Protocol

Chromatin Immunoprecipitation

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Chromatin immunoprecipitation, commonly referred to as ChIP, is a powerful technique for the evaluation of in vivo interactions of proteins with specific regions of genomic DNA. Formaldehyde is used in this technique to cross-link proteins to DNA in vivo, followed by the extraction of chromatin from cross-linked cells and tissues. Harvested chromatin is sheared and subsequently used in an immunoprecipitation incorporating antibodies specific to protein(s) of interest and thus coprecipitating and enriching the cross-linked, protein-associated DNA. The cross-linking process can be reversed, and protein-bound DNA fragments of optimal length ranging from 200 to 1000 base pairs (bp) can subsequently be purified and measured or sequenced by numerous analytical methods. In this protocol, two different fixation methods are described in detail. The first involves the standard fixation of cells and tissue by formaldehyde if the target antigen is highly abundant. The dual cross-linking procedure presented at the end includes an additional preformaldehyde cross-linking step and can be especially useful when the target protein is in low abundance or if it is indirectly associated with chromatin DNA through another protein.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Agarose gel (1%)
Antibody (target dependent)
Block solution (PBS containing 0.5% BSA)
Cells, 10^8 healthy viable cells

- Monolayer-cultured cells: 10×15 cm^2 plates
- Suspension-cultured cells: 0.5×10^6 to 1.0×10^6 cells/mL

Cells, monolayer cultured (5×15-cm^2 plates) (for dual cross-linking procedure only)
Disuccinimidyl glutarate (DSG; 0.5 M) <R> (for dual cross-linking procedure only)
DNA loading buffer (6×) <R>
EBC lysis buffer for ChIP <R>
Elution buffer for ChIP <R>

From the Antibodies collection, edited by Edward A. Greenfield.

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Formaldehyde (37%)
Formaldehyde solution (11%) <R>
Glycine (2.5 M)
N-Lauroyl-sarcosine (10% stock solution)
Liquid nitrogen (LN_2)
Lysis Buffer 1 for ChIP <R>
Lysis Buffer 2 for ChIP <R>
Lysis Buffer 3 for ChIP <R>
PBS (pH 7.2)
PMSF (for dual cross-linking procedure only)
Protease inhibitors (Set I, Calbiochem catalog no. 539131; or similar)
Proteinase K (Roche)
RIPA buffer solution for ChIP <R>
RNase A (Sigma-Aldrich)
Salmon sperm DNA (sonicated)
$T_{50}E_{10}$ (without SDS)
$T_{50}E_{10}S_1$ mixture
TE buffer containing 50 mM NaCl

Equipment

Aspirator
Cell scraper
Conical tube (50 mL; Falcon)
Dynabeads and magnetic stand
Eppendorf tubes (1.5 mL)
Gel electrophoresis equipment
Humidified incubator set at 37°C, 5% CO_2
Hybridization oven
Magnetic racks
Microcentrifuge
NanoDrop Spectrophotometer
PCR apparatus
PCR purification columns (QIAGEN catalog no. 28104)
Rotating platform
Rotating wheel (for dual cross-linking procedure only)
Sonicator
Vortex

METHOD

Chromatin Immunoprecipitation

This protocol details individual steps sequentially performed during the chromatin immunoprecipitation procedure and is ideally suited for the formaldehyde fixation of chromatin to highly abundant proteins. The initial steps of chromatin preparation and evaluation before the performance of the immunoprecipitation of the target antigen(s) are described in this protocol, followed by step-by-step guidance in the washing and elution of target antigen(s) and the reverse cross-linking of the protein-associated DNA fragments for purification, amplification, and sequencing purposes.

Cross-Linking of DNA and Protein

For Monolayer-Cultured Cells

1. Remove the growth medium from ten 15-cm^2 cell culture dishes of proliferating cells totaling ~10^8 cells, and wash the adherent cells once with PBS before aspiration.

   Avoid the use of overconfluent or dead cells.

