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An optimized chromatin immunoprecipitation protocol using Staph-seq for analyzing genome-wide protein-DNA interactions.

Fang Tao
Children's Mercy Hospital

Egidy Rhonda

Xi He

John M. Perry
Children's Mercy Hospital

Linheng Li

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Protocol

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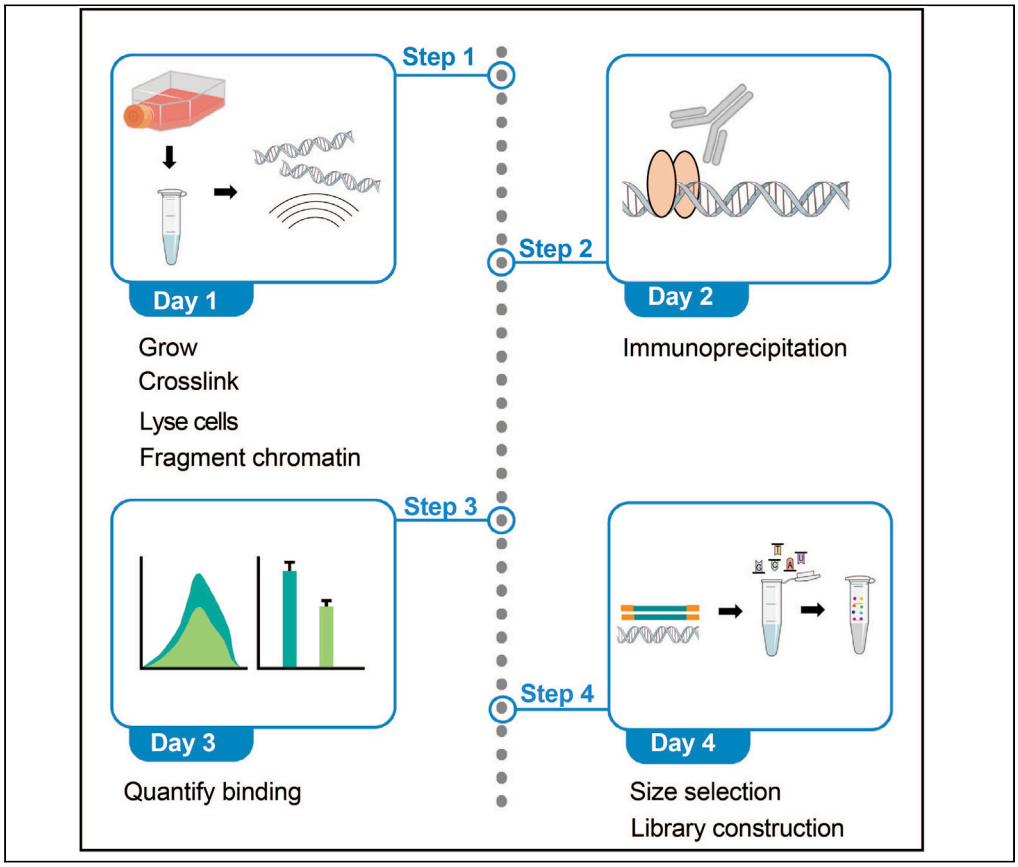

Genome-wide mapping of transcription factors (TFs) is critical to understand their functions. In chromatin immunoprecipitation (ChIP)-seq assay, it's challenging to study recruitment of low-abundant TFs transiently bound to the genome. Here, we present an optimized protocol using ChIP Next-Gen Seq Sepharose (Staph-seq) to efficiently pull down chromatin complexes. The double size selection promotes sensitive capture of genome-wide protein-DNA associations while eliminating potential Staph A contamination, which is a common problem in protocols using Staph A cells.

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

Fang Tao, Egidy Rhonda, Xi He, John M. Perry, Linheng Li

ftao@cmh.edu (F.T.)
lil@stowers.org (L.L.)

Highlights

A protocol for analyzing protein-DNA association of low-abundant transcription factors

Full details for sonication, immunoprecipitation, and library preparation

Double size selection optimizes signal with minimal background noise

The protocol describes steps for mouse ESC but can be applied to other cell types

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An optimized chromatin immunoprecipitation protocol using Staph-seq for analyzing genome-wide protein-DNA interactions

Fang Tao,[1,2,3,*] Egidy Rhonda,[2] Xi He,[2] John M. Perry,[1] and Linheng Li[2,4,*]

[1]Children's Research Institute, Children's Mercy Kansas City, Kansas City, MO 64108, USA

[2]Stowers Institute for Medical Research, Kansas City, MO 64111, USA

[3]Technical contact

[4]Lead contact

*Correspondence: ftao@cmh.edu (F.T.), lil@stowers.org (L.L.)
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SUMMARY

Genome-wide mapping of transcription factors (TFs) is critical to understand their functions. In chromatin immunoprecipitation (ChIP)-seq assay, it's challenging to study recruitment of low-abundant TFs transiently boud to the genome. Here, we present an optimized protocol using ChIP Next-Gen Seq Sepharose (Staph-seq) to efficiently pull down chromatin complexes. The double size selection promotes sensitive capture of genome-wide protein-DNA associations while eliminating potential Staph A contamination, which is a common problem in protocols using Staph A cells.

