Analyzer-to-Analyzer Variations in Assaying Ultralow Concentrated Biomarkers Associated with Neurodegenerative Diseases Using Immunomagnetic Reduction

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High consistence in assaying biomarkers among three IMR analyzers
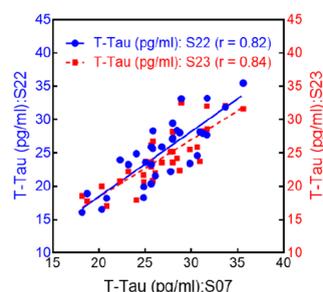
ABSTRACT: By utilizing a high-temperature superconducting quantum interference device (high-T_c SQUID) magnetometer, an alternating current (AC) magnetosusceptometer, referred to as an analyzer, was developed for ultrasensitive immunoassays. The analyzer has been applied to assay biomarkers in human plasma associated with Alzheimer's disease (AD) and Parkinson's disease (PD). The involved assay methodology is the so-called immunomagnetic reduction (IMR). Such an analyzer has been approved for clinical use in Taiwan and Europe. The mass production of the analyzer is needed for clinical utilities. The issue of exploring analyzer-to-analyzer variations in the performances becomes critical. Unfortunately, there is no standard characterization to determine the variations in performances among analyzers. In this study, key characterizations, such as output signal stability, signal-to-noise ratio, measured concentrations of a control sample, etc., are proposed. In total, three analyzers are characterized in this work. The detected biomarkers include amyloid peptides, total tau protein, phosphorylated tau protein, and α-synuclein protein for AD and PD. Through one-way ANOVA for any of the characterizations among the three analyzers, it was found that there was no significant difference in any of these characterizations among the analyzers ($p > 0.05$). Furthermore, the three analyzers are applied to assay biomolecules for AD and PD in reference samples. High correlations ($r > 0.8$) in measured concentrations of any of these biomarkers in reference samples were obtained among the three analyzers. The results demonstrate that the proposed characterizations are feasible for achieving consistent performance among high-T_c SQUID-based AC magnetosusceptometers for assaying biomolecules.

KEYWORDS: magnetosusceptometer, immunomagnetic reduction, biomarkers, Alzheimer's disease, Parkinson's disease

INTRODUCTION

Assaying fluid biomarkers plays a role in the assessment of neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD).[1−3] The pathological biomarkers are amyloid β 1-40 ($A\beta_{1-40}$) or 1-42 ($A\beta_{1-42}$) peptide, total tau protein (T-Tau), or phosphorylated tau protein (threonine 181) (pTau181) for AD.[4−7] The pathological biomarker of PD is α-synuclein protein.[8,9] The correlations between the concentrations of these biomarkers in cerebrospinal fluid (CSF) and neuropathology in AD and PD have been evidenced.[10−13] Assay of CSF biomarkers is suggested to be done in assessing AD and PD in clinical practices. However,

lumbar puncture for CSF biomarker assays is invasive and leads to several side effects causing pain and discomfort to the subject. Noninvasive assays of fluid biomarkers, such as blood tests, are expected. Unfortunately, the concentrations of these biomarkers in human plasma are extremely low, ~pg/mL or
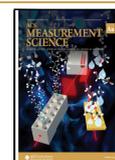
lower, which is 1−10% of the concentrations in CSF. Conventional assay technologies are not sensitive enough to precisely assay these biomarkers in human plasma. Ultrasensitive assay technologies are needed.

In the early 2000s, authors developed ultrasensitive assay technology called immunomagnetic reduction (IMR).[14−16] The IMR reagent is a pH 7.4 phosphate-buffered saline (PBS) solution containing well-dispersed magnetic nanoparticles with diameters of approximately 55 nm that are functionalized with antibodies.[17] The characterizations of the IMR reagent were reported in published papers.[17,18] The first-generation IMR analyzer was issued in 2008.[14] Intuitively, the IMR analyzer is a high-temperature superconducting quantum interference device (high-T_c SQUID) magnetosusceptometer. Because of the demands of high throughput for clinical use, second-, third-, and fourth-generation IMR analyzers were developed in 2010, 2018, and 2022, respectively.[19−21] The evolution from the first-generation to the fourth-generation high-T_c SQUID magnetosusceptometer was discussed in ref 21. Currently, the IMR analyzer is a 36-channel high-T_c SQUID magnetosusceptometer. More than 13 000 vials of plasma biomarkers are available with an IMR analyzer.

With the reagent and the analyzer, tremendous clinical evidence has been obtained.[22−27] The results demonstrate the feasibility of using IMR to precisely identify plasma biomarkers of AD and PD for clinical practice. In the early 2020s, the high-T_c SQUID magnetosusceptometer and IMR reagent for assaying biomarkers associated with AD or PD were approved for routine clinical practice in Europe and Taiwan. Because of the unmet demands of plasma tests for the assessment of AD and PD, many fourth-generation high-T_c SQUID magnetosusceptometers are needed. This stirs up the mass production of the fourth-generation high-T_c SQUID magnetosusceptometer for clinical markets.

