes at 4°C in a microcentrifuge at maximum speed for 15 minutes.
10. Carefully remove the supernatant taking care not to disturb the pellet. Wash the pellet with 500 µl 70% ethanol and spin at 4°C in a microcentrifuge at maximum speed for 5 minutes.
11. Carefully remove the supernatant taking care not to disturb the pellet. Remove residual ethanol with a pipet tip. Leave the tubes uncapped and air dry for 10-15 minutes.
12. When the pellets are dry, add 25 µl DNA Purification Elution Buffer to each tube. Incubate at room temperature for 10 minutes. Then vortex to ensure the pellet is completely resuspended. This solution contains your Input DNA.
13. Read the absorbance of each sample on a NanoDrop or other spectrophotometer at 260 nm to determine the DNA concentration of each chromatin preparation. Set aside 500 ng of DNA for analysis as described in Step 14. Store the remaining Input DNA at -20°C.
14. Analyze each chromatin preparation on an agarose gel. Add gel loading buffer to each sample and run on a 1.5% agarose gel. Include 100 bp and 1 kb DNA ladders to analyze chromatin size. DNA should appear as a smear anywhere between 200-2000 bp.
15. If chromatin preparations were successful, the aliquots stored at -80°C from Section B, Step 4 can be used to perform enChIP.

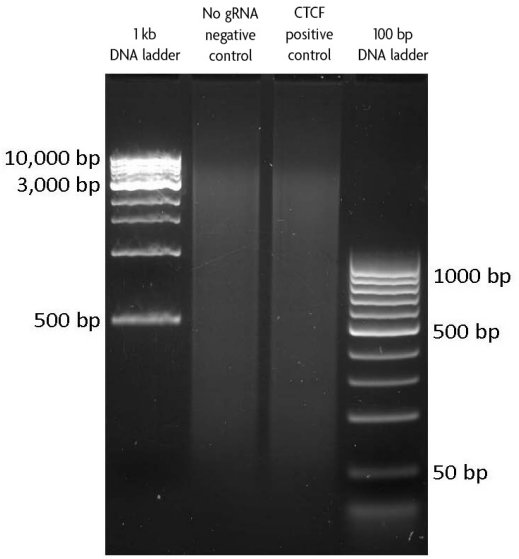


Figure 4: Validation of chromatin shearing efficiency.

Chromatin preparations of HEK293T cells transfected with either pAM_dCas9 alone (no gRNA negative control) or a dual transfection of the pAM_gRNA_CTCF vector with the pAM_dCas9 vector (CTCF positive control) were run on a 1.5% agarose gel. The enChIP Kit manual was used to formaldehyde fix and extract chromatin from each transfected cell line. Chromatin was then fragmented using Active Motif's EpiShear™ Probe Sonicator and EpiShear™ Cooled Sonication Platform to generate the desired fragmentation range of 200-2000 bp. A portion of the chromatin was used for reversal of cross-links and DNA purification. Following quantification, 500 ng of each chromatin sample was run on the agarose gel to confirm chromatin shearing efficiency. If the protocol steps were followed and the DNA fragments fall outside of the recommended range, sonication conditions should be further optimized.

Section C. Protein G Magnetic Bead Preparation

This section describes the processing of the Protein G Magnetic beads prior to use in enChIP.

1. In 1.5 ml microcentrifuge tubes prepare the Protein G magnetic beads. Set up separate tubes for each IP reaction and prepare a bulk reaction for pre-clearing. If more than 4 IP reactions will be performed, we suggest setting up multiple pre-clearing reactions 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 (no gRNA) and positive control IPs into the calculation for the number of IP reactions.

Reagent	IP Reactions	Pre-clearing Rxn
Protein G Magnetic Beads	50 µl per tube	35 µl x _____ IPs*

* We do not recommend pre-clearing more than 4 IP reactions in a single tube. If more than 4 IPs are to be performed in a single experiment, set up multiple tubes for pre-clearing.

2. Place the tubes on a magnetic stand to pellet beads on the tube side. Wait 2 minutes and carefully remove and discard supernatant.
3. Remove tubes from the magnetic stand. Add 1 ml Equilibration Buffer AM2 to each tube. Invert the tubes to mix. Quick spin to collect contents to the bottom of the tube.
4. Place the tubes on a magnetic stand to pellet beads on the tube side. Wait 2 minutes and carefully remove and discard supernatant.
5. Repeat steps 3-4.
6. Set up antibody pre-binding reactions with Protein G magnetic beads according to the table below. For IP reactions, add 5 µg AM-Tag monoclonal antibody to each tube. For pre-clearing bulk reactions, add 3.5 µg anti-mouse IgG per IP.