For complete details on the use and execution of this protocol, please refer to Tao et al. (2020).[1]

BEFORE YOU BEGIN

This protocol describes the specific steps for using mouse embryonic stem cells (ESCs). However, in principle this method should be applicable to other cell lines. Regular ChIP performed with agarose protein A/G beads or Dynabeads often are inefficient for rare TF-associated chromatin complexes.[2] Staphylococcus aureus (Staph A) with high density of protein A on the cell wall has been used to pull-down protein-DNA complexes.[3] However, we found substantial carry-over of Staph A DNA in the sequencing result that over 80% reads were mapped to bacteria genome. In this protocol, we use ChIP Next Gen Seq Sepharose (Staph-seq), which is modified Staph A to prevent Staph A DNA contamination and achieve improved signal-to-noise ratio.

Preparing buffers

Timing: 0.5–2 h

1. Make sure all required solutions are prepared and ready to use.
2. Use fresh formaldehyde that is < 3 months old.

Designing primers

Timing: 1–2 h

3. Initial check by qPCR is recommended to make sure that the procedure worked. Use known target genes or loci that the protein of interest binds to design primers. Make sure the size of amplicons

is smaller than the sheared genomic DNA. Optimally, the amplicons should be around 100–150 bp so the PCR amplified region does not span the nucleosome-free region.

⚠ CRITICAL: Use primer blast https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi to make sure primer pairs are specific to input template as no other targets are found in selected database:

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-E2F6	Abcam	Cat#AB53061
Chemicals, peptides, and recombinant proteins		
37% formaldehyde	Sigma-Aldrich	Cat#252549
Proteinase K solution	Invitrogen	Cat#25530-049
RNase A	Sigma-Aldrich	Cat#R6513
HEPES pH7.5	Self-Made	N/A
500 mM EDTA	Invitrogen	Cat#15575-38
500 mM EGTA	Sigma-Aldrich	Cat#E3889
2.5 M Glycine	Sigma-Aldrich	Cat#G8790
20 mg/mL Glycogen	Roche	Cat#901393
Neurobasal medium	Invitrogen	Cat#21103-049
DMEM/F12 medium	Invitrogen	Cat#10565-018
N2	Invitrogen	Cat#17502-408
B27	Invitrogen	Cat#17504044
CHIR99021	Tocris	Cat#4423
PD0325901	Stem Cell Technologies	Cat#72184
β-mercaptoethanol	Millipore	Cat#ES-007-E
L-glutamine	Invitrogen	Cat# 25030081
Non-essential amino acid (NEAA)	Stem Cell Technologies	Cat# 07600
Bovine serum albumin fraction V (7.5% solution)	Invitrogen	Cat#15260037
Bovine serum albumin	Sigma-Aldrich	Cat#B6917
Proteinase inhibitor cocktail	Sigma-Aldrich	Cat#4693159001
Critical commercial assays		
Power SYBR™ Green PCR Master Mix	Applied Biosystems	Cat#4367659
ChIP Next Gen Seq Sepharose	Sigma-Aldrich	Cat#S6576
KAPA HTP Library Prep Kit	Roche	Cat#KK8234
Sciclone G3 NGS Workstation	Perkin Elmer	Cat#134386
NEXTflex DNA barcodes	Perkin Elmer	Cat#NOVA-514104
Agencourt AMPure XP system	Beckman Counter	Cat#A63880
Pippin prep 2% Agarose Gel Cassette	Sage Science	Cat#CDF-2010
Agilent High Sensitivity DNA kit	Agilent	Cat#5067-4626
Qiagen PCR purification kit	Qiagen	Cat#28104
Accutase	Stem Cell Technologies	Cat#07920
Deposited data		
Raw data	This paper	GSE108620
Experimental models: Cell lines		
V6.5 mouse embryonic stem cells	Novus Biologicals	Cat#NBP1-41162
Oligonucleotides		
Zcwpw1 Forward:AAAAGTTGCTCGAGGTGGAA	N/A	N/A
Zcwpw1 Reverse:TTCCTCTGCAGTCGCATAAA	N/A	N/A
Rab8a Forward:CGAAAATCCTCCTCAGAAGC	N/A	N/A
Rab8a Reverse:CAGAGAACGCCTCGTGTTTC	N/A	N/A
Gene desert Forward:CACGGGAGCTCAAGACAAGA	N/A	N/A
Gene desert Reverse:AGGGGAAAGAGGCACTTGTG	N/A	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
HiSeq Control Software 2.2.58	Illumina	Cat#FC-401-4002
Other		
1.5 mL Bioruptor® Pico Microtubes with Caps	Diagenode	Cat#C30010016
Covaris S220 focused-ultrasonicator	Covaris	Cat#500217
Biorupter pico sonicator device	Diagenode	Cat#B01060010
MicroAmp fast optical 96-well reaction plate	Life Technologies	Cat#4346906
StepOne™ Real-Time PCR System	Applied Biosystems	Cat#4376357
Bioanalyzer	Agilent	Cat#G2939B
Qubit 4 Fluorometer	Fisher Scientific	Cat#Q33238
Cellometer Auto T4 cell counter	Nexcelom	N/A
Eppendorf Thermomixer	Fisher Scientific	Cat#05-400-205
Eppendorf Safe-Lock microcentrifuge tubes	Eppendorf	Cat#022363204
Cooled microcentrifuge for 1.5–2.0 mL tubes	Eppendorf	Cat#5425R

MATERIALS AND EQUIPMENT

ChIP lysis buffer		
Reagent	Final concentration	Amount
1 M HEPES pH 7.5	15 mM	3.75 mL
5 M NaCl	140 mM	7 mL
0.5 M EDTA	1 mM	0.5 mL
0.5 M EGTA	0.5 mM	250 µL
Triton X-100	1%	2.5 mL
10% sodium deoxycholate	0.1%	2.5 mL
10% SDS	1%	2.5 mL
10% N-lauroylsarcosine	0.5%	12.5 mL
Total	**N/A**	**Add ddH_2O to 250 mL**
Add protease inhibitors just before use, filter, and store at 4°C for 6 months maximum.		

