RESEARCH ARTICLE

The Impact of Decreased Bead Count to Determine Concentrations of Amyloid beta1-42, Total-tau, and Phosphorylated-tau181 in Human Cerebrospinal Fluid Using xMAP Technology

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ABSTRACT: Alzheimer's disease is the leading cause of human dementia. The lack of diagnostic tests and limited therapeutic options has driven the search for endogenous biomarkers. The INNO-BIA AlzBio3 assay is a multiplex flow-based immunoassay measuring Aβ42, tau, and p-tau in cerebrospinal fluid (CSF). This study assesses assays performance under varying bead count (BC) parameters. Original method validation parameters at 100 BC were acceptable. Reanalyses performed at 3, 10, 25, and 50 BCs were compared to 100 BC data by ANOVA, Bland–Altman analysis, evaluation of concordance correlation coefficients, and frequency distribution of coefficient of variation (CV) ranges. Method validation characteristics were acceptable with 100 BCs. Equivalency for 25 and 50 versus 100 BCs was demonstrated, but not for 3 and 10 BCs. A general trend of decreasing agreement between decreasing BCs and the 100 BC reference resulted in decreases in concordance coefficients ρ_c. The frequency of CV values greater than 20% increased with decreasing BCs, and CV values of 5% or less decreased with decreased BCs. Statistical analyses demonstrate that BCs of 3 and 10 are not equivalent with the reference and should not be used as a basis for determination of Aβ42, tau, and p-tau concentration in human CSF. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: flow-based immunoassay; protein aggregation; cerebrospinal fluid; Alzheimer's disease; analytical biochemistry; biomarker; protein structure; bioanalysis; biotechnology; disease effects

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia. More than 25 million patients worldwide are affected by this ultimately fatal disease, and its prevalence is expected to reach 100 million in the next 40 years.[1] Currently, a probable AD diagnosis can be made with approximately 90% accuracy based upon neurological and mental status assessments in conjunction with an evaluation of risk factors, family history, and an evaluation of the gradual onset of progressive symptoms. Such symptoms include memory loss, changes in personality, decline in cognitive abilities, etc.[2] Definitive diagnosis is only confirmable at autopsy based upon the presence of amyloid plaques and neurofibrillary tangles in the brain.

Several diagnostic approaches are being investigated to facilitate earlier detection of AD, including imaging and risk factors (e.g., genetics, inflammation markers) algorithms.[3] In addition, therapy-responsive markers in biological fluids are being evaluated as indicators of AD.[4–6] Ideally, a noninvasive, inexpensive, readily available, rapidly changing plasma pharmacodynamic biomarker with high sensitivity and specificity is needed to follow clinical trials and for diagnosis. The National Institutes of Health's Alzheimer's Disease Neuroimaging Initiative (ADNI)

Abbreviations used: AD, Alzheimer's disease; Aβ42, amyloid beta1-42; Tau, total tau; p-Tau, phospho-tau$_{181}$; CSF, cerebrospinal fluid; BC, bead count; CV, coefficient of variation; ADNI, Alzheimer's Disease Neuroimaging Initiative; LLOQ, lower limit of quantitation; ULOQ, upper limit of quantitation; FTS, freeze/thaw stability

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was launched in 2004 and is the largest public–private partnership in Alzheimer's disease research.[7,8] The primary goal of ADNI has been to recruit and follow 800 adults in different stages of dementia (healthy elderly, mild cognitive impairment (MCI), and AD) and test whether serial MRI, positron emission tomography, clinical and mental assessment, and other biological markers can be combined to measure the progression of MCI and early AD.

One of the tasks of the biomarker core of ADNI is to utilize biological samples from ADNI participants to measure protein biomarkers from biological fluids. cerebrospinal fluid (CSF) Aβ42, tau, and p-tau have been determined using product A, a multiplex commercial test kit. Evaluation of CSF obtained at baseline evaluation of 416 of the 819 ADNI subjects has been completed.[9,10] Bioanalysis is on-going and in October 2010 the Foundation for the National Institutes of Health announced that ADNI-2 has been renewed for an additional 5 years.[11]

The search for novel exploratory biomarkers to support basic research and for new diagnostic markers has increased in large part due to the availability of prepackaged commercial test kits capable of measuring practically any desired analyte. In addition, demand for efficacy biomarkers measured during the course of preclinical and clinical trials has increased in an effort to expedite decision making on candidate therapeutics. This is highlighted in the Critical Path Initiative[12] and the U.S. FDA Strategic Priorities 2011 −2015.[13]