Reagent	IP Reactions	Pre-clearing Rxn
Blocking Buffer AM4	500 µl per tube	300 µl x _____ IPs
AM-Tag monoclonal antibody	5 µg per tube	–
Anti-mouse IgG	–	3.5 µg x _____ IPs
Incubate with rotation at 4 ° C	3 hours	2 hours

7. Cap tubes and incubate on an end-to-end rotator at 4°C for 2-3 hours. Follow the recommendations in the chart above for the incubation time.
8. Remove the **pre-clearing reactions** from the rotator and quick spin to collect contents to the bottom of the tube. Allow the IP reactions to continue to incubate on the rotator.
9. Place the pre-clearing tube(s) on a magnetic stand to pellet beads on the tube side. Wait 2

minutes and carefully remove and discard supernatant

10. Remove tubes from the magnetic stand. Add 1 ml Equilibration Buffer AM2 to each tube. Invert the tubes to mix. Quick spin to collect contents to the bottom of the tube.
11. Place the tubes on a magnetic stand to pellet beads on the tube side. Wait 2 minutes and carefully remove and discard supernatant.
12. Repeat steps 10-11.
13. Resuspend the beads with 35 µl x _____ IPs of Equilibration Buffer AM2. Pipette up and down to completely resuspend the beads into a uniform solution. If multiple pre-clearing reactions were used, add the Equilibration Buffer AM2 to each tube. Then, combine the pre-clearing reactions into a single tube. Pipette up and down to completely resuspend the beads into a uniform solution.

Section D. Immunoprecipitation

1. If working with large-scale transfections, place ChIP Buffer on ice to chill.
2. Prepare labeled microcentrifuge tubes corresponding to each IP reaction to be performed. Mark each tube as "pre-cleared". Pipette bead solution up and down to ensure the beads have not settled. Dispense 30 µl of the resuspended pre-cleared bead solution per tube.
3. Thaw chromatin on ice. Add chromatin to its corresponding labeled pre-cleared bead tube:

   For small scale transfections: Add 500 µl chromatin.

   For large scale transfections: Add 200 µl chromatin and 300 µl ice-cold ChIP Buffer supplemented with 3 µl PIC and 3 µl PMSF.
4. Return tubes to the rotator and incubate at 4°C for 1 hour.
5. Approximately 10 minutes before the end of the pre-cleared incubation, remove the **IP reactions** from the rotator and quick spin to collect contents to the bottom of the tube.
6. Place the IP tubes on a magnetic stand to pellet beads on the tube side. Wait 2 minutes and carefully remove and discard supernatant
7. Remove tubes from the magnetic stand. Add 1 ml Equilibration Buffer AM2 to each tube. Invert the tubes to mix. Quick spin to collect contents to the bottom of the tube.
8. Place the tubes on a magnetic stand to pellet beads on the tube side. Wait 2 minutes and carefully remove and discard supernatant.
9. Repeat steps 6-7.
10. Place washed IP beads on ice.
11. Following the 1 hour incubation of the pre-cleared beads with the chromatin, remove the tubes from the rotator and quick spin to collect contents to the bottom of the tube.
12. Place the **pre-cleared** reactions on a magnetic stand for 2 minutes to pellet beads on the tube side.

13. Carefully transfer the supernatant from the pre-cleared beads with the chromatin to the corresponding IP reactions containing the AM-tag antibody-conjugated beads on ice.
14. Return the reactions to an end-to-end rotator and incubate overnight at 4°C.

Section E. Washing of IP reactions

We recommend using chilled buffers for the IP wash steps. Place buffers on ice prior to use.

