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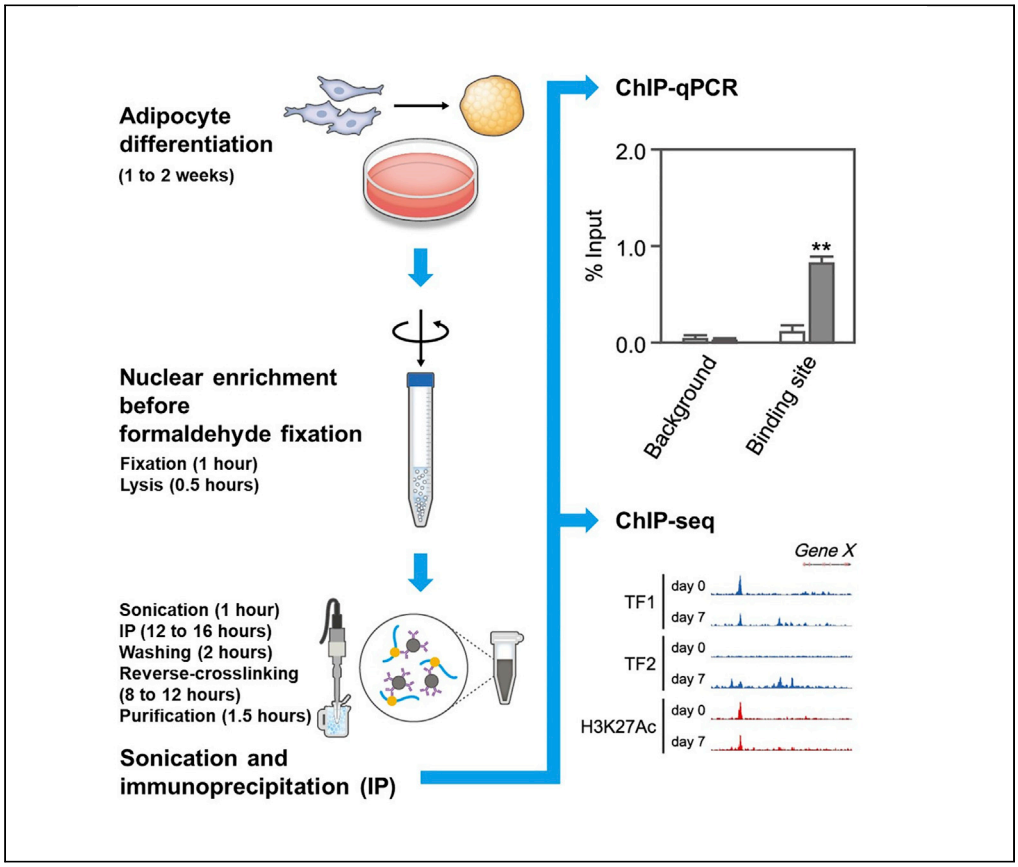
Protocol

Chromatin immunoprecipitation with mouse adipocytes using hypotonic buffer to enrich nuclear fraction before fixation



Chromatin immunoprecipitation (ChIP) experiments with differentiated adipocytes are challenging because lipid droplets interfere with immunoprecipitation efficiency. Here, the author describes optimized procedures to minimize the burden of lipid droplets by using hypotonic buffer to enrich nuclear fraction before formaldehyde crosslinking, thus increasing the sensitivity and specificity of ChIP experiments with differentiated adipocytes. The author also describes steps for after fixation, including sonication, immunoprecipitation, washing, reverse-crosslinking, and purification. This protocol is compatible with ChIP-qPCR and ChIP-seq of various transcription factors and histone modifications.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Optimized ChIP protocol for differentiated adipocytes to minimize the burden of lipids

Using hypotonic buffer to enrich nuclear fraction before fixation

Compatible with ChIP-seq of various transcription factors and histone modifications

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Chromatin immunoprecipitation with mouse adipocytes using hypotonic buffer to enrich nuclear fraction before fixation

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SUMMARY

Chromatin immunoprecipitation (ChIP) experiments with differentiated adipocytes are challenging because lipid droplets interfere with immunoprecipitation efficiency. Here, the author describes optimized procedures to minimize the burden of lipid droplets by using hypotonic buffer to enrich nuclear fraction before formaldehyde crosslinking, thus increasing the sensitivity and specificity of ChIP experiments with differentiated adipocytes. The author also describes steps for after fixation, including sonication, immunoprecipitation, washing, reverse-crosslinking, and purification. This protocol is compatible with ChIP-qPCR and ChIP-seq of various transcription factors and histone modifications.

