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Western Blot V.1

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Chelsie Steele[1], Yujie Fan[1]

[1]Caltech

Chelsie Steele
Caltech

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We use this protocol and it's working

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Abstract

Western Blot

Materials

10x RIPA buffer (Millipore 20-188)
Protease inhibitor cocktail (AbCAM, ab271306)
Qubit Protein BR Assay kit (A50668)
Qubit4

Collecting cells from the plate

1 This protocol is written for a 24-well plate, please adjust the medium/buffer volume based on plate size.

2 Aspirate the medium in the wells and wash once with pre-cold PBS. Add 0.5mL of Trypsin to each well, and incubate the plate for 5 minutes at 37°C.

3 Add 0.5mL of 5% FBS to each well to neutralize the Trypsin. Collect the cells with the cell-scraper into a 1.5mL Eppendorf tube. Make sure all the cells are scrapped off the plate.

4 Centrifuge the tubes at 300g for 5 minutes.

5 Discard the supernatant. If not performing the WB immediately, store pellet at -80°C.

Preparing Western Blot lysate with RIPA buffer

6 Prepare 1x RIPA buffer to lysis the cells. Use 10x RIPA buffer (Millipore 20-188) to prepare 1X RIPA buffer (2mL RPA buffer + 18mL H2O).

7 Add protease inhibitor cocktail (1x RIPA buffer + Protease inhibitor cocktail (1:100). At this step, 1x RIPA buffer with inhibitor can be aliquoted and frozen down at -20°C.

8 Add 100 ul RIPA buffer to the cell pellet and re-suspend the cells. Leave the RIPA lysate on ice for 15 minutes. If there are cell clusters, continue pipetting until the lysate is clear.

9 Centrifuge the lysate for 10 minutes at 15000g at 4°C (important that it is done at 4°C). Transfer the supernatant to a new 1.5mL Eppendorf tube. Make sure that the transferring is performed on ice. The supernatant will be your samples for the Western Blot.

10 At this step, cell lysis can be stored at -80°C for long-term storage.

Measuring Protein concentration Using Qubit Protein BR Assay

11 Use the Qubit Protein BR Assay kit (A50668) and Qubit4 to measure the protein concentration. In the 0.5mL Qubit Invitrogen tubes, prepare a Blank, Standard 1, Standard

2, and samples according to the following, ensuring that the order added is Sample (or Standard),, followed by the BR buffer, followed by the kit reagent:

Blank: Add 170uL of BR buffer + 30uL of Reagent
Standard 1: Add 20uL of S1 + 150uL BR Buffer + 30uL Reagent
Standard 2: Add 20uL of S2 + 150uL BR Buffer + 30uL Reagent
Sample: Add 2uL of Sample + 168uL of BR Buffer + 30uL of Reagant

12 Vortex each of the blank, standards, and samples, and leave at room temperature for 10 minutes. Measure the protein concentration.

Sample Preparation and Boiling

13 In a separate 1.5mL Eppendorf tube, prepare a 1:9 ratio of 4x Laemmli Sample Buffer to 2-mercaptoethanol. Prepare enough for 34uL of this mix per sample.

14 Add 34uL of the loading mix to 100uL of lysis sample, and boil the samples for 3-5 minutes. At this point, prior to boiling, samples can be stored long-term at -80°C.

Gel Preparation and Running

15 After boiling samples, prepare 1X running buffer: mix 100mL of 10X Tris/Glycine/SDS Buffer with 900mL of Milli-Q water.

16 Prepare mini-protein TGX stain-free gels (4-20%), in the BIO-RAD running cassette, and add running buffer.

17 Load samples onto wells depending on protein concentration (up to 20uL/well). Add 8uL of loading marker dye to each end.

18 Run the gel at 100V for 10 minutes, and then at 150V for 30-40 minutes.

Transfer with Trans-Blot Turbo Transfer System and iBind Antibody Incubation

19 Transfer gel according to the Trans-Blot Turbo Transfer System. Transfer for 30 minutes.

20 Following transfer, prepare 1X iBind Solution by adding: 300uL of 100X Additive + 6mL iBind 5X buffer + 23.7mL H2O to a 50mL falcon tube.

21 Prepare primary antibody and secondary antibodies according to iBind kit, and add solution buffer in appropriate amounts.