ChIP wash buffer		
Reagent	Final concentration	Amount
1 M HEPES pH 7.5	50 mM	25 mL
0.5 M EDTA	1 mM	1 mL
10% sodium deoxycholate	0.7%	35 mL
10% NP-40 (IGEPAL CA-630)	1%	50 mL
8 M LiCl	0.5 M	31.25 mL
Total	**N/A**	**Add ddH_2O to 500 mL**
Filter and store at 4°C for 6 months maximum.		

ChIP elution buffer		
Reagent	Final concentration	Amount
1 M Tris pH 8	50 mM	5 mL
0.5 M EDTA	10 mM	2 mL
10% SDS	1%	10 mL
Total	**N/A**	**Add ddH_2O to 100 mL**
Filter and store at 4°C for 6 months maximum.		

TE buffer		
Reagent	Final concentration	Amount
1 M Tris pH 8	10 mM	1 mL
0.5 M EDTA	1 mM	200 μL
Total	N/A	Add ddH_2O to 100 mL

Filter and store at 4°C for 6 months maximum.

Alternatives: This protocol uses a Bioruptor to fragment genomic DNA. Alternatives are Misonix 3000 with Microtips, which allows higher volume of cell lysis buffer when working with large number of cells. We typically begin with power setting 5.5 and 8 cycles (where each cycle is a 30 s burst of sonication, followed by a 90 s pause).

STEP-BY-STEP METHOD DETAILS

Blocking ChIP Next Gen Seq Sepharose

Timing: Day 0, 0.5–1 h

1. Prepare BSA: reconstitute 250 mg pure BSA powder with 12.5 mL of cold molecular biology grade water (DNase-, RNase-, Protease- free) water, mix by vortexing and inverting to make a 20 mg/mL solution. Make 10 μL aliquots and freeze at −20°C.
2. Add 200 μL molecular biology grade water and 10 μL of 20 mg/mL BSA to each vial of Staph-seq. Incubate on rotating platform at 4°C for 16 h and store at 4°C until step 19.

Note: For best results, we recommend preparing the Staph-seq on Day 0, the evening before you start the ChIP. If time constrained, the incubation may be shorted to 3 h at 4°C or 2 h at 23°C. The blocked Staph-seq can be used up to 2 weeks.

Harvesting and cross-linking cells

Timing: day 1, 3 h

Mouse embryonic stem cells are harvested in single cell suspension and cross-linked.

3. Harvesting cultured ESCs.
   a. V6.5 mouse ESCs are plated without feeder in serum-free N2B27 complete media. For serum-free 2i culture, ESCs were cultured without feeders or serum in N2B27 medium (1 × neurobasal medium, 1 × DMEM/F12, 0.5 × N2, 0.5 × B27,1 × β-mercaptoethanol, 2 mM L-glutamine, 100 μM non-essential amino acid, 0.033% BSA, 3 μM CHIRON99021, 1 μM PD03).
   b. Use ∼1–2 × 10^7 ESCs for every Staph-seq immunoprecipitation.
   c. Add 5 mL Accutase per 15-cm plate of ESCs and incubate at 37°C for 5 min.
   d. Add 10 mL 1 × PBS to dilute and stop Accutase activity, followed by counting cell number.
   e. Centrifuge at 500 *g* for 5 min at 23°C and resuspend in 1 × PBS volume of PBS to a concentration of 1 × 10^6 ESCs/mL.
4. Cross-linking ESCs.
   a. Dilute fresh 37% formaldehyde to 2%.
   b. Add equal volume of freshly prepared 2% formaldehyde to the cell suspension in a 50 mL conical tube. Swirl briefly.
   c. Incubate by gentle mixing on a nutator for 10 min at 23°C.
   d. Quench formaldehyde with 1/20 volume of 2.5 M glycine for 5 min at 23°C by gentle mixing on a nutator.

e. Centrifuge at 700 *g* for 10 min at 4°C.
f. Wash cells by resuspending the pellet with glycine in PBS twice (50 mL 1 × PBS with 1 mL 2.5 M glycine). At the second wash, keep cells in glycine for 1 min.
g. Centrifuge at 700 *g* for 10 min at 4°C. Discard the formaldehyde and glycine mix following the hazardous chemical waste disposal guideline.
h. Resuspend the cell pellet in 20 mL 1 × PBS (Cells may stick to the tube or pipette at this stage).
i. Add 20 mL 1 × PBS to wash off any carry over cells stuck to the side of tube or pipette.
j. Centrifuge at 700 *g* for 10 min at 4°C and discard the supernatant.
k. Repeat h-j once more, total of two times.

Pause point: Cells can be used immediately or snap frozen in liquid nitrogen and stored at −80°C. However, freezing cells at this stage inevitably results in rupture of some nuclei and partial loss of chromatin.

