Western Blot for phosphoprotein samples - Lucho Fuentealba

(Samples of phosphorylated proteins)

- Mix samples (1 embryo/ 10 AC / dissAC from 20 AC) in 60 µl of Phospho ready solution.
- Keep mixture on ice for 15 min.
- Centrifuge at 5000 rpm for 5min at 4ºC.
- Save supernatant.
- Put 60 µl of Freon solution and mix very well this extracts lipids.
- Centrifuge at 5000 rpm for 5 min.
- Save supernatant and keep samples in -80°C until use.

(Electrophoresis)

- Combine 15 µl of the protein solution with 5 µl of 4 x Loading buffer.
- Prepare 3 µl of protein marker solution.
- Boil for 5 min, and cool down at RT (Don't put tube on ice!!).
- Apply 20 µl of solution into each well.
- Electrophoresis in the polyacrylamide gel at 150 V for 90 min.

(Transfer)

- Activate PDVF membrane (5.5 x 8.5 cm, and cut top right corner) in MtOH.
- Wash membrane in 1 x Transfer buffer.
- Use membrane with cut corner always located at top right corner in the following steps to ensure proper orientation.
- Make 4 papers (5.5 x 8.5 cm) and wet in 1 x Transfer buffer.
- Put the papers, membrane, and gel on the wet electrode plate in the following order:
  From bottom to top: 1) wet paper, 2) wet paper, 3) PDVF membrane, 4) gel, 5) wet paper, 6) wet paper.
- Electro transfer at 100-110 mA for 1 h.

(Blotting)

- Block membrane with BSA-TBST at least for 1 h at RT or overnight at 4 ºC.
- Wash membrane 3 times for 10 min in 1x TBST.
- Put membrane into 1st antibody solution (diluted with BSA-TBST) and shake vigorously for 1 hour at RT.
  Note: Never dry membrane during this step!!!
  Note: We can store 1st antibody solution at -20ºC and re-use 5 times.
- Wash membrane 3 times for 10 min in TBST.
- Put membrane with 2nd antibody solution (diluted with BSA-TBST) and shake vigorously for 1 h at RT.
  Note: Never dry membrane in this step!!!
  Note: We can store 2nd antibody solution at 4ºC and re-use for 2 weeks.
- Wash 4 times for 10 min in TBST.
- Just before next step, mix 500 µl of substrate reaction and 500 µl of Pico substrate.
- Put the membrane on the SaranWrap, apply 1 ml of substrate solution on the top of the membrane, and close completely with SaranWrap.
- Expose film for a very short time.
- Develop film.
  Note: If you do not get a band with the *Pico substrate*, you can use a much more sensitive substrate, *Femto*.

EDTA-free RIPA (RIPA[-]):
0.1% NP40, 20mM Tris/HCL pH 8,10% Glycerol, store at 4ºC (Usually RIPA buffer contains 1 mM EDTA)

10x Protein inhibitor solution (PI):
Put 1 tablet of Protein inhibitor cocktail tablets (Roche) into 1 ml of RIPA[-]. Vortex very well and store at -20ºC.

Phospho Stay Solution (PSS):
Buy *Novagen* Phospho Stay Solution (#71296), then make aliquots (900 µl each), and store in -80ºC.

Phospho Ready Soln:
(Make just before using!!)
Mix 900 µl of PSS and 100 µl of PI and store at 4ºC.

Freon Soln:
1, 1, 2-Trichloro-1, 2, 2-trifluorethene (Kodak) **1 x Transfer buffer:**
2.9g of Glycine, 5.9 g of Tris, 1.8 ml of 20% SDS, 200 ml of MtOH / 1L, store at RT.
10 x TBST (1L):
Tris-Buffered Saline Tween-20 (TBST). Dissolve 88 g of NaCl, 2 g of KCl, and 30 g of Tris in 800ml of distilled H_2O. Add 5 ml of Tween-20. Adjust the pH to 7.4 with HCl.
BSA-TBST (100 ml):
1 g of BSA powder into TBST (Usually use 5% Milk for normal Western Blot).

Pico substrate:
Mix 1 & 2 just before using.
1: SuperSignal® West Pico Stable Peroxide Solution (PIERCE: #1856135)
2: SuperSignal® West Pico Luminol/ Enhancer Solution (PIERCE: #1856136)

Femto substrate:
Mix 1 & 2 just before using.
1: SuperSignal® West Femto Stable Peroxide Solution (PIERCE: #1856190)
2: SuperSignal® West Femto Luminol/ Enhancer Solution (PIERCE: #1856189)
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