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CERTIFICATION REPORT

The certification of Amyloid β_{1-42} in CSF in ERM®-DA480/IFCC, ERM®-DA481/IFCC and ERM®-DA482/IFCC

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Abstract

This report describes the production of ERM®-DA480/IFCC, ERM®-DA481/IFCC and ERM®-DA482/IFCC, which are human cerebrospinal fluid (CSF) materials certified for the mass concentration of amyloid β_{1-42} peptide ($A\beta_{1-42}$). These materials were produced by the European Commission's Joint Research Centre (EC-JRC) in collaboration with the International Federation for Clinical Chemistry and Laboratory Medicine (IFCC) following ISO Guide 34:2009 and are certified in accordance with ISO Guide 35:2006.
The starting material used to prepare ERM-DA480/IFCC, ERM-DA481/IFCC and ERM-DA482/IFCC was human CSF collected from normal pressure hydrocephalus patients by continuous lumbar drainage. After collection, the CSF was aliquoted and frozen at -80 °C. For the preparation of each certified reference material (CRM) a selected number of CSF donations were thawed, pooled, mixed, filled in microvials and stored at (-70 ± 10) °C immediately thereafter.
Between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006.
The material was characterised by an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025. Technically invalid results were removed but no outlier was eliminated on statistical grounds only.
Uncertainties of the certified values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) and include uncertainties related to possible inhomogeneity, instability and characterisation.
The materials are intended for the calibration of methods, quality control and/or the assessment of method performance. As with any reference material, they can be used for establishing control charts or in validation studies. The CRMs are available in microvials containing at least 0.5 mL of frozen liquid. The minimum amount of sample to be used is 15 µL.

The certification of Amyloid β_{1-42} in CSF in ERM®-DA480/IFCC, ERM®-DA481/IFCC and ERM®-DA482/IFCC

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Summary

This report describes the production of ERM®-DA480/IFCC, ERM®-DA481/IFCC and ERM®-DA482/IFCC, which are human cerebrospinal fluid (CSF) materials certified for the mass concentration of amyloid β_{1-42} peptide ($A\beta_{1-42}$). These materials were produced by the European Commission's Joint Research Centre (EC-JRC) in collaboration with the International Federation for Clinical Chemistry and Laboratory Medicine (IFCC) following ISO Guide 34:2009 [1] and are certified in accordance with ISO Guide 35:2006 [2].

The starting material used to prepare ERM-DA480/IFCC, ERM-DA481/IFCC and ERM-DA482/IFCC was human CSF collected from normal pressure hydrocephalus patients by continuous lumbar drainage. After collection, the CSF was aliquoted and frozen at -80 °C. For the preparation of each certified reference material (CRM) a selected number of CSF donations were thawed, pooled, mixed, filled in microvials and stored at (-70 ± 10) °C immediately thereafter.

Between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006 [2].

The material was characterised by an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025 [3]. Technically invalid results were removed but no outlier was eliminated on statistical grounds only.

Uncertainties of the certified values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties related to possible inhomogeneity, instability and characterisation.

The materials are intended for the calibration of methods, quality control and/or the assessment of method performance. As with any reference material, they can be used for establishing control charts or in validation studies. The CRMs are available in microvials containing at least 0.5 mL of frozen liquid. The minimum amount of sample to be used is 15 µL.

The following values were assigned:

Amyloid β_{1-42} peptide in human CSF[1)]	Mass concentration	
	Certified value [2)] [µg/L]	Uncertainty [3)] [µg/L]
ERM-DA480/IFCC	0.45	0.07
ERM-DA481/IFCC	0.72	0.11
ERM-DA482/IFCC	1.22	0.18

[1)] As obtained by solid phase extraction and subsequent quantification by liquid chromatography with mass spectrometry detection, according to the reference methods (Leinenbach *et al.* Clin. Chem. 60 (2014) 987-94; Korecka *et al.* J. Alzheimers Dis. 41 (2014) 441-451) [5,6].

[2)] Certified values are values that fulfil the highest standards of accuracy and represent the unweighted mean value of the means of 5 accepted sets of data, each set being obtained in a different laboratory. The certified value and its uncertainty are traceable to the International System of Units (SI).

[3)] The uncertainty is the expanded uncertainty of the certified value with a coverage factor $k = 2$ corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008 [4].