For complete details on the use and execution of this protocol, please refer to Hiraike et al. (2022).[1]

BEFORE YOU BEGIN

The protocol presented below has been optimized for differentiated, lipid-filled adipocytes. The author and colleagues successfully performed ChIP-seq experiments of brown, beige and white adipocytes.[1,2] This protocol works well also for preadipocytes.[3] However, other protocols might yield better results for different types of cells. Also, the success of ChIP experiments strongly depends on the use of the appropriate antibody. Checking the sensitivity and specificity of the antibody is encouraged.

Institutional permissions

All animal work related to this protocol, which was required to isolate stromal vascular fractions (SVFs) from mouse adipose tissue to obtain preadipocytes, was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Tokyo and conducted according to the institutional guidelines of the University of Tokyo.

Preparation of differentiated adipocytes

Timing: 1–2 weeks

Prepare preadipocytes and induce adipocyte differentiation *in vitro*.

1. Prepare preadipocytes (primary, immortalized, or established cell lines) and plate them to induce adipocyte differentiation by using the protocol of your choice. Usually, the author uses two to five 10 cm dishes to obtain approximately 1×10^7 to 2×10^7 differentiated adipocytes (Since brown adipocytes are smaller than white adipocytes, two 10 cm dishes might be enough to obtain above-mentioned cell counts. For large-scale cell culture, preadipocytes isolated from newborn mice might be easier than that isolated from adult mice because they grow faster.).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-NFI	Santa Cruz Biotechnology	sc-30198; RRID: AB_2287441
Anti-PPARγ	Santa Cruz Biotechnology	sc-7273; RRID: AB_628115
Anti-PPARγ	Perseus Proteomics	A3409A; RRID: AB_843370
Antt-H3K27Ac	Abcam	ab4729; RRID: AB_2118291
Chemicals, peptides, and recombinant proteins		
Phosphate buffered salts (PBS) tablets	Takara	T900
Complete protease inhibitor cocktail tablets	Sigma	11697498001
Trizma (Tris base)	Sigma	T4661
5 mol/L hydrochloric acid	FUJIFILM Wako Chemicals	081-05435
Sodium chloride	FUJIFILM Wako Chemicals	191-01665
Magnesium chloride	FUJIFILM Wako Chemicals	136-03995
Nonidet(R) P40 Substitute	NACALAI TESQUE, INC.	23640-94
(Alternative) IGEPAL CA-630	N/A	N/A
Sodium dodecyl sulfate	FUJIFILM Wako Chemicals	191-07145
Polyoxyethylene(10) octylphenyl ether (Triton X-100)	FUJIFILM Wako Chemicals	160-24751
2NA(EDTA • 2Na)	Dojindo	345-01865
Lithium chloride BioXtra, ≧99.0% (titration)	Sigma	L4408
Sodium Deoxycholate	FUJIFILM Wako Chemicals	192-08312
Sodium hydrogen carbonate	FUJIFILM Wako Chemicals	191-01305
37% Formaldehyde	FUJIFILM Wako Chemicals	061-00416
Dynabeads Protein A	Thermo Fisher	10002D
Dynabeads Protein G	Thermo Fisher	10003D
(Alternative) Protein A Sepharose 4 Fast Flow	GE	17-1279-02
(Alternative) Protein G Sepharose 4 Fast Flow	GE	17-0618-02
RNAase A	Qiagen	1007885
Proteinase K	Thermo Fisher	EO0491
Critical commercial assays		
QIAquick PCR purification kit	Qiagen	28104
Power SYBR Green PCR Master Mix	Applied Biosystems	4367659
(Optional) KAPA Hyper prep kit	KAPA biosystems	KK8502
(Alternative) SPIN-X 0.45 μm column	Corning Costar	CLS8162
Experimental models: Cell lines		
BAT SVF, C57BL6/J, new born	Hiraike et al.[2]	N/A
iWAT SVF, C57BL6/J, 8–10 weeks old male	Hiraike et al.[1]	N/A
Oligonucleotides		
Ins -0.2 kb Fwd: CTTCAGCCCAGTTGACCAAT Rev: AGGGAGGAGGAAAGCAGAAC	N/A	N/A
Fabp4 -13 kb Fwd: TGTTTTCTATTGAGGCTTGCAG Rev: AAGTTGTCTTCAGGGACTCTGG	N/A	N/A
Fabp4 -5.4 kb Fwd: TGGGAACTCCATTTGCTCTC Rev: GAACAGAATTCCCAGCAGGA	N/A	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ucp1 9.5 kb Fwd: ACCGTGTAGCTTCCTCTCAG Rev: TTGTCTGACTGAGTCCTGGC	N/A	N/A
Ucp1 -12 kb Fwd: ACCGTGTAGCTTCCTCTCAG Rev: TTGTCTGACTGAGTCCTGGC	N/A	N/A
Ucp1 -4.5 kb Fwd: TCCCAGTGTCACCTTAATTTCTC Rev: AAAGGACTTGGTAAAAGGTGAGG	N/A	N/A
Software and algorithms		
FIMO ver. 4.12.0	Grant et al.[4]	N/A
Other		
Digital Sonifier 450	Branson	N/A
(Alternative) Other sonicator system that can be used for chromatin fragmentation	N/A	N/A
DynaMag-2 Magnet	Invitrogen	DB12321
(Alternative) Other magnetic stand that is compatible with 1.5 mL tubes	N/A	N/A
Hybridization oven	Taitec	HB-100
(Alternatives) Other hybridization oven	N/A	N/A
Qubit 2.0 fluorometer	Thermo Fisher	Q32866
Qubit dsDNA HS assay kit	Thermo Fisher	Q32851
Alternatives: Other fluorospectrometer, such as Nanodrop	N/A	N/A
2100 Bioanalyzer Instrument	Agilent	G2939B
High sensitivity DNA kit	Agilent	5067-4626
Alternatives: Other on-chip electrophoresis system such as Agilent Tape Station	N/A	N/A
QuantStudio 7 Flex Real-Time PCR System	Applied Biosystems	44-856-98
Alternatives: Other Real-Time PCR system	N/A	N/A
LoBind tubes 1.5 mL	Eppendorf	0030108051