One important issue with mass production is how to control variations in performance among high-T_c SQUID magnetosusceptometers. Unfortunately, there is no standard characterization to determine the variations in performance among IMR analyzers. In this study, we propose some key characterizations of a high-T_c SQUID magnetosusceptometer, such as output signal stability, signal-to-noise ratio, and measured concentrations of a control sample, as checkpoints for quality control of mass production. In addition, the variations in the characterizations among high-T_c SQUID magnetosusceptometers are investigated.

EXPERIMENTAL SECTION

Output Signal Stability

The 5 h output AC magnetosusceptibility χ_{ac} of a calibrator is recorded. The calibrator was a PBS solution composed of 55 nm diameter Fe_3O_4 magnetic nanoparticles (MF-DEX-0080, MagQu). The magnetic concentration of the calibrator was 10 mg of Fe/mL. A 120 μL calibrator is put into a glass tube, referred to as a sample tube, followed by placement of the sample tube into a channel of the IMR analyzer. An IMR analyzer has 36 channels, so 36 identical calibrators are used in one measurement run. The mean value and the standard deviation of the 5 h χ_{ac} signals of a channel are calculated.

Signal-to-Noise Ratio

The mean value of the 5 h χ_{ac} signals of a channel with the calibrator is referred to as a signal, while the mean value of the 5 h χ_{ac} signals of the channel without anything is referred to as a noise level. The ratio of the signal to the noise is calculated, referred to as the signal-to-noise ratio of a channel.

Measurements of Concentrations of a Control Sample

PBS solutions spiked with various biomarkers of known concentrations, which are referred to as control samples, were prepared, as listed in Table 1. The IMR reagent model for each kind of control

Table 1. Specifications of Control Samples Spiked with Various Biomarkers and the Reagent Models Used for IMR Measurements

spiked biomarker	spiked concentration (pg/mL)	reagent model	volume in μL(sample/reagent)
$A\beta_{1-40}$	50	MF-AB0−0060	40/80
$A\beta_{1-42}$	20	MF-AB2−0060	60/60
T-Tau	50	MF-TAU-0060	40/80
pTau181	5	MF-PT1−0060	40/80
α-synuclein	10	MF-ASC-0060	40/80

sample is also listed in Table 1. For assaying $A\beta_{1-40}$, T-Tau, pTau181, and α-synuclein, a 40 μL control sample was mixed with 80 μL of reagent. For assaying $A\beta_{1-42}$, a 60 μL control sample was mixed with 60 μL of reagent.

Assay of Biomarkers in Plasma Substitutes

Thirty-two human plasma substitutes (Gelofusine, B. Braun) spiked with various concentrations of $A\beta_{1-40}$, $A\beta_{1-42}$, T-Tau, pTau181, or α-synuclein were prepared and referred to as SK samples. The 32 SK samples spiked with a given biomarker at various concentrations were assayed with IMR reagent and three IMR analyzers (XacPro-S, MagQu) labeled with S07, S22, and S23. The volumes of sample and reagent used for one measurement are the same as in the case of control samples.

Statistical Methods

Continuous variables are presented as the mean ± standard deviation. The coefficient of variation (CV) is calculated via the ratio of the standard deviation to the mean value. Continuous variables were compared using one-way ANOVA to determine the differences ($p < 0.05$). The Pearson coefficient r was calculated to investigate the correlations between continuous variables of two groups.

RESULTS AND DISCUSSION

The 5 h real-time χ_{ac} signals of the calibrator detected with a channel of the analyzer S07 are shown in Figure 1a. There was no significant change in χ_{ac} with time ($p > 0.05$). The time-averaged value of χ_{ac} was found to be 995.3, as guided by the gray solid line. The standard deviation of the data shown in Figure 1a was 10.3. The coefficient of variation (CV) was 1.03%.

The distribution of CV of the 5 h χ_{ac} among the 36 channels in analyzer S07 is plotted in Figure 1b. The CV of χ_{ac} among the 36 channels in analyzer S07 ranges from 0.76% to 1.65%, thereby resulting in 1.18% for the mean value and 0.23% for the standard deviation. Thus, the channel-to-channel variation in CV of the 5 h χ_{ac} of the calibrator is 19.5% for analyzer S07.

The distribution of CV of the 5 h χ_{ac} among the 36 channels in analyzer S07 was recorded weekly for approximately one month from December 17, 2020, to January 12, 2021, as shown in Figure 1c. Obviously, the distribution of CV of the 5 h χ_{ac} among the 36 channels in analyzer S07 did not vary significantly for one month. This implies that the output χ_{ac} signals of the calibrator from analyzer S07 are stable.