2. Fix the cells by addition of 10 mL of 11% Formaldehyde solution, and gently incubate the cell culture dishes for 10 min at room temperature using a rotating platform.
3. Stop the cross-linking reaction by adding 0.5 mL of 2.5 M glycine to a final concentration of 125 mM. Incubate the cell culture dishes for 5 min at room temperature on a rotating platform, and aspirate the solution completely from each dish.
4. Add 5 mL of ice-cold PBS to individual dishes, and scrape the cells from all plates into two 50-mL conical tubes. Collect cells by centrifugation at 400*g* for 5 min at 4°C, and aspirate the supernatant.
5. Wash the fixed cell pellet with 5 mL of ice-cold PBS, and pellet cells by centrifugation at 400*g* for 5 min at 4°C. Aspirate the PBS completely.
6. Proceed to Step 14 or snap-freeze the cells in LN_2 for storage at −80°C.

For Suspension-Cultured Cells

7. Collect cells from a 100-mL culture (0.5×10^6 to 1.0×10^6 cells/mL) by centrifugation at 400*g* for 5 min, and aspirate the growth medium.
8. Wash the cells once with PBS and pellet by centrifugation at 400*g* for 5 min. Aspirate the Wash buffer.
9. Resuspend the cell pellet in 20 mL of 11% Formaldehyde solution and gently incubate for 15 min at room temperature using a rotating platform.
10. Stop the cross-linking reaction by adding 1.0 mL of 2.5 M glycine to a final concentration of 125 mM, and incubate the cells for 5 min at room temperature on the rotating platform.
11. Collect the cells by centrifugation at 400*g* for 5 min at 4°C, and aspirate the supernatant.
12. Wash the fixed cell pellet with 5 mL of ice-cold PBS, and pellet the cells by centrifugation at 400*g* for 5 min at 4°C before complete aspiration of the PBS.
13. Proceed to Step 14 or snap-freeze the cells in LN_2 for storage at −80°C.

Chromatin Preparation

14. Resuspend cell pellets in 10 mL of Lysis Buffer 1 containing protease inhibitors and incubate for 10 min at 4°C using a rotating platform.
15. Collect cells by centrifuging at 400*g* for 5 min at 4°C, and resuspend the pellet in 10 mL of Lysis Buffer 2 containing protease inhibitors. Incubate the tube for 10 min at room temperature on a rotating platform.
16. Pellet the cells by centrifuging at 400*g* for 5 min at 4°C, and resuspend the pellet in 3–4 mL of Lysis Buffer 3 containing protease inhibitors.
17. Sonicate the cells on ice using a 20% duty cycle for 2 min followed by a 1-min cooling period. Repeat this 10–15 times.

    The solution will start to appear clear when looking through the tube. The goal is to shear chromatin DNA into fragments ranging 100–600 bp in length. Although chromatin from some cell lines is easy to shear into this range, the shearing of chromatin from other lines is extremely difficult and requires more extensive sonication at higher power settings over more cycles.

18. Add 150 µL of *N*-lauroyl-sarcosine (10% stock solution) and incubate for 10 min at room temperature on a rotating platform.

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19. Distribute the mixture into three 1.5-mL Eppendorf tubes and centrifuge at 15,000*g* for 5 min at 4°C using a microcentrifuge. Collect the supernatant containing the chromatin, and pool it into a new 15-mL tube.
20. Prepare single-use 100-µL aliquots of the pooled supernatant containing the chromatin, and snap-freeze them in LN_2 for storage at −80°C.

Chromatin Quality and Quantity Determination and Input Chromatin Preparation

21. To determine the quality and quantity of the chromatin preparation, take a single 100-µL aliquot and reverse cross-link by incubation overnight at 65°C in a hybridization oven.
22. Clean up the test sample using DNA purification spin columns (QIAGEN catalog no. 28104).

    Use only 50 µL per column because the amount of DNA may sometimes exceed the column capacity.
23. Collect the chromatin upon elution using 30 µL of Elution buffer, and repeat this step twice before combining both eluates into a single Eppendorf tube.
24. Mix 25 µL of the eluted chromatin with 5 µL of DNA loading buffer, and load 5, 10, and 15 µL of this mixture into the separate wells of a 1% agarose gel. Load sonicated salmon sperm DNA as a control to determine the amount of extracted DNA.
    i. Mix 2.5 µL of a 1:10 diluted salmon sperm DNA stock of 1 mg/mL with 22.5 µL of ddH_2O and 5 µL of DNA loading dye.
    ii. Load 5, 10, and 15 µL of the sonicated salmon sperm DNA mixture into separate wells of a 1% agarose gel.
25. Separate the sonicated chromatin and salmon sperm DNA by gel electrophoresis.

