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Immunoblotting of macrophages and microglia

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We use this protocol and it's working

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Abstract

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

Attachments

DOCX

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17KB

Materials

Solutions to prepare

RIPA buffer:

	A	B
	NaCl	150 mM
	Tris	10 mM
	EDTA	0.5 mM
	0.5% NP40	

supplemented immediately before use with Protease Inhibitor Cocktail (Roche) and PhosStop phosphatase inhibitor (Roche).

TBS:

	A	B
	Tris-Cl	50 mM
	NaCl	150 mM

adjust pH to 7.5

TBST: TBS with 0.1% TWEEN-20 (Sigma-Aldrich)

4x Laemmli buffer:

	A	B
	Tris-Cl	188 mM
	3% SDS	
	30% glycerol	
	0.01% bromophenol blue	
	15% β-mercaptoethanol	

Tris-glycine running buffer:

	A	B
	tris base	25 mM
	glycine	192 mM
	0.1% SDS in milliQ milliQ water	

Tris-glycine transfer buffer:

	A	B
	Tris	25 mM
	glycine	192 mM
	20% methanol in milliQ water	

Chill to 4 °C .

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.

Human Cathepsin D Antibody **R&D Systems Catalog #**AF1014

Mouse Monoclonal Antibody to GAPDH **Encor Biotechnology Catalog #**MCA-1D4

Human Cathepsin B Antibody **R&D Systems** **Catalog #**AF953

Human Cathepsin L Antibody **R&D Systems** **Catalog #**AF952

Mouse/Rat Cathepsin L Antibody **R&D Systems** **Catalog #**AF1515

Human Cathepsin C/DPPI Antibody **R&D Systems** **Catalog #**AF1071

Human Glucosylceramidase/GBA Antibody **R&D Systems** **Catalog #**MAB7410

MITF (D5G7V) Rabbit mAb **Cell Signaling Technology** **Catalog #**12590

Recombinant Anti-MiTF antibody **Abcam** **Catalog #**ab303530

p70 S6 Kinase Antibody **Cell Signaling Technology** **Catalog #**9202

Phospho-p70 S6 Kinase (Thr389) Antibody **Cell Signaling Technology** **Catalog #**9205

TFEB (D2O7D) Rabbit mAb **Cell Signaling Technology** **Catalog #**37785

TFEB Polyclonal antibody **Proteintech** **Catalog #**13372-1-AP

Anti-TFE3 antibody produced in rabbit **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**HPA023881

Troubleshooting

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 00:05:00 . 5m

6 Spin down at 14000 rpm, 4°C, 00:05:00 . 5m

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.

Gel electrophoresis and immunoblotting (Tris-glycine buffer system) 4h 48m

10 Incubate samples at 95 °C for 00:03:00 . 3m

11 During this incubation, prepare gel apparatus with Mini PROTEAN TGX 4-15% tris-glycine gels (Bio-Rad) and running buffer.

12 Load samples into gel and run until dye front reaches bottom (250 V).

13 Remove gel and set up transfer cassette with nitrocellulose membrane.

14 Transfer at 100 V for 01:00:00 in tris-glycine transfer buffer. 1h

15 Remove nitrocellulose membrane and stain for total protein with ponceau stain.

16 Wash with milliQ water.

17 Block membrane with 5% milk in TBST for 01:00:00 at Room temperature . 1h

18 Incubate membrane with primary antibodies in 2.5% milk in TBST Overnight at 4 °C . 1h

Note

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

19 Wash membrane.

19.1 Wash membrane for 00:10:00 with TBST. (1/3) 10m

19.2 Wash membrane for 00:10:00 with TBST. (2/3) 10m

19.3 Wash membrane for 00:10:00 with TBST. (3/3) 10m

20 Incubate membrane with secondary antibodies conjugated HRP for 01:00:00 . 1h

21 Wash membrane.

21.1 Wash membrane for 00:05:00 with TBST. (1/3) 5m

21.2 Wash membrane for 00:05:00 with TBST. (2/3) 5m

21.3 Wash membrane for 00:05:00 with TBST. (3/3) 5m

22 Image membranes using a Biorad Chemidco.