(a)

(b)

(c)
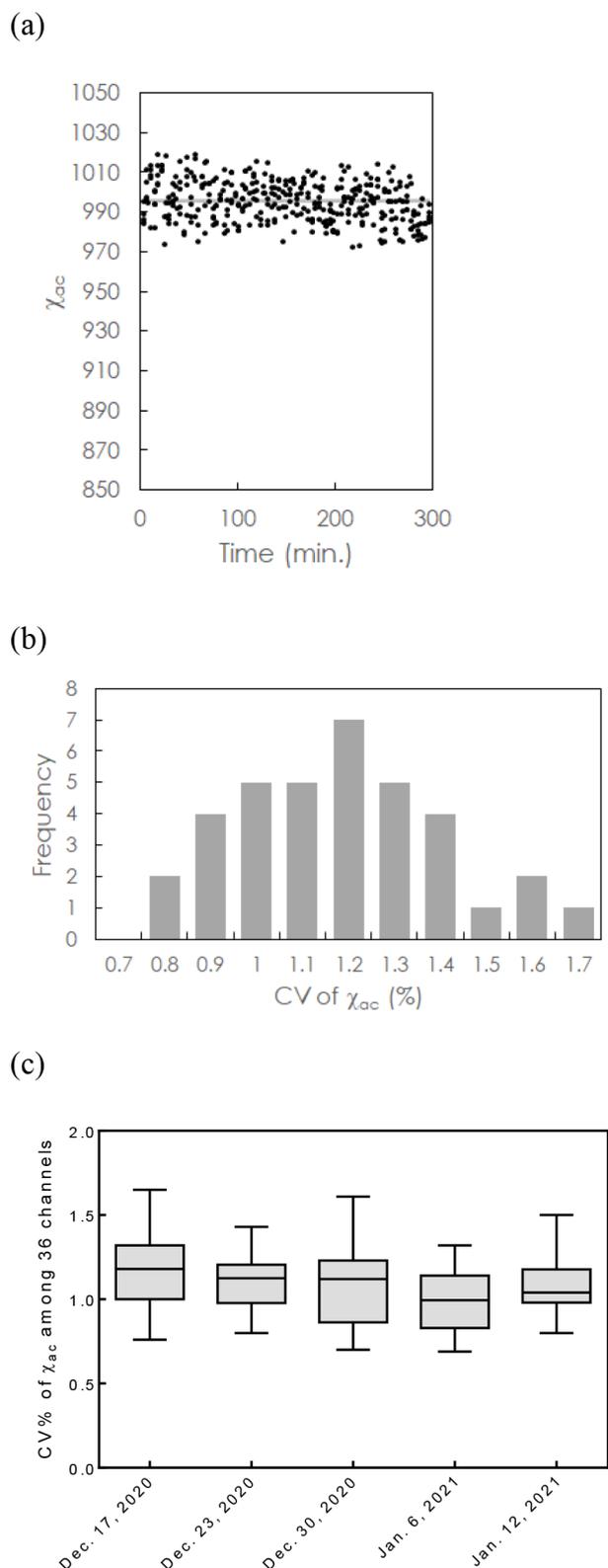
Figure 1. (a) 5 h real-time χ_{ac} signals of a calibrator detected with a channel of the analyzer S07, (b) histogram of CV values of the real-time χ_{ac} signals in (a) among the 36 channels of S07, and (c) distributions of CV values of the real-time χ_{ac} signals in (a) among the 36 channels of S07 over approximately one month.

The analyzer-to-analyzer variation in the distributions of CV of the 5 h χ_{ac} among the 36 channels of an analyzer is

investigated. There are three analyzers to be investigated, referred to as S07, S22, and S23. The results are shown in Figure 2a and are listed in Table 2. S07 showed $1.18 \pm 0.23\%$

(a)

