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Western Blot Assay

Preparing Protein Lysates for Western blot from organoid co-culture (Cici's Protocol)

1. Remove media from the well. Do not disturb the Matrigel while removing media.
2. Wash each well with 1 mL of ice cold 1X PBS and re-suspend repeatedly. This will help dissolve the Matrigel.
3. Pipette the re-suspended solution in the 15 ml falcon tube. Repeat steps 1-3 for all other wells.
4. Fill up the falcon tube up to 10 ml for washing purposes. Centrifuge at 1500 rpm for 10 minutes at 4ºC. Depending on whether the organoids have pelleted to the bottom or not, you need to repeat the centrifugation step.
5. Take out the supernatant and blot the tube on a paper towel. If Matrigel is not dissolved, repeatedly re-suspend with a smaller volume (approximately 1 ml) of cold PBS. Fill to the 10 ml mark with ice cold PBS.
6. Centrifuge at 1500 rpm for 10 minutes at 4ºC.
7. Remove supernatant and re-suspend pellet in 1 mL of cold PBS. Transfer into a 1.5 ml Eppendorf tube.
8. Centrifuge at 1500 rpm for 10 minutes at 4ºC.
9. Completely remove the supernatant. Let is settle down for 3-5 minutes.
10. Prepare 4X Lammeli buffer with 50mM DTT (Store concentration: 1M), 1mM PMSF (Store concentration: 100mM), 1% of protease inhibitor cocktail and 1% of phosphatase inhibitor cocktail in a hood.
11. Before adding Lammeli buffer, gently resuspend each tube.
12. Depending on the volume left in the Eppendorf tube, calculate how much 4X Lammeli buffer you need to use.
13. Boil for 20 minutes. Let it cool down.
14. Spin down at full speed (14000 rpm) for 5 minutes.
15. Keep protein containing supernatant in Eppendorf tubes, and store in -80ºC for future use.

RC DC microplate protein assay

1. Add 125 µl of RC Reagent I to 1.5-ml Eppendorf tubes.
2. Thaw the samples and dilute at 1:5 or 1:10 (depending on how much sample you have) and add 25 µl of sample to the Eppendorf tube at room temperature. (Sample: RC Reagent = 1:5)
3. Add 25 µl BSA to separate set of Eppendorf tubes as standards with concentration: (*0), 25 µg/ml, 125 µg/ml, 250 µg /ml, 500 µg /ml, 750 µg /ml, 1500 µg /ml in triplicates

4. Add 125 µl of RC Reagent II to each tube.
5. Mix the samples and spin in a microfuge at max speed at RT for 5 min.
Discard the supernatant without losing any of the precipitates. To maximize supernatant removal, discard supernatant by aspiration.
6. During the spinning, mix 250 µl of Reagent A with 5 µl of Assay Reagent S to make mixture A/S (Reagent S: Reagent A= 1:50). Make as much as needed according to this ratio.
7. Resuspend the precipitates in 30 µl of mixture A/S.
8. Incubate the suspension at RT for 5 min.
9. Transfer the samples into the wells of a 96-well plate (Flat bottom).
10. Add 200 µl of Reagent B. Make sure there are no air bubbles.
10. Incubate at RT for 10 to 15 min.
11. Read OD at 650-750 nm in a spectrophotometer. Use 30 µl of mixture A/S + 200 µl Reagent B as blank.
12. Calculate the protein concentration according to the standard curve.

Western Blot Assay

(Preparation)

(1) Keep buffers in 4 °C refrigerator 1 day before starting Western Blot

(2) 1X Laemmli buffer (1L): use 10X Laemmli Electrode stock

(3) 1X Transfer buffer (w/o SDS) (2L): 200ml of 10X Stock + 400 ml of methanol (20%) + 1400 ml of D.I water.

(4) TBST buffer (1L): 100 ml of 10x TBS + 900 ml of D.I water + 1 ml of Tween-20

(5) Blocking buffer 1: 5g of BSA (5%) + 100ml of 1X TBST, then filter it.

