Chapter 4

Isolation of Specific Genomic Regions and Identification of Associated Molecules by Engineered DNA-Binding Molecule-Mediated Chromatin Immunoprecipitation (enChIP) Using CRISPR

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Abstract

Isolation of specific genomic regions retaining molecular interactions is necessary for their biochemical analysis. Here, we describe engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) using the CRISPR system, for purification of specific genomic regions retaining molecular interactions. In this form of enChIP, specific genomic regions are immunoprecipitated with antibody against a tag(s), which is fused to a catalytically inactive form of Cas9 (dCas9), which is co-expressed with a guide RNA (gRNA) and recognizes endogenous DNA sequence in the genomic regions of interest. enChIP combined with mass spectrometry (enChIP-MS), next-generation sequencing (enChIP-Seq), and RNA-Seq (enChIP-RNA-Seq) can identify proteins, other genomic regions, and RNA, respectively, that interact with the target genomic region.

Key words enChIP, CRISPR, dCas9, gRNA, Mass spectrometry, Locus-specific chromatin immunoprecipitation

1 Introduction

Function of eukaryotic genome is mediated by molecular complexes in the context of chromatin [1]. Elucidation of molecular mechanisms of genome functions requires identification of components mediating the genome function. To purify specific genomic regions retaining molecular interactions in vivo, we recently developed a novel method, engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP), for purification of specific genomic regions [2, 3]. In enChIP, a tagged engineered DNA-binding molecule recognizing an endogenous target DNA sequence such as a zinc-finger protein [4], a transcription activator-like (TAL) protein [5], and the CRISPR (clustered regularly interspaced short palindromic repeats) system consisting of a catalytically inactive form

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of Cas9 endonuclease (dCas9) and small guide RNA (gRNA) [6] is expressed into the cell to be analyzed. Subsequently, the target genomic region is subjected to affinity purification such as immunoprecipitation with an antibody against the tag.

Here, we describe enChIP using the CRISPR system, for purification of specific genomic regions retaining molecular interactions (Fig. 1).

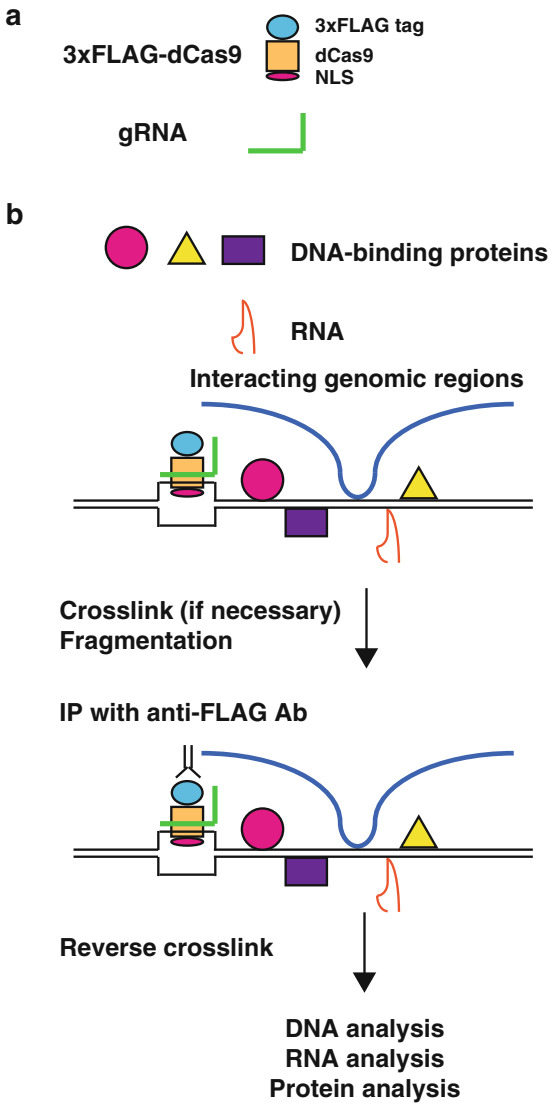


Fig. 1 Overview of engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) using the CRISPR system consisting of dCas9 and gRNA. (**a**) The system is composed of a fusion protein, 3xFLAG-dCas9, consisting of 3xFLAG-tag, dCas9, and the nuclear localization signal (NLS) of SV40 T-antigen, and a guide RNA (gRNA). (**b**) 3xFLAG-dCas9 and gRNA are expressed in appropriate cells. The cells are cross-linked, if necessary, lysed, and fragmented by sonication or other methods. The complexes are immunoprecipitated with anti-FLAG Ab, and cross-link is reversed when cross-linkers are used. Molecules (DNA, RNA, proteins, and others) associated with the target genomic region are isolated and characterized

