Jun 15, 2023

Western Blotting

DOI
dx.doi.org/10.17504/protocols.io.36wgq3rzklk5/v1

Eric ECS Cordeiro-Spinetti[1]

[1]University of Miami

Eric ECS Cordeiro-Spinetti
University of Miami

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Protocol Citation: Eric ECS Cordeiro-Spinetti 2023. Western Blotting. **protocols.io** https://dx.doi.org/10.17504/protocols.io.36wgq3rzklk5/v1

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

Created: June 15, 2023

Last Modified: June 15, 2023

Protocol Integer ID: 83418

Keywords: western blotting

Abstract

It's a classic

Troubleshooting

Before Starting

2h 40m 45s

1

Make RIPA buffer (to be used in 3 months after adding Nuclease)

	A	B
	50mM Tris HCl, PH 7.4	5mL (1M stock)
	150mM NaCl	5mL (3M stock)
	1% Triton X-100 or NP-40	1 mL
	0.1% SDS	1mL (10% supply center solution)
	1mM EDTA	0.2 mL (0.5M stock)
	10mM Naf	0.042g (@L5P1 chemical room)
	0.5% Sodium deoxylcholate	0.5g (@L5P1 chemical room)
		Add ddH2O to 100 mL
		Add PMSF [final] = 1mM and any other protease inhibitors immediately before use
		Add 1uL Pierce™ Universal Nuclease per 1mL

2 SDS Running Buffer

Cell lysis

3 Trypsinize cells

4 Wash pellet with PBS

5 Remove all supernatant with micropipette

6 Freeze pellet in liquid N2

7 Thaw quickly at 37 °C

8 Resuspend cell pellet on buffer 100 µL

9 Vortex 00:00:15 15s

10 Incubate on Ice 00:20:00 20m

11 Vortex 00:00:15

12 Collect supernatant

13 Centrifuge 10000 RPM for 00:05:00 5m

Running, Transfer and Blot 2h 40m 45s

14 Collect supernatant without disturbing the pellet

15 Quantify protein

15.1 799 uL water + 1 uL lysate
+ 200 uL 5x Bradford reagent

15.2 Read at 595 nm

16 Load 50 µg of protein/well

17 Loading buffer 50 µL β-mercaptoethanol + 950 µL 2x Laemli Buffer

18 Water up to 15 µL

19 Boil at 95 °C before loading 00:00:15 15s

20 Add 10 µL of ladder (Precision Plus Protein - Dual Color Standards)

21 Run at 120-150 volts 01:00:00 1h

22 Activate PVDF membrane in methanol

23 Soak filter paper in Towbin Buffer

24 Equilibrate gel in Towbin buffer 00:10:00 10m

25 Semi-dry transfer

25.1 90' (<50KDa) or 120' (>50KDa)

25.2 Mount sandwich (paper-membrane-gel-paper)

25.3 45 mA/gel (constant current)

26 Cut out LEFT UPPER corner of the membrane

27 Block membrane with 5% BSA in PBS 0.1% Tween (PBST) for 00:30:00 30m

28 Add primary antibody in BSA-PBST and incubate overnight 18:00:00 at 4 °C 18h

29 Wash 3x with PBST for 00:05:00 5m

30 Add HRP secondary antibody in BSA-PBST and incubate for 01:00:00 at room temperature on a shaker 1h

31 Wash 3x with PBST for 00:05:00 5m

32 Add 500 µL ECL (enhanced chemilumescent) per membrane strip

Protocol references

https://www.ptglab.com/news/blog/lysate-preparation-why-is-ripa-buffer-best-for-western-blot/

https://docs.abcam.com/pdf/protocols/sample-preparation-for-western-blot.pdf
RIPA buffer is useful for whole cell extracts and membrane-bound proteins, and may be preferable to NP-40 or Triton X-100-only buffers for extracting nuclear proteins. It will disrupt protein-protein interactions and may therefore be problematic for immunoprecipitations and pull-down assays.

https://www.usbio.net/protocols/immunoprecipitation

- We recommend using 1.0 mL of RIPA Lysis Buffer to lyse 0.5 to 5 × 10E7 adherent mammalian cells.
- 1mL of buffer per 75cm2 flask containing 5 × 10ˆ6 HeLa or A431 cells.

	RIPA buffer	100mL
	50mM Tris HCl, PH 7.4	5mL (1M stock)
	150mM NaCl	5mL (3M stock)
	1% Triton X-100 or NP-40	1 mL
	0.1% SDS	1mL (10% supply center solution)

	1mM EDTA	0.2 mL (0.5M stock)
	10mM Naf	0.042g (@L5P1 chemical room)
	0.5% Sodium deoxylcholate	0.5g (@L5P1 chemical room)
	Add ddH2O to 1000ml	
		Add PMSF to a final concentration of 1mM and any other protease inhibitors immediately before use.
		Add 1 uL Pierce™ Universal Nuclease per 1mL

	4 x SDS sample buffer	For 1000ml
	12% SDS	120 g
	25% Glycerol	250 ml
	150 mM Tris.HCl (pH 7.0 1M stock)	150 ml
	0.03% Bromophenol Blue	300 mg
	20% β-mercaptoethanol	200 ml
	Add ddH2O to 50ml, aliquot	

	and store at -20°C	
	20% β-mercaptoethanol, (or 500 mM DTT replaced) should be added freshly before use.	

Nonidet P-40 (IGEPAL® CA-630) 0.5%

Pierce™ Universal Nuclease for Cell Lysis added to RIPA NP40 1% buffer
0.1uL nuclease per 1 mL buffer = 25U/mL [final]
https://assets.thermofisher.com/TFS-Assets/LSG/certificate/Certificates-of-Analysis/00492617_88700.pdf