Analytical Feasibility of Plasma Phospho-Tau for Predicting Amyloid and Tau PET Positivity Study

Foundation for the National Institutes of Health, Biomarkers Consortium Plasma Aβ as a Predictor of Amyloid Positivity in Alzheimer's Disease Project

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Introduction

Developing new diagnostics and therapeutics for Alzheimer's disease (AD) critically relies on the proper characterization of patient populations and disease staging. To facilitate successful therapeutic development, the National Institute on Aging (NIA) and the Alzheimer's Association® have created a research framework based on defining vulnerable populations according to their amyloid, tau, and neurodegeneration state (A/T/N, Jack et al. 2018). The success of implementing the A/T/N framework relies on the ease, sensitivity, and accuracy with which each of the three components can be assessed. The primary methods for measuring A/T/N potential biomarkers requires costly neuroimaging (positron emission tomography (PET) and invasive (CSF lumbar puncture) techniques. The combination of expensive biomarkers and high screen failure rates generates exceptionally high costs for many AD-related clinical trials. Ideally, a much simpler blood-based test for biomarker status would help simplify clinical trials, reduce patient burden and reduce the costs required to identify and characterize Alzheimer's patients, particularly as clinical research moves into earlier stages of the disease where patients need to be identified in a pre-symptomatic state (no cognitive impairments).

The Biomarkers Consortium Plasma Aβ Project Team conducted a study to assess plasma Aβ as a predictor of amyloid PET positivity in AD. The evaluated assays, when combined with genotype and age data, performed similarly to previous studies with AUROCs for predicting amyloid PET positivity ranging from to 0.77 to 0.84 (a modest improvement on Age and APOE status alone, AUROC= 0.75, Zicha et al. 2021). At the outset of the study, new evidence had shown that a multi-component biomarker approach for selecting persons with a likelihood of progressing can be even more effective using algorithms that increase the predictability of conversion to AD in two, four, or six years to above 0.90. (unpublished data, https://www.alzforum.org/news/conference-coverage/where-now-phospho-tau).

The emergence of new sensitive methods for phospo-tau (pTau) detection in blood presented a unique opportunity to extend the Plasma Aβ project to generate critically needed information on plasma pTau using the most promising high performing pTau assays. The Analytical Feasibility of Plasma Phospho-Tau for Predicting Amyloid and Tau PET Positivity study evaluated whether the plasma concentrations of pTau-181, when combined with Aβ, improve the accuracy of Aβ alone to predict amyloid PET positivity in AD. Additionally, the data generated in the study was used to determine each plasma pTau-181 assay's ability to differentiate between tau-positive and tau-negative subjects.

Summary (or Abstract)

The project team selected four plasma pTau-181 immunoassays and one plasma pTau-231 immunoassay for inclusion in the Analytical Feasibility of Plasma Phospho-Tau for Predicting Amyloid and Tau PET Positivity study based on internal validation data and preanalytical requirements. Plasma samples collected within three months of an amyloid PET scan were selected from ADNI n=130 (50% Aβ+): cognitively normal n=54 (37% Aβ+), mild cognitive impairment n=54 (46% Aβ+), and AD n=22 (91% Aβ+). Each participant's sample was tested in

a blinded fashion by all four assays with analytical controls. Nine (9) duplicate ADNI participants (i.e., 9 pairs of repeated measures) were identified in the sample set.

To assess intra- and inter-run variability for each assay, 2 plasma endogenous quality control (eQC) samples were included in each analytical run. The assay providers were supplied with healthy control plasma eQC samples (BioIVT or University of Pennsylvania/ADNI biorepository) and plasma eQC samples selected from individuals whose CSF tested positive for pTau-181 and A**β**42 (University of Pennsylvania/ADNI biorepository).

Methodology

Plasma pTau-181 Assays

	ADx NeuroSciences	**Fujirebio**	**Quanterix**	**Roche Diagnostics**
Immunoassay	pTau181 Simoa	Lumipulse G pTau 181 Plasma	Simoa® pTau 181V2 Advantage	Roche Elecsys plasma Phospho-Tau(181P)
Platform	Single molecule array (Simoa) HD-X	LUMIPULSE G1200 (FDA/CE)	HD-1	Cobas e601
Performing Laboratory	Teunissen laboratory, Amsterdam UMC	University of Pennsylvania Biomarkers Core Laboratory	Quanterix Accelerator Laboratory Services	Microcoat Biotechnologie GmbH

Plasma pTau-231 Assay

	University of Gothenburg
Immunoassay	pTau231 Simoa
Platform	Single molecule array (Simoa) HD-X
Performing Laboratory	Henrik Zetterberg and Nicholas Ashton, University of Gothenburg

Project Sample Preanalytical Processing

The ADNI plasma samples were collected in 10 mL K2-EDTA tubes and centrifuged within 1 hour of collection at room temperature in a clinical centrifuge at 1300 g for 10 minutes. All plasma samples were frozen on dry ice within 90 minutes of collection at ADNI sites and shipped to the University of Pennsylvania on dry ice. Aliquoting was performed in the Biomarker Core laboratory into 0.5 mL polypropylene tubes, and samples were frozen and stored at -80°C until shipped on dry ice to the assay providers.

Datasets

Sample analysis results for the four plasma pTau-181immunoassays generated by ADx Neurosciences in partnership with Amsterdam University Medical Centers (VUmc), Fujirebio in partnership with the University of Pennsylvania Biomarkers Core Laboratory, Quanterix by its Accelerator Laboratory, and Roche Diagnostics in partnership with Microcoat Biotechnologie GmbH are within the following datasheets:
FNIH Biomarkers Consortium Plasma Abeta Project_ADx Neurosciences_VUmc_ plasma pTau-181
FNIH Biomarkers Consortium Plasma Abeta Project_Fujirebio_plasma pTau-181
FNIH Biomarkers Consortium Plasma Abeta Project_Quanterix_plasma pTau-181
FNIH Biomarkers Consortium Plasma Abeta Project_Roche Diagnostics_plasma pTau-181

Sample analysis results for the plasma pTau-231 immunoassay generated by the University of Gothenburg with oversight from Drs. Henrik Zetterberg and Nicholas Ashton are within the following datasheets:
FNIH Biomarkers Consortium Plasma Abeta Project_U of Gothenburg_plasma pTau-231

References

Jack CR, Bennett DA, Blennow K, Carrillo MC, Dunn B, Haeberlein SB, et al. NIA-AA Research Framework: Toward a biological definition of Alzheimer's disease. Alzheimers Dement 2018; 14: 535–62.

Zicha S, Bateman RJ, Shaw LM, Zetterberg H, Bannon AW, Horton WA, et al. Comparative analytical performance of multiple plasma Aβ42 and Aβ40 assays and their ability to predict positron emission tomography amyloid positivity. Alzheimers Dement 2022 Jul 12. doi: 10.1002/alz.12697. Epub ahead of print.

About the Authors

This data was generated through a public-private partnership involving the NIH, FDA, industry, and advocacy groups and contributed for use by any investigator interested in measures that characterize aspects of ADNI participants using repositories of fluid samples. Acknowledgement of the Foundation for the National Institutes of Health (FNIH) Biomarkers Consortium "Plasma Aβ as a Predictor of Amyloid Positivity in Alzheimer's Disease" project in any publications using this data would be appreciated and serve the purpose of supporting such public-private partnerships dedicated to providing information that can be used to further our understanding of a disease and the development of novel treatments.

It is requested that articles published utilizing the project data include the following acknowledgement:

The results of the study represent the work of the Foundation for the National Institutes of Health (FNIH) Biomarkers Consortium "Plasma Aβ as a Predictor of Amyloid Positivity in Alzheimer's Disease" project. The study was made possible through a public-private partnership managed by the FNIH and funded by AbbVie Inc., Alzheimer's Association®, Diagnostics Accelerator at the Alzheimer's Drug Discovery Foundation, Biogen MA Inc., Janssen Research & Development, LLC, Takeda Pharmaceutical Company Limited. The Project Investigators have not participated in reviewing the data analysis or content of the manuscript.

Additional information is posted at https://fnih.org/our-programs/biomarkers-consortium/programs/plasma-abeta.

This document was prepared by the Plasma Aβ as a Predictor of Amyloid Positivity in Alzheimer's Disease Project Team. For more information, please contact Wesley Horton at 301-402-5311 or by email at whorton@fnih.org, copying foundation@fnih.org with the email subject line "To the attention of Neuroscience Research Partnerships".

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ADx NeuroSciences

Sample analysis performed by Amsterdam University Medical Centers (VUmc), laboratory of Charlotte Teunissen, Ph.D.
Assay: pTau181 Simoa

Platform	Single molecule array (Simoa) HD-X
Capture antibody	ADx252
Detector antibody	ADx204
Calibrator	Synthetic peptide
Calibrator matrix	PBS based matrix with detergents and serum replacer
Sample diluent	PBS based matrix with detergents
Dilution factor	1/5
LLoD or LLoQ	LLOQ (LOD + 10 SD): 2.36 pg/ml

Sample Storage and Preparation

Project samples were stored in -80°C freezer upon receipt. In preparation for measurement, the samples were thawed on ice and centrifuged for 8 minutes at 10,000 g in a refrigerated microcentrifuge (5°C). The samples were kept on ice until diluted in a Simoa assay plate.

Dilution protocol for test specimens in the working micro-plate (for duplicate measurement):

1) Add the required volume of dilution buffer to the working microplate wells before adding the required volume of sample. Required volumes for plasma analysis is 184 µL diluent + 46 µL plasma sample (dilution factor 5).
2) Mix well by gently pipetting up and down min. 5 times. Try to avoid air bubbles.

Sample addition should be done by direct pipetting.

Assay Procedure and Principles

Test procedure:
Determine the size of the assay by considering the total number of wells required. Include the 7-point calibrator curve, blank and 3 control samples in duplicate.

1. Allow ready-to-use Simoa reagents to reach room temperature. Mix thoroughly.
2. Prepare the capture bead, detector and enzyme conjugate solution as described in the Instructions for Use.
3. Incubate the RGP substrate solution as described in the Instructions for Use.
4. Add 230 µL of the 7-point calibrator + blank into the assay microplate (no predilution required).
5. Control samples are prediluted in the working microplate by adding dilution buffer and QC sample as described in the reagent preparation of the Instructions for Use.
6. Cover the plate with an adhesive sealer and store at 4°C until use.

7. The Simoa HD-1 or HD-X analyzer is used for analysis. Refer to the Simoa HD-1/HD-X Analyzer User Guide and Simoa Homebrew Assay Development Kit package insert for instrument operation guidelines.
8. Assay set-up is programmed into the Simoa software. Both assays are 2-step neat 2.0 Homebrew protocols and programmed as described in the Instructions for Use.
9. Beads (10 seconds vortexed immediately prior to loading), detector, SβG and RGP are loaded into the appropriate lanes of the Simoa HD-1/HD-X analyzer and assigned by scanning the appropriate Homebrew barcodes.
10. Sealed Simoa sample plate (step 6) is loaded on the Simoa device and wells are assigned to the corresponding assay set-up.
11. Consumables (cuvettes, discs, pipettor tips), wash buffers and system liquid are filled when requested by the instrument. Liquid and solid waste are discarded when requested by the instrument.

