Mar 01, 2025 Version 2

Western Blot V.2

DOI
https://dx.doi.org/10.17504/protocols.io.j8nlk96p6v5r/v2

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DOI: https://dx.doi.org/10.17504/protocols.io.j8nlk96p6v5r/v2

Protocol Citation: Chelsie Steele, Yujie Fan 2025. Western Blot . **protocols.io** https://dx.doi.org/10.17504/protocols.io.j8nlk96p6v5r/v2Version created by Yujie Fan

Protocol status: Working
We use this protocol and it's working

Created: March 01, 2025

Last Modified: March 01, 2025

Protocol Integer ID: 123609

Keywords: western blot, ibind automated western system

Funders Acknowledgements:
Aligning Science Across Parkinson's
Grant ID: ASAP-020495

Abstract

Western Blot using the iBind Automated Western Systems

Materials

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

Collecting cells from the plate

15m

1 This protocol is written for a 6-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 DPBS. Add 1 mL of cold DPBS to each well, and collect the cells with cell lifter.

10m

3 Centrifuge the tubes at 300g for 5 minutes. Discard the supernatant. If the WB is not performed immediately, the pellet can be stored at -80°C.

5m

Preparing Western Blot lysate with RIPA buffer

55m

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

5m

5 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.

5m

6 Add 100 ul RIPA buffer to the cell pellet and re-suspend the cells. Leave the RIPA lysate on ice for 30 minutes. Pipetting to mix every 10 minutes until the lysate is clear.

30m

7 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 step is performed on ice. The supernatant will be your samples for the Western Blot.

15m

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

Measuring Protein concentration Using Qubit Protein BR Assay

40m

9 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:

20m

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

10 Vortex each of the blank, standards, and samples, and leave at room temperature for 10 minutes. Measure the protein concentration. 20m

Sample Preparation and Boiling 5m

11 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.

12 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. 5m

Gel Preparation and Running 1h 20m

13 After boiling samples, prepare 1X running buffer: mix 100mL of 10X Tris/Glycine/SDS Buffer with 900mL of Milli-Q water. 5m

14 Prepare mini-protein TGX stain-free gels (4-20%), in the BIO-RAD running cassette, and add running buffer. 5m

15 Load samples onto wells depending on protein concentration (up to 20uL/well). Add 8uL of loading marker dye to each end. 10m

16 Run the gel at 100V for 10 minutes, and then at 150V for 30-40 minutes. 1h

Transfer with Trans-Blot Turbo Transfer System 1h 30m

17 Transfer gel to PVDF membrane using the Trans-Blot Turbo Mini 0.2 um PVDF Transfer Packs. Transfer for 30 minutes. 30m

18 After the transfer, membranes are placed in iBind buffer. If alpha-synuclein and pS129-alpha-synuclein are to be blotted, fix the membrane with 4% PFA in PBS for 10 minutes and wash with PBS for 30 minutes before blotting. 1h

Protein Immunodetection with iBind Automated Western Systems

8h 40m

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

10m

20 Prepare primary antibodies, secondary antibodies and washing buffer according to iBind kit protocol. Add them to iBind Automated Western Systems and run overnight.

8h

21 Detect the signal with Max Western ECL and image with Biorad ChemiDoc Imaging system

30m