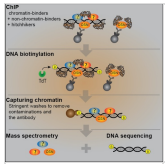

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ChIP-SICAP

Molecular Systems Biology

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1 Works for me dx.doi.org/10.17504/protocols.io.bcrriv56

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ABSTRACT

Chromatin immunoprecipitation combined with mass spectrometry (ChIP-MS) suffers from unexpected proteins that bind to chromatin during the cell lysis and immunoprecipitation (IP). The so-called hitchhikers of chromatin are hardly washed away by gentle washing steps applied to a normal IP protocol. We employed DNA end-labeling by Terminal Transferase (TdT) to purify proteins specifically bound to chromatin. Using this double purification strategy, chromatin fragments are released from IP beads, and re-captured by streptavidin beads. Thus, we can apply stringent conditions to remove the hitchhikers of chromatin, and to identify authentic proteins co-localized with the protein of interest. We call this protocol Selective Isolation of Chromatin-Associated Proteins (SICAP). So far, ChIP-SICAP was successfully applied to different cell lines such mouse ES cells, mouse fibroblast cells, HEK 293T, HeLa, U2OS as well as mouse and human tissues. Here, we share the most updated version of our protocol. If you have any questions please feel free to email us.

EXTERNAL LINK

https://doi.org/10.15252/msb.20199370

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Rafiee MR, Girardot C, Sigismondo G, Krijgsveld J (2016) Expanding the Circuitry of Pluripotency by Selective Isolation of Chromatin-Associated Proteins. Mol Cell 64: 624-635
Rafiee MR, Sigismondo G, Kalxdorf M, Förster L, Brügger B, Béthune J, Krijgsveld J. (2020) Protease-resistant streptavidin for interaction proteomics. Mol Syst Biol (In press)

GUIDELINES

This protocol has 4 parts. Part #1 and #2 are essentially ChIP. If you already have a optimised ChIP protocol you may also use your protocol. Part #1 has two alternative approaches for crosslinking the cells by formaldehyde. So you may choose either approach A or B. Approach A is detaching the cells, and then counting, fixing, and freezing them. The approach B is crosslinking the cells in the plate, and then harvesting and freezing them.
There are 2 appendices in this protocol. Appendix #1 is a protocol for retrieving DNA to use it for qPCR or sequencing. Appendix #2 is a protocol for modification of streptavidin beads to avoid streptavidin contamination in mass spectrometry. Before you start, please use Appendix #2 to modify your streptavidin beads. You may also do this during Part #2: ChIP (Step 47).

For each replicate, you may need 4-24 million cells. The negative control for this assay is a no-antibody control, or normal IgG. Non-biotinylated control is not necessary, however, you may include a non-biotinylated control to demonstrate the identified proteins are chromatin-binders. The best negative control is a knock-out control to assess the specificity of the antibody. Also in order to compare with the negative control, you don't need SILAC or other types of peptide labelling. However, labeling helps when you compare two or more cellular conditions.

MATERIALS

NAME	CATALOG #	VENDOR
IP Buffer		
LB3 buffer for sonication		
BW2x wash buffer		

Citation: Sina Rafiee, Jeroen Krijgsveld (05/28/2020). ChIP-SICAP. https://dx.doi.org/10.17504/protocols.io.bcrriv56

NAME	CATALOG #	VENDOR
SDS wash buffer		
2-Propanol wash buffer		
Acetonitril wash buffer		
Sodium cyanoborohydride	156159	Sigma Aldrich

STEPS MATERIALS

NAME	CATALOG #	VENDOR
T4 Polynucleotide Kinase	M0201S	New England Biolabs
Terminal Deoxynucleotidyl Transferase (20 U/µL)	EP0162	Thermo Fisher
Streptavidin Magnetic Beads - 5 ml	S1420S	New England Biolabs
ZipTipC18		Merck Millipore
Pierce BCA Protein Assay Kit	23225	Thermo Fisher Scientific
Pierce™ 16% Formaldehyde (w/v), Methanol-free	28906	Thermo Fisher
cOmplete™, Mini Protease Inhibitor Cocktail	11836153001	Roche
Biotin-7dATP	NU-835-BIO-S	
Biotin-dCTP	NU-809-BIOX-S	
Klenow Fragment (3'-5' exo-) - 200 units	M0212S	New England Biolabs
Deoxynucleotide Solution Set - 25 umol of each	N0446S	New England Biolabs
Biotin-ddUTP	NU-1619-BIOX-L	Jena Bioscience
Benzonase® Nuclease	E1014 SIGMA	Sigma-aldrich