1. Remove the IP reactions from the rotator and quick spin to collect contents to the bottom of the tube.
2. Place the tubes on a magnetic stand to pellet beads on the tube side. Wait 2 minutes and carefully remove and discard supernatant.
3. Remove tubes from the magnetic stand. Add 1 ml ice-cold **LS Buffer** to each tube and invert to mix.
4. Incubate on an end-to-end rotator at 4°C for 10 minutes. Remove tubes from the rotator and quick spin to collect contents to the bottom of the tube.
5. Place the tubes on a magnetic stand to pellet beads on the tube side. Wait 2 minutes and carefully remove and discard supernatant.
6. Repeat steps 3-5.
7. Remove tubes from the magnetic stand. Add 1 ml ice-cold **Wash Buffer AM1** to each tube and invert to mix.
8. Incubate on an end-to-end rotator at 4°C for 10 minutes. Remove tubes from the rotator and quick spin to collect contents to the bottom of the tube.
9. Place the tubes on a magnetic stand to pellet beads on the tube side. Wait 2 minutes and carefully remove and discard supernatant.
10. Repeat steps 7-9.
11. Remove tubes from the magnetic stand. Add 1 ml ice-cold **LiCl Buffer** to each tube and invert to mix.
12. Incubate on an end-to-end rotator at 4°C for 10 minutes. Remove tubes from the rotator and quick spin to collect contents to the bottom of the tube.
13. Place the tubes on a magnetic stand to pellet beads on the tube side. Wait 2 minutes and carefully remove and discard supernatant.
14. Repeat steps 11-13.
15. Remove tubes from the magnetic stand. Add 1 ml ice-cold **TE** to each tube and invert to mix.
16. Incubate on an end-to-end rotator at 4°C for 10 minutes. Remove tubes from the rotator and quick spin to collect contents to the bottom of the tube.
17. Place the tubes on a magnetic stand to pellet beads on the tube side. Wait 2 minutes and carefully remove and discard supernatant.

18. Repeat steps 15-17.

Section F. Elution, Reversal of Cross-links and DNA Purification

1. Remove tubes from the magnetic stand. Resuspend each IP in 100 µl Elution Buffer AM4. Pipette up and down to mix.
2. Briefly vortex samples and incubate at room temperature for 15 minutes without rotation.
3. Briefly vortex samples and quick spin to collect contents to the bottom of the tube. Place the tubes on a magnetic stand for 2 minutes to pellet beads on the tube side.
4. Transfer supernatant to 0.2 ml PCR tubes.
5. Add 2 µl Proteinase K. Vortex to mix. Heat samples in a thermocycler at 55°C for 30 minutes. Then increase the temperature to 80°C for 2 hours.
6. Transfer the DNA to a 1.5 ml microcentrifuge tube and add 5 volumes (500 µl) DNA Purification Binding Buffer to each tube. Vortex to mix. Adjust the pH with 5 µl 3 M Sodium Acetate. The sample should be bright yellow in color to indicate proper pH. If your sample is not bright yellow, please refer to the Troubleshooting guide in the Appendix on page 26 for details to adjust pH prior to loading the sample into the purification column.
7. For each sample, place a DNA purification column (AM# 103928) in a collection tube and add each pH adjusted sample to its own column. Close the cap on each column and centrifuge at 14,000 rpm for 1 minute.
8. Remove the column from the collection tube, then remove and discard the flow through from the collection tube. Return the column to the collection tube.
9. Prepare DNA Purification Wash Buffer (AM #103497) before the first use. Follow the instruction on page 14 for the addition of ethanol prior to using the solution. Add 750 µl DNA Purification Wash Buffer to each column and cap the column.
10. Spin at 14,000 rpm for 1 minute in a microcentrifuge.
11. Pre-warm DNA Purification Elution Buffer (36 µl x _____IPs) at 37°C for 5 minutes prior to use.
12. Remove the column from the collection tube, then remove and discard the flow through from the collection tube. Return the column to the collection tube.
13. With the column cap open, spin at 14,000 rpm for 2 minutes in a microcentrifuge to remove any residual Wash Buffer from the column.
14. Transfer the column to a clean microcentrifuge tube. Add 36 µl pre-warmed elution buffer directly to the center of each column. Let sit for 1 minute.
15. Spin at 14,000 rpm for 1 minute in a microcentrifuge.
16. Discard column. Purified DNA may be stored at -20°C for future use. We recommend preparing a DNA dilution for use in qPCR (Add 6 µl eluted DNA to 94 µl sterile water). The remaining 30 µl DNA sample can be used for library generation and sequencing.

Section G: qPCR Primer Design

Guidelines are provided below to design primers for use in qPCR analysis of the enriched DNA.

