

Supplementary Material

An automated clinical mass spectrometric method for identification and quantification of variant and wild-type amyloid- β 1-40 and 1-42 peptides in CSF

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1 Material Sources and Equipment

The following materials were obtained from the indicated commercial sources: Beta-Amyloid (1-42) (A β 42), Ultra Pure, TFA [A-1002-2; rPeptide], Beta-Amyloid (1-40) (A β 40), Ultra Pure, TFA [A-1001-2; rPeptide], ¹⁵N Beta-Amyloid (1-42) (A β 42), Uniformly labeled [A-1102-2; rPeptide], ¹⁵N Beta-Amyloid (1-40) (A β 40), Uniformly labeled [A-1101-2; rPeptide], E22G variant A β 40 [H-6694.0500; Bachem], E22Q variant A β 40 [H-6696.0500; Bachem], D23N variant A β 40 [H-7332.0500; Bachem], D7N variant A β 40 [H-7334.0500; Bachem], H6R variant A β 40 [H-7336.0500; Bachem], L34V variant A β 40 [H-7414.0500; Bachem], E22 Δ variant A β 40 [H-7474.0500; Bachem], E22K variant A β 40 [H-6698.0500; Bachem], A21G variant A β 40 [H-6702.0500; Bachem], bovine serum albumin (BSA) [2930-100 GM; Fisher EMD Millipore Brand], guanidine hydrochloride (GdnHCl) [G-4506-1006; Sigma-Aldrich], INNOTEST hTau Ag (RUO) [81579; Fujirebio], INNOTEST β -Amyloid (1-42) (RUO) [81583; Fujirebio], 15 mL conical sterile polypropylene centrifuge tubes [12-565-269; Fisher Scientific], Axygen MAXYMum Recovery 1.7 mL Snaplock microcentrifuge tubes [MCT-175-L-C], Axygen MAXYMum Recovery 0.6 mL Snaplock microcentrifuge tubes [MCT-060-L-C], NUNC pre-slit capmats [12-565-570; Fisher Scientific], 0.5 mL AntiBIND 96WP [02 911 700; Fisher Scientific], MCX μ Elution plate [186001830BA; Waters Oasis], eppendorf ThermoMixer [CA11028-280], 2.6 μ m EVO C18 100 x 3.0 mm [00D-4725-Y0; Phenomenex Kinetex], Phenomenex KrudKatcher Classic HPLC In-Line Filter [AFO-5727, Phenomenex], SCIEX 5500 QTRAP mass spectrometer, Shimadzu HPLC, and Hamilton STARlet.

2 Methods

2.1 Preparation of A β calibrators

Stock Peptide Solutions. The 1 mg/vial or 0.5 mg/vial of lyophilized A β peptides from rPeptide were stored at -70 °C until use. Lyophilized stocks were brought to room temperature for 10 min and then dissolved in DMSO in the original vial to produce 0.5 mg/mL stock solutions. After sonication (2 min) and inversion, 100 μ L aliquots were made in MAXYMum Recovery tubes, capped, sealed with Parafilm and stored immediately at -70 °C.

Intermediate Peptide Solutions. A 1/10 dilution was performed on the 0.5 mg/mL stock solutions with DMSO producing an intermediate 50 μ g/mL working solution. The solution was aliquoted in 100 μ L aliquots in MAXYMum Recovery tubes, sealed with Parafilm and stored immediately at -70 °C.

Working Peptide Solution. The 50 μ g/mL intermediate peptide solutions were diluted 1/10 in 30% ACN in DI water + 1% NH₄OH to make 5000 ng/mL, 500 ng/mL and 50 ng/mL solutions.

Spiking Peptide Solutions. The spiking solutions were prepared as follows in Supplementary Table 1 using the stock and working peptide solutions.

Supplemental Table 1. Spiking solutions preparation scheme.