(6) Blocking buffer 2: 5g of fat-free milk powder (5%) + 100ml TBST

1. Preheat samples on 60°C for 5min before running the gel. Wash each lane on the gel with 3ml syringe + 22G needle for cleaning purpose.
2. Load 25-100 µg samples in each well. For protein marker, use 5ul for loading.
※Empty wells should be filled with 1×SDS loading dye to keep balance purpose.
Run the SDS-PAGE gel at 100V for 1 hour until samples and markers running to the bottom.

3. Transfer the protein on the gel onto nitrocellulose membrane at 110V (current) for 90 minutes (Figure #1). ※Make sure gel direction on the membrane. Keep the transfer apparatus it on 4°C; prepare small white ice bucket for protecting purpose from high temperature and insert it near apparatus.
4. After transferring, mark the protein marker line with permanent marker and also up/down side on the membrane before adding blocking buffer.
5. (Blocking) Incubate membrane in 5% milk in TBST (0.1% Tween-20) for 1hr with gentle shaking at room temperature. ※if over the 2 hours, keep it in 4°C refrigerator
6. Wash the membrane 1X TBST for one time.
7. Incubate the membrane with primary antibodies for 1 hour with gentle shaking at room temperature. ※if overnight, keep in 4°C refrigerator

   (1st antibodies)

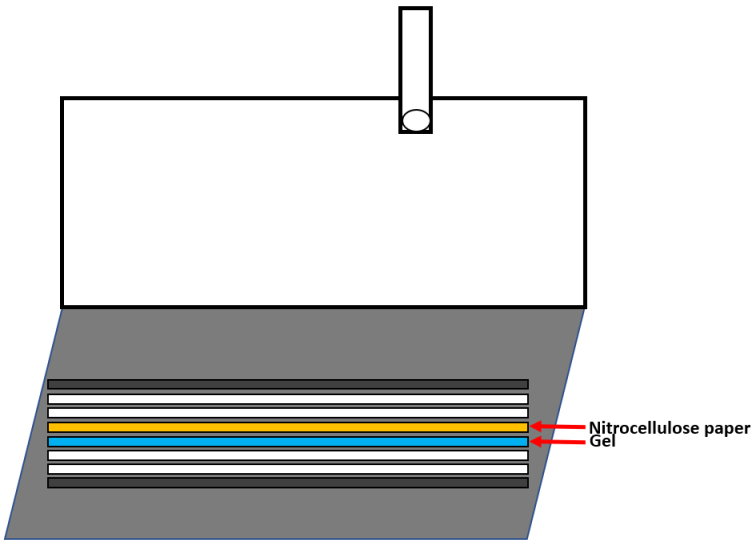
   5% BSA in TBST: for p-β-catenin (Rabbit) antibody (use 1:800; 16ml TBST + 20ul of Ab)

   5% milk in TBST: for β-actin (Mouse) antibody (use 1:1,000; 16ml TBST + 16ul of Ab)
8. Wash the membrane with 1X TBST for 5-10 minutes with strong shaking 3 times.
9. Incubate the membrane with 2nd antibody with gentle shaking for 1 hour.

   (2nd antibodies)

   anti-rabbit 2nd antibody for p-β-catenin (use 1:1,000; 16ml TBST + 16ul of Ab)

   anti-mouse 2nd antibody for β-actin (use 1:1,000; 16ml TBST + 16ul of Ab)
10. Wash the membrane with 1X TBST for 10 minutes 3 times.
11. Add 1ml of chemiluminescent reagent to the membrane and develop with Kodak film. ※for p-β-catenin, use forte strong reagent



(Figure #1)

(References)

1. https://www.abcam.com/protocols/general-western-blot-protocol
2. https://www.cellsignal.com/contents/resources-protocols/western-blotting-protocol/western