- Design and analyze your potential primer pairs using an *in silico* PCR program (*i.e.* Primer3 at http://frodo.wi.mit.edu/ or the UCSC Genome Browser at http://genome.ucsc.edu/cgi-bin/hgPcr).
- Primers that dimerize should be avoided, as they will be bound by SYBR Green, which will compromise accurate quantitation. You can test your primers for self-complementarity and secondary structure at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi.
- Ideally, the amplicons should be 75-150 bp in length.
- For use with the ChIP-IT qPCR Analysis Kit (Catalog No. 53029), primers should be designed to anneal optimally at 58°C with a recommended length of 18-22 bp.

Section H. Quantitative PCR (qPCR)

Active Motif recommends the use of its ChIP-IT® qPCR Analysis Kit (Catalog No. 53029) for the analysis of qPCR data. The ChIP-IT qPCR Analysis Kit contains positive and negative control primer pairs, standard curve DNA, standard curve primers and a qPCR Analysis spreadsheet to perform the analysis calculations. Active Motif's analysis strategy determines primer efficiencies and the ChIP sample values are normalized according to input, primer efficiency, chromatin amount used in the ChIP reaction and resuspension volume. The ChIP-IT qPCR Analysis Kit provides consistency in data analysis and allows direct comparison across samples and experiments.

If using the ChIP-IT qPCR Analysis Kit, please refer to the product manual for instructions.

If the data analysis will not be performed using Active Motif's ChIP-IT qPCR Analysis Kit, prepare a standard curve using known amount of Input DNA for each primer pair being used in the experiment. Calculate the fold enrichment of positive primers (known binding sites) relative to negative control primers (regions of the genome not bound by your gRNA of interest).

1. Produce a standard curve by performing qPCR with your primer set on known DNA quantities of Input DNA (from Section B) in triplicate. Run three to five samples that are 10-fold dilutions, *e.g.* 0.005 ng, 0.05 ng, 0.5 ng, 5 ng and 50 ng.
2. Run the enChIP and no gRNA samples along with the dilution series of the Input DNA standards using both positive control primers and negative control primers.
   Input DNA: Prepare dilution series based on input DNA concentration. Use 5 µl per PCR reaction.
   enChIP / no gRNA DNA: Dilute 6 µl of the 36 µl elution volume into 94 µl sterile water. Use 5 µl of diluted sample per PCR reaction.

3. 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.

Reagent	20 µl PCR reactions
2X SYBR Green master mix	10 µl
Primer mix (2.5 µM each primer)	4 µl
Sterile water	1 µl
DNA sample (enChIP or Input)	5 µl
Total volume	**20 µl**

4. 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

5. Your qPCR instrument will assign values (in ng) to each qPCR reaction based on the standard curve. If your machine does not average your triplicate reactions automatically, you will need to calculate these averages.

6. Divide the average value from the positive control primer set by the average value of the negative control primer set to obtain your fold enrichment.

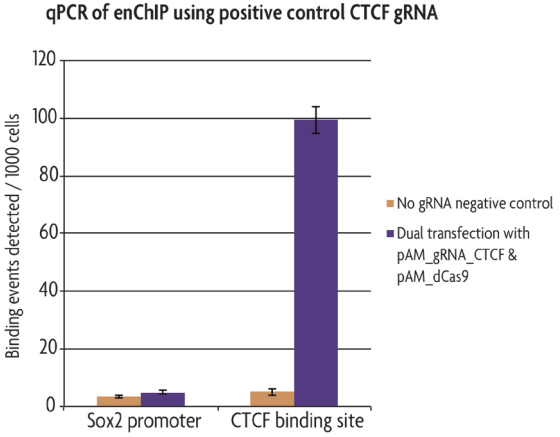
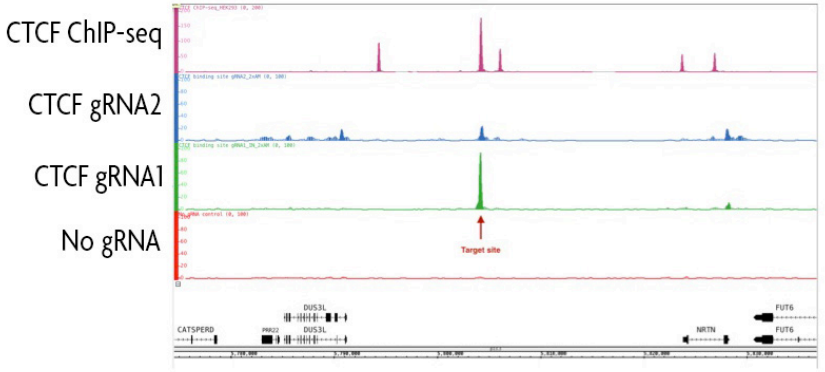


Figure 5: qPCR analysis of positive control CTCF binding site using the enChIP Kit.