The analyte concentration is determined by detecting the number of wells containing both a bead and fluorescent signal relative to the total number of wells containing beads (AEB – average enzymes per bead). If the target has been captured and labeled on the bead, ß-galactosidase hydrolyzes the RGP substrate in the microwell into a fluorescent product that provides the signal for detection.
At low target concentrations, the percentage of bead-containing wells in the array that have a positive signal is proportional to the amount of target present in the sample. At higher target concentrations, when most of the bead-containing wells have one or more labeled target molecules, the total fluorescence signal is proportional to the amount of target present in the sample. The concentration of target in unknown samples is interpolated from the calibration curve.

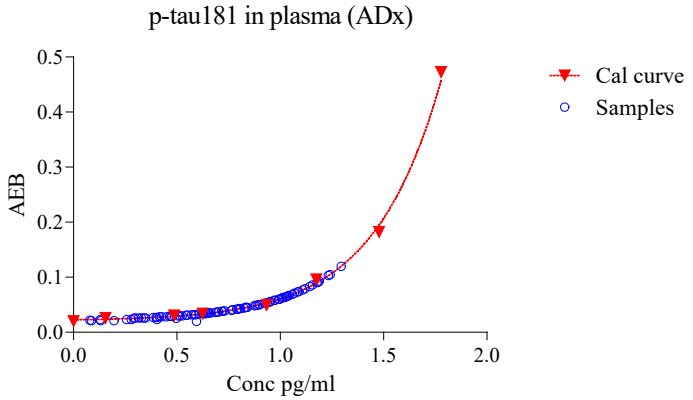
Calibrators and Internal QCs

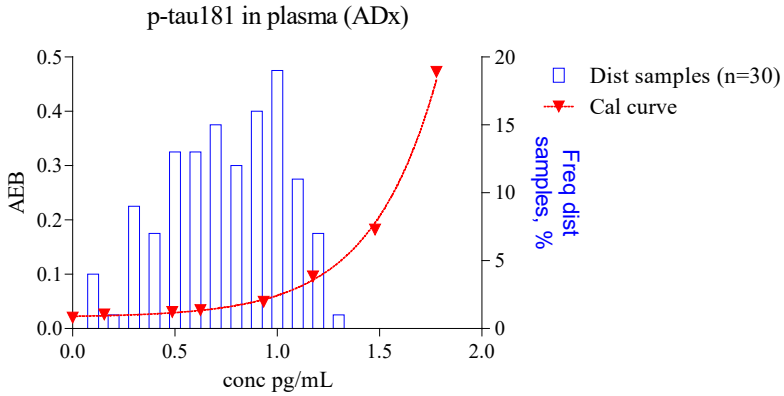
Calibrators were supplied in kit as ready-to-use calibrator series: 7 calibration points + 1 blank in Qiagen collection microtubes (Qiagen – Cat N° 19560). 1 strip contains 250 µL of 60.09 – 30.05 – 14.95 – 8.58 – 4.22 – 3.07 – 1.43 and 0 pg/mL peptide calibrator in calibrator diluent. No additional internal QCs were included in the analytical runs.

Calibrator Concentrations and Replicates

			Run 1		Run 2		Run 3	All runs	
Calibrator	CONC (pg/ml)	Log conc	Rep AEB	Rep AEB	Rep AEB	Rep AEB	Rep AEB	Rep AEB	Avg
A	60.09	1.7788	0.4024	0.3949	0.5169	0.5043	0.5174	0.5006	0.4727
B	30.05	1.4778	0.1544	0.1580	0.2119	0.1860	0.1917	0.1914	0.1822
C	14.95	1.1746	0.0374	0.0399	0.1021	0.1042	0.0929	0.0852	0.0961
D	8.58	0.9335	0.0437	0.0478	0.0552	0.0466	0.0520	0.0498	0.0492
E	4.22	0.6253	0.0347	0.0339	0.0329	0.0308	0.0366	0.0355	0.0341
F	3.07	0.4871	0.0317	0.0268	0.0340	0.0314	0.0339	0.0247	0.0304
G	1.43	0.1553	0.0239	0.0240	0.0263	0.0296	0.0150	0.0159	0.0260
H	0		0.0182	0.0203	0.0239	0.0190	0.0251	0.0289	0.0203

ADNI plasma sample concentrations (in blue) plotted on the pTau-181 calibration curve (in red)





Plasma Endogenous QC Results

Two plasma endogenous control samples were run twice in a separate run from the ADNI study samples at the same dilution factor (DF) as plasma (DF5)

Sample Barcode (DF=5)	AEB Rep 1	AEB Rep 2	Conc Rep 1	Conc Rep2	SD conc	%CV	Conc	Conc* DF
Healthy control	0.031	0.04	2.194	3.47	0.902	31.86	2.8	14.2
Positive eQC*	0.159	0.163	15.116	15.438	0.228	1.49	15.3	76.4
Healthy control	0.023	0.023	1.044	1.032	0.008	0.82	1.0	5.2
Positive eQC*	0.151	0.17	14.384	16.043	1.173	7.71	15.2	76.1

*Pooled plasma eQC samples selected from individuals whose CSF tested positive for pTau-181 and Aβ42 (University of Pennsylvania/ADNI biorepository).

Plasma Endogenous QC Data Summary Table

Control	n	mean	median	sd	CV
Negative eQC	4	9.67500	8.095	5.796979	59.92%
Positive eQC	4	76.22625	76.385	3.454483	4.53%

Validation Report

pTau-181			
	Experimental Design	**Results**	**Acceptance Criteria**
Dilution Linearity and MRD Analysis	1/5; 1/25;1/125 The concentration of ptau spiked for dilutional linearity is 150 pg/ml as described in Bayoumy et al, 2021, PMID 34863295	54%, 139%, 127%	80-120%
Standard Linearity and Assay Range		1.91-77.29	2-100 pg/ml
Accuracy	ND	ND	ND
Repeatability	high, med, low QCs*	7%, 12.3%, 24%	<20% CV
Intermediate Precision	high, med, low QCs*	8.2%, 13.4%, 24%	<20% CV
Limit of Quantification	Mean signal (16 blanks)+10*SD	LLOQ 2.36 pg/ml	2-100 pg/ml
Selectivity (matrix effects)		ND	ND
Controls	QCs, KCs		
Analyte Stability	As described in Verberk et al, 2022, PMID 34845818, the RT stability of ptau-181 is not affected within the tested range. Efforts to determine stability data with this assay configuration are ongoing.		
Parallelism (as defined in Andreasson et al., 2019) with Clinical Samples	4 plasma samples serially diluted (1/5; 1/10; 1/20; 1/40) Refer to Fig 2 of Bayoumy et al, 2021 for individual sample parallelism	110.30%	80-120%
Cut point for amyloid PET positivity and/or tau PET positivity, if evaluated, and the relevant references or supporting data	Centiloid >23.5	12.6 pg/ml	De Meyer et al, PMID 35502634

*Plasma samples ran in 4 independent runs
The concentration of the high, med, low QCs are 28.8, 13.3 and 9.6 using the ADx plasma ptau181 assay and described in Bayoumy et al, 2021.

Fujirebio Diagnostics

Sample analysis performed at the University of Pennsylvania with oversight by Leslie Shaw, Ph.D.
Assay: Lumipulse G pTau 181 Plasma

Kit and reagents used in study: Fujirebio IRC/Cartridge Lot No. T9B2071, Fujirebio Calibrator Lot No.U1B3011.

Platform	LUMIPULSE G1200 (FDA/CE)
Capture antibody	AT270
Detector antibody	HT7 and BT2
Calibrator	0, 3, 10, 30, 60 pg/ml (CAL 1,2,3,4,5) Calibration curve measurement range: 0.05 - 60 pg/mL.
Calibrator matrix	Contains Tris buffer with protein (bovine) and chemical stabilizers. Preservative: 0.1% ProClin 300, 0.05% ProClin 950
Sample diluent	NA
Dilution factor	NA
Use of protease inhibitor	No
LLoD or LLoQ	0.261 pg/mL (CV<10%) Eight (8) filtrated low concentrated plasma samples + 3 blanks (2x CAL 0 + 1 pTau181 depleted plasma). Evaluated in 4 different runs at different days. Five (5) replicates for each sample.

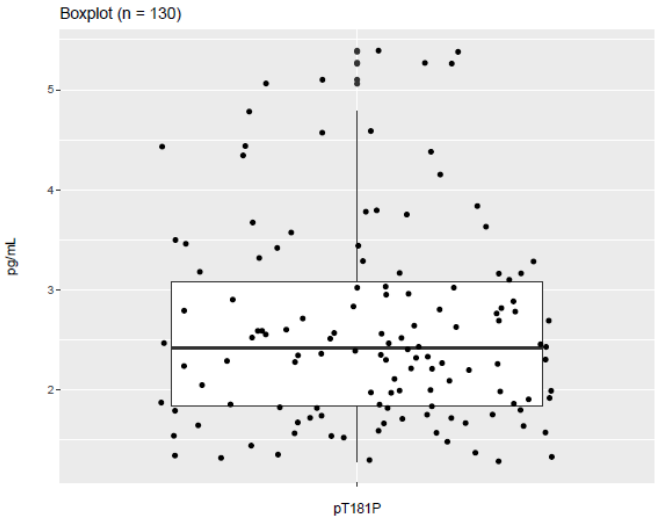
Sample Storage and Preparation

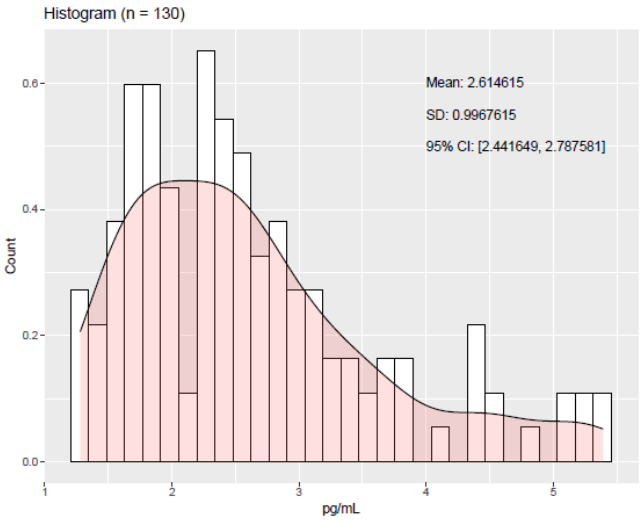
Project samples were stored in -80°C freezer upon receipt. After thawing in room temperature for a minimum of 30 minutes 0.5 mL plasma samples were vortexed for 10 seconds and centrifuged according to instructions in Fujirebio IRC insert.