MATERIALS AND EQUIPMENT

Buffers

- When preparing stock solutions such as 10% NP-40 (or IGEPAL CA-630), 20% SDS, 20% Triton X-100 and 5 M LiCl, use magnetic stirrer while warming since they are hard to dissolve.
- Phosphate buffered salts (PBS) buffer: add 5 PBS tablets in 500 mL ddH_2O. Store at 20°C–25°C for up to 3 months. When applicable, add 25× stock solution of complete protease inhibitor just before use.

Hypotonic buffer		
Reagent	Final concentration	Amount
1 M Tris-HCl pH 7.5	10 mM	0.5 mL
5 M NaCl	10 mM	0.1 mL
1 M $MgCl_2$	3 mM	0.15 mL
10% NP-40 or IGEPAL CA-630	0.1%	0.5 mL
ddH_2O	N/A	48.75 mL
Total		**50 mL**

Store at 20°C–25°C for up to 3 months.
Take required volume and add 25× stock solution of complete protease inhibitor just before use.

MC lysis buffer		
Reagent	Final concentration	Amount
1 M Tris-HCl pH 7.5	10 mM	0.50 mL
5 M NaCl	10 mM	0.10 mL
1 M $MgCl_2$	3 mM	0.15 mL
10% NP-40 or IGEPAL CA-630	0.5%	0.25 mL
ddH_2O	N/A	49 mL
Total		**50 mL**
Store at 20°C–25°C for up to 3 months.		

SDS lysis buffer		
Reagent	Final concentration	Amount
20% SDS	1%	2.5 mL
0.5 M EDTA	10 mM	1.0 mL
1 M Tris-HCl pH 8.0	50 mM	2.5 mL
ddH_2O	N/A	44 mL
Total		**50 mL**
Store at 20°C–25°C for up to 3 months.		

