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Immunoblotting

Forked from Immunoblotting

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We use this protocol and it's working

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Abstract

This protocol describes collection of protein from cultured cells and immunoblotting

Materials

Cell culture materials:
DMEM (Thermo Fisher Scientific, 11965-092)
FBS (Thermo Fisher Scientific, 16140-071)
Penicillin/Streptomicin (10,000 U/mL; Thermo Fisher Scientific, 15140122)
PBS (Thermo Fisher Scientific, 10010023)
Earle's Balanced Salt Solution (EBSS; Thermo Fisher Scientific, 24010043)

Transfection Reagents:
Opti-Mem (Thermo Fisher Scientific, 31985062)
Lipofectamine 3000 (Thermo Fisher Scientific, L3000008)
Dharmafect (Horizon Discovery, T-2001-01)

Lysis Buffer:
lysis buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40) with Protease Inhibitor Cocktail
Tris (American Bio, AB02000-05000)
NaCl (Sigma-Aldrich, S9888-25G)
EDTA (Sigma-Aldrich, 3690)
Nonidet P40 Substitute (Roche, 11754599001)
COmplete mini EDTA Free (Roche, 11836170001)

Cell culture and treatments

2d

1 Culture the HEK293 cells at 37 °C in 5% CO_2 and DMEM containing 10% FBS, 1000 U/mL penicillin, a [M] 1 mg/mL streptomycin.

2 For any given experiment, plate the cells at such density so as to be approximately 90% confluent at the time of lysis.

3 For experiments using siRNA, transfect a final concentration of 25 nM of the indicated siRNA using 4 µL Dharmafect (Horizon Discovery) in Opti-MEM (Gibco) per well of a six well dish according to manufacturer protocol. Lyse the cells 48:00:00 after siRNA transfection. 2d

Cell lysis and sample preparation

18m

4 Supplement lysis buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40) with Protease Inhibitor Cocktail (Roche) and chill On ice .

5 Aspirate media from cells, add PBS, and scrape cells using a cell scraper (Corning) On ice

6 Pipette the resuspended cells into Eppendorf tube.

7 Centrifuge at 500 x g, 4°C, 00:03:00 , aspirate the excess PBS. 3m

8 Pipette 150 uL lysis buffer onto cell pellet, pipette mixture up and down 10 times with a P-200 pipette tip

Note

NOTE: Take care not to introduce bubbles.

9 Incubate Eppendorf tube On ice for 00:05:00 . 5m

10 Determine protein concentration in sample using Bradford Assay.

11 Prepare samples at desired concentration, add 1 mM DTT and 1x LDS loading buffer.

Gel electrophoresis and immunoblotting 5h 20m

12 Incubate samples at 95 °C for 00:05:00 . 5m

13 During this incubation, prepare gel apparatus with NuPAGE™ Bis-Tris Mini Protein Gels, 4–12%, 1.0–1.5 mm (ThermoFisher) and 1x MOPS running buffer.

14 Load samples into gel and run until dye front reaches bottom (180 V, roughly 60 min).

15 Remove gel and set up transfer cassette with nitrocellulose membrane.

16 Transfer at 30-35 V for 01:30:00 in 1x transfer buffer. 1h 30m

17 Block membrane with 5% BSA in PBST for 01:00:00 at 22 °C . 1h

18 Incubate membrane with primary antibodies in 5% BSA and 0.03% sodium azide in PBST Overnight at 4 °C . 1h

Note

NOTE: Optimal primary antibody incubation time and temperature can be determined empirically for a given primary antibody

19 Wash membrane with PBST for five minutes. Repeat a total of 3 times.

19.1 Wash membrane for 00:05:00 with PBST (1/3). 5m

19.2 Wash membrane for 00:05:00 with PBST (2/3). 5m

19.3 Wash membrane for 00:05:00 with PBST (3/3). 5m

20 Incubate membrane with secondary antibodies (1:5,000) in 5% milk in PBST for 01:00:00 at 22 °C . 1h

21 Wash membrane with PBST. Repeat a total of 3 times.

21.1 Wash membrane for 00:05:00 with PBST (1/3). 5m

21.2 Wash membrane for 00:05:00 with PBST (2/3). 5m

21.3 Wash membrane for 00:05:00 with PBST (3/3). 5m

22 Image membranes using a BioRad VersaDoc imaging system.

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