2 Materials

2.1 Construction of gRNA Expression Vectors and Generation of Cells for enChIP Analysis

1. 3xFLAG-dCas9/pCMV-7.1 (catalog number 47948) (Addgene, Cambridge, MA, USA).
2. gRNA Cloning vector (catalog number 41824) (Addgene).
3. Phusion polymerase (catalog number M0530) (New England Biolabs Inc., Ipswich, MA, USA).
4. Gibson Assembly Master Mix (catalog number E2611) (New England Biolabs Inc.).
5. Anti-FLAG M2 antibody (catalog number F1804) (Sigma-Aldrich, St. Louis, MO, USA).
6. Fluorescein isothiocyanate (FITC)-conjugated anti-FLAG M2 antibody (catalog number F4049) (Sigma-Aldrich).

2.2 Formaldehyde Cross-Linking of Cells

1. 37 % Formaldehyde.
2. 1.25 M Glycine solution: Dissolve 18.8 g of glycine in 200 ml water.

2.3 Preparation of Chromatin

1. Cell lysis buffer (CLB): 10 mM Tris (pH 8.0), 1 mM EDTA, 0.5 % IGEPAL CA-630, 1× protease inhibitors (Complete, Mini, EDTA-free (catalog number 04 693 159 001) (Roche Diagnostics GmbH, Mannheim, Germany), 1 tablet/10 ml). Protease inhibitors should be added immediately before use for all solutions.
2. Nuclear lysis buffer (NLB): 10 mM Tris (pH 8.0), 1 mM EDTA, 0.5 M NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.5 % lauroylsarcosine, 1× protease inhibitors.
3. Modified lysis buffer 3 (MLB3): 10 mM Tris (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 0.1 % sodium deoxycholate, 0.1 % SDS, 1× protease inhibitors.

2.4 enChIP

1. Dynabeads-Protein G (catalog number DB10004) (Life Technologies, Carlsbad, CA, USA).
2. Normal mouse IgG (catalog number sc-2025) (Santa Cruz Biotechnology, Dallas, TX, USA).
3. Phosphate buffered saline (PBS) (pH 7.4).
4. PBS-T: PBS (pH 7.4), 0.01 % Tween-20.
5. BSA fraction V (7.5 %) (catalog number 15260) (Life Technologies).
6. PBS-T-BSA: PBS (pH 7.4), 0.01 % Tween-20, 0.1 % BSA.
7. Low-salt buffer (LSB): 20 mM Tris (pH 8.0), 2 mM EDTA, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS.
8. High-salt buffer (HSB): 20 mM Tris (pH 8.0), 2 mM EDTA, 500 mM NaCl, 1 % Triton X-100, 0.1 % SDS.

9. LiCl buffer: 10 mM Tris (pH 8.0), 1 mM EDTA, 0.25 M LiCl, 0.5 % IGEPAL CA-630, 0.5 % sodium deoxycholate.
10. TBS-IGEPAL CA-630: 50 mM Tris (pH 7.5), 150 mM NaCl, 0.1 % IGEPAL CA-630.
11. 3xFLAG peptide (catalog number: F4799) (Sigma-Aldrich).
12. Elution buffer: 50 mM Tris (pH 7.5), 150 mM NaCl, 0.1 % IGEPAL CA-630, 500 μg/ml 3xFLAG peptide.
13. 2-propanol.
14. 3 M sodium acetate buffer solution (pH 5.2).
15. 20 mg/ml glycogen.
16. 70 % ethanol.
17. 2× sample buffer: 125 mM Tris (pH 6.8), 10 % 2-mercaptoethanol, 4 % SDS, 10 % sucrose, 0.004 % bromophenol blue.
18. 4–20 % Mini-PROTEAN TGX Gel, 10 well, 50 μl (catalog number 456-1094) (Bio-Rad).
19. Running buffer solution for SDS-PAGE, Tris-Glycine.
20. ChIP DNA clean & concentrator (catalog number D5205) (Zymo Research, Irvine, CA, USA).
21. RNasin Plus RNase Inhibitor (catalog number N2611) (Promega, Madison, WI, USA).
22. Isogen II (catalog number 311-07361) (Nippon Gene, Tokyo, Japan).

3 Methods

3.1 Design of gRNA and Construction of gRNA Expression Vectors

Construction of gRNA expression vectors is done basically according to the protocol described in Mali et al. [7] with some modifications.