The positive control pAM_gRNA_CTCF Vector (Catalog No. 53123), which contains a gRNA sequence corresponding to a CTCF binding site on chromosome 19 (5,804,115–5,804,209), and the pAM_dCas9 (no gRNA) negative control vector were transfected into HEK293T cells. Chromatin was prepared and immunoprecipitated using the AM-tag antibody according to the instructions in the enChIP Kit. Enriched DNA was analyzed by qPCR to evaluate a negative control (Sox2 promoter) and positive control (CTCF) gene locus. Results show enrichment of CTCF at the expected gene locus and little to no enrichment of the no gRNA negative control or Sox2 promoter.

Section I. ChIP-Seq

ChIP-seq analysis involves the preparation of libraries from ChIP DNA by the addition of adapter sequences to the ends of the DNA fragments. The library is then PCR amplified and validated prior to sequencing. For enChIP, we recommend using the Illumina® platform. Create TruSeq libraries and perform sequencing using Illumina NextSeq or HiSeq. We recommend sequencing 50-75 bp single end reads for a total of at least 20 million reads. The no gRNA samples should be sequenced as a control reaction in order to identify false "peaks" associated with the dCas9 protein and also to reveal regions of the genome that have been duplicated. Subtracting the no gRNA peaks from the experimental peaks will help eliminate false data.



	CTCF gRNA1	CTCF gRNA2	No gRNA
Total number of reads	39,114,618	40,036,875	38,208,604
Total number of alignments	31,486,919	29,584,781	31,765,380
Unique alignments	28,729,643	26,987,217	28,634,570
Unique alignments (without duplicate reads)	27,418,487	24,458,100	26,837,038
Peak numbers	100	30,822	N/A

Figure 6: ChIP-Seq analysis of CTCF binding site using the enChIP Kit.

Two gRNAs were designed using CRISPRdirect targeting different sequences of a CTCF binding site on chromosome 19. Each gRNA sequence was cloned into the pAM_dCas9 Vector and transfected into HEK293T cells along with a no gRNA negative control transfection. Chromatin was prepared and immunoprecipitated according to the instructions in the enChIP Kit. Enriched DNA was analyzed by ChIP-seq and background was subtracted using the no gRNA control. Data was compared to traditional ChIP-seq data for CTCF in the same cell line. Results show gRNA 1 had a strong peak at the desired target site and had little to no off-target binding events. Data for gRNA 2 revealed a larger number of peaks, which represented non-specific binding of the gRNA outside of the target locus. A comparison of overlapping peak data for gRNA 1, gRNA 2 and the ChIP-seq data set could provide information about potential looping interactions.

