Immunoblotting of macrophages and microglia:

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**Abstract**

This protocol describes the preparation from cell lysate from cultured cells and immunoblotting procedure.

**Keywords**

Immunoblot, antibody, tris-glycine

**Solutions to prepare**
**RIPA buffer:** 150 mM NaCl, 10 mM Tris, 0.5 mM EDTA, 0.5% NP40 supplemented immediately before use with Protease Inhibitor Cocktail (Roche) and PhosStop phosphatase inhibitor (Roche)
**TBS:** 50 mM Tris-Cl, 150 mM NaCl, adjust pH to 7.5
**TBST:** TBS with 0.1% TWEEN-20 (Sigma-Aldrich)
**4x Laemmli buffer:** 188 mM Tris-HCl, 3% SDS, 30% glycerol, 0.01% bromophenol blue, 15% β-mercaptoethanol
**Tris-glycine running buffer:** 25 mM tris base, 192 mM glycine, 0.1% SDS in milliQ water
**Tris-glycine transfer buffer:** 25 mM Tris, 192 mM glycine, 20% methanol in milliQ water. Chill to 4 ̊C.

**A. Cell culture and treatments**

1. Supplement RIPA buffer with Protease Inhibitor Cocktail (Roche) and PhosStop phosphatase inhibitor (Roche) and chill on ice.
2. Aspirate media from cells and rinse cells with PBS on ice. Aspirate PBS thoroughly.
3. Pipette RIPA lysis buffer onto cells and scrape cells using a cell lifter (Corning).
4. Pipette lysis buffer containing cell mass into Eppendorf tube.
5. Treat with 25 units of benzinase for 5 minutes.
6. Spin down at 14000 RPM for 5 minutes at 4 ̊C.
7. Collect supernatant.
8. Determine protein concentration in sample using Pierce BCA assay (ThermoFisher)
9. Prepare samples at desired concentration and add 4x Laemmli buffer.

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

1. Incubate samples at 95 ̊C for 3 minutes.
2. During this incubation, prepare gel apparatus with Mini PROTEAN TGX 4-15% tris-

glycine gels (Bio-Rad) and running buffer.

1. Load samples into gel and run until dye front reaches bottom (250 V).
2. Remove gel and set up transfer cassette with nitrocellulose membrane.
3. Transfer at 100 V for 1 hour in tris-glycine transfer buffer.
4. Remove nitrocellulose membrane and stain for total protein with ponceau stain.
5. Wash with milliQ water.
6. Block membrane with 5% milk in TBST for 1 hour at RT.
7. Incubate membrane with primary antibodies in 2.5% milk in TBST overnight at 4 ̊C .

**NOTE:** Optimal primary antibody incubation time and temperature can be determined empirically for a given primary antibody

1. Wash membrane 3X for 10 minutes with TBST.
2. Incubate membrane with secondary antibodies conjugated HRP for 1 hour.
3. Wash membrane 3X for 5 minutes with TBST. 13.
4. Image membranes using a Biorad Chemidco.

Antibody dilutions:

Primary antibodies:

GAPDH,1:10000 dilution (EnCor biotechnology Inc, # MCA-1D4), human specific cathepsin D, 1:4000 dilution (R&D systems #AF1014), mouse specific cathepsin D, 1:5000 dilution (R&D systems #AF1061), human cathepsin B, 1:1000 dilution (R&D systems #AF953), human cathepsin L, 1:10000 dilution (R&D systems # AF952), mouse cathepsin L, 1:2000 dilution (R&D systems # AF1515), human cathepsin C, 1:1000 dilution (R&D systems #AF1071), human GBA, 1:1000 dilution (R&D systems #MAB7410), hLAMP1, 1:2000 dilution (Cell signaling Technology # 9091), mouse LAMP1, 1:2000 dilution (DSHB #ID4B), MITF, 1:1000 dilution (Cell signaling Technology # 125903), MITF, 1:1000 dilution (Abcam #Ab303530), S6K, 1:1000 dilution (Cell signaling Technology #9202 ), S6K-Phospho-T389, 1:1000 dilution (Cell signaling Technology #9205), human TFEB, 1:1000 dilution (Cell signaling Technology #37785), mouse TFEB, 1:1000 dilution (Proteintech #13372-1-AP), TFE3, 1:1000 dilution (Sigma #HPA023881).

Secondary antibodies:

All the HRP tagged secondary antibodies are purchased from Cell signaling Technology and diluted at 1:2000 ratio.