STAR Protocols

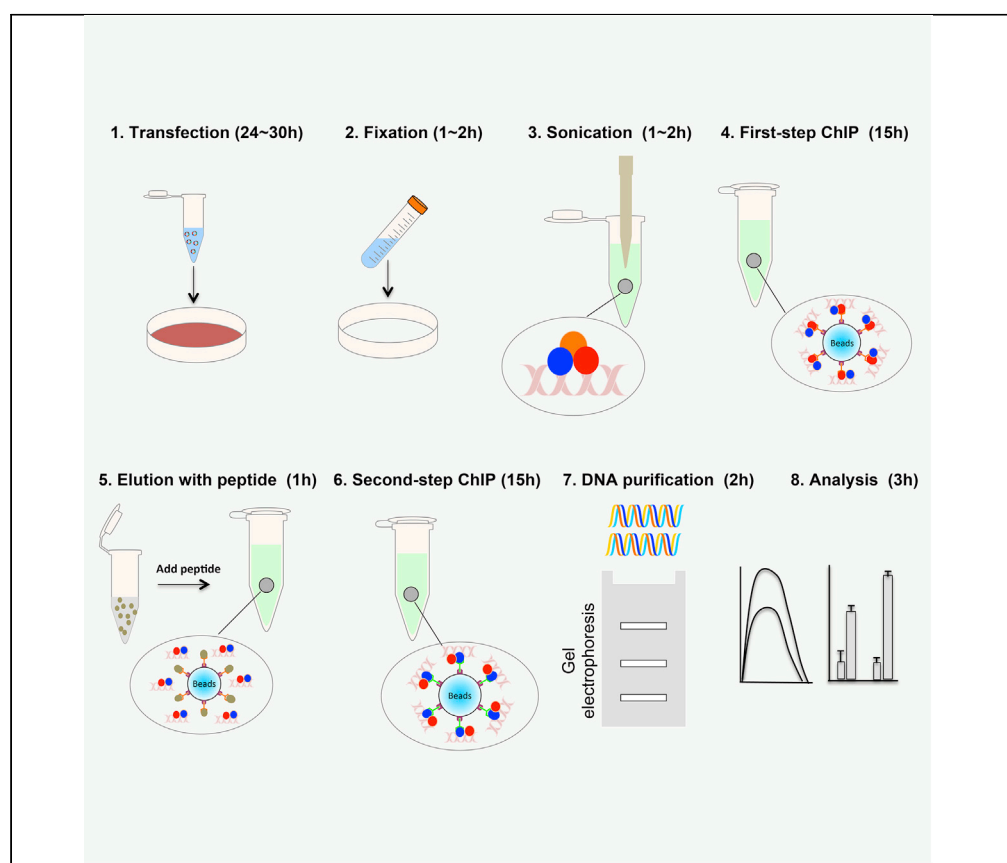**Protocol**

An optimized two-step chromatin immunoprecipitation protocol to quantify the associations of two separate proteins and their common target DNA

1. Transfection (24~30h)   2. Fixation (1~2h)   3. Sonication (1~2h)   4. First-step ChIP (15h)

5. Elution with peptide (1h)   6. Second-step ChIP (15h)   7. DNA purification (2h)   8. Analysis (3h)

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Highlights

Optimized two-step chromatin immunoprecipitation protocol

Useful to study *in vivo* association of multi-proteins to DNA regulatory elements

Physical interaction between two proteins is determined at specific genomic loci

Applicable to common mammalian cell lines and transcription factors

Sequential chromatin immunoprecipitation (ChIP) is commonly used to investigate DNA-protein and protein-protein interactions to a specific genomic region. However, it can be tricky to achieve a robust and reproducible signal with sequential ChIP. Here, we provide an optimized two-step ChIP protocol to quantify the *in vivo* associates of multiple proteins with the same DNA regulatory element.

An optimized two-step chromatin immunoprecipitation protocol to quantify the associations of two separate proteins and their common target DNA

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SUMMARY

Sequential chromatin immunoprecipitation (ChIP) is commonly used to investigate DNA-protein and protein-protein interactions to a specific genomic region. However, it can be tricky to achieve a robust and reproducible signal with sequential ChIP. Here, we provide an optimized two-step ChIP protocol to quantify the *in vivo* associates of multiple proteins with the same DNA regulatory element.

For complete details on the use and execution of this protocol, please refer to He et al. (2020).

BEFORE YOU BEGIN

Design primers

⏱ Timing: 20 min

1. Design the ChIP-quantitative PCR (qPCR) primers targeting genes of interest with an annealing temperature around 60°C and an amplicon length of 100–180 bp.
2. Test qPCR primers by amplifying targeted DNA sequence with single melting curve peak.