There are more than 20 vendors offering commercial assay kits for the measurement of beta amyloid and tau proteins on at least four immunoassay platforms. The quality of available commercial test kits depends upon the affinity and specificity of the antibodies for the analyte and the extent of method optimization conducted by the kit manufacturer.[14]

Product A, a commercial assay that is being utilized in the ADNI studies, is a fluorimetric assay for the determination of Aβ42, tau, and p-tau in human CSF.[15,16] The method is performed on the Luminex platform (Luminex Corporation, Austin, TX). This technology consists of a family of 100 fluorescently dyed (analyte discriminator) 5.6-μm polystyrene microspheres and an analyzer capable of distinguishing each of the 100 microspheres. The analyzer integrates detection components such as lasers, optics, fluidics, and high-speed digital signal processors to detect and report fluorescence intensity data resulting from the binding of analytes to biological reactants at the microsphere surfaces. The xPONENT® (Luminex Corporation, Austin, TX) analyzer control software is designed for protocol-based data acquisition and data regression analysis. For commercial test kits, instrument protocols, including instructions for acquiring raw data counts and bead count (BC) acquisition specifications, are provided by the reagent manufacturer. Raw data are expressed as median fluorescent intensity (MFI): the median of individual fluorescence readings for a given bead set collected from manufacturer-specified BCs. MFI values of calibrators and biological samples are mathematically regressed to generate analyte concentrations.

Recent experiments in our laboratory utilizing product A resulted in a series of analytical run failures. Upon investigation, it was determined that the run failures were the result of an insufficient quantity of reagent beads (in a lot-specific production) to satisfy the acquisition of 100 BC during analysis. Recommendations from the manufacturer were to reduce the BC acquisition number from 100. This article evaluates the validity of reducing the 100 BC acquisition parameter for product A when measuring Aβ42, t-tau, and p-tau in human CSF.

MATERIALS AND METHODS

CSF Validation Samples

CSF was obtained from Bioreclamation (Jericho, NY). Aliquots were prepared in approximately 10 mL volumes and stored in polypropylene conical tubes frozen at −20°C. CSF lots underwent one freeze–thaw cycle to prepare pooled QC 1 and pooled QC 2, respectively.

Buffer Validation Samples

Kit standards 1–6 and Controls A and B (Innogenetics lot #181286) were acquired components of product A. A seventh standard (Standard 7) was prepared by diluting Standard 6 1:2 in a sample diluent. All standards and controls were received frozen on dry ice, and subsequently stored at −20°C.

Description of xMAP Technology

The analyzer used in these experiments functions by utilizing xMAP technology (Luminex Corporation, Austin, TX), a flow-based immunoassay format in which the stationary phase is a polystyrene microsphere coated with a unique combination of red and ultraviolet pigment. Each bead region in an assay is coated with a capture antibody specific for an analyte, resulting in the bead region's specificity for that analyte. After incubation with analytical sample, the beads are washed and incubated with a biotinylated secondary and/or detection antibody, then with a streptavidin–phycoerythrin conjugate. Alternatively, a detection antibody can be directly labeled with phycoerythrin; however, this method of detection is not as common.

During acquisition, a designated volume (typically 50 μL) of sample (beads suspended in the appropriate read buffer) is aspirated through a sample. The

mobile buffer phase is responsible for transporting the sample through the fluidics of the system. Samples are carried through the system's fluidics to the bottom of the cuvette. The beads in a sample enter the cuvette at 1 μL/s, aligning in a narrow column as they pass through the path of the system's red (λ = 635 nm) and green (λ = 532) excitation lasers. The red laser excites the dye embedded in the bead, and the green laser excites phycoerythrin associated with analyte bound to the surface of the beads. Beads of different regions are distinguished by detection of their respective emission wavelength. Microspheres for a particular assay will have a known diameter and resulting side scatter, which determines the gate acquisition setting. During acquisition, the analyzer will only take into account those events that fall within the designated gate, thus discriminating beads from other possible interference.

