

# LICOR Western Blot Protocol – Reed Lab

## UIC College of Dentistry

### Reagents:

#### Matrix EXTRACTION BUFFER, per sample

- 70 µl dH<sub>2</sub>O
- 30 µl glycerol
- 187.5 mM tris
- 6% SDS
- 100 mM DTT
- PROTEASE INHIBITORS (HALT 100x; pn 78440)
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#### EXTRACTION BUFFER

- NP-40 (Life Technologies; FNN0021)
- HALT PI, 100x

#### RUNNING GEL:

- 3.4 ml dH<sub>2</sub>O
- 4 ml 30% acrylamide (pn 161-0107)/ 0.8% bis (pn 161-0201) in dH<sub>2</sub>O
- 2.5 ml 1.5 M Tris, pH 8.8 (BR pn: 161-0798)
- 75 µl 10% SDS
- 75 µl 10% Ammonium Persulfate (BR pn: 161-0700)
- 15 µl TEMED (BR pn: 161-0801)

#### STACKING GEL:

- 1.68 ml, dH<sub>2</sub>O
- 2.0 ml, 30% Acrylamide (BR pn 161-0107)/ 0.8% bis (BR pn 161-0201) in dH<sub>2</sub>O
- 1.25 ml, 0.5 mM Tris, pH 6.8 (BR pn: 161-0799)
- 50 µl 10% SDS
- 25 µl 10% Ammonium Persulfate (BR pn: 161-0700)
- 15 µl TEMED (BR pn: 161-0801)

#### LICOR LOADING BUFFER (4x)

- Licor pn 928-40004 (4C freezer, orange bottle)

#### LOADING BUFFER WS 1X, per sample

- 1.8 µl of LICOR LOADING BUFFER (4x)
- 0.2 µl B-mercaptoethanol (fume hood, Fluka pn 63689)

#### LICOR RUNNING BUFFER (10x)

- Licor Tris-Glycine-SDS (pn 928-40016)

#### RUNNING BUFFER WS 1X

- 900 ml dH<sub>2</sub>O
- 100 ml LICRO RUNNING BUFFER (10x)

#### LICOR TRANSFER BUFFER (10x)

- Licor Tris-Glycine (pn 928-40012)

#### TRANSFER BUFFER WS 1X

- 1020 ml dH<sub>2</sub>O
- 300 ml MEOH (under fume hood)
- 150 ml LICOR TRANSFER BUFFER 10x
- 15 ml of 10% SDS (to make 0.1% SDS)

#### PBST (1:1000)

- 900 ml dH<sub>2</sub>O
  - 100 ml 10x PBS
  - 1 ml tween-20 (under fume hood)
- \*heat a little in the microwave before adding tween-20

#### BLOCKING SOLUTION

- Licor Blocking Buffer (927-40000)

#### PRIMARY ANTIBODY SOLUTION

- 5 ml Licor Blocking Buffer (927-40000)
- 0.01% Tween 20 (5 ul)
- 2.5 µl antibody target (for NG2\_R 1:500)
- 5 µl Actin control (-20 freezer; Licor)

#### SECONDARY ANTIBODY SOLUTION:

- 5 ml Licor Blocking Buffer (927-40000)
- 0.01% Tween 20 (5 ul)
- 1 µl Donkey-PRIMARY ANIMAL 800C (4C fridge)
- 1 µl Donkey-ACTIN ANIMAL 680RD (4C fridge)

#### COOMASSIE FIX SOLUTION:

- 50% MEOH
- 10% Acetic Acid
- 40% dH<sub>2</sub>O

#### COOMASSIE DESTAIN SOLUTION:

- 5% MEOH
- 7% Acetic Acid
- 88% dH<sub>2</sub>O

### **A. MATRIX PROTEIN Extraction from FRESH FROZEN Tissue:**

1. Mechanically homogenize tissue using liquid nitrogen and mortar and pestle
2. Scrap tissue into EXTRACTION BUFFER
3. For MATRIX, place tube in boiling water of 10 minutes
4. Spin tube at 14,000 g at room temperature (cold will congeal glycerol)
5. Remove supernatant and place in new tube
6. Add Chondroitinase ABC 0.025 U/ml (AMSbio, AMS.E1028-02) for 3 hours at 37 C