(b)
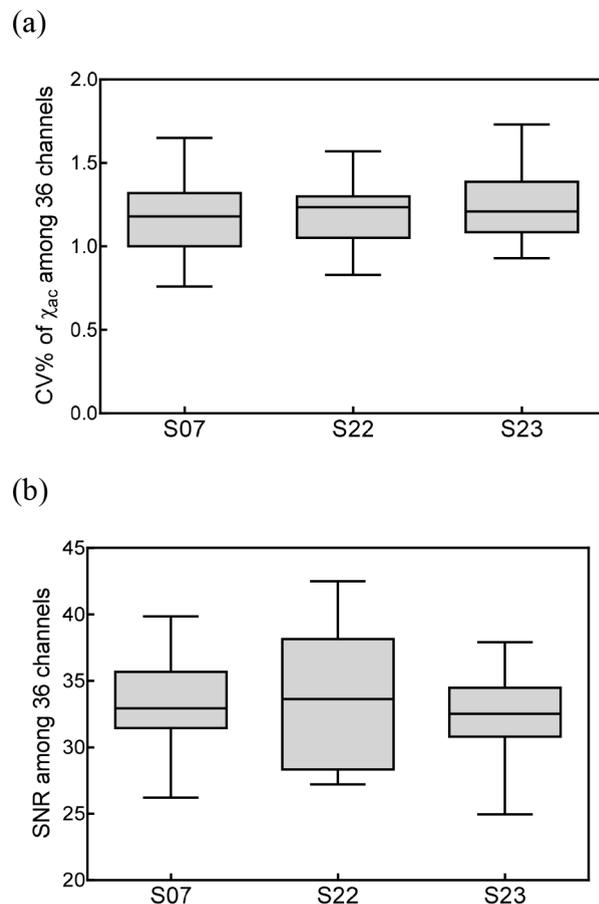
Figure 2. Distributions of (a) CV values of the real-time χ_{ac} signals in Figure 1a,b signal-to-noise ratios of a calibrator among the 36 channels of three analyzers S07, S22, and S23.

Table 2. Coefficient of Variation (CV) of 5 h χ_{ac} Signals and Signal-to-Noise Ratio (SNR) among 36 Channels of an Analyzer for the Calibrator

analyzer	S07	S22	S23	p value
CV of χ_{ac} among 36 channels (%)	1.18 ± 0.23	1.21 ± 0.18	1.24 ± 0.19	>0.05
signal-to-noise ratio (SNR)	33.30 ± 3.54	33.63 ± 4.79	32.43 ± 3.04	>0.05

for the CV of the 5 h χ_{ac} among 36 channels. S22 and S23 show $1.21 \pm 0.18\%$ and $1.24 \pm 0.19\%$, respectively. Through ANOVA, the p value in the analyzer-to-analyzer CV of the 5 h χ_{ac} among the 36 channels is higher than 0.05. This implies that the three analyzers perform identically in the distributions of CV of the 5 h χ_{ac} among the 36 channels.

In addition to the output signal stability, the signal-to-noise ratios among 36 channels of an analyzer are investigated. The measurement of the signal-to-noise ratio (SNR) of a channel in an analyzer is described in the Experimental Section. Among the 36 channels in analyzer S07, the average SNR is 33.30, and the standard deviation is 3.54, as listed in Table 2. The other

(a)

(b)

(c)

(d)

(e)
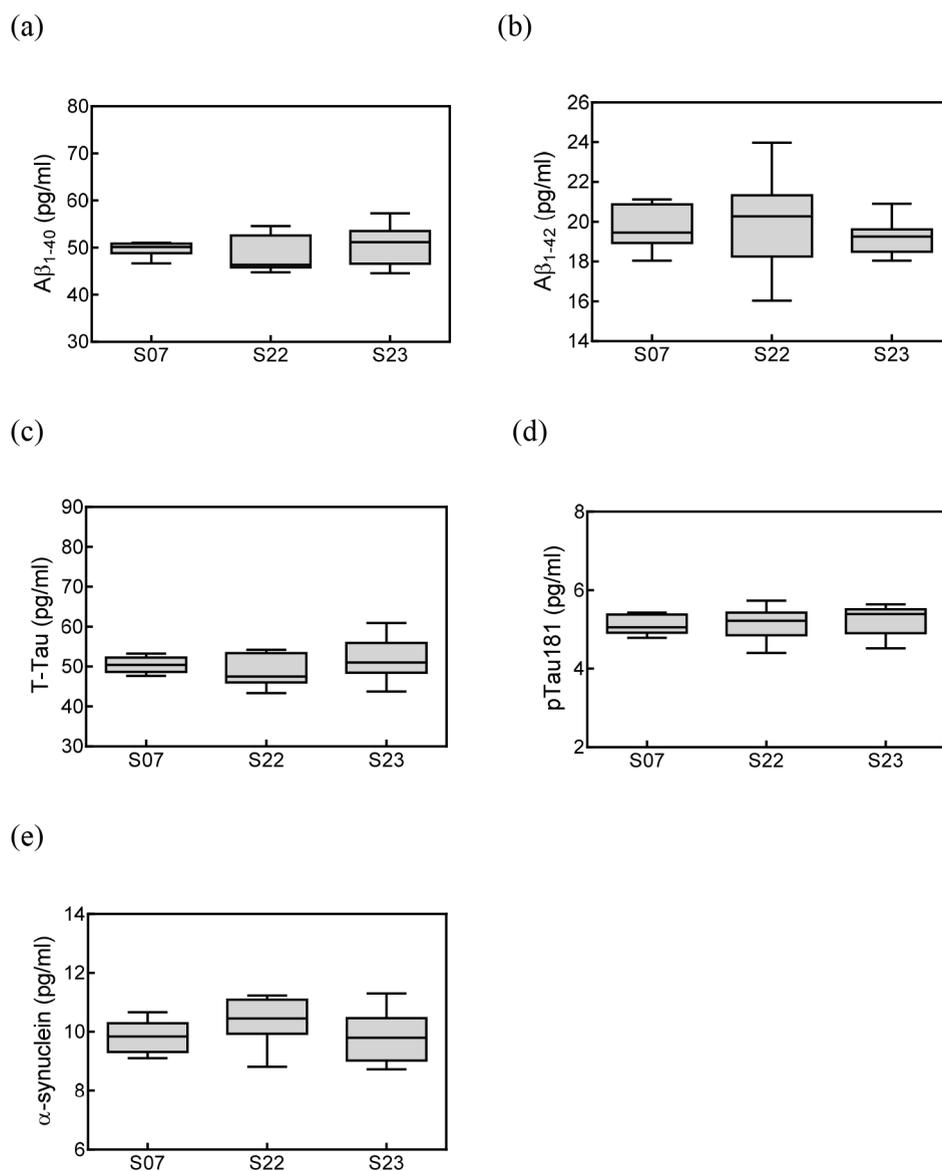
Figure 3. Measured (a) $A\beta_{1-40}$, (b) $A\beta_{1-42}$, (c) T-Tau, (d) pTau181, and (e) α-synuclein concentrations of control solutions using three analyzers, S07, S22, and S23.

two analyzers, S22 and S23, show 33.63 ± 4.97 and 32.43 ± 3.04 for SNR among 36 channels, as listed in Table 2 and shown in Figure 2b. Through ANOVA, the p value in the distribution of the 36-channel SNR among the three analyzers is higher than 0.05. This means that there is no significant difference in SNR among the 36 channels for these three analyzers.

The measured concentrations of control solutions, which are spiked with various proteins $A\beta_{1-40}$, $A\beta_{1-42}$, T-Tau, pTau181, and α-synuclein, detected with the three analyzers are shown in Figure 3a−e. The spiked concentration of the control solution was 50 pg/mL for $A\beta_{1-40}$, 20 pg/mL for $A\beta_{1-42}$, 50 pg/mL for T-Tau, 5 pg/mL for pTau181, and 10 pg/mL for α-synuclein, as listed in Table 1. The mean value and the standard deviation of the measured $A\beta_{1-40}$ concentrations among the 36 channels of S07 were found to be 49.72 and 1.42 pg/mL, respectively, as listed in Table 3. The channel-to-channel variation in assaying $A\beta_{1-40}$ using S07 is 2.8% (= 1.42/49.72 × 100%). The recovery rate, which is defined by the ratio of the mean value of measured $A\beta_{1-42}$ concentrations among 36 channels to the

spiked concentration, is obtained to be 99.4% (= 49.72/50 × 100%).

The measured $A\beta_{1-40}$ concentrations among the 36 channels using S22 and S23 are 48.17 ± 3.62 and 50.57 ± 3.89 pg/mL, respectively, as listed in Table 3 and shown in Figure 3a. The results reveal 7.5% and 7.7% for the channel-to-channel variation in assaying $A\beta_{1-40}$ with S22 and S23, respectively. Meanwhile, the recovery rate is 96.3% for S22 and 101.1% for S23. With mean values of 49.72, 48.17, and 50.57 pg/mL for the measured $A\beta_{1-40}$ concentrations among 36 channels with S07, S22, and S23, the analyzer-to-analyzer variation is found to be 2.46%.

In the case of $A\beta_{1-42}$, the measured concentrations among the 36 channels are 19.74 ± 1.06 pg/mL for S07, 19.99 ± 2.28 pg/mL for S22, and 19.18 ± 0.87 pg/mL for S23, as listed in Table 3 and shown in Figure 3b. There was no significant difference in the measured $A\beta_{1-42}$ concentrations among the three analyzers ($p > 0.05$). The channel-to-channel variation in the measured $A\beta_{1-42}$ concentration ranges from 4.5% to 11.4%. The recovery rate in assaying $A\beta_{1-42}$ ranged from 95.9% to

Table 3. Measured Concentrations of Aβ_{1-40}, Aβ_{1-42}, T-Tau, pTau181, and α-Synuclein Control Samples among 36 Channels of an Analyzer

analyzer biomarker		S07	S22	S23	*p* value
Aβ_{1-40}	mean ± SD (pg/mL)[a]	49.72 ± 1.42	48.17 ± 3.62	50.57 ± 3.89	>0.05
	channel-to-channel variation	2.8%	7.5%	7.7%	
	recovery rate	99.4%	96.3%	101.1%	
	analyzer-to-analyzer variation	2.46%			
Aβ_{1-42} (pg/mL)	mean ± SD (pg/mL)[a]	19.74 ± 1.06	19.99 ± 2.28	19.18 ± 0.87	>0.05
	channel-to-channel variation	5.4%	11.4%	4.5%	
	recovery rate	98.7%	99.9%	95.9%	
	analyzer-to-analyzer variation	2.11%			
T-Tau (pg/mL)	mean ± SD (pg/mL)[a]	50.48 ± 2.03	48.81 ± 3.79	51.76 ± 5.1	>0.05
	channel-to-channel variation	4.0%	7.8%	9.8%	
	recovery rate	101.0%	97.6%	103.5%	
	analyzer-to-analyzer variation	2.94%			
pTau181 (pg/mL)	mean ± SD (pg/mL)[a]	5.10 ± 0.24	5.14 ± 0.43	5.24 ± 0.39	>0.05
	channel-to-channel variation	4.7%	8.4%	7.4%	
	recovery rate	102.0%	102.8%	104.8%	
	analyzer-to-analyzer variation	1.40%			
α-synuclein (pg/mL)	mean ± SD (pg/mL)[a]	9.81 ± 0.52	10.43 ± 0.77	9.82 ± 0.88	>0.05
	channel-to-channel variation	5.3%	7.4%	9.0%	
	recovery rate	98.1%	104.3%	98.2%	
	analyzer-to-analyzer variation	3.54%			

[a]Mean value and standard deviation of measured concentrations among 36 channels.

98.7% among analyzers. The analyzer-to-analyzer variation in assaying Aβ_{1-42} is found to be 2.11%.

The results of assaying 50 pg/mL of T-Tau control solution using S07, S22, and S23 are listed in Table 3 and shown in Figure 3c. Results of 50.48 ± 2.03, 48.81 ± 3.79, and 51.76 ± 5.1 pg/mL were obtained for the measured T-Tau concentrations among the 36 channels using S07, S22, and S33, respectively. Through one-way ANOVA, the *p* value was found to be higher than 0.05, thereby indicating a nonsignificant difference in the measured T-Tau concentrations among the three analyzers. For each analyzer, the channel-to-channel variation in assaying T-Tau is within the range from 4.0% to 9.8%. The recovery rate ranges from 97.6% to 103.5%. The analyzer-to-analyzer variation in the mean values of measured T-Tau concentrations among 36 channels is found to be 2.94%.

For assaying pTau181, the mean values and standard deviations among 36 channels using S07, S22, and S33 are listed in Table 3 and shown in Figure 3d. The fact that the *p* value is higher than 0.05 reveals the nonsignificant difference in the measured pTau181 concentrations among analyzers S07, S22, and S23. The channel-to-channel variation in the measured pTau181 concentrations is 4.7% for S07, 8.4% for S23, and 7.4% for S23. The recovery rate of assaying pTau181 is 102.0% for S07, 102.8% for S22, and 104.8% for S23. The analyzer-to-analyzer variation in the mean values of the measured pTau181 concentrations among the 36 channels is calculated to be 1.40%.

The measured α-synuclein concentration among the 36 channels of S07 was 9.81 ± 0.52 pg/mL, which resulted in 5.3% channel-to-channel variation and 98.1% recovery rate, as listed in Table 3 and shown in Figure 3e. The measured α-synuclein concentration for S22 of 10.43 ± 0.77 pg/mL is obtained among 36 channels, thereby resulting in 7.4% channel-to-channel variation and 104.3% recovery rate. S23 shows 9.82 ± 0.88 pg/mL for the measured α-synuclein concentrations among 36 channels, thereby resulting in 9.0%

channel-to-channel variation and 98.2% recovery rate. The analyzer-to-analyzer variation in assaying α-synuclein is found to be 3.54%.

The channel-to-channel variation in assaying a biomarker of an analyzer corresponds to the assay irrepeatability. In studies on bioanalytical method validation,[28−30] the acceptable irrepeatability for clinical use should be 20−30%. According to the results in Table 3, the irrepeatability of assaying biomarkers associated with neurodegenerative disease using IMR exists within the range from 2.8% to 11.4%, which is lower than 20%, thereby indicating that the measured results for Aβ_{1-40}, Aβ_{1-42}, T-Tau, pTau181, and α-synuclein are reliable, even at ultralow concentrations in the pg/mL range.

The recovery rate in assaying biomarkers of control solutions denotes the assay accuracy. The results in Table 3 reveal that the precision of assaying these biomarkers is 95.9−104.8%. The Clinical & Laboratory Standards Institute (CLSI) provides standards and guidelines for acceptable ranges of recovery rates, i.e., CLSI EP34. The acceptable range of recovery rates is from 90% to 110%. All the values of the recovery rate in Table 3 are distributed from 90 to 110%. This evidence indicates the high accuracy of assaying Aβ_{1-40}, Aβ_{1-42}, T-Tau, pTau181, and α-synuclein for IMR.