ChIP dilution buffer		
Reagent	Final concentration	Amount
20% SDS	0.00033%	0.001 mL
20% Triton X-100	0.037%	0.095 mL
0.5 M EDTA	0.0377 mM	0.004 mL
1 M Tris-HCl pH 8.0	0.566 mM	0.030 mL
5 M NaCl	167 mM	1.77 mL
ddH_2O	N/A	51.1 mL
Total		**53 mL**
Store at 20°C–25°C for up to 3 months.		

Wash buffer 1 (Low Salt Immune Complex Wash Buffer)		
Reagent	Final concentration	Amount
20% SDS	0.1%	0.25 mL
20% Triton X-100	1%	2.5 mL
0.5 M EDTA	2 mM	0.2 mL
1 M Tris-HCl pH 8.0	20 mM	1 mL
5 M NaCl	150 mM	1.5 mL
ddH_2O	N/A	44.55 mL
Total		**50 mL**
Store at 20°C–25°C for up to 3 months.		

Wash buffer 2 (High Salt Immune Complex Wash Buffer)		
Reagent	Final concentration	Amount
20% SDS	0.1%	0.25 mL
20% Triton X-100	1%	2.5 mL
0.5 M EDTA	2 mM	0.2 mL
1 M Tris-HCl pH 8.0	20 mM	1 mL
5 M NaCl	500 mM	5 mL
ddH_2O	N/A	41.05 mL
Total		**50 mL**
Store at 20°C–25°C for up to 3 months.		

Wash buffer 3 (LiCl Immune Complex Wash Buffer)		
Reagent	Final concentration	Amount
5 M LiCl	0.25 M	2.5 mL
10% NP-40 or IGEPAL CA-630	1%	5 mL
10% Na-deoxycholate	1%	5 mL
0.5 M EDTA	1 mM	0.1 mL
1 M Tris-HCl pH 8.0	10 mM	0.5 mL
ddH_2O	N/A	36.9 mL
Total		**50 mL**
Store at 20°C–25°C for up to 3 months.		

Elution buffer		
Reagent	Final concentration	Amount
20% SDS	1%	0.25 mL
1 M $NaHCO_3$	0.1 M	0.5 mL
ddH_2O	N/A	4.25 mL
Total		**5 mL**
Store at 20°C–25°C for up to 3 months.		

⚠ CRITICAL: SDS and formaldehyde are hazardous and toxic chemicals. Follow your institutional guidelines for safe use and disposal.

STEP-BY-STEP METHOD DETAILS

Fixation of differentiated adipocytes

Timing: 1 h

Many protocols suggest adding formaldehyde directly to the culture dish to fix the cells. However, if the cells are differentiated adipocytes, this will result in contamination of many lipid droplets in ChIP samples, which will interfere with immunoprecipitation efficiency. To minimize the burden of lipid droplets such as reduced sensitivity and increased non-specific signals, the author developed a protocol to enrich the nuclear fraction and reduce the lipid content by using hypotonic buffer before fixation. Hypotonic buffer is commonly used for nuclear enrichment in many experiments including ATAC-seq (Assay for Transposase-Accessible Chromatin coupled with high-throughput sequencing).[5]

1. Aspirate culture media, wash the cells with PBS (20°C–25°C), and aspirate PBS.
2. Add pre-warmed 0.05% trypsin to the dish (1 mL for a 10 cm dish), incubate for 3 min in the incubator, and collect the cells into 50 mL corning tubes with culture media (9 mL for a 10 cm dish).
3. Count the cells with a hematocytometer. Usually, the author uses two to five 10 cm dishes to obtain approximately 1×10^7 to 2×10^7 differentiated adipocytes.
4. Centrifuge at 440 × *g*, 4°C, for 5 min. Adipocytes will form loose pellet.
5. Suspend the cells in 5 mL of ice-cold PBS.
6. Centrifuge at 440 × *g*, 4°C, for 5 min.
7. Suspend the cells in 5 mL of ice-cold hypotonic buffer with protease inhibitor.
8. Incubate at 4°C for 10 min. During the incubation, gently shake the corning tube by hand a few times.
9. Add 135 μL of 37% formaldehyde to achieve a final concentration of 1% and incubate at 20°C–25°C for 7 min and 30 s on a rotary shaker. During the incubation, gently shake the corning tube by hand a few times.

