P2-036

Development and Analytical Validation of a Novel Assay for Detection of Aβ42 Peptide in Human CSF

Flora Berisha,[1] Jill Dunty,[2] Leonid Dzantiev,[2] Adam Simon,[3] Carol Gleason,[1] Oi Wong,[1] Mwanatumu Mbwana,[2] Sara Hapip,[2] Qian Ning,[2] Franklin Braffett,[2] Sarah Robles,[2] George Green,[1] Robert Neely,[1] Holly Soares,[1] James Wilbur,[2] Pankaj Oberoi,[2] Paul Rhyne,[1] David Stewart,[2] and Jacob N. Wohlstadter[2]

[1]**Bristol-Myers Squibb Company, Princeton, NJ;** [2]MESO SCALE DISCOVERY® (MSD), Gaithersburg, MD; [3]AJ Simon Enterprises LLC, Yardley, PA

1 Abstract

Background: Amyloid Beta 1–42 peptide (Aβ42) in human cerebrospinal fluid (CSF) has been a useful biomarker for the study of Alzheimer's disease (AD).[1-3] However, the majority of assays present challenges due to inter-lot variability and matrix intolerance.[4] Here we describe the development and analytical validation of a novel Aβ42 assay for analysis of human CSF using "Fit-for-Purpose" and Clinical and Laboratory Standards Institute (CLSI) principles. Elements of assay development are presented with results of multi-lot validation demonstrating robust performance and consistency.

Methods: Antibodies against both the C-terminal and N-Terminal regions of Aβ42 were selected based on sensitivity, specificity, physical properties, and CSF sample discrimination. A set of bioanalytical tests were performed on the critical reagents (antibodies, peptides, and controls) to ensure purity, integrity, performance, and lot-to-lot consistency. The assay was developed using MESO SCALE DISCOVERY MULTI-ARRAY® technology and has been optimized to minimize CSF matrix effects and interferences. Analytical validation was performed across three independent kit lots to verify consistency, sensitivity, accuracy, and precision. Human CSF samples were used to establish the dynamic range of the assay and sample performance characteristics.

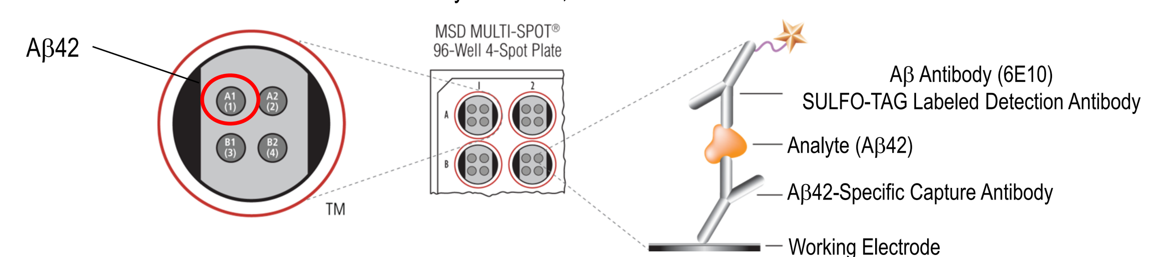
Results: The Aβ42 assay demonstrated excellent sensitivity, performance, and inter-lot reproducibility and was able to measure differences in Aβ42 levels between normal and AD samples. The results showed an average LLOD (66 runs, 3 lots) of 0.35 pg/mL with a quantitative range of 3 to 2000 pg/mL. The precision, accuracy, and total error were determined from human CSF control samples with typical inter- and intra-plate precision of <12% CV. Dilution linearity and spike recovery testing demonstrated minimal matrix effects at 1:8 dilution of CSF as well as accurate quantitation of Aβ42 peptide over the range of the assay. The assay exhibited tolerance of CSF contamination with hemolyzed blood and showed no significant cross-reactivity to closely related Aβ peptides, suggesting the assay was highly specific for Aβ42.

Conclusion: A new assay was developed and analytically validated to measure Aβ42 in human CSF. The Aβ42 assay had good analytical performance characteristics, inter-lot consistency, and the ability to distinguish between normal and AD CSF samples based on Aβ42 levels. This assay will support ongoing efforts to standardize AD biomarker testing.

2 Assay Development and Validation

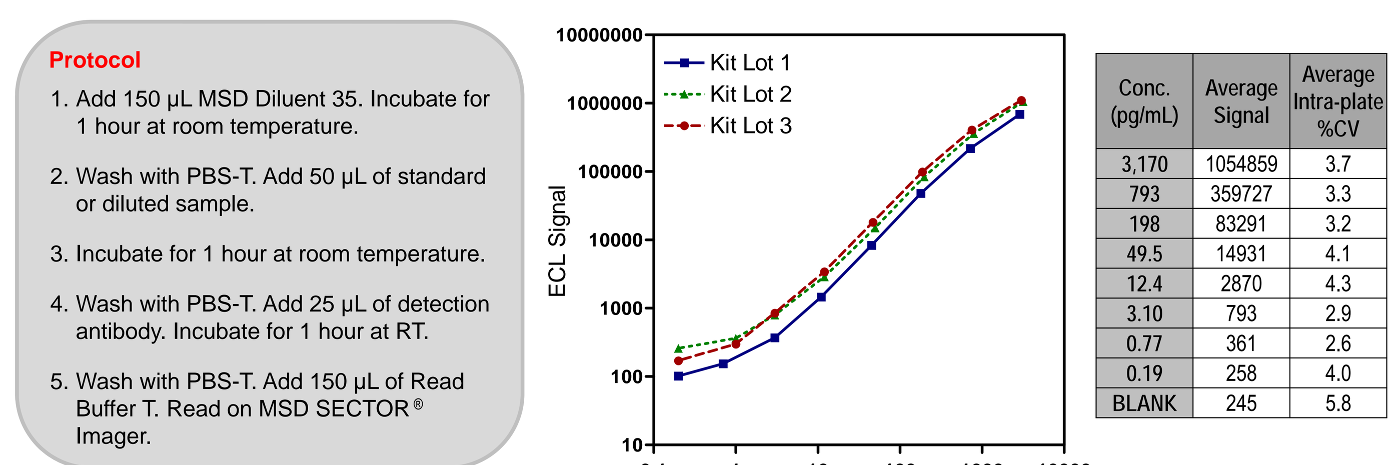
Development of the MSD® Human Aβ42 Kit* was tailored to address shortcomings of existing kits on the market and was conducted under design and development controls. The assay was built on MSD's electrochemiluminescence detection technology platform. SULFO-TAG™ labels emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY and MULTI-SPOT® microplates. The assay uses an Aβ-specific capture antibody (clone 12F4**), synthetic Aβ42 calibrator, and a total Aβ (clone 6E10**) detection antibody. The assay diluent, Diluent 35, was optimized to minimize matrix effects in human CSF. MSD recommends a minimum 8-fold sample dilution; given the assay's good sensitivity and linearity, a higher dilution factor may be used if desired. Calibrator handling and assay protocol are optimized for human CSF.

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 Aβ42 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 of this kit for longitudinal studies. The kit performance 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 Aβ42 assay protocol is described; this is a washed assay. **Middle:** Standard curves from three independently built 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 kit.



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
3,170	1054859	3.7
793	359727	3.3
198	83291	3.2
49.5	14931	4.1
12.4	2870	4.3
3.10	793	2.9
0.77	361	2.6
0.19	258	4.0
BLANK	245	5.8

*MSD offers the Human Aβ42 Kit for purchase in 1-, 5-, and 25-plate kit sizes (catalog numbers K151LBE-1, K151LBE-2, K151LBE-4, respectively).

**The 6E10 and 12F4 antibodies used in MSD Alzheimer's disease kits are supplied by Covance Research Products, Inc.

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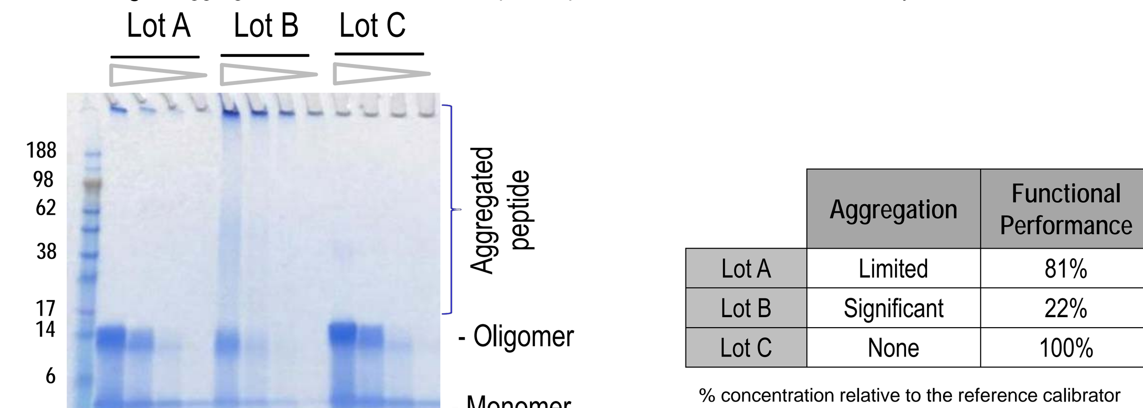
4 Peptide Calibrator Development

Lyophilized Aβ42 synthetic peptides purchased from three vendors were screened using analytical and functional characterization. One vendor was selected, and multiple lots of the material from this vendor were evaluated for consistency and performance. **Left:** Representative data from three vendor lots of Aβ42 synthetic peptide calibrator were characterized by gel electrophoresis. Differences in the degree of aggregation were observed across lots. **Right:** Aggregation state correlates with poorer performance in the functional assay.

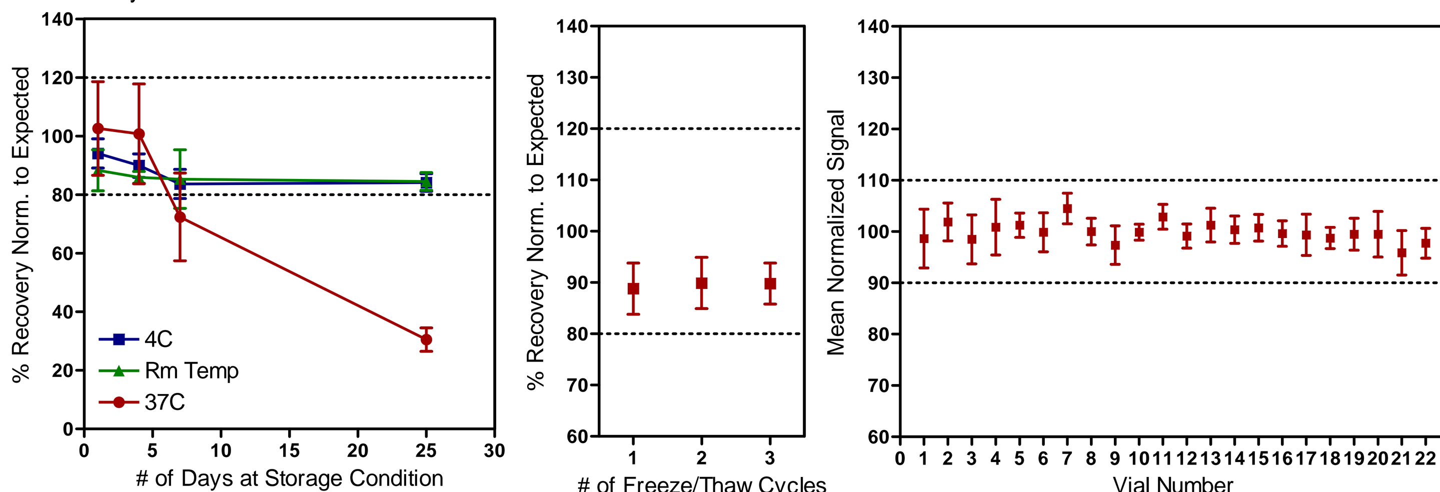


	Aggregation	Functional Performance
Lot A	Limited	81%
Lot B	Significant	22%
Lot C	None	100%

% concentration relative to the reference calibrator

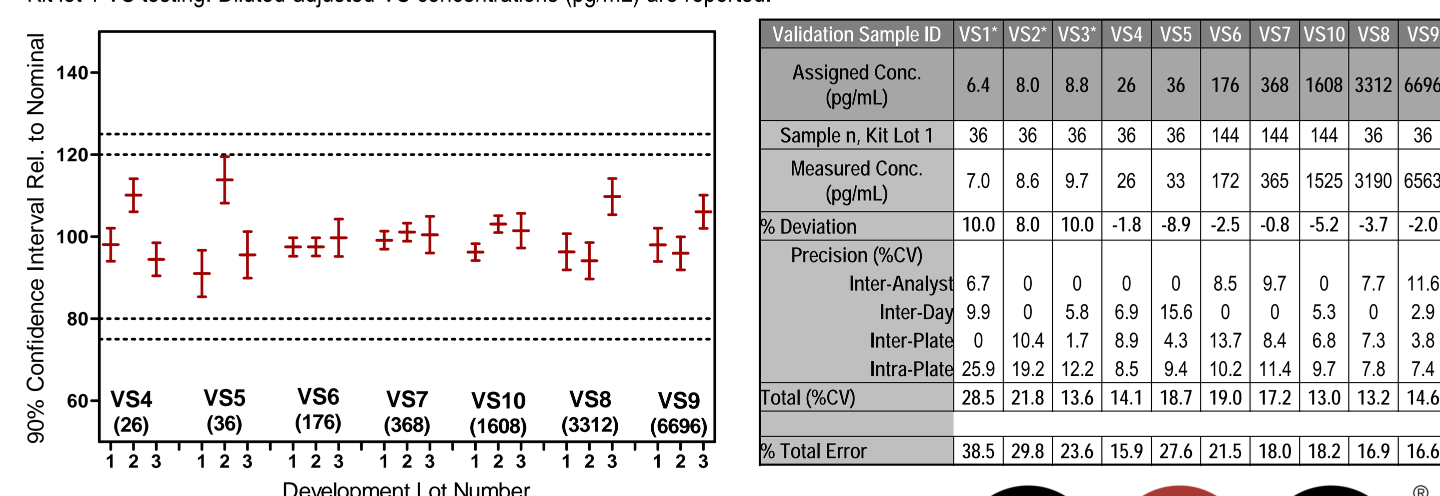
5 Peptide Calibrator Robustness

The handling strategy for reconstitution of Aβ42 peptide was optimized to minimize aggregate formation and maximize stability and consistency. Kit calibrator vials were provided as single-use aliquots that should be stored at ≤-70°C. A reference stock of calibrator was created for use in bridging kit lots. **Left:** Kit calibrator vials were stored at 4°C, room temperature, or 37°C for the indicated time periods. Kit calibrator vials were stable at 4°C and room temperature for greater than one week. **Middle:** Kit calibrator vials were robust through at least three freeze-thaw cycles. **Right:** Twenty-two vials pulled throughout the course of a 400-vial production run were diluted to Std03 (198 pg/mL) and measured for inter-vial consistency.



6 Measurement of Validation Samples

Ten Validation samples (VS) built in human pooled CSF (6.4-6696 pg/mL Aβ42) were used to survey the lower limit of quantitation (LLOQ). VS1-3(*) were built using immunodepleted matrix spiked with calibrator, and were purposely built below the predicted LLOQ. The remaining VS contained endogenous Aβ42 and some were spiked with calibrator to achieve the desired range. VS 1-10 were measured across runs, analysts, and kit lots. **Left:** Summary of measured concentrations across kit lots for VS that fall within the quantitative range of the assay (VS 4-10). Variation (%CV) among the three development lots ranged from 0.1-11% across VSs (data not shown). **Right:** Representative ANOVA analysis: Kit Lot 1 VS testing. Diluted-adjusted VS concentrations (pg/mL) are reported.



Validation Sample ID	VS1*	VS2*	VS3*	VS4	VS5	VS6	VS7	VS10	VS8	VS9
Assigned Conc. (pg/mL)	6.4	8.0	8.8	26	36	176	368	1608	3312	6696
Sample n, Kit Lot 1	36	36	36	36	36	144	144	144	36	36
Measured Conc. (pg/mL)	7.0	8.6	9.7	26	33	172	365	1525	3190	6563
% Deviation	10.0	8.0	10.0	-1.8	-8.9	-2.5	-0.8	-5.2	-3.7	-2.0
Precision (%CV)										
Inter-Analyst	6.7	0	0	0	0	8.5	9.7	0	7.7	11.6
Inter-Day	9.9	0	5.8	6.9	15.6	0	0	5.3	0	2.9
Inter-Plate	0	10.4	1.7	8.9	4.3	13.7	8.4	6.8	7.3	3.8
Intra-Plate	25.9	19.2	12.2	8.5	9.4	10.2	11.4	9.7	7.8	7.4
Total (%CV)	28.5	21.8	13.6	14.1	18.1	19.0	17.2	13.0	13.2	14.6
% Total Error	38.5	29.8	23.6	15.9	27.6	21.5	18.0	18.2	16.9	16.6

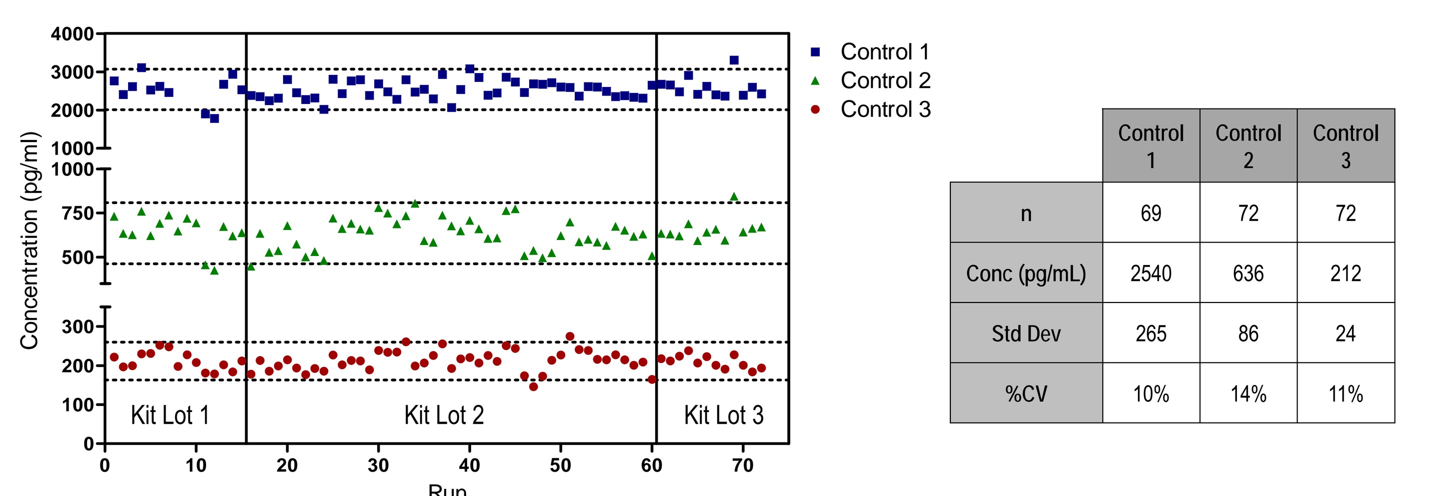
7 Assay Sensitivity

The Human Aβ42 Kit is sensitive and measures Aβ42 over a wide dynamic range. Assay sensitivity (LLOD), lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) were determined for each of three independent kit lots. The LLOD is a calculated concentration based on a signal 2.5 standard deviations above the blank (zero calibrator). The range of LLODs measured across three kit lots (N=66 plates) is presented; the average was 0.35 pg/mL. The ULOQ and LLOQ samples were created by spiking a known value of Aβ42 calibrator into diluent. 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. The quantitative range is 3.0-2700 pg/mL. Testing for each kit involved a minimum of 12 runs conducted by three analysts across at least three days of testing (N=42 runs across 3 kit lots). 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	2700	2559	14.4	95%	2748	8.1	102%	2,799	15.4	104%
LLOQ	3.0	3.23	17.0	108%	3.53	7.9	118%	2.95	16.2	98%
Average LLOD (pg/mL)		0.08-0.7			0.12-0.83			0.07-0.96		

8 Real Time Stability of Controls

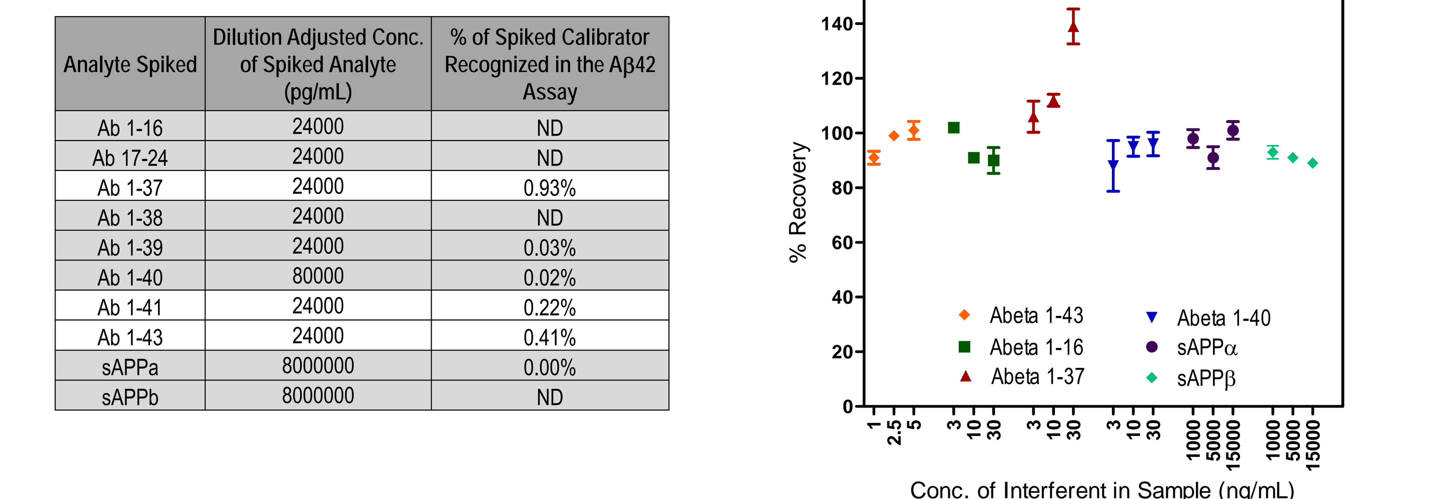
Aβ42 levels were measured in matrix-based controls (n=3). Measurements were made across three kit lots using multiple analysts and plates during runs over a five month period. **Left:** Results for the three controls, tested on kit lots 1–3, are presented. Dotted lines represent two standard deviations above and below the assigned concentration. **Right:** The precision results for the controls are presented. Reported concentrations are adjusted for sample dilution.



	Control 1	Control 2	Control 3
n	69	72	72
Conc (pg/mL)	2540	636	212
Std Dev	265	86	24
%CV	10%	14%	11%

9 Specificity and Interference

The Human Aβ42 assay is designed to recognize the Aβ42 peptide specifically, and to minimize interferences in Aβ42 measurement. **Left:** Different Aβ peptides and amyloid precursor proteins were spiked into Diluent 35 and tested with the Human Aβ42 assay. Concentrations shown in the table have been corrected for sample dilution. The assay detects very low levels of Aβ1-41, 1-43, and 1-37. Grey shading indicates values outside the LOQ of the Aβ42 assay. ND indicates not detected. **Right:** Various Aβ peptides and amyloid precursor proteins were spiked into human CSF at levels that exceed the expected endogenous levels for these analytes.[6] 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. Comparable results were obtained when Aβ42 calibrator was co-spiked with excess Aβ peptides and amyloid precursor proteins in Diluent 35 (data not shown).



Analyte Spiked	Dilution Adjusted Conc. of Spiked Analyte (pg/mL)	% of Spiked Calibrator Recognized in the Aβ42 Assay
Ab 1-16	24000	ND
Ab 17-24	24000	ND
Ab 1-37	24000	0.93%
Ab 1-38	24000	ND
Ab 1-39	24000	0.03%
Ab 1-40	80000	0.02%
Ab 1-41	24000	0.22%
Ab 1-43	24000	0.41%
sAPPα	8000000	0.00%
sAPPβ	8000000	ND

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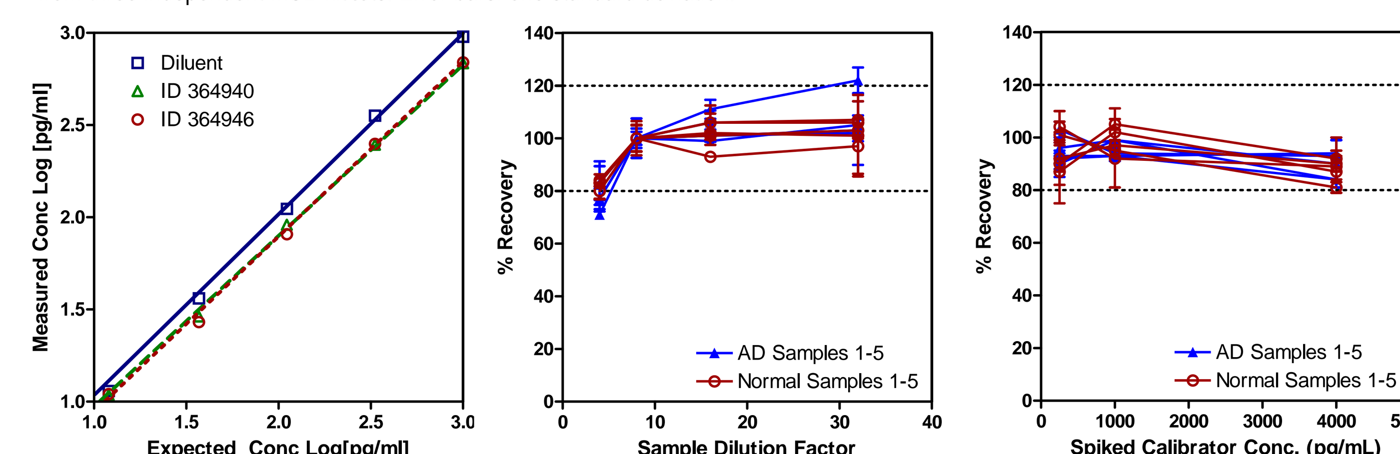
10 Matrix Tolerance

Left: Matrix tolerance using the optimized assay diluent, Diluent 35, was evaluated using immunodepleted (ID), pooled human CSF (n=2). Recovery of Aβ42 calibrator spiked into ID CSF and diluent was parallel, although there is a bias. **Middle:** CSF samples from well-curated normal and AD individuals were diluted 4-, 8-, 16-, and 32-fold with Diluent 35. Measured concentrations were corrected for dilution factor. Recovery at each dilution was calculated relative to the optimal sample dilution (8-fold). **Right:** Well-curated CSF samples were spiked with calibrator at multiple levels, diluted 8-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.

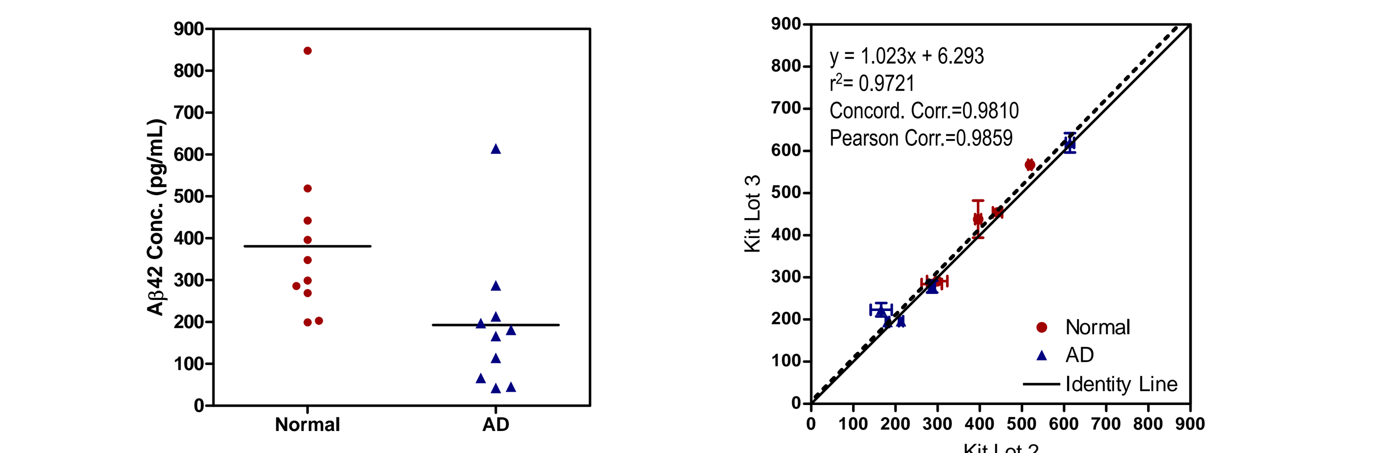


11 Tolerance to Blood Interference

The Human Aβ42 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 Aβ42 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 8-fold and tested with the Human Aβ42 Kit. The measured Ab42 concentration relative to the unspiked sample is plotted. Results are representative of data collected across three independent MSD kit lots. Error bars: one standard deviation. **Right/Bottom:** Samples with 0.1% contamination are tinged slightly pink; samples with 1% contamination are red and easily identified as contaminated.

12 Measurement of Patient Samples

Left: Aβ42 levels were measured in well-curated individual normal and AD patient samples (n=10 each). The Aβ42 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. A good correlation in measured concentration and agreement was observed between the two kit lots. Reported concentrations are adjusted for an 8-fold dilution. Error bars: one standard deviation.



13 Conclusions

The MSD Human Aβ42 Kit has been analytically validated for Aβ42 measurement in human CSF. The kit was built using highly characterized, critical reagents and improved handling methods. The Human Aβ42 Kit exhibits improved robustness and reliability, good analytical performance, inter-lot consistency, and the ability to distinguish between normal and AD samples based on Aβ42 levels.

Acknowledgements

Current affiliation information is: Paul Rhyne, Tandem Labs, 115 Silvia Street, West Trenton, NJ 08628; Oi Wong, Merck, 90 East Scott Avenue Rahway, NJ 07065.

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