Development of Analytically Validated Assays for Amyloid Beta 1-42 and Tau in Human Cerebrospinal Fluid

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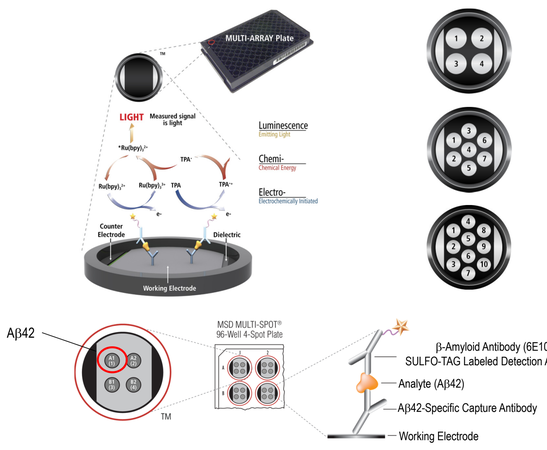
1 Abstract

Amyloid beta 1-42 peptide (Aβ42) and tau have been identified as core biomarkers of Alzheimer's disease (AD). Their levels in cerebrospinal fluid (CSF) may be used to distinguish normal and AD patients, and in combination, the two biomarkers may be useful in identifying patients with mild cognitive impairment (MCI).[1-3] Standardized assays with minimal variability across manufacturing runs, users, and platforms are needed in the field to provide accurate analysis of AD markers.[4] We have developed and analytically validated assays for the detection of Aβ42 and tau in human CSF. Both assays meet 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] The assays were validated using three independently-built kit lots. Testing for each kit involved a minimum of twelve runs conducted by three analysts across at least three days (N=54 runs across three kit lots). Each kit lot was built using different lots of raw materials that were characterized using multiple bioanalytical methods. Limit of quantification samples, matrix-based validation samples, and controls were measured using multiple kit lots, plates, and analysts over multiple days to establish sensitivity, accuracy, precision, and assay calibration curves. Spike recovery and dilution linearity were evaluated using individual normal and AD patient samples. An additional element of the development and validation work involved identification and elimination of potential causes of assay variability such as calibrator and sample handling, including pre-analytical factors that influence sample quantitation. Assay specificity and tolerance to sample contamination with hemolyzed blood were evaluated. Assay robustness and stability were assessed through freeze-thaw testing and accelerated stability studies. This poster presents the results of the analytical validation process.

2 Methods

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.



Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

Protocol for Human Aβ42 Kit

1. Add 150 µL MSD® Diluent 35. Incubate for 1 hour at room temperature.
2. Wash with PBS-T. Add 50 µL of calibrator 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.

* MSD recommends a minimum 8-fold sample dilution for the Human Aβ42 Kit.

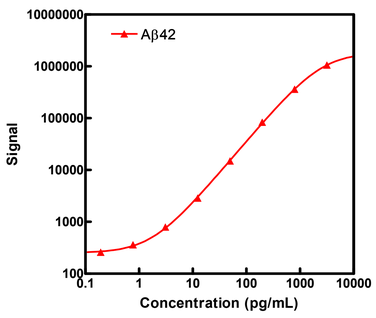
Protocol for Human Total Tau Kit

1. Add 150 µL MSD Diluent 35. Incubate for 1 hour at room temperature.
2. Wash with PBS-T. Add 50 µL of calibrator 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.

* MSD recommends a minimum 2-fold sample dilution for the Human Total Tau Kit.

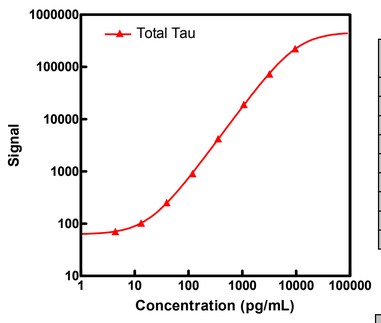
3 Typical Standard Curve Performance and Sensitivity

Standard curve accuracy and precision were assessed for three kit lots. Representative standard curve data from one kit lot are presented below. The data were collected over six days of testing by three analysts (23 runs in total). An 8-point curve plus a blank were used to validate the assay over a broad dynamic range.