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Glossary

$A\beta_{1\text{-}42}$	Amyloid $\beta_{1\text{-}42}$ peptide
AAA	Amino Acid Analysis
ACN	Acetonitrile
AD	Alzheimer's disease
ANOVA	Analysis of variance
BE	Belgium
CRM	Certified reference material
CSF	Cerebrospinal fluid
DE	Germany
DK	Denmark
EC	European Commission
ELISA	Enzyme – linked immunosorbent assay
ERM®	Trademark of European Reference Materials
EU	European Union
g	Gravitational acceleration
GUM	Guide to the Expression of Uncertainty in Measurement
HFIP	Hexafluoroisopropanol
HIV	Human immunodeficiency virus
HPLC-MS	High performance liquid chromatography mass spectrometry
ID	Isotope dilution
IDMS	Isotope dilution mass spectrometry
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
ISO	International Organization for Standardization
IVD	In Vitro Diagnostics
IVD-MD	In vitro diagnostic medical devices
JCTLM	Joint Committee for Traceability in Laboratory Medicine
JRC	Joint Research Centre of the European Commission
k	Coverage factor
LC-MS	Liquid chromatography-mass spectrometry
MeOH	Methanol
MS	Mass spectrometry
$MS_{between}$	Mean of squares between-unit from an ANOVA
MS_{within}	Mean of squares within-unit from an ANOVA
n	Number of replicates per unit

N	Number of samples (units) analysed
NH_4OH	Ammonium hydroxide
p	Number of accepted datasets
PP	Polypropylene
rel	Index denoting relative figures (uncertainties etc.)
RMPs	Reference measurement procedures
rpm	Revolutions per minute
s	Standard deviation
s_{bb}	Between-unit standard deviation; an additional index "rel" is added when appropriate
$s_{between}$	Standard deviation between groups as obtained from ANOVA; an additional index "rel" is added as appropriate
SE	Sweden
SI	International System of Units
SPE	Solid phase extraction
s_{within}	Standard deviation within groups as obtained from ANOVA; an additional index "rel" is added as appropriate
s_{wb}	Within-unit standard deviation
T	Temperature
TFE	2,2,2,-trifluoroethanol
t	Time
t_i	Time point for each replicate
t_{sl}	Proposed shelf life
u	Standard uncertainty
U	Expanded uncertainty
$u^*_{bb, rel}$	Relative standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by method repeatability/intermediate precision
u_{bb}	Standard uncertainty related to a possible between-unit inhomogeneity; an additional index "rel" is added as appropriate
u_c	Combined standard uncertainty; an additional index "rel" is added as appropriate
u_{cal}	Standard uncertainty of calibration
u_{char}	Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate
u_{CRM}	Combined standard uncertainty of the certified value; an additional index "rel" is added as appropriate
U_{CRM}	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
u_{lts}	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate

u_{meas}	Standard measurement uncertainty
U_{meas}	Expanded measurement uncertainty
US	United States of America
u_{sts}	Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
UV	Ultraviolet
V	Volume
WG-CSF	(IFCC) Working group for CSF proteins
$\bar{x}$	Arithmetic mean
Δ_{meas}	Absolute difference between mean measured value and the certified value
$\nu_{s,meas}$	Degrees of freedom for the determination of the standard deviation s_{meas}
$\nu_{MSwithin}$	Degrees of freedom of MS_{within}

1 Introduction

1.1 Background

The early diagnosis and treatment of Alzheimer's disease (AD) remain challenging and the use of biomarkers like the $A\beta_{1-42}$ peptide could contribute to improve early diagnosis. However, analysis results from different routine measurement procedures show large variability for this biomarker.

In 2011 a working group for cerebrospinal fluid (CSF) proteins (WG-CSF) was created by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), with the purpose of developing certified reference materials (CRMs) and reference measurement procedures (RMPs) for international use. The WG-CSF decided to first focus on $A\beta_{1-42}$ as a biomarker for AD. After a commutability study including immunoassays for $A\beta_{1-42}$ from different manufacturers and a ring trial using different liquid chromatography-mass spectrometry (LC-MS) methods among four laboratories showed promising results, the JRC committed to the production of a CRM for $A\beta_{1-42}$ in human CSF. In the meantime, two reference measurement procedures using selected reaction monitoring LC-MS for quantification of $A\beta_{1-42}$ in human CSF were developed [5, 6] and subsequently listed by the Joint Committee for Traceability in Laboratory Medicine (JCTLM).

The EU Directive on *in vitro* diagnostic medical devices (IVD-MD) (Directive 98/79/EC) requires traceability of calibrants and control materials to reference measurement procedures and/or reference materials of higher order.

A CRM is required to have an assigned value that is metrologically traceable, and accompanied by an uncertainty statement. The stability and homogeneity with respect to the certified property must be verified.

In collaboration with the IFCC WG-CSF, the JRC produced three CRMs certified for the mass concentration of $A\beta_{1-42}$ in CSF. The materials, ERM-DA480/IFCC, ERM-DA481/IFCC, ERM-DA482/IFCC, are intended to be used as calibrators or quality control materials for methods for the measurement of $A\beta_{1-42}$ in CSF. The homogeneity and the stability of the CRMs were demonstrated and the certified value was assigned using the reference measurement procedures in an interlaboratory comparison among expert laboratories. The commutability of the CRMs was assessed in a study including six routine measurement procedures.

1.2 Choice of the material

Human CSF was chosen as the starting material for the CRM because artificial matrices were found not to be commutable for the methods tested in a first commutability study [7]. Furthermore, three concentration levels were produced to allow users to prepare calibration curves. A frozen liquid material was