Western Blot (JG protocol)

I. Prepare protein lysates

Prepare beforehand:

Thaw RIPA buffer (1x) bottle/aliquoted tubes at 4C overnight before use.
25X Proteinase inhibitor, PIC stock (1 tablet dissolved in 2 mL water, aliquot & store at -20C)
Freeze mortar/pestle in -20 C overnight (autoclaved)
Freeze spatulas at -20C overnight (autoclaved)
Cool microcentrifuge to 4C.

Obtain:

Dry ice for tissue handling.
Ice for cooling solutions & tubes.
Cool tubes (eppendorf and regular, 50 mL/5 mL tubes) on ice before use.
Gather 11G Needles and 3 mL syringes

On day:

Prepare fresh lysis buffer on ice:

1X RIPA	1 mL	20 mL
Na orthovanadate (#3)	10 uL	200 uL
Protease inhibitor cocktail (#2)	20 uL	400 uL
25X PIC (1:24)	40 uL	900 uL

a) Lysates from mouse tissues

chill 50 mL tubes, -30C (for homogenizer)
chill eppendorf tubes, -30C (1.5 mL/2 mL)
get 2 buckets of ice
chill microcentrifuge

method 1 homogenizer (pancreas, liver, etc)

wash homogenizer tip/blade with water, acetone, water and RIPA buffer (have 15 mL tubes with each)

place tissue (frozen/fresh) in 50 mL tube (because homogenizer tip bottom can reach 50 mL)

add 1-2 mL RIPA

homogenize while placing tube in ice in a beaker, 5-6 bursts x 10 sec

transfer homogenized lysate to chilled eppendorf tubes; keep on ice

centrifuge **15rpm x 15 minutes x 4C**

transfer supernatant to fresh eppendorf, aliquot into tubes, freeze -80C

save ~5-10 uL of lysate in another tube for protein determination

b) Lysates from cell lines

Wash cell lines 2x in PBS. Remove PBS.

Place plate on ice. Add 1 mL lysis buffer directly on plate.

Scrape lysate into 5 mL tube (on ice).

Use needle and syringe to shear lysate. Vortex and keep on ice.

Aliquot into eppendorf. Spin at 14K x 10 min at 4C.

Remove supernatant into fresh tube. Freeze lysates at -20C.

Save an aliquot (~20 uL) in another tube to perform protein determination.

II. Protein determination

a. Dilute Bio-Rad protein assay dye, 1:4 dilution (1 part Bio-Rad to 4 parts PCR water).
   Volume of diluted Bio-Rad = number of standard curve points + number of samples
b. To cuvette, add **1mL** diluted Bio-Rad solution.
   Add 1-10 uL BSA (1 ug/ul stock; standard curve: 1- 10 ug) or 2 -3 ul protein lysate (undiluted or diluted). Lysate may have to be diluted 2-100-fold. Add RIPA buffer to control and standards.
c. Cover cuvette with parafilm, invert to mix, incubate at RT for 5 minutes, read at 595nm.

See Protein Calculator and Spectophotometer protocol

Table 1. Example of set up: Use at least 3 points for the standard curve.

Standard curve points	uL of BSA (1 ug/uL)	Diluted BioRad	Absorbance (595nm)		
0	0	1 mL			
1 ug	1 uL	1 mL			
3 ug	3 uL	1 mL			
5 ug	5 uL	1 mL			
Protein lysates (diluted X fold)	uL of lysate	Diluted BioRad	Absorbance (595nm)	Concentration (ug/uL)	30 ug protein
a	2 uL	1 mL			uL
b	2 uL	1 mL			
c	2 uL	1 mL			

Note: Spectophotometer in Dr. Rangana's lab uses 2 blanks: well #0 and well # 6 (reading from top)

III. Prepare samples for loading

a. Place 20-30 ug protein sample in tube. Note volume. Add equal volume 2X Laemmli sample buffer. Heat 90-100C x 10 min. Place on ice for 5 min. Load on gel or store samples at -20C. Heat & cool samples again before loading.

b. *Alternately:* Place 20-30 ug protein sample in tube. Add water to make up to 15 ul.
Add 15 uL 2x Laemmli sample buffer, total volume 30 uL.
Heat 90-100C x 10 min. Place on ice for 5 min. Load on gel or store samples at -20C. Heat & cool samples again before loading.

Sample #	Volume for 30 ug	Water, uL	Total volume to 15 uL
1			
2			
3			

IV. Cast Gel *(Mini-Protean Tetra Cell)*

*(reminder: **freeze blue, cool pack at -20C for transfer step; chill transfer buffer**)*

Assemble Glass Plates

a. Clean glass plates and combs with EtOH.
b. Stack short plate on top of long spacer plate (with attached spacer, 1 mm)
c. Place assembled plates in green casting frame. **Level the plates on hard bench tops**. Clamp plates. Place casting frame+plates on grey gasket. Engage top lever to secure the plates.
d. Pour water halfway up the plates to check for leaks. Remove water with filter paper. Place comb between glass plates, mark comb bottom with marker, remove comb.