Section J. Troubleshooting Guide

Problem/question	Recommendation
At what points in the protocol can I stop?	The protocol may be stopped and samples stored at the times and temperatures below: 1. After formaldehyde fixation and centrifugation (intact cell pellet), -80°C. 2. After dounce homogenization and centrifugation (intact nuclear pellet), -80°C. 3. After chromatin shearing, -80°C. 4. After DNA clean up, -20°C.
Poor yield of sheared chromatin.	Insufficient cell numbers were used. Repeat chromatin preparation using a larger number of cells.
	Nuclei not released. It is highly recommended to perform dounce homogenization, even when using sonication. Use a dounce homogenizer with a small clearance pestle (Active Motif Catalog Nos. 40401 & 40415). Monitor cell lysis under a microscope. Generally, the more cells that are lysed, the higher the sheared chromatin yield.
	Sonication samples were emulsified. Avoid emulsification by turning up the power of the sonicator gradually. If a chromatin preparation becomes emulsified inadvertently, discontinue shearing and centrifuge the sample for 4 minutes at 8,000 rpm in a 4°C microcentrifuge to remove trapped air.
	Use fresh formaldehyde when preparing Complete Cell Fixation Solution.
	Buffers were not scaled proportionally to the size of the sample. Use the recommendations for cell culture size.
Shearing efficiency is not clear from gel analysis.	Material is stuck in the wells, and smears or streaks are seen from the top to bottom of the lane. The sheared chromatin needs to have the cross-links reversed, protein removed (Proteinase K) and RNA removed (RNase), followed by DNA purification.
	Chromatin should be between 200-2,000 bp in size. If you observe higher molecular weight products, we recommend to decrease the size of the fragments by re-sonicating the sample. If chromatin falls within the lower end of the size range (*e.g.* 200-700 bp) this is still sufficient for determining gRNA specificity. However, these shorter fragments may not provide reliable information regarding chromatin looping.
Performing enChIP with a large volume of chromatin.	This is not recommended. Follow the instructions in the manual for chromatin quantities based on the cell culture volumes. Do not perform a single scaled-up reaction, as the capture efficiency is lower.
enChIP DNA does not turn bright yellow following the addition of 3 M sodium acetate	If the color is light orange or violet, this indicates the pH is too high. Add more 3 M sodium acetate 5 µl at a time, mixing after addition until the color is bright yellow. This step is crucial to the success of DNA binding and purification. For a full color image please see the manual for Active Motif's Chromatin IP DNA Purification Kit Catalog No. 58002 available online at our website www.activemotif.com.
High background.	Chromatin not sheared enough. Shearing should produce DNA fragments that are small enough to exclude background from neighboring chromosomal sequences, but still large enough that there is a good possibility your amplicon remains intact. We recommend 200-2,000 bp fragments. If the DNA fragments are too large, the background is increased. Consider increasing the number of pulses for sonication. Check the fragment size on a gel to assess your shearing efficiency.
	Non-specific gRNA design. Some gRNAs display off-target binding, resulting in high background. It is recommended to design multiple gRNAs to a specific genomic locus and test them in enChIP to identify the most specific gRNA. Use a software program to design a new gRNA target sequence.

Problem/question	Recommendation
Poor or no enrichment with AM-Tag monoclonal antibody.	Ensure that you are using the correct antibody. The AM-Tag antibody provided in the kit is a mouse monoclonal antibody. The antibody recommended for WB analysis of protein expression (Catalog No. 61677) is a rabbit polyclonal antibody. The rabbit polyclonal antibody is not validated for use in enChIP. Use the AM-Tag monoclonal antibody.
	Inefficient transfection efficiency or poor expression of the gRNA and dCas9 vectors. Prepare a duplicate transfection reaction. Prepare cell lysates and analyze for expression of the AM-tagged dCas9 protein by Western blot using Active Motif's AM-Tag polyclonal antibody (Catalog No. 61677). If the tagged dCas9 protein is not detected, continue to optimize transfection conditions.
	Problems with PCR. Confirm the amplified sequence for the positive control primer set contains the genomic region targeted by the gRNA. Design new PCR primers.
	Run a positive control reaction using Active Motif's pAM_gRNA_CTCF vector (Catalog No. 53123) or the pAM_dCas9_CTCF vector (Catalog No. 53124) to confirm all other assay reagents are working.
No PCR products with real-time PCR	Confirm the species specificity and efficiency of your primers. You may need to redesign your primers. Primers that work in end point PCR do not always work in qPCR.
	Ensure that your PCR amplicon is designed to include your gRNA target sequence. Refer to your gRNA design. Make sure your gRNA target sequence does not contain any known binding sites of DNA binding proteins. Protein binding may interfere with the ability of your gRNA to bind its genomic locus.
	No ethanol in DNA Purification Wash Buffer. Make sure that ethanol has been added to the DNA Purification Wash Buffer prior to first use.