1. Using the **CRISPRdirect** web tool (http://crispr.dbcls.jp), find candidate gRNA target sequences in the genomic region of interest. The output window shows 23 bp genomic sites of the form 5′-N_{20}NGG-3′ within your target region. These sites may reside on the + or – strand (*see* **Note 1**).

5′-NNNNN NNNNN NNNNN NNNNN NGG-3′
PAM

2. Synthesize gBlock as follows. Appropriate sites of restriction enzymes should be added for subcloning purpose.
TGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCA
GTCGACTGGATCCGGTACCAAGGTCGGGCA
GGAAGAGGGCCTATTTCCCATGATTCCTTCATATTT
GCATATACGATACAAGGCTGTTAGAGAGATAATT

AGAATTAATTTGACTGTAAACACAAAGATATTAGT
ACAAAATACGTGACGTAGAAAGTAATAATTTCTTG
GGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATG
GACTATCATATGCTTACCGTAACTTGAAAGTATTT
CGATTTCTTGGCTTTATATATCTTGTGGAAAGGA
CGAAACACCGNNNNNNNNNNNNNNNNNNNNGTT
TTAGAGCTAGAAATAGCAAGTTAAAATAAGGC
TAGTCCGTTATCAACTTGAAAAAGTGGCACCG
AGTCGGTGCTTTTTTTT

3. Alternatively, incorporate 20 bp of the selected target sequence 5′ to PAM: 5′-NNNNN NNNNN NNNNN NNNNN NGG-3′ into two 61-mer oligonucleotides as indicated below. The region of N_{20} in Insert_F corresponds to the abovementioned 20-mer. The region of N_{20} in Insert_R corresponds to reverse complements of the 20-mer.

   Insert_F: 5′-TTTCTTGGCTTTATATATCTTGTGGAAAGG ACGAAACACC *G*<u>*NNNNNNNNNNNNNNNNNNNN*</u>-3′

   Insert_R: 5′-GACTAGCCTTATTTTAACTTGCTATTTCTA GCTCTAAAAC <u>*NNNNNNNNNNNNNNNNNNNN*</u>*C*-3′

4. Anneal the two oligos and extend these to make a 101 bp double-strand DNA fragment using Phusion polymerase.
5. Linearize the gRNA cloning vector with *Afl* II and incorporate the 101 bp DNA fragment into the linearized vector using Gibson assembly Master Mix.

3.2 Generation of Cells for enChIP Analysis

1. Express 3xFLAG-dCas9 and gRNA into cells to be analyzed. If transfection efficiency is high, transient transfection can be used. Alternatively, stable transformant clones can be established by co-transfection of 3xFLAG-dCas9/pCMV-7.1 and the gRNA expression vector together with suitable selection marker genes. Sequential transfection of 3xFLAG-dCas9/pCMV-7.1 and the gRNA expression vector would be preferable because different gRNA can be expressed in the clones expressing 3xFLAG-dCas9 (*see* **Note 2**).
2. Confirm expression of 3xFLAG-dCas9 by immunoblot analysis using anti-FLAG Ab. Expression of 3xFLAG-dCas9 can also be detected by intracellular staining using FITC-conjugated anti-FLAG Ab and flow-cytometric analysis (*see* **Note 3**).
3. Confirm expression of gRNA by RT-PCR.

3.3 Formaldehyde Cross-Linking of Cells

1. Culture target cells (*see* **Note 4**). Use 2×10^7 cells × 2 (total 4×10^7 cells) (e.g., Ba/F3) for chromatin preparation. Collect 2×10^7 cells in a 50 ml centrifuge tube × 2. (If necessary, increase cell number).

2. Suspend cell pellets (2×10^7 cells) in 30 ml of medium and add 810 μl of 37 % formaldehyde to 1 % final concentration in the cell suspension. Incubate at 37 °C for 5–10 min (usually 5 min).
3. Stop cross-linking by adding 3 ml of 1.25 M Glycine solution to 127 mM final concentration. Incubate at room temperature for 10 min.
4. Collect cells by centrifugation (200 × *g*, 4 °C for 5 min).
5. Wash the pellet with PBS twice (30 ml × 2). Collect the pellet (cells). The cells can be stored at −80 °C.

3.4 Preparation of Chromatin (per 2×10^7 Cells)

1. Suspend the fixed cells in 10 ml of CLB. Incubate on ice for 10 min.
2. Centrifuge at 830 × *g* at 4 °C for 8 min. Discard carefully the supernatant.
3. Suspend the pellet in 10 ml of NLB. Incubate on ice for 10 min. Vortex every 2–3 min.
4. Centrifuge at 830 × *g* at 4 °C for 8 min. Discard carefully the supernatant.
5. Suspend the pellet in 10 ml of PBS. Centrifuge at 830 × *g* at 4 °C for 10 min. Collect the pellet as the chromatin fraction. The chromatin fraction can be stored at −80 °C after immediate freezing in liquid nitrogen.