Prepare resolving gel: *lower gel* : acrylamide (12% Gel) (see list at end for other acrylamide %)

In tube, prepare for 1 gel (10 mL):

Water	3.35 ml
Tris-HCL pH 8.8	2.5 ml
10% SDS	100 µL
30% Acrylamide:Bis	4 ml
10% APS	50 µL (add 2nd last)
TEMED	8 µL (add last)

Mix well by inversion. Using blue 1mL tip/plastic pipette, pour solution between glass plates until ~ 1cm from bottom of marked gel well.
Overlay acrylamide solution with water.
Allow gel to polymerize for 45min - 1hr
Remove excess water with filter paper before pouring Upper stacking gel.

Prepare stacking gel: *upper gel* :

In tube, prepare for 1 gel (5 mL):

Water	3.05 mL
0.5M Tris-HCL pH 6.8	1.25 mL
10% SDS	50 µL
30% Acrylamide Bis	0.65 mL
10% APS	50 µL (add 2nd last)
TEMED	12.5 µL (add last)

Mix well by inversion. Add on top of polymerized lower gel.
Place comb in the gel. Allow to polymerize for 30 min - 1hr.
Gel can be stored at 4 degrees for up to 1 week, after wrapping top in saran,

V. Prepare Samples for Electrophoresis

a. Set heating block to 90^0 C (check T with thermometer!)
b. Heat samples+loading dye at 90-100^0 C x 10min
c. Place tubes on ice, 5 min; spin before loading. Samples can be frozen at -20C.

VI. Run Gel (Electrophoresis)

a. Dilute 10X running buffer (Tris/glycine/SDS) to 1x : 70mL 10x running buffer+water to 700mL
b. Rinse and flush the wells of gels with running buffer to remove excess acrylamide.
c. Carefully remove gel from casting unit and place in clamping frame (with electrodes), **short plate** facing **inward**. (clamping frame must have 2 gels. If using 1 gel, place **dummy plate** on opposite side).
d. Set clamping frame+gels inside Mini-PROTEAN Tetra cell tank.
e. Fill inside of clamping frame with running buffer **to the top**.
f. Fill the Mini-PROTEAN Tetra cell tank with running buffer up to the 2-gel mark
g. Load marker+samples.
h. Run gel(s) at constant voltage: eg. 150V constant x ~1hr (or run at 50 V for 30 min (until dye crosses stacking gel) and 150 V after that)

i. After run, remove gels, pour buffer in tank. Buffer may be reused 2-3 times.

VII. Transfer/Blotting

***[Prepare ahead ~1 day**: Store blue cooling unit in -20 ^{0} C.*
Chill Transfer buffer components: water (~2 L), 10X transfer buffer; 100% MeOH]

Prepare chilled (**TB**) Transfer Buffer (~ 2L):

10X Tris/glycine	200 mL
water	1400 mL
100% MeOH	400 mL

a. Lay out 3 trays on bench.
b. Remove gel after electrophoresis. Remove upper plate with plastic chisel. Cut out stacking gel + any unnecessary lanes. Measure gel dimensions.
c. **Soak gel in TB** in tray (~ 15 min); **Soak fiber pads in TB.**
d. Cut 2 filter papers and 1 blotting membrane (exact size). Minimize touching the blotting membrane with fingers.
e. **Soak filter papers in TB**.
f. **Soak blotting membrane in 100% MeOH for 20 sec**. **Transfer membrane to TB, soak 5 min**.
g. Prepare the blotting sandwich.
   Place the cassette, with the black side down, on tray.
   Place 1 fiber pad on the cassette (black side)
   Place 1 sheet of filter paper on the fiber pad - or use filter paper to lift gel onto pad.
   Place the gel on the filter paper. Roll tube to rid any trapped bubbles between layers.
   Place the pre-wetted membrane on the gel. Roll tube to remove any air bubbles.
   Placing 2nd filter paper on top of membrane. Remove any air bubbles.
   Add the last fiber pad.
   Close and lock the cassette.
h. Place the cassette in the module (black cassette side to black electrode)
i. Add frozen blue cooling unit to tank. **Add TB to the blotting mark on the tank**.
j. Add stir bar to tank to maintain even buffer temperature. Place unit on stirrer.
k. Run at constant 100V constant for 1 – 2 h (if small gel, ~ 1hr)
l. After completion of the run, disassemble the blotting sandwich to remove the membrane. Rinse the cell, fiber pads, and cassettes with deionized water (*do not use detergent* to wash fiber pads).

VIII. Immunoblotting

a. Remove membrane from the cassette with forceps and rinse in water. Lay **gel side up**.
b. **Air dry membrane** on filter paper for 30 min – 1 h. **Air dry for Licor membrane only**. Check instructions for each membrane type used.
c. **Wet membrane in MeOH** for 15 sec until membrane is translucent. Rinse with 1xTBST.
d. Add blocking buffer to membrane (**5%** non-fat dry milk in 1X TBST). Shake 1 hour x RT or O/N at 4^0 C on shaker
e. Prepare primary antibody in 5%milk-TBST.
f. Place membrane in plastic. Seal 3 ends. Add antibody solution. Remove air bubbles. Seal 4th end.
g. Incubate membrane overnight at 4^0 C on shaker.
h. Remove primary antibody (store and save at 4^0 C). Wash membrane in 1X TBST x 20min x RT, 3 times with shaking
i. Prepare secondary antibody, Incubate membrane with secondary antibody for 1hr x RT, on shaker.
j. Remove secondary antibody. Wash membrane with 1X TBST x 3 x 20min x RT, with shaking.

