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Immunological detection of autophagy and mTORC1-related proteins

Forked from Immunological detection of APP and proteins of the endolysosomal system

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DISCLAIMER

ABSTRACT

Here we present a general protocol for immunological detection by Western blotting MTOR, MTOR (pS2448), ULK1, ULK1 (pS757), p70S6K, p70S6K (pT389), SQSTM1, CALCOCO2, MAP1LC3B, GABARAP, TFEB, TFE3, PGRN, HSP90, and PCNA

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FORK NOTE

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MATERIALS TEXT

A	B	C
REAGENT or RESOURCE	**SOURCE**	**IDENTIFIER**
Antibodies		
human anti-progranulin	R&D Systems	AF2420
anti-PCNA	Santa Cruz Biotechnology	sc56
anti-HSP90	Proteintech	60318-1-lg
anti-TFEB	Cell Signaling Technology	4240
anti-TFE3	Proteintech	14480-1-AP
anti-SQSTM1	Proteintech	18420-1-AP
anti-Calcoco2	Proteintech	12229-1-AP
anti-MAP1LC3B	Cell Signaling Technologies	2775
anti-GABARAP	Proteintech	18723-1-AP
anti-MTOR	Cell Signaling Technologies	2983
anti-p70S6K	Cell Signaling Technologies	2708
anti-MTOR (pS2448)	Cell Signaling Technologies	2971
anti-ULK1	Cell Signaling Technologies	8054
anti-ULK1 (pS757)	Cell Signaling technologies	14202
IRDye 680RD Goat a-Rabbit IgG secondary antibody	Li-Cor	926-68071

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IRDye 680RD Goat a-Mouse IgG secondary antibody	Li-Cor	926-68070
IRDye 800CW Goat a-Rabbit IgG secondary antibody	Li-Cor	926-32211
IRDye 800CW Goat a-Mouse IgG secondary antibody	Li-Cor	926-32210
Goat a-Rabbit IgG, HRP-linked antibody	Cell Signaling Technology	7474P2
Goat a-Rabbit IgG HRP conjugate	Bio-Rad	1706515
Goat a-Mouse IgG HRP conjugate	Bio-Rad	1706516
Chemicals, peptides, and recombinant proteins		
PhosSTOP	Roche	04906845001
Complete EDTA-free protease inhibitor cocktail	Sigma-Aldrich	11873580001
REVERT 700 total protein stain kit	Li-Cor	926-11016
NuPAGE LDS sample buffer (4X)	Thermo Fisher Scientific	NP0007
NuPAGE sample reducing agent (10X)	Thermo Fisher Scientific	NP0009
Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad	5000006

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NuPAGE MES SDS Running Buffer (20X)	Thermo Fisher Scientific	NP0002
Immobilon-FL PVDF Membrane	Millipore	IPFL00010
WHEATON Dounce Tissue Grinder, 7 mL	DWK Life Sciences	357542
KIMBLE KONTES Dounce Tissue Grinder, 2 mL	DWK Life Sciences	885300-0002
Nonidet P40 substitute	Sigma-Aldrich	74385
Urea	Sigma-Aldrich	U5378
RIPA lysis and extraction buffer	Thermo Fisher Scientific	89900
Experimental models: Cell lines		
293T cells	ATCC	CRL-3216
293 cells	ATCC	CRL-1573
HeLa cells	ATCC	CCL-2
HeLa cells; GRN-/-	This study and DOI: dx.doi.org/10.17504/protocols.io.4r3l2oxqqv1y/v1	
Software and algorithms		
ImageLab v6.0.1	Biorad	https://www.bio-rad.com/en-us/product/image-lab-software?ID=KRE6P5E8Z&source_wt=imagelabsoftware_surl

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Western blotting

1 Lyse cell pellets by homogenization in KPBS buffer, urea buffer, or RIPA buffer with protease and phosphatase inhibitors. FOr some experiments, we employ 293 cells or alternatively HeLa cells with or without the GRN gene, created by CRISPR-based gene editing (DOI: dx.doi.org/10.17504/protocols.io.4r3l2oxqqv1y/v1).

2 Determined total protein concentration by BCA or Bradford assay. Normalize samples within a set of samples with additional buffer. Add NuPAGE LDS buffer (4X) plus NuPAGE reducing agent (10X).

3 Load samples onto 4-12% NuPAGE Bis-Tris gels (ThermoFisher), and separate by electrophoresis in MES buffer.

4 Transfer proteins to PVDF or nitrocellulose membranes by standard wet transfer in 20% methanol.

5 Stain membranes with REVERT 700 total protein stain following manufacturer's instructions, and image total protein with a ChemiDoc MP (Bio-Rad) at 680 nm.

6 De-stain with REVERT reversal solution for 5 min. Block membranes with tris-buffered saline (TBS) (5% non-fat dry milk) at room temperature for 30-60 min.

7 Incubate membranes overnight at 4ºC with primary antibody solution in TBS with 0.1% Tween-20 (TBST). Wash six times with TBST for 5 min each. Incubate in secondary antibody solution in TBST (plus 0.01% SDS) for 1h at room temperature.

8 Wash membranes four times with TBST for 5 min each.

9 When using HRP-conjugated secondary antibodies (Bio-Rad or Cell Signaling Technology), apply luminol and hydrogen peroxide solution to membrane for 2 min, and image membrane with a ChemiDoc MP using the chemiluminescent setting.

10 When using Li-Cor fluorescent secondary antibodies, blot membranes dry and image with a ChemiDoc MP at either 800 nm or 680 nm, depending on the secondary antibody.

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