3.5 Sonication of Chromatin (per 2×10^7 Cells)

1. Suspend the collected chromatin fraction in 800 μl of MLB3. Transfer the suspension into a 1.5 ml microtube.
2. Sonicate the chromatin by using Ultrasonic disruptor UD-201 (TOMY SEIKO, Japan). Keep the position of the tip of the sonication bar approximately 0.5 cm away from the tube bottom to avoid foaming. Conditions are as follows:
   - Output: 3; Duty: 100 % (continuous); Time: Free
   - 10–18 cycles of sonication for 10 s and cooling on ice for 20 s
   - 2 min on ice after 5–6 cycles to avoid excessive heating
3. Centrifuge at 16,000 × *g* at 4 °C for 10 min. Collect the supernatant (800 μl). The supernatant can be stored at −80 °C after immediate freezing in liquid nitrogen.

3.6 Evaluation of Fragmentation of Chromatin

1. Suspend 10 μl of the fragmented chromatin in 85 μl of distilled water.
2. Add 4 μl of 5 M NaCl. Incubate at 65 °C overnight.
3. Add 1 μl of 10 mg/ml RNase A. Incubate at 37 °C for 45 min.
4. Prepare 1 % agarose gel without staining dye.
5. Add 2 μl of 0.5 M EDTA (pH 8.0), 4 μl of 1 M Tris (pH 6.8), and 1 μl of 20 mg/ml Proteinase K. Incubate at 45 °C for 1.5 h.

6. Use 10 μl for electrophoresis in 1 % agarose gel without staining dye. Run the gel at 100 V for 30 min.
7. Stain the gel with staining dye (e.g., ethidium bromide) for 0.5–1 h.
8. The remaining samples can be subjected to purification of DNA (*see* Subheading 3.10). The purified DNA can be used as input DNA to calculate yield of enChIP procedure.

3.7 Preparation of Dynabeads Conjugated with Antibody (Anti-FLAG Antibody or Normal Mouse IgG) (per 2×10^7 Cells)

1. Transfer 300 μl of Dynabeads-Protein G in a 2 ml tube.
2. Put the tube on a magnet stand and wait for 3 min. Discard the supernatant by pipetting.
3. Suspend the pellet in 1 ml of PBS-T. Put the tube on a magnet stand and wait for 2 min. Discard the supernatant by pipetting.
4. Repeat the step (Subheading 3.7, **step 3**).
5. Suspend the pellet in 1.5 ml of PBS-T-BSA.
6. Add 30 μg of antibody (anti-FLAG antibody or normal mouse IgG). Rotate at 4 °C overnight.
7. Spin down briefly. Put the tube on a magnet stand and wait for 3 min. Discard the supernatant by pipetting.
8. Suspend the pellet in 1.5 ml of PBS-T. Invert several times and spin down briefly. Put the tube on a magnet stand and wait for 3 min. Discard the supernatant by pipetting.
9. Repeat the step (Subheading 3.7, **step 8**), twice. The Dynabeads are ready for the next step.

3.8 Chromatin Immunoprecipitation

1. Transfer 1.6 ml of the fragmented chromatin, which corresponds to chromatin extracted from 4×10^7 cells, into a 2 ml tube.
2. Add 400 μl of 5 % Triton X-100 (in MLB3) to 1 % final concentration.
3. Transfer all (2 ml) of the chromatin solution into the tube, in which the Dynabeads conjugated with normal mouse IgG were prepared. Rotate at 4 °C for 1 h.
4. Put the tube on a magnet stand and wait for 3 min.
5. Transfer the supernatant into the tube, in which the Dynabeads conjugated with anti-FLAG antibody were prepared. Rotate at 4 °C overnight.
6. Put the tube on a magnet stand and wait for 3 min. Discard the supernatant by pipetting.
7. Wash one and two: Add 1.8 ml of LSB. Rotate at 4 °C for 5 min. Put the tube on a magnet stand and wait for 3 min. Discard the supernatant by pipetting. Repeat again (total two times).
8. Wash three and four: Repeat the step (Subheading 3.8, **step 7**) with HSB (two times).