MATERIALS TEXT

Pierce™ 16% Formaldehyde (w/v), Methanol-free
by Thermo Fisher
Catalog #: 28906

cOmplete™, Mini Protease Inhibitor Cocktail
by Roche
Catalog #: 11836153001

Terminal Deoxynucleotidyl Transferase (20 U/µL)
by Thermo Fisher
Catalog #: EP0162

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Biotin-ddUTP
by Jena Bioscience
Catalog #: NU-1619-BIOX-L

Streptavidin Magnetic Beads - 5 ml
by New England Biolabs
Catalog #: S1420S

Benzonase® Nuclease
by Sigma-aldrich
Catalog #: E1014 SIGMA

ZipTipC18
by Merck Millipore

Pierce BCA Protein Assay Kit
by Thermo Fisher Scientific
Catalog #: 23225

Biotin-dCTP
Catalog #: NU-809-BIOX-S

Klenow Fragment (3'-5' exo-) - 200 units
by New England Biolabs
Catalog #: M0212S

Biotin-7dATP
Catalog #: NU-835-BIO-S

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Deoxynucleotide Solution Set - 25 umol of each
by New England Biolabs
Catalog #: N0446S

T4 Polynucleotide Kinase
by New England Biolabs
Catalog #: M0201S

SAFETY WARNINGS

Please work with formaldehyde and Sodium borohydride in a fume hood.

BEFORE STARTING

- Please read the Guidelines.

Part #1-A: detach and crosslink the cells 2h

1 Detach the cells by Accutase. Accutase doesn't lyse cells, so it is more suitable for single cell preparation. After detaching the cells, make sure that they are single cell by pipetting in the medium.

2 Count the cells.

3 Spin the cells at 200g for 5min, and remove the medium.

4 Resuspend the cells in formaldehyde 1.5% in PBS. Roughly for every 10 million mammalian cells, add 10ml formaldehyde 1.5%. Pipette up and down to disrupt the cell pellet completely.

5 Rotate occasionally, and keep it 15min at room temperature.

6 Add Glycine 1M to stop the crosslinking, so the final concentration will be 130mM (1.5ml Glycine for every 10ml of formaldehyde).

7 Rotate occasionally 5min at room temperature.

Spin the cells at 2000RPM, 2min. Discard the supernatant (SN).

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8

9 Resuspend the cells with PBS-T, equal volume to the formaldehyde volume that you'd used in step 4

10 Spin the cells at 2000RPM, 2min, and discard the SN.

11 Resuspend the cells in some PBS-T

12 Count the cells, and Aliqoute them. e.g. every 24 millions in a 15-ml tube

- Use polypropylen (soft) tubes. Do not use polystyrene (rigid) tubes, as a lot of cells bind to the walls.

13 Spin the cells at 2000 RPM 2min, and discard the SN. Freeze the cells in liquid nitrogen

Pause point: You may freeze the cells in -80°C for months.

Part #1-B: crosslinking the cells in a plate, and then harvesting them 2h

14 Inside a fume hood, remove the medium, and pour 10ml of formaldehyde 1.5% in a 10cm dish.

15 Wait 15min, and rotate the plate gently.

16 Add Glycine to the final concentration of 130mM

17 Rotate the plate gently, and wait 5min.

18 Discard the liquid, gently pour 10ml of PBS on the cells, rotate, and discard it

19 Repeat the last step once again.

20 Pour 3ml of PBS-T (Tween 0.1%) on the plate.
Triton allows to collect the cells more efficiently. While, using PBS you will lose quite some cells.
Adding Complete protease inhibitor is optional. Apparantely, endogenous proteases are inactivated by formaldehyde.

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21 Scrap the cells with a cell lifter, and collect them in one side of the plate. Then transfer them by a pipet to a tube (e.g. 15ml or 50ml depending on the number of the plates)

22 Repeat the last two steps, once again for each plate.

23 Spin the cells at 2000RPM for 2 min, and discard the supernatant

24 Resuspend the cells in PBS-T, and aliqoute the them if you wish

25 Spin the cells at 2000RPM 2 min, and discard the supernatant

26 Freeze the cells in liquid nitrogen, and keep them in -80 ºC

Pause point: You may freeze the cells in -80°C for months.

Part #2: Chromatin immunoprecipitation (ChIP) 1d

27 Resuspend the cells in 0.9ml Tris-Cl 10mM. Then add 100ul Tritone X100 10%(v/v). Leave the cells on ice for 5 min

Optional: You may also add Complete protease inhibitor.

