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Western blotting for Rubicon and Pacer expression

In 1 collection

Dan Tudorica[1]

[1]University of California, Berkeley

Dan Tudorica
Hurley Lab, QB3, UC Berkeley

ABSTRACT

Traditional.

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1 Wash adherent cells to be analyzed twice with PBS, then scrape into microcentrifuge tubes. Pellet cells via tabletop centrifuge, aspirate supernatant, and lyse for 30 minutes on a rocker at 4 C (lysis buffer: 50 mM HEPES 7.4, 150 mM NaCl, 0.5% NP-40 detergent, 1 mM TCEP, cOmplete protease inhibitor tablet).

2 Pellet cells post lysis and recover supernatant

3 Measure protein content via a BCA assay. Briefly, reconstitute BCA detection reagent, dispense ~100 uL into each well of a 96 well plate, and prepare BSA stock solutions at 1, 2, and 3 mg/mL diluted in PBS.

Set up a standard curve using the BSA samples, as well as blanks for lysis buffer and PBS. To prepare samples, add 2 uL of sample to the prepared 100 uL of BCA detection reagent.

Incubate sample at 37 C for 30 minutes, then scan on a plate reader using a BCA detection protocol. Calculate standard curve, and use to determine protein concentration in each sample. Check that BSA standard curve is linear, and that analyzed samples fall within the standard curve linear range.

4 Load 20 µg of samples in each lane of an SDS-PAGE, and run at 120 V for about 1.5 H. Run a prestained ladder

5 Recover gels and incubate in transfer buffer (48 mM Tris Base, 39 mM glycine, 0.037% SDS, 20% methanol) for 5 minutes. Also incubate a nitrocellulose sheet in transfer buffer.

6 Set up TransBlot apparatus, keeping transfer sandwich moist in transfer buffer. Transfer using standard protocol for 1 gel.

7 Post transfer, use MW markers to cut strip at expected MW

8 Incubate strips in 5% (m/v) powdered milk in TBST at RT for 1 H or at 4 C while rocking to block.

9 Dilute primary antibodies according to manufacturer instructions in blocker buffer, incubate at RT for 1 H in a rocker.

10 Wash strips 3 x 5 min in TBST

11 Dilute secondary antibodies conjugated to HRP according to manufacturer instructions in blocking buffer, incubate at RT for 1 H in a rocker.

12 Wash strips 3x5 min in TBST.

13 Pick up strips and dry corner using kimwipe. Lays strips on saran wrap when excess buffer has been removed.

14 Reconstitute ECL substrate and pippete onto strips. Incubate for 5 min before dabbing off excess substrate and imaging using a chemiluminescent protocol on a chemidoc.