Spiking soln ID	A β 42 soln (ng/L)	Volume of A β 42 soln (μ L)	A β 40 soln (ng/L)	Volume of A β 40 soln (μ L)	30% ACN in DI water + 1% NH ₄ OH (mL)	A β 42 in spiking soln (ng/L)	A β 40 in spiking soln (ng/L)
1	5.0E+04	100	5.0E+04	100	0.800	5.0E+03	5.0E+03
2	5.0E+05	20	5.0E+05	100	0.880	1.0E+04	5.0E+04
3	5.0E+05	50	5.0E+06	50	0.900	2.5E+04	2.5E+05
4	5.0E+05	75	5.0E+06	100	0.825	3.8E+04	5.0E+05
5	5.0E+05	100	5.0E+07	15	0.885	5.0E+04	7.5E+05
6	5.0E+06	30	5.0E+07	20	0.950	1.5E+05	1.0E+06

Calibrators were used immediately after preparation for each experimental run. Calibrators were prepared in artificial CSF (1 mg/mL BSA in phosphate-buffer saline [PBS]) to create the 6-point calibrators (Supplemental Table 2).

Supplemental Table 2. Calibrator preparation and resulting calibrator concentrations.

Calibrator	A β Spiking Solution ID	Standard solution (μ L)	Artificial CSF (μ L)	Final A β 42 concentration (ng/L)	Final A β 40 concentration (ng/L)
n/a	n/a	n/a	500	-	-
S1	1	10	490	100	100
S2	2	10	490	200	1,000
S3	3	10	490	500	5,000
S4	4	10	490	750	10,000
S5	5	10	490	1,000	15,000
S6	6	10	490	3,000	20,000

2.2 Internal Standards (IS)

Stock IS Solutions. One milliliter of 1% NH₄OH was added to the lyophilized ¹⁵N-uniformly labelled A β 42 and ¹⁵N-uniformly labelled A β 40 vials to produce 1 mg/mL concentrated solutions. The solution was sonicated for 1 minute, aliquoted in 100 μ L aliquots in MAXYMum Recovery tubes, sealed with Parafilm and stored immediately at -70 °C.

Working IS Solutions. A 1/10 dilution was performed in 1% NH₄OH in 30:70 ACN: DI water to make a 100 μ g/mL solution. The solution was aliquoted to labelled tubes and stored immediately at -70 °C. The ¹⁵N-A β 42 was diluted by 1/10 further in 1% NH₄OH in 30:70 ACN: DI water to

make a 10 µg/mL solution. This solution was aliquoted in 100 µL aliquots and stored immediately at -70 °C.

IS Mix. One hundred microliters of the 100 µg/mL ¹⁵N-Aβ40 and 100 µL of the 10 µg/mL ¹⁵N-Aβ42 were added into 800 µL of 1% NH₄OH in 30:70 ACN: DI water producing a mixed solution of 10 µg/mL ¹⁵N-Aβ40 and 1 µg/mL ¹⁵N-Aβ42. The solution was aliquoted in 50 µL aliquots and stored immediately at -70 °C.

IS in 5 M GdnHCl. Ten microliters of the IS mix was added to 10 mL of 5 M GdnHCl and vortexed to mix. This solution was prepared fresh on the day of analysis.

2.3 Sample preparation

Two hundred microliters each CSF standard, human CSF sample or QC was aliquoted to a 2 mL AntiBIND 96 well plate. Two hundred microliters of the IS mix in 5 M GdnHCl was then added to each sample. The 96 well plate was sealed and placed on the ThermoMixer to mix at 1000 rpm for 40 minutes at room temperature. Following denaturation, 200 µL of 4% H₃PO₄ was added to each sample followed by SPE extraction of the Aβ peptides.