⚠ CRITICAL: Efficient cross-linking is achieved by mixing 2% formaldehyde with single-cell ESC suspension to get a 1% final concentration of formaldehyde. Make sure ESCs colonies are separated into single-cell suspension by pipetting gently for at least 10 times with minimal bubbles while pipetting.

Chromatin solubilization and shearing

Timing: day 1, 3 h

Cells are lysed by sonication to obtain sheared chromatin. The efficiency of sonication varies with cell type, quantity, volume of sonication and specifics of the sonicator used. We suggest optimizing sonication conditions for each experimental situation before processing large quantities of cells.

5. Add protease inhibitor to ChIP lysis buffer before use.
6. Resuspend each cell pellet of ~1–2 × 10^7 cells in 300 μL cold ChIP lysis buffer. Incubate on platform rocker at 4°C for 10 min.

Optional: Save a 50 μL aliquot as a pre-sonication control.

7. Transfer lysates to Bioruptor Pico microtubes and sonicate with Bioruptor with the setting of 30 s on, 30 s off for 16 cycles. The lysate should become as clear as water with no visible debris after sonication. Troubleshooting 1.

⚠ CRITICAL: When loading samples into the tube holder of Bioruptor, make sure all slots are filled with microtubes containing equal volume of samples or water for balance.

8. Transfer sonicated lysates to Eppendorf 1.5 mL polypropylene tubes and centrifuge at 20,000 *g* at 4°C for 10 min.

Note: The Bioruptor Pico microtubes may crack at maximum speed of centrifuging.

9. Carefully transfer supernatant (sonicated chromatin) to a new tube and store at 4°C. Save the cell debris pellet in case of under sonication to recombine with the supernatant and repeat sonication until desired results are achieved.

⚠ CRITICAL: From here on, keep lysates on ice.

Note: Wear hearing protection while working with sonicator. For larger quantity of cells with sample volume between 0.5 mL and 2 mL, we prefer to use Misonix sonicator with 15 mL

tubes. Use a clamp to secure the tube with sonicator probe centered and 0.5–1 cm above the bottom of the tube. The sonicator probe should never be in contact with the sides of the tube. Immerse tube in the ice-water bath during sonication. Avoid foaming, which indicates poor fragmentation of DNA. If large amount of foam is observed during sonication, stop, and readjust the sonicator probe. Foam can be removed by centrifuging at 20,000 *g* followed by resuspension of samples.

Determining the degree of sonication

This step quickly estimates the chromatin size to determine if further sonication is needed.

10. Take chromatin equivalent of approximately 1 × 10^6 cells (15 μL out of the 300 μL sonicated lysate).
11. Add ChIP elution buffer up to 100 μL and add 12 μL 5 M NaCl.
12. Boil samples at 95°C for 15 min to reverse crosslink.
13. After samples are cooled down, add 1 μL RNase A and incubate at 37°C for 20 min to remove RNA.
14. Purify DNA using a PCR purification kit and elute in 25 μL water.
15. Add 10 × orange G loading dye to 5–10 μL of purified DNA and run on a 2% agarose gel. The desired rage of DNA size is 200–600 bp (Figure 1).
16. If the majority of sheared chromatin is over 600 bp, recombine the cell debris pellet with the sonicated chromatin to continue sonication to achieve the desired size fragments.

Pause point: Sonicated chromatin can be snap frozen in liquid nitrogen and stored at −80°C for use the next day.

Chromatin immunoprecipitation

Timing: day 1 or 2 (15 min and 16 h incubation)

This step captures and pulls down the antibody/chromatin complex.

17. Save 50 μL sonicated chromatin in −20°C as input DNA.
18. Add antibody specific to the protein of interest to capture the protein/chromatin complex. Use ~5–10 μg antibody for each immunoprecipitation. Incubate on platform rocker at 4°C for 16 h.
19. The next day, add 5 μL prepared Staph-seq per 1 μg of primary antibody used to each chromatin-antibody sample.
20. Incubate on the rotating platform for 15 min at 23°C.
21. Centrifuge at 17,000 *g* for 5 min at 23°C, remove supernatant.

⚠ CRITICAL: The incubation time in step 20 should not exceed 15 min at 23°C.

Wash, elution, and crosslink reversal

Timing: day 2 or 3 (8 h or up to 16 h)

22. Wash the Staph-seq pellet with 800 μL ChIP wash buffer for 30 s.
23. Centrifuge at 17,000 *g* at 4°C for 5 min, discard supernatant.
24. Repeat step 22 and 23.
25. Wash once using 1 mL 1 × TE with 50 mM NaCl.
26. Transfer to a new tube, centrifuge at 17,000 *g* at 4°C for 5 min, discard supernatant.

Note: During washes, a lot of DNA may stick to the walls of the tube. This step will help remove the carryover of the DNA into the elution process and reduce noise in result.

