**TGF-B Stimulation**

Day 1: Plate

1. Cell type: IMR90 on T75 flask
2. Aspirate media from cells
3. Wash 1x HBSS ~8mL
4. 1 mL trypsin 37C 2-5min
5. Quench typsin with 9 mL full media
6. Count cells
   1. Count: \_\_\_\_\_E5, \_\_% viability
7. Plate 5x105 cell/well (6 well plate) in 3 ml per well
8. Incubate overnight

**Cell type IMR90 / Passage 11 / Date 20230522**

Day 2: Serum Starve

1. Aspirate media
2. Wash x2 with 3ml of PBS
3. Incubate with 3ml serum starve media (0.25% FBS) overnight
   1. 50mL DMEM+pen-strep + 125uL FBS

Day 3: Stimulation

1. Make TGF-B stimulation media 5ng/ml, 3 ml per well

4 ml media + 1 µl TGF-B for stim wells

4 ml media + 1 µl HCl-BSA for unstim wells

1. Replace media with +TGF-B at -120’, -60’, -30’, -15’, -5’ and +HCl-BSA at -5’ before harvest.

**Cell harvest**

1. Label tubes (initial collection, final collection, BCA tube for each sample)
2. Make 1x RIPA
3. 1X RIPA Recipe (2.5 mL):
   1. 125 uL 1 M Tris Base pH 7.4
   2. 250 uL 10% NP-40 (in the small fridge under PCR machines)
   3. 25 uL 10% SDS
   4. 250 uL 5% DOC
   5. 375 uL 1 M NaCl
   6. 1475 uL milliQ Water (737.5X2)
   7. 25 uL PICIII\*\*\*
   8. 12.5 uL NaOrt\*\*\*
   9. 2.5 uL NaF\*\*\*
   10. \*\*\*Add fresh for each harvest
4. Aspirate media from cultures.
5. Wash 2x cold PBS on ice on bench.
6. Add 100uL RIPA to each well.
7. Scrape wells and pipet lysate into microcentrifuge tubes on ice.
8. Spin 10 min 4C 14,000 x g.
9. Transfer soluble phase to new tube.
10. Place 40 uL PBS + 10 uL sample in BCA tubes.

**BCA**

1. Load plate as below:

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| **A** | Blank | Blank | S8 | S8 |  |  |  |  |  |  |  |  |
| **B** | S1 | S1 | S9 | S9 |  |  |  |  |  |  |  |  |
| **C** | S2 | S2 |  |  |  |  |  |  |  |  |  |  |
| **D** | S3 | S3 |  |  |  |  |  |  |  |  |  |  |
| **E** | S4 | S4 |  |  |  |  |  |  |  |  |  |  |
| **F** | S5 | S5 |  |  |  |  |  |  |  |  |  |  |
| **G** | S6 | S6 |  |  |  |  |  |  |  |  |  |  |
| **H** | S7 | S7 |  |  |  |  |  |  |  |  |  |  |

1. Vortex and load 20 µl samples in duplicate according to plate
2. Prepare BCA WR reagent A:B 50:1 (example 100 µl B to 5 ml A)
   1. 26 samples \* 200uL WR \* 1.2 = need 6240 uL. Round up to 8000 for pipetting.
   2. 160uL A + 7840uL B
3. Add 200 µl WR to each well with a sample
4. Cover with lid and incubate at 37°C for 30 min
5. Allow to cool to room temp
6. Read on plate reader at 460nm abs

**Western blot**

1. Make 7.5% or 12% gels according to BioRad protocols
   1. Gels Made \_\_\_\_\_\_\_\_\_\_\_\_
2. Rinse wells with 1X running buffer
3. Pre wet green gasket in the Running Cassette with 1X Running Buffer
4. Secure the gels in the Cassette and fill the chamber with 1X Running Buffer
5. Fill the outer chamber to the correct volume for the number of gels you are running (2 or 4)
6. Load 5 uL of size marker to the first lane on the gel
7. Load 40 uL of sample/lane, load remaining wells (if any) with 1X Loading buffer in RIPA
8. Put on top of tank and run at 100V for about 2 hours at RT (watch for tracking dye to exit gel) Can run at 200V for 1 hour but it tends to morph the gel at the lower size…

**Loading Layout:**

Gel 1:

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |

Gel 2:

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |

Turbo Transfer

1. Equilibrate PDVF membrane in MeOH for 2 mins
2. Transfer membrane to blot box with 1X Transfer buffer, incubate for 4 mins
3. Transfer 2 transfer stacks per gel transfer and soak with 20 mL of turbo transfer buffer on to each stack
4. Place 1 transfer stack onto transfer tray, roll to remove bubbles
5. Add the pre-equilibrated membrane on top of that
6. Remove gel from running box and cut off extra lane separation gel
7. Orient the gel on top of the membrane with the size marker on the left
8. Roll to remove bubbles
9. Add last transfer stack on top of all and roll again to remove bubbles
10. Lock Transfer Box and insert into turbo transfer block
11. Press BioRad, 1 (or 2) mini gel, high m.v., run, choose drawer A or B depending which one you put it in, 10 minutes to transfer
12. Once transfer is finished move blot to TBS, throw polyacrylamide gel into hazardous waste, and clean electrodes immediately.
13. If doing a Total Protein Stain, do it now:
14. If no Total Protein Stain is needed, Dry Blot over night between two filters to lock in the protein

Rehydrate and probe with antibodies:

1. Rehydrate Membrane with MeOH ~30s
2. Transfer to TBS and wash 3X 5 min
3. Block with Odyssey Blocking buffer for at least 1 hour
4. Incubate Overnight with Primary Antibody:
   1. BLOT 1:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ (dilution? \_\_\_\_\_) catalog number \_\_\_\_\_\_\_\_made\_\_\_\_\_\_\_ used \_\_\_\_times before
   2. BLOT 2:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ (dilution? \_\_\_\_\_) catalog number \_\_\_\_\_\_\_\_made\_\_\_\_\_\_\_ used \_\_\_\_times before
5. Wash 3X 5 min in TTBS
6. Move Blot into pink or black container
7. Incubate with Secondary antibody 1 hr (can go longer)
   * 1. (Licor Fluorescent secondary recommended dilution 1:15,000)
   1. BLOT 1:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_(dilution?\_\_\_\_\_\_\_)
      * 1. made \_\_\_\_\_\_\_\_\_ used \_\_\_\_\_\_ times before
   2. BLOT 2:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_(dilution?\_\_\_\_\_\_\_)
      * 1. made \_\_\_\_\_\_\_\_\_ used \_\_\_\_\_\_ times before
8. Wash 3X 5 min TTBS
9. Wash 3X 5 min TBS, then read on odyssey machine