**Immunoblotting of I3Neurons and dopaminergic neurons**

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**Abstract**

This protocol describes the preparation of cell lysate from and iPSC-derived neurons (i3Neurons, dopaminergic) and the immunoblotting procedure.

**Keywords**

Immunoblot, antibody, RIPA buffer

**Solutions to prepare**

**A. Cell culture and treatments**

1. i3Neurons and dopaminergic (DA) neurons were grown on six-well plates (3-5 × 105 cells/well).
2. After differentiation in their respective maturation media, the neurons were washed with 1 ml ice-cold PBS.
3. Cells were then lysed in 200ul 1xRIPA lysis buffer (10X RIPA lysis buffer, Sigma-Aldrich) supplemented with cOmplete™ EDTA-free protease inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitor cocktail (Roche).
4. Cells were then centrifuged at 13,000 × g for 10 min.
5. The supernatant was collected and store at -20°C.
6. Protein concentration was determined in sample using Pierce BCA assay (ThermoFisher)

**B. Gel electrophoresis and immunoblotting (Tris-glycine buffer system)**

1. Appropriate volume of cell lysate solution was incubated at 95 °C for 5 min in SDS sample buffer containing 1% 2-mercaptoethanol (Sigma).
2. The extracted proteins were separated by SDS-PAGE in Mini-PROTEAN TGX precast polyacrylamide gels (Bio-Rad) at 250V and transferred to nitrocellulose membranes (Bio-Rad) at 100 V for 1 h or 75 V for 2 h (for high molecular weight proteins: >150 kDa).
3. The nitrocellulose membranes were blocked for 1 h with 5% non-fat milk (AmericanBIO) in TBST (tris-buffered saline [TBS] + 0.1% tween 20), then incubated overnight at 4 °C with primary antibodies. Antibody dilutions can be found in Table S1.

**TBS:** 50 mM Tris-Cl, 150 mM NaCl, adjust pH to 7.5
**TBST:** TBS with 0.1% TWEEN-20 (Sigma-Aldrich)

1. Blots were washed with TBST, thrice, each 5 minutes.
2. Blots were incubated with IRDye 680RD or 800CW (LI-COR) secondary antibodies (1:8000) for 1h at room temperature in TBST.
3. Blots were imaged using the Gel Doc imaging system (Bio-Rad) using manufacturer’s protocols.