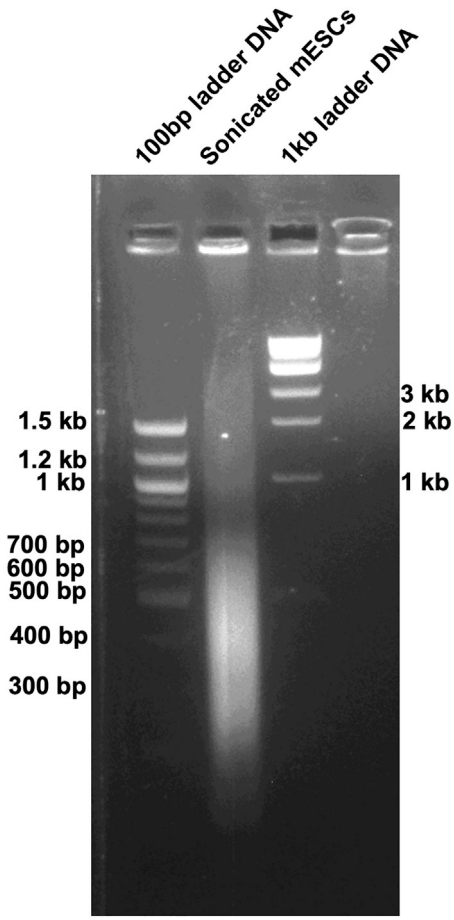

Figure 1. Gel image of sonicated DNA

Electropherogram of a 2% agarose gel showing sonication of genomic DNA from mouse ESCs. The sheared chromatin shows desired distribution ranged between 200 and 600 bp.

⚠ CRITICAL: Return to 23°C. From this point on, barrier filter tips are used to avoid DNA contamination from pipettes.

27. Thaw the input samples from the previous day and bring to 150 µL with ChIP elution buffer.
28. Add 150 µL 1× TE to the input sample.
29. Add 150 µL elution buffer and 150 µL 1× TE to Staph-seq pellet.
30. Add 4 µL of 10 mg/mL RNase (final concentration 0.2 µg/µL) to every sample. Incubate at 37°C on a thermomixer at 950 rpm for 2 h.
31. Add 2 µL 20 mg/mL Protease K to each sample.
32. Incubate for 2 h at 55°C for protease-K digestion.
33. Incubate at 65°C for at least 6 h to reverse crosslinking.

Note: Step 33 can be done in the PCR machine (6 h at 65°C and then a 4°C hold).

Pause point: The material can be frozen at −20°C and stored for up to 16 h. Alternatively, the crosslink reversal can be extended for up to 16 h. A crosslink reversal longer than 16 h may result in increased noise.

⚠ CRITICAL: Use Eppendorf Safe-Lock tubes to avoid drying out samples.

34. Add 300 μL phenol:chloroform:isoamyl alcohol (25:24:1) to each sample, and vortex or shake by hand thoroughly for approximately 20 s.
35. Centrifuge at 23°C for 5 min at 16,000 *g*.
36. While centrifuging, prepare new microfuge tube containing 12 μL 5 M NaCl and 2 μL 20 mg/mL glycogen.
37. Carefully remove the upper aqueous phase containing DNA and transfer the layer to the tubes prepared in step 36. Make sure not to carry over any phenol during pipetting.
38. Precipitate DNA by adding 750 μL cold 100% Ethanol.

Pause point: Samples can be frozen at −20°C at this point.

39. Mix well and incubate at −80°C for 30 min to 1 h.
40. Centrifuge at 20,000 *g* for 20 min at 4°C and discard supernatant.

⚠ CRITICAL: The precipitated DNA appears as a white flake loosely attached to the bottom of tubes. Avoid decanting or using vacuum to aspirate the supernatant. Instead, use pipette to carefully remove the supernatant.

41. Centrifuge at 20,000 *g* for another 5 min at 4°C and remove supernatant.
42. Add 800 μL 70% ethanol. Vortex to mix and centrifuge at 20,000 *g* for 5 min at 23°C.
43. Remove supernatant. Spin briefly at 23°C and remove remaining liquid with a pipette.
44. Position tubes upside down to air dry at 23°C until the pellets are dry but still have moist appearance.

⚠ CRITICAL: Avoid over drying samples by incubating at 37°C which may lead to difficulty in dissolving them in water.

45. Add 55 μL molecular grade water to each sample, mix well and allow DNA to dissolve for at least 10 min at 23°C.

Pause point: Material can be frozen at −80°C and stored indefinitely.

Quantification of ChIP results with qPCR

Timing: day 3 (2–4 h)

Assess ChIP results by qPCR before proceeding to Library preparation and Sequencing. Use at least 2 positive and 1 negative target sites to verify enrichment at positive sites compared to negative sites. For example, two E2F6 target genes, *Zcwpw1* and *Rab8a* were used as the positive controls and at least one "gene desert" loci from an intronic locus is used as the negative control (Figure 2). ChIP signals obtained from the positive gene loci are compared with the negative gene loci, and further normalized by ChIP signals from input samples because background signal levels vary among primer sets.

46. Dilute ChIP DNA by adding 17 μL Nuclease-free (NF) water to 5 μL ChIP DNA.
47. Set up PCR reaction master mix as table below for each reaction:

Reagent	Amount
DNA template (diluted)	5 μL
Primer mix (2 μM forward and 2 μM reverse primer)	5 μL
Power SYBR green master mix	10 μL

48. Aliquot the samples with qPCR master mix reaction in a qPCR plate, make sure not to create and bubbles. Seal with clear qPCR thermal seal and spin briefly to remove the bubbles if any.
49. Set up the qPCR and run the below PCR cycling conditions:

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	2 min	1
Denaturation	98°C	10 s	40 cycles
Annealing	60°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	10 min	1
Hold	75°C–95°C with increment 0.5°C		
Hold	4°C	forever	

Pre-library preparation size selection

Timing: day 4 (2 h) (for step 50)

This section describes how to prepare the Staph-Seq ChIP DNA for library preparation. The ChIP DNA is first quantified then a pre-amplification size selection step is added to eliminate potential Staph A DNA contamination using the Pippin Prep instrument.

