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Western blot analysis and immunoprecipitation assay

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

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1 Cells are washed in cold PBS and lysed on ice in a buffer containing 1.6 mM NaH2PO4, 8.6 mM Na2HPO4, 1% Triton X-100, 0.1% SDS; 0.1% NaCl, 0.5% NaDoc, 2 mM AEBSF, 20 mg/mL each of aprotinin and leupeptin.

2 Cell lysates are centrifuged in a microfuge at maximum speed for 10 min to eliminate debris.

3 Protein content is evaluated by bicinchoninic acid assay (BCA Protein assay kit by Thermo Fisher) in a spectrophotometer.

4 Lysates (100 µg each) are subjected to SDS-PAGE electrophoresis and transferred to PVDF membrane.

5 For the IMMUNOPRECIPITATION ASSAY, cells are lysed in a buffer containing 20 mM Tris-HCl, 1% Triton X-100, 10% Glycerol, 2 mM AEBSF, 20 mg/mL each of aprotinin and leupeptin.

6 Lysates (1000 µg) are incubated overnight at 4˚C with the desired antibody under constant rotation.

7 Gammabind G-Sepharose (40 µl) is added to each sample and let rotate for 1 hour and 30 min.

8 Samples are washed three times with the lysis buffer in a microfuge at 4300 g for 1 min.

9 After eliminating the last supernatant, samples are eluted in 40 µl of 1x Laemmli's buffer, subjected to SDS-PAGE and transferred to PVDF membrane.

10 PVDF membrane is incubated in blocking buffer (4% nonfat milk in T-TBS -Tris Buffer Solution 1x with 0,05%Tween) for 2 hr at room temperature.

11 Membrane is incubated in primary antibody diluted in 0,5%BSA in T-TBS, overnight at 4°C.

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12 Membrane is washed in T-TBS, 5 times, 5 min each.

13 Membrane is incubated in HRP-conjugated secondary antibody diluted in 0,5%BSA in T-TBS, 2 hr, at room temperature.

14 Membrane is washed in T-TBS, 5 times, 5 min each.

15 Proteins are visualized by enhanced chemiluminescence (ECL) detection, through the Bio-Rad ChemiDoc instrument. Signal intensity was measured by densitometry using the Bio-Rad Image Lab 6.0 software.

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