

Mostoslavsky's Lab Protocols

Chromatin Immunoprecipitation (ChIP)

Materials

1. Formaldehyde: 36.5% (Sigma Aldrich)
2. 1xPBS (Invitrogen)
3. glycine: 2.5 M. For 1 L, dissolve 187.5 g glycine in 1 L of distilled water. Filter to sterilize. Store at room temperature.
4. Lysis Buffer: 150 mM NaCl, 1% v/v Nonidet P-40, 0.5% w/v Sodium deoxycholate, 0.1% w/v SDS, 50 mM Tris-Cl pH 8.0, and 5 mM EDTA. For 500 ml, mix: 15 mL of 5 M NaCl (For 1 L of 5 M NaCl, dissolve 292 g of NaCl in 800 ml of distilled water. Adjust the volume to 1 L with distilled water), 5 ml of 100% Nonidet P-40, 2.5 g Sodium deoxycholate, 2.5 ml of 20% SDS (For 1 L of 20% SDS, dissolve 200 g of SDS in 900 ml of distilled water. Heat to 68°C with constant stir. Adjust the volume to 1 L with distilled water), 5 ml of 1 M Tris-Cl pH 8.0 (For 1 L of 1 M Tris-Cl pH 8.0, dissolve 121.1 g of Tris base in 800 ml distilled water. Adjust the pH to 8.0 with concentrated HCl. Adjust the volume to 1 L with distilled water), 5 ml of 0.5 M EDTA (For 1 L of 0.5 M EDTA, add 186.1 g of EDTA.2H₂O to 800 ml distilled water. Stir constantly. Adjust the pH to 8.0 with NaOH. Adjust the volume to 1 L with distilled water). Adjust the volume to 500 ml with distilled water. Store at 4°C.

Right before use, add: Protease Inhibitors Cocktail (Roche Applied Science), Phosphatase Inhibitors: 50 mM NaF (For 5 ml of 500 mM NaF, dissolve 0.1 g in 5 ml distilled water. Aliquot and store at -20°C.), 0.2 mM sodium orthovanadate (For 5 ml of 200 mM sodium orthovanadate, dissolve 0.18 g in 5 ml distilled water. Aliquot and store at -20°C), HDAC inhibitors: 5 µM trichostatin A (For 5 mM TSA, dissolve 5 mg in 3.3 ml DMSO), 5 mM sodium butyrate (For 5 M, dissolve 2.75 g in 5 ml of distilled water. Aliquot and store at -20°C), 0.5 mM PMSF (For 100 mM PMSF, dissolve 87.09 mg in 5 ml ethanol. Aliquot and store at -20°C).
5. Protein A agarose beads (Roche Applied Science)
6. Salmon sperm DNA (Invitrogen)
7. BSA (Fisher Scientific). For 10 mg/ml stock solution, dissolve 100 mg in 8 ml of distilled water. Adjust the volume to 10 ml with distilled water. Aliquot and store at -20°C.
8. SIRT6 antibody (See Note 1)
9. High Salt Buffer. 100 mM Tris-Cl pH 8.5, 500 mM LiCl, 1% Nonidet P-40 and 1% w/v Sodium deoxycholate. For 500 ml, add: 50 ml of 1 M Tris-Cl pH 8.5, 50 ml of 5 M LiCl (For 5 M LiCl, dissolve 21.2 g LiCl in 90 ml distilled water. Adjust the volume to

100 ml. Store at 4°C), 5 ml of 100% Nonidet P-40, and 5 g of Sodium deoxycholate. Adjust the volume to 500 ml with distilled water. Store at 4°C.

10. Low Salt Buffer. 150 mM NaCl, 0.5% w/v Sodium deoxycholate, 0.1% w/v SDS, 1% Nonidet P-40, 1 mM EDTA pH 8.0, Tris-Cl pH 8.0. For 500 ml, add: 15 ml 5 M NaCl (Final concentration: 150 mM), 2.5 g Sodium deoxycholate, 2.5 ml 20% SDS, 5 ml 100% Nonidet P-40, 1 ml 0.5 M EDTA, pH 8.0, 25 ml 1M Tris-Cl, pH 8.0. Adjust the volume to 500 ml with distilled water. Store at 4°C.

11. Tris-EDTA (TE, pH 8.0). 100 mM Tris-Cl pH 8.0 and 10 mM EDTA pH 8.0. For 500 ml, mix 50 ml 1M Tris-Cl pH 8.0 and 10 ml 0.5 M EDTA pH 8.0. Adjust the volume to 500 ml with distilled water.

12. Talianidis Elution Buffer. 70 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0, 1.5% w/v SDS. For 50 ml, mix 3.5 ml 1M Tris HCl pH 8.0, 100 ul 0.5 M EDTA pH 8.0, and 3.75 ml 20% w/v SDS. Adjust the volume to 50 ml with distilled water. Store at room temperature.

13. DNA purification Kit (Qiagen)

14. Real-time PCR kit (Roche)

Methods

This Sirt6 ChIP protocol is adapted with modifications from Joaquin Espinosa's laboratory.