2.4 Solid-phase extraction

Following denaturation, Aβ peptides were extracted from CSF samples by SPE. The MCX µElution plate was conditioned with 200 µL of 100% MeOH, followed by 200 µL of 4% H₃PO₄ under ~200 mbar vacuum. Six hundred microliters of the sample was then added to the plate under ~200 mbar vacuum. The column was washed twice with 200 µL of 4% H₃PO₄. Three elution steps were then performed with: 25 µL of 75:15:10 (v:v:v) ACN: H₂O: NH₄OH, followed by 50 µL of 75:15:10 (v:v:v) ACN: H₂O: NH₄OH, followed 75 µL of deionized water. Eluted samples were collected in 500 µL AntiBIND 96 well plate.

2.5 HPLC-MS/MS

For each sample, 40 µL of the eluate was injected onto a reverse phase column. Mobile phase A was 0.3% NH₄OH in water (prepared fresh daily) and mobile phase B was 25:75 IPA:ACN. The gradient was: 1.00 min = 15% B; 5.00 min = 55% B; 5.50 min = 95% B; 8.90 min = 95% B; 9.00 min = 15% B. Total run time was 11.5 minutes.

The SCIEX 5500 QTRAP mass spectrometer was operated in ESI+ mode, with curtain gas set to 35 V, collision gas set to high, ionspray voltage set to 4250 V, source temperature set to 700 °C, gas 1 and gas 2 both set to 50. A dwell time of 45 ms, a declustering potential of 100 V, and an entrance potential of 10 V was used for all MRM transitions. Data was acquired for each of the MRM transitions outlined in Supplemental Table 3.

Supplemental Table 3. MRM Transitions for A β peptides.

Analyte	Q1 (m/z)	Q3 (m/z)	Collision Energy (V)	Collision Cell Exit Potential (V)
¹⁵ N-A β 42	1143.0	1092.1	35	24
A β 42 1	1129.2	1107.1	35	24
A β 42 2	1129.2	1078.6	32	24
A β 42 3	1129.2	1053.8	39	24
¹⁵ N-A β 40	1096.0	1066.9	35	22
A β 40 1	1083.4	1054.1	33	23
A β 40 2	1083.4	1029.1	37	22
A β 40 3	1083.4	1000.8	36	22

2.6 Figures of Merit

Linearity of A β 42 and A β 40 was assessed in a mixing study using human CSF pools (see manuscript for details).

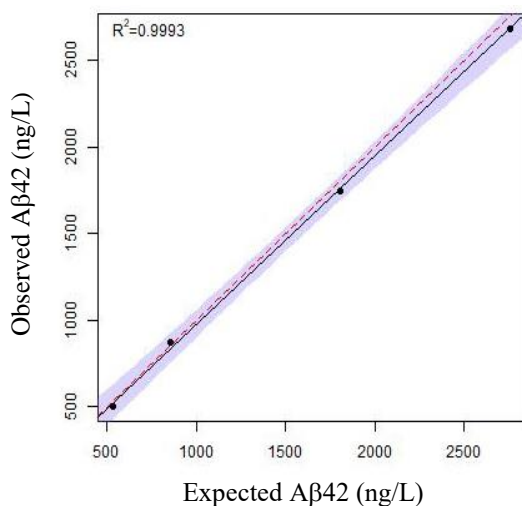
Supplemental Table 4. Linearity results for A β 42.

Human CSF low: high pool ratios	Observed average of triplicate analyses (ng/L)	Expected (ng/L)	Recovery (%)
100:0	220	N/A	N/A
90:10	502	537	93.5
80:20	872	853	102.1
50:50	1750	1803	97.0
20:80	2687	2753	97.6
0:100	3387	N/A	N/A

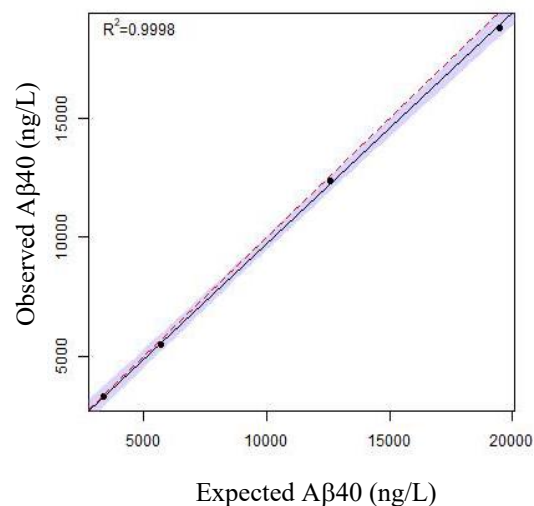
Supplemental Table 5. Linearity results for A β 40.

Human CSF (low: high pool ratios)	Observed average of triplicate analyses (ng/L)	Expected (ng/L)	Recovery (%)
100:0	1067	N/A	N/A
90:10	3303	3367	98.1
80:20	5503	5667	97.1
50:50	12400	12567	98.7
20:80	18833	19467	96.7
0:100	24067	N/A	N/A

A)



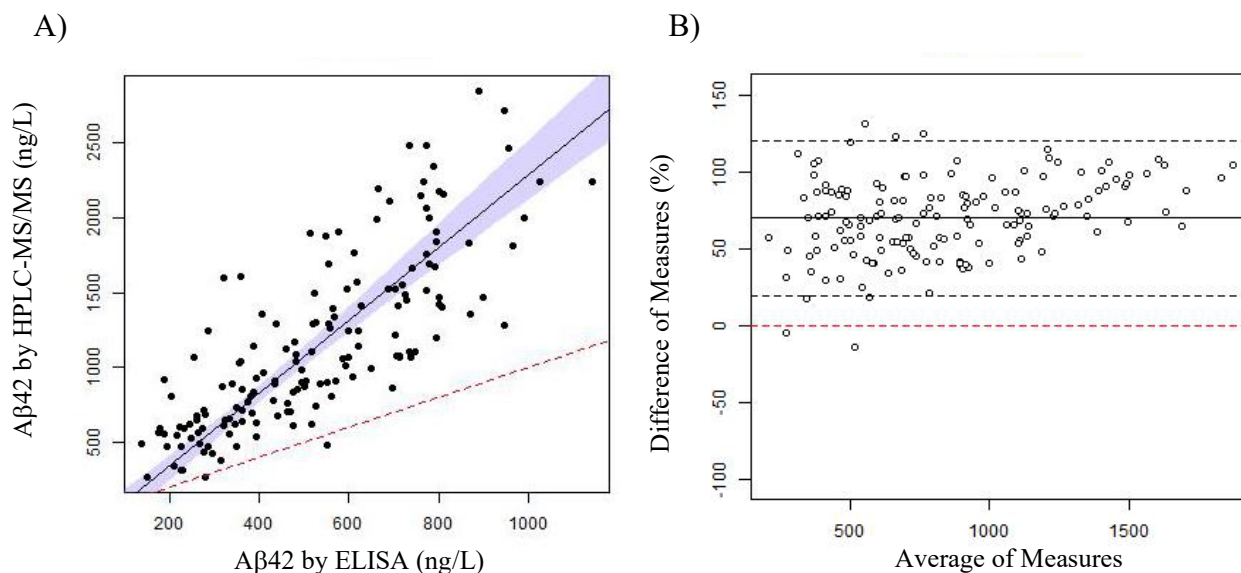
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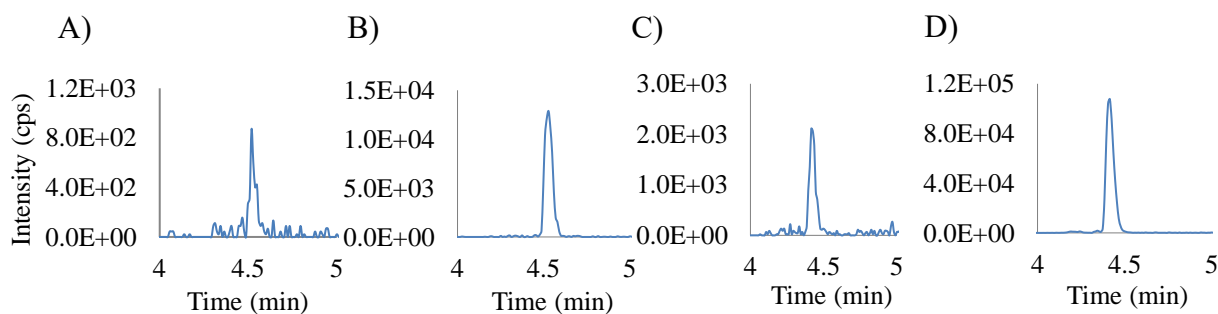
Supplemental Figure 1. Linear regression analysis for the observed HPLC-MS/MS results versus the expected (A) A β 42 and (B) A β 40 concentrations.

Supplemental Table 6. Precision of manual versus automated sample preparation for HPLC-MS/MS analysis.

Sample preparation	QC pool	Nominal concentration (ng/L)	Within-run CV (%)	Between-run CV (%)	Total CV (%)	
Manual	A β 40	Low (n = 19)	2020	4.60	6.20	7.70
		Medium (n = 20)	3900	6.60	2.70	7.10
		High (n = 19)	10100	4.70	4.50	6.50
	A β 42	Low (n = 19)	273	9.00	13.0	16.0
		Medium (n = 19)	522	9.00	0.0	8.00
		High (n = 19)	1290	7.00	6.00	9.00
Automated	A β 40	Low (n = 24)	840	6.00	3.30	7.70
		Medium (n = 25)	3500	8.50	6.80	10.9
		High (n = 25)	10000	9.50	7.80	12.3
	A β 42	Low (n = 24)	113	10.0	0.0	9.00
		Medium (n = 25)	452	12.0	15.0	19.0
		High (n = 24)	1270	8.00	6.00	10.0



Supplementary Figure 2. Method comparison of the HPLC-MS/MS method and the INNOTEST ELISA (n =155) including (A) linear regression analysis: $Y_{\text{HPLC-MS/MS}} = 2.64 \times X_{\text{ELISA}} - 247.4$, $R_2 = 0.63$, $R_2 = 0.63$ (shaded region represents the 95% CI of the slope, red dashed line is the line of identity) and (B) the difference plot (black dashed lines represent the 95% CI of the mean difference).



Supplemental Figure 3. Representative chromatograms of A) Aβ42 MRM-1, B) ^{15}N - Aβ42, C) Aβ40 MRM-1, D) ^{15}N - Aβ40, all from Aβ 100 ng/L solutions.

2.7 APP Variants

Supplemental Table 7. Published variants in A β 42 region, phenotype (if reported), relevant disease (i.e., AD, cerebral amyloid angiopathy [CAA] and/or Parkinson's Disease Dementia [PDD]) and the global population frequency from Database searches (Genome Aggregation Database (GnomAD- Exomes), Trans-Omics for Precision Medicine (TOPMed), Exome Aggregation Consortium (ExAC), and the NHLBI GO Exome Sequencing Project (GO-ESP) Exome Variant Server.

Variant	Phenotype	Disease Relevance	Frequency				Average Frequency
			GnomAD-Exomes	TOPMED	ExAC	GO-ESP	
Icelandic (A2T)	Protective	AD	4.6E-04	1.1E-04	4.7E-04	1.0E-04	4.6E-04
A2V	Pathogenic	AD					
E3Q	Unknown		4.0E-05	2.0E-05	1.0E-05		2.3E-05
E3K	Unknown		4.0E-05	2.0E-05	1.0E-05		2.3E-05
F4I	Unknown				1.0E-05		1.0E-05
R5Q	Unknown		2.0E-05	3.0E-05	4.0E-05		3.0E-05
English (H6R)	Pathogenic	AD	1.0E-05				1.0E-05
Taiwanese (D7H)	Pathogenic	AD					
Tottori (D7N)	Pathogenic	AD					
D7E	Unknown			1.0E-05			1.0E-05
G9A	Unknown			1.0E-05	1.0E-05	1.0E-04	4.0E-05
G9V	Unknown			1.0E-05	1.0E-05	1.0E-04	4.0E-05
G9E	Unknown			1.0E-05	1.0E-05	1.0E-04	4.0E-05
Leuven (E11K)	Pathogenic	AD					
V12I	Unknown		1.0E-05	6.0E-05	2.0E-05		3.0E-05
H13P	Unknown						
H14R	Unknown			1.0E-05	1.0E-05		1.0E-05
Q15E	Unknown			1.0E-05			1.0E-05
K16N	Pathogenic	AD					

V18M	Unknown		1.0E-05	1.0E-05	2.0E-05		1.3E-05
F19L	Unknown						
Flemish (A21G)	Pathogenic (AD)	AD & CAA					
E22D	Unknown				1.0E-05		1.0E-05
Osaka (E22Δ)	Pathogenic	AD					
Arctic (E22G)	Pathogenic (AD)	AD & CAA					
Dutch (E22Q)	Pathogenic	CAA					
Italian (E22K)	Pathogenic	CAA					
Iowa (D23N)	Pathogenic	AD & CAA					
V24G	Unknown			2.0E-05			2.0E-05
V24A	Unknown			2.0E-05			2.0E-05
G25D	Unknown						
K28R	Unknown		1.0E-05				1.0E-05
I31V	Unknown			2.0E-05	1.0E-05		1.5E-05
I32V	Unknown		1.0E-05		1.0E-05		1.0E-05
Piedmont (L34V)	Pathogenic	CAA					
V36M	Unknown				1.0E-05		1.0E-05
G38C	Unknown				8.2E-06		8.2E-06
G38S	Not Pathogenic (AD)	PDD			4.1E-05		4.1E-05
G38V	Unknown				1.0E-05		1.0E-05
V39I	Unknown		2.0E-05		3.3E-05	1.0E-04	5.1E-05
I41V	Unknown						
A42T	Unknown	AD	1.0E-04	3.0E-05	5.8E-05		6.3E-05
A42V	Not Pathogenic (AD)		4.0E-05	2.0E-05	6.6E-06		4.2E-05

Supplemental Table 8. MRM transitions of wt-A β peptides and var-A β peptides meeting the selection criteria in the multiplex HPLC-MS/MS method.

Peptides	Precursor ion (m/z)	Product ions (m/z)
wt-A β 40	1083.0	1054.1, 1029.1, 1000.8
wt-A β 42	1129.4	1107.1, 1078.6, 1053.8
¹⁵ N-IS-A β 40	1096.0	1066.9
¹⁵ N-IS-A β 42	1143.0	1092.1
E22K-, D7N-, E22Q-, D23N-, E11K-A β 40 ^a	1083.0	1054.1, 1029.1, 1000.8
H6R-A β 40	1088.2	1058.9, 1033.9, 1005.4
L34V-, A21G-, K16N-A β 40	1079.9	1050.5, 1025.8, 997.4
E22G-A β 40	1065.5	1036.2, 1011.5, 997.1
E22 Δ -A β 40	1051.2	943.9, 914.8, 882.7
A2V-A β 40	1090.4	1054.1, 1029.1, 1000.8
D7H-A β 40	1089.0	1054.1, 1029.1, 948
G9A-A β 40	1087.0	1050.5, 1029.1, 943.9
G9V-A β 40	1093.3	1054.1, 1029.1, 943.5
G9E-A β 40	1101.5	1054.1, 1029.1, 1025.8
G38S-A β 40	1090.9	1054.1, 1029.1, 1000.8
V39I-A β 40	1087.0	1029.1, 1025.8, 1000.8
A42T-A β 42	1137.0	1107.1, 1078.6, 1053.8
A42V-A β 42	1136.5	1107.1, 1078.6, 1053.8

^a Variants separated chromatographically from wt-A β 40