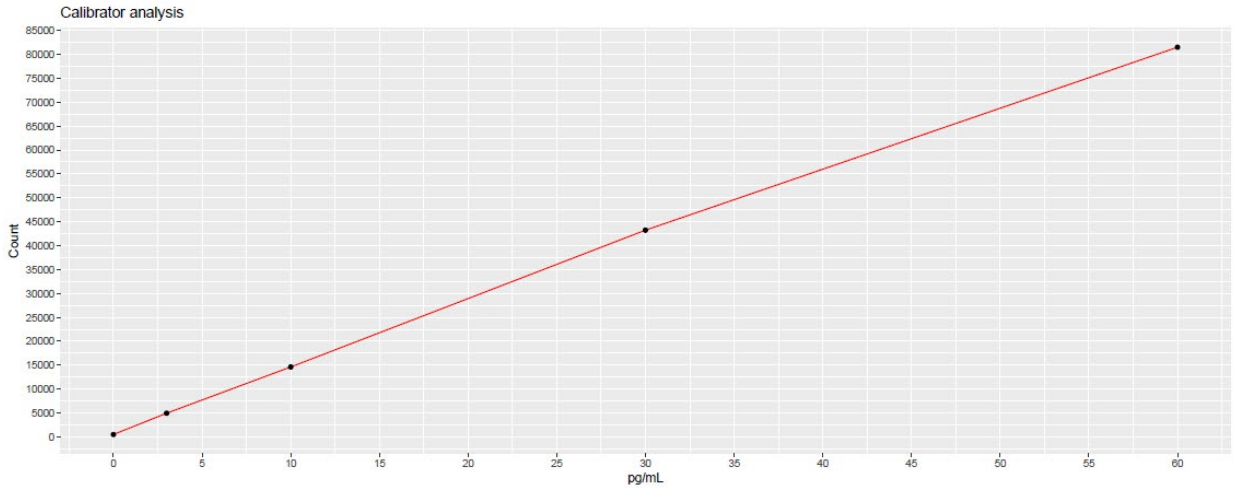
Calibration and Internal QC Data

Internal QC	**Analyte**	**Conc. in pg/ml** **Date of Analysis 4/12/2022**	**Conc. in pg/ml** **Date of Analysis 4/13/2022**	**Average in pg/ml**
L1 (range pg/mL: 4.02 - 6.04)	pT181P	4.73	4.94	4.84
L2 (range pg/mL: 35.33 - 52.99)	pT181P	40.95	42.48	41.72

L1 and L2 are commercially available QC samples.







Plasma Endogenous QC Results

Control	Analyte	Conc. in pg/ml Date of Analysis 4/12/2022	Conc. in pg/ml Date of Analysis 4/13/2022	Average in pg/ml
QCA	pT181P	3.88	4.06	4.09
QCA	pT181P	4.65	3.78	
QCB	pT181P	2.3	2.14	2.63
QCB	pT181P	3.87	2.21	

QCA is an ADNI abnormal pool prepared from individuals from individuals whose CSF tested positive for pTau-181 and Aβ42 (positive eQC, University of Pennsylvania/ADNI biorepository)

QCB is an ADNI normal plasma pool from individuals with normal CSF ptau181 concentration values (negative eQC).

Plasma Endogenous QC Data Summary Table

Control	n	mean	median	sd	CV
Negative eQC	4	2.6300	2.255	0.8292567	31.53%
Positive eQC	4	4.0925	3.970	0.3893049	9.51%

Validation Report

pTau-181			
	Experimental Design	**Results**	**Acceptance Criteria**
Dilution Linearity and MRD Analysis	Two plasma samples spiked with CSF samples with a high pTau181 concentration used as starting material. Diluted using Specimen Diluent 1.	Up to 20x using SD1 %Diff between -3% and 6%	+/- 10%
Standard Linearity and Assay Range	Proportional mixing of high and low concentrated pTau181 plasma samples (CLSI Protocol EP6-A)	Between 0.5 and 56.8 pg/mL	
Accuracy	NA	No CRM Assay is standardized to internal reference materials	
Repeatability	Four (4) plasma K2EDTA samples and 4 buffer-based control samples were tested over 8 runs. Sample testing in triplicate per run using one LUMIPULSE G1200 System. Analysis of variability was done cfr. CLSI Guideline EP5-A3.	%CV range: 1.3% - 3.0%	+/- 10%

Intermediate Precision	Four (4) plasma K2EDTA samples and 4 buffer-based control samples were tested over 8 runs. Sample testing in triplicate per run using one LUMIPULSE G1200 System. Analysis of variability was done cfr. CLSI Guideline EP5-A3.	Total variability (8 runs; plasma & buffer samples): %CV < 4.0% ; Inter-run variability (using clinical samples, n = 40): Y = 0.1363 + 1.010x r = 0.990 ; Lot-to-lot variation: %CV = 0.4% - 7.5%	
Limit of Quantification	Eight (8) filtrated low concentrated plasma samples + 3 blanks (2x CAL 0 + 1 pTau181 depleted plasma). Evaluated in 4 different runs at different days. Five (5) replicates for each sample.	0.261 pg/mL (CV<10%)	
Selectivity (matrix effects)	Used mAbs with previously characterized epitopes are used : AT270 (capturing), and HT7 + BT2 (detection)	No cross reactivity with non-phophorytated Tau at T181 was detected. No reaction with other phosphorylation sites (217 and 231)	
Controls	Synthetic peptide diluted in buffer (stored frozen)	2 levels of controls tested in singlicate; 5 calibration points tested in duplicate	
Analyte Stability	Pooled samples (n=3) were assessed at RT 4h and 8h, 2-8ºC 1d, 3d and 8d, and -20ºC for F/T	Up to 3 days at 2-8ºC; samples failed at 4h RT, Stable up to 3 F/T	+/- 10%
Parallelism (as defined in Andreasson et al., 2019) with Clinical Samples	Searching for sufficiently high concentrated clinical samples in large volume, see dilutional linearity at current	NA	
Cut point for amyloid PET positivity and/or tau PET positivity, if evaluated, and the relevant references or supporting data	Still being investigated		

Quanterix

Sample analysis performed by Quanterix Accelerator Laboratory Services.
Assay: Simoa® pTau 181V2 Advantage

Platform	HD-X
Capture antibody	Proprietary
Detector antibody	Proprietary
Calibrator	Antigen in buffer with protein stabilizers
Calibrator matrix	Antigen in buffer with protein stabilizers
Sample diluent	Buffer with protein stabilizers, a heterophilic blocker
Dilution factor	4X
LLoD or LLoQ	LOD - 0.028 pg/mL LLOQ - 0.085 pg/mL

Sample Storage and Preparation

Project samples were stored in -80°C freezer upon arrival. In preparation for measurement, the samples were completely thawed and mixed thoroughly before preparing assay plate then returned to -80°C.

Assay Procedure

The digital immunoassay was performed according to Quanterix's Simoa® pTau-181 Advantage V2 Kit instructions for HD-1/HD-X. In the incubation procedure, target antibody coated paramagnetic beads were combined with sample and biotinylated detector antibody in the same incubation.

Briefly, the appropriate volume of calibrators, controls, and samples were pipetted into the plate wells. The bead reagent (capture antibody coated beads in buffer with protein stabilizers) was vortexed for a minimum of 30 seconds. Prepared reagents (Bead Reagent; Detector Reagent; Streptavidin-ß-galactosidase, SBG, Reagent; Plasma Sample Diluent) were loaded into the reagent rack and Resorufin ß-D-galactopyranoside (RGP) reagent into the RGP. The assay was configured using Simoa software, entering Neat or the standard 4x Dilution protocol for samples and controls, and the plate was loaded on a sample plate rack and into the sample bay.

The analyte concentration is determined by detecting the number of wells containing both a bead and fluorescent signal relative to the total number of wells containing beads (AEB – average enzymes per bead). If the target has been captured and labeled on the bead, ß-galactosidase hydrolyzes the RGP substrate in the microwell into a fluorescent product that provides the signal for measurement.

At low target concentration, the percentage of bead-containing wells in the array that have a positive signal is proportional to the amount of target present in the sample. At higher target concentration, when most of the bead-containing wells have one or more labeled target molecules, the total fluorescence signal is proportional to the amount of target present in the sample. The concentration of target in unknown samples is interpolated from the calibration curve.

Calibrator and Internal QCs

Six calibrator points were prepared from concentrate provided in the assay kit. Two controls were included in each analytical run. The calibrators are run using the Neat protocol, whereas the controls are run using the same protocol as samples.

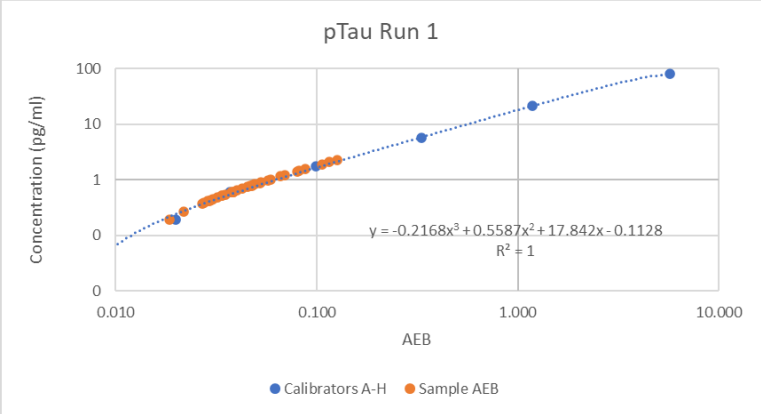
NA = not applicable

pTau-181 Run 1

Calibrator	Conc. (pg/mL)	Weight	AEB Rep1	AEB Rep2	AEB Rep3	Ave. AEB	CV	S/B	Backfit Conc. (pg/mL)	% Bias
Calibrator A	0	1	0.009	0.009	0.012	0.010	19%			
Calibrator B	0.196	1	0.020	0.016	0.024	0.020	20%	1.9	0.221	-12.69
Calibrator C	0.606	1	0.035	0.039	0.037	0.037	4%	3.6	0.585	3.39
Calibrator D	1.76	1	0.100	0.104	0.094	0.099	5%	9.7	1.79	-1.94
Calibrator E	5.80	1	0.352	0.319	0.322	0.331	6%	32.3	5.9	-1.68
Calibrator F	21.5	1	1.189	1.223	1.151	1.187	3%	115.9	19.8	8.11
Calibrator G	79.7	1	5.267	5.989	5.948	5.734	7%	559.6	86	-7.9

Control	AEB Rep1	AEB Rep2	Ave. AEB	CV	Conc. Rep1 (pg/mL)	Conc. Rep2 (pg/mL)	Ave. Conc. (pg/mL)	CV	Dilution Factor	Dilution Corrected Conc. (pg/mL)	Control Range Lower Limit (pg/mL)	Control Range Upper Limit (pg/mL)	PASS/FAIL
Control 1	0.058	0.055	0.057	3%	0.997	0.954	0.975	3%	4	3.90	2.88	4.77	PASS
Control 2	0.826	0.857	0.841	3%	14.0	14.5	14.3	2%	4	57.2	44.2	66.3	PASS

ADNI plasma sample concentrations (in red) plotted on the calibration curve (in blue)

