P2-037

Development and Analytical Validation of a Novel Assay for Measurement of Total Tau in Human CSF

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1 Abstract

Background: Measurements of Alzheimer's disease (AD) biomarkers in clinical samples present challenges when using existing assays due to inter-lot variability, matrix interferences, and inconsistent quantitation. Here we describe the development and analytical validation of an assay to measure total tau in human cerebrospinal fluid (CSF) using "Fit-for-Purpose" and Clinical and Laboratory Standards Institute (CLSI) principles. Elements of assay development and performance are presented as well as results of multi-lot validation.

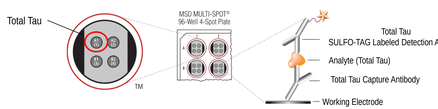
Methods: Monoclonal antibodies were evaluated and selected based on sensitivity, specificity, affinity, and performance characteristics in human CSF. Critical reagents were subjected to biochemical and functional characterization to verify purity, integrity, and lot-to-lot consistency. The assay was developed using MESO SCALE DISCOVERY MULTI-ARRAY® technology and have been optimized to minimize CSF matrix effects and interferences. Analytical validation was assessed across three independent kit lots to verify performance—including sensitivity, accuracy, and precision—using matrix-based validation samples. Tests were conducted by multiple analysts over multiple runs and days. Dilution linearity and spike recovery were measured using well-curated normal and AD CSF samples.

Results: The assay demonstrated good sensitivity, performance, and inter-lot reproducibility and differentiated between normal and AD CSF samples. The average LLOD, determined from 54 runs over three independent kit lots, was 10 pg/mL with a quantitative range of 30 to 8000 pg/mL. The precision, accuracy, and total error were determined from matrix-based controls with typical precision of <15% CV (inter-plate) and <5% CV (intra-plate). Dilution linearity and spike recovery testing demonstrated quantitation of tau protein over the range of the assay as well as minimal interference from human CSF matrix. Furthermore, the assay detected six isoforms of human tau and was tolerant of significant CSF contamination with hemolyzed blood. Data also showed that the assay measured elevated levels of tau in AD samples compared to healthy samples.

Conclusion: A new assay was developed and analytically validated to measure tau in human CSF. The result is an assay with good analytical performance characteristics and lot-to-lot reproducibility with the ability to distinguish between normal and AD CSF samples based on tau levels. This assay will support ongoing efforts to standardize testing of biomarkers for AD and other tauopathies.

2 Assay Development and Validation

Development of the MSD® Human Total Tau Kit* was tailored to address shortcomings of existing kits on the market and was conducted under design and development controls. The assay was built on MSD's electrochemiluminescence detection technology platform. SULFO-TAG™ labels emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY and MULTI-SPOT® microplates. The assay utilizes capture and detection antibodies which recognize all six isoforms of human tau. The calibrator is an E. coli-expressed recombinant protein representing the longest tau isoform, tau441. The assay diluent, Diluent 35, was optimized to minimize matrix effects in human CSF. MSD recommends a 4-fold sample dilution; however, given the good linearity of the assay, a lower dilution factor may be used to achieve greater sensitivity. The assay was validated using three independently-built kit lots tested by multiple analysts across multiple runs and days. Each lot was built using different lots of raw materials. Human CSF-based validation samples with total tau concentrations that spanned the standard curve were built and used to validate the dynamic range of the assay. Long-term stability studies with inter-lot bridging demonstrated the utility for this kit in longitudinal studies. The kit performance met the levels of consistency and robustness outlined in "Fit-for-Purpose Method Development and Validation for Successful Biomarker Measurement" by J. W. Lee, et al.[5]

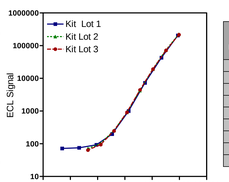


3 Assay Protocol and Standard Curve

Left: The Human Total Tau assay protocol is described; this is a washed assay. **Middle:** Standard curves from three independently build kit lots are presented, illustrating the range of the assay and the reproducibility of standard curve signals across manufactured kit lots. Each curve represents the average signals from a multi-run, multi-analyst, multi-day data set. **Right:** Representative data from one lot.

