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# Fully automated chemiluminescence enzyme immunoassays showing high correlation with immunoprecipitation mass spectrometry assays for β-amyloid (1–40) and (1–42) in plasma samples

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ABSTRACT

Blood based β-amyloid (Aβ) assays that can predict amyloid positivity in the brain are in high demand. Current studies that utilize immunoprecipitation mass spectrometry assay (IP-MS), which has high specificity for measuring analytes, have revealed that precise plasma Aβ assays have the potential to detect amyloid positivity in the brain. In this study, we developed plasma Aβ40 and Aβ42 immunoassays using a fully automated immunoassay platform that is used in routine clinical practice. Our assays showed high sensitivity (limit of quantification: 2.46 pg/mL [Aβ40] and 0.16 pg/mL [Aβ42]) and high reproducibility within-run (coefficients of variation [CVs]: <3.7% [Aβ40] and <2.0% [Aβ42]) and within-laboratory (CVs: <4.6% [Aβ40] and <5.3% [Aβ42]). The interference from plasma components was less than 10%, and the cross-reactivity with various lengths of Aβ peptides was less than 0.5%. In addition, we found a significant correlation between the IP-MS method and our immunoassay (correlation coefficients of Pearson's r: 0.91 [Aβ40] and 0.82 [Aβ42]). Our new method to quantify plasma Aβ40 and Aβ42 provides clinicians and patients with a way to continuously monitor disease progression.
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## 1. Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia and has a significant effect on global public health [1,2]. A key pathological hallmark of AD is the accumulation of β-amyloid protein (Aβ) in the brain [3]. This accumulation starts approximately 20 years before the onset of symptoms, such as cognitive decline [4,5]. Therefore, early detection of amyloid pathology is important for diagnosis and facilitates the recruitment of patients into AD clinical trials [6].

Currently, amyloid positron emission tomography (PET) and cerebrospinal fluid (CSF) tests, which measure the concentration of $Aβ_{1–42}$ (Aβ42) or the ratio of Aβ42 to $Aβ_{1–40}$ (Aβ40), are used to confirm amyloid pathology [7–9], but their use may be limited due to their cost, accessibility, and invasiveness [10,11]. To overcome these difficulties, there is a great need for immunoassays that can detect plasma Aβ levels, and numerous immunoassays have been developed for this purpose [12–15]. Despite these efforts, the development of plasma Aβ assays that can assess amyloid pathology in the brain has not been successful, because the levels of plasma Aβs are approximately 10 times lower than those of CSF, and high levels of assay interferants are contained in the plasma [16–18].

Nevertheless, owing to the advancement of analytical technology for detecting analytes, recent studies have shown that the ratio of plasma Aβ42 to Aβ40 has the potential to predict amyloid positivity in the brain as determined by amyloid PET [19,20]. In these

*Abbreviations:* Aβ, β-amyloid; AD, Alzheimer's disease; B/F, bound/free; CSF, cerebrospinal fluid; CV, Coefficients of variation; HISCL, High Sensitivity Chemiluminescence Enzyme Immunoassay; IP-MS, immunoprecipitation mass spectrometry assay; LoB, Limit of blank; LoD, Limit of detection; LoQ, Limit of quantification; MMSE, Mini-Mental State Examination; PET, Positron emission tomography.

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studies, plasma Aβs were measured using immunoprecipitation mass spectrometry assay (IP-MS), which has high specificity for measuring analytes because it identifies the target analytes directly based on their molecular weight [21].

In this study, we developed highly specific plasma Aβ40 and Aβ42 immunoassays using a fully automated immunochemistry analyzer that is used in routine clinical practice. The analyzer, High Sensitivity Chemiluminescence Enzyme Immunoassay (HISCL™) series, employs the chemiluminescence enzyme immunoassay methodology. The HISCL™ series are superior to conventional enzyme-linked immunosorbent assays because of their high reproducibility, wide dynamic range, and rapid reaction time, requiring only 17 min to complete [22–24]. Here, we describe the analytical performance of Aβ40 and Aβ42 assays. To verify the specificities of our assays, we assessed the correlation between the Aβ40 and Aβ42 assays and the IP-MS assays using plasma samples.

## 2. Materials and methods

### 2.1. Assay description

Aβ40 and Aβ42 immunoassays were developed using the HISCL™ series. In this system, the peptides were captured by a biotinylated antibody, which is specific to the N-terminus of Aβ, and immobilized on magnetic beads. After bound/free (B/F) separation, the antigen-antibody complex was bound to an alkaline phosphatase-conjugated antibody, which is specific to the C-terminus of Aβ40 or Aβ42, to form a sandwich immunocomplex. After a second B/F separation, the immunocomplex was incubated with a luminescent substrate, and the luminescence intensity was measured. The entire reaction was performed at 42 °C, and luminescence intensity was acquired within 17 min.

### 2.2. Standard curve

Chemically synthesized Aβ40 and Aβ42 peptides were used as calibration samples. These peptides were purchased from Peptide Institute (Osaka, Japan). The concentrations used were 2400, 600, 150, 37.5, and 9.38 pg/mL for Aβ40 and 4000, 500, 62.5, 7.81, and 0.98 pg/mL for Aβ42.

### 2.3. Sensitivity

The limit of blank (LoB), limit of detection (LoD), and limit of quantification (LoQ) values were determined according to the Clinical and Laboratory Standards Institute EP17-A2 guideline [25]. A calibration buffer was used for LoB analysis. For the LoD and LoQ analyses, five different concentrations of peptides diluted with calibration buffer were used.

### 2.4. High-dose hook effect

The high-dose hook effect was assessed using high-concentration peptides diluted with a calibration buffer. The samples had 10, 5, 2.5, and 1.25 times higher concentrations than the upper end of the calibration samples.

### 2.5. Precision

Three control samples (low, middle, and high) were used to assess repeatability (within-run) and intermediate precision (within-laboratory). These control samples were prepared using peptides and measured over 5 days with two runs per day and duplicate measurement per run.

### 2.6. Dilution linearity

Peptide-spiked plasma was diluted with a calibration buffer at ratios of 4:1, 3:2, 2:3, and 1:4. Percentage changes were evaluated using the expected concentration calculated based on the undiluted sample value.

### 2.7. Interference from endogenous substances

Possible interfering substances were spiked into plasma samples up to the following concentrations: hemoglobin (100 mg/dL), free bilirubin (20 mg/dL), conjugated bilirubin (20 mg/dL), chyle (1610 FTU), rheumatoid factor (500 IU/mL), and biotin (100 ng/mL). Biotin was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other substances were obtained from Interference Check A Plus and Interference Check RF (Sysmex, Kobe, Japan). The interference was evaluated as a percentage difference calculated using the following formula:

$$\left(\frac{\textit{Concentration of the spiked sample}}{\textit{Concentration of the non}-\textit{spiked sample}}-1\right)\times 100$$

Absence of interference was declared if the percentage difference was less than 10%.

### 2.8. Cross-reactivity

Plasma samples were spiked with different types of Aβ peptides at 1000 pg/mL. $A\beta_{1-38}$, $A\beta_{2-40}$, $A\beta_{3-40}$, $A\beta_{pyr3-40}$, $A\beta_{pyr3-42}$, $A\beta_{4-42}$, $A\beta_{11-40}$, $A\beta_{pyr11-40}$, $A\beta_{17-40}$, and $A\beta_{22-42}$ were used for this analysis. $A\beta_{1\text{-}38}$ was purchased from Peptide Institute (Osaka, Japan). All the other peptides were obtained from AnaSpec (Fremont, CA, USA). The cross-reactivity was calculated based on the following formula:

$$\frac{\textit{Concentration of the spiked sample}-\textit{Concentration of the non}-\textit{spiked sample}}{\textit{Cross}-\textit{reactant concentration}}\times 100$$

The absence of cross-reactivity was declared if the cross-reactivity was less than 10%.

### 2.9. Correlation with immunoprecipitation mass spectrometry assay (IP-MS)

For the correlation analysis, 20 commercially available plasma samples were purchased from PrecisionMed (Solana Beach, CA, USA) and BioIVT (Westbury, NY, USA) and assessed using Aβ immunoassays and an in-house IP-MS assay. Briefly, samples were first bound to anti-Aβ antibody-coated magnetic beads. After washing, Aβs were eluted and analyzed by liquid chromatography with tandem mass spectrometry. The detailed procedure of the in-house IP-MS assay has been described previously [26].

### 2.10. Statistical analysis

Statistical analysis was performed using StatFlex version 7.0 software (Artech Co. Ltd., Osaka, Japan). The Pearson correlation coefficient (Pearson's r) was calculated to analyze the correlation between the immunoassay and IP-MS assay. $P$ values $< 0.05$ were considered significant.

## 3. Results

### 3.1. Standard curve

Aβ40 and Aβ42 assays were standardized using the synthesized peptides. Chemiluminescent signals were measured as relative photon counts and directly correlated with the concentrations of Aβ40 and Aβ42. The standard curves were well fitted by a four-parameter logistic regression model with a coefficient of determination of $R^2 > 0.99$ (Fig. 1).

### 3.2. Sensitivity

LoB, LoD, and LoQ were evaluated to confirm the sensitivity of the Aβ40 and Aβ42 assays. For the Aβ40 assay, LoB, LoD, and LoQ were 0.57, 1.31, and 2.46 pg/mL, respectively. For the Aβ42 assay, LoB, LoD, and LoQ were 0.07, 0.13, and 0.16 pg/mL, respectively.

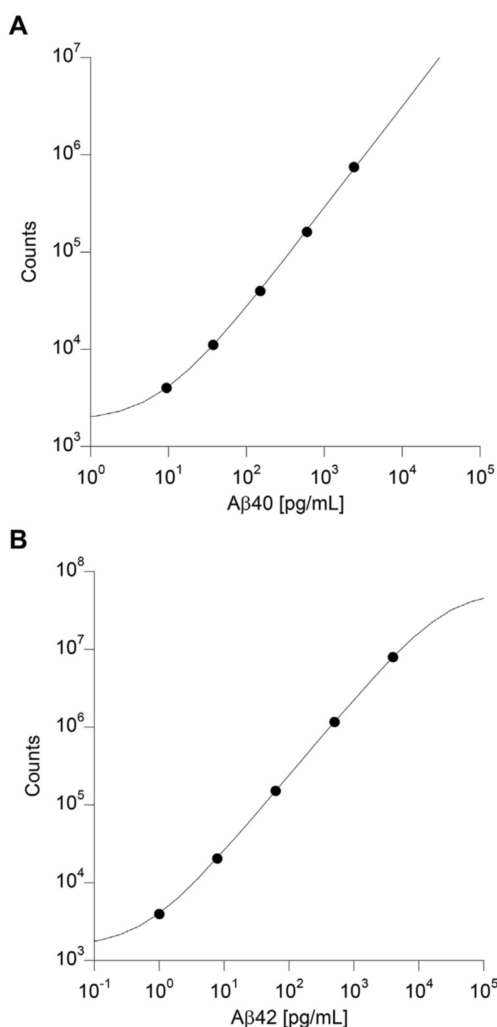


**Fig. 1.** Calibration curves of (A) β-amyloid (Aβ) 40 and (B) Aβ42. The association between relative photon counts and concentration of Aβs.

### 3.3. High-dose hook effect

The high-dose hook effect was evaluated by measuring the concentration of each peptide. No falsely low results were observed up to 24000 and 40000 pg/mL for Aβ40 and Aβ42, respectively. These concentrations exceeded the upper end of the calibration samples by tenfold.

### 3.4. Precision

Repeatability (within-run) and intermediate precision (within-laboratory) were determined using the low, middle, and high control samples. The repeatability coefficients of variation (CVs) of Aβ40 and Aβ42 were 2.0–3.7% and 1.7–2.0%, respectively. The intermediate CVs of Aβ40 and Aβ42 were 2.3–4.6% and 4.2–5.3%, respectively (Table 1).

### 3.5. Dilution linearity

Dilution linearity was assessed using plasma samples spiked with the peptides. Percentage changes from undiluted sample were 0.1–10% for Aβ40 and 6.9–18% for Aβ42 (Table S1).

### 3.6. Interference from endogenous substances

Interference of common blood components was assessed by measuring plasma samples spiked with interfering substances. For both assays, the percentage differences were less than 10% up to the tested concentrations (Table S2).

### 3.7. Cross-reactivity

Table 2 shows the cross-reactivity of various Aβ peptides. In the Aβ40 assay, the cross-reactivity was less than 0.5% for various lengths of Aβ peptides (−0.4–0.4% with the 1000 pg/mL peptides). The cross-reactivity of the Aβ42 assay was also less than 0.5% for various lengths of Aβ peptides (−0.1–0.1% with the 1000 pg/mL peptides).

### 3.8. Correlation with IP-MS

To verify the specificity of our immunoassays based on HISCL™ series, the correlation with IP-MS was assessed by measuring plasma samples. Both Aβ40 and Aβ42 assays were significantly correlated with IP-MS (Fig. 2). The correlation coefficients of Pearson's r were 0.91 (Aβ40) and 0.82 (Aβ42).

## 4. Discussion

In this study, we described the analytical performance of our fully automated immunoassays that allow the quantification of Aβ40 and Aβ42 in plasma. The dynamic ranges of our assays are sufficiently wide to cover the possible concentration distributions of Aβs in plasma without a high-dose hook effect [27,28]. We achieved high sensitivity comparable to previously reported highly sensitive assays with a small amount of sample (30 μL) and rapid measurement (17 min) [29,30]. Our assays showed high reproducibility within-run and within-laboratory. Furthermore, the results of dilution linearity and interference from endogenous substances indicate that our assays are robust. We also showed that there was significantly little cross-reactivity (<0.5%) with Aβ peptides of various lengths. Finally, we confirmed the correlation between our immunoassays and IP-MS assays.

**Table 1**
Analytical performance data for repeatability and intermediate precision of our immunoassay.

| | Control sample | Mean measured concentration (pg/mL) | Repeatability (%CV) | Intermediate precision (%CV) |
|---|---|---|---|---|
| Aβ40 | Low | 27.6 | 3.7 | 4.6 |
| | Middle | 104 | 2.0 | 2.3 |
| | High | 378 | 2.9 | 3.4 |
| Aβ42 | Low | 8.73 | 1.7 | 4.2 |
| | Middle | 79.4 | 1.8 | 4.9 |
| | High | 537 | 2.0 | 5.3 |

CV, coefficients of variation.

**Table 2**
Cross-reactivity against various lengths of β-amyloid peptides.

| | Cross-reactivity (%) | |
|---|---|---|
| Length of Aβ | Aβ40 assay | Aβ42 assay |
| 1–40 | - | 0.1 |
| 1–42 | −0.1 | - |
| 1–38 | −0.2 | 0.1 |
| 2–40 | 0.4 | 0.0 |
| 3–40 | 0.2 | 0.0 |
| Pyr3–40 | 0.1 | 0.0 |
| Pyr3–42 | −0.3 | 0.0 |
| 4–42 | −0.2 | 0.0 |
| 11–40 | −0.3 | 0.0 |
| Pyr11–40 | −0.1 | 0.0 |
| 17–40 | −0.2 | −0.1 |
| 22–42 | −0.4 | −0.1 |

Our automated Aβ immunoassays are easy to use for high-throughput testing of a small amount of plasma, suggesting that our assays have great potential for use in clinical practice. In fact, conventional biomarkers, such as the hepatitis B virus antigen, have been measured using our immunoassay analyzer in clinical settings.

We verified the specificities of our plasma Aβ assays by evaluating their correlation with the IP-MS method. The significant correlations between our immunoassays and IP-MS assays suggested that our immunoassays have a high specificity that is comparable to the IP-MS assays. Our immunoassays can accurately quantify analytes in plasma, indicating that our plasma Aβ assays may predict amyloid deposition in the brain.

The potential availability of our method improves the efficiency of the evaluation in patients with suspected dementia based on cognitive assessments, such as the Mini-Mental State Examination (MMSE). This leads to a shortened time to integrate patients with early-stage AD into primary care workflows or assessment by specialists to support AD diagnosis that does not require PET or CSF tests. In addition, the combination of MMSE and immunoassay could likely lead to reduced medical costs associated with employing specialists when an immunoassay becomes a routine clinical practice [31].

To assess the clinical utility of our immunoassays, future studies are needed to measure clinical samples from patients with amyloid PET information. We will evaluate the correlation between our immunoassays and amyloid PET data to clarify that our blood test has a potential to predict amyloid positivity in the brain.

In conclusion, we have developed a highly sensitive blood Aβ assay that is feasible for application in routine clinical tests. Currently, drugs are available, and this method can allow a high-throughput analysis for the prescreening and monitoring of Aβ40 and Aβ42. Although further validation is needed, this method may provide rapid and effective identification of patients for PET and CSF testing or treatment with approved drugs in the future.

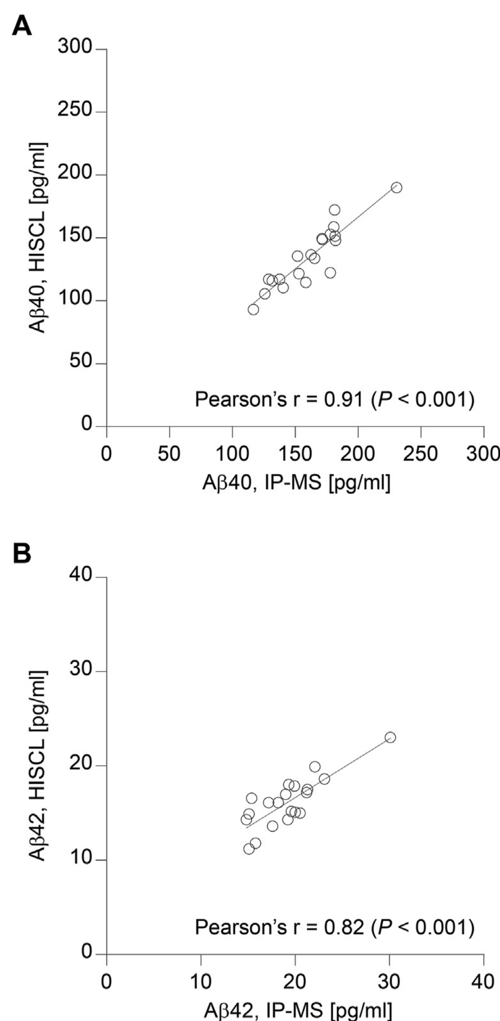


**Fig. 2.** Correlation between High Sensitivity Chemiluminescence Enzyme Immunoassay (HISCL) and the immunoprecipitation mass spectrometry (IP-MS) assay. Twenty plasma samples were measured with HISCL assay and IP-MS for (A) β-amyloid (Aβ) 40 and (B) Aβ42, respectively. The Pearson correlation coefficient (Pearson's r) was calculated to analyze the correlation.

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## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: KY, SW, KI, MM, YI, JM, KH, and SI are employees of

Sysmex Corporation. TK is an employee of Sysmex R&D Center Americas, Inc. TY is a board member of Sysmex Corporation.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.08.066.

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