Immunological detection of APP and proteins of the endolysosomal system V.2

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Here we present a general protocol for immunological detection by Western blotting of APP and proteins of the endolysosomal system, including EEA1, RAB5, PSEN1, LAMP1, LAMP2, TMEM192, and BACE1.

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
a-EEA1 (C45B10) rabbit mAb	Cell Signaling Technology	3288
a-RAB5 (C8B1) rabbit mAb	Cell Signaling Technology	3547
a-PSEN1 (D39D1) rabbit mAb	Cell Signaling Technology	5643
a-PSEN2/AD5 (EP1515Y) rabbit mAb	Abcam	ab51249
a-LAMP1 (D2D11) rabbit mAb	Cell Signaling Technology	9091
a-LAMP2 (D5C2P) rabbit mAb	Cell Signaling Technology	49067
a-TMEM192 rabbit pAb	Proteintech	28263-1-AP
a-HA	Biolegend	901513
a-HA (6E2) mouse mAb	Cell Signaling Technology	2367
a-FLAG M2 mouse mAb	Sigma-Aldrich	F1804
a-ZO-1 rabbit pAb	Proteintech	21773-1-AP
a-Golga1 rabbit pAb	Proteintech	12640-1-AP
a-Calreticulin rabbit pAb	Proteintech	10292-1-AP
a-S6K rabbit pAb	Proteintech	14485-1-AP
a-RAB11 (D4F5) rabbit mAb	Cell Signaling Technology	5589
a-Lamin A/C (4C11) mouse mAb	Cell Signaling Technology	4777
a-VDAC1/Porin rabbit pAb	Proteintech	55259-1-AP
a-RAB7 (D95F2) rabbit mAb	Proteintech	9367

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a-DYKDDDDK tag, mouse mAb (FG4R)	Thermo Fisher Scientific	MA1-91878
a-GAPDH (D16H11) XP rabbit mAb	Cell Signaling Technology	5174
a-APP CTF (C1/6.1) mouse mAb	BioLegend	802801
a-APP A4 (22C11) mouse mAb	Sigma	MAB348
a-PEX19 rabbit pAb	Proteintech	14713-1-AP
a-CD71/TFR1 (D7G9X) rabbit mAb	Cell Signaling Technology	13113
a-HSP90 (3F11C1) mouse mAb	Proteintech	60318-1-Ig
a-BACE1 (D10E5) rabbit mAb	Cell Signaling Technology	5606
IRDye 680RD Goat a-Rabbit IgG secondary antibody	Li-Cor	926-68071
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

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NuPAGE sample reducing agent (10X)	Thermo Fisher Scientific	NP0009
Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad	5000006
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
293L: TMEM192-3xHA	This study	
293L-APP-/-: TMEM192-3xHA; APP-/-	This study	
293EL-APP-/-: TMEM192-3xHA; APP-/-; FLAG-EEA1	This study	
293EL-APP*: TMEM192-3xHA; APP-/-; FLAG-EEA1; APPSw;T700N	This study	
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 293^{EL} APP^{Sw,T700N} cells expressing 3xFLAG-EEA1, TMEM192-3xHA, and APP harboring Swedish and T700N mutations as described in dx.doi.org/10.17504/protocols.io.byi7puhn.

2 Determined total protein concentration by BCA or Bradford assay. Normalize samples within a set of samples with additional lysis 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) plus 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) and 5% non-fat dry milk. Wash six times with TBST for 5 min each. Incubate in secondary antibody solution in TBST (plus 0.01% SDS and 5% non-fat dry milk) for 1h at room temperature.

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

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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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