Conc. (pg/mL)	Aβ42 Average Signal	Aβ42 %CV
0	245	5.8
0.193	258	4.0
0.774	361	2.6
3.10	793	2.9
12.4	2870	4.3
49.5	14931	4.1
198	83291	3.2
793	359727	3.3
3170	1054859	3.7

	Aβ42 (pg/mL)
LLOD Range	0.070–0.96
LLOQ	3.0
ULOQ	2000



Conc. (pg/mL)	Total Tau Average Signal	Total Tau %CV
0	57	17.1
4.39	71	8.7
13.2	103	6.8
39.5	253	6.0
119	913	6.5
356	4230	7.3
1067	19164	6.2
3200	73507	7.3
9600	223102	6.2

	Total Tau (pg/mL)
LLOD Range	1.07– 23.7
LLOQ	30.0
ULOQ	8000

Assay sensitivity and dynamic range were assessed by testing across multiple kit lots, analysts, and runs. The lower limit of detection (LLOD) and upper and lower limits of quantification (ULOQ and LLOQ, respectively) were determined for each of three independent kit lots. Testing for each kit involved a minimum of twelve runs conducted by three analysts across at least three days of testing (N=54 runs across three Tau kit lots; N=42 runs across three Aβ42 kit lots). A summary of the sensitivity and dynamic range is presented in the table above. In-well concentrations are reported.

The lower limit of detection (LLOD) is a calculated concentration based on a signal 2.5 standard deviations above the blank (zero calibrator). The ULOQ and LLOQ were determined by spiking a known value of calibrator into diluent to assess the accuracy and precision of the samples. The ULOQ is the highest concentration 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. The LLOQ is the lowest concentration 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. The LLOQ and ULOQ are verified for each kit lot and the results are provided in the lot-specific certificate of analysis that is included with each kit and available for download at WWW.MESOSCALE.COM.®

4 Precision and Accuracy

Control samples using pooled human CSF with or without spiked Tau calibrator and Human Aβ42 were built. Two sets of control samples were independently prepared and tested in the Human Total Tau and Human Aβ42 Kits. Each set contained three controls with levels spanning the expected range of each assay in human CSF samples. Controls were diluted 4-fold for total tau and 8-fold for Aβ42. Concentrations for all controls were measured using three independent kit lots. Representative data from one set of controls are presented in the tables below. For this study, four analysts ran tests over ten days (N=26 runs across three kit lots). The control data for each kit lot and an inter-kit lot summary are presented in the upper tables. Concentrations presented in the tables below have been dilution-adjusted. Avg. Intra-plate Calc. Conc. %CV is the average concentration %CV of the control replicates on an individual plate. Inter-plate Calc. Conc. %CV is the variability of measured control concentration across plates, with replicate information as indicated in the tables. Percent Total Error was calculated as the (Inter-plate Calc. Conc. %CV) + (absolute value of % Conc. Recovery Relative to Final Expected Concentration-100%). The concentrations presented in the inter-lot summary represent the expected concentrations for each control. Measured concentrations for each kit relative to the final expected concentrations are presented in the lower tables.

Aβ42

	Sample ID	Calc. Conc. (pg/mL)	Inter-plate Calc. Conc. %CV	Avg. Intra-plate Calc. Conc. %CV	% Total Error
Kit Lot 1 N=2	Control 1	2586	9.7	5.1	13
	Control 2	683	10.1	7.0	19
	Control 3	210	8.5	8.0	9
Kit Lot 2 N=4	Control 1	2719	9.7	8.5	18
	Control 2	675	9.6	7.8	18
	Control 3	228	9.4	5.4	19
Kit Lot 3 N=23	Control 1	2453	12.4	6.8	14
	Control 2	611	16.6	10.0	19
	Control 3	205	10.9	6.6	13
Inter-Lot Summary N=29	Control 1	2499	12.1	7.0	
	Control 2	625	15.8	9.5	
	Control 3	208	11.1	6.5	

	% Conc. Recovery Relative to Final Expected Concentration		
	Kit Lot 1	Kit Lot 2	Kit Lot 3
Control 1	103	109	98
Control 2	109	108	98
Control 3	101	110	98

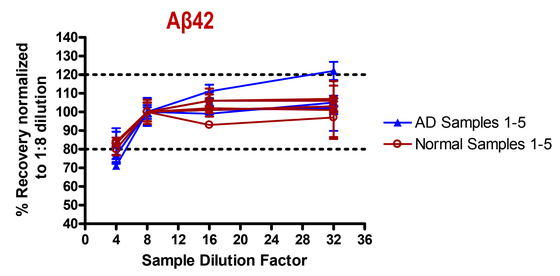
Total Tau

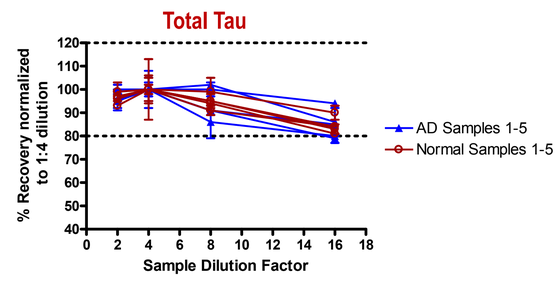
	Sample ID	Calc. Conc. (pg/mL)	Inter-plate Calc. Conc. %CV	Avg. Intra-plate Calc. Conc. %CV	% Total Error
Kit Lot 1 N = 3	Control 1	4928	5.0	3.3	12
	Control 2	1167	3.0	2.5	3
	Control 3	304	6.3	3.2	9
Kit Lot 2 N = 5	Control 1	4734	7.2	3.1	10
	Control 2	1118	2.8	3.3	7
	Control 3	260	4.8	5.4	17
Kit Lot 3 N = 18	Control 1	4504	9.5	2.6	12
	Control 2	1184	7.2	5.3	8
	Control 3	305	18.6	5.0	21
Inter-Lot Summary N = 26	Control 1	4598	9.0	2.4	
	Control 2	1170	6.5	4.3	
	Control 3	296	17.1	4.5	

	% Conc. Recovery Relative to Final Expected Concentration		
	Kit Lot 1	Kit Lot 2	Kit Lot 3
Control 1	107	103	98
Control 2	100	96	101
Control 3	103	88	103

5 Matrix Tolerance

To assess linearity, CSF samples from individual normal and AD patient samples were diluted 2-fold, 4-fold, 8-fold, and 16-fold with Diluent 35. Measured concentrations were corrected for dilution factor to determine the actual levels of Aβ42 and tau in the samples. Percent recovery at each dilution was calculated relative to the optimal sample dilution (4-fold). Average % Recovery and % Recovery Range for normal and AD samples at each dilution are presented in the graph below. The total tau graph of % Recovery versus Sample Dilution Factor shows that a 2-fold dilution may be used for higher sensitivity with minimal effect on recovery (% Recovery=(measured∗dilution factor)/(measured at 4-fold dilution∗4)∗100). A minimum sample dilution of 2-fold is recommended.





CSF from individual normal and AD patient samples were spiked with calibrator at multiple levels throughout the range of the assay. The samples were then diluted 8-fold (Aβ42) or 4-fold (Total Tau) and tested for recovery (% Recovery=measured/expected∗100).

Sample	Aβ42 Spike Conc. (pg/mL)	Aβ42 Average %Recovery	Aβ42 %Recovery Range
Normal CSF (N=5)	4000	88	81–92
	1000	98	92–105
	250	95	87–104
AD CSF (N=5)	4000	89	84–94
	1000	95	93–99
	250	95	90–103

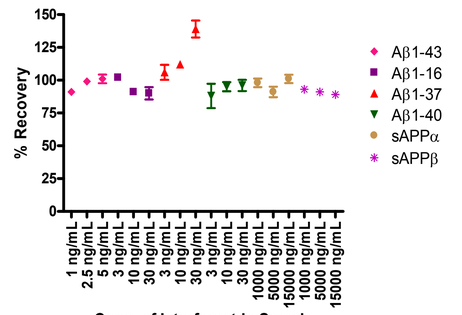
Sample	Total Tau Spike Conc. (pg/mL)	Total Tau Average %Recovery	Total Tau %Recovery Range
Normal CSF (N=5)	4000	101	93–107
	1000	110	106–113
	250	105	99–109
AD CSF (N=5)	4000	101	97–104
	1000	112	106–116
	250	106	104–107

6 Interference and Specificity

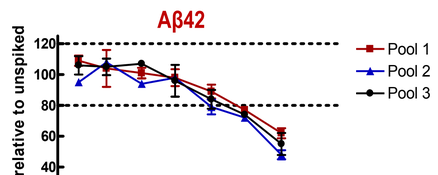
Different Amyloid beta (Aβ) peptides and amyloid precursor proteins were spiked into Diluent 35 and tested with the Human Aβ42 assay. Concentrations shown in the table below have been corrected for sample dilution. The assay detected very low levels of Aβ 1-41, 1-43, and 1-37. Grey shading indicates values below the LLOD of the Aβ42 assay. ND indicates not detected.

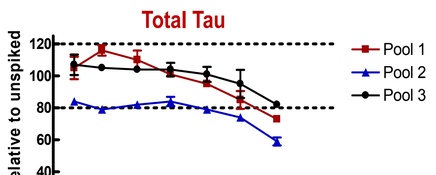
Analyte Spiked	Dilution Adjusted Conc. of Spiked Analyte (pg/mL)	% of Spiked Calibrator Recognized in the Aβ42 Assay
Aβ1-16	24000	ND
Aβ17-24	24000	ND
Aβ1-37	24000	0.93
Aβ1-38	24000	ND
Aβ1-39	24000	0.03
Aβ1-40	80000	0.02
Aβ1-41	24000	0.22
Aβ1-43	24000	0.41
sAPPα	8000000	ND
sAPPβ	8000000	ND

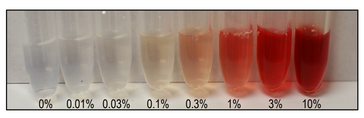
The Human Aβ42 Kit was designed to minimize interferences in Aβ42 measurement. Various Aβ peptides and amyloid precursor proteins were spiked into human CSF at levels that exceed the expected endogenous levels for these analytes as noted below. The endogenous Aβ42 levels were measured in the parent and spiked samples. Measured Aβ42 levels were largely within 20% of the parent sample, regardless of the spiked analyte or concentration.

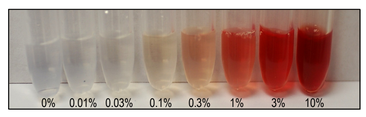


Assay tolerance to blood contamination was assessed by measuring Aβ42 and tau levels in CSF spiked with hemolyzed clarified blood. The hemoglobin concentration in the hemolyzed blood sample was estimated through absorbance measurement at 414 nm (extinction coefficient 524,280 cm^{-1}/M). The measured concentration was 160 g/L hemoglobin, consistent with expected hemoglobin levels in normal whole blood. Hemolyzed blood was titrated into three human CSF pools. The resulting contaminated samples contained 0.02–16 mg/mL hemoglobin, which is equivalent to 0.01–10% blood in the sample. Samples spiked with blood were diluted the appropriate amount and measured. The measured Aβ42 and total tau concentrations relative to the unspiked sample are plotted below. Samples with 0.1% contamination are tinged slightly pink; samples with 1% contamination are dark pink and easily identified as contaminated. The assays tolerated up to 1.6 mg/mL hemoglobin in CSF, which is equivalent to 1% blood contamination in the sample.



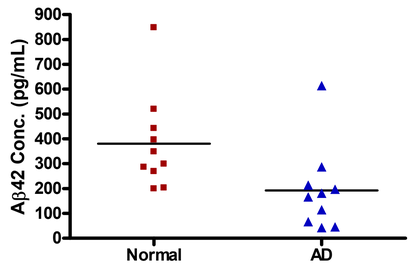


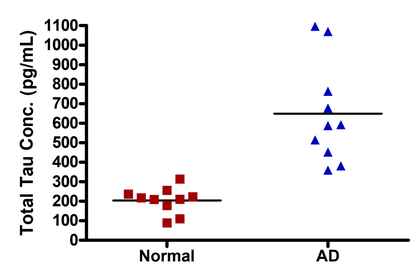




7 Samples

Individual normal and AD patient CSF samples and pooled human CSF samples were purchased from commercial vendors. Sample collection methods and pre-analytical variables may cause variability in the measured range of normal and diseased samples. The individual patient samples were well-curated; handling was consistent with accepted protocols. The commercial vendors that supplied the pooled CSF samples were not able to adhere to stringent collection and handling procedures. Samples were diluted 8-fold prior to measuring with the Human Aβ42 Kit. Samples were diluted 4-fold prior to measuring with the Human Total Tau Kit. The graph displays median and range of concentrations for each sample set. Concentrations have been corrected for sample dilution.





8 Conclusion

Analytical validation is essential to the development of biomarker assays that provide the accuracy, precision, and sensitivity required to measure endogenous levels of Human Aβ42 and Total Tau in normal and AD CSF samples. The impact of matrix interference factors and blood contamination is mitigated through adherence to this robust process. Analytical validation assures both assays meet the level of reproducibility and robustness consistent with "Fit-for-Purpose Method Development."

References

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