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Performance Characteristics of Plasma Amyloid β 40 and 42 Assays

Olivia I. Okereke[1,2], **Weiming Xia**[3], **Michael C. Irizarry**[4,*], **Xiaoyan Sun**[5], **Wei Q. Qiu**[5], **Anne M. Fagan**[6], **Pankaj D. Mehta**[7], **Bradley T. Hyman**[4], **Dennis J. Selkoe**[3], and **Francine Grodstein**[1,2,8]

[1]Division of Aging, Harvard Medical School, Boston, MA, USA

[2]Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, and Harvard Medical School, Boston, MA, USA

[3]Center for Neurologic Diseases, Department of Neurology, Brigham and Women's Hospital, and Harvard Medical School, Boston, MA, USA

[4]Alzheimer Disease Research Unit, Department of Neurology, Massachusetts General Hospital, Boston, MA, USA

[5]Department of Psychiatry, Tufts-New England Medical Center, Tufts University School of Medicine, Boston, MA, USA

[6]Department of Neurology, Washington University School of Medicine, St. Louis, MO, USA

[7]Department of Immunology, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, NY, USA

[8]Department of Epidemiology, Harvard School of Public Health, Boston, MA, USA

Abstract

Background—Identifying biomarkers of Alzheimer disease (AD) risk will be critical to effective AD prevention. Levels of circulating amyloid β (Aβ) 40 and 42 may be candidate biomarkers. However, properties of plasma Aβ assays must be established.

Methods—Using five different protocols, blinded samples were used to assess: intra-assay reproducibility; impact of EDTA vs. heparin anticoagulant tubes; and effect of time-to-blood processing. In addition, percent recovery of known Aβ concentrations in spiked samples was assessed.

Results—Median intra-assay coefficients of variation (CVs) for the assay protocols ranged from 6–24% for Aβ-40, and 8–14% for Aβ-42. There were no systematic differences in reproducibility by collection method. Plasma concentrations of Aβ (particularly Aβ-42) appeared stable in whole blood kept in ice packs and processed as long as 24 hours after collection. Recovery of expected concentrations was modest, ranging from -24% to 44% recovery of Aβ-40, and 17% to 61% of Aβ-42.

Address correspondence to Dr. Olivia Okereke, Channing Laboratory 3rd floor, 181 Longwood Avenue, Boston, MA 02115. Email: ookereke@partners.org. Phone: (617) 525-2027. Fax: (617) 525-2008.
*Dr. Irizarry's new affiliation is GlaxoSmithKline, Research Triangle Park, NC, USA.
Reprint requests to: Dr. Olivia Okereke, Channing Laboratory 3rd floor, 181 Longwood Avenue, Boston, MA 02115.

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The authors have no actual or potential conflicts of interest pertaining to this manuscript.

Conclusions—Across five protocols, plasma Aβ-40 and Aβ-42 levels were measured with generally low error, and measurements appeared similar in blood collected in EDTA vs. heparin. While these preliminary findings suggest that measuring plasma Aβ-40 and Aβ-42 may be feasible in varied research settings, additional work in this area is necessary.

Keywords

Alzheimer disease; amyloid; assay reliability; biomarker; quality control

Introduction

Alzheimer disease (AD) may take years, if not decades, to develop. Thus, identifying markers that can predict AD risk prior to clinical onset will be critical to effective prevention and early intervention [3]. However, for maximum utility in large-scale research studies, it is important that such biomarkers are easily-measured, at low-cost and with high precision. Levels of circulating amyloid β (Aβ) peptide ending at amino acid 40 (Aβ-40) and 42 (Aβ-42) are potential biomarkers of AD risk, both due to the central role of Aβ accumulation in AD pathology [6], and the recent availability of sensitive assays to detect the low Aβ levels that are in plasma. Cross-sectional reports [4,9,15] have demonstrated inconsistent associations between plasma Aβ levels and AD [7]. Limited prospective data relating plasma Aβ to risk of AD and dementia have also yielded mixed results: studies generally have reported significant associations between plasma Aβ and risk of AD and dementia, but the direction of the associations have varied with respect to the levels of Aβ-40 and/or Aβ-42 [5,8,17,20]. Still, very few studies have been conducted, and the ease of acquiring venous blood samples clearly renders plasma Aβ a worthwhile candidate biomarker for further inquiry. However, before plasma Aβ can be considered for any broad applications, including additional large-scale epidemiologic studies, it is essential to investigate performance of the assay in a variety of research settings.

We investigated characteristics of five assay protocols for plasma Aβ-40 and Aβ-42 that were performed by several U.S. research laboratories. The aims of this study were to determine: reproducibility of assay results; comparability of assay results across blood samples collected in different anticoagulant media; performance of the assay for assessing absolute levels of Aβ in spiked plasma samples with known amounts of peptide; and stability of Aβ in plasma after varying delays in processing of whole blood samples.

Materials and Methods

Specimen collection and preparation

We conducted several studies to evaluate performance characteristics of the plasma Aβ assays. Not all protocols were examined in all aspects of these studies. All participating labs received blinded blood samples that had been processed in identical fashion. Unless otherwise specified, venous blood was centrifuged (2500 × *g* for 15 minutes) within a few hours of collection; plasma was then aliquotted into cryotubes and stored in liquid nitrogen freezers for less than one year until thawed for the current studies.

First, to evaluate error in measurements of plasma Aβ-40 and Aβ-42, as well as the extent to which error may differ for specimens collected in EDTA vs. heparin tubes, we distributed a series of blinded replicate plasma samples to all labs. Specifically, a total of 28 samples were included: 3 replicate samples of pooled quality control plasma; 5 replicates of a second pooled plasma quality control group; and 10 duplicate pairs of plasma samples from 5 individuals (one pair of EDTA tubes and one pair of heparin tubes collected on the same occasion from each

of the 5 persons). In addition to evaluating measurement error, we used these samples to assess differences in reported Aβ levels by EDTA vs. heparin tubes.

Next, to further examine the ability of the different assays to identify absolute levels of Aβ in plasma, we evaluated three separate plasma samples for recovery testing. These consisted of one "blank" or background plasma sample, the same sample spiked with 400 pg/ml Aβ-40, and the same sample spiked with 400 pg/ml Aβ-42. The plasma was provided by the blood bank at our institution, Brigham and Women's Hospital (blood bank specimens are collected as whole blood in EDTA tubes and processed immediately).

Finally, to test how delays between blood collection and processing might affect Aβ assays, we distributed 42 blinded plasma samples from 14 individuals (i.e., triplicate samples for 14 unique persons) for testing under two different assay protocols. Blood from each individual was collected in heparin Vacutainer tubes, and split into three samples, processed immediately after venipuncture (0 hours), 24 hours later, or 48 hours later. Differences across processing times were evaluated with two of the five assay protocols (see Protocols A and D, below) due to limited availability of personnel and other practical issues at this final stage of the study (e.g., one laboratory had moved during the study).

This study was approved by the Institutional Review Board of Brigham and Women's Hospital (Boston, MA).

Detailed laboratory protocols for Aβ assays

Five different assay protocols for measuring Aβ (including the preparation and inclusion of known Aβ standards on ELISA [enzyme-linked immunosorbent assay] plates) were conducted by the labs involved in this study. Details of each protocol are provided below and summarized in Table 1. (N.B.: Aβ-42 measurement was not performed using protocols C and E.)

Protocol A—Aβ-40 and Aβ-42 were assayed by sandwich ELISA. Nunc MaxiSorp 96-well immunoassay plates were coated with capture antibodies (2G3 for Aβ40 and 21F12 for Aβ42) in phosphate buffered saline (PBS) and incubated overnight at 4° C. Plates were then blocked with Block Ace (BA; Dainippon Pharmaceutical, Osaka, Japan; 1:4 dilution of original solution) for 2 hours at room temperature (RT) and washed briefly with PBS containing Tween 20 (PBS-T). Samples were then loaded and incubated overnight at 4° C followed by incubation with a biotinylated monoclonal anti-N terminus Aβ antibody (3D6B) overnight at 4° C. Finally, plates were washed twice with PBS-T, treated with alkaline phosphatase (AP) (Amersham, UK) for 1.5 hours, and washed with Tris-buffered saline (TBS) with Tween 20. The signal was amplified by adding AttoPhos (Promega, Madison, WI, USA), and was measured with a Victor3 (PerkinElmer, Boston, MA, USA).

Protocol B—Levels of Aβ were measured using monoclonal antibody 6E10 (specific to an epitope present on 1–16 amino acid residues of Aβ), as well as pooled rabbit antisera specific to Aβ-40 and Aβ-42 in a double-antibody sandwich ELISA. Briefly, monoclonal antibody 6E10 diluted in carbonate-bicarbonate buffer (pH 9.6) was coated in plate wells and incubated at 4°C overnight. After washing the plates with PBS-T, the wells were blocked for an hour with 10% normal sheep serum in PBS; plates were washed again, and samples were loaded and incubated 2 hours at RT and then at 4°C overnight. After washing, the plates were incubated with the pooled antibodies diluted in PBS-T with 0.5% bovine serum albumin (BSA) at RT for 1 hour and 15 minutes. After washing, NeutrAvidin–horseradish peroxidase (HRP) conjugated (Pierce, Rockford, IL, USA) diluted in PBS-T was added into the wells, and plates were incubated 1 hour at RT. Plates were washed again, and OPD (o-phenylenediamine dihydrochloride) (Sigma-Aldrich, St. Louis, MO, USA) in citric acid and sodium phosphate

buffer (pH 5.0) was added to each well; the reaction was stopped by adding 1N sulfuric acid. The optical density was measured at 490 nm in a micro-ELISA reader.

Protocol C—Aβ-40 was assayed by sandwich ELISA. Nunc MaxiSorp 96-well plates were coated with capture antibody (2G3) in carbonate coating buffer (pH 9.6) and incubated overnight at 4° C. Plates were washed 5 times with PBS, blocked with 1% milk-PBS for one hour at 37° C, and then washed 5 times with PBS. Following denaturation in 1.4M guanidine/ Tris (pH 8.0) for 0.5 hours to disrupt the binding of endogenous Aβ to binding proteins in plasma, samples and standards (spiked with mouse plasma [Lampire Biological Laboratories, Pipersville, PA, USA] at the same concentration as the experimental samples, 1:5) were incubated with antibodies (in loading buffer containing 400mM guanidine/Tris, pH 7.4) overnight at 4° C with gentle shaking; plates were then washed 8 times with PBS. Samples were incubated with detector antibody 3D6-Biotin in 1% BSA-PBS for 2.5 hours at 37° C, and then washed 8 times with PBS. Finally, samples were incubated with Strep-poly HRP 20 (RDI, Concord, MA, USA) in 1% BSA-PBS for 1.5 hours at RT, and then washed 8 times with PBS. Color development was in Slow TMB (tetramethylbenzidine) for ELISA (Sigma-Aldrich, St. Louis, MO, USA); plates were read at 650nm at various time intervals.

Protocol D—Aβ-40 and Aβ-42 were assayed by sandwich ELISA. Nunc MaxiSorp 384-well plates were coated with capture antibodies (2G3 for Aβ-40 and 21F12 for Aβ-42) in PBS and incubated for 4 hours at RT, then blocked with BA overnight at 4° C. Plates were washed 3 times with PBS-T, and samples were freshly diluted in BA, loaded into the wells, and incubated with antibodies for 2 hours at RT. Samples were then re-incubated in solution of detector antibodies (266B) for 2 hours at RT. Finally, samples were incubated with streptavidin AP (Promega, Madison, WI, USA), in PBS, for 1 hour at RT and washed 3 times with TBS. The signal was amplified, by adding AttoPhos (Promega, Madison, WI, USA), and measured with a Victor2 (PerkinElmer, Boston, MA, USA).

Protocol E—Plasma was pretreated prior to the ELISA. To block cross-reaction of unidentified components of human plasma with the ELISA, plasma was precleared with mouse IgG1 κ (Sigma-Aldrich, St. Louis, MO, USA) cross-linked to agarose beads (CNBr-activated Sepharose 4B; Amersham Biosciences, Piscataway, NJ, USA). Preclearing was performed by diluting 300 μL of each plasma sample with 525 μL of sample buffer (20mM phosphate, 400mM sodium chloride, 2mM EDTA, 10% blocking agent [BA Liquid; Dainippon Pharmaceutical, Osaka, Japan], 0.2% BSA, 0.0765% 3-{[3-cholamidopropyl] dimethylammonio}-1-propanesulfonate [CHAPS], pH 7.2), and 75 μL of the agarose beads covalently cross-linked to nonspecific mouse IgG1 κ. After incubation for 2 hours at 4°C, the beads were removed by centrifugation.

Aβ-40 was assayed by sandwich ELISA. The 96-well microtiter plates (Maxisorp Black; Nalge Nunc, Rochester, NY, USA) were coated with the capture antibody – 5 μg/mL BNT77 (mouse IgA antibody to 11–28 amino acid residues of Aβ; Takeda Chemical Industries, Osaka, Japan) – and blocked with blocking buffer (25% BA Liquid in PBS) for 6 hours. Pretreated plasma samples (100 μL, in triplicate) were incubated in BNT77-coated wells containing 50 μL of sample buffer overnight at 4°C. The plates were washed 4 times with PBS, then reacted with HRP-conjugated detector antibodies (BA27 mouse IgG2 anti-Aβ40, 1:1000; Takeda Chemical Industries, Osaka, Japan) in 75 μL of sample buffer for 4 hours at RT. After 6 washes with PBS, HRP enzyme activity was measured with a fluorogenic substrate (Quanta Blu; Pierce, Rockford, IL, USA) on a fluorometer (Wallac Victor2 1420 Multi-label Counter; PerkinElmer, Boston, MA, USA) with a 320-nm excitation filter and 400-nm emission filter.

Statistical analyses

To evaluate intra-assay reliability of the plasma Aβ assays, we calculated percent intra-assay coefficients of variation (CVs), using the replicate samples. We then used the median CV across all sets of relevant replicate samples to represent a single, best approximation of laboratory error. The CV is calculated as: (standard deviation / mean) ×100 %. Lower CVs indicate better reproducibility, or less laboratory error. In general, CVs <10% are considered very good; CVs from 10–15% are considered good; CVs from 15–20% are typically acceptable; and CVs >20% are considered poor [2,18].

We also calculated percent recovery of a known Aβ concentration under each protocol condition. All the labs were provided with the identical background plasma sample, plus two spiked plasma samples (one spiked with 400 pg/ml Aβ-40 and another spiked with 400 pg/ml Aβ-42); we calculated percent recovery of Aβ by subtracting the level of Aβ detected in the background sample from the level of Aβ detected in the spiked sample, dividing this result by 400, and then multiplying by 100. (Thus, perfect measurement of the Aβ added to the spiked sample would yield a percent recovery of 100%.)

Finally, to analyze the stability of Aβ-40 and Aβ-42 in plasma over 0-, 24- and 48-hour processing time delays, we first created natural log transformations of Aβ-40 and Aβ-42 values, as these were non-normally distributed. Then, intraclass correlation coefficients (ICCs) and their 95% confidence intervals (CIs) were calculated by analysis of variance using the MIXED procedure in SAS (SAS Institute Inc., Cary, NC, USA) to assess the stability of Aβ-40 and Aβ-42 over the repeated measurements of samples. Higher ICCs indicate better agreement of values: an ICC ≥ 0.75 is considered excellent, $0.4 \leq \text{ICC} < 0.75$ is considered fair to good, and ICC <0.4 is considered poor [13].

Results

Reproducibility of Aβ Assays

Combining data from all replicate samples, we found moderate to low intra-assay CVs (Table 2), indicating that reproducibility was generally acceptable. For Aβ-40, the median CVs from the samples tested ranged from 6.0–23.8% across the lab protocols, with the CV below 20% for four out of five. For Aβ-42, median CVs ranged from 7.5–14.1% across protocols, with CVs below 15% for all three that were used to measure Aβ-42. Although the range of CVs included isolated extreme values (e.g., the CV was 85% for one set of replicates under protocol A), this reflected outliers, and average CVs were generally low.

When we separately evaluated CVs for the replicates collected in EDTA vs. heparin tubes, we found no systematic differences in levels of error for both Aβ-40 and Aβ-42 (Table 3). For example, in protocol B, for Aβ-40, the median CV was 11.7% for samples collected in EDTA vs. 9.3% for heparin; in protocol B, for Aβ-42, the median CV was 15.7% for samples collected in EDTA vs. 8.7% for heparin. For the two assay protocols with reported median CVs >20% for Aβ-40, the difference in error did not appear driven by collection using one medium vs. another.

Absolute Levels of Aβ Detected

Table 2 provides a qualitative comparison of the absolute levels of Aβ detected in the test samples across lab protocols. As expected, there were large differences in the absolute Aβ concentrations reported, due to variations in the materials and procedures involved in each protocol. Mean Aβ-40 levels in the test samples ranged from 127.1 pg/ml in protocol A to 625.8 pg/ml in protocol D. For Aβ-42, mean levels in the test samples ranged from 12.8 pg/ml

in protocol A to 208.2 pg/ml in protocol D; measurements of Aβ-42 were not obtained using protocols C or E.

Nonetheless, although there was wide variation across different protocols in reported Aβ concentrations, we found no evidence of differences in absolute levels of Aβ-40 or Aβ-42 by anti-coagulation medium (Table 3). Within each protocol condition, Aβ values were generally similar for EDTA and heparin samples, and there was no clear pattern across protocols of consistently lower or higher Aβ values from samples collected in EDTA versus heparin.

To further evaluate the ability of the assays to identify absolute Aβ levels, we examined the percent recovery in the spiked samples of 400 pg/ml of Aβ-40 and 400 pg/ml of Aβ-42, under each protocol condition. In general, the percent recovery was low for both Aβ-40 and Aβ-42. Specifically, for Aβ-40, the percent recovery ranged from -24% to 44%; recovery was slightly higher for Aβ-42, with a range from 17% to 61% (Table 4).

Stability of Aβ in plasma over processing delays

In this preliminary examination of the stability of Aβ levels across various delays in blood processing, we found that levels of plasma Aβ-42 were very similar in specimens processed after 24 hours compared to those processed immediately, but there was less consistency for Aβ-40 (Table 5). Comparing samples processed at 0 and 24 hours, the ICC for Aβ-40 was 0.30 (95% CI 0.04, 0.83) in protocol A and 0.96 (95% CI 0.89, 0.99) in protocol D; for Aβ-42, the ICCs were >0.9 in both protocols. Over a 48-hour processing delay, values for Aβ-40 were more variable. Values tended to be lower after longer processing delays in protocol A, and the ICC was 0.09 (95% CI 0.00, 0.87); in protocol D, there was little impact of a 48-hour processing delay on Aβ-40 values, with an ICC of 0.95 (95% CI 0.89, 0.98). Similar values of Aβ-42 were observed across the 48-hour processing delay using either protocol, with ICCs of 0.83 (95% CI 0.64, 0.93) in protocol A and 0.96 (95% CI 0.90, 0.98) in protocol D.

Discussion

This report provides data on a variety of performance characteristics of plasma amyloid β assays. Overall, we found low or modest laboratory error in measuring levels of plasma Aβ-40 and Aβ-42. Assay reproducibility was generally similar whether EDTA or heparin was used as the anticoagulant in blood collection tubes; furthermore, mean Aβ values did not appear consistently higher or lower in specimens collected in either anticoagulant. As expected, due to the considerable variation in assay procedures, there were large differences across the various protocols in the reported absolute concentrations of Aβ measured in the same samples. In addition, the percent recovery of Aβ species in spiked plasma samples was low; however, this was not unexpected, as free Aβ (i.e., unbound to plasma proteins) accounts for a low percentage of total Aβ in human plasma – possibly as low as 15–20% [14]. Indeed, because the plasma Aβ ELISA protocols measure free Aβ, the low recovery percentages reported in this study must be considered in this context. Finally, when we tested the effects of delays of up to 48 hours in processing of whole blood samples, there appeared to be little variation in Aβ-42 concentrations according to processing time, especially for delays of 24 hours; however, for Aβ-40 concentrations, the extent of variation in values across blood processing times was less consistent.

Several important issues are raised by our findings. The reproducibility results demonstrate that acceptable within-person reliability can generally be achieved for plasma Aβ-40 and Aβ-42, regardless of EDTA vs. heparin blood collection method. The demonstration of low intra-assay CVs is a critical step before any biomarker assay can be broadly applied in large-scale epidemiologic studies. However, the broad range of assay methods and the large differences in absolute Aβ values reported on identical samples tested *across* the different

protocols, as well as the low percent recovery of Aβ in spiked samples, suggest that comparisons of absolute plasma Aβ values across studies is probably of little value at this time.

Numerous factors might affect the measurement of plasma Aβ levels, and explain the variations that we found across the different laboratory protocols. Overall, concentrations of Aβ in plasma are very low – approximately 100-fold lower than those measured in cerebrospinal fluid [10] – and achieving sufficient sensitivity for detecting such low levels is a challenge. Specifically, differences in assay materials and procedures, including differences in the synthetic Aβ peptides used as the standards for calculation of absolute values, likely contributed to variation in reported values (see Table 1). For example, many of the protocols involved in the current study utilized different antibodies to measure plasma Aβ-40 and Aβ-42, with varying clonality (polyclonal vs. monoclonal) and Aβ measurement targets (1–40/42 vs. x-40/42). In addition, there is evidence that the aggregation of Aβ monomers is dependent on temperature, with aggregation tending to increase between 10 and 37 °C [16]; this might result in the formation of differing amounts of Aβ aggregates that fail to be recognized by the anti-Aβ monomer antibody under the temperature conditions involved in a given protocol. Finally, plasma is known to contain an abundance of proteins that bind to Aβ *in vivo* (e.g., albumin and transferrin), and this could mask the binding of Aβ to anti-Aβ antibodies under certain assay conditions. Indeed, a recent report [14] demonstrated that the amount of Aβ bound to soluble circulating low-density lipoprotein receptor-related protein-1 may be as high as 5–6 times that of free Aβ in plasma. With regard to the protocols tested in this study, there is variability across most of these factors – standards and antibodies used, temperature conditions, incubation times – such that it is very difficult to definitively pinpoint any single greatest determinant of differences in absolute values; moreover, it seems most likely that a combination of factors best explains the variations we found.

Recently, commercial assays have been developed for measuring plasma Aβ (Covance Research Products, Inc.; Innogenetics NV [19]), and use of such assays has the potential to decrease some of the observed variation in absolute Aβ values (e.g., through uniform use of antibody types or prescribed incubation conditions). However, even in previous experiences with assays for which commercial kits were widely available (e.g., C-reactive protein, cholesterol), substantial work was needed on assay standardization across laboratories before studies could begin to establish cutpoints associated with disease risk [11,12]; such standardization work will likely be needed with commercial assays of plasma Aβ, and we hope to conduct such investigations in the future.

Nevertheless, the apparent stability of Aβ values over processing delays of up to 24, and potentially as many as 48, hours is a promising finding. Processing delays are common in the conditions in which blood samples are typically collected in large studies – that is, blood specimens may be collected at one location, shipped on ice and received at a central laboratory within 24 hours, and then processed and placed in deep frozen storage. Thus, our findings on processing delays suggest there is potential for plasma Aβ assays to be utilized in large-scale epidemiologic settings. It is notable, however, that the ICCs for Aβ values were not consistent in the two protocols. These differences might be explained by numerous variables, including variations in methods, as detailed in Table 1. For example, absolute Aβ-40 values appeared to decrease with increasing processing delays in Protocol A; this observed decrease may be explained by the longer incubation time under this protocol. However, it is unclear why longer incubation times would not have similarly affected absolute Aβ-42 values. In contrast to the differing findings for the ICCs of Aβ-40 concentrations, the absolute values of Aβ-42 were relatively stable across processing times under both protocols. The stability of Aβ-42 values indicates that extensive Aβ-42 oligomerization does not occur in human plasma and is less of a concern with processing delays, as reported values would be expected to fall sharply with increased oligomer formation. Another major difference between protocol A and D is the Aβ

species detected by ELISA: Aβ 1–40/42 vs. Aβ x-40/42. It is conceivable that some Aβ 1–40 peptides underwent N-terminal truncation over time, as observed in human brains during aging; those newly generated Aβ x-40 peptides would not be recognized by ELISA using protocol A, but would still be measurable by ELISA under protocol D. Thus, although such an explanation remains speculative, these differences in Aβ targets may account in part for the divergent pattern in absolute Aβ-40 values across processing times.

Strengths and limitations of our work should be noted. This study provides some of the first evidence on a wide variety of essential performance characteristics for plasma Aβ-40 and Aβ-42 assays performed in U.S. research laboratories. Another advantage was that several laboratories with experience in Aβ research performed the different protocols and provided their expertise in the conduct of this project. Nevertheless, it would be have been interesting to conduct the same performance and reliability tests using newly-developed commercial assay protocols (e.g., Innogenetics NV [19]), in addition to the five research protocols; however, such protocols were not available at the time our study was initiated. Similarly, it would have been desirable for each lab in our study to conduct plasma Aβ assays using multiple different protocols, allowing more direct assessment of laboratory variations. However, this was not possible due to practical constraints for the labs (e.g., the cost of purchasing multiple different sets of reagents or additional instruments to perform the various protocols), but is certainly an appropriate goal for future research. Another limitation was that the total number of samples used in this study was relatively small; however, if there were systematic differences in Aβ concentration or measurement error by blood anticoagulant, repeatedly examining this issue using several different protocols would likely have been sufficient to provide some suggestion of such differences, despite the small number of samples. Finally, although the current study is fairly comprehensive, we did not address all issues, such as the effects of long-term frozen storage (i.e., >10 years) on assay reliability, the influence of repeat freeze-thaw cycles on reproducibility of results from stored specimens, or the within-person reproducibility of plasma Aβ levels over longer periods (e.g., up to 5 years). Other factors, such as fasting status or diurnal variation [1], will also require further inquiry in order to determine their potential influences on measured plasma Aβ levels. Such issues should be addressed in future research, including tests of the influence of these factors on results generated by recently-developed commercial plasma Aβ assays.

In summary, our results demonstrate that, although absolute plasma Aβ-40 and Aβ-42 values vary *across* different protocols, the reproducibility of values is generally good *within* each protocol. Furthermore, there do not appear to be systematic differences in results by different anticoagulants commonly used in blood collection. Our results also show that it is possible to attain stability of plasma Aβ values across processing time delays that are typical of those occurring in many large population-based studies. Overall, these preliminary results offer some promise of feasible measurement of plasma Aβ-40 and Aβ-42 in varied research settings. In concluding, it is critical to emphasize that, however favorable initial reproducibility results may be, strong quality control procedures must be established and repeated over time to ensure continued reliability of the assay.

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Table 1

Characteristics of Protocols Used for Assays of Plasma Amyloid β [*].

Characteristic	Protocol A	Protocol B	Protocol C	Protocol D	Protocol E
Antibody Type					
Capturer - *Name*	2G3 (Aβ-40)	6E10	2G3 (Aβ-40)	2G3 (Aβ-40)	BNT77
	21F12 (Aβ-42)			21F12 (Aβ-42)	
Capturer - *Clonality*	Monoclonal	Monoclonal	Monoclonal	Monoclonal	Monoclonal
Detector - *Name*	3D6B	Pooled antisera to	3D6B	266B	BA27 (Aβ-40)
		Aβ-40 and Aβ-42			
Detector - *Clonality*	Monoclonal	Polyclonal	Monoclonal	Monoclonal	Monoclonal
Aβ Measurement Target	1–40	1–40	1–40	x-40	x-40
	1–42	1–42		x-42	
Incubation Conditions					
Capture step - *Duration*	O/N	2 hours at RT, and	O/N	2 hours	O/N
Capture step - *Temperature*	4° C	then O/N at 4° C	4° C	RT	4° C
Detection step - *Duration*	O/N	1.25 hours	2.5 hours	2 hours	4 hours
Detection step - *Temperature*	4° C	RT	37° C	RT	RT

[*] Aβ = amyloid β; O/N = overnight; RT = room temperature. Amyloid β 42 measurement was not performed using protocols C and E.

Table 2

Mean values and median intra-assay coefficients of variation for plasma amyloid β 40 and 42, by protocol *

	Protocol A	Protocol B	Protocol C	Protocol D	Protocol E
Amyloid β 40					
Mean (pg/ml)	127.1	132.1	565.5	625.8	303.2
Median (range) CV†(%)	6.0 (1.2–11.9)	10.0 (1.6–27.9)	23.8 (3.2–83.9)	6.3 (0.4–23.8)	16.6 (2.9–48.0)
Amyloid β 42					
Mean (pg/ml)	12.8	30.6	N/A	208.2	N/A
Median (range) CV†(%)	7.5 (0.7–85.0)	14.1 (3.8–27.5)	N/A	9.5 (4.7–62.3)	N/A

*Medians and ranges were determined across 7 sets of replicate samples. Amyloid β 42 measurement was not performed using protocols C and E.

†CV = coefficient of variation.

Table 3

Mean values and median intra-assay coefficients of variation for plasma amyloid β 40 and 42, by protocol and blood collection method*

	Protocol A	Protocol B	Protocol C	Protocol D	Protocol E
Amyloid β 40					
Mean, EDTA (pg/ml)	160.1	123.1	694.1	623.2	319.6
Mean, Heparin (pg/ml)	138.6	171.5	602.9	626.0	331.4
Median CV† EDTA (%)	6.6	11.7	29.9	6.3	14.3
Median CV†, heparin (%)	4.3	9.3	12.6	1.0	24.8
Amyloid β 42					
Mean, EDTA (pg/ml)	18.7	17.7	N/A	264.4	N/A
Mean, Heparin (pg/ml)	6.6	25.9	N/A	128.7	N/A
Median CV†, EDTA (%)	3.1	15.7	N/A	9.6	N/A
Median CV†, heparin (%)	7.5	8.7	N/A	13.7	N/A

*Medians were determined from 5 sets of replicate samples from blood collected in both EDTA and heparin tubes. Amyloid β 42 measurement was not performed using protocols C and E.

†CV = coefficient of variation.

Table 4

Percent recovery of known concentrations of plasma amyloid β 40 and 42 in spiked plasma samples, by protocol*

	Protocol A	Protocol B	Protocol C	Protocol D	Protocol E
Amyloid β 40					
Background plasma sample (pg/ml)	108.5	130.9	460.7	615.3	226.5
Sample spiked with 400 pg/ml Aβ-40† (pg/ml)	129.0	202.7	363.6	718.4	403.4
Recovery	5.1%	18.0%	−24.3%	25.8%	44.2%
Amyloid β 42					
Background plasma sample (pg/ml)	3.9	27.6	N/A	138.3	N/A
Sample spiked with 400 pg/ml Aβ-42† (pg/ml)	72.1	270.4	N/A	227.7	N/A
Recovery	17.1%	60.7%	N/A	22.4%	N/A

*Amyloid β 42 measurement was not performed using protocols C and E.

†Aβ = amyloid β.

Table 5

Stability of amyloid β species in plasma over 0-, 24- and 48-hour processing delays*.

	Mean Amyloid β values			Overall ICC (95% CI)	0–24 hour ICC (95%CI)
	0 hours	24 hours	48 hours		
Protocol A					
Amyloid β 40 (pg/ml)	155.8	94.4	51.9	0.09 (0.00, 0.087)	0.30 (0.04, 0.83)
Amyloid β 42 (pg/ml)	71.1	63.2	64.1	0.83 (0.64, 0.93)	0.93 (0.81, 0.98)
Protocol D					
Amyloid β 40 (pg/ml)	1101.6	1266.3	1332.2	0.95 (0.89, 0.98)	0.96 (0.89, 0.99)
Amyloid β 42 (pg/ml)	316.7	387.3	449.8	0.96 (0.90, 0.98)	0.98 (0.96, 0.99)

*ICC = intraclass correlation coefficient; CI = confidence interval.