28 Spin the cells 2min at 2000RPM. Discard the supernatant.

29 Resuspend the cells in 1ml LB3 buffer. Spin the cells 2min at 2000RPM. Discard the supernatant.

30 Repeat Step 31, twice again.

31 Resuspend the cells in LB3 buffer.

- Resuspend every 8 million cells in 250ul LB3 buffer, and-so-forth.
- One 10cm dish of confluent mES cells is about 20 million cells. Therefore, you may resuspend the cells in 750ul LB3 buffer.

32 Aliqoute the resuspended cells into Biorupotor Pico 1.5ml special tubes.

- Pour every 250ul of the resuspended cells in a Biorupotor Pico 1.5ml special tube.

33 Sonicating conditions depends on the cell lines. For mES cells using Bioruptor Pico, you may apply 7 cycles ON and OFF

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(30''-30'').

- Bioruptor Pico is faster, however, Covaris is more precise and reproducible.
- Adding SDS to the final concentration of 0.1% improves the efficiency of sonication. However, some antibodies are sensitive to it. You may add 0.1% sodium deoxycholate or 0.1% Sarkosyl.
- Too many cycles of sonication blast the proteins from chromatin.

34 After the sonication spin the sonication tubes 10min at 12000g

35 Collect the supernatant, and transfer it into a 2ml epi-tube or a 15ml tube. You may pool the sheared chromatin obtained from one replicate.

- LoBind tubes slightly improve the results.

36 Measure the protein concentration using a BCA protein assay kit.

- Briefly, take 5-10ul of the sheared chromatin, and pour it into a PCR tube. Add 25ul of PBS-SDS 1%. Do the same with your standard controls, and the negative control. Heat the samples at 95 ºC for 5min. Add 0.5ul of Benzonase, and gently vortex. Take 25ul of each sample, and pour it in a 96-w plate. Follow the kit manual to measure the protein concentration.

37 Pour up to 1 mg protein (chromatin) in a 2-ml epi-tube as one replicate.

- If you are using SILAC to compare two/three cellular states using one antibody you may mix the samples at this step. However, if you want to use SILAC to compare your antibody with normal IgG or no-antibody control, please mix the samples in step

38 Add Triton X100 from a 10% (v/v) stock to make the final conc. 1%

Pause point: You may freeze the samples in -80°C for weeks.

39 Keep 1%, as the input control for ChIP-qPCR/seq

40 Add proper amounts of your antibody to the samples.
- Depending on the antibody you may need to add between 2-10 ug or 1:50 – 1:100 v/v.

41 Agitate at 800RPM overnight in a Thermomixer (eppendorf) at 4°C or cold room

42 After the overnight incubation, spin the samples at 12000g for 10min at 4°C

43 Collect 95% of the liquid and transfer it into a new 2-ml epi-tube

- You may not see the pellet easily, however, sometimes there is a transparent precipitation
- A 2-ml epi-tube is necessary for efficient agitation in the next steps.

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44 Adjust the volume to 1ml by adding IP buffer.

45 For each tube, you will need to prepare 40ul of ProteinA or ProteinG Dynabeads (depending on the antibody).

- Wash the magnetic beads once with IP buffer. More washing is not necessary.

46 Add 40ul magnetic Dynabeads to each tube

47 Rotate the samples 2-4 hours at 4°C (cold room) on a rotating wheel.

- Alternatively, you may rotate the samples overnight at 4°C.

Part #3: Selective isolation of Chromatin-Asscotiated Proteins (SICAP) 2h

48 Put the tubes on the magnet, after 1min remove the liquid, and take the tubes off the magnet. Immidiately pour 1000ul of Tris-HCl 10mM pH 7.5 (without EDTA) on the beads.

- Process the tubes one by one. Never allow the beads to dry.
- Here the idea is to remove the inhibitors of the enzymatic reactions (e.g. EDTA). Stringent washes come later.

49 Resuspend the beads by rotating end to end, and transfer them to a new 2ml epi-tube.

- 2ml epi-tube is necessary because agitation is more efficient, and beads do not precipitate in the subsequent steps

50 Resuspend the beads in 200ul of NEB Cutsmart buffer 1x. Don't pipet the beads. Gently swirl the tube to resuspend the beads.

51 **Optional**: If you wish to improve the efficiency of DNA end-labeling you may perform the folloing step. Although TdT adds nucleotides to 3'- and 5'-overhangs and blunt ends, Klenow 3'exo- adds biotinylated dATP to only 5'overhangs or blunt ends. Combination of these two enzymes improves DNA end-labelling about 10-20%. In addition, T4 PNK removes 3'-phosphates, and prepares the 3'-OH ends for TdT and Klenow.