### **B. Preparing Western Blot gel:**

*\*SKIP IF USING PRECAST GELS*

1. Clean glass plates
2. Set glass plates into plastic holder
3. Wash with 1000 µl of PBS
4. Remove PBS
5. Mix RUNNING GEL
6. Mix STACKING GEL (sans TEMED)
7. Add 4500 µl of RUNNING GEL mix
8. Add 1000 µl of 70% ETOH to the top of the mix
9. Let sit for 30 min
10. Add TEMED to the STACKING GEL mix
11. Remove the 70% ETOH from the gel using paper towel
12. Add the STACKING GEL on top
13. Place the comb in the STACKING GEL. Make sure there are no bubbles
14. Wipe the overrun from the glass
15. Allow to solidify for 45 minutes
16. Place unused gels in TGS (RUNNING BUFFER), and store in 4 degree

### **C. Preparing Western Blot Sample:**

1. For every sample you will use 2 µl of LOADING BUFFER WS 1x and 6 µl of sample – total 8 µl
2. **Critical - Remember to add B-mercaptoethanol to the LICOR LOADING BUFFER (4x)**
3. Label PCR tubes for each sample
4. Pipette 2 µl of LOADING BUFFER WS 1x into each tube
5. Pipette 6 µl of sample into the correct tube
6. Pipette up and down several times to mix
7. Heat up tap water in microwave, 6 min
8. Put the square glass pyrex on the heating unit in the fume hood
9. Put heated water into pyrex and allow to boil
10. Place PCR tubes with Sample/Loading buffer into a PCR tube holder
11. Place holder in boiling water for 7 minutes
12. Place holder on ice for 5 minutes
13. Centrifuge tubes for 2 min

#### **D. Preparing the Gel:**

1. Take a pre-cast gel (or gel you just made) out of the 4C fridge (Biorad 7.5% mini-protean TGX, pn: 456-1024)
2. FOR PREMADE: Remove from packaging
3. FOR PREMADE: **Critical - Make sure to remove the green tape on the bottom of the gel**
4. Do not remove the green comb yet
5. Clip the gel into the U-shaped holder with the buffer dam on the other side
6. The gel goes on the side with the pointed green rubber piece, the ledge goes on the inside
7. Fill up the u-shaped holder with RUNNING BUFFER WORKING SOLUTION (1x)
8. Place the u-shaped holder in the MINI-PROTEAN TETRA CELL with the red side of the holder on the red side of TETRA CELL
9. Fill the TETRA CELL up with RUNNING BUFFER WORKING SOLUTION (1x) to the '2-gel' line
10. **Critical – remove the bubbles at the bottom of the u-shaped holder**
11. Remove the green comb from the gel
12. Now use a transfer pipette to get rid of the bubbles at the bottom of the gel
13. Add 8  $\mu$ l of each sample/loading buffer starting at well #2
14. Add 5  $\mu$ l of the LICOR MOLECULAR WEIGHT MARKER (-20 freezer, Licor pn: 64045412) to well #1
15. Plug the TETRA CELL into the POWERPAC unit
16. Set the POWERPAC to 200V, and hit the 'running man'. Should take about 30 minutes. Stop when marker is about 1 cm below the water line
17. Stop and let the gel sit for 5 min

#### **E. Transferring the gel:**

1. Fill the small square glass pyrex with pure MEOH (under the fume hood)
2. **Critical – make sure to wipe out all of the water from the pyrex**
3. Make fresh TRANSFER BUFFER WORKING SOLUTION (1x)
4. Fill the larger pyrex with ~500 ml of TRANSFER BUFFER WORKING SOLUTION (1x)
5. Take 2 pieces of filter paper out and place in MEOH
6. Transfer filter papers to the container with the TRANSFER BUFFER WORKING SOLUTION 1x
7. Cut a transfer membrane to the size of the gel (Immobilon-FL pn: IPL00010 )
8. **Critical -make sure that it is the fluorescent membrane labeled FL (NOT P)**
9. **Critical -do not touch the transfer membrane, only use clean forceps, cut the top corner in the position of the ladder**
10. Place membrane in the MEOH, turn over several times with forceps
11. Place the membrane/filter paper in the TRANSFER BUFFER WORKING SOLUTION (1x)
12. Using the green wedge, open the gel casing into the TRANSFER BUFFER WORKING SOLUTION 1x
13. Assemble the transfer stack in the transfer cassette: black side down
  - a. Sponge
  - b. Filter paper
  - c. Gel
  - d. Membrane
  - e. Filter paper
  - f. sponge