1. Grow Cells in 150 mm plates until cells are 50-70% confluent.
2. Cross-linking: Remove media from the plates completely. Add 19 ml of 1% Formaldehyde in 1xPBS. Incubate on a shaking platform at room temperature for 15 minutes (See Note 2).
3. Quench formaldehyde with 125 mM glycine (1 ml of 2.5 M glycine per 19 ml of 1% formaldehyde in 1xPBS) on a shaking platform at room temperature for 5 minutes.
4. Remove the formaldehyde solution completely from the plates.
5. Wash the plates twice with ice-cold 1xPBS. After the second wash, place the plates vertically on ice for 1 minute to collect the leftover PBS at the bottom of the plates. Remove PBS by aspiration.
6. Put the plates on ice or at 4°C. Add lysis buffer (1 ml per plate) to the first plate (See Note 3). Scrape the plate and transfer the lysate to the next plate, until all the plates are collected (See Note 4).
7. Sonicate the lysate to shear chromatin (See Note 5).

8. Centrifuge at 12,000 rpm for 20 minutes at 4°C to clear the lysate. Transfer the supernatant to a fresh tube. The lysate can be snap frozen and stored at -80°C (See Note 4).
9. Measure protein concentration and dilute the lysate to 2 mg/ml with complete lysis buffer. Aliquot ~1.1 ml/tube and snap freeze the lysate if it is not used immediately. Store aliquots at -80°C.
10. Wash 2 sets of protein A agarose beads (50 µl per IP sample) 3 times with lysis buffer to remove ethanol. A wash consists of resuspending the beads with 1 ml lysis buffer, centrifuging at 4,000 rpm for 1 minute at 4°C, and removing the supernatant. One set of protein A agarose beads will be used to pre-clear the lysate. The other set will be blocked and used in the IP.
11. Pre-clear lysate with 50 µl of 50% slurry protein A agarose beads previously washed in lysis buffer, not blocked with salmon sperm DNA nor BSA. Rotate at 4°C for 2 hours. Centrifuge 1 minute at 4,000 rpm at 4°C. Transfer supernatant to a fresh tube.
12. During step 11, block the other set of washed agarose beads with 1 mg/ml BSA and 0.3 mg/ml Salmon Sperm DNA. Rotate for 2 hours at 4°C. Wash 3 times with lysis buffer.
13. Transfer 10 µl of pre-cleared lysate to a new tube and store at -20°C for later use. This is the Input sample.
14. Transfer 1 ml of pre-cleared lysate to a new tube and add Sirt6 antibody or equivalent amount of non-specific serum or IgGs. The amount of Sirt6 antibody added should be determined empirically (See Note 1). Add 50 µl of blocked protein A agarose beads from Step 12. Rotate at 4°C overnight.
15. Wash 2 times with lysis buffer, 4 times with high salt buffer (See Note 6), 2 times with lysis buffer, and 2 times with TE.
16. Aspirate supernatant from the last wash down to the 100 µl mark in the microcentrifuge tube (Take special care to be consistent between samples).
17. Add 200 µl of Talianidis Elution Buffer.
18. Elute immunocomplexes by incubating 10 minutes at 65°C, vortex samples occasionally.
19. Transfer 240 µl to a fresh tube and add 10 µl of 5M NaCl (200 mM final).
20. Take out the Input samples from -20°C (from step 13). Add 10 µl of 5M NaCl and 230 µl of Talianidis Elution Buffer.
21. Reverse-crosslink all the samples (including Inputs) by incubating them in a 65°C water bath for 5 hours. Spin occasionally to bring down liquid condensation formed on the lids of the tubes.

22. Purify DNA with commercially available DNA purification kit of your choice. Purified DNA can be stored at -20°C.

23. Setup real time PCR with the primer pairs designed to amplify genomic region of interest. Follow the PCR protocol of the PCR kit of your choice. For data analysis, follow standard procedure (for example: <http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/epigenetics-noncoding-rna-research/Chromatin-Remodeling/Chromatin-Immunoprecipitation-ChIP/chip-analysis.html>)

Notes

1. The quality of the Sirt6 antibody is critical to the success of the ChIP. A desirable Sirt6 antibody would give one single clear band on a whole cell lysate western blot. It should also work well in an immunoprecipitation experiment. If such Sirt6 antibody is not available, proper controls are necessary to differentiate real Sirt6 binding signals from non-specific binding. Always include an IgG control when planning the ChIP experiment. Another important control is to use Sirt6 deficient cells or Sirt6 knockdown cells.

2. The cross-linking time and the formaldehyde concentration are the key factors that affect cross-linking efficiency. Longer cross-linking time or higher formaldehyde concentration is more efficient, but may cause detection of non-specific binding. On the other hand, shorter cross-linking time or lower formaldehyde concentration may increase chromatin shearing efficiency, but this may decrease the yield of precipitated chromatin for certain proteins, especially those that do not directly bind to DNA.

3. The protease/HDAC/phosphatase inhibitors should be added right before the harvest.

4. The lysate can be snap-frozen by dropping the tubes into liquid nitrogen and stored at -80°C until ready to proceed. To thaw, put tubes in room temperature water bath, then put on ice.

5. The sonication conditions should be determined empirically for each cell type or sonicator model. To check the sonication efficiency, take an aliquot of 50 µl from the sonicated chromatin, add 3 µl of 5 M NaCl and put on a 95°C heatblock for 5 minutes. Run the mixture on a 1.5% agarose gel. The fragment should be between 300-1000 bp.

6. If the Sirt6 antibody is not strong, substitute High Salt Buffer with Low Salt Buffer.

References

Donner, A.J., Ebmeier, C.C., Taatjes, D.J., and Espinosa, J.M. (2010) CDK8 is a positive regulator of transcriptional elongation within the serum response network. *Nat Struct Mol Biol.* 17, 194-201.