

Quantification of Higher Order Forms of Amyloid Beta and Total Abeta Peptides in CSF

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1 Abstract

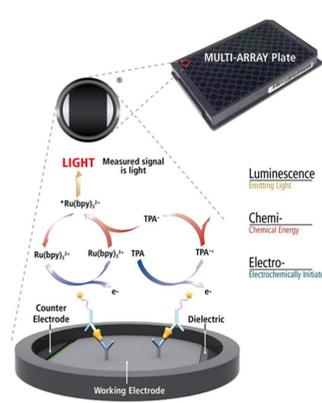
Background: The Aβ hypothesis of Alzheimer's disease (AD) posits that oligomers and/or aggregates, but not monomers or plaques, are the toxic species that result in neuronal loss. Therefore, assays are needed that can monitor higher order forms in patient samples. Assays that quantify total Aβ are also in demand for monitoring response to therapeutic treatment. We report the feasibility of a sandwich immunoassay that binds higher order forms of amyloid beta but not monomers. We also developed an assay that measures total Aβ. Both assays have been used to characterize CSF samples from healthy controls and patients with various neurological diseases.

Methods: We tested a variety of assay formats with N-terminal, C-terminal, and mid-epitope antibodies against Aβ peptides. Monomeric and oligomeric forms of Aβ40 and Aβ42 peptides were generated and used to develop the assays. The assay that was designed to measure oligomeric forms used a common N-terminal Aβ antibody for both capture and detection; the total Aβ assay used that same antibody for capture but used a mid-epitope antibody for detection. Standard curves presented here show the functionality and specificity of each assay. Aβ40, Aβ42, total Aβ, and oligomeric forms of Aβ peptide were measured in human CSF.

Results: The total Aβ assay had a sensitivity of <5 pg/mL of oligomerized Aβ42. The oligomeric assay did not detect monomeric Aβ40 but had a sensitivity below approximately 10 pg/mL of oligomerized Aβ42. (The precise concentration of the oligomerized peptide was difficult to measure.) Although oligomeric forms of Aβ were not detectable in the CSF samples, roughly 5–20 ng/mL of total Aβ was detected in the same samples. The total Aβ levels were greater than the sum of the individual Aβ40 and Aβ42 peptides measured independently.

Conclusion: We have developed assays for total and oligomeric forms of Aβ peptides. The total Aβ assay was extremely sensitive and could measure differences within a common set of human CSF samples. We were unable to measure oligomeric forms of Aβ in normal CSF. The development of these assays should enable the evaluation of relative levels Aβ peptide forms in normal and AD cohorts.

2 Methods



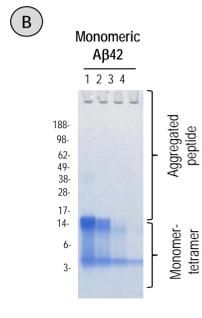
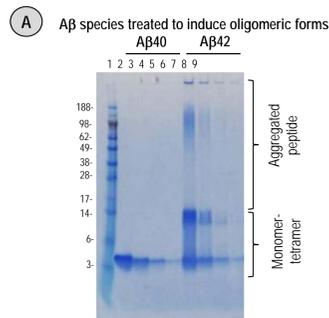
- MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels, which emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT™ microplates.
- Higher order forms of Aβ42 were generated and several assay formats with N-terminal, C-terminal, and mid-epitope antibodies were examined for their ability to measure oligomerized Aβ42.
- All assays used the protocol described below.
- Human CSF pools (remnant samples) and well-cured human CSF from individual donors were tested with selected assays.
- Standard curves were analyzed using a 4-parameter logistic model with 1/y² weighting. Error bars = 1 standard deviation (SD).

Protocol

- Add 150 μL of MSD® Diluent 35. Incubate for 1 hour at room temperature (RT) with shaking.
- Wash 3X with PBS-T. Add 50 μL of standard or diluted sample. Incubate for 1 hour at RT with shaking.
- Wash 3X with PBS-T. Add 25 μL of detection antibody. Incubate for 1 hour at RT with shaking.
- Wash 3X with PBS-T. Add 150 μL of MSD Read Buffer T. Read on MSD instrument.

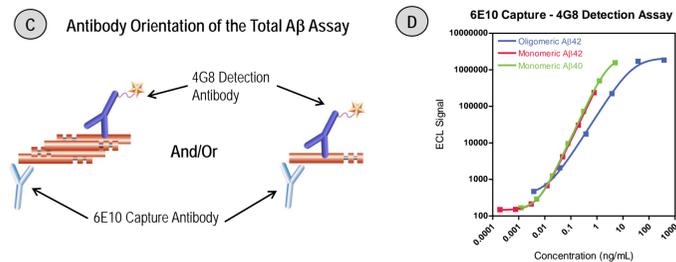
3 Higher Order Forms of Aβ Peptides

Agitation and heat were applied to Aβ40 and Aβ42 peptides to induce higher order forms. Aβ peptides were serially diluted (0.375, 0.125, 0.042, and 0.014 mg/mL) and characterized by gel electrophoresis. The SDS-Page gel (Figure A) shows aggregated peptide was formed for Aβ42, but not for Aβ40. In contrast, Figure B indicates that Aβ42 peptides prepared according to MSD manufacturing conditions do not form soluble oligomers but instead display bands at the monomer/tetramer molecular weight marker. The oligomeric Aβ42 species was used in subsequent assays and the concentration was calculated as 378 ug/mL by a BCA Protein Assay. No other method was available to accurately measure the number of aggregated particles.



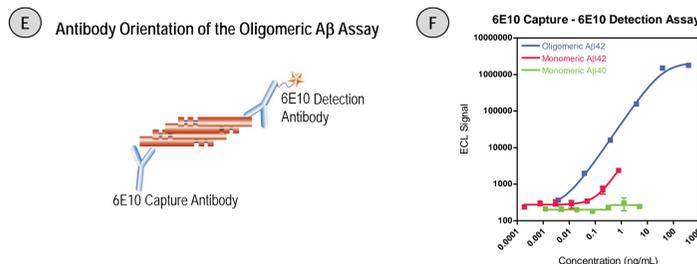
4 Total Aβ Assay

The total Aβ assay uses an N-terminal capture antibody (6E10*) and a mid-epitope detection antibody (4G8*) (Figure C). The assay detects and measures both monomeric Aβ and oligomeric Aβ species (Figure D). The Hill slopes and sensitivities for oligomeric Aβ42, monomeric Aβ42, and monomeric Aβ40 are: 1.1 and <5 pg/mL; 1.5 and 1.53 pg/mL; and 1.5 and 1.57 pg/mL, respectively.



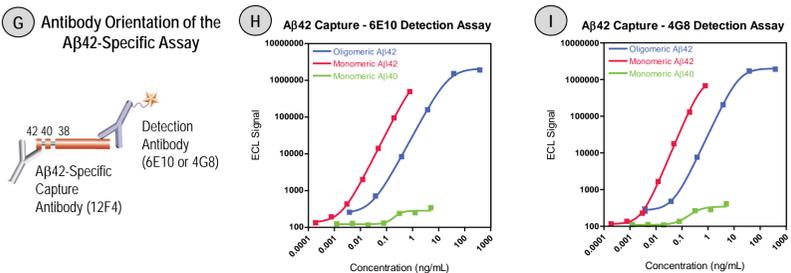
5 Oligomeric Aβ Assay

The oligomeric Aβ assay format uses the same N-terminal antibody (6E10) as both the capture and detection antibody (Figure E). This assay format can detect soluble Aβ oligomers that expose multiple Aβ N-termini within the higher order structural conformation of aggregated Aβ. As shown in Figure F, the oligomeric assay detects <10 pg/mL of oligomeric Aβ42 with a Hill slope of 1.0. The monomeric Aβ species did not detect monomeric Aβ40, but did appear to detect trace amounts of monomeric Aβ42 with a sensitivity of >100 pg/mL.



6 Peptide-Specific Aβ Assay: Aβ42-Specific Capture

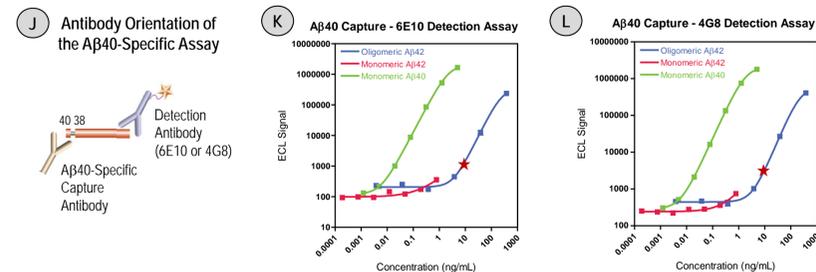
The Aβ42-specific assay format uses a capture antibody (12F4*) that is reactive to the C-terminus of Aβ42. The sandwich immunoassay is completed using a detection antibody that is reactive to either the N-terminus (6E10) or mid-region (4G8) of Aβ peptides (Figure G). Both assay formats measured oligomeric Aβ42 and monomeric Aβ42, but detected negligible levels of monomeric Aβ40 (Figures H and I). For the Aβ42-specific assay that used 6E10 detection, Hill slopes and sensitivities are: 1.3 and <10 pg/mL (oligomeric) and 1.4 and 0.67 pg/mL (monomeric). For the Aβ42-specific assay using 4G8 detection, Hill slopes and sensitivities are: 1.5 and <100 pg/mL (oligomeric) and 1.7 and 1.77 pg/mL (monomeric).



*NOTE: The 6E10, 4G8, and 12F4 antibodies used in this study were supplied by Covance Research Products, Inc.

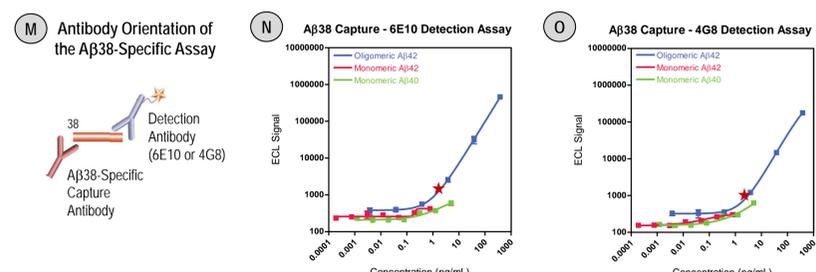
7 Peptide-Specific Aβ Assay: Aβ40-Specific Capture

The Aβ40-specific assay format uses a capture antibody that is reactive to the C-terminus of Aβ40. The sandwich immunoassay is completed using a detection antibody that is reactive to either the N-terminus (6E10) or mid-region (4G8) of Aβ peptides (Figure J). Both assay formats measured monomeric Aβ40, but did not detect monomeric Aβ42 (Figures K and L). Aβ42 oligomers are less than 0.01% detectable compared to monomeric Aβ40. In the graphs below, the red star designates the upper limit of physiological levels of Aβ40 in human CSF. For the Aβ40-specific assay, the Hill slopes and sensitivities for monomeric Aβ40 are: 1.6 and 1.96 pg/mL (6E10 detection) and 1.5 and 2.52 pg/mL (4G8 detection).



8 Peptide-Specific Aβ Assay: Aβ38-Specific Capture

The Aβ38-specific assay format uses a capture antibody that is reactive to the C-terminus of Aβ38. The sandwich immunoassay is completed using a detection antibody that is reactive to either the N-terminus (6E10) or mid-region (4G8) of Aβ peptides (Figure M). Neither assay detected monomeric Aβ42 or monomeric Aβ40 (Figures N and O). Aβ42 oligomers are not detected within physiologically relevant concentrations: the red star designates the upper limit of physiological levels of Aβ38 in human CSF. We speculate that high concentrations of aggregated Aβ42 bind non-specifically in both the Aβ38- and Aβ40-specific capture assays.



9 Total Aβ Assay: Aβ Peptide Titration Study

An Aβ peptide titration study was used to assess the ability of the total Aβ assay to detect various combinations of monomeric Aβ42 and monomeric Aβ40 peptides. The measured concentration (i.e., apparent Aβ40, ng/mL) was determined for: (1) a constant level of Aβ42 (0.3 ng/mL) spiked with various concentrations of Aβ40 (0, 0.5, 2.5, and 10 ng/mL) and (2) a constant level of Aβ40 (0.3 ng/mL) spiked with various concentrations of Aβ42 (0, 0.5, 2.5, and 10 ng/mL). In both cases, total Aβ, as determined by the sum of monomeric Aβ42 and monomeric Aβ40, increased in an additive manner, as measured with the total Aβ assay. NOTE: The concentrations designated with an asterisk in the table below represent measured concentrations that are above the limit of quantitation.

Spike Conc. (ng/mL)		Measured Conc. (ng/mL)
Aβ42	Aβ40	
0.3	0	0.3
0.3	0.5	0.8
0.3	2.5	3.0
0.3	10	6.3*
0	0.3	0.3
0.5	0.3	0.8
2.5	0.3	2.8
10	0.3	6.7*

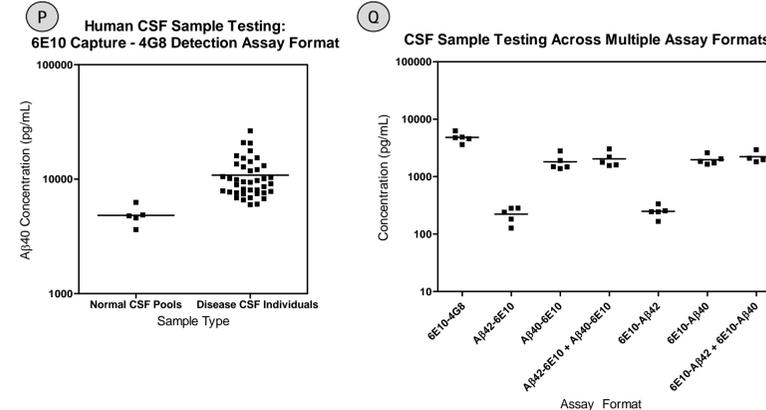
10 Oligomeric Aβ Assay: Interference Study

The ability of the oligomeric Aβ assay to measure interference from various combinations of monomeric Aβ42 and monomeric Aβ40 was examined. The measured ECL signal was compared for: (1) a constant level of Aβ42 (0.3 ng/mL) spiked with various concentrations of Aβ40 (0, 0.5, 2.5, and 10 ng/mL) and (2) a constant level of Aβ40 (0.3 ng/mL) spiked with various concentrations of Aβ42 (0, 0.5, 2.5, and 10 ng/mL). Varying the Aβ40 spike concentrations in a constant level of Aβ42 (0.3 ng/mL) did not impact the signal measured in the oligomeric assay. In contrast, the signal increases appreciably when increasing concentrations of Aβ42 are spiked into a constant level of Aβ40 (0.3 ng/mL). This may represent a change from monomeric Aβ42 to an aggregated state within the context of the experiment. The spiked levels of Aβ42 were >10 times the maximum physiological levels.

Spike Conc. (ng/mL)		Measured Signal
Aβ42	Aβ40	
0.3	0	1048
0.3	0.5	1239
0.3	2.5	1113
0.3	10	1051
0	0.3	176
0.5	0.3	1379
2.5	0.3	5987
10	0.3	18970

11 Human CSF Sample Testing

Five remnant CSF pools from normal donors and 40 well-cured CSF individual samples from patients with various neurological diseases were measured using the total Aβ and oligomeric Aβ assays. Higher order Aβ was not detectable in the oligomeric Aβ assay (data not shown). In contrast, total Aβ was detectable in the total Aβ assay and assessed in terms of apparent Aβ40 concentration (Figure P). Total Aβ levels were elevated in disease CSF samples compared to normal CSF pools. The mean, median, and range for the disease CSF samples are: 10.8 ng/mL, 9.4 ng/mL, and 6.0–26.5 ng/mL, respectively. The mean, median, and range for the normal CSF pools are: 4.8 ng/mL, 4.8 ng/mL, and 3.6–6.3 ng/mL, respectively. Figure Q compares Aβ peptide concentration detected in 5 remnant CSF pools measured using several assay formats. Interestingly, the sum of the mean concentrations of Aβ42 and Aβ40 detected in the peptide-specific assays is less than the total Aβ measured concentration. This result suggests that the total Aβ assay detects additional Aβ-related species in human CSF.



12 Conclusion

We present the development of two novel Aβ sandwich immunoassays for the measurement of either total Aβ or oligomeric Aβ. While higher order forms of Aβ were not detected in human CSF samples, substantial levels of total Aβ (5–20 ng/mL) were detected in the same set of samples. The total Aβ assay was extremely sensitive and discerned a difference in total Aβ between normal and disease CSF. The observation that total Aβ levels were greater than the sum of Aβ40 and Aβ42 measured in the peptide-specific assays suggests that the total Aβ assay detects additional Aβ isoforms. We anticipate that these assays will prove useful in advancing the understanding of Aβ pathogenesis and in evaluating the efficacy of novel therapeutics that target Aβ clearance.

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