10. To quench the fixation reaction, add 270 μL of 2.5 M glycine to achieve a final concentration of 125 mM, then incubate at 20°C–25°C for 5 min on a rotary shaker. During the incubation, gently shake the corning tube by hand a few times.
11. Centrifuge at 440 × *g*, 4°C, for 5 min.
12. Transfer the pellet to a 1.5 mL tube with 500 μL of ice-cold PBS with protease inhibitor.
13. Centrifuge at 5,800 × *g*, 4°C, for 5 min.
14. Discard the supernatant, then snap-freeze the 1.5 mL tube with liquid nitrogen.

⚠ CRITICAL: The fixation time is critical for obtaining optimal results, and further optimization might be required depending on your proteins and cell types of interest.

Pause point: You can store the sample at −80°C in a freezer and re-start the experiment at any time.

Lyse the cells and isolate chromatin

Timing: 30 min

In preparation for shearing the chromatin with the sonicator, lyse the cells with two different buffers to isolate chromatin.

15. Resuspend the pellet in 800 μL of ice-cold MC lysis buffer with protease inhibitor, pipetting up and down 10 times but try not to be foamed. The pellet will not dissolve completely.
16. Incubate for 10 min on ice.
17. Centrifuge at 5,800 × *g*, 4°C, for 5 min.
18. Discard the supernatant and resuspend the nuclei in 600 μL of ice-cold SDS lysis buffer with protease inhibitor. Pipetting up and down 10 times but try not to be foamed. The pellet will not dissolve completely.
19. Transfer to a new 1.5 mL tube. If you are going to compare different samples with different cell counts upon sample harvesting (step 3 in this protocol), adjust the count in this step by using ice-cold SDS lysis buffer with protease inhibitor.
20. Incubate for 10 min on ice.

Sonication to shear chromatin

Timing: 1 h

Successful sonication is critical for obtaining optimal results. Shearing DNA to 300 to 600 base pairs is optimal for most proteins and cell types. The settings shown below are the optimized parameters for the Branson Digital Sonifier 450, but different sonicators will require different parameters to obtain good results. Figure 1 shows the arrangement of the sonicator, beaker and sample.

21. Turn on the Branson Digital Sonifier 450. Use the following settings: amplitude, 0.2; pulse on for 0.5 s; pulse off for 0.5 s; time, 20 s.
22. Set the tube in a glass or plastic beaker filled with ice and water, and insert the tip of the probe to approximately 0.5 cm from the bottom of the 1.5 mL tube (the volume is 600 μL).
23. Perform sonication for 5–7 cycles, depending on the proteins and cell types of interest. Be careful not to be foamed.
24. Centrifuge at 20,400 × *g*, 4°C, for 5 min.
25. Transfer supernatant to a 15 mL corning tube.
26. Dilute the sample 10-fold with ChIP dilution buffer.

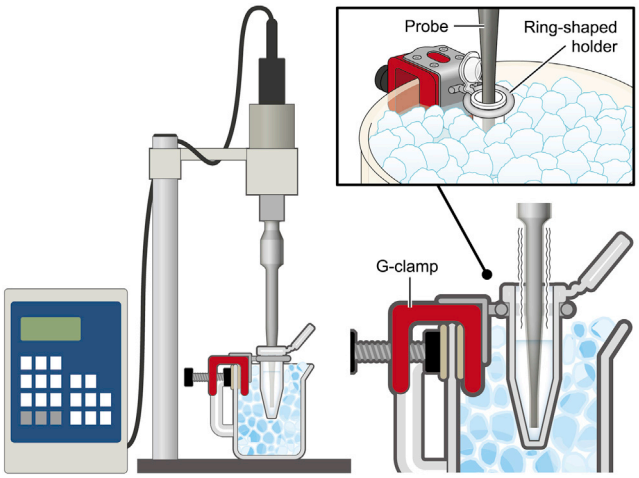

Figure 1. An illustration showing the arrangement of the sonicator, beaker, and sample

⚠ CRITICAL: The sonication is critical for obtaining optimal results, and further optimization might be required depending on your proteins and cell types of interest.

Optional: The size distribution of the sample can be analyzed by DNA electrophoresis using 2.0% agarose gel.