Finally, a photomultiplier tube detects fluorescence emission of phycoerythrin bound to the biological reactants at the surface of the beads. Typically, at least 100 beads of a particular region are counted and measured for fluorescence, resulting in at least 100 individual fluorescence measurements per region per sample. An MFI is assigned to each bead region within a sample and analyte, where the individual fluorescence signals below this value are equal to those above this value. This MFI value is reported as the representative value for the respective analyte in an acquired sample.

Product A Description

All validation assays were performed using a sandwich-type immunoassay format with the INNOBIA AlzBio3 kit (product A; Innogenetics NV, Gent, Belgium; Cat. #80584, Lot #19113). Product A is a fluorimetric assay intended to simultaneously determine levels of Aβ42, total tau, and p-tau in human CSF using the prior mentioned bead-based immunoassay technology. The range of quantitation is approximately 45–1500 pg/mL for Aβ42 and tau, and approximately 10–225 pg/mL p-tau. Aβ42, tau, and p-tau are selectively captured on three uniquely colored microspheres, which have been coated with monoclonal antibodies (4D7A3 for Aβ42, AT120 for tau and p-tau). Samples are simultaneously incubated with antibody-coated microspheres and biotinylated detector antibodies (conjugate 1; 3D6 for Aβ42, HT7 for tau and p-tau). A streptavidin–phycoerythrin conjugate is used to detect analyte bound to the surface of the xMAP microspheres. A unique ratio of red and ultraviolet dye embedded within each of the three microsphere regions (2 for tau, 56 for Aβ42, and 69 for p-tau) is excited by a 635-nm laser. The corresponding emission is detected by two avalanche photodiodes (each specific for excitation of ultraviolet and red pigment). Phycoerythrin bound to biological reactants at the microsphere surface is excited by a 532-nm reporter laser. The resulting emission is detected by a photomultiplier tube.

The procedure for determination of Aβ42, t-tau, and p-tau in human CSF by product A was followed according to manufacturer's specifications (with the addition of one standard, Standard 7 as described previously). Only polypropylene tubes (Sarstedt Ref 72.730 and 72.694) were used in sample handling and storage, as glass and polystyrene can absorb Aβ42. All assays employed 96-well filter plates (2 μm; Millipore Ref. #25343). Filter plates were washed with 225 μL wash buffer per well a total of three times per wash step. Aspiration steps were performed using a Millipore multiscreen vacuum manifold. All incubations were administered at room temperature (approximately 22—25∞C) with shaking using an orbital microtiter plate shaker (Barnstead/Lab Line model #4625) at setting 6. All reagents and samples were delivered using Rainin Pipet-Lite L-8 adjustable multichannel pipettes. Wash buffer was dispensed by a Matrix equalizer digital multichannel repeater. All reagents were kept at 4°C until use. All buffer standards and controls were kept at −20°C until use.

Antibody-coated beads 100X (Ref. #57823), Diluent (Ref. #57826), conjugate 1 100X (Ref. #57824), and diluted wash solution (Ref. #57882) was allowed to reach room temperature approximately 30 min before use. Standards (Ref. #57828-57832, 57874), controls (Ref. #57833, 57835), and human CSF pooled QC samples were removed from −20 or −70°C and placed at room temperature approximately 15 min prior to use. Coated beads were vortexed briefly, sonicated for 3 min, vortexed again, and diluted 1:100 in the diluent. Working bead solution was protected from light until use. Conjugate 1 working solution was prepared by diluting 100X conjugate 1 1:100 in the diluent. Filter plates were prewetted with 225 μL wash buffer per well and aspirated prior to use in assays. Working bead solution was vortexed to ensure suspension of beads, then 100 μL dispensed into all wells to be used in the assay. Bead suspension in filter plate was aspirated, then resuspended in 25 μL of conjugate 1 working solution. All standards, controls, and pooled QC samples were vortexed briefly, and 75 μL of each was aliquoted in duplicate into the appropriate wells. The mean of two duplicate wells was reported as one result. Antibody-coated beads, conjugate 1, and standards/controls/pooled QC samples were incubated 14–18 h (overnight). Thirty minutes prior to the end of overnight incubation, diluted wash solution, diluent, reading solution (Ref. #57827), and detection conjugate (100X, Ref. #57825) were allowed to come to room temperature. A detection conjugate working solution was made by diluting detection conjugate (100X) 1:100 in the diluent. The filter plate was

aspirated and washed, and 100 μL of detection conjugate working solution dispensed to each well. The filter plate was protected from light and incubated for 50–70 min. After incubation with detection conjugate, the filter plate was aspirated and washed. Beads were resuspended in 100 μL reading solution and placed on an orbital shaker for 2–10 min. Fifty microliter of each reaction was analyzed. One hundred beads per analyte were acquired at a gating of 7500–15,000, as specified by the manufacturer.

Instrumentation and Software

Samples analysis and acquisition was performed using a Luminex 200 analyzer (described previously) and the corresponding control software. All data regression were performed by StatLIA® Immunoassay Workflow and Analysis Software (Brendan Technologies, Carlsbad, CA).

Method Validation

A fit-for-purpose approach[17,18] for conducting method validation was used in product A method qualification. The intended purpose of the method was to support drug development programs. Two lots of CSF, buffer lower limit of quantitation (LLOQ; Standard 7) and upper limit of quantitation (ULOQ; Standard 1), and buffer controls A and B were analyzed in seven accuracy and precision runs using a weighted 5-PL analysis. Additional data were collected to evaluate method performance characteristics, including linearity, recovery, stability, etc.

Statistical Methods

The accuracy and precision data collected during the 100 BC setting was used as the reference data set. Software manipulation allowed for virtual generation of additional data at defined n BC settings, based upon the collection of the first n beads. Three statistical approaches were used to assess the equivalence of 3, 10, 25, and 50 BC to the 100 BC reference while determining the concentrations of three analytes, Aβ42, tau, and p-tau:

1 *ANOVA*. Differences between least-squares means (LSM) expressed as differences or percent ratios were calculated and tested. p values and 99% confidence intervals (CI) were also reported.
2 *Bland–Altman analysis*.[19] Differences between the paired concentration values were used to calculate the mean differences (D) and the standard deviation of the differences (S). The limits ($D \pm 2S$) were calculated and reported.
3 *Concordance correlation coefficients approach*.[20] Pearson correlation coefficients, the bias correlation factors, concordance correlation coefficients, and the associated 95% CI were calculated and reported.

In addition, the correlation coefficient (CV) for each sample was categorized into four groups, 0–5%, 6–10%, 11–20%, and >20%. The frequency percentages based on the total frequency for each category within a BC were plotted to reflect the changes in the concentration variation as the number in BC increased.

RESULTS

Method Validation, 100 BC Reference

Assay characteristics, a priori acceptance criteria, and a summary of the results of the validation are described in Table 1. All analytes were acceptable for accuracy and inter- and intrarun precision.

Method Comparisons—Impact of Decreased Bead Count

ANOVA results (Table 2) indicated that differences between 50 or 25 and 100 BC for all three analytes were consistently small with test/ref ratios (%) ranging from 99.6 to 100.1 and p values ranging from 0.8095 to 0.9781. Greater differences and inconsistency were indicated when 3 or 10 BC was compared to 100 BC with test/ref ratios (%) ranging from 95.9 to 101.4 and p values from 0.0267 to 0.5936. Because of the closeness in the test/ref ratios, a higher percent CI (99%) was used to widen the CI ranges for better comparisons. In addition, the p values (a larger p value indicates a less significant difference between two methods and vice versa) are included.

ANOVA Bland–Altman analysis (Table 3) demonstrated general trends for all three analytes. Greater agreements were indicated between 25 or 50 and 100 BC with mean of differences (D) ranging from–1.6 to 0.4, standard deviation of differences (S) from 1.5 to 35.6, the lower limit (D–2S) from–71 to–3.2, and the upper limit ($D + 2S$) from 2.8 to 71.5 for all three analytes. Less agreement was found between 3 or 10 and 100 BC as indicated by greater values in D (–14.7 to 0.8), S (4.7–94.7), D–2S (–202.5 to–9.7), and $D + 2S$ (9.0–176.4).

Bland–Altman plots were omitted in this analysis due to the large number of comparisons performed in this study; however, the key statistics of the analysis, the mean differences (D), the standard deviation of the differences (S), and the limits ($D \pm 2S$) are summarized in Table 3.

Although high concordance correlation coefficients or ρ_c (0.9716–0.9999) were found for all three

Table 1. Validation Summary, 100 BC Reference

Analyte	Assay Characteristic	A Priori Acceptance Criteria	Method Summary
Aβ42	Calibration model	±15% bias (≤20% at the LLOQ and ULOQ) ≤20% CV	% bias:–1.84 to 3.50 % CV: 1.41–3.21
	Accuracy (interrun) and relative accuracy	±30% bias ±35% bias for the LLOQ and ULOQ validation samples	% bias:–8.57 to 5.33 % bias (RA):–16.57 to 0.92
	Precision: Intrarun (repeatability)	≤20% CV for pooled QCs	% CV: 0.40–10.26
	Precision: Interrun (intermediate precision)	≤25% CV for all validation samples	% CV: 8.56–22.95
	Total error (interrun)	[|% bias| + % CV] ≤40% for all validation samples	% total error: 12.72–31.52
	Hemoglobin interference (mg/dL)	±30% bias of 0 mg/dL Hb sample (pooled QC 1)	% bias:–57.40 to–10.95 (unacceptable)
	Stability: Freeze/thaw Short term Long term	2/3 of the pooled QCs must be within ±30% of the pooled QC interassay mean for each test	FTS (Four cycle): All within ±30% 22°C (51 h): All within ±30% 4°C (51 h): All within ±30% –70°C (237 days): All within ±30% −20°C (22 days): All within ±30%
T-Tau	Calibration model	±15% bias (≤20% at the LLOQ and ULOQ) ≤20% CV	% bias:–2.43 to 13.04 % CV: 0.00–4.17
	Accuracy (interrun) and relative accuracy	±30% bias ±35% bias for the LLOQ and ULOQ validation samples	% bias:–9.52 to 4.91 % bias (RA):–8.49 to 1.88
	Precision: Intrarun (repeatability)	≤ 20% CV for Pooled QCs	% CV: 0.64–12.31
	Precision: interrun (intermediate precision)	≤25% CV for all validation samples	% CV: 6.05–13.45
	Total error (Interrun):	[|% bias| + % CV] ≤40% for all validation samples	% total error: 6.13–22.97
	Hemoglobin interference (mg/dL)	± 30% bias of 0 mg/dL Hb sample (pooled QC 1)	% bias:–88.24 to–68.38 (unacceptable)
	Stability: Freeze/thaw Short term Long term	2/3 of the pooled QCs must be within ±30% of the pooled QC interassay mean for each test	FTS (four cycle): All within ±30% 22°C (51 h): All within ±30% 4°C (51 h): All within ±30% –70°C (237 days): All within ±30% −20°C (22 days): All within ±30%
P-Tau	Calibration model	± 15% bias (≤20% at the LLOQ and ULOQ) ≤ 20% CV	% bias:–1.50 to 2.53 % CV: 0.00–2.41
	Accuracy (interrun) and relative accuracy	±30% bias ±35% bias for the LLOQ and ULOQ validation samples	% bias:–4.72 to–0.27 % bias (RA):–9.28 to 4.48
	Precision: Intrarun (repeatability)	≤20% CV for pooled QCs	% CV: 0.00–14.43
	Precision: Interrun (intermediate precision)	≤25% CV for all validation samples	% CV: 2.97–9.91
	Total error (interrun):	[|% Bias| + % CV] ≤40% for all validation samples	% total error: 3.76–10.53
	Hemoglobin interference (mg/dL)	± 30% bias of 0 mg/dL Hb sample (pooled QC 1)	% bias: 9.80–13.73
	Stability: Freeze/thaw Short term Long term	2/3 of the pooled QCs must be within ±30% of the pooled QC interassay mean for each test	FTS (four cycle): All within ±30% 22°C (51 h): 2/3 within ±30% 4°C (24 h): 2/3 within ±30% –70°C (192 days): All within ±30% −20°C: None established

analytes when 3, 10, 25, or 50 BC were compared with 100 BC, it was clearly indicated that ρ_c was becoming smaller as the comparison BC number decreased from 50 to 3 (Table 4). Further analysis indicated that this decrease in ρ_c was primarily caused by the Pearson correlation coefficient ρ, a measure of deviation from each concentration point to the best-fitted line.

Table 2. ANOVA Results

Analyte	Comparisons	LSMean[a] Test	LSMean[a] Ref	Difference[b]	Ratio (%)[c] (Test/Ref)	99% CI[d] Lower	99% CI[d] Upper	p Value[e]
Aβ42	3 vs. 100 BC	346.2	353.6	–7.4	97.9	93.1	102.7	0.3126
	10 vs. 100 BC	344.3	353.6	–9.3	97.4	92.5	102.2	0.2027
	25 vs. 100 BC	353.9	353.6	0.3	100.1	95.3	104.9	0.9686
	50 vs. 100 BC	352.0	353.6	–1.6	99.6	94.7	104.4	0.8261
T-Tau	3 vs. 100 BC	348.7	361.8	–13.1	96.4	92.1	100.7	0.0501
	10 vs. 100 BC	347.0	361.8	–14.8	95.9	91.6	100.2	0.0267
	25 vs. 100 BC	361.6	361.8	–0.2	100.0	95.7	104.2	0.9781
	50 vs. 100 BC	362.2	361.8	0.4	100.1	95.8	104.4	0.9527
P-Tau	3 vs. 100 BC	62.5	61.6	0.9	101.4	98.1	104.6	0.3249
	10 vs. 100 BC	61.2	61.6	–0.4	99.3	96.0	102.5	0.5936
	25 vs. 100 BC	61.4	61.6	–0.2	99.7	96.4	102.9	0.8095
	50 vs. 100 BC	61.4	61.6	–0.2	99.7	96.4	103.0	0.8361

[a]Least squares mean for the test (3, 10, 25, or 50 BC) and Ref (100 BC).
[b]Difference = LS Mean (Test)–LS Mean (Ref).
[c]Ratio (%) = LS Mean (Test)/LS Mean (Ref).
[d]99% confidence interval.
[e]p value for the difference; Significant difference defined a priori as $p < 0.05$.

Table 3. Bland–Altman Analysis

Analyte	Statistical Parameters	50 vs. 100 BC	25 vs. 100 BC	10 vs. 100 BC	3 vs. 100 BC
Aβ42	N	139	139	139	139
	Mean of differences (D)	–1.6	0.3	–9.3	–7.4
	Standard deviation of differences (S)	11.0	35.6	38.0	57.0
	D–2S	–23.5	–71.0	–85.4	–121.4
	$D + 2S$	20.3	71.5	66.7	106.6
T-Tau	N	137	137	137	137
	D	0.4	–0.2	–14.7	–13.0
	S	6.1	21.5	91.1	94.7
	D–2S	–11.9	–43.2	–197.0	–202.5
	$D + 2S$	12.7	42.9	167.5	176.4
P-Tau	N	138	138	138	138
	D	–0.2	–0.3	–0.4	0.8
	S	1.5	2.6	4.7	7.3
	D–2S	–3.2	–5.6	–9.7	–13.9
	$D + 2S$	2.8	5.0	9.0	15.4

Table 4. Concordance Correlation Coefficients

Analyte	Comparisons	ρ^a	Cb[b]	$\rho_c{}^c$	95% CI Lower[d]	95% CI Upper[d]
Aβ42	3 vs. 100 BC	0.9900	0.9993	0.9894	0.9891	0.9897
	10 vs. 100 BC	0.9961	0.9990	0.9950	0.9949	0.9952
	25 vs. 100 BC	0.9961	1.0000	0.9961	0.9959	0.9962
	50 vs. 100 BC	0.9997	0.9999	0.9996	0.9996	0.9996
T-Tau	3 vs. 100 BC	0.9741	0.9974	0.9716	0.9708	0.9723
	10 vs. 100 BC	0.9781	0.9944	0.9726	0.9720	0.9733
	25 vs. 100 BC	0.9986	1.0000	0.9986	0.9986	0.9987
	50 vs. 100 BC	0.9999	1.0000	0.9999	0.9999	0.9999
P-Tau	3 vs. 100 BC	0.9932	0.9990	0.9922	0.9920	0.9924
	10 vs. 100 BC	0.9967	1.0000	0.9967	0.9966	0.9968
	25 vs. 100 BC	0.9990	0.9999	0.9989	0.9989	0.9990
	50 vs. 100 BC	0.9997	1.0000	0.9997	0.9996	0.9997

[a]Pearson correlation coefficient.
[b]The bias correction factor.
[c]Concordance correlation coefficient.
[d]95% CI for ρ_c.

The concordance correlation coefficients (ρ_c) together with their 95% CI are presented in Table 4. To further understand ρ_c, the two components used in calculating ρ_c are also presented in Table 4.

The variability of duplicate measures on a sample is represented in Figure 1. The frequency distribution of correlation coefficient (CV) in four range groups, 0–5, 6—10%, 11–20%, and >20% for each BC with all three analytes is summarized (see Table 5). As expected, a trend is observed, such that, a decrease in the number of BCs correlates with increased variability between duplicate measurements.

DISCUSSION

Product A is designed to measure three biomarkers, which have previously been implicated in AD: Aβ42, the primary marker for plaque formation; tau (total), thought of as a reflection of neuronal damage resulting from the disease state; and tau protein phosphorylated at Thr-181. The assay is a popular platform for AD biomarker detection in CSF. Prior to in-house method validation, a priori acceptance criteria were defined in accordance with suggestions for biomarker method validation to support drug discovery. Acceptance criteria for the initial validation (100 BC) were acceptable and are described in Table 1. During a qualification of a subsequent commercial kit lot release, a dramatic loss in BC for xMAP region 2 (t-tau) was observed, resulting in assay run failures. A substantial number of assay plate wells were unable to reach maximum count (100 beads) for bead region 2. The manufacturer's suggestion to avoid assay run failures was to adjust the software parameters and decrease the required number of acquired beads counted per analyte per well. An assessment was conducted to determine whether altering the number of beads used to determine MFI reporter values had a detrimental effect on assay performance.

There was a trend that as BC number acquisition was decreased from 100 to 3, the percentage of results that had more imprecision between duplicate wells increased. Results with a %CV from 0–5% decreased, but the 11–20% and ≥21% groups increased (Figure 1A–1C). The interpretation is that decreased BC results in more variability in MFI between identical samples. Equivalence in raw data generation and subsequent regression was strengthened between 50 and 100 BC and to a lesser degree between 25 and 100 BC, based on the results from the three statistical methods.

Observation of changes in CV with decreased BC is important to future applications using all technologies associated with product A, as it demonstrates a need to expound limits on minimum BC used to determine reporter MFI values. These data have also been helpful in defining a connection between problems with assay components, and how this translates to actual assay performance characteristics (e.g., events per second during acquisition, acquisition time, %CV

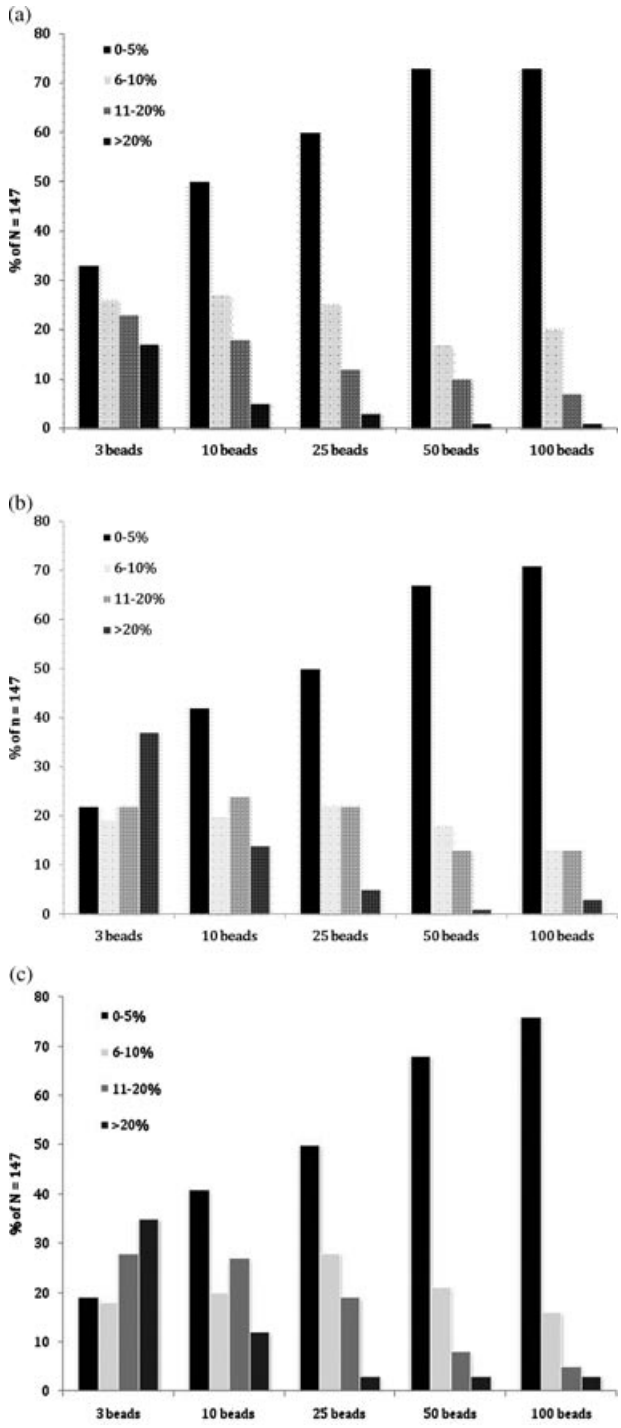

Figure 1. Representative plot describing precision between duplicate microtiter plate wells at 3, 10, 25, 50, and 100 BC parameters. (A) Aβ42 frequency analysis of CV ranges: percentage of $N = 147$. (B) t-Tau frequency analysis of CV ranges: percentage of $N = 147$. (C) p-Tau frequency analysis of CV ranges: percentage of $N = 147$.

Table 5. Frequency Distribution of CV Ranges

Analyte	Bead Count	CV Range (%)	Frequency	Percentage of Total (%)
Aβ42	3	0–5	43	32
		6–10	32	24
		11–20	31	23
		> 20	30	22
	10	0–5	64	49
		6–10	34	26
		11–20	25	19
		> 20	8	6
	25	0–5	79	59
		6–10	33	25
		11–20	18	13
		> 20	4	3
	50	0–5	86	70
		6–10	22	18
		11–20	13	11
		> 20	2	2
	100	0–5	94	70
		6–10	29	22
		11–20	10	7
		> 20	1	1
T-Tau	3	0–5	29	21
		6–10	25	18
		11–20	33	23
		> 20	54	38
	10	0–5	55	40
		6–10	26	19
		11–20	31	23
		> 20	25	18
	25	0–5	69	50
		6–10	27	20
		11–20	30	22
		> 20	11	8
	50	0–5	84	64
		6–10	26	20
		11–20	18	14
		> 20	3	2
	100	0–5	93	72
		6–10	16	12
		11–20	19	14
		> 20	4	3
P-Tau	3	0–5	27	22
		6–10	23	19
		11–20	35	29
		> 20	57	47
	10	0–5	54	40
		6–10	22	17
		11–20	39	29
		> 20	18	14
	25	0–5	62	47
		6–10	37	28
		11–20	27	20
		> 20	6	5
	50	0–5	87	67
		6–10	26	20
		11–20	11	9
		> 20	5	4
	100	0–5	101	75
		6–10	20	14
		11–20	8	6
		> 20	5	4

between duplicate wells). The study data include all data points without regard for failing criteria. This approach was taken because as the validation was unsuccessful when recalculating the data for bead counts of less than 50 (with original acceptance criteria). Reduced bead counts produced resulted in an increase in intraassay CV values greater than 20%. At BC of 3 and 10, validation runs would not have been acceptable simply because the calibration curve models alone would fail %CV criteria. In spite of the apparent equivalence demonstrated by the statistical analyses performed in this study, reducing bead counts to determine representative MFIs also decreases precision between replicates of the same sample. Consequently, our analysis suggests bead counts of least 25 per analyte per well are needed to obtain sound data to support a biomarker method validation.

Discussions with the developers of the microsphere and analyzer components of this immunoassay technology suggest the use of the median fluorescent value, as opposed to the mean, for statistical data analysis. This is because the median is relatively insensitive to any outliers existing in a given bead set. As shown in this study, the number of beads on which to base this median can vary in its capacity to deliver reliable data. Typically, 50–100 beads are read to determine MFI, but actual BCs vary depending on the manufacturer of kits, changes in lots of reagent components in kits (antibodies conjugated to microspheres, detection antibodies, etc.), and between scientists developing analytical methods these technologies. A BC of 35 is suggested by developers because any fewer events can no longer guarantee the exclusion of outliers in the data.[21] However, these data demonstrate that product A may be able to use a minimum BC threshold acquisition number of 25 and still generate data that are comparable to that obtained at 100 BC.

For purposes of sample analysis, a good laboratory practices revalidation of the assay was performed by recomputing the original 100 BC data using a 50 BC protocol and regressing the data using StatLIA® according to a priori acceptance criteria. Based on these statistical analyses, equivalency was observed between 50 and 100 BC methods when determining the concentrations of Aβ42, t-tau, and p-tau in human CSF.

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