The coefficient of variation for the measured concentrations among the three analyzers ranges from 1.40% to 3.54%, as listed in Table 3. Obviously, the analyzer-to-analyzer variations in assaying biomarkers are lower than 5% at the concentration level of pg/mL. A high consistency in assaying pg/mL biomarkers among IMR analyzers was obtained.

Thirty-two SK samples with various concentrations of Aβ_{1-40}, Aβ_{1-42}, T-Tau, pTau181, and α-synuclein were assayed using S07, S22, and S23 with the aid of the IMR reagents listed in Table 1. The measured concentrations of the SK samples using one of the three analyzers, say S07, are used as a reference. The other measured concentrations of SK samples using S22 and S23 are compared with those using S07, as shown in Figure 4a−e.
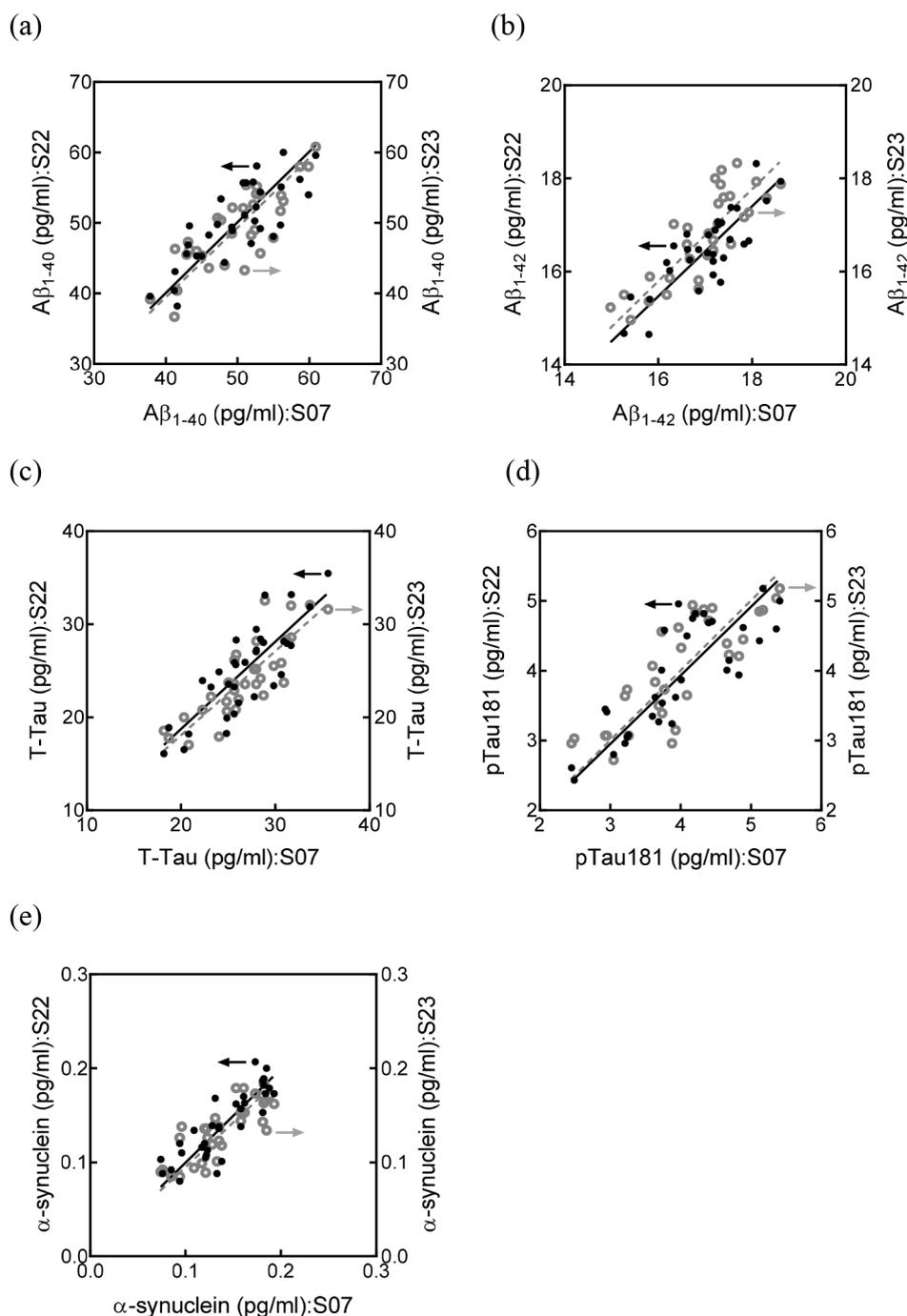
Figure 4. Relationships in measured (a) $A\beta_{1-40}$, (b) $A\beta_{1-42}$, (c) T-Tau, (d) pTau181, and (e) α-synuclein concentrations of SK samples between S22 and S07 (dots) and S23 and S07 (circles).