Immunoprecipitation

Timing: 8–12 h

The authors usually use Protein A and/or G magnetic beads for ChIP experiments. However, some antibodies yield more sensitive and specific results when used in combination with Sepharose beads. If Sepharose beads are used, the sample must be precleared before immunoprecipitation to reduce non-specific binding of any proteins in the sample to the Sepharose beads. Whichever beads are used, a mixture of protein A and G might yield better results. The author usually use 2–4 μg of antibody for each immunoprecipitation reaction.

Alternatives: If you use sepharose beads rather than magnetic beads, preclearing is required before immunoprecipitation. Sepharose beads will tend to stick to the sides of the tip upon pipetting, so cut the tip 5 mm from the end to minimize the loss of beads. (1) Add 30 μL of Sepharose beads to the diluted sample in a 15 mL tube. (2) Rotate for 30 min at 4°C. (3) Spin at 700 × *g* for 2 min at 4°C. (4) Transfer the supernatant to a new 15 mL tube.

27. Take 10% input from the sample and store at 4°C.
28. Divide the sample between several 1.5 mL tubes. Usually, the author use approximately 1400 μL of the sample for each immunoprecipitation reaction in a 1.5 mL tube.
29. Add the antibody of interest to each 1.5 mL tube.
30. Incubate for 8–12 h on a rotator at 4°C.

Incubation with beads

Timing: 4.5 h

Incubate the chromatin-antibody complex with magnetic beads.

31. Add 40 μL of magnetic beads to a new 1.5 mL tube.
32. Wash the beads by adding 500 μL of ChIP dilution buffer to the 1.5 mL tube.
33. Place the tube on a magnetic stand for 2 min to separate the beads from the solution.
34. Discard the supernatant.
35. Remove the tube from the stand and repeat the washing with 500 μL of ChIP dilution buffer twice for a total of three washings.
36. Transfer the immunoprecipitation product (incubated chromatin and antibody in the 1.5 mL tube) to the 1.5 mL tube with the washed magnetic beads.
37. Incubate for 4 h on a rotator at 4°C.

Alternatives: If you use sepharose beads rather than magnetic beads, the beads should be washed with a Spin-X column. Sepharose beads will tend to stick to the sides of the tip upon pipetting, so cut the tip 5 mm from the end to minimize the loss of beads. (1) Put 40 μL of sepharose beads into Corning Costar Spin-X Centrifuge Tube Filters. (2) Wash the beads by adding 500 μL of ChIP dilution buffer to the tube filter. (3) Spin at 700 × *g* for 2 min at 4°C. (4) Discard the waste, and repeat the washing with 500 μL of ChIP dilution buffer twice for a total of three washings. (5) Transfer the washed sepharose beads to a new 1.5 mL tube with ChIP dilution buffer. (6) Transfer the immunoprecipitation product (incubated chromatin and antibody in 1.5 mL tube) to the 1.5 mL tube with the washed sepharose beads. (7) Incubate for 4 h on a rotator at 4°C.

Washing antibody-beads complex

Timing: 2 h

Extensively wash the antibody-beads complex using multiple buffers with different salt concentrations.

38. Place the tube on a magnetic stand for 2 min to separate the beads from the solution.
39. Discard the supernatant.
40. Remove the tube from the stand and add 600 μL of ice-cold Wash buffer 1 (Low Salt Immune Complex Wash Buffer). Make sure that the beads are well-mixed with the wash buffer.
41. Place the tube on a magnetic stand for 2 min to separate the beads from the solution.
42. Discard the supernatant.
43. Remove the tube from the stand and repeat the washing with Wash buffer 1 twice for a total of three washings.
44. Repeat steps 40–43 for ice-cold Wash buffer 2 (High Salt Immune Complex Wash Buffer).
45. Repeat steps 40–43 for ice-cold Wash buffer 3 (LiCl Immune Complex Wash Buffer).
46. Add 600 μL of ice-cold TE. Make sure that the beads are well-mixed with the wash buffer.
47. Place the tube on a magnetic stand for 2 min to separate the beads from the solution.
48. Discard the supernatant.
49. Remove the tube from the stand, transfer the beads to a new 1.5 mL tube with 200 μL of elution buffer.

Alternatives: If you use sepharose beads rather than magnetic beads, the beads should be washed with Spin-X column instead of magnetic stand. Sepharose beads will tend to stick to the sides of the tip upon pipetting, so cut the tip 5 mm from the end to minimize the loss of beads.