Protocol

1. Add 150 µL MSD Diluent 35. Incubate for 1 hour at room temperature.
2. Wash with PBS-T. Add 50 µL of standard or diluted sample.
3. Incubate for 1 hour at room temperature.
4. Wash with PBS-T. Add 25 µL of detection antibody. Incubate for 1 hour at RT.
5. Wash with PBS-T. Add 150 µL of Read Buffer T. Read on MSD SECTOR® Imager.



Conc. (pg/mL)	Average Signal	Average Intra-plate %CV
9,600	223102	6.2
3,200	73507	7.3
1,067	19164	6.2
356	4230	7.3
119	913	6.5
39.5	253	6.0
13.2	103	6.8
4.39	71	8.7
Blank	57	17.1

*MSD offers the Human Total Tau Kit for purchase in 1-, 5-, and 25-plate kit sizes (catalog numbers K151LAE-1, K151LAE-2, K151LAE-4, respectively).

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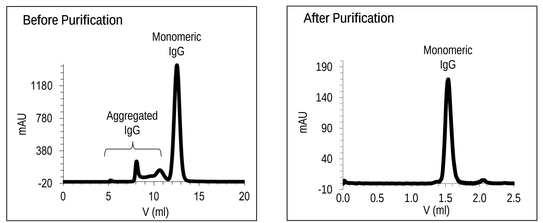
4 Evaluation of Antibody Critical Reagents

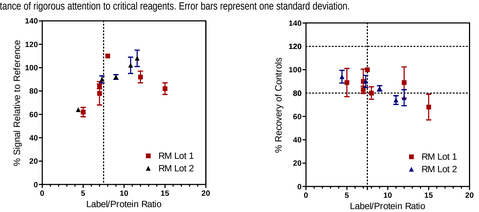
Consistency across manufactured kit lots requires consistency across the raw material lots used to build the kit. A critical aspect of MSD assay development and validation is characterization of critical reagents including antibodies, calibrator, and diluent components. Incoming raw materials were evaluated using a variety of analytical approaches including Experion, dynamic light scattering (DLS), and capillary isoelectric focusing (cIEF). Multiple lots of vendor raw material were studied to build a database of information about the material, allowing for identification of changes in the raw material which may impact performance. In some cases, the material may be processed in order to remove contaminants and/or improve consistency. Data for the detection antibody are summarized below. **Top:** Size-exclusion chromatogram reveals significant aggregation in the raw material (left) which can be removed (right). **Bottom:** Summary of analytical characterization of the detection antibody raw material before and after processing at MSD. Data from three processing runs are presented. Good consistency across lots is achieved through introduction of the processing step.



Characterization Method		Metric	Raw Material	Processed Lot 1	Processed Lot 2	Processed Lot 3
Experion	Non-reducing	Ab % Total Mass	not done	96%	97%	96%
	Reducing	% Total Mass H + L	not done	100%	100%	100%
		H/L Ratio	not done	2.2	2.1	2.2
DLS		Ab % Polydispersity	61%	8%	14%	8%
		Ab % Intensity	75%	99%	95%	93%
		Ab % Mass	63%	83%	93%	98%
		Radius of Ab peak	10.4	5.1	5.3	5.0
cIEF		pI of main peak	6.1	6.1	6.1	6.1
		pI range	5.8-6.2	5.8-6.2	5.9-6.2	5.9-6.2

5 Optimization of Critical Reagents: Detection Antibody Conjugation

Conjugated detection antibody optimization includes optimization of conjugation conditions and assay concentration. Detection antibodies were conjugated with SULFO-TAG to establish a set of antibodies with a range of labels per protein (L/P). These antibodies, conjugated from separate raw material (RM) lots were tested in functional assays to measure the signal response and quantitation of control samples. The target L/P is 7.5 (vertical dotted line). **Left:** Assay signal begins to plateau at the highest L/P ratios. **Right:** Tau quantitation in human CSF-based controls. Data are a summary of results from three controls. At the target L/P, the controls recover at appropriate levels. These results demonstrate the importance of rigorous attention to critical reagents. Error bars represent one standard deviation.



