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Proteomics workflow for APP/Aβ TOMAHAQ analysis in endosomal and lysosomal fractions V.2

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The ability to detect processing of APP to the Ab amyloid peptide is challenging. This protocols describes methods for analysis of Ab "half-tryptic" peptides from purified organelles (endosomes and lysosomes). The targeted proteomics approach using TOMAHAQ coupled with Tomahto, which is an API for use on a Thermo orbitrap instrument that facilitates detection of trigger peptides and fragmentation of target peptide reporter ions.

full peptide spreadsheet for protocols.io.xlsx

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A	B	C
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		
a-FLAG M2 magnetic beads	Sigma-Aldrich	M8823
Pierce a-HA magnetic beads	Thermo Fisher Scientific	88837
TMT10plex Isobaric Label Reagent Set plus TMT11-131C Label Reagent	Thermo Fisher Scientific	A34808
TMTProTM 16Plex Label Reagent set	Thermo Fisher Scientific	A44520
Super Heavy TMT Label Reagent	Thermo Fisher Scientific	A43073
Pierce™ High pH Reversed-Phase Peptide Fractionation Kit	Thermo Fisher Scientific	84868

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HyClone Fetal bovine serum	GE Healthcare	SB30910
Puromycin	Sigma-Aldrich	P9620
G418 (Geneticin)	Invivogen	ant-gn-2
Dulbecco's MEM (DMEM), high glucose, pyruvate	GIBCO / Invitrogen	11995
PhosSTOP	Roche	04906845001
Complete EDTA-free protease inhibitor cocktail	Sigma-Aldrich	11873580001
Tris(2-carboxyethyl)phosphine hydrochloride solution	Sigma-Aldrich	646547
Iodoacetamide	Sigma-Aldrich	I1149
Trichloroacetic acid solution 6.1 N	Sigma-Aldrich	T0699
Trifluoroacetic acid	fisher scientific	A11650
Hydroxylamine solution 50 wt. %	Sigma-Aldrich	438227
Formic Acid	Sigma-Aldrich	5330020050
Pierce Trypsin Protease, MS grade	Thermo Fisher Scientific	90305
Lysyl endopeptidaseR (Lys-C)	Wako	129-02541
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
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
EPPS 0.2M buffer solution, pH 8.5	Alfa Aesar	J61476.AE
Empore C18 47 mm Extraction Disc, Model 2215	3M	98060402173
Sep-Pak C18 1 cc Vac Cartridge	Waters	WAT054955

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Dyngo4a	Cayman Chemical	29479
Lanabecestat (AZD3293)	Selleckchem	S8193
Semagacestat	Cayman Chemical	16713
BPN-15606	MedChemExpress	HY-117482
RIPA lysis and extraction buffer	Thermo Fisher Scientific	89900
Reference peptides for APP/Ab (see Supplemental Data Table S7)	Biomatik Thermo Fisher Scientific	Custom order
Experimental models: Cell lines		
293 cells	ATCC	CRL-1573
293EL-APP-/-: TMEM192-3xHA; APP-/-; FLAG-EEA1	This study	
293EL-APP*: TMEM192-3xHA; APP-/-; FLAG-EEA1; APPSw;T700N	This study	

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Sample preraration

1 Prepare all samples (unfiltered PNS, Lyso, and Endo; Amicon-filtered PNS_LMW, Lyso_LMW, and Endo_LMW) as described (dx.doi.org/10.17504/protocols.io.byjfpujn). For full peptide sequences and associated proteomic parameters, see the attached document.

2 Reduce all samples by adding TCEP to 5 mM final and incubate at 25 ºC for 30 min with shaking.

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3 Alkylate cysteines by adding iodoacetamide to15 mM final and incubate at 25 ºC for 30 min with shaking and protected from light.

4 Protein precipitation:

4.1 Dilute samples with EPPS buffer for 1.2 M urea final concentration.

4.2 Add 6.1 N TCA solution to 20% final and incubate at 4 ºC for 1.5h. Centrifuge samples at 21,000xg for 15 min at 4 ºC and remove supernatants.

4.3 Wash twice with ice-cold acetone by centrifuging at 21,000xg for 10 min at 4 ºC. After final wash, briefly dry protein pellets in a SpeedVac.

5 Resuspend pellets in 10 µL of 8 M urea buffer, sonicate in a water bath sonicator, and dilute urea by adding 10 µL of 200 mM EPPS.

6 Peptide digestion:

6.1 Add 0.3 µg of LysC and incubate at 37 ºC for 2h with shaking. Further dilute urea to 1.6 M final by adding 200 mM EPPS.

6.2 Further digest peptides by adding 0.4 µg trypsin and incubate at 37 ºC overnight with shaking.

7 The next day, add acetonitrile (ACN) to 30% final, and label peptides by adding 3.6-5.3 µL of TMT 11-plex reagents (10 µg/µL in anhydrous ACN) for 1h at 25 ºC with shaking. Quench labeling reaction by adding hydroxylamine to 0.5% final followed by incubation at room temperature for 15 min.

8 Dry pooled sample by SpeedVac, and desalt by C18 StageTip.

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9 Add synthetic reference trigger peptides labeled with super-heavy TMTsh as described (dx.doi.org/10.17504/protocols.io.byjcpuiw). The peptides employed and their associated ionization and fragmentation properties are provided in the attached spreadsheet.

TOMAHAQ experiment

10 Perform TOMAHAQ experiments using the Tomahto software package on a Thermo Scientific Orbitrap Eclipse Tribrid mass spectrometer coupled to an Easy-nLC 1200 UHPLC system. Load each sample on a C18 column (30 cm, 2.6 µm Accucore [Thermo Fisher], 100 µm I.D.), and eluted using a 150-min method over a gradient from 5% to 38% B (95% ACN/0.125% formic acid). The instrument method only controlls Orbitrap MS^1 scans (resolution at 120,000; mass range 300–1500 m/z; automatic gain control (AGC) target $2*10^5$, maximum injection time 50 ms). Peptide targets are imported into Tomahto, and the following decisions are made by Tomahto in real-time.

11 Export summed signal-to-noise ratio (S/N) to csv file, and analyze by R 3.6.3. Adjust S/N of each channel using the isotopic impurity table of TMT reagents provided by the vendor. Normalize the adjusted S/N values according to the total reporter values in each channel according to a SPS-MS^3 analysis, assuming equal amount of loading. Test a statistical significance between DMSO- and compound-treated group using two-sided Student's *t*-test with *t_test()* function in the *rstatix* package version 0.7.0.

SIM experiment

12 For the absolute quantification of the target peptides, apply selected ion monitoring (SIM) experiments. Perform SIM experiments on a Thermo Scientific Orbitrap Eclipse Tribrid mass spectrometer coupled to an Easy-nLC 1200 UHPLC system. Load each sample on an in-house packed C18 column (30 cm, 2.6 um Accucore [Thermo Fisher], 100 µm I.D.), and elute using a 150-min method over a gradient from 5% to 38% B (95% acetonitrile, 0.125% formic acid). Monitor target peptides within a 50 min window around the scheduled retention time. Isolate a pair of trigger and target peptides, and accumulated separately targeting same AGC value ($5*10^4$; resolution at 240,000) and subsequently analyze in a single Orbitrap SIM scan. If two target peptides share similar retention time and same FAIMS CV values, multiplex their detections into a single SIM scan.

13 Import RAW files from the SIM experiments into Skyline, and extract the precursor ion peaks with 10 ppm accuracy. Because the TMT-labeled target peptides and TMTsh-labeled trigger peptides have the same retention time, measure the area under each peak, and calculate the ratio between target and trigger. Derive absolute amount the target peptide by multiplying the ratio by the known amount of the trigger peptide. Divide the absolute amount of target peptide by the relative quantitation from TOMAHAQ to calculate the absolute amount from each channel.

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