Western Blotting Protocol ­­– **SDS-PAGE**

**NOTES:**

**--Do not reuse running buffer--**

--10% polyacrylamide gel: 10-150 kDa--

--10-20% gradiant gel: 6 to 150 kDa--

--4-12% tris-glycine gel (30 to 200 kDa) 10 well, 30 uL volume--

--8.6 x 6.7 x 0.1 cm mini-PROTEAN TGX Pre-cast gel

1) Quantify protein samples, must have ≥ 5 ug (≥ 0.25 ug/uL)

2) Prepare buffers:

 **Sample loading buffer** (125 uL)

 6 uL βME

 119 uL Laemmli buffer (contains bromophenol blue)

 **Running buffer** (750 mL)

 675 mL water

 75 mL 10x TGS w/SDS (Tris-glycine pH 8.3, w/ 0.1% SDS)

 750mg SDS (if needed)

3) Prepare protein samples

 -Mix equal amount of protein (5-25ug) with loading buffer / water

 -Max of 28uL for 30uL well gels

 I.E. 5-18 uL sample/water + 5-12 uL loading buffer

 -Spin, then heat at 95°C 5', then spin

3) Electrophoresis

 -Rinse wells

 -Pre-run empty gel 5'

 -Load samples

 -Load 5-10uL Chameleon duo pre-stained ladder in center well (ready to use)

 -Run gel 5-10V/cm first 10' (100V)

 -Run gel 10-20V/cm 25' (200V)

 -Watch dye front, keep temperature stable @ 15°C

4) Open cassette and remove gel by floatation

Western Blotting Protocol – **TRANSFER**

**NOTES:**

--PVDF membrane is hydrophobic, binding capacity is 155 ug/cm2 (Insulin)--

1) Prepare buffers:

 **Transfer buffer** (1,000 mL **CHILLED**)

 100 mL 10x Tris-glycine buffer, pH 8.3

 800 mL Water

 100 mL Methanol (10 % final)

2) Equilibrate gel **15**-30'

 -Large volume transfer buffer allows for SDS stripping

 -Preserves dimensional equilibrium

3) Prepare PVDF membrane

 -Cut to size 8.6 x 6.7 cm

 -Wet PVDF in ≥ 50% metOH (turns transparent)

 -Submerse in water, do not allow to float 2'

 -Rinse in transfer buffer 5'

4) Setup transfer sandwhich (all in transfer buffer):

 (black) **­– cathode**

soaked sponge ­– push out airbubbles (clean in 100% methanol)

soakedfilter paper

 ***Gel***

 ***Membrane***

 soakedfilter paper

 soaked sponge – push out airbubbles (clean in 100% methanol)

 (clear/red) **+ anode**

5) **Transfer** 60-120' in ice bath

 -6-8 V/cm interelectrode distance (32V)

6) **Dry** membrane

 -Rinse in water

 -Air dry at room temp 2 hours

 -Store up to 2 weeks at 4°C

Western Blotting Protocol – **STAINING**

1) **Wetting**

 -100% MetOH 1'

 -Rinse in water 2'

 -Rinse in TBS (NO tween) 5'

2) **Block** 1 hour (shaking)

 -100 % LiCor block (or 0.2 to 5 % BSA in TBS)

 -0.4 mL/cm2 (full 58 cm2 gel = 23 mL)

 -NO tween

3) Prepare solutions:

 **TBST Wash Buffer** (500 mL, 0.1 % tween)

50 mL 10x TBS buffer

 450 mL Water

 0.5 mL Tween 20

 **TBST Blocking Buffer** (500 mL, 0.1 % tween)

 500 mL LiCor TBS blocking buffer

 0.5 mL Tween 20

 **TBST Blocking Buffer w/SDS** (100 mL, 0.01 % SDS)

 100 mL TBST Blocking Buffer

 10 mg SDS

3) Incubate **primary** 1-4 hours @ room temp shaking (overnight 4C)

 -1:200 to 1:5,000 in **TBST Blocking Buffer** (1:1,000 ERK)

 - ≥ 0.1 mL solution/cm2 membrane (full 58 cm2 gel = 5.8 mL)

4) Wash 3x in **TBST Wash Buffer**

5) Incubate **secondary** 1 hour @ room temp shaking

 -1:5,000 to 1:25,000 (1:20,000 starting)

 -**TBST Blocking Buffer w/SDS**

6) Wash 3x in **TBST Wash Buffer**

 -Final wash in TBS (NO tween)

7) Scan within 1 week if wet, 2-3 years if dry

 -Cannot strip once dried

Western Blotting Protocol – **ANTIBODIES**

**Primary**

Rabbit-Anti-NefH 200 kDa 1:1,000 IgG (Chp+) Sigma

Rabbit-Anti-Phospho-IGF-I 95 kDa 1:1,000 IgG (Ch?) CST

\*Mouse-Anti-MAP2 70 kDa 1:1,000 IgG1 (Ch+) Sigma

\*Rabbit-Anti-PhosphoAKT 60 kDa 1:1,000 IgG (Ch+) Sigma

\*Mouse-Anti-beta3 Tub 50 kDa 1:1,000 IgG2a (Ch?) Therm

Rabbit-Anti-beta3 Tub 50 kDa 1:1,000 IgG (Ch+) Therm

\*Rabbit-Anti-Phospho-ERK1/2 42 & 44 kDa 1:1,000 (Chp+) CST

\*Mouse-Anti-ERK1/2 42 & 44 kDa 1:1,000 IgG1 (Ch+) Millip

\*Mouse-Anti-COX IV 17 kDa 1:1,000 IgG1 (Ch-) CST

**Secondary**

Goat-Anti-Mouse IRDye 680RD For abundant proteins

Goat-Anti-Rabbit IRDye 800CW For low abundance proteins