    Chromatin DNA should appear as a smear ranging from 100 bp to 1 kb, with a maximum intensity around 500 bp.
26. If the chromatin has not been sufficiently sheared to the desired size, repeat the sample sonication as described in Step 17.
27. Quantify the reverse cross-linked and purified chromatin using a NanoDrop Spectrophotometer.

Blocking and Antibody Coating of Magnetic Beads

28. Add 1 mL of Block solution (PBS containing 0.5% BSA) to 100 µL of Dynabeads in a 1.5-mL Eppendorf tube.
29. Collect the Dynabeads using a magnetic rack; remove the supernatant, and wash the beads in 1.5 mL of Block solution for a total of three times.
30. Add 10 µg of antibody to the Dynabeads resuspended in 250 µL of Block solution and incubate overnight at 4°C.
31. Wash the Dynabeads three times in Block solution, and discard the Block solution.
32. Wash the blocked Dynabeads with RIPA buffer and aspirate. Store the Dynabeads in 100 µL of RIPA buffer at 4°C.

Setting Up the Chromatin Immunoprecipitation

33. Centrifuge the chromatin from Step 20 at 16,000*g* for 15 min at 4°C, and transfer it to a new tube.
34. Set up the chromatin immunoprecipitation as described in Protocol: **Immunoprecipitation** (DeCaprio and Kohl 2017), and incubate it overnight at 4°C.

    Generally, 1 µg of antibody is added to 12.5 µg of chromatin DNA per immunoprecipitation, translating into the use of 10 µL of antibody prebound to Protein A magnetic beads.

Washing

35. Collect the Dynabeads using a magnetic rack, and wash the Dynabeads three times with 1 mL of RIPA buffer washes allowing for 1–2 min between washes.
36. Collect the Dynabeads using a magnetic rack, and wash them three times with 1 mL of EBC lysis buffer.
37. Collect the Dynabeads using a magnetic rack, and wash them twice with 1 mL of TE buffer containing 50 mM NaCl.
38. Collect the Dynabeads by centrifugation at 1000*g* for 3 min at 4°C, and pipette off any remaining TE buffer.

    Dynabead collection using the magnetic rack does not ensure for complete removal of TE buffer stuck to the sides of the tube. Thus, centrifugation of the beads is recommended for complete buffer collection followed by removal.

Elution

39. Elute the sample from the Dynabeads by addition of 100 µL of $T_{50}E_{10}S_1$. Resuspend the beads by pipetting up and down about 10 times.

    Be careful not to lose any beads in the pipette tip.

40. Incubate the resuspended Dynabeads for 30 min at 65°C while vortexing every 5–10 min.

    Do not allow the beads to settle down.

41. Centrifuge the sample at 16,000*g* for 1 min at room temperature, and transfer the supernatant to a new 1.5-mL Eppendorf tube.
42. Elute the sample from the Dynabeads by addition of 100 µL of $T_{50}E_{10}$ (no SDS), and incubate the resuspended Dynabeads for 30 min at 65°C while vortexing every 5–10 min.

    Do not allow the beads to settle down.

43. Centrifuge the sample at 16,000*g* for 1 min at room temperature, and transfer the supernatant to the Eppendorf tube containing the first elution (Step 41) to obtain a total volume of 200 µL.

Reverse Cross-Linking

44. Incubate the sample collected in Step 43 overnight at 65°C in a hybridization oven to reverse the cross-linking.
45. Add 20 µg of RNase A to the sample and incubate for 1 h at 37°C.
46. Add 40 µg of Proteinase K and incubate for 2 h at 65°C.

DNA Purification, Amplification, and Sequencing

47. Purify the sample using polymerase chain reaction (PCR) purification spin columns, and elute the DNA in 50 µL of $T_{50}E_{10}$ buffer. The eluted DNA can be analyzed by PCR or real-time PCR or stored at −80°C for subsequent use.

    For Troubleshooting, see Table 1.

Dual Cross-Linking for Chromatin Immunoprecipitation

The dual cross-linking procedure serves the purpose of cross-linking proteins to proteins before the formaldehyde fixation of chromatin to protein and is particularly valuable for low-abundance protein antigens or when proteins interact indirectly with chromatin through associated proteins (Schmidt et al. 1977; Choe et al. 2002; Zeng et al. 2006; Adelmant and Marto 2009). This method, however, is of lesser value when protein targets are highly abundant, and, in this case, the main protocol is the better choice for a chromatin precipitation. The dual cross-linking procedure described here incorporates the use of disuccinimidyl glutarate [di(N-succinimidyl)] glutarate (DSG) during the initial cross-linking step before formaldehyde fixation.

TABLE 1. Troubleshooting

Problem	Possible cause	Solution
No specific signal observed	Inefficient precipitation of the cross-linked target protein	Increase the amount of antibody. Use a monoclonal antibody recognizing a different epitope. Use a different antibody with higher affinity to the target. Use an affinity-purified polyclonal antibody.
	Inefficient chromatin/protein cross-linking	Increase the cross-linking time. If protein/chromatin interaction is transient, explore the dual cross-linking method.
	Antibody epitope is masked by cross-linked chromatin.	Use a monoclonal antibody recognizing a different epitope. Use an affinity-purified polyclonal antibody.
	DNA fragments too large or too small	Monitor DNA fragment sizes on an agarose gel.
	Formaldehyde cross-linking time has been too short or too long.	Evaluate various cross-linking time periods.
	Wash buffer disrupted antibody–antigen interaction	Use milder Wash buffer. Reduce the salt and detergent concentration, or use a different detergent and reduce the number of washes.
High background in negative controls	Nonspecific sample binding to control antibodies	Clarify the lysate before addition of the specific antibody by centrifugation at 10,000*g* for 10 min at 4°C, transfer the supernatant to a clean tube without disturbing the pellet, and repeat the centrifugation.
	Genomic or plasmid contamination of reagents/ buffers	Perform control PCRs without DNA templates.

For additional information, please refer to Table 1 in Protocol: **Immunoprecipitation** (DeCaprio and Kohl 2017).

48. Remove the growth medium from five 15-cm^2 cell culture dishes of proliferating cells by aspiration.

    Avoid the use of overconfluent or dead cells.

49. Add 2 mL of PBS containing 1 mM PMSF to each cell culture dish; scrape and transfer the cells to a 50-mL conical tube before cell pelletting by centrifugation at 400*g* for 5 min at room temperature.

50. Wash the pellet in 20 mL of PBS with 1 mM PMSF and centrifuge at 400*g* for 5 min at room temperature. Repeat this step for a total of three washes. Resuspend the cell pellet in 20 mL of PBS with 1 mM PMSF by gentle pipetting.

    Ensure that no cell clumps are present and that all cells have been properly resuspended.

51. Add 80 µL of 0.5 M disuccinimidyl glutarate (DSG) to the 20 mL of PBS/PMSF-resuspended cells, resulting in a final DSG concentration of 2 mM, and mix immediately.

52. Incubate the sample for 45 min at room temperature on a rotating wheel set to medium speed (5–10 rpm).

53. Collect the cells by centrifugation at 400*g* for 10 min at room temperature.

54. Wash the cell pellet in 20 mL of PBS, and centrifuge it at 400*g* for 5 min at room temperature. Repeat this step for a total of three washes.

    It is extremely important to wash off and remove the DSG completely.

55. Resuspend the cell pellet in 20 mL of PBS at room temperature.

    Ensure that no cell clumps are present and that all cells have been properly resuspended.

56. Add 540 µL of 37% formaldehyde. Mix immediately and incubate the sample for 15 min at room temperature on a rotating wheel set to medium speed (5–10 rpm).

57. Quench the reaction by addition of 1 mL of 2.5 M glycine. Mix immediately, and incubate the sample for 10 min at room temperature on a rotating wheel set to medium speed (5–10 rpm).

58. Collect the cells by centrifugation at 400*g* for 5 min at 4°C, and aspirate the supernatant.

59. Wash the cell pellet twice with 10 mL of ice-cold PBS while keeping the samples on ice.

60. Snap-freeze the cells in LN_2 for storage at −80°C.

61. Proceed with the chromatin preparation and immunoprecipitation as described above, starting at Step 14.

For Troubleshooting, see Table 1.

DISCUSSION

The chromatin immunoprecipitation technique represents the most commonly applied method for investigating specific interactions between proteins and DNA on a genome-wide scale. Developed and refined in the mid-1980s, this technique is capable of elucidating the various underlying mechanisms of gene regulation, replication, and DNA damage repair (Takahashi et al. 2000; Wang et al. 2002; Weinmann and Farnham 2002; Aparicio et al. 2004; Viens et al. 2004; Breiling and Orlando 2006; Havis et al. 2006; Jin et al. 2006; Nelson et al. 2006; O'Neill et al. 2006; Sandmann et al. 2006; Haring et al. 2007; Tansey 2007; Brand et al. 2008; Cho et al. 2008; Dahl and Collas 2008a,b; Gossett and Lieb 2008; Jiang et al. 2008; Matevossian and Akbarian 2008; Mukhopadhyay et al. 2008; Saleh et al. 2008; Bermejo et al. 2009; Caby and Pierce 2009; Fode and Gatz 2009; Furlan-Magaril et al. 2009; Gaillard et al. 2009; Lindeman et al. 2009; Magdalena and Goval 2009; Papantonis and Lecanidou 2009; Percipalle and Obrdlik 2009; Sikes et al. 2009; Sorensen and Collas 2009; Tong and Falk 2009; Buro et al. 2010; Grably and Engelberg 2010; Haudry et al. 2010; Jayani et al. 2010; Kaufmann et al. 2010; Pien and Grossniklaus 2010; Ricardi et al. 2010; Villar and Kohler 2010; Adli and Bernstein 2011; Chernukhin et al. 2011; Collas 2011; Cosseau and Grunau 2011; de Folter 2011; de Medeiros 2011; Fanelli et al. 2011; Floriddia et al. 2011; Kulyyassov et al. 2011; Strenkert et al. 2011; Akkers et al. 2012; Bonn et al. 2012; Cheung et al. 2012; Duc et al. 2012; Gilfillan et al. 2012; Heliot and Cereghini 2012; Jung and Adjaye 2012; Lin et al. 2012; Micsinai et al. 2012; Murgatroyd et al. 2012; Ramaswami et al. 2012; Sailaja et al. 2012; Schuch et al. 2012; Singh and Szabo 2012; Wu et al. 2012; Adhikary and Muller 2013; Bolli et al. 2013; Blecher-Gonen et al. 2013; Cheaib and Simon 2013; Le May and Li 2013; Liao and Lu 2013; Lopez-Rubio et al. 2013; Peng and Chen 2013; van den Boogaard et al. 2013).

Numerous ChIP-validated polyclonal and monoclonal antibodies have been developed specific to the epigenetic ("control from above") regulation of gene expression and DNA replication that target DNA and histone modifications, including methylation, acetylation, ubiquitylation, phosphorylation, and sumoylation. Although the quality and specificity of these and other antibodies as well as other aspects of the immunoprecipitation process are important to the success of a chromatin immunoprecipitation, the quality of the sheared chromatin material extracted from cells or tissues is essential to the overall goal of a chromatin immunoprecipitation. Various methods for the effective shearing of chromatin have been developed and include the traditional sonication and enzymatic digestion approaches and the recently developed controllable isothermal shearing. The latter overcomes the shortcomings of sonication or enzymatic digestion by foregoing epitope damage using excessive heating and the introduction of shearing biases, thus consistently delivering reproducible results.

Although ChIP provides valuable information, the limitations of the assay need to be taken into consideration. Polyclonal or monoclonal antibodies used for the immunoprecipitation of specific target proteins might cross-react with other nuclear proteins, resulting in the misinterpretation of the results. The ChIP assay is not a functional assay and does not elucidate the function of the protein associated with the immunoprecipitated DNA, nor can it reveal the exact binding site of the protein during its interaction with the DNA. Furthermore, ChIP assays often deliver low signals in comparison with the negative controls and could result in possible misinterpretation.

Nonetheless, since its development, refinement, and modification, ChIP technology has resulted in the development of two further assays, commonly referred to as ChIP-chip (or ChIP-on-chip) and ChIP-Seq. The ChIP-chip assay requires the amplification of purified DNA fragments followed by the annealing of fragments to DNA chips or microarrays composed of immobilized DNA oligonucleotides covering the entire genome, thus providing valuable information about the genomic region(s) specifically interacting with the immunoprecipitated protein. Unlike the ChIP-chip assay, the ChIP-sequence (ChIP-Seq) assay is not influenced by variable or weak DNA annealing efficiencies and is

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considered to be more accurate by providing quantitative data, including the actual DNA sequences specifically interacting with the immunoprecipitated protein. Together, all three assays—ChIP, ChIP-chip, and ChIP-Seq—can provide valuable data and information about the proteins associated with DNA and their function in gene regulation.