Prepare the following mixture for each tube. You may prepare a master mix for all the tubes:

- Cutsmart buffer 10x 10 ul
- Klenow 3'exo- 2 ul
- dBTP (C+G+T) 10mM 1 ul
- Biotin-dATP 1mM 10 ul
- T4 PNK 1ul

Fill up to 100ul with H2O

Note: dBTP means al nucleotides except dATP.

52 Put the samples one by one on the magnet, remove the liquid, and take the tubes off the magnet. Pour the mixture prepared in the previous step on the beads. Gently swirl the beads to resuspend the beads.

- Be careful not to dry the beads. samples should be treated one by one to avoid drying the beads.

53 Incubate at 37°C in a thermomixer with agitation at 700 RPM for 30 min.

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54 Put the samples one by one on the magnet, after 1min remove the liquid, and take the tubes off the magnet. Resuspend the beads in 100ul of TdT buffer 1x.

- Do not vortex the beads. Gently swirl the beads to resuspend the beads

55 Prepare the following mixture for each tube. You may prepare a master mix for all tubes:

- TdT buffer (including CoCl2) 20ul
- Biotin-dCTP 5ul
- Biotin-ddUTP 5ul
- Fill up to 95ul with H2O

Note: Biotin-dCTP was used to reduce the cost. You may use 10ul of biotin-ddUTP.

56 Put the samples one by one on the magnet, remove the liquid, and take the tubes off the magnet. Resuspend the beads in the mixture prepared in step 55. Gently swirl the beads to resuspend the beads.

57 To each tube add :

- TdT 4ul
- Optional: RNase A 1ul

TdT is a quite specific enzyme for DNA labelling, as evident by TUNEL assay. Adding RNase A reassures specific DNA labelling.

58 Incubate at 37°C in a thermomixer with agitation at 800 RPM for 30min.

59 Wash the beads 4 times with 1ml ice-cold IP buffer at room temp.

- During each wash, invert the tubes to resuspend the beads. Then spin them briefly. Put them on the magnet, and remove the liquid. Take the tube off the magnet, and pour 1ml wash buffer. Treat the tubes one by one on the magent to avoid drying the beads.

60 Remove the last IP wash buffer

61 Resuspend the beads in 100ul, SDS 7.5% + DTT 200mM, and vortex vigorously

62 Incubate 15min at 37°C in a thermomixer, with 1000 RPM

63 Put the samples on the magnet, collect the liquid, and discard the beads

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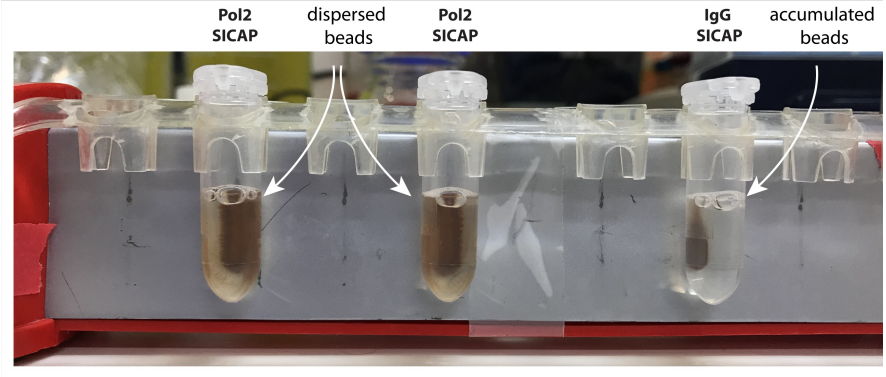
64 Dilute the liquid with 1000ul IP buffer

65 Add 70ul of Streptavidin magnetic beads to each tube. Rotate the tubes for 30-60min at room temptrature.

66 Put the tubes on the magnet after 2 min discard the solution.

67 Wash the beads 3 times by SDS wash buffer

- Each washing consists of resuspending the beads in the washing solution by vortexing, briefly spinning, and putting on the magnet for 2 min to remove the solution
- After the second wash, normally you should be able to see the beads are dispersed, except the negative control.



68 Wash with BW2x buffer, once

69 Wash the beads with Iso-propanol wash buffer, once

70 Wash the beads with acetonitrile wash buffer, twice

71 Resuspend the beads in 80ul acetonitrile wash buffer, and transfer it into a PCR tubes.
- Make sure that all the beads have been transferred

72 Put the tubes on the magnet, and remove the wash buffer.

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73 Spin the tubes briefly, and remove the residues of the wash buffer

Part #4: Digesting proteins and sample preparation for mass spectrometry 1d

74 Resuspend the beads in 20ul Ambic 50mM + 10mM DTT (or TEAB 50mM for dimethylation or TMT labelling)

Note: freshly prepared Ambic should be used.