Section K. 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® qPCR Analysis Kit	10 rxns	53029
ChIP-IT® ChIP-Seq	10 libraries	53041
ChIP-IT® FFPE	16 rxns	53045
ChIP-IT® FFPE Chromatin Preparation Kit	5 rxns	53030
ChIP-IT® Express HT	96 rxns	53018
Re-ChIP-IT®	25 rxns	53016
RNA ChIP-IT®	25 rxns	53024
Chromatin IP DNA Purification Kit	50 rxns	58002
EpiShear™ Probe Sonicator	110 V	53051
EpiShear™ Cooled Sonication Platform, 1.5 ml	1 platform	53080
ChIP-IT® Protein G Magnetic Beads	25 rxns	53014
Protein G Agarose Columns	30 rxns	53039
Siliconized Tubes, 1.7 ml	25 tubes	53036
ChIP-IT® Control qPCR Kit – Human	5 rxns	53026
ChIP-IT® Control qPCR Kit – Mouse	5 rxns	53027
ChIP-IT® Control qPCR Kit – Rat	5 rxns	53028
ChIP-IT® Control Kit – Human	5 rxns	53010
ChIP-IT® Control Kit – Mouse	5 rxns	53011
ChIP-IT® Control Kit – Rat	5 rxns	53012
Ready-to-ChIP HeLa Chromatin	10 rxns	53015
Ready-to-ChIP Hep G2 Chromatin	10 rxns	53019
Ready-to-ChIP K-562 Chromatin	10 rxns	53020
Ready-to-ChIP NIH/3T3 Chromatin	10 rxns	53021
Bridging Antibody for Mouse IgG	500 µg	53017
Dounce Homogenizer	1 ml	40401
Dounce Homogenizer	15 ml	40415

ChIP-validated Antibodies

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

Whole Genome Amplification	Format	Catalog No.
GenoMatrix™ Whole Genome Amplification Kit	1 kit	58001

Co-Immunoprecipitation	Format	Catalog No.
Nuclear Complex Co-IP Kit	50 rxns	54001
Universal Magnetic Co-IP Kit	25 rxns	54002

Modified Histones Array	Format	Catalog No.
MODified™ Histone Peptide Array	1 array	13001

Histone Modification FP Binding Assay	Format	Catalog No.
HiLite™ Histone H3 Methyl-Lys9 / Lys27 FP Binding Assay	1 kit	57001

Histone ELISAs	Format	Catalog No.
Histone H3 monomethyl Lys4 ELISA	1 x 96 rxns	53101
Histone H3 dimethyl Lys4 ELISA	1 x 96 rxns	53112
Histone H3 trimethyl Lys4 ELISA	1 x 96 rxns	53113
Histone H3 acetyl Lys9 ELISA	1 x 96 rxns	53114
Histone H3 dimethyl Lys9 ELISA	1 x 96 rxns	53108
Histone H3 trimethyl Lys9 ELISA	1 x 96 rxns	53109
Histone H3 phospho Ser10 ELISA	1 x 96 rxns	53111
Histone H3 acetyl Lys14 ELISA	1 x 96 rxns	53115
Histone H3 monomethyl Lys27 ELISA	1 x 96 rxns	53104
Histone H3 trimethyl Lys27 ELISA	1 x 96 rxns	53106
Histone H3 phospho Ser28 ELISA	1 x 96 rxns	53100

Histone Purification & Chromatin Assembly	Format	Catalog No.
Histone Purification Kit	10 rxns	40025
Histone Purification Mini Kit	10 rxns	40026
Chromatin Assembly Kit	10 rxns	53500
HeLa Core Histones	36 µg	53501

Recombinant Methylated, Acetylated and Phosphorylated Histone Proteins

For an up-to-date list of Recombinant Histone Proteins, please visit **www.activemotif.com/recombhis**.

Histone Acetyltransferase and Deacetylase Activity	Format	Catalog No.
HAT Assay Kit (Fluorescent)	1 x 96 rxns	56100
Recombinant p300 protein, catalytic domain	5 µg	31205
Recombinant GCN5 protein, active	5 µg	31204
HDAC Assay Kit (Fluorescent)	1 x 96 rxns	56200
HDAC Assay Kit (Colorimetric)	1 x 96 rxns	56210

Histone Demethylase Activity	Format	Catalog No.
Histone Demethylase Assay (Fluorescent)	48 rxns	53200

DNA Methylation	Format	Catalog No.
hMeDIP	10 rxns	55010
MeDIP	10 rxns	55009
MethylDetector™	50 rxns	55001
MethylCollector™	25 rxns	55002
MethylCollector™ Ultra	30 rxns	55005
UnMethylCollector™	30 rxns	55004
Hydroxymethyl Collector™	25 rxns	55013
DNMT Activity / Inhibition Assay	96 rxns	55006
Methylated DNA Standard Kit	3 x 2.5 µg	55008
Fully Methylated Jurkat DNA	10 µg	55003
Jurkat genomic DNA	10 µg	55007

Technical Services

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

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