RECIPES

Disuccinimidyl Glutarate (DSG; 0.5 M)

Reagent	Amount per 300 µL of solution (w/v)	Final concentration
Disuccinimidyl glutarate	50 mg	0.5 M
DMSO	300 µL	

Store at −20°C.

DNA Loading Buffer (6×)

30% (v/v) glycerol
0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF

Store at 4°C.

EBC Lysis Buffer for ChIP

Reagent	Volume per 100 mL of solution (v/v)	Final concentration
NaCl (5 M)	2.4 mL	120 mM
Nonidet P-40 (10%)	5.0 mL	0.5%
Leupeptin (10 mg/mL)	50 µL	5 µg/mL
Aprotinin (10 mg/mL)	100 µL	10 µg/mL
PMSF (0.1 M)	1 mL	1 mM
Na-vanadate (0.2 M)	100 µL	0.2 mM
NaF (1 M)	10 mL	100 mM
Tris-HCl (1 M, pH 8.0)	5.0 mL	50 mM
ddH_2O	Add to 100 mL	

Store at 4°C. Add protease inhibitors immediately before use.

Elution Buffer for ChIP

50 mM Tris-Cl (pH 8.0)
10 mM EDTA
1% SDS

Store at room temperature.

Formaldehyde Solution (11%)

Reagent	Volume per 50 mL of solution (v/v)	Final concentration
HEPES-KOH (1 M, pH 7.5)	2.5 mL	50 mM
NaCl (5 M)	1.0 mL	100 mM
EDTA (0.5 M)	100 µL	1 mM
EGTA (0.5 M)	50 µL	0.5 mM
Formaldehyde (37%)	14.9 mL	11%
ddH_2O	Add to 100 mL	

Store at room temperature.

Lysis Buffer 1 for ChIP

Reagent	Volume per 100 mL of solution (v/v)	Final concentration
HEPES-KOH (1 M, pH 7.5)	5 mL	50 mM
NaCl (5 M)	2.8 mL	140 mM
EDTA (0.5 M)	0.2 mL	1 mM
Glycerol (50%)	20 mL	10%
Nonidet P-40 (10%)	5.0 mL	0.5%
Triton X-100 (10%)	2.5 mL	0.25%
ddH_2O	Add to 100 mL	

Store at 4°C.

Lysis Buffer 2 for ChIP

Reagent	Volume per 100 mL of solution (v/v)	Final concentration
Tris-HCl (1 M, pH 8.0)	1.0 mL	10 mM
NaCl (5 M)	4.0 mL	200 mM
EDTA (0.5 M)	0.2 mL	1 mM
EGTA (0.5 M)	0.1 mL	0.5 mM
ddH_2O	Add to 100 mL	

Store at 4°C.

Lysis Buffer 3 for ChIP

Reagent	Volume per 100 mL of solution (v/v)	Final concentration
Tris-HCl (1 M, pH 8.0)	1.0 mL	10 mM
NaCl (5 M)	2.0 mL	100 mM
EDTA (0.5 M)	0.2 mL	1 mM
EGTA (0.5 M)	0.1 mL	0.5 mM
Na-deoxycholate (10%)	1.0 mL	0.1%
N-Lauroyl-sarcosine (20%)	2.5 mL	0.5%
ddH_2O	Add to 100 mL	

Store at 4°C.

RIPA Buffer Solution for ChIP

Reagent	Volume per 500 mL of solution (v/v)	Final concentration
HEPES-KOH (1 M, pH 7.6)	25 mL	50 mM
LiCl (5 M)	50 mL	500 mM
EDTA (0.5 M)	1 mL	1 mM
Nonidet P-40 (10%)	50 mL	1%
Na-deoxycholate (10%)	35 mL	0.7%
ddH_2O	Add to 500 mL	

Prepared RIPA buffer should be aliquoted and stored at −20°C. Add protease and/or phosphatase inhibitors to a thawed aliquot before immediate use. Discard and do not freeze again.

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