75 Incubate the beads at 50°C for 15min

76 Chill the beads, and add 2ul of IAA 0.4M

IAA: Iodoacetimide

77 Vortex, and keep the tubes in a drawer for 15-30min

78 Add 0.5ul of DTT 1M to neutralize IAA

79 Add 300ng of LysC (3ul of 100ng/ul), vortex, and spin briefly

80 Incubate overnight at 37 °C (12-16h)

81 Remove the beads, and transfer the liquid into new PCR tubes.

- Do not discard the beads as they contain the DNA. Follow the DNA purification steps to prepare the DNA for qPCR or sequencing.

82 Heat the samples (without the beads) at 95 °C for 5min to reverse the formaldehyde crosslinking

83 Chill the samples briefly

84 Add 100ng of Trypsin (1ul of 100ng/ul)

85 Incubate at 37°C for 8h.

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86 Add 1ul TFA 10% to the digested samples to acidify the pH

87 Pour 50ul of Acetonitrile 80% + formic acid 0.1% in a glass insert (tubes for auto-sampler)

88 Set a P20 on 20ul, and pick up a zip-tip

89 Pipete 20ul Acetonitrile 100%, and discard the liquid. Repeat once again.

90 Equilibrate the zip-tip by pipetting 20ul TFA 0.1%, and discard the liquid. Repeat twice again.

91 Pipet one sample 10 times. Do not generate bubbles.

92 Wash the zip-tip by pipetting 20ul TFA 0.1%, and discard the liquid. Repeat once again.

- The aliqoute of TFA 0.1% should be discarded to avoid carry over between the samples.

93 Elute the peptides by pipetting Acetonitrile 80% + formic acid 0.1% in the glass insert

94 Dry out the eluent in an appropriate speed vac.

95 Reconstitute the peptides in 8ul DMSO 2% + formic acid 0.1%

96 Inject 7 out of 8 ul of the samples to the mass spec

Appendix #1: Modification of streptavidin beads to avoid streptavidin contamination 2h

97 Pour 5ml of streptavidin beads (e.g. NEB S1420S) into a 15mL tube

98 Transfer the tube on the magnet, wait 5min and then discard the liquid

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99 Wash the beads once again with 10mL of PBS-T

PBS-T : PBS + Tween20 0.1% (v/v)

100 Put the tube on the magnet, wait 5min and then discard the liquid

- From here work in a fume hood

101 Resuspend the beads in 7ml of 0.2M Sodium cyanoborohydride in PBS-T.

- In a fume hood, weight 0.125 g Sodium cyanoborohydride. Then dissolve it in 10ml PBS-T

102 Add 7ml of Formaldehyde 4%

- In a fume hood, add 2.5ml formaldehyde 16% to 7.5 ml PBS-T

103 Rotate 2 hours at room temperature

104 Put the tube on the magnet, wait 5min and then discard the liquid

105 Wash the beads with 10ml Tris.Cl 0.1M pH 7.5

106 Put the tube on the magnet, wait 5min and then discard the liquid

107 Wash the beads with 10ml PBS-T

108 Put the tube on the magnet, wait 5min and then discard the liquid

109 Resuspend the beads in 5mL PBS-T

- You may wash the original bottle of the beads, and transfer the modified beads to the original bottle.

110 14-Keep the beads in the fridge. The beads are stable at +4^{0}C for months

Appendix #2: Clean up the DNA by Ampure XP beads 30m

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111 Following step 81, resuspend the beads in 23ul of SDS wash buffer

112 Add 2ul of Proteinase K (20mg/ul)

113 Incubate the beads at 55 °C for 15 min

114 Incubate the beads at 95 °C for 10min

115 Put the tubes on a magnetic rack, and remove the beads.

116 Collect the liquid, and add 50ul of Ampure XP beads to the samples. Vortex, and wait 10min.

117 Spin the samples, and put them on a magnetic rack to separate the beads.

118 discard the liquid, and add 200ul of freshly prepared EtOH 80%, and wait a few second

119 10-discard the liquid, repeat the previous step once again.

120 Spin the samples, and put them on a magnetic rack to separate the beads.

121 Discard the residual ethanol.

122 Resuspend the beads in 20ul of Tris-Cl 10mM pH 8.0

123 Spin the samples, and put them on a magnetic rack to separate the beads.

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124 Collect the samples, and transfer them to new tubes. The samples can be used for qPCR or library preparation.

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