50. Quantify the precipitated Staph-Seq ChIP DNA with Qubit® dsDNA HS Assay Kit for DNA using their standard protocol.

Note: Add 1 μL of ChIP DNA into prepared sample tubes containing 199 μL Qubit master mix according to the user guide.

51. Use results from sample quantification to determine how much of the ChIP DNA material to use for size selection. The Pippin Prep size selection cassette has an input range from low nanograms to 5 μg of DNA for a selection range of 100–600 bp. For best results, we recommend using at least 100 ng DNA. In this protocol, we used 300 ng to 1,000 ng for the ChIP DNA samples.
52. Size select the quantified Staph-Seq ChIP DNA using internal standards.
    a. Bring loading/marker mix (Marker L) to room temperature.
    b. Bring the DNA samples up to 30 μL in 1× TE buffer.
    c. Combine each DNA sample with 10 μL of loading/marker mix (Marker L) to make a total sample volume of 40 μL.
    d. Setup size selection parameters on the Pippin Prep for a selection range of 150–350 bp before loading.
    e. Load samples according to manufacturer's instructions and press start.
    f. Retrieve samples from the elution wells after 70 min.

Note: The Sample starting masses for pre-library preparation size selection may need optimization on a case-by-case basis. We use Sage Science Pippin Prep instrument for DNA size selection using their standard protocol (Pippin Prep 2% Agarose Gel Cassette (CDF-2010, 2% DF Marker L) quick guide for more information: https://sagescience.com/wp-content/uploads/2022/01/Quick-Guide-CDF2010-marker-L-460058-RevA-9_7_21.pdf).

Pause point: Material can be frozen at −20°C and stored indefinitely, no additional purification is required for future use.

Library preparation & sequencing

Timing: day 4 (6–8 h)

This section describes how to prepare a Staph-Seq library using KAPA HTP Library Preparation Kit (Roche) and Bioo Scientific NEXTflex DNA Barcodes (Perkin Elmer). The Staph-Seq ChIP DNA was size selection prior to library construction in the previous steps. The size selected ChIP DNA is then prepared into libraries as noted below and size-selected again.

This section describes how to prepare a Staph-Seq library using KAPA HTP Library Preparation Kit (Roche) and BiooScientific NEXTflex DNA Barcodes (Perkin Elmer). The Staph-Seq ChIP DNA was size selection prior to library construction in the previous step. The size selected ChIP DNA is then prepared into libraries as noted below.

53. Library preparation.
    a. Prepare libraries from size selected ChIP DNA according to manufacturer's instructions for most steps.
    b. Modifications to these instructions are as follows:
        i. Bring size selected ChIP DNA to 50 μL in nuclease free water, up from 40 μL eluted from the Pippin Prep.
        ii. Begin protocol at end repair, followed by remaining steps; a-tailing, adapter ligation and PCR.
        iii. Use Agencourt Ampure XP beads for all bead purification steps.
        iv. At adapter ligation, use 5 μL of diluted 1:125 NEXTflex DNA barcodes.
        v. Perform the standard two post-ligation cleanups without size selection.
        vi. Perform library amplification for 15 cycles with standard thermocycling parameters as noted.
        vii. Proceed with standard library bead purification and elution.

Pause point: Material can be frozen at −20°C and stored indefinitely.

Note: We use KAPA HTP Library Preparation Kit for the library preparation using the manufacturer's standard protocol in combination with the Scicline G3 liquid handling workstation (Perkin Elmer) Alternative kits and processing methods could be utilized at user's discretion (see https://resources.perkinelmer.com/lab-solutions/resources/docs/prd_htplibrarypreppkapabiosystems.pdf, https://rochesequencingstore.com/wp-content/uploads/2022/07/KAPA-HTP-Library-Preparation-Kit-Technical-Data-Sheet.pdf and https://www.mediray.co.nz/media/18825/514104-nextflex-dna-barcodes-48.pdf for more information).

54. Quantify post-amplification libraries.
    a. Quantify stock post PCR libraries using the HS dsDNA Qubit assay as before.
    b. Dilute stock libraries to 10 nM and assay libraries on the Agilent Bioanalyzer HS DNA assay to determine sizing information and determine if adapter dimer contamination is present.
    c. Quantify diluted 10 nM libraries using the HS Qubit assay to determine concentrations of diluted libraries.
    d. Normalize and pool libraries for sequencing using 10 nM dilutions and sizing information from the Bioanalyzer.
55. Using the Pippin Prep cassette for an additional round of size selection following instructions from step 52.

⚠ CRITICAL: The second round of size selection does not promote the alignment percentage to the target genome (Figure 3), but helps improve the signal-to-noise ratio (Figure 4).

