Protein carbonylation assay

This is a modified protein carbonylation assay using Western blot membrane using Millipore OxyBlot protein oxidation detection kit (cat# S7150).

Procedures

1. Prepare 10% SDS-PAGE gel for Western blot.
2. Thaw lysates in 2X sample buffer complete (SBC) to room temperature quickly and keep in room temperature until after the loading (Note: Samples should not be frequently frozen and thawed). If you see precipitates at the bottom of the tube, heat the sample at 100°C briefly (1 min).
3. Load 40 μg lysate per lane on the SDS-PAGE gel, run at 100V till the blue dye to the bottom of the gel.
4. Transfer the proteins to nitrocellulose membrane at 36V overnight (20% methanol in 1X Tris-Glycine buffer) followed by high voltage transfer at 90V for 1.5 hrs.
5. Stain the membrane with Ponceau S. dye for 5 min on a shaker and acquire images.
6. Destain with ddH2O for 5 times.
7. Block the membrane with 5% milk (in PBST) for 2 hr.
8. Wash membrane twice with 1X PBST.
9. Make 1X DNPH right before use (600 μl is enough for a 15-well membrane. If needed, adjust the volume based on membrane size): 60 μl 10X DNPH (mix well before use) + 540 μl ddH2O. Transfer 1X DNPH to a 150 mm-cell culture plate.
10. Place the membrane face down by touching the 1X DNPH solution from one side to expose the whole membrane gradually to avoid air bubbles. Move the membrane side to side to ensure complete exposure.
11. Incubate at RT for 15 min on a shaker.
12. Wash membrane once with ddH2O.
13. Repeat stem 10 with 300 μl neutralization solution.
14. Incubate for 5 min at RT on a shaker.
15. Wash with 10 ml of 5% milk in PBST once for 2 min.
16. Make 6 ml of 1st antibody (1:150 Rabbit anti-DNP from OxyBlot protein oxidation detection kit (cat# S7150, Millipore) in 5% milk in PBST).
17. Apply 1st Ab to membrane in a plastic bag and incubate overnight at 4°C on a shaker.
18. Wash in 1X PBST twice for 10 min each.
19. Apply 2nd Ab (Goat anti-Rabbit IRDye 800CW 1:20,000 in 10 ml 5% milk in PBST) and incubate for 1 hr at RT.
20. Wash membrane with 1X PBST twice for 5 min each and with 1X PBS 3 times for 5 min each.
21. Scan for images with a gel documentation system (e.g. Licor).

Reagents

10X DNPH

Millipore OxyBlot protein oxidation detection kit, cat# S7150

1st Ab

Rabbit anti-DNPH in Millipore OxyBlot protein oxidation detection kit, cat# S7150

Nitrocellulose membrane
BioRad, cat#162-0097

Sample buffer stock solution (4.5X) 44 ml
(Tris-HCl 0.23 M, pH 6.8, SDS 4.5%, Glycerol 45%, Bromophenol blue 0.04%)
1. Pipette 20 ml of Tris-HCl (1 M, pH 6.8) into a 100-ml beaker containing a stir bar.
2. Transfer 20 ml of glycerol (ICN 800688, 100%) to the stirring solution with a 25-ml pipette. Rinse pipette repeatedly with liquid from the beaker to completely transfer the glycerol.
3. Add SDS (Fisher Biotech BP166-500 MW 289) and Bromophenol blue (Fisher Biotech BP115-25 MW 670) to the solution.
4. Transfer solution to a graduated cylinder. Use purified water to rinse beaker and adjust volume to 44 ml in the graduated cylinder.
5. Mix by gentle inversion to avoid formation of foam.
6. Divide into 5-ml aliquots and store at 4°C.

Sample buffer (4X) 5 ml
(Sample buffer stock 4X, DTT 80 mM, 2-Mercaptoethanol 0.57 M)
1. Allow 4.5X sample buffer stock solution (stored at 4 °C, crystals form) and DTT (stored at -20 °C) to warm to room temperature and dissolve completely.
2. Pipette 4.4 ml of 4.5X Sample buffer stock into a 15-ml conical tube.
3. Add 0.4 ml of DTT (1 M).
4. Add 0.2 ml of 2-Mercptoethanol (EM Science 6010, 14.2 M).
5. Cap the tube and mix by gentle inversion to avoid formation of foam.
6. Use it fresh to prepare Sample buffer complete. Store the remaining buffer at -20 °C.

Sample buffer complete (2X) 5 ml
(Protease inhibitor cocktail 2X, Sample buffer 2X, Phosphatase inhibitor cocktails 2X)
1. Allow phosphatase inhibitor cocktails 1&2 (Sigma-Aldrich P2850, 100X, and Sigma-Aldrich P5726, 100X, stored at 4°C) to thaw and dissolve completely. This step will vary in time, depending on volume of aliquot.
2. Add a tablet of protease inhibitor (Roche 1836153) to 2.3 ml purified water in a 15-ml conical tube, and vortex to dissolve completely.
3. Add 2.5 ml of freshly made 4X sample buffer (containing reducing agents), 100 μl phosphatase inhibitor cocktail 1, and 100 μl phosphatase inhibitor cocktail 2 to the solution.
4. Cap tube and mix slowly by inversion (to avoid formation of foam).
5. Divide into 0.5-ml aliquots and store at -20°C.