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

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We use this protocol and it's working

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Disclaimer

Abstract

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.

Materials

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

	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
	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

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.

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.