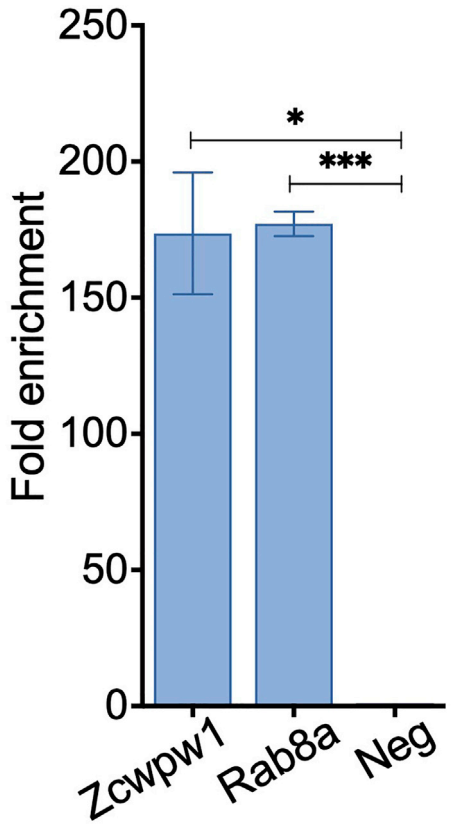

Figure 2. E2F6 binding at target genes shown by ChIP-qPCR

ChIP-qPCR showing enrichment of E2F6 at target genes *Zcwpw2* and *Rab8a* as shown by fold enrichment over the negative region. *p < 0.05, **p < 0.01, ***p < 0.001. Data are represented as mean ± SEM.

Note: We recommend size selecting at least 100 ng of stock post PCR library material with a selection range of 200–600 bp using the same Pippin Prep cassette (CDF-2010, Marker L) and the loading instructions from before. Re-quantify post amplification size selected libraries again using the HS dsDNA Qubit assay and the Agilent Bioanalyzer HS DNA assay.

56. Pool and sequence libraries.
    a. Normalize and pool libraries for sequencing using 10 nM dilutions and information from Qubit and Bioanalyzer.
    b. Sequence libraries as 50 bp single read on HiSeq 2500 instrument using HiSeq Control Software 2.2.58.
57. Following sequencing, run Illumina Primary Analysis version RTA 1.18.54 and Secondary Analysis version CASAVA-1.82 to demultiplex reads for all libraries and generate FASTQ files.

EXPECTED OUTCOMES

For less abundant TFs that are difficult to study using regular ChIP method using protein A/G beads or magnetic beads, a successful Staph-seq ChIP will show at least 5-to-50-fold enrichment of its expected binding sites over background controls. An example of how these values were calculated can be found in Table 1.

LIMITATIONS

First, chromatin immunoprecipitation measures the relative enrichment of TF binding to a chosen reference. Using unenriched input DNA and a "gene desert" region[4] is both critical in qPCR to

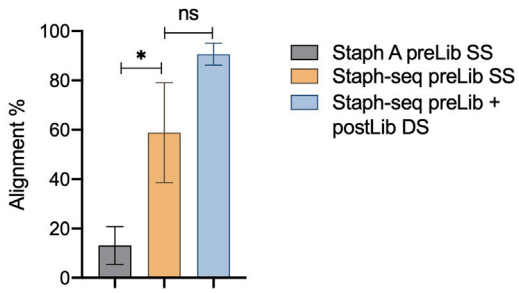

Figure 3. Alignment percentage to the mouse genome with size selection

Genome browser tracks showing improved alignment percentage to the mouse genome in Staph-Seq samples with double size selection (DS) including pre-library and post-library size selection (prelib + postLib DS) compared with Staph A and Staph-Seq samples with single size selection. PreLib SS indicates single size selection before library amplification. "PreLib + postLib DS" indicates double size selection before and after library amplification. *p < 0.05, **p < 0.01, ***p < 0.001. Data are represented as mean ± SEM.

successfully detect the binding of TFs before proceeding to massively parallel next-generation sequencing. However, the target genes and the expected binding sites of the TF of interest are often unknown, making it challenging to troubleshoot when the ChIP signal in the sequencing results turns out low.

Second, the Staph-seq Sepharose binds efficiently to rabbit polyclonal antibodies. However, a rabbit primary antibody that is specific for an epitope is not always available. When working with monoclonal antibody from mouse or rat, a rabbit host bridging antibody should be used.

Third, although Staph A DNA is blocked in Staph-seq Sepharose, cautions need to be taken when pipetting to remove any supernatant from the Staph-seq pellet. Although traces of carry-over of Staph-seq will not affect the quality of data, accidental contamination of substantial amount of Staph-seq into ChIP DNA will and result in low alignment of the target genome.

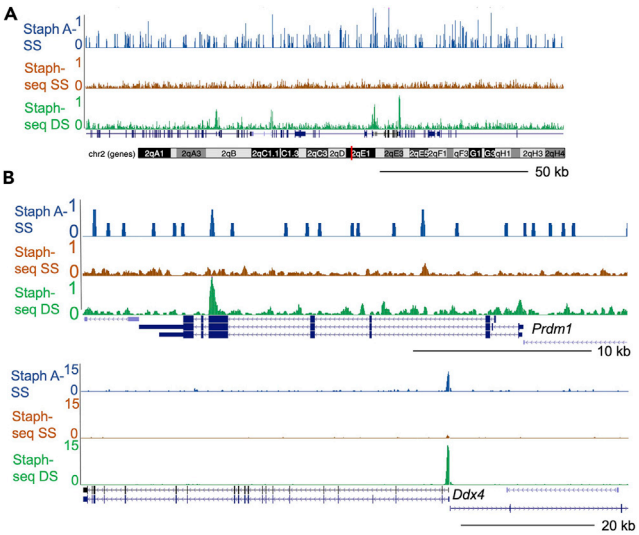

Figure 4. Improved ChIP-seq signal of E2F6 binding at target genes

Genome browser tracks of E2F6 ChIP using the Staph-seq method in this protocol.

(A) Overall improved signal-to-noise ratio as indicated on a segment of Chromosome 2.