9. Wash five and six: Repeat the step (Subheading 3.8, **step 7**) with LiCl buffer (two times).
10. Add 1.8 ml of TBS-IGEPAL CA-630. Rotate at 4 °C for 5 min. Put the tube on a magnet stand and wait for 3 min. Discard the supernatant by pipetting.
11. Elution: Add 200 μl of elution buffer. Incubate at 37 °C for 20 min. Put the tube on a magnet stand and wait for 3 min.
12. Transfer the supernatant (200 μl) into the 1.5 ml microtube (*see* **Note 5**). The eluate can be used for purification of proteins (Subheading 3.9), DNA (Subheading 3.10), or RNA (Subheading 3.11).

3.9 SDS-PAGE, Staining, Mass Spectrometric Analysis

1. Mix eluate (200 μl) with 500 μl of 2-propanol, 25 μl of 3 M sodium acetate buffer solution (pH 5.2) and 5 μl of 20 mg/ml glycogen. Precipitate proteins at −20 °C overnight.
2. Centrifuge at 16,000 × *g* at 4 °C for 30 min. Discard the supernatant.
3. Rinse with 1 ml of 70 % ethanol. Centrifuge at 1,600 × *g* for 10 min at 4 °C. Discard the supernatant completely by pipetting.
4. Add 40 μl of 2× sample buffer. Vortex for 5 min to completely solve the precipitant. Incubate at 100 °C for 30 min (protein denaturing and reverse-cross-linking).
5. SDS-PAGE. Run the eluate on polyacrylamide gel until the dye reaches 1 cm from the well.
6. Coomassie Brilliant Blue (CBB) staining or silver staining.
7. Cut the gel into 5 pieces × 2 mm. An example of gel image is shown in Fig. 2 in the ref. [2].
8. In gel digestion and mass analysis. Our current system is:

   A nanoLC-MS/MS system, composed of LTQ Orbitrap Velos (Thermo Fisher Scientific) coupled with nanoLC (Advance, Michrom BioResources) and HTC-PAL autosampler (CTC Analytics).

3.10 Purification of DNA

1. Add 8 μl of 5 M NaCl and 0.4 μl of 0.5 M EDTA to the eluate (200 μl) (Subheading 3.8, **step 12**). Incubate at 65 °C, overnight.
2. Add 3 μl of 10 mg/ml RNase A. Incubate at 37 °C for 1 h.
3. Add 10 μl of 10 % SDS and 5 μl of 20 mg/ml Proteinase K per 200 μl of eluate. Incubate at 45 °C for 2 h.
4. Purify DNA using ChIP DNA Clean & Concentrator kit (Zymo Research, D5205).
5. The purified DNA is ready for downstream analyses such as PCR, microarray analysis, and next-generation sequencing.

3.11 Purification of RNA

1. For purification of RNA, the enChIP procedure is performed as described above with 5 U/ml of rRNasin Plus in all the buffer solution except for MLB3 in which 40 U/ml of rRNasin Plus is added.
2. Add 8 μl of 5 M NaCl and 0.4 μl of 0.5 M EDTA to the eluate (200 μl) (Subheading 3.8, **step 12**). Incubate at 65 °C, overnight.
3. Add 10 μl of 10 % SDS and 5 μl of 20 mg/ml Proteinase K per 200 μl of eluate. Incubate at 45 °C for 2 h.
4. Purify RNA with 1 ml of Isogen II.
5. The purified RNA is ready for downstream analyses such as RT-PCR, microarray analysis, and next-generation sequencing (RNA-Seq).

4 Notes

1. Binding of the CRISPR complex may affect chromatin structure such as nucleosome positioning and abrogate normal genome activities such as gene expression. Although the effects of binding need to be tested empirically for each locus, the author's group has guidelines to avoid potential aberrant effects caused by binding of the CRISPR complex. (a) For analysis of promoter regions near transcription start sites (TSSs), the gRNA binding site should be several hundred base 5′ to the TSS so that the binding would not inhibit transcription or disrupt nucleosome positioning. In contrast, for identification of binding molecules of genomic regions with distinct boundaries such as enhancer or silencer, the binding site of gRNA can directly be juxtaposed to the regions because of less probability of inhibition of their function. (b) The binding site should not be conserved among species because conserved regions are often binding sites of conserved binding molecules. In this regard, however, since multiple gRNA can be generated easily, a trial-and-error approach can work.
2. Retroviral systems are available through Addgene.
3. The protocol of intracellular staining and flow-cytometric detection of the FLAG epitope can be downloaded from our laboratory website:
   http://www.biken.osaka-u.ac.jp/lab/microimm/fujii/top/index_e.html
4. For SILAC (stable isotope labeling using amino acids in cell culture) experiments, culture a set of comparison group cells in light medium and heavy medium. SILAC media can be purchased from Thermo Fisher Scientific and other vendors.
5. Elution step can be repeated to increase the yield.

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