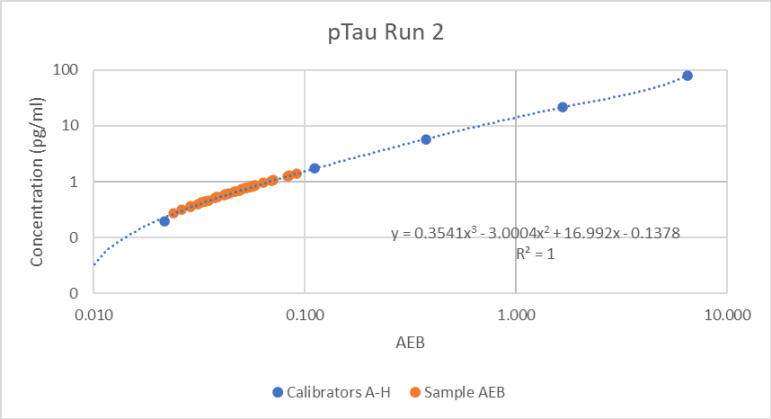


pTau-181 Run 2

Calibrator	Conc. (pg/mL)	Weight	AEB Rep1	AEB Rep2	AEB Rep3	Ave. AEB	CV	S/B	Backfit Conc. (pg/mL)	% Bias
Calibrator A	0	1	0.010	0.010	0.009	0.009	4%			
Calibrator B	0.196	1	0.021	0.022	0.022	0.022	3%	2.3	0.233	-18.86
Calibrator C	0.606	1	0.045	0.038	0.044	0.042	9%	4.4	0.591	2.54
Calibrator D	1.76	1	0.111	0.111	0.114	0.112	2%	11.8	1.71	2.75
Calibrator E	5.80	1	0.383	0.370	0.370	0.374	2%	39.4	5.57	3.96
Calibrator F	21.5	1	1.671	1.604	1.728	1.668	4%	175.6	22.7	-5.53
Calibrator G	79.7	1	6.431	6.681	6.242	6.451	3%	679.3	79.9	-0.21

Control	AEB Rep1	AEB Rep2	Ave. AEB	CV	Conc. Rep1 (pg/mL)	Conc. Rep2 (pg/mL)	Ave. Conc. (pg/mL)	CV	Dilution Factor	Dilution Corrected Conc. (pg/mL)	Control Range Lower Limit (pg/mL)	Control Range Upper Limit (pg/mL)	PASS/FAIL
Control 1	0.068	0.063	0.065	6%	1.02	0.932	0.977	6%	4	3.91	2.88	4.77	PASS
Control 2	0.877	0.934	0.906	4%	12.5	13.2	12.8	4%	4	51.3	44.2	66.3	PASS

ADNI plasma sample concentrations (in red) plotted on the calibration curve (in blue)

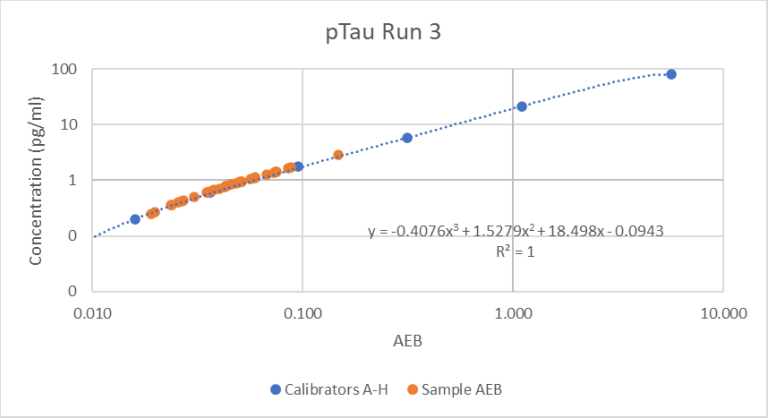


pTau-181 Run 3

Calibrator	Conc. (pg/mL)	Weight	AEB Rep1	AEB Rep2	AEB Rep3	Ave. AEB	CV	S/B	Backfit Conc. (pg/mL)	% Bias
Calibrator A	0	1	0.010	0.008	0.009	0.009	10%			
Calibrator B	0.196	1	0.015	0.016	0.017	0.016	4%	1.8	0.183	6.48
Calibrator C	0.606	1	0.035	0.038	0.036	0.036	4%	4.1	0.637	-5.16
Calibrator D	1.76	1	0.093	0.099	0.093	0.095	4%	10.6	1.82	-3.6
Calibrator E	5.80	1	0.316	0.305	0.318	0.313	2%	35.2	5.88	-1.45
Calibrator F	21.5	1	1.100	1.063	1.128	1.097	3%	123.2	19.2	10.85
Calibrator G	79.7	1	5.542	5.845	5.537	5.642	3%	633.7	88	-10.41

Control	AEB Rep1	AEB Rep2	Ave. AEB	CV	Conc. Rep1 (pg/mL)	Conc. Rep2 (pg/mL)	Ave. Conc. (pg/mL)	CV	Dilution Factor	Dilution Corrected Conc. (pg/mL)	Control Range Lower Limit (pg/mL)	Control Range Upper Limit (pg/mL)	PASS/FAIL
Control 1	0.060	0.058	0.059	3%	1.13	1.08	1.11	3%	4	4.43	2.88	4.77	PASS
Control 2	0.818	0.811	0.814	1%	14.6	14.4	14.5	1%	4	58.0	44.2	66.3	PASS

ADNI plasma sample concentrations (in red) plotted on the calibration curve (in blue)

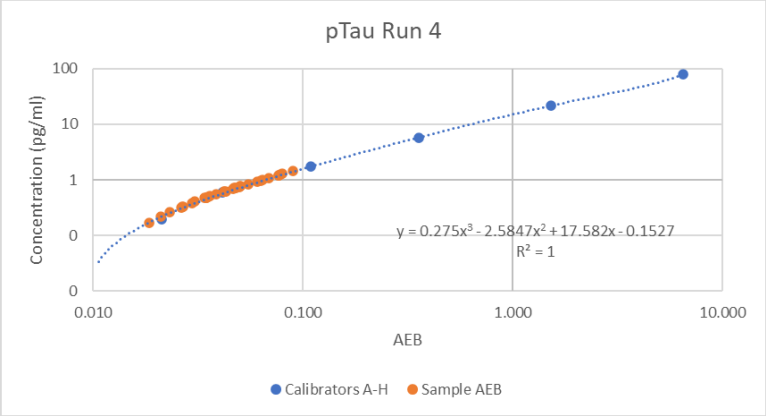


pTau-181 Run 4

Calibrator	Conc. (pg/mL)	Weight	AEB Rep1	AEB Rep2	AEB Rep3	Ave. AEB	CV	S/B	Backfit Conc. (pg/mL)	% Bias
Calibrator A	0	1	NaN	0.010	0.011	0.011	3%			
Calibrator B	0.196	1	0.022	0.019	0.022	0.021	8%	2.0	0.222	-13.51
Calibrator C	0.606	1	0.041	0.044	0.039	0.041	5%	3.9	0.6	0.98
Calibrator D	1.76	1	0.113	0.107	0.107	0.109	3%	10.3	1.75	0.34
Calibrator E	5.80	1	0.351	0.351	0.370	0.357	3%	33.7	5.59	3.56
Calibrator F	21.5	1	1.509	1.506	1.528	1.514	1%	142.9	21.5	0.07
Calibrator G	79.7	1	6.441	6.560	6.377	6.459	1%	609.5	82	-2.83

Control	AEB Rep1	AEB Rep2	Ave. AEB	CV	Conc. Rep1 (pg/mL)	Conc. Rep2 (pg/mL)	Ave. Conc. (pg/mL)	CV	Dilution Factor	Dilution Corrected Conc. (pg/mL)	Control Range Lower Limit (pg/mL)	Control Range Upper Limit (pg/mL)	PASS/FAIL
Control 1	0.070	0.067	0.069	3%	1.11	1.06	1.08	3%	4	4.32	2.88	4.77	PASS
Control 2	0.860	0.949	0.905	7%	12.7	13.9	13.3	6%	4	53.3	44.2	66.3	PASS

ADNI plasma sample concentrations (in red) plotted on the calibration curve (in blue)

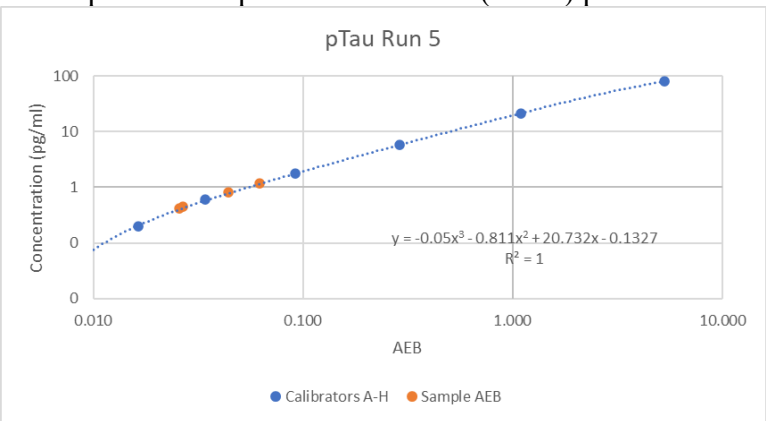


pTau-181 Run 5

Calibrator	Conc. (pg/mL)	Weight	AEB Rep1	AEB Rep2	AEB Rep3	Ave. AEB	CV	S/B	Backfit Conc. (pg/mL)	% Bias
Calibrator A	0	1	0.007	0.008	0.007	0.007	5%			
Calibrator B	0.196	1	0.017	0.017	0.015	0.016	5%	2.3	0.218	-11.18
Calibrator C	0.606	1	0.037	0.033	0.033	0.034	7%	4.7	0.607	-0.13
Calibrator D	1.76	1	0.095	0.091	0.089	0.092	3%	12.7	1.79	-1.94
Calibrator E	5.80	1	0.284	0.302	0.283	0.290	4%	40.2	5.58	3.71
Calibrator F	21.5	1	1.077	1.121	1.082	1.093	2%	151.6	19.8	7.7
Calibrator G	79.7	1	5.503	5.068	5.394	5.322	4%	738.1	88.4	-10.94

Control	AEB Rep1	AEB Rep2	Ave. AEB	CV	Conc. Rep1 (pg/mL)	Conc. Rep2 (pg/mL)	Ave. Conc. (pg/mL)	CV	Dilution Factor	Dilution Corrected Conc. (pg/mL)	Control Range Lower Limit (pg/mL)	Control Range Upper Limit (pg/mL)	PASS/FAIL
Control 1	0.059	0.064	0.062	6%	1.13	1.24	1.19	7%	4	4.74	2.88	4.77	PASS
Control 2	0.768	0.759	0.764	1%	14.2	14.0	14.1	1%	4	56.5	44.2	66.3	PASS

ADNI plasma sample concentrations (in red) plotted on the calibration curve (in blue)