(B) Gene-specific examples of the E2F6 target genes *Prdm1* and *Ddx4* showing the successful capture of E2F6 binding at its target genes in Staph-seq DS (double selection) samples, but not in Staph A SS (single selection) or Staph-seq SS (single selection) samples.

Table 1. Example of calculation of relative fold enrichment

Sample name	Target name	Reporter	CT	#	Adjusted CT to negative control	# #	Adjustment to input	Fold enrichment	Average fold enrichment
E2F6 IP	Zcwpw1-a	SYBR	23.6		6.9		7.1	137.4	158.1
E2F6 IP	Zcwpw1-a	SYBR	23.3		7.2		7.5	178.8	
E2F6 IP	Zcwpw1-b	SYBR	24.0		6.5		7.6	196.5	175.0
E2F6 IP	Zcwpw1-b	SYBR	24.4		6.1		7.3	153.6	
E2F6 IP	Rab8a	SYBR	26.9		3.6		6.9	116.6	114.6
E2F6 IP	Rab8a	SYBR	27.0		3.5		6.8	112.6	
E2F6 IP	RNF130	SYBR	24.9		5.7		7.0	125.2	121.4
E2F6 IP	RNF130	SYBR	24.9		5.6		6.9	117.6	
E2F6 IP	Neg control	SYBR	30.9	30.5	-0.4		-0.4	0.8	1.0
E2F6 IP	Neg control	SYBR	30.1		0.4		0.4	1.3	
Input	Zcwpw1-a	SYBR	25.0		-0.5	-0.2			
Input	Zcwpw1-a	SYBR	24.5		0.0				
Input	Zcwpw1-b	SYBR	25.5		-1.0	-1.1			
Input	Zcwpw1-b	SYBR	25.8		-1.3				
Input	Rab8a	SYBR	27.8		-3.3	-3.3			
Input	Rab8a	SYBR	27.8		-3.3				
Input	RNF130	SYBR	25.9		-1.4	-1.3			
Input	RNF130	SYBR	25.7		-1.2				
Input	Neg control	SYBR	24.6	24.5	-0.1	0.0			
Input	Neg control	SYBR	24.4		0.1				
# Average of negative control									
# # Average of input									

TROUBLESHOOTING

Problem 1

Low ChIP signal.

Potential solution

Low ChIP signal can be a complicated problem influenced by numerous factors including affinity and specificity of primary antibody, cell number, crosslinking, or chromatin fragmentation. Before looking into individual factors, we recommend setting up a positive control using a high-quality ChIP grade antibody against a protein that strongly binds to chromatin. The most common positive control we use is anti-RNA polymerase II antibody or anti-histone 3 (tri methyl K4) that allow detection of bindings to chromatin without substantial background noise. When conducting small-scale pilot experiment to test the enrichment of protein of interest at expected locus, an appropriate control is critical to avoid artifacts. Both non-specific IgG that matches the isotype of primary antibody and chromatin input are often used. However, non-specific IgG usually pulls down less DNA than the primary antibody, resulting in insufficient reads that covers the genome when compared with reads generated by binding of specific antibodies. We prefer chromatin input that serves as a background to measure the relative enrichment of the protein of interest.

Problem 2

Undersonication or oversonication (step 7).

Potential solution

The effect of sonication varies depending on the cell type, cell numbers, crosslinking conditions and specifics of sonicators used. For different cell types, sonication specifics need to be optimized individually. Undersonication can be determined by the large DNA fragments in DNA electrophoresis. However, oversonication is more difficult to identify because small DNA fragments are not visually

increased in an oversonicated sample. Thus, we recommend optimizing the sonication condition by conducting a time course starting from the lowest power output with minimum duration of sonication time.

Problem 3

A low signal-to-noise ratio.

Potential solution

A low signal-to-noise ratio may be solved by enhancing the specific signals and dampening background noise. Antibody affinity and specificity is directly related with signal-to-noise ratio. When a high-quality ChIP grade antibody is used but the signal-to-noise ratio is still low, we recommend starting by optimizing the starting cell number for ChIP. Abundant proteins such as RNA polymerase II usually does not require more than one million cells. However, for rare proteins or TFs that bind at limited gene loci, a greater cell number need to be used to increase the signal-to-noise ratio.

To reduce the background noise, we suggest starting with adjusting the salt strength in both the immunoprecipitation and washing. A range of salt concentration including 50, 100 and 250 mM can be tested to optimize the salt strength in ChIP lysis buffer.

If both signal and noise are high, increase washes, enhance salt strength in washing buffer, and prolong wash time may help reduce non-specific binding events. However, overwashing is a common problem reflected by an overall low signal and background noise. Over 5 washes are generally not recommended to avoid overwashing. Washing with multiple buffers such as low salt, high salt and TE wash often help remove non-specific signals. Here, we used two buffers including high salt (0.5 M LiCl) and TE wash to achieve minimal noise.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Linheng Li (lil@stower.org).

Materials availability

This study did not generate gene unique reagents.

Data and code availability

The ChIP-seq data generated using this protocol in the study[1] are available on the GEO repository with the accession number GSE108620.

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AUTHOR CONTRIBUTIONS

Conceptualization, F.T. and L.L.; Investigation, F.T. and E.R.; Writing – Original draft, F.T. and E.R.; Writing – Review and editing, F.T., J.M.P., and L.L.; Supervision, X.H.; Funding acquisition, L.L.; Supervision, L.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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