6 Assay Sensitivity

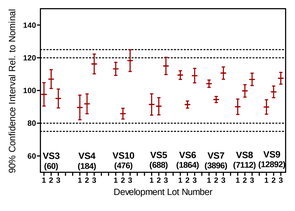
The Human Total Tau kit is sensitive and measures total tau over a wide dynamic range. The lower limit of detection (LLOD) is a calculated concentration based on a signal of 2.5 standard deviations above the blank (zero calibrator). The ULOQ and LLOQ are the highest and lowest concentration, respectively, at which the %CV of the calculated concentration is <20% and the percent recovery of the standard is within 80–120% of the known value. Assay sensitivity (LLOD) and dynamic range (upper limit of quantification [ULOQ] to lower limit of quantification [LLOQ]) were determined for each of three independent kit lots. Samples for determining ULOQ and LLOQ were created by spiking a known value of total tau calibrator into diluent. Testing for each kit involved a minimum of 12 runs conducted by three analysts across at least three days of testing (N=36 runs across 3 kit lots). The range of LLODs measured across three kit lots (N=54 plates) is presented. The average LLOD is 10 pg/mL. The quantitative range of the assay is 30-8000 pg/mL. In-well concentrations are reported.

	Expected Conc (pg/mL)	Kit Lot 1 Calc. Conc. (pg/mL)	Kit Lot 1 Calc Conc %CV	Kit Lot 1 % Recovery	Kit Lot 2 Calc. Conc (pg/mL)	Kit Lot 2 Calc Conc %CV	Kit Lot 2 % Recovery	Kit Lot 3 Calc. Conc. (pg/mL)	Kit Lot 3 Calc Conc %CV	Kit Lot 3 % Recovery
ULOQ	8,000	7,317	18.7	91%	7,484	11.0	94%	8,481	8.4	106%
LLOQ	30.0	29.3	17.3	98%	32.2	13.7	107%	31.5	10.2	105%
LLOD Range (pg/mL)		7.90 – 23.7			3.99 – 15.3			1.07 – 18.7		

7 Measurement of Validation Samples

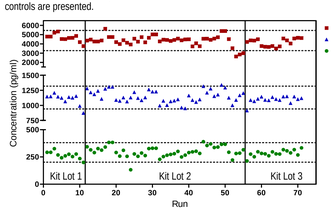
Validation samples (VS) were used to define the limits of detection and to demonstrate reproducibility across kits lots. Ten validation samples were prepared in human pooled CSF (24–2892 pg/mL total tau). VS1-3(*) were built using immunodepleted matrix spiked with calibrator, and were purposely built below the predicted LLOQ. The remaining VS contained endogenous total tau; some were spiked with calibrator to achieve the desired range. VS 1–10 were measured across runs, analysts, and kit lots. **Left:** Representative ANOVA analysis from VS testing on Kit Lot 1. **Right:** Measured concentrations across three independent kit lots. Variation (% CV range) among the three development lots was 4–14% for total tau across all validation samples (not shown).

Validation Sample ID	VS1*	VS2*	VS3*	VS4	VS10	VS5	VS6	VS7	VS8	VS9
Assigned Dilution-Adjusted Conc. (pg/mL)	24	48	60	184	476	688	1864	3896	7112	12892
Sample n Kit Lot 1	15	24	24	24	96	24	96	96	24	24
Measured Dilution-Adjusted Conc. (pg/mL)	26	47	59	165	539	629	2040	4059	6407	11594
% Deviation	8.8	-1.8	-2.3	-10.3	13.2	-8.5	9.4	4.2	-9.9	-10.1
Precision (%CV)										
Inter-Analyst	0	0	0	0	0	0	0	0	0	0
Inter-Day	41	0	0	0	0	17	4	4	9	3
Inter-Plate	0	16	10	15	16	5	10	7	3	0
Intra-Plate	56	19	20	6	7	4	6	6	5	4
Total (%CV)	68.8	25	21.8	16.4	17.3	18.2	12.2	9.6	10.9	5.5
% Total Error	77.6	26.8	24.1	26.7	30.5	26.7	21.6	13.8	20.8	15.6



8 Real Time Stability of Controls

Total tau levels were measured in matrix-based controls (n=3) across three kits lots, and multiple analysts, plates, and runs over a period of seven months. Reported sample concentrations are adjusted for sample dilution. **Left:** Results for the three controls, tested on kit lots 1–3, are presented. Dotted lines represent two standard deviations above and below the assigned concentration. **Right:** The precision results for the controls are presented.