Note: About selection of the target genes and designing of qPCR primers, it's easy to obtain target genes from published ChIP-seq data, but the detailed binding sites of the antigens are difficult to get for one who are not skilled in bioinformatics. So we recommend ChIP-atlas tool (http://chip-atlas.org/peak_browser) in combination with IGV (integrative genomics viewer) (http://software.broadinstitute.org/software/igv/home). By using this, we can select the antigen and cell type of interest, look for novel target genes, and get the detailed binding regions.

Note: Besides several pairs of qPCR primers amplifying genes of interest are required, you also need to design a pair of primers as positive control, whose amplicon has been validated to be bound by your proteins of interest, and a pair of primers as negative control, whose amplicon is far away from the positive control's or proved not to be bound by your proteins.

Prepare 293T cells

⏱ Timing: 2–3 days

3. This protocol has been validated in 293T and MDA-MB-231 cells. These cells are cultured in Dulbecco modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin antibiotics at 37°C in 5% CO_2 (v/v). All cell lines have been tested for mycoplasma contamination.

Note: This protocol could also be reasonably applied to other common cell lines. Please see troubleshooting 4 for tips on different cell types.

Note: In terms of different transcription factors or co-factors, cell density would probably influence the proteins-DNA binding pattern. We recommend to optimize cell density before proceeding to the next step. Here, the ideal cell density for ChIP-PCR should be between 60%–80%.

Check equipment

⏱ Timing: 30 min

4. Make sure that all of the equipment work well and the centrifuges have been pre-chilled.

Note: Before performing the following experiments, it is strongly recommended to optimize the cell number, sonication cycles and SDS concentration (0.1%–0.5%). The DNA shearing length will decide the specificity and efficiency of ChIP. See troubleshooting 4.

Prepare antibodies and overexpressing plasmids

⏱ Timing: 1∼2 weeks

5. Prepare Flag-tagged (or other common tags) plasmid for overexpression of interested protein (1st step ChIP), anti-Flag antibody (1st step ChIP) and validated antibody against your protein of interest (2nd step ChIP) suitable for ChIP assay.

Note: In this protocol, we use an exogenous tagged protein as bait for the first-step ChIP, and use endogenous protein as bait for the second-step ChIP. If users use endogenous protein as bait for the first-step ChIP, it is feasible, but the IP efficiency will not be as good as the exogenous one. Besides, this will also reduce the efficiency of the second-step ChIP.

Note: Using a non-specific antibody (the same species with your primary antibody) as a control ChIP group in parallel is necessary.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
37% Formaldehyde (w/v)	Sigma-Aldrich	Cat# 252549
Dulbecco's phosphate-buffered saline (DPBS)	Gibco	Cat# C14190500CP
Glycine	Sigma	Cat# G7126; CAS: 56-40-6
Hydrochloric acid (HCl)	Sinopharm Chemical Reagent	Cat# 10011018; CAS: 2647-01-0
Sodium hydroxide (NaOH)	China Reagent	Cat# 1310-73-2; CAS: 1310-73-2

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sodium chloride (NaCl)	Sinopharm Chemical ReagentCo	Cat#10019318; CAS: 7647-14-5
Tris base	Sigma	Cat# 648311; CAS: 77-86-1
Sodium bicarbonate (NaHCO3)	Sigma	Cat# S5761; CAS: 144-55-8
Potassium hydroxide (KOH)	Sinopharm Chemical ReagentCo	Cat#10017018; CAS: 1310-58-3
EDTA	Sigma-Aldrich	Cat#U3620; CAS: 60-00-4
Triton X-100	SSCB	Cat#0694; CAS: 9002-93-1
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	Cat#L3771;CAS: 151-21-3
HEPES	Sigma	Cat#H4034; CAS: 7365-45-9
Sodium deoxycholate	Sigma-Aldrich	Cat#D6750; CAS: 302-95-4
Protease inhibitor cocktail	MCE	Cat#HY-K0010
Proteinase K	Abcone	Cat#P78893
DMEM medium	Gibco	Cat#C11995500CP
FBS	ExCell Bio	Cat#FSP500
BSA	Sigma-Aldrich	Cat#V900933
Sperm DNA	Sigma-Aldrich	Cat#31149
3xFlag peptide	APExBIO	Cat#A6001
Highgene	ABclonal	Cat#RM09014
Antibodies		
Mouse anti-CHD4	Abcam	Cat#ab70469; RRID: AB_2229454
Mouse anti-FLAG	Sigma-Aldrich	Cat#F3165; RRID: AB_259529
Critical commercial assays		
Protein A/G Plus Agarose beads	Santa Cruz Biotechnology	Cat# Sc-2003
QIAquick PCR Purification Kit (250)	QIAGEN	Cat# 28106
SYBR Green Mix	Toyobo	Cat# QPK-201
Experimental models: Cell lines		
HEK293T cell ATCC	ATCC	ATCC ACS-4500
Oligonucleotides		
Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
NR4A1	CTCTGGCTCTT GGA TCTGCCG	AGTCTGCAGTCT CTAAACCCTGC
Other		
Ultrasonic cell grinder	Shanghai Zhisun Equipment	JYD-900L
Real-time PCR machine	Roche	LightCycler® 96
Incubator	VWR	INCU-Line IL23

MATERIALS AND EQUIPMENT

The chemicals listed in the key resources table can all be replaced with identical chemicals from different suppliers with the same grade.

