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Version 1

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Western Blot V.1

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2 Works for me dx.doi.org/10.17504/protocols.io.bfiijkce

Bethany Rozeboom

MATERIALS

NAME	CATALOG #	VENDOR
2x Laemmli Sample Buffer	1610737	BioRad Sciences
TE buffer		Thermo Fisher Scientific
Bio Rad Precision Plus protein Standard	161-0374	Bio-rad Laboratories
Tryptic Soy Broth, E - 10mL	R07226	Thermo Fisher
SignalFire™ ECL Reagent	#6883S	Cell Signaling Technology
Tris Buffered Saline (TBS-10X)	#12498	Cell Signaling Technology
Tris-Glycine Transfer Buffer (10X)	#12539	Cell Signaling Technology
Tris-Glycine SDS Running Buffer (10X)	#4050	Cell Signaling Technology
Tris Buffered Saline with Tween® 20 (TBST-10X)	#9997	Cell Signaling Technology
Nonfat Dry Milk	#9999	Cell Signaling Technology
BSA	#9998	Cell Signaling Technology
Anti-mouse IgG HRP-linked Antibody	#7076	Cell Signaling Technology

Cell Preparation

1 Grow cell **50 ml** of TSB, let incubate **Overnight**

2 Distributed **17 ml** of culture into two **50 ml** centrifuge tubes, and **10 ml** into one **20 ml** centrifuge tube

3 Centrifuge down for **00:20:00** at 4300rpm (4303 xg) **4 °C**

4 Aspirate media from cultures; wash cells 3x with TE aspirate.

5 Lyse cells by resuspending in **1 ml** of the different lysis buffers

5.1 [M]**20 Milimolar (mM) Tris Cl** , [M]**15 Milimolar (mM) NaCl** , 0.9696% Triton pH**7.5** (labelled A)
Phosphatase Inhibitor: [M]**50 Milimolar (mM) NaF** ,
[M]**1 Milimolar (mM) Sodium Orthovanadate** ,
[M]**10 Milimolar (mM) Sodium Pyrophosphate** and

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[M]10 Milimolar (mM) B-glycerophosphate

EDTA-free Protease tablet (1 tablet per **10 ml**)

5.2 1% SDS, **10 ml Tris Cl** , **[M]1 Milimolar (mM) EDTA** (labelled B)

Phosphatase Inhibitor: **[M]50 Milimolar (mM) NaF** ,

[M]1 Milimolar (mM) Sodium Orthovanadate ,

[M]10 Milimolar (mM) Sodium Pyrophosphate and

[M]10 Milimolar (mM) B-glycerophosphate

EDTA-free Protease tablet (1 tablet per **10 ml**)

6 Freeze thaw 3 times

7 Sonicated for **00:01:00** , repeat 5 times

8 Centrifuged at 13000 rpm for **00:05:00**

9 Bradford Assay

10 Combine **40 µl** 4X Laemmli to cell lysate, **40 µl** of Lysis A and Lysis B

11 Load **40 µl** onto SDS-PAGE gel and **40 µl** of All Blue Precision Plus for ladder

12 Soaked sponges and transfer paper in Transfer Buffer

13 Soak PVDF membrane in Methanol

14 Soak fiber pads thoroughly in a Transfer Buffer

15 Make the blotting sandwich

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15.1 Add **1 cm** depth of Transfer Buffer to container and insert plastic cassette with black side down, lay a wet fiber pad on the black side of the cassette

15.2 Lay one wet blotting paper on the fiber pad and roll out air bubbles

15.3 Lay gel squarely on blotting paper and roll out air bubbles

15.4 Lay wet PVDF on the gel and roll out air bubbles

15.5 Lay one wet blotting paper on the membrane and roll out air bubbles

15.6 Lay a wet fiber pad on top of the blotting paper and close the cassette and clamp together with the white clip.

16 Electrotransfer to PVDF membrane at 30 mV **Overnight**

Membrane Blocking

17 After transfer, wash PVDF membrane with **25 ml TBS** for **00:05:00** at **Room temperature**

18 Incubate membrane in **25 ml Blocking Buffer** for **01:00:00** at **Room temperature**

18.1 Combine **7.5 g Nonfat dry Milk** with **150 ml TBST** to make Blocking Buffer

19 Wash three times for **00:05:00** each with **15 ml TBST**

20 Incubate membrane in Primary Antibody (at 1:2000) in a **10 ml Primary Antibody Dilution Buffer** with gentle agitation **Overnight**

20.1 Combine **1 g 5% BSA** to **20 ml 1X TBST** and mix well

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21 Wash three times for **00:05:00** each with **15 ml TBST**

22 Incubate membrane with Anti-mouse IgG, HRP-linked Antibody (1:1000) to detect biotinylated protein markers in **10 ml Blocking Buffer** with gentle agitation for **01:00:00** at **Room temperature**

23 Wash three times for **00:05:00** each with **15 ml TBST**

Detection of Proteins

24 Prepare 1X SignalFire™ ECL Reagent (#6883) by diluting one part 2X Reagent A and one part 2X Reagent B (based off of the size of our membrane we mixed **1 ml** of each together)

25 Incubate substrate with membrane for **00:07:00** , remove excess solution (membrane remains wet) expose to X-ray film using an imager

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