14. Use serological pipette to roll out air bubbles
15. Load the transfer cassette into the RED/BLACK CENTRAL CORE (Clear side of cassette faces the RED side of the central core)
16. Place the TRANSFER CELL in large pyrex surrounded by ice
- 17. Critical – make sure to unplug the TETRA CELL before plugging in the TRANSFER CELL**
- 18.** Fill the TRANSFER CELL all the way to the top with the TRANSFER BUFFER (you can use the extra transfer buffer in the pyrex to fill)
19. Set the POWERPAC to 25V
20. Hit the 'running man' and run overnight
21. After O/N transfer, fill a plastic container with regular 1x PBS
22. Wash the membrane in PBS for 5-10 minutes
23. Place the gel in COOMASSIE FIX SOLUTION

#### **F. Testing the gel with COOMASSIE BLUE:**

1. Put the gel in COOMASSIE FIX SOLUTION for 2 hours
2. Place gel in COOMASSIE BLUE STAIN for 4 hours
3. Wash gel with COOMASSIE FIX SOLUTION for 5 min
4. Destain the gel with COOMASSIE DESTAINING SOLUTION for 2 hours
5. Image the gel

#### **G. Blocking and primary antibody:**

6. Put MEMBRANE in a plastic sleeve, seal on three sides
7. Make the BLOCKING SOLUTION
8. Pour the BLOCKING SOLUTION into the plastic sleeve
- 9. Critical – make sure to get almost all of the air bubbles out**
10. Seal the remaining side
11. Put on the shaker in the 37C *incubator* for 60 min, shaker goes on ~ the 60 speed
12. 10 min before blocking step ends, make the PRIMARY ANTIBODY SOLUTION
13. After blocking, remove membrane from plastic sleeve
14. Put into new plastic sleeve
15. Seal three sides
16. Pour in the PRIMARY ANTIBODY SOLUTION
17. Put on the shaker in the 4C *fridge* overnight

#### **H. Secondary antibody:**

1. Remove the membrane from the plastic sleeve and drain solution
2. Pour PBST into plastic container
3. 4 – 5 min washes in PBST
4. On last wash, make the SECONDARY ANTIBODY SOLUTION
- 5. Critical – turn out the lights to minimize photo-exposure**
6. Take membrane out of PBST and place in new plastic sleeve
7. Seal three sides
8. Pour in SECONDARY ANTIBODY SOLUTION
9. Seal fourth side
10. Place on shaker in 37C *incubator* for 60 min
11. Pour off solution
12. four 5 min washes in PBST

13. one 3 min wash in PBS
14. Place membrane on filter paper, and put in drawer to dry

### Gel Percentage Table

Percentage	14%	12%		10%		7.5%	
<b>Total</b>	40 ml	<b>10 ml</b>	<b>5 ml</b>	<b>10 ml</b>	<b>5 ml</b>	<b>10 ml</b>	<b>5 ml</b>
<b>D.Water</b>	10.33 ml	3.35 ml	1.68 ml	4.0 ml	2.0 ml	4.85 ml	2.43 ml
<b>Tris buffer (1.5M, pH 8.8)</b>	10 ml	2.5 ml	1.25 ml	2.5 ml	1.25 ml	2.5 ml	1.25 ml
<b>Acrylamide : Bis acrylamide</b>	18.67 ml	4.0 ml	2.0 ml	3.33 ml	1.67 ml	2.5 ml	1.25 ml
<b>10% SDS</b>	400 µl	100 µl	50 µl	100 µl	50 µl	100 µl	50 µl
<b>10% APS</b>	200 µl	50 µl	25 µl	50 µl	25 µl	50 µl	25 µl
<b>TEMED</b>	40 µl	15 µl	15 µl	15 µl	15 µl	15 µl	15 µl