> *Note:* All stock solutions and buffers are pH adjusted with either 1 M HCl or 1 M NaOH solutions as required.

> *Note:* High concentration of HCl or 1 M NaOH are corrosive. Use them in the fume hood carefully.

Stock Chemical Solutions:

0.5 M EDTA, pH 8 (14.612 g EDTA, to 100 mL with ddH$_2$O; store at 4°C for six months).

Note: Adjust pH of solution with NaOH while stirring in order to dissolve EDTA powder.

1 M Tris, pH 8 (12.114 g Tris base, to 100 mL with ddH$_2$O; store at 4°C for six months)

5 M NaCl (29.22 g NaCl, to 100 mL with ddH$_2$O; store at 4°C for six months)

1 M HEPES pH 7.9 (23.83 g HEPES, to 100 mL with ddH$_2$O; store at 4°C for six months).

2.5 M Glycine (18.768 g Glycine, to 100 mL with ddH$_2$O; store at 20°C~25°C for six months)

Formaldehyde Stock Solution (37% Formaldehyde (w/v))

Note: Formaldehyde is acutely toxic. Use them in the fume hood carefully.

ChIP Lysis Buffer 500mL

Reagent	Final concentration	Amount
HEPES-KOH pH7.5	50 mM	1 M HEPES 25 mL
NaCl	140 mM	5 M NaCl 14 mL
EDTA pH8.0	1 mM	0.5 M EDTA 1 mL
Triton X-100	1%	Triton X-100 5 mL
Sodium deoxycholate	0.1%	Sodium deoxycholate 0.5 g
SDS	0.1%	SDS 0.5 g

Add fresh protease inhibitor before use. The buffer can be stored at 4°C for six months.

ChIP Wash Buffer 1 L

Reagent	Final concentration	Amount
NaCl	150 mM	5 M NaCl 30 mL
EDTA pH8.0	2 mM	0.5 M EDTA 4 mL
Triton X-100	1%	Triton X-100 10 mL
SDS	0.1%	SDS 1g
Tris-HCL	20 mM	1 M Tris-HCL 20 mL

The buffer can be stored at 4°C for six months.

ChIP Final Wash Buffer 500 mL

Reagent	Final concentration	Amount
NaCl	500 mM	5 M NaCl 50 mL
EDTA pH8.0	2 mM	0.5 M EDTA 2 mL
Triton X-100	1%	Triton X-100 5 mL
SDS	0.1%	SDS 0.5g
Tris-HCL	20 mM	1 M Tris-HCL 10 mL

The buffer can be stored at 4°C for six months.

ChIP Elution Buffer 100 mL

Reagent	Final concentration	Amount
SDS	1%	1 g
NaHCO$_3$	100 mM	0.84 g

The buffer can be stored at 20°C~25°C for one month.

STEP-BY-STEP METHOD DETAILS
Transfecting the cells with Flag-tagged expression plasmid

⏱ Timing: 24 h–30 h

1. Seed about two million 293T cells each 10 cm dish. 12–15 h later, the cell density will be up to 30%–50%, and this is an ideal density range for the transfection. Then transfect 293T cells with 10 μg Flag-tagged expression plasmid (For example, pcDNA 3.1-Flag-TEAD4) by the Lipofect-amine 2000 according to the transfection protocol (https://www.thermofisher.com).

   Note: 293T cells are easy to be suspended, so gentle operation is required for each step.

   Note: We need at least 10^7 cells, so preparing a 10 cm dish cells is recommended.

   ⚠ CRITICAL: Test the expression level of your protein of interest prior to ChIP assay. See troubleshooting 1.

   ⚠ CRITICAL: The cell density will affect the expression profile of some proteins. Controlling the cell density to an ideal range is strongly recommended. Please see troubleshooting 2.

Fixing the cells

⏱ Timing: 1 h∼2 h

2. 30 h after transfection, remove medium and wash once with 2 mL DPBS (at about 25°C) per 10 cm dish.
3. Dilute 37% Formaldehyde to a final concentration of 1% with DPBS (at about 25°C) and shake to mix.
4. Immediately add 1% formaldehyde 4 mL to cell culture dish and cover the cells.

   Note: If the users use 15 cm dish, we recommend that you should add 1% formaldehyde 8 mL. Just remember, whatever size of dish you use, ensure that all of the cells are covered by the formaldehyde solution.

   ⚠ CRITICAL: Do not add high concentration formaldehyde to the cell culture dish directly! This operation will make a damage to the antigen.

   ⚠ CRITICAL: In order to avoid any change in expression profiles, we fix the cells in natural or physiological status before digesting or scraping off the cells.

5. Incubate at 37°C for 15 min.

   ⚠ CRITICAL: The duration of fixation is very important as over-fixation will affect the antigen recognition, while under-fixation will reduce the cross-linking efficiency. See troubleshooting 5.

6. Stop the fixation by adding 1:20 volume (200 μL) of 2.5M Glycine. Shake the cell culture dish gently to mix it. Put the dish back to 37°C incubator for 5 min.
7. Pellet cells in 1.5 mL tube by scraping off the cells from the dish and spinning down at 1000 × *g* for 5 min at 4°C.

   Note: Some cell lines are difficult to scrape off. See troubleshooting 3.

Note: After this step, keep the cells on ice or 4°C unless otherwise indicated.

8. Carefully tip off supernatant, wash pellet with 1 mL cold DPBS with pipetting gently, and centrifuge the tube at 1000 × *g* for 5 min at 4°C.
9. Repeat step 8 twice then make a cell suspension in DPBS.
10. Take 10 μL of cell suspension for cell counting.

Note: If the cell density is too high, that will result in inaccurate cell counting, so you may need to dilute the sample to bring the concentration within an appropriate range. The cell number will affect the efficiency of sonication. See troubleshooting 4.

11. Centrifuge at 1000 × *g* for 5 min at 4°C and discard the supernatant.

▐▐ **Pause point:** This step you can record cell number and store the pellets at −80°C for several months.

Shearing the chromatin into a suitable size range

⏱ Timing: 1 h∼2 h

12. Add ChIP Lysis Buffer with fresh protease inhibitor cocktail (1:100) to get a cell concentration of $2×10^6$/mL, and rotate the samples for 30 min at 4°C.

⚠ CRITICAL: Cell concentration affects the following chromatin shearing efficiency, so the volume of ChIP Lysis Buffer added should be optimized. See troubleshooting 4. Besides, this step ensures the equal cell concentration of input aliquots among samples.

Note: Take 10 μL aliquot per sample for western blotting using Flag-tagged primary antibody (1:5000) to confirm the expression of the proteins that you over-expressed by transfection.

13. Take another 5 mL aliquot per sample and divide it equally into 5*1.5 mL Axygen tubes (each tube contains 1 mL sample).
14. Shear them for 25 cycles one by one by sonication (10 s on, 20 s off with power of 25% at 4°C).

Note: In order to ensure the shearing efficiency, we may need to adjust the parameters in terms of different cell lines used and different sonication equipment.

⚠ CRITICAL: This step we must make sure all the sample tubes are placed on a mixture of ice and water to protect the protein from degradation (Figure 1A). Long time continuous sonication would heat samples, so the interval between ''on'' and ''off'' should not less than 20 s to recover the temperature of the samples. See troubleshooting 5.

Optional: If your proteins of interest are easy to degrade, enzymatic fragmentation may be a good option instead of sonication.

15. Spin down all samples for 15 min at 16,800 × *g* speed at 4°C.

16. Combine the supernatants of 5 tubes from the same sample into a new 5 mL tube.
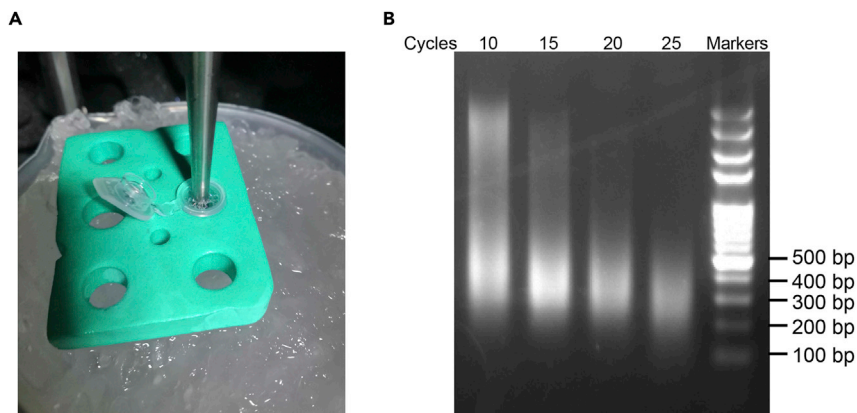
Figure 1. Schematic diagram of shearing the fixed chromatin into a suitable size-range
(A) Picture showing the sonication tube put on ice.
(B) Agarose gel picture showing the distribution of DNA fragment after chromatin sonication of different cycles.

17. Take 50 μL aliquot as 5% Input 1 control sample (in comparison with the volume of step 18) and store at −20°C.

   Note: Before performing immunoprecipitation, it is required to check the fragmentation pattern of the chromatin. The ideal DNA fragmentation for ChIP is that most of the DNA fragments are ranging from 200∼600 bp after sonication. If not, we need to optimize the cell concentration, sonication time and cycle (Figure 1B), or SDS concentration until it shows an allowable range. See troubleshooting 4.

Performing the first-step ChIP

   ⊙ **Timing: 15 h**

18. Take four 1.5 mL EP tubes for the following IP, and each is approximately 1 mL.
19. Add 2 μg primary antibody targeting Flag-tagged fusion protein A and IgG (In this study, three tubes with mouse anti-Flag and one with mouse IgG as a control). Rotate overnight at 4°C.

   Optional: Cell samples transfected with an empty vector or not-transfected can be also used as a negative control.

   Note: The species for IgG antibody is recommended the same with Flag tag antibody.

   Optional: As appropriate, use other tag antibodies as control, such as mouse anti-V5.

20. Prepare ChIP Lysis Buffer with sperm DNA and BSA to 1 mg/mL final concentration, and block Protein A/G Plus Agarose beads by adding prepared ChIP Lysis Buffer with same volume as beads and incubating for one hour at 4°C.

   Note: This step is to block the beads and avoid the nonspecific binding between agarose beads and proteins or genomic DNA fragments.

21. Prepare four 1.5 mL tubes added with 60 μL buffer (containing 30 μL beads) for each tube, centrifuge the agarose beads at 1000 × *g* for 1 min, and remove the supernatant from the tubes. Then add the cell lysate-antibody to the tube loaded with 30 μL beads, and rotate at 4°C for 2 h.
22. Centrifuge the tube for 1 min at 2,000 × *g* and remove the supernatant.

23. Wash beads three times with 1 mL ChIP Wash Buffer at 2,000 × *g* for 2 min.
24. Wash beads with 1 mL ChIP Final Wash Buffer at 2,000 × *g* for 2 min.

   Note: The ChIP Final Wash Buffer with high salt concentration can remove the nonspecific binding.

Eluting the immunocomplexes from the first step and performing the second-step ChIP

   ⏱ Timing: 15 h

25. Elute two tubes of the immunocomplexes (added with anti-Flag antibody) from beads by adding 0.5 mg/mL 3×Flag peptide (Sigma) in 100 μL ChIP Lysis Buffer rotating at 4°C for one hour. The rest two tubes have finished their process of first-step ChIP. Please store them at −20°C for elusion step (step 32).

   Note: If you used the other tags instead of Flag, please elute the immunocomplexes from the beads by adding the same concentration (0.5 mg/mL) peptide of your tag in 100 μL ChIP Lysis Buffer.

   Note: In this step, elution by rotating the tubes is required to sufficiently elute the proteins-DNA complex from the beads.

26. Centrifuge at 2,000 × *g* for 2 min and collect the supernatant.
27. Two sequential elutions are performed as steps 25 and 26.
28. Collect the eluent together from three times and take 15 μL aliquot as 5% Input 2 sample.
29. Dilute the eluted 285 μL protein-DNA complex by adding 855 μL ChIP Lysis Buffer (1:3), supplemented with 1% Triton X-100.
30. Incubate the samples in the two tubes with 3 μg of normal IgG or anti-Protein B antibody (such as mouse anti-CHD4 antibody) respectively, and rotate overnight at 4°C.
31. Repeat steps 20–24 for these two tubes.

Eluting and reversing cross-link

   ⏱ Timing: 1 day

32. Add 400 μL ChIP Elution Buffer to the beads and incubate at 65°C for 30 min.

   Note: From this step, take all the samples out including Input 1 from step 17, first-step ChIP immunocomplexes from step 24, Input 2 from step 28, and second-step ChIP immunocomplexes from step 31.

   Note: Supplement the Input samples to a final volume of 400 μL with ChIP Elution Buffer.

33. Add 16.8 μL NaCl (from 5 M stock) to each tube, and incubate at 65°C for at least 4 h or overnight.

   Note: Ensure sufficient incubation for at least 4 h.

34. Add 4 μL EDTA (from 0.5 M stock) and 5 μL Proteinase K (from 20 mg/mL stock) to each tube and incubate at 55°C for 2 h.

35. Centrifuge at 16,800 × *g* for 15 min and collect the supernatant which contains the eluted DNA from immunoprecipitation.

Purifying the DNA from the immunocomplexes

⊙ Timing: 2 h

36. Purify DNA from immunoprecipitation or input with QIAQUICK PCR Purification Kit (250) (cat N.O., 28106, QIAGEN) according to the manual's instruction.
37. The purified DNA are resuspended in 30 μL ddH$_2$O and the concentration is measured by NanoDrop.
38. Take 5 μL aliquot of Input 1 sample and run DNA agarose gel electrophoresis to verify the shearing efficiency before qPCR.

    Note: We check the shearing efficiency again just for verifying the shearing efficiency of this time to ensure the reliability of our results.

    ⚠ CRITICAL: The ideal DNA fragment size should range between 200 and 600 bp. Do not test chromatin fragmentation by running the crosslinked chromatin-protein complex. See troubleshooting 4.

    Note: The DNA concentration of Input 2 and ChIP is possibly less than 2 ng/μL, which is acceptable.

    Note: Purified samples can be stored at −20°C.

Running ChIP-qPCR and analyzing the data

⊙ Timing: 3 h

39. Dilute DNA sample from Input 1 with more 120 μL ddH$_2$O to get a 1% input 1.

    Note: The DNA concentration of Input 1 sample should be higher than ChIPed DNA with many folds, so we need to dilute the DNA from Input 1 accordingly.

40. Run qPCR of all of the samples in the following system with qPCR Machine (Roche, LC96).

Sample	Volume
Template DNA	1 μL
2*SYBR SYBR Green Master mix	10 μL
Primers (forward, 10 μM)	1 μL
Primers (reverse, 10 μM)	1 μL
ddH$_2$O	7 μL

    ⚠ CRITICAL: Besides investigating the gene loci that you interested in, it is necessary to verify your ChIP quality by checking the positive loci that can be enriched by the protein of interest which has been demonstrated, as well as the negative loci that is far away from the positive one.

41. Assessing the enrichment of the proteins of interest on the targeting region by calculating the value of "fold over IgG" of each step ChIP using the following equations:

$$\text{Fold over IgG (first-step)} = 2^{\text{Ct (IgG from first-step ChIP)} - \text{Ct (first-step ChIP)}}$$
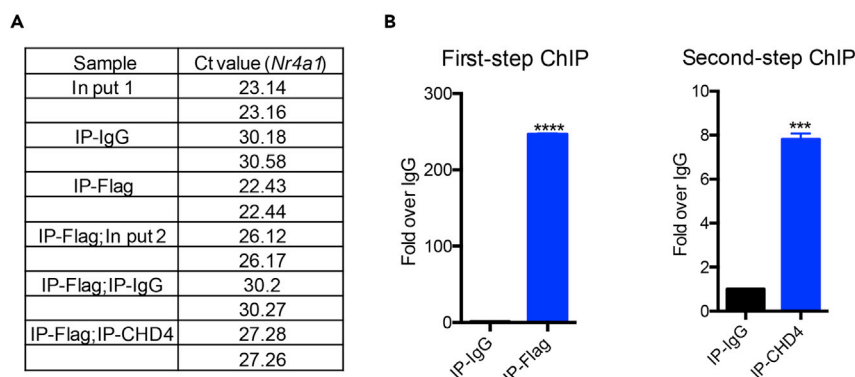
Figure 2. Analysis of ChIP-PCR data
(A) The Cq value of ChIP-PCR from the first step and second step.
(B) Two-step ChIP-qPCR analysis both of TEAD4 and CHD4 enrichment at the *nr4a1* promoter in HEK293T cells.

$$\text{Fold over IgG (second-step)} = 2^{\text{Ct (IgG from second-step ChIP) - Ct (second-step ChIP)}}$$

Note: The value of fold over IgG is used to assess the ChIP efficiency. Usually, the fold over IgG of positive control is supposed to be more than 10, and the negative control is approximately 1 (barely enriched). Additionally, the Ct value is also an important parameter to verify the ChIP quality. The negative control should ideally have a C_t value with more than 30, whereas the Ct value of the target genes for 1% Input 1 is in the range of 20–26, and ideally <28 for the positive control. The Ct value of the target genes for Input 2 is in the range from IgG to Flag of step one ChIP (Figures 2A and 2B). Please see troubleshooting 5.

Optional: We can also assess the enrichment of the proteins of interest on the targeting region by calculating the value of ''% of input'' of each step ChIP using the following equations:

$$\text{\% of input (first-step)} = 1\% * 2^{\text{Ct (Input 1) - Ct (first-step ChIP)}}$$

$$\text{\% of input (second-step)} = 5\% * 2^{\text{Ct (Input 2) - Ct (second-step ChIP)}}$$

Note: If you choose this equation, usually, the % of input absolute value of the positive control is supposed to be more than 0.5, and the negative control is less than 0.1. The relative value of positive loci signal/IgG signal should be more than 10 times, and the relative value of negative loci signal/ IgG signal should be about 1.

EXPECTED OUTCOMES

The sheared genomic DNA fragments are observed as expected. The first and second steps of IP work well. Positive gene loci are well enriched in both the ChIPed samples but not the negative loci. To ensure the efficiency of subsequent immunoprecipitation, it is critical to control the DNA fragments mostly in the range of 200–600 bp. See troubleshooting 4 for approaches to improve this result. To a great extent, the primary antibody decides the affinity of DNA binding. Ideally, the positive control should be well enriched, while the negative control should not be so. The IP efficiency of the first step largely affects the output of the second step. In general, the enrichment fold over IgG (first step) on the positive gene loci is supposed to be more than 30 in the experimental group. The Ct value is also an important

indicator. The Ct value of the negative control should be more than 30. The positive control should be less than 28. See troubleshooting 5 for approaches to improve this result.

LIMITATIONS

This protocol has been tested with some transcription factors/cofactors (including TEAD4-CHD4 and TEAD4-GR) (He et al., 2019; He et al., 2020) in several cell lines. So it should also work in any couple of proteins (protein A and B) and cell lines. However, the users still need to optimize their specific experiment with different cell lines and interested proteins. Firstly, in different cell lines, the users should optimize the initial cell number, sonication condition, and SDS concentration in ChIP Lysis Buffer until get DNA fragments in the range of 200–600 bp (see troubleshooting 4). Secondly, for different protein of interest, the users should construct it in a recombinant protein-A expressing-plasmid with tag, such as Flag tag. The primary tag and anti-protein B antibodies should be ChIP-grade. Which one is chosen as protein A in the coupled proteins depends on the users.

TROUBLESHOOTING

Problem 1

The expression level of Flag-tagged protein A is not good (step 1).

Potential solution

The expression level of Flag-tagged protein A depends on vector, transfection efficiency and the antibody recognition efficiency. We recommend using pcDNA3.1 vector for the recombinant plasmid. And transfection efficiency needs to be verified in a new cell line. One should check the transfection efficiency by using different transfection methods, such as lipofectamine, calcium phosphate transfection, electroporation. To enhance the recognition efficiency of antibody to Flag, clone a three repeated Flag tag and use the validated anti-Flag antibody (Sigma, F3165) will be helpful.

Problem 2

The cell density is too high or too low (step 1).

Potential solution

Cell density has a great influence on the binding pattern of some proteins on genomic DNA. Before cell fixation, it's important to check the cell density to see whether it's in a suitable range according to the experimental requirements, For example, the binding of TEAD4 on the *Nr4a1* promoter region is largely affected by density—the enrichment of TEAD4 on the *Nr4a1* promoter region is decreased in high cell density (He et al., 2020). A very low cell density may be due to poor cell condition caused by transfection. Changing the medium 8 h after transfection can reduce the cytotoxicity. In addition, adjusting cell number when seeding can effectively control cell density.

Problem 3

It is difficult to scrape off cells from dish after fixation (step 7).

Potential solution

To avoid altering the binding pattern of the protein of interest, we strongly recommend fixing the cells in their natural state: fix adherent cells when they are attached but not digest them; Fix suspended cells after centrifugation. However, some adherent cells are difficult to scrape off from the cell culture dish after fixation. In this case, we recommend digesting and collecting cells before fixing. Please note that this process must be performed quickly to avoid alteration of gene expression profile in cells. For suspension cells, centrifuge the cells in a low speed (100 × g) and short time (less that two minutes) before fixation in case that quick and long-time centrifugation lead to binding pattern change of the protein of interest. Then discard the supernatant and add 4% formaldehyde solution to re-suspend and fix the cell.

Problem 4

The chromatin fragmentation size is not in the recommended range (step 14).

Potential solution

To get appropriate fragment size in different cell lines, usually, we need to optimize the following conditions.

The cell number: Too many cells will result in incomplete sonication and long DNA fragments with more than 600 bp, while too few cells result in short fragments below 100 bp. Cell counting in step 10 is critical to decide the cell concentration of sonication. Adjusting the cell number will be helpful.

The power and number of cycles of sonication: High power will cause too many samples loss, while low power will cause incomplete sonication. When the concentration of cells and the power of sonication are determined, the size of DNA fragments usually decreases as the number of sonication cycles increases. Adjusting the power and number of cycles of sonication according your cell types will bring the DNA fragment size to an ideal range.

SDS concentration in the ChIP Lysis Buffer: In some special cells, such as C2C12 and 3T3-L1 (Feng et al., 2019; Zhang et al., 2018), reducing the cell number or increasing the sonication cycles may not be useful. In this situation, increasing the SDS concentration in the ChIP Lysis Buffer may be beneficial to the DNA shearing. Please note that the SDS concentration should not exceed 0.5%. Too high SDS concentration will affect antigen-antibody binding in the IP steps, so after shearing DNA, the buffer containing high concentration of SDS needs to be diluted with normal ChIP Lysis Buffer.

Before performing the full ChIP protocols, we highly recommend to optimize the above conditions first. To save time, users can collect DNA samples from the following pipeline: chromatin shearing, reverse crosslinking, and the DNA purification. Then check the DNA fragments size by DNA gel electrophoresis. After confirming the optimal conditions, thaw the remaining samples and complete the whole protocols.

Problem 5

The positive gene loci are not enriched well in the first-step ChIP sample (the fold over IgG is less than 10), or the negative loci are enriched (the fold over IgG is more than 2 and Ct value is less than 28) (step 42).

Potential solution

The following reasons will lead to the controls do not work in the first-step ChIP.

Fixation time is longer than 15 min: Generally, 15 min fixation time at 37°C can be applied to all transcription factors we have studied, so we set this as a default time. Longer fixation times will mask the antigen and increase the risk of non-specific binding. Users can optimize the fixation times between 10 min and 15 min. To note that the crosslink time will affect the subsequent steps, so re-optimization is required. For some higher-order interactions, such as transcription co-factors-DNA, longer cross-linkers such as EGS (16.1 Å) or DSG (7.7 Å) can be used in combination with formaldehyde solution to capture larger protein complexes. Please note that the DSG is water-insoluble non-storable in solution.

The target gene locus is of low abundance: Increasing the Proteins-DNA complexes by enhancing the reaction samples of ChIP system may be helpful, but the relative volume of Input does not change. Do not change the optimized cell number used in sonication, but just increase the IP volume after shearing. Be aware that this operation will also bring a high binding background, so increasing time of the ChIP Final Wash buffer with high salt concentration in step 24 will reduce non-specific binding.

The DNA fragments are below 100 bp: This will bring two bad results: much DNA loss when purification and reversing crosslink of proteins and DNA before IPs. Please see troubleshooting 4 to get optimized DNA fragments.

The proteins are degraded during sonication: Sonication makes the temperature rise, and the proteins are more easily to be degraded. Firstly, try to raise the concentration of protease inhibitors cocktail, and secondly, extend the ''off ''time of sonication to 30 s to allow the sample cool down. It is also very important to put the tube in the ice-water mixture when sonication. To preliminarily judge whether the proteins are degraded, check the liquid. Normally, the liquid is clear after sonication, while the turbid liquid may indicate the proteins have been degraded.

Poor specificity of antibodies: To enhance the specificity of antibodies, block the beads with sperm DNA and BSA prior to addition of them (step 20). Washing the beads with ChIP Final Wash Buffer twice according to step 24 will be also helpful to reduce non-specific binding.

Problem 6
The positive control DNA region is not enriched in the step-two ChIP (step 42).

Potential solution
Low enrichment of positive gene loci in the second-step ChIP may due to a poor elution efficiency. A good option is to enhance the concentration of Flag peptide, extend elution time and dilute the eluted chromatin with 1:5 ChIP Lysis Buffer. Besides, increasing the sample of first-step ChIP will also help to enhance the IP yield of second step.

Problem 7
There is not an alternative primary antibody for second-step ChIP (Prepare antibodies).

Potential solution
Not all antibodies work well for ChIP. Sometimes you need to identify the antibodies from some candidates that can be used for ChIP. To verify that the protocol work well as intended, we suggest verifying by doing a ChIP-qPCR on a positive gene locus. Nevertheless, there may be still no good antibodies to choose. Alternatively, we recommend constructing a tagged-recombinant plasmid and use an overexpression system. Thus, you just need a ChIP-grade antibody targeting tag protein.

Advantages of two-step ChIP compared to the classical ChIP:

This protocol has a wider applicability compared with classical ChIP. By following this protocol, we can study the binding region of multiple related proteins from one experiment.

The results can be verified by changing the ChIP order of these two proteins to enhance to reliability of the conclusion.

We can use two-step ChIP to further confirm the genetically physical association between two proteins.

Compared with two independent ChIP, the second step ChIP of this process has been enriched and noise-reduced by the first step ChIP. Therefore, the sensitivity and specificity of the second one are improved.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Lei Zhang (rayzhang@sibcb.ac.cn).

Materials availability
This study did not generate any new unique reagents.

Data and code availability

This study did not generate any unique datasets or code.

AUTHOR CONTRIBUTIONS

L.H., W.Y., and W.Z. optimized the protocol. L.H. wrote the manuscript. W.Y. and W.Z. edited the manuscript. L.H. and L.Z. supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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