	Control 1	Control 2	Control 3
n	71	71	71
Conc (pg/mL)	4355	1130	292
Std Dev	543	95	45
%CV	12%	8%	15%

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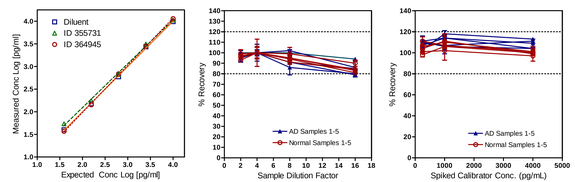
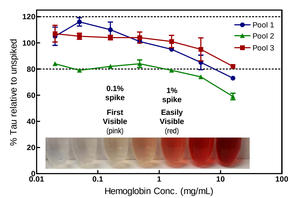
9 Matrix Tolerance

Left: Matrix tolerance using the optimized assay diluent, Diluent 35, was evaluated using immunodepleted (ID), pooled human CSF. Recovery of Tau calibrator spiked into ID CSF (n=2) and diluent was parallel. **Middle:** CSF from well-curated normal and AD individuals were diluted 2-, 4-, 8-, and 16-fold with Diluent 35. Measured concentrations were corrected for dilution factor. Recovery at each dilution was calculated relative to the optimal sample dilution (4-fold). **Right:** Well-curated CSF samples were spiked with calibrator at multiple levels, diluted 4-fold, and tested for recovery. % Recovery=measured/expected*100. Results are representative of data from three independent MSD kit lots. Error bars: one standard deviation.



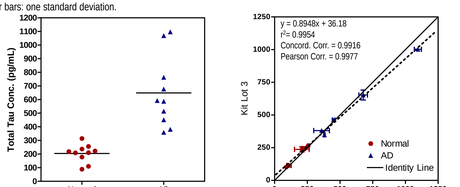
10 Tolerance to Blood Interference

The Human Total Tau Kit is tolerant of up to 1.6 mg/mL hemoglobin in CSF, which is equivalent to 1% blood contamination in the sample. **Right/Top:** Assay tolerance to blood contamination was assessed by measuring total tau levels in CSF (n=3 pools) spiked with a titration of clarified hemolyzed blood. The resulting contaminated samples contained 0.02–16 mg/mL hemoglobin, which is equivalent to 0.01–10% blood in the sample. Spiked samples were diluted 4-fold and tested with the Human Total Tau Kit. The measured total tau concentration relative to the unspiked sample is plotted. Results are representative of data collected across three independent MSD kit lots. **Right/Bottom:** Samples with 0.1% contamination are tinged slightly pink; samples with 1% contamination are red and easily identified as contaminated.



11 Measurement of Patient Samples

Left: Total tau levels were measured in well-curated individual normal and AD patient samples (n=10 each). Total tau level distinguishes normal and AD samples. **Right:** A subset of the AD and normal samples (n=5 each) was measured on a second kit lot. Good correlation and agreement in measured concentration was observed between the two kit lots. Reported concentrations are adjusted for a 4-fold dilution. Error bars: one standard deviation.



12 Conclusions

MSD's Human Total Tau Kit has been analytically validated for measurement of total tau in human CSF. The assay was built using highly characterized, critical reagents and enhanced handling methods which improved robustness and reliability. The Human Total Tau kit exhibits good analytical performance, inter-lot consistency, and the ability to distinguish between normal and AD samples based on total tau levels.

Acknowledgements

Current affiliation information is: Paul Rhyne, Tandem Labs, West Trenton, NJ; Oi Wong, Merck, Rahway, NJ.

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