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NB21-1252 Protocol

Protocol specific for H3K36me2 antibody (NB21-1252)

Histone H3 [Dimethyl Lys36] Antibody: https://www.novusbio.com/products/histone-h3-antibody_nb21-1252
Western Blot Protocol

1. Perform SDS-PAGE on samples to be analyzed, loading 10 ug of histone preps per lane.

2. Transfer proteins to membrane according to the instructions provided by the manufacturer of the membrane and transfer apparatus.

3. Stain according to standard Ponceau S procedure (or similar product) to assess transfer success, and mark molecular weight standards where appropriate.

4. Rinse the blot.

5. Block the membrane using standard blocking buffer for at least 1 hour.

6. Wash the membrane in wash buffer three times for 10 minutes each.

7. Dilute primary antibody in blocking buffer and incubate 1 hour at room temperature.

8. Wash the membrane in wash buffer three times for 10 minutes each.

9. Apply the diluted HRP conjugated secondary antibody in blocking buffer (as per manufacturers instructions) and incubate 1 hour at room temperature.

10. Wash the blot in wash buffer three times for 10 minutes each (this step can be repeated as required to reduce background).

11. Apply the detection reagent of choice in accordance with the manufacturers instructions.

Note: Tween-20 can be added to the blocking or antibody dilution buffer at a final concentration of 0.05-0.2%.

Chromatin Immunoprecipitation Protocol

Cell Fixation and Preparation

1. Begin with a cell culture that has reached 80% confluency.

2. Add formaldehyde to a final concentration of 1% in growth media and incubate for 10 minutes at room temperature.

3. Add glycine to reach a final concentration of 125 mM in the media. Incubate for 5 minutes at room temperature.

4. Remove all media and wash twice with 20 mL of ice cold PBS.

5. Add 2 mL of ice cold PBS with protease inhibitors*. Scrape cells into microcentrifuge tube.

6. Spin cells at 4 C for 5 minutes at 800 x g.

7. Remove supernatant and resuspend cells in 750 ul of RIPA lysis buffer containing protease inhibitors* per 10,000,000 cells (enough for 10 IPs). Incubate at 4 C for 15 minutes.

8. Spin cells at 4 C for 5 minutes at 800 x g.

DNA Shearing by Sonication

1. Sonicate crosslinked DNA to fragments sizes of 200-1000 base pairs. Keep samples ice cold to prevent denaturing of chromatin. Conditions for fragmenting must be empirically derived, and vary depending on equipment, cell type, cell density, and cross-linking efficiency.

2. Centrifuge samples to remove debris at 4 C for 10 minutes at 12,500 x g. Remove supernatant and transfer to a new tube. Discard pellet. Set aside 75 ul of sample for input fraction, which will not go through the subsequent IP steps. The remaining sample can be moved into 75 ul aliquots, each of which is sufficient for a single IP. Although it is preferable to proceed directly to the following steps, sheared chromatin can now be frozen at -80 C for up to 1 month.

a. Optional: Test the efficiency of the shearing by running 5-10 ul of sample on a 2% agarose gel after reversal of crosslinking, RNase treatment (0.5 mg/ml) and proteinase K treatment (0.1 ug/ul) as described below.

Chromatin Immunoprecipitation

Recommended controls include: No antibody negative control OR normal IgG negative control, positive control antibody.

1. Dilute each IP sample 1:10 by adding 75 ul sheared chromatin to 675 ul dilution buffer, along with appropriate protease inhibitors*. Save undiluted input fraction for step 10.

2. Add 25 ul of fully suspended protein A/G magnetic bead slurry. Do not allow the beads to dry.

3. Add antibody of interest to the diluted sample. For best results, incubate tubes with rotation at 4 C overnight. Alternatively, incubate at room temperature for 1-2 hours.

4. Pellet magnetic beads with a magnetic separator and remove the supernatant.

5. Add 750 ul cold low salt buffer and wash for 5 minutes with rotation. Pellet beads with separator and discard supernatant.

6. Add 750 ul cold high salt buffer and wash for 5 minutes with rotation. Pellet beads with separator and discard supernatant.

7. Add 750 ul cold LiCl buffer and wash for 5 minutes with rotation. Pellet beads with separator and discard supernatant.

8. Add 750 ul cold TE buffer and wash for 5 minutes with rotation. Pellet beads with separator and discard supernatant.

9. Elute complex by adding 200 ul elution buffer and agitate at RT for 15 minutes. Pellet beads with separator and discard beads. Keep the supernatant.

10. Add 8 ul of 5M NaCl and 2 ul of proteinase K (10 ug/ul) to each sample to reverse cross-linking. For the input fraction, add 125 ul of elution buffer along with NaCl and proteinase K. Incubate at 62 C for 4 hours or overnight. Incubate at 95 C for 10 minutes to deactivate proteinase K.

a. Optional: To decrease time, this step may also be performed at 95 C for 20 minutes without proteinase K treatment.

DNA Purification and Amplification

1. DNA can now be purified with either a commercially available column kit or by phenol/chloroform extraction.

2. Perform real-time PCR with 2 ul of purified DNA and primers (catalog numbers NBP1-71650, NBP1-71651, NBP1-71652, NBP1-71653, NBP1-71654 and NBP1-71655) per reaction. Dilute input fraction to 1% before PCR. Normalize all IPs and no antibody control IP to adjusted input fraction.

a. Ex. Raw input CT=30, adjusted input: 30 - 6.44 = 23.4

b. Antibody CT=28

c. Normalized signal relative to input: 2 ^ (23.4-28) = 0.04

Buffers

Dilution Buffer: 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCL (pH8.1), 167 mM NaCl

Low Salt Wash Buffer: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl.

High Salt Wash Buffer: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl.

LiCl Wash Buffer: 250mM LiCl, 1% NP-40, 1% Na-deoxycholate, 1mM EDTA, 10mM Tris, pH 8.1.

TE Buffer: 10mM Tris-HCL pH 8.1, 1 mM EDTA

Elution buffer: 1% SDS, 100mM NaHCO3

* Please note that protease inhibitors have variable half-lives and should be freshly prepared as applicable.

**The above information is only intended as a guide. The researcher should determine what protocol best meets their needs. Please follow safe laboratory procedures.