Digestion and reverse-crosslinking

Timing: 8–12 h

Perform proteinase K digestion and reverse-crosslinking.

50. Add 2 μL of 5 mg/mL proteinase K to a final concentration of 50 μg/mL.
51. Rotate both the ChIP sample and the input in a hybridization oven for 2 h at 42°C.
52. Rotate both the ChIP sample and the input in a hybridization oven for 6–10 h at 65°C.

Alternatives: in step 52, you can also use a heat block incubator instead of the hybridization oven.

RNase A treatment and DNA purification

Timing: 1.5 h

Purify the ChIP DNA for subsequent ChIP-qPCR and ChIP-seq.

53. Place the tube on a magnetic stand for 2 min to separate the beads from the solution.
54. Transfer the supernatant to a new 1.5 mL tube.
55. Add 10 μL of 1 mg/mL RNase A to a final concentration of 50 μg/mL.
56. Incubate for 1 h at 37°C.
57. Purify DNA with a QIAquick PCR purification kit. Follow the manufacturer's instruction (Add 3 M NaOAc to adjust the pH and use 30 μL of EB buffer).

Evaluation using Qubit, Bioanalyzer, and qPCR

Timing: 3 h

Evaluate the sample using ChIP-qPCR and proceed to ChIP-seq if applicable.

58. Check the concentrations of the samples with a Qubit fluorometer. Follow the manufacturer's instructions.
59. Check the size distribution of DNA with a Bioanalyzer and Agilent High Sensitivity DNA kit. The author usually use 1 ng of DNA for this process.
60. Run a qPCR to evaluate signal enrichment. Include the positive and negative control primers.

Optional: If applicable, proceed to ChIP-seq library preparation. The author usually use 1–2 ng of DNA as a starting material and follow the manufacturer's instruction of KAPA Hyper prep kit KK8502.

EXPECTED OUTCOMES

The author usually uses 1 × 10^7 to 2 × 10^7 differentiated adipocytes for ChIP experiments with 3–4 proteins of interest. In case of representative transcription factors involved in adipocyte differentiation such as peroxisome proliferator-activated receptor γ (PPARγ) and nuclear factor I-A (NFIA), obtained DNA mass would be approximately 1–10 ng. Importantly, the obtained yield would not correlate with signal to noise ratio as evaluated by qPCR. Figure 2 shows a representative size distribution of DNA as evaluated by Bioanalyzer (Figure 2A) and a representative ChIP-qPCR result using NFI antibody in brown adipocytes (Figure 2B), and a representative ChIP-qPCR result using PPARγ antibody (Figure 2C) and H3K27Ac antibody (Figure 2D) in white adipocytes.

LIMITATIONS

This protocol has been optimized for ChIP experiment with differentiated adipocytes. However, the success of ChIP experiments strongly depends on the sensitivity and specificity of the antibody. Also, some optimization of experimental procedures might be required for your specific experiment.

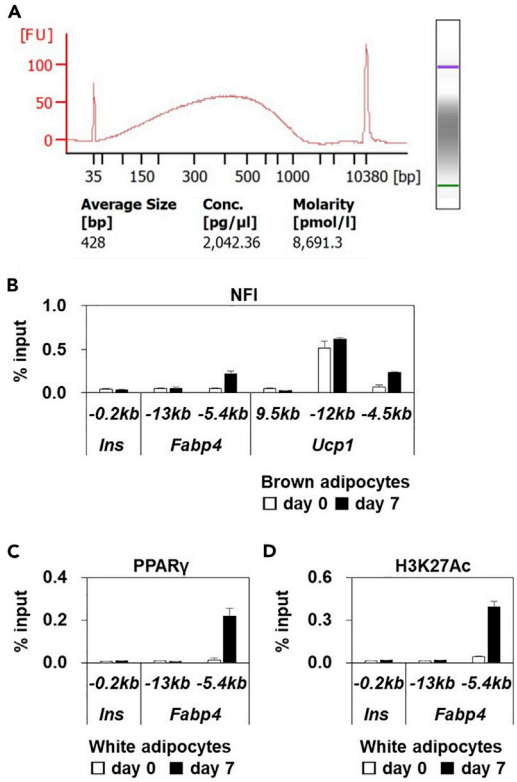

Figure 2. Representative size distribution of DNA and ChIP-qPCR

(A) Size distribution of DNA evaluated by Bioanalyzer and High Sensitivity DNA kit.