IX. ECL Procedure

a. After last wash, keep membrane in the wash buffer while preparing the substrate mixture.
b. In dark box, mix substrate components in 1:1 ratio. Prepare ~ 0.1ml of solution per cm^2
c. Incubate membrane in the substrate solution for 5 min with shaking.

d. View membrane in the Chemidoc digital imager
(UV tray; blot; chemiluminesence. To photograph marker use UV tray; blot; colorimetric)

X. Ponceau staining (to visualize total protein)

a. Rinse off ECL solution with water.
b. Add Ponceau to cover membrane. Shake ~ 5 min.
c. Rinse membrane in water for 5 min.
d. Visualize protein bands on membrane in Chemidoc (white tray, gel; silver staining)

XI. Stripping membrane (to reprobe with another antibody)

a. Soak membrane in 1X stripping buffer for 20 min x RT with shaking.
b. Wash with PBS, 3 x RT.
c. Check membrane in Chemidoc to see if any bands are visible.

XII. Reprobe membrane with another antibody

a. Block membrane with 5%milk-TBST
b. Add primary antibody.
c. Proceed as step VIIIg.

TGX- Stain Free, 10% (Bio-Rad)

Lower Gel
Prepare resolving gel by combining 3ml of Resolving solution A and 3ml Resolving solution B.
Add 3ul TEMED and 30ul 10% APS and immediately pour lower gel solution.

Upper Gel; no waiting required
Prepare resolving gel by combining 1ml of Stacking solution A and 1ml Staking solution B.
Add 2ul TEMED and 10ul 10% APS and immediately pour to top of the short plate. Ensure lower gel is not disturbed. Place the comb in the gel cassette. Allow to polymerize for 1hr.

Solutions:

RIPA (1X) (Santa Cruz), Lysis buffer; sc-364162A
1X TBS
1% Nonidet p-40
0.5% sodium deoxycholate
0.1% SDS
0.004% sodium azide

suggested use: 3 mL RIPA/g tissue; 1 mL RIPA/2x10^7 cells; 0.6 mL RIPA /100 mm plate

Alternate: **Lysis buffer**: from older protocol (MZ):
Prepare fresh **lysis buffer + PIC** (keep cold on ice)
Ratio:

Protein lysis buffer	480 uL
PIC (25x)	20 uL
Total	500 uL

1. Protein lysis buffer (stored at 4 degrees)
20 mM Tris-Cl (pH 7.4)
2 mM EDTA
250 mM NaCL
1% Triton-X-100
1 mM DTT

2. 25 x stock Proteinase inhibitor (PIC) (stored at -20)
25X stock; 1 tablet PIC dissolved in 2 mL water (aliquot and freeze at -20)
or directly add 1 tablet to 50 mL lysis buffer (use fresh!)

2x Laemmli sample buffer (Santa Cruz) sc-286962
4%SDS
20% glycerol
10% 2-mercaptoethanol
0.004% bromphenol blue
0.125 M Tris HCl, pH 6.8

Runnning buffer (1X): Tris/glycine/SDS
25 mM Tris base 3.03 g
192 mM glycine 14.41 g
0.1% w/v SDS 1 g
Water to 1 L

Transfer buffer (1X): Tris/glycine/MeOH
25 mM Tris base 3.03 g
192 mM glycine 14.41 g
Methanol 20% v/v 200 mL
Water to 1 L

Blocking solution: 5% milk-TBST

1X TBST solution (1L): [Genesee: 18-235B: TBST (TBS-Tween 20), 10X]
200mL of 5X TBS
1mL of Tween 20

5X TBS solution (1L):
Tris 30.3g
NaCl 146.1g
HCl ~ 22mL to pH 7.4. Test with pH paper.

10% SDS: 5 g/50 mL water

10% APS: 0.5 g /5 mL water; good for 1-2 months

Immobilon-P membrane (IPVH00010), 0.45um pores size, Licor

BioRad
1610156: 30% Acrylamide/Bis Solution. 500 ml,
1610700: Ammonium Persulfate (APS). 10 g,
1610771: 10x Tris/Glycine. 5 L cube, 10x
1610772 : 10x Tris/Glycine/SDS. 5 L cube,
1610801: TEMED. 50 ml
1705060: Clarity Western ECL Subs,200ml
5000002: Bio-Rad Protein Assay Kit II.

Miscellaneous:
Amersham blotting: RPN6101M paper
Amersham PUDF RPN303F
BioRad 1.5 spacer 1653308

Loading tips VWR 37001-150
Abcam goat a-rabbit–hrp ab97051
Santocruz sc-2048

Resolving & Stacking gel composition:

