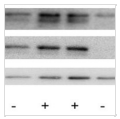

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Brooks Lab Western Blotting Protocol

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1 Works for me dx.doi.org/10.17504/protocols.io.bcsmiwc6

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ABSTRACT

This is a Western Blotting Protocol for Brooks Lab, Department of Biomolecular Engineering, University of California, Santa Cruz.

ATTACHMENTS

Brooks_Lab_Western_Protocol.pdf

MATERIALS

NAME	CATALOG #	VENDOR
Pierce BCA Protein Assay Kit	23225	Thermo Fisher Scientific
4–15% Mini-PROTEAN® TGX™ Precast Protein Gels, 10-well, 30 µl	4561083	BioRad Sciences
Pierce RIPA Lysis and Extraction Buffer	P189900	Thermo Fisher Scientific
cOmplete™ Mini Protease Inhibitor Cocktail (Roche)	04693124001	Millipore Sigma
4x Laemmli Sample Buffer	1610747	Bio-rad Laboratories
2-Mercaptoethanol	1610710	Bio-rad Laboratories
Precision Plus Protein™ Dual Color Standards 500 µl	1610374	Bio-rad Laboratories
Precision Plus Protein™ Dual Color Standards 500 µl	1610374	Bio-rad Laboratories
Western Blot Box (black size 8.9 cm × 6.5 cm × 2.5 cm)	Z742099-5EA	Millipore Sigma
β-Actin Antibody (C4)	sc-47778	Santa Cruz Biotechnology
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	34577	Thermo Fisher Scientific

MATERIALS TEXT

Note: You can use Pierce BCA Protein Assay Kit #23225 or #23227

Additional Reagents required:

- 100 mM Tris-Cl, pH 8.0
- 300 mM NaCl
- 10% NP-40 in ddH_2O
- 10% Na-deoxycholate in ddH_2O (light sensitive!)
- 10% SDS
- 10x PBS
- Tween-20

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- 190 proof ethanol
- 10x TGX running buffer
- Safeway brand non-fat milk pouch
- PBST

- BioRad Transblot kit

- LoBind microcentrifuge tubes Protein, 1.5 ml

Additional Equipment

- Sonicator or Bioruptor
- Microplate reader
- 200 µl pipettor
- Varioskan
- BioRad Gel Doc EZ Gel Documentation System
- BioRad ChemiDoc XRS+

SAFETY WARNINGS

Please refer to the Safety Data Sheets (SDS) for safety and environmental hazards.

RIPA Lysis Buffer

1 Make **100 ml RIPA w/o protease inhibitor** , cover, and freeze in *10 ml aliquots* at **-20 °C** .

OR

Use pre-made Pierce RIPA buffer, 100 ml, which is aliquoted in **10 ml** aliquots and stored in **-20 °C**

2 Before use, thaw and add **1 tablet** of protease inhibitor (PI) **per 10 ml aliquot**. (Roche cOmplete™, Mini Protease Inhibitor Cocktail).

Harvesting Cells and Preparing Lysate

3

Always keep everything on ice, unless otherwise indicated.

Wash confluent 10 cm plate of cells **2X** in *ice cold* PBS.

4 Add **1 ml cold RIPA with PI** to cells, and scrape the cells to remove them from the dish. Transfer to a pre-chilled 2 ml tube **On ice** .

5

Incubate **On ice** **00:00:20** , with periodic vortexing.

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6 Pellet the insoluble material by spinning at max speed in refrigerated (**4 °C**) microcentrifuge **00:00:10** .

7 Transfer supernatant to a clean LoBind Protein 1.5 ml tube as lysate. To avoid multiple freeze-thaw cycles, make aliquots (generally **200 µl** each).

Additional Sonicator or BioEruptor sonication step often needed here for more complete nucleic acid removal. See below.

8 Store lysates aliquots at **-80 °C** .

Sonication of Lysate to break up nucleic acids

9 Place samples in a 1.5 ml LoBind Protein microfuge tube and prepare a volume balance tube.

10 Bring a timer, ear cuffs, **On ice** samples, and balance to second floor to Kamakaka Lab to get the plastic tube adaptor (in back of second drawer in 2nd bay from the back of the lab.)

11 Sonicator is in cold room down the hall. Put the outflow tube up on the shelf and fill glass reservoir with water to top of white line.

12 Sonicator settings: timer on hold, use max setting (10), constant, then flip on.

13 Hold tubes in the adaptor in the sonicating water bath for **00:00:30** .

14 Place tubes **On ice** to cool for **00:01:00** .

15 Repeat the 30’ sonication (= total of two rounds on max at **00:00:30** .)

BCA Assay for Protein Concentration Determination

16 **Pierce BCA Protein Assay Kit** #23225 or #23227, Thermo Sci with 2 mg/ml BSA standard.

Use instructions from kit.
Equilibrate reagents, samples, and standards to **Room temperature** . Use the *Microplate reader instructions.*

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17 Prepare a dilution series of BSA in working range of [M]**20 µg/ml** – [M]**2000 µg/ml** from 1 glass vial of stock 2 mg/ml BSA in the kit for the standard curve:

Vial	RIPA buffer, µl	2 mg/ml BSA or standard, µl	Final con µg/ml
A	0	300 of stock	2000
B	125	375 of stock	1500
C	325	325 of stock	1000
D	175	175 of B	750
E	325	325 of C	500
F	325	325 of E	250
G	325	325 of F	125
H	400	100 of G	25
I	400	0	0

18 Prepare fresh working reagent 50:1 of A:B, enough for all standards, unknowns, and replicates of the unknowns (should have n=3).

19 Add **25 µl** of unknown or standard to 96-well plate well followed by **200 µl working reagent** using 200 µl pipettor and *gently mix avoiding spillover*.

20

Cover plate with foil and incubate **37 °C** for **00:30:00** in shaking incubator, gently shaking.

21 Cool to **Room temperature** .

22 Measure Absorbance on the VarioSkan at 562 nm.

23

Determine protein concentration of your unknown from the standard curve (the Skanit software will plot the curve and the unknowns on it if you edit the standards in the plate layout to include the concentrations above in the table.)

24 Export report as an excel doc.

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SDS PAGE

25 Prepare the following solutions for SDS-PAGE, Transfer, and Antibody Incubations:

Running Buffer (Tris-Glycine)	
100 ml	10x TGX running buffer
900 ml	dd water

Transfer Buffer	
200ml	5x Biorad Transfer Buffer
200ml	190 proof ethanol
600ml	dd water

PBST	
50 ml	10x PBS
0.5 ml	Tween-20
bring vol to 500 ml with dd water	

5 % Milk Block (prepare immediately before use!)	
5 g	Safeway brand non-fat milk pouch
100 ml	PBST

SDS PAGE: *Denaturing samples in Laemmli reducing buffer*

26 In a fume hood, Add **100 µl 2-mercaptoethanol** to **900 µl 4x Laemmli sample buffer** to make MLB.

27 Dilute samples with MLB at a ratio of *3 parts sample to 1 part MLB*. So for **30 µl** final volume, add **7.5 µl MLB** to **22.5 µl sample in RIPA** in 1.5 ml LoBind safety lock tube.

28 Denature at **95 °C** for **00:05:00** .

29 Transfer to ice.

SDS PAGE: *Loading and Running Gel*

30

Remove gel from package, take off green strip at the bottom, and rinse the wells three times with about **1 ml running buffer** (use 1 ml pipettor).

31 Place gel, tall plate facing out in outer side of a holder and the reservoir block at the other to make a running buffer reservoir. Put the other gel holder in place to take up space.

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32 Fill the chamber to the 2-gel mark. Make sure there are no leaks before testing the circuit. Test the circuit by checking for bubbles after turning on power to **70 volts**.

33 Using a 20 ul conte-tipped pipettor, load **30 µl** of the samples in MLB onto gel, as well as **10 µl** of Biorad Precision Plus protein ladder Dual Color.

34 Run at 100 – 120V, depending on desired resolution, until adequate separation of ladder lanes in sizes regions of interest.