(B) ChIP-qPCR with NFI antibody, which reacts primarily with NFIA in brown adipocytes[2] at day 0 and day 7 of differentiation. *Ins* -0.2 kb, *Fabp4* -13 kb, and *Ucp1* 9.5 kb loci are shown as background sites. The binding of NFI to *Fabp4* -5.4 kb enhancer and *Ucp1* -4.5 kb enhancer can be observed in differentiated brown adipocytes. For *Ucp1* -12 kb enhancer, the binding of NFI can be observed even before differentiation (Mean ± S.E.M.; N = 2).

(C) ChIP-qPCR with PPARγ antibody in white adipocytes at day 0 and day 7 of differentiation. *Ins* -0.2 kb and *Fabp4* -13 kb loci are shown as background sites (Mean ± S.E.M.; N = 2).

(D) ChIP-qPCR with H3K27Ac antibody in white adipocytes at day 0 and day 7 of differentiation. *Ins* -0.2 kb and *Fabp4* -13 kb loci are shown as background sites (Mean ± S.E.M.; N = 2).

In particullar, the number of sonication cycles needed to achieve the optimal fragment size will vary with the model and setting of the sonicator in your lab or institute.

TROUBLESHOOTING

Problem 1

The signal of ChIP-qPCR is low (i.e., The enrichment of the positive control regions is less than 5-fold to that of the negative control regions).

Potential solution

There are multiple possible reasons for low signal (or low signal to noise ratio), but the potential solutions are as follows:

- Increase the amount of antibody used. Also, try different antibodies. The author sometimes uses a combination of multiple antibodies (related to step 29). For example, the author uses combination of two antibodies for PPARγ (Santa Cruz Biotechnology, sc-7273, and Perseus Proteomics, A3409A) and obtains good binding signals (Figure 2C).
- Increase the amount of starting material to 2×10^7 cells (related to step 3).

- Inadequate chromatin searing and longer DNA fragment might increase non-specific qPCR amplification. On the other hand, if DNA fragments are too short, they may be lost during the purification step. In many cases, 300 to 600 base pairs of DNA is optimal (related to steps 21–23). If you are comparing multiple groups in your experiment, make sure that the DNA size distribution is similar across groups (related to step 59).

Problem 2

The background noise of ChIP-qPCR is high.

Potential solution

There are multiple possible reasons for high background noise, but the potential solutions are as follows:

- Increase the repetitions of the wash steps (related to steps 40–45).
- Decrease the amount of antibody used (related to step 29).

Problem 3

Almost no signal is obtained in ChIP-qPCR.

Potential solution

The potential solutions are as follows:

- Try experiments using adipocytes that overexpress your protein of interest. Usually, the author uses retroviral vector to overexpress 3×FLAG-tagged proteins and try ChIP experiments using FLAG antibody (related to "preparation of differentiated adipocytes" in "before you begin").
- Crosslinking can affect the sensitivity and specificity of ChIP experiments. Over-fixation can mask the epitope, while under-fixation might lead to the loss of the DNA-protein interaction during the sonication (related to steps 9 and 10).
- Additional crosslinking may be required in case of proteins that do not directly bind to DNA[6,7] (related to steps 9 and 10).

Problem 4

Positive and negative control regions are unknown.

Potential solution

If the ChIP-seq in adipocytes has been published for your protein of interest, you can refer to the dataset and define the positive and negative control regions. If your protein of interest has a known DNA binding motif, you can scan a set of candidate sequences for matches to the motif, for example using FIMO online software.[4] Considering the accessible chromatin regions in adipocytes or adipose tissue[2] helps to narrow down the candidate sequences (related to step 60).

Problem 5

The obtained DNA mass is inadequate for ChIP-seq library preparation.

Potential solution

The author usually use 1–2 ng of DNA as the starting material. If necessary, consider merging samples from multiple experiments (related to step 58).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yuta Hiraike (hiraike-tky@umin.net).

Materials availability

This protocol does not include unique materials.

Data and code availability

This protocol did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

Y.H. performed the experiments, developed and optimized the protocol, and wrote the manuscript.

DECLARATION OF INTERESTS

The author declares no competing interests.

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