Plasma Endogenous QC Results

FA80B8MN-03	Pooled plasma selected from individuals whose CSF tested positive for pTau-181 and Aβ42 (University of Pennsylvania/ADNI biorepository).
HMN500278	Healthy control plasma supplied by BioIVT

pTau-181 Run 1

Sample ID	AEB Rep1	AEB Rep2	Ave. AEB	CV	Conc. Rep1 (pg/mL)	Conc. Rep2 (pg/mL)	Ave. Conc. (pg/mL)	CV	Dilution Factor	Dilution Corrected Conc. (pg/mL)	Notes
FA80B8MN-03	0.079	0.082	0.080	2%	1.41	1.46	1.43	3%	4	5.74	
HMN500278	0.030	0.030	0.030	1%	0.429	0.442	0.436	2%	4	1.74	

pTau-181 Run 2

Sample ID	AEB Rep1	AEB Rep2	Ave. AEB	CV	Conc. Rep1 (pg/mL)	Conc. Rep2 (pg/mL)	Ave. Conc. (pg/mL)	CV	Dilution Factor	Dilution Corrected Conc. (pg/mL)	Notes
FA80B8MN-03	0.082	0.087	0.085	5%	1.24	1.33	1.29	5%	4	5.14	
HMN500278	0.029	0.029	0.029	1%	0.362	0.357	0.360	1%	4	1.44	

pTau-181 Run 3

Sample ID	AEB Rep1	AEB Rep2	Ave. AEB	CV	Conc. Rep1 (pg/mL)	Conc. Rep2 (pg/mL)	Ave. Conc. (pg/mL)	CV	Dilution Factor	Dilution Corrected Conc. (pg/mL)	Notes
FA80B8MN-03	0.074	0.072	0.073	2%	1.41	1.37	1.39	2%	4	5.55	
HMN500278	0.027	0.028	0.027	2%	0.425	0.444	0.435	3%	4	1.74	

pTau-181 Run 4

Sample ID	AEB Rep1	AEB Rep2	Ave. AEB	CV	Conc. Rep1 (pg/mL)	Conc. Rep2 (pg/mL)	Ave. Conc. (pg/mL)	CV	Dilution Factor	Dilution Corrected Conc. (pg/mL)	Notes
FA80B8MN-03	0.072	0.081	0.076	8%	1.14	1.29	1.22	8%	4	4.86	
HMN500278	0.027	0.032	0.030	10%	0.344	0.424	0.384	15%	4	1.54	

pTau-181 Run 5

Sample ID	AEB Rep1	AEB Rep2	Ave. AEB	CV	Conc. Rep1 (pg/mL)	Conc. Rep2 (pg/mL)	Ave. Conc. (pg/mL)	CV	Dilution Factor	Dilution Corrected Conc. (pg/mL)	Notes
FA80B8MN-03	0.058	0.065	0.062	8%	1.12	1.26	1.19	9%	4	4.76	
HMN500278	0.026	0.025	0.026	3%	0.438	0.410	0.424	5%	4	1.70	

Plasma Endogenous QC Data Summary Table

Control	n	mean	median	sd	Inter-run CV	Intra-run CV	Total CV
Negative eQC	10	1.630637	1.698176	0.1530517	0.0686349	0.0679881	0.0966081
Positive eQC	10	5.211327	5.236606	0.4593236	0.0714849	0.0568002	0.0913036

Simoa® pTau-181 Advantage V2 Kit Validation Report for HD-1/HD-X

pTau-181 Advantage V2 Kit Summary

Test	Claim
LOD	0.028 ± 0.009 pg/mL Range: 0.019 – 0.052 pg/mL
Analytical LLOQ (Fit for Purpose)	0.085 pg/mL
Functional LLOQ	Serum and EDTA Plasma (4x): 0.338 pg/mL CSF (10x): 0.85 pg/mL
Calibrator B/A	2.66 ± 0.324 Range: 2.06 – 3.16
Calibrator A	0.013 ± 0.003 Range: 0.009 – 0.016
Calibrator G	10.4 ± 1.91 Range: 8.11 – 13.1
Precision	Within Run CV: 6.5% Between Run CV: 7.7% Between Instrument CV: 3.7% Between Lot CV: 12.7%
Spike and Recovery	Serum: 84.3%, Range: 69.2 – 95.2% EDTA Plasma: 98.0%, Range: 87.8 – 112%
Dilution Linearity	Endogenous Serum (16x): 114%, Range: 103 – 130% Spiked Serum (128x): 110%, Range: 72.4 – 144% Endogenous EDTA Plasma (16x): 108%, Range: 85.7 – 140% Spiked EDTA Plasma (128x): 127%, Range: 107 – 187% Endogenous CSF (160x): 104%, Range: 72.7 – 117%
Curve Storage	Bias: 3.1%, Range: 2.0 – 5.7%
Drift	Three-plate Variance Plate 1: 5.9%; Plate 1+2: 10.7%; Plate 1+2+3: 11.8% Three-plate Precision: 9.5%, Range: 5.9 – 13.4%
Sample Freeze-Thaw Stability	EDTA Plasma and CSF: 103%, Range: 98.1 - 107%
Calibrator and Sample Stability	Calibrator: 92.0%, Range: 91.2 – 92.4% EDTA Plasma: 101%, Range: 97.1 – 107%
Normal Samples	Serum: 0.479 pg/mL, Range: 0.115 – 1.97 pg/mL, Detectability: 100%, Quantifiability: 60% EDTA Plasma: 1.02 pg/mL, Range: 0.523 – 2.88 pg/mL, Detectability: 100%, Quantifiability: 100% CSF: 25.2 pg/mL, Range: 6.40 – 158 pg/mL, Detectability: 100%, Quantifiability: 100% Heparin Plasma: 0.473 pg/mL, Range: ND* – 0.638 pg/mL, Detectability: 40%, Quantifiability: 40% Mouse EDTA Plasma: 12.0 pg/mL, Range: ND* – 17.6 pg/mL, Detectability: 60%, Quantifiability: 60%
Kit Stability	6 months

*ND: Not detectable

Limit of Detection:

The table below shows the calculated LOD (Lower Limit of Detection). LOD is calculated as 2.5 Standard Deviations above the background (mean of calibrator blanks).

Test Conditions		Claim
Replicates/Run (each test sample)	3	Grand Mean LOD: 0.028 pg/mL Range: 0.019 – 0.052 pg/mL
# Instruments	2	
Runs/Instrument	3	
# Lots Tested	2	
Total Runs	12	

Limit of Quantification:

The table below shows the calculated LLOQ (Lower Limit of Quantification). Analytical LLOQ is the lowest concentration of calibrator with ≤ 20% pooled CV and 80 – 120% mean recovery over all runs. Functional LLOQ is the Analytical LLOQ multiplied by the Minimum Required Dilution.

Test Conditions		Claim
Samples	Calibrator B diluted to 0.085 and 0.042 pg/mL Calibrator C diluted to 0.134 and 0.067 pg/mL	Analytical LLOQ: 0.085 pg/mL Functional LLOQ (Serum and EDTA Plasma) (4x): 0.338 pg/mL Functional LLOQ (CSF) (10x): 0.85 pg/mL
Replicates/Run (each test sample)	3	
# Instruments	2	
Runs/Instrument	5 – 7	
# Lots Tested	2	
Total Runs	12	

Precision Profile:

3 Panels and 2 controls were diluted 1:4 in sample diluent with on board dilution. 12 runs were run on 2 instruments comparing 2 reagent lots. The mean concentration corrected for MRD (pg/mL) and % CV based on concentration are reported in the table below.

Test Conditions		Claim
Test Samples	Control 1: Low Concentration Calibrator spiked into calibrator diluent Control 2: High Concentration Calibrator spiked into calibrator diluent Panel 1: Normal Human Serum Sample spiked with calibrator Panel 2: Normal Human EDTA Plasma Sample Panel 3: Normal Human CSF Sample	Mean Total CV: 7.6%
Specifications	Replicates/Sample: 3 Instruments: 2 Runs/Instrument: 3 Lots Tested: 2 Total Runs: 12; Total Measurements/Sample: 36	
Within Run CV		6.5%
Between Run CV		7.7%
Between Instrument CV		3.7%
Between Lot CV		12.7%

Spike Recovery (Serum/Plasma):

8 Normal samples (4 serum and 4 plasma) and a diluent control were spiked at two concentrations (high and low) with pTau-181 V2 antigen and then diluted 1:4 with sample diluent. Percent recovery is defined as the measured concentration of pTau-181 V2 in the spiked sample less the measured concentration in unspiked sample relative to the concentration of pTau-181 V2 in spiked calibrator diluent.

Test Conditions		Claim
Test Samples	Normal Human Samples – 4 Serum + 4 EDTA Plasma	Buffer Corrected Grand Mean Serum: 84.3% Range: 69.2 – 95.2% Grand Mean EDTA Plasma: 98.0% Range: 87.8 – 112%
Spike Concentrations	Control: Calibrator Diluent Lot 1 – Low: 14 pg/mL; High: 97 pg/mL Lot 2 – Low: 11 pg/mL; High: 70 pg/mL	
Replicates/Run (each test sample)	3	

Dilution Linearity (EDTA Plasma):

4 Normal human EDTA plasma samples spiked with recombinant analyte were diluted 2x serially with sample diluent from MRD (4x) to 128x. 4 Unspiked normal human EDTA plasma samples were diluted 2x serially with sample diluent from MRD (4x) to 16x. The average percent recovery across the entire dilution series is displayed for each sample type.

Test Conditions		Claim
Test Samples	4 Endogenous Normal Human EDTA Plasma 4 Spiked Normal Human EDTA Plasma	Endogenous EDTA Plasma Dilution Linearity (16x total dilution): 108% Range: 85.7 – 140%
Dilutions	2x serial from MRD (4x)	Spiked EDTA Plasma Dilution Linearity (128x total dilution): 127% Range: 107 – 187%
Replicates/Run (each test sample)	3	

Drift:

3 Panels and 2 controls were diluted 1:4 in sample diluent. Controls, panels 1 and 2 were run across three plates with a total of 54 replicates per sample. The Plate 1, Plate 1+2 and Plate 1+2+3 variance values are determined by the absolute bias of end job/beginning job for each sample at each period. The three-plate precision is determined by the within run CV for each sample (all controls and panels).

Test Conditions		Claim
Test Samples	Control 1: Low Concentration Calibrator spiked into calibrator diluent Control 2: High Concentration Calibrator spiked into calibrator diluent Panel 1: Normal Human Serum Sample spiked with kit calibrator Panel 2: Normal Human EDTA Plasma Sample Panel 3: Normal Human CSF Sample	Three-plate Variance: Plate 1:5.9% Plate 1+2: 10.7% Plate 1+2+3: 11.8%
Specifications	Replicates/Sample: 3 Replicates of Sample/Plate: 6 Plates: 3 Total Replicates: 54	Three-plate Precision: 9.5% Range: 5.9 – 13.4%

Sample Freeze-Thaw Stability:

2 Panels and 2 normal human EDTA plasma samples were diluted 1:4 in sample diluent. 3 Freeze-Thaw cycles were evaluated on the same instrument using one lot of reagents. Normalized sample concentrations are calculated as of the set of samples prepared right before run for each freeze-thaw cycle.

Test Conditions		Claim
Test Samples	Panel 2: Normal Human EDTA Plasma Sample Panel 3: Normal Human CSF Sample Sample 1 and 2: Normal Human EDTA Plasma Sample	EDTA Plasma and CSF: 103% Range: 98.1 - 107%
Freeze-Thaw Cycles	1, 2 and 3 cycles. Each thaw period was less than 1-hr and each freeze period was more than 12-hr.	

Calibrator and Sample Stability:

4 Normal human EDTA plasma samples were diluted 1:4 in sample diluent. 3-hr and 7-hr stability at room temperature and 24-hr stability at 4 ˚C of calibrators and samples were evaluated on the same instrument using one lot of reagents. Normalized calibrator signals and sample concentrations are calculated as of the set of calibrators and samples prepared right before run for each time point.