35 Photograph the gel after the run with your phone to help keep track of orientation.

Semi-dry Transfer to PVDF Membrane

36 Pre-soak blotting stacks in 1X transfer buffer made according to directions on the bottle.

37 Pre-wet PVDF membrane from *Biorad Transblot kit* in 190 proof ethanol, then in transfer buffer.

38 Layer into Transblot drawer (stack, membrane, gel, second stack) before rolling out bubbles gently with conical tube

39 Run the transfer on Biorad setting, standard Mininigel TGX, 25mA, 25V, **00:03:00** .

40 While running, pour 5% milk block in 1X PBST directly into cleaned small black Western blot box.

41

Incubate transferred blot for **01:00:00** at **Room temperature** in 5% milk block in 1X PBST.

42 Photograph the blot after the transfer to help keep track of orientation.

Antibody Incubations and ECL visualization of target protein bands: *Primary and secondary antibody binding*

43 *Primary and secondary antibody binding*

- β-Actin (C4): sc-47778
- ECL SuperSignal West Pico Chemiluminescent Substrate

Use 1:500 Dilutions for primary antibodies, in 5% milk/PBST

Note: Primary antibody (**unconjugated with HRP**) can be re-used 3x if kept frozen.

44 Drain milk block from blots and add primary Ab (in milk block).

45

Incubate in cold room **Overnight** covered and shaking on orbital shaker, covered with plastic.

46

The next day, wash blot **3x**/ **00:15:00** in PBST.

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47 Freshly prepare secondary antibody at 1:10,000 dilution (**2 µl** to **20 ml 5% milk/PBST**).

48 

Incubate in secondary antibody for **01:00:00** , **Room temperature** .

49 

Discard secondary and wash blot **3x**/ **00:05:00** in 1xPBST.

50 Discard secondary and in the same box or moving the blot to a new box, add ECL working solution (**1 ml** of both reagents mixed in a **15 ml** conical and applied to blot.)

51 

Incubate in ECL for **00:05:00** , shaking occasionally by hand, covered.

52 Drain blot and place between plastic sheets for imaging using Biorad GelDoc, as below. After imaging, the blot can be probed for actin as a protein loading control without stripping the blot, using anti-actin-HRP as described below.

Antibody Incubations and ECL visualization of target protein bands: *Actin staining with anti-actin-HRP primary*

53 

Actin staining with anti-actin-HRP primary

Wash blot 3x with 1X PBST < **00:10:00** .

54 

Incubate **00:20:00** , **Room temperature** in the primary actin antibody 1:500 freshly made dilution in 5% Milk block.

55 

Wash 2 – 3x in 1x PBST **00:05:00** each.

56 

Incubate in ECL as above (step 51) and image.

Antibody Incubations and ECL visualization of target protein bands: *Imaging Blot on BioRad ChemiDoc XRS+ (Vollmers lab)*

57 *Imaging Blot on BioRad ChemiDoc XRS+ (Vollmers lab)*

Place blot in between sheet protector plastic and transport to imager in cassette to keep dark.

58 Place blot on white screen.

59 Place screen into chamber drawer.

60 Select new protocol, then blot, then chem hi sensitivity and position blot under live focus setting.

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61 Under Applications, select chemidoc hi sensitivity to photograph target bands. Note the image size, the gain (2X), and bin (2x2) to make sure the image size is the same when the protein standards are photographed in order to be able to merge the images.

62 Under Live Acquire, select acquisition settings: 1, 600, for range and total =100 acquisitions (the 100 can be lowered for longer exposure), starting at 0.25 start time for high expression target.

63 Freeze and save the image of your choice as it comes up.

64 **Without moving the blot**, take a picture to visualize the protein standards by selecting Custom under Applications, then create an Epi illumination protocol with the same gain and binning as used for photographing the bands (double check the image size prediction under the options…)

65 Merge the target bands and the protein standards pictures in Image Lab software using Image tools.

Note: Imaged blots can be stored at **4ºC in PBST** for stripping and re-probing. Some folks also store them frozen in **-20ºC** flat in plastic bag.

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