The relationship in the measured $A\beta_{1-40}$ concentrations of SK samples between S22 and S07 is investigated, as plotted with dots in Figure 4a. A significantly positive correlation ($r = 0.801$, $p < 0.0001$) is obtained, as listed in Table 4. The proportionality in the measured $A\beta_{1-40}$ concentrations between S07 and S22 is guided by the solid line in Figure 4a. The slope of the solid line is found to be 1.002. The coefficient of determination R^2 of the solid line fitted to the dots in Figure 4a is 0.9949. The measured $A\beta_{1-40}$ concentrations with S23 versus those with S07 are plotted with circles in Figure 4a. The Pearson correlation coefficient r is found to be 0.816 ($p < 0.0001$), which results in a high correlation in the measured

$A\beta_{1-40}$ concentrations of SK samples between S23 and S07. The proportionality of the circles in Figure 4a is guided with the dashed line. The slope of the dashed line is 0.987. The coefficient of determination R^2 of the dashed line fitted to the circles in Figure 4a is 0.9952.

According to the 510k guidelines issued by the US Food and Drug Administration (FDA), the two kinds of assays are identical, with a Pearson correlation coefficient r higher than 0.8 and a slope of the proportionality ranging between 0.9 and 1.1 for the relationship in measured concentrations. Clearly, the Pearson correlation coefficients r and the slopes listed in Table 4 for the $A\beta_{1-40}$ assay with S22 versus S07 and S23

Table 4. Pearson Correlation Coefficient r, Slope, and Coefficient of Determination R^2 of the Relationships in the Measured Concentrations for Various Biomarkers between Analyzers S07 and S22 and S07 and S23

S07 vs biomarker		S22	S23
$A\beta_{1-40}$	r (p value)	0.801 (<0.0001)	0.816 (<0.0001)
	slope	1.002	0.987
	R^2	0.9949	0.9952
$A\beta_{1-42}$	r (p value)	0.850 (<0.0001)	0.812 (<0.0001)
	slope	0.9668	0.9871
	R^2	0.9991	0.9989
T-Tau	r (p value)	0.824 (<0.0001)	0.836 (<0.0001)
	slope	0.9375	0.9037
	R^2	0.9886	0.9913
pTau181	r (p value)	0.812 (<0.0001)	0.810 (<0.0001)
	slope	0.9830	1.0013
	R^2	0.9861	0.9865
α-synuclein	r (p value)	0.856 (<0.0001)	0.807 (<0.0001)
	slope	0.9972	0.9484
	R^2	0.9829	0.9791

versus S07 meet the requirements of the US FDA 510k guidelines.

The relationships in measured $A\beta_{1-42}$, T-Tau, pTau181, and α-synuclein concentrations between S22 and S07 and S23 and S07 are plotted with dots and circles, respectively, in Figure 4b−e. The Pearson correlation coefficients r between the measured concentrations, slopes, and coefficients of determination R^2 of the proportionality are listed in Table 4. Remarkably, all values of the Pearson correlation coefficients are higher than 0.8. All slope values are in the range from 0.9 to 1.1. These results meet the requirements of the FDA 510k guideline for the equivalence of medical devices. Hence, a high consistency in assaying $A\beta_{1-40}$, $A\beta_{1-42}$, T-Tau, pTau181, and α-synuclein at levels of pg/mL is demonstrated among IMR analyzers.

CONCLUSIONS

Key specifications, such as output signal stability and signal-to-noise ratio of calibrators, measurements of concentrations of control solutions, etc., are suggested to characterize the operating performances of the IMR analyzers for in vitro protein assays. By well controlling these specifications of analyzers, a high consistency in assaying proteins, such as biomarkers associated with neurodegenerative diseases, among analyzers is achieved. These results lay out the protocol and checkpoints for the mass production of IMR analyzers in clinical uses.

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Author Contributions

K.-H.L. = methodology (equal) and software (equal); M.-H.H. = data curation (equal) and formal analysis (equal); H.-H.C. = formal analysis (equal), investigation (equal), and validation (equal); and S.-Y.Y. = conceptualization (equal), resources (equal), supervision (equal), and writing-original draft (equal). CRediT: **Kun-Hung Lee** methodology, software; **Ming-Hung Hsu** data curation; **Hsin-Hsien Chen** formal analysis, investigation, validation; **Shieh-Yueh Yang** conceptualization, resources, supervision, writing-original draft.

Notes

The authors declare the following competing financial interest(s): All the authors are employees of MagQu Co., Ltd. H.-H.C. and S.-Y.Y. are shareholders of MagQu Co., Ltd.

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