	Test Conditions	Claim
Test Samples	Calibrators: Cal A to Cal G Samples: 4 Normal Human EDTA Plasma Samples	Calibrators: 92.0% Range: 91.2 – 92.4%
Time points	3-hour, 7-hour, and 24-hour prior to the run	Samples: 101% Range: 97.1 – 107%

Roche Diagnostics

Sample analysis performed at Microcoat Biotechnologie GmbH
Assay: Roche Elecsys Plasma Phospho-Tau(181P)

Study samples were analyzed by the electrochemiluminescence immunoassay (ECLIA) on the fully automated cobas e 601 with the plasma Phospho-Tau(181P) (assay variant C2, internal arbitrary nomenclature) Roche Elecsys prototype immunoassay.

The Roche NeuroToolKit is a panel of exploratory prototype assays designed to robustly evaluate biomarkers associated with key pathologic events characteristic of AD and other neurological disorders. It is used for research purposes only and it is not approved for clinical use.

Sample Storage and Preparation

The 130 patient aliquots were tested with the Roche Elecsys plasma prototype immunoassay at Microcoat Biotechnologie GmbH, according to the preliminary kit manufacturer's instructions and as described by Roche's Exploratory Study Protocol, "Measurements of Samples with the Elecsys Neuro Toolkit Immunoassays; version 3". Five aliquots of two plasma controls (low and high) were also measured with the samples.

The samples remained frozen at -70°C or below until the time of measurement. Upon which the samples were thawed for 30 minutes at room temperature in an upright position. After thawing, roller mixer was used to mix the samples for 20 minutes, avoiding foam formation. Elecsys measurements were performed in one run. Each sample was run in singlicate. The samples were not diluted prior to measurements and dilution is not recommended.

Prior to sample measurements the Elecsys rackpacks to be used for the measurements were calibrated using 1 thawed aliquot of each of the 2 calibration levels. The cobas e601 instrument was calibrated and the daily quality controls were run.

Table 1: Table showing antibodies used for the biomarker in the respective Elecsys Immunoassay

Assay	Capture Antibody	Detector Antibody
Plasma Phospho-Tau (181P), assay variant C2 (internal arbitrary nomenclature) (pTauC2)	monoclonal antibody linked to biotin*	monoclonal antibody linked ruthenium complex*

Table 2: Table showing calibrator information for the Elecsys Immunoassay

Calibrator	Spiked material	Matrix
Plasma pTau(181P) Cal1	Synthetic pTau peptide	Equine serum based matrix
Plasma pTau(181P) Cal2	Synthetic pTau peptide	Equine serum based matrix

Table 3: Table showing calibrator ranges for the Plasma pTau(181P)Elecsys Immunoassays

Calibrator	Plasma pTau(181P) Analyte concentration [pg/ml]
Cal 1	0.64
Cal 2	26.0

Table 4: Table showing quality control information for the Elecsys Immunoassays

Precicontrol	Spiked material	Matrix
Plasma pTau(181P) PC1	Synthetic pTau peptide	Equine serum based matrix
Plasma pTau(181P) PC2	Synthetic pTau peptide	Equine serum based matrix

Table 5: Table showing Plasma pTau(181P) lot number used

				QC Acceptance Criteria (Kit Lot Specific)	
Unabbreviated Name	Unit of Measure	Measuring Range	Lot #	Lower Control Range	Upper Control Range
Plasma Phospho-Tau(181P) (Plasma pTau(181P)	pg/mL	0.100 – 33.5	**DR01**	0.80 – 1.22	2.65 – 4.05

Method Summary

The 130 ADNI sample aliquots were tested with the Roche Elecsys plasma immunoassays at Covance Greenfield Laboratories (Translation Biomarker Solutions), according to the preliminary kit manufacturer's instructions and as described by Roche's Exploratory Study Protocol, "Measurements of Samples with the Elecsys Neuro Toolkit Immunoassays; version 3". Five controls (3 CSF controls (low, medium, and high) as well as 2 plasma controls (low and high) were also included in each run of the samples.

The samples remained frozen at -70°C or below until the time of measurement. Upon

which the samples were thawed for 30 minutes at room temperature in an upright position. After thawing, a roller mixer was used to mix the samples for 20 minutes, avoiding foam formation. Elecsys measurements were performed in a series of runs. Each sample was run in singlicate for both assays. The samples were not diluted prior to measurements and dilution is not recommended.

Prior to sample measurements the Elecsys rackpacks to be used for the measurements were calibrated using 1 thawed aliquot of each of the 5 calibration levels. The Cobas e601 instrument was calibrated and the daily quality controls were run.

Table 6: Table showing Precicontrol results for the Plasma pTau(181P) Elecsys Immunoassay

Precicontrol	Rackpack 1		Rackpack 2	
	MC 1	MC 2	MC1	MC2
Plasma pTau(181P) PC1	0.995	0.948	0.975	0.936
Plasma pTau(181P) PC2	3.27	3.33	3.30	3.24

The results from the measurements from the UPenn-provided plasma controls (QC A and QC B) are shown in Table 7.

QC A	Pooled plasma selected from individuals whose CSF tested positive for pTau-181 and Aβ42 (University of Pennsylvania/ADNI biorepository).
QC B	Pooled healthy control plasma

Table 7: Results of the provided controls measured with the Plasma pTau(181P) Elecsys Immunoassay

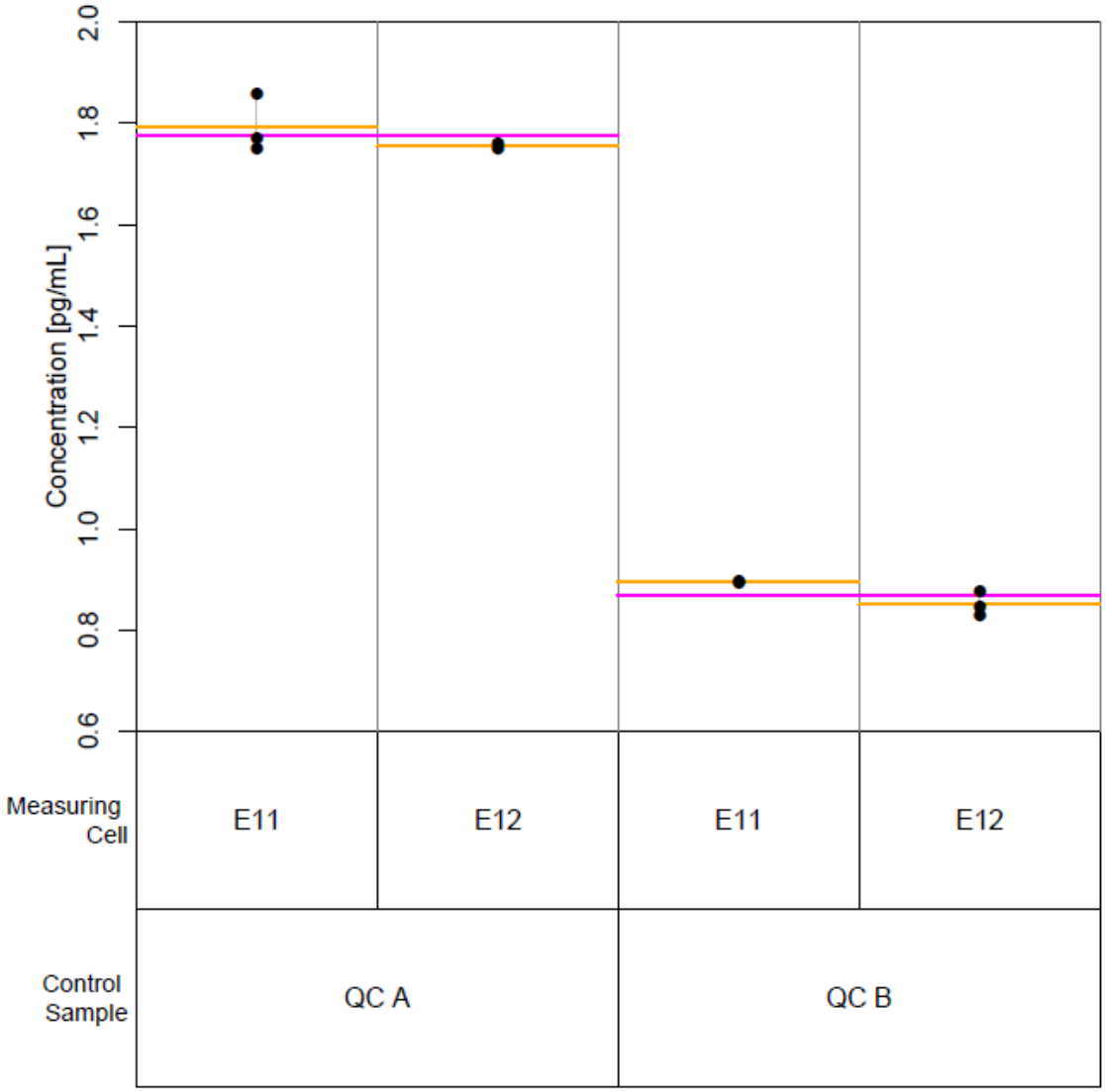
Control	Results (pg/mL)				
QC A	1.770	1.750	1.860	1.760	1.750
QC B	0.894	0.877	0.830	0.898	0.845

Plasma Endogenous QC Data Summary Table

Control	n	mean	median	sd	CV
Negative eQC	5	0.8688	0.877	0.0301115	3.47%
Positive eQC	5	1.7780	1.760	0.0465833	2.62%

Precision of Control Samples

Intra-assay (within-run) precision was calculated for five measurements of QCA and Five measurements of QCB.



Variability plots showing the distribution of the measurements of control samples QCA and QCB. The red line indicates the mean per control sample, with the orange line indicating the mean per measuring cell, respectively.

E11 and E12

	QCA	QCB
N	5	5
Mean [pg/mL]	1.78	0.869
Min [pg/mL]	1.75	0.830
Max [pg/mL]	1.86	0.898
SD [pg/mL]	0.0466	0.0301
Conc. CV [%]	2.62	3.47

E11

	QCA	QCB
N	3	2
Mean [pg/mL]	1.79	0.896
Min [pg/mL]	1.75	0.894
Max [pg/mL]	1.86	0.898
SD [pg/mL]	0.0586	0.00283
Conc. CV [%]	3.27	0.316

E12

	QCA	QCB
N	2	3
Mean [pg/mL]	1.76	0.851
Min [pg/mL]	1.75	0.830
Max [pg/mL]	1.76	0.877
SD [pg/mL]	0.00707	0.0240
Conc. CV [%]	0.403	2.82

Distribution of Samples and Calibrators

Two calibrators with concentration target values of 0.640 mg/mL and 26.00 pg/mL were used. Each calibrator was measured twice on measuring cell E11 and E12, respectively, providing eight signal measurements per calibrator. A linear regression line was calculated using the measured signals and the respective target values of the calibrators.

Figure 2 shows all calibrator and all sample measurements. As there is a large distance between the sample measurements and the upper calibrator, Figure 3 only shows the part of Figure 2, which contains all sample measurements.

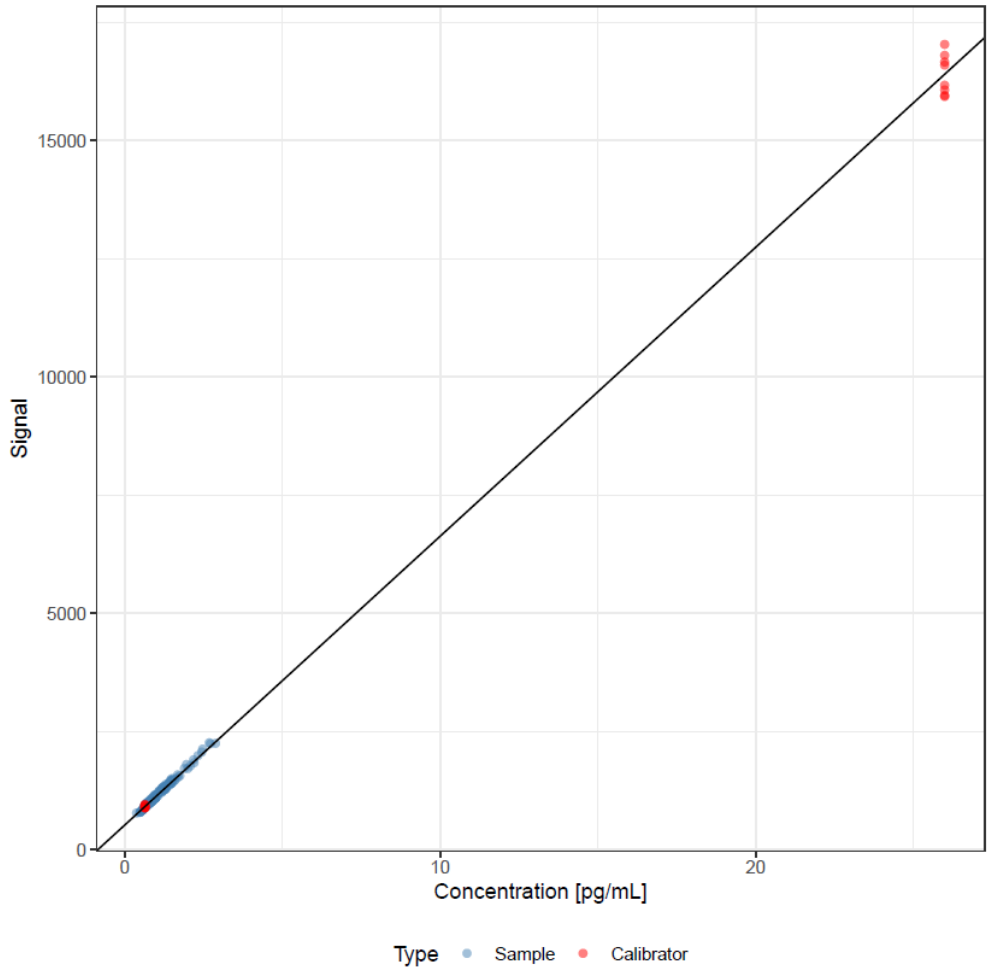


Figure 2: Calibration curve with sample measurements and regression line of calibrator samples, using the linear regression equation: Signal = 522 + 610:943 * Target Values).

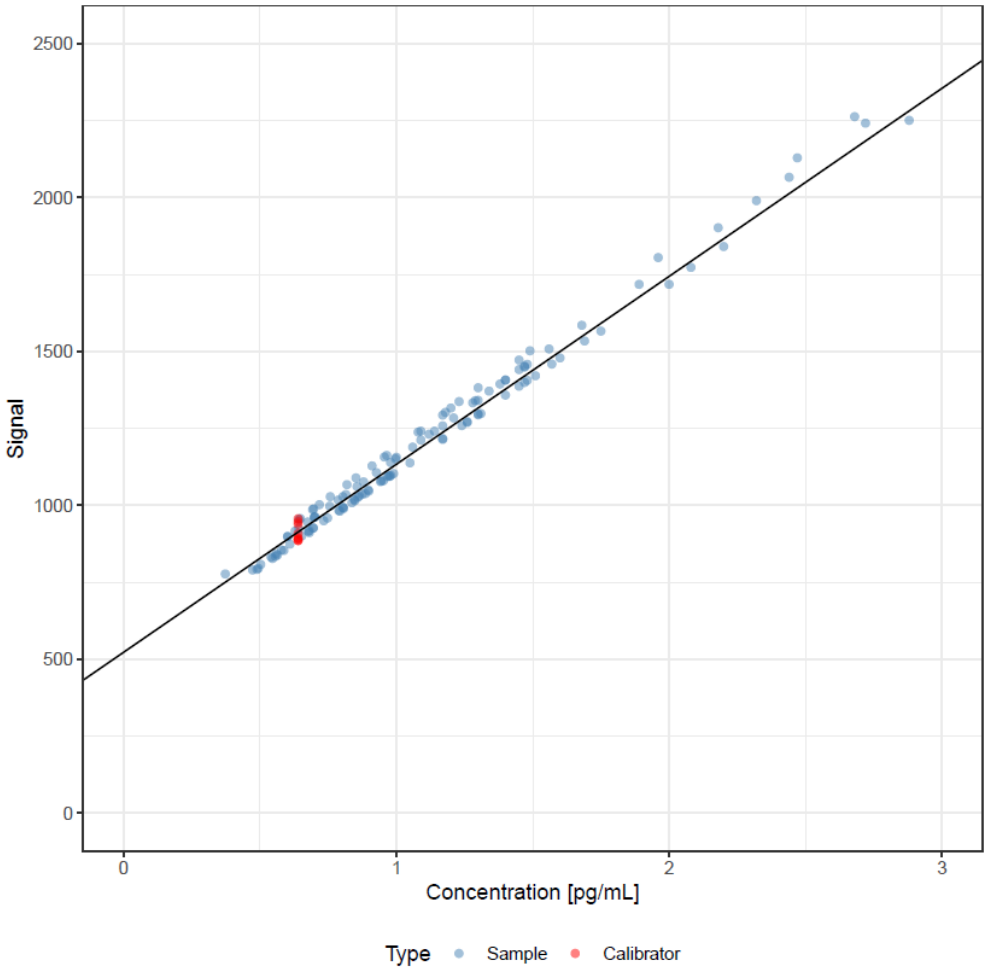


Figure 3: Calibration curve with sample measurements and regression line of calibrator samples, using the linear regression equation: Signal = 522 + 610:943 * Target Values. The x-axis is restricted to the concentration range of sample measurements (0-3 pg/mL).

Elecsys standard was used for curve fitting, i.e. results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve (fitted via Rodbard function) provided via the Cobas link.

Freeze Thaw Stability

The plasma samples for analysis may have up to 4 freeze/thaw cycles. This was determined by looking at the recovery of the analytes following multiple freeze/thaw cycles.

Preliminary internal data shows stability of pTau181 in EDTA plasma for up to 7 days at 25°C, indicating that this analyte is stable. However, it is not recommended to store samples for long periods at room temperature. Cooled storage at 4°C is the preferred practice.

Precision

Inter-module/inter-instrument precision:

Inter-module precision was performed on 4 instruments (e601) on 2 measuring cells each. In total, 30 plasma samples (8 native, 22 spiked) and 2 controls were measured covering the measuring range from low to high (Table 9).
The CV (%) of the concentration was calculated to be ≤ 8.57 %.

Table 8: Measurements of Inter-module/inter-instrument precision for Plasma pTau(181P)

e601									
Sample		Run Device Measuring cell	G1_MC1 1155-05 MC1	G1_MC2 1155-05 MC2	G2_MC1 1227-02 MC1	G2_MC2 1227-02 MC2	G3_MC1 1153-05 MC1	G3_MC2 1153-05 MC2	G4_MC1 1246-01 MC1	G4_MC2 1246-01 MC2
Control 1			1.03	1.01	1.06	1.02	1.00	1.02	0.992	1.07
			0.948	0.978	1.01	1.02	1.01	1.01	0.986	1.02
Control 2			3.31	3.34	3.31	3.37	3.30	3.28	3.46	3.40
			3.35	3.25	3.27	3.32	3.19	3.26	3.39	3.25
IKP_01	*native*		0.838	0.798	0.837	0.845	0.864	0.910	0.830	0.923
IKP_02	*native*		0.930	0.875	0.951	0.923	0.958	0.945	0.923	0.978
IKP_03	*native*		0.689	0.631	0.721	0.671	0.775	0.747	0.694	0.774
IKP_04	*spiked*		2.72	2.63	2.71	2.67	2.80	2.71	2.77	2.87
IKP_05	*spiked*		4.86	4.96	4.92	4.97	5.42	5.46	5.29	5.58
IKP_06	*spiked*		30.1	29.2	30.0	29.5	31.2	30.5	31.8	31.9
PK_01	*native*		0.604	0.565	0.628	0.594	0.677	0.684	0.602	0.654
PK_02	*native*		0.750	0.718	0.802	0.751	0.825	0.833	0.756	0.820
PK_03	*native*		0.922	0.842	0.945	0.899	1.01	0.951	0.945	0.998
PK_04	*native*		0.609	0.539	0.654	0.607	0.681	0.686	0.630	0.709
PK_05	*native*		1.18	1.15	1.26	1.20	1.27	1.30	1.26	1.30
PK_06	*spiked*		1.62	1.51	1.62	1.60	1.69	1.68	1.66	1.67
PK_07	*spiked*		1.81	1.73	1.80	1.76	1.86	1.87	1.85	1.91
PK_08	*spiked*		2.58	2.45	2.55	2.52	2.71	2.64	2.70	2.74
PK_09	*spiked*		3.10	3.00	3.13	3.03	3.19	3.16	3.16	3.21
PK_10	*spiked*		3.29	3.08	3.14	3.23	3.37	3.29	3.40	3.43
PK_11	*spiked*		6.32	6.02	6.17	6.09	6.43	6.30	6.57	6.57
PK_12	*spiked*		6.20	6.08	6.36	6.30	6.78	6.55	6.71	6.82
PK_13	*spiked*		11.5	11.2	11.4	11.2	11.9	11.6	12.2	12.1
PK_14	*spiked*		16.9	16.3	16.3	16.6	17.7	17.3	17.5	17.6
PK_15	*spiked*		3.71	3.58	3.60	3.60	3.86	3.66	3.72	3.85
PK_16	*spiked*		5.42	5.23	5.40	5.40	5.69	5.56	5.75	5.74
PK_17	*spiked*		5.90	5.90	5.58	5.74	6.22	6.14	6.13	5.99
PK_18	*spiked*		7.71	7.42	7.63	7.66	8.16	7.88	8.23	8.20
PK_19	*spiked*		9.23	8.80	9.17	9.00	9.46	9.40	9.74	9.80
PK_20	*spiked*		9.83	9.54	9.80	9.54	10.4	10.1	10.6	10.5
PK_21	*spiked*		11.6	11.6	11.2	11.4	12.4	12.2	12.6	12.5
PK_22	*spiked*		19.9	19.1	19.7	19.8	20.6	19.9	21.0	20.7
PK_23	*spiked*		23.3	23.0	23.1	23.3	24.7	24.2	24.6	24.7
PK_24	*spiked*		30.4	29.3	29.2	30.3	31.5	30.4	31.5	31.7

Table 9: Determination of Inter-module/inter-instrument precision for Plasma pTau(181P)

e601					
Sample	MED$_{conc}$	MIN$_{conc}$	MAX$_{conc}$	Stde V$_{conc}$	%CV$_{conc}$
Control 1	1.01	0.948	1.07	0.0291	2.88
Control 2	3.31	3.19	3.46	0.0685	2.07
IKP_01	0.842	0.798	0.923	0.0419	4.90
IKP_02	0.937	0.875	0.978	0.0309	3.31
IKP_03	0.708	0.631	0.775	0.0509	7.15
IKP_04	2.72	2.63	2.87	0.0758	2.77
IKP_05	5.13	4.86	5.58	0.284	5.48
IKP_06	30.3	29.2	31.9	1.031	3.38
PK_01	0.616	0.565	0.684	0.0423	6.75
PK_02	0.779	0.718	0.833	0.0431	5.51
PK_03	0.945	0.842	1.005	0.0528	5.63
PK_04	0.642	0.539	0.709	0.0548	8.57
PK_05	1.26	1.15	1.30	0.0555	4.48
PK_06	1.64	1.51	1.69	0.0590	3.62
PK_07	1.83	1.73	1.91	0.0572	3.14
PK_08	2.61	2.45	2.74	0.1017	3.89
PK_09	3.14	3.00	3.21	0.0752	2.41
PK_10	3.29	3.08	3.43	0.1254	3.83
PK_11	6.31	6.02	6.57	0.207	3.29
PK_12	6.45	6.08	6.82	0.279	4.32
PK_13	11.5	11.2	12.2	0.376	3.23
PK_14	17.1	16.3	17.7	0.585	3.44
PK_15	3.68	3.58	3.86	0.1098	2.97
PK_16	5.49	5.23	5.75	0.190	3.44
PK_17	5.94	5.58	6.22	0.217	3.65
PK_18	7.80	7.42	8.23	0.305	3.88
PK_19	9.31	8.80	9.80	0.345	3.70
PK_20	9.96	9.54	10.6	0.420	4.19
PK_21	11.9	11.2	12.6	0.533	4.46
PK_22	19.9	19.1	21.0	0.617	3.07
PK_23	23.8	23.0	24.7	0.749	3.14
PK_24	30.4	29.2	31.7	0.962	3.15

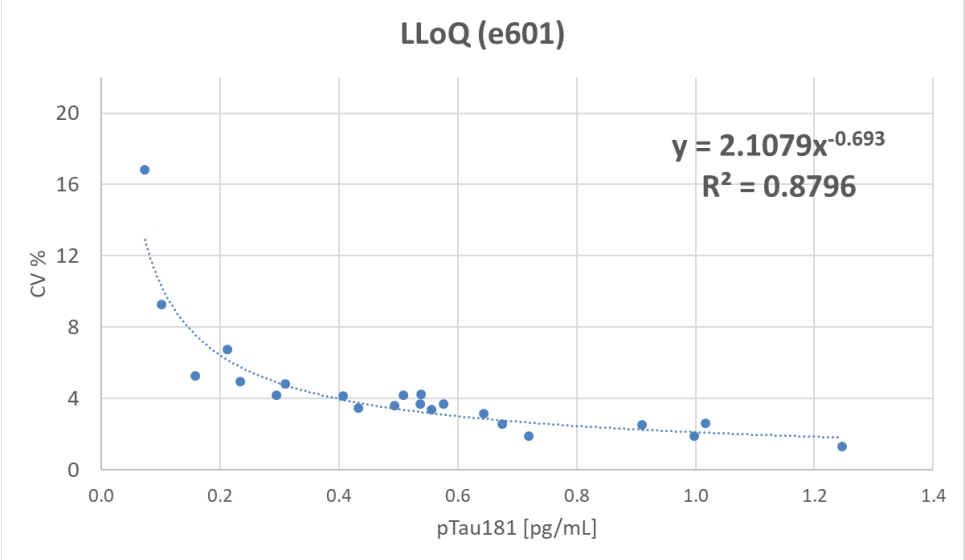
LLoQ (functional sensitivity)
10 native plasma samples, 6-7 diluted plasma samples and 6-7 artificial samples spiked with antigen were measured in at least 4 independent runs on the same instrument and the CV (%) of the measurements was calculated. Concentrations are plotted against the CV (%).
LLoQ was defined as the lowest sample concentration with a CV ≤ 20%.

Acceptance criteria: not applicable, value will be determined.

Table 10: LLoQ for Plasma pTau(181P)

Assay	Functional sensitivity (LLoQ)
Plasma pTau(181P)	0.073 pg/mL

Non-linear regression model for LLoQ determination for Plasma pTau(181P)



About the Authors

This document was prepared by Alexander Jethwa (Development Lead) and Gwendlyn Kollmorgen (Study Manager) - all employees of Roche Diagnostics. For more information, please contact Gwendlyn Kollmorgen at +49 (0)152 36913830 or by email at gwendlyn.kollmorgen@roche.com.

University of Gothenburg

Sample analysis performed at the University of Gothenburg with oversight by Henrik Zetterberg, M.D., Ph.D. and Nicholas Ashton, Ph.D.
Assay: pTau231 Simoa

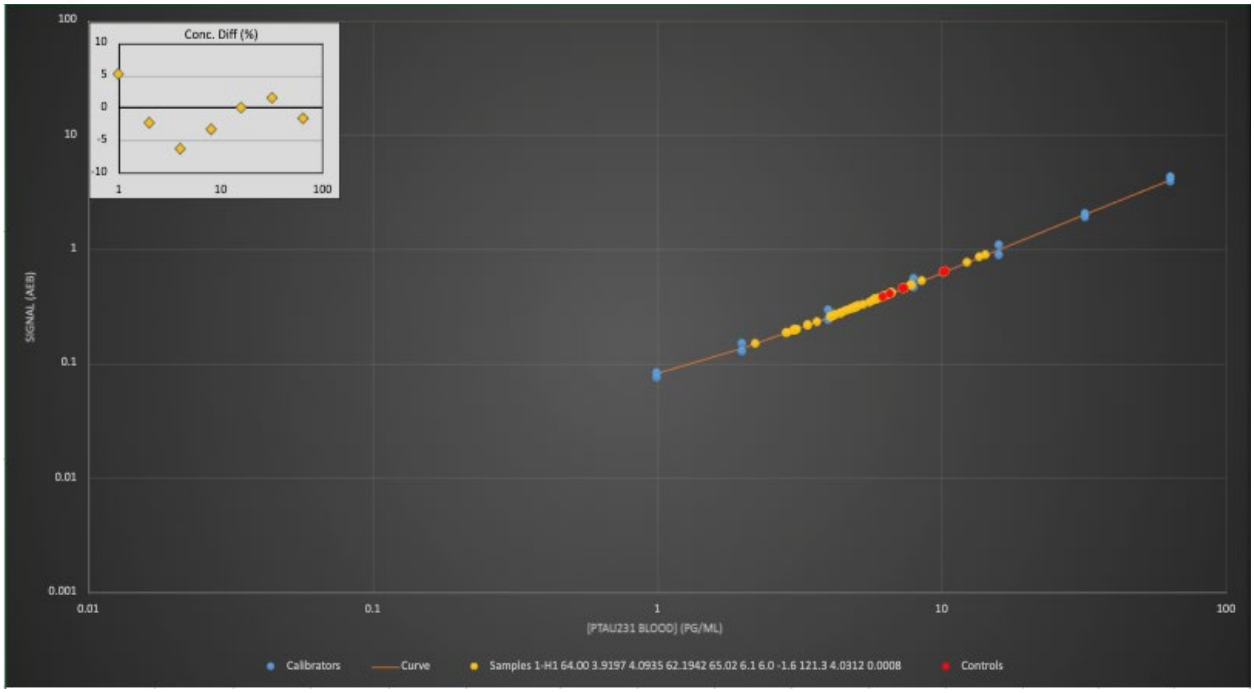
Platform	Single molecule array (Simoa) HD-X
Capture antibody	ADx253
Detector antibody	Tau12
Calibrator	Sythetic peptide
Calibrator matrix	PBS based matrix with detergents
Sample diluent	PBS based matrix with detergents
Dilution factor	x2
Use of protease inhibitor	No
LLoD or LLoQ	LLOQ: 1pg/mL (calibrator replicate >20%)

Sample Storage and Preparation

Project samples were stored in -80°C freezer upon receipt. In preparation for measurement, the samples were thawed on ice and centrifuged for 10 minutes at 4000 g in a refrigerated microcentrifuge (RT). The samples were kept on ice until diluted in a Simoa assay plate.

Calibrator Results

	Concentration	AEB Run 1	AEB Run 2	AEB Run 3
Rep 1	0	0.0357	0.0330	0.0266
Rep 2	0	0.0318	0.0288	0.0279
Rep 1	1.0000	0.0818	0.1296	0.1111
Rep 2	1.0000	0.0763	0.1246	0.1009
Rep 1	2.000	0.1480	0.2244	0.1739
Rep 2	2.000	0.1291	0.2271	0.1788
Rep 1	4.000	0.2911	0.3880	0.3235
Rep 2	4.000	0.2390	0.4035	0.3362
Rep 1	8.00	0.5522	0.6968	0.6245
Rep 2	8.00	0.4608	0.7332	0.6028
Rep 1	16.00	1.0846	1.1482	1.1491
Rep 2	16.00	0.8943	1.2117	1.1785
Rep 1	32.00	2.0536	2.2869	2.0132
Rep 2	32.00	1.9060	2.2328	2.1500
Rep 1	64.00	4.2673	4.0716	4.0610
Rep 2	64.00	3.9197	4.4329	4.1748



Plasma Endogenous QC Results

Sample Code	Analyte	[pTau231] pg/ml	% CV
FA80B8MN-03	pTau-231	14.30	1.81
HMN500278	pTau-231	8.94	4.94
FA80B8MN-03	pTau-231	14.77	2.16
HMN500278	pTau-231	8.15	2.16
FA80B8MN-03	pTau-231	14.42	1.78
HMN500278	pTau-231	8.21	9.37

HMN500278 is the pooled plasma from healthy young donors (negative eQC samples supplied by BioIVT)

FA80B8MN-03 (positive eQC samples) is the pooled plasma from individuals whose corresponding CSF values for pTau-181 and Aβ42 were abnormal (> 24 pg/mL for pTau181, positive eQC provided by the University of Pennsylvania/ADNI biorepository)

Validation Report

pTau-231			
	Experimental Design	**Results**	**Acceptance Criteria**
Dilution Linearity and MRD Analysis	Not available		
Standard Linearity and Assay Range	LOD to highest measured standard using calibrators	1-128pg/mL	
Accuracy	Not available		
Repeatability	Andresson et al., 2015, *Frontiers in Neurology*	4%	
Intermediate Precision	Andresson et al., 2015, *Frontiers in Neurology*	15%	
Limit of Quantification		1 pg/mL	
Selectivity (matrix effects)	Not available		
Controls	low, middle and high plasma samples of p-tau181	Not reported in this study	
Analyte Stability	Not available		
Parallelism (as defined in Andreasson et al., 2019) with Clinical Samples	Ashton et al., *Acta Neuropathologica*	84–111.2%	80-120%
Cut point for amyloid PET positivity and/or tau PET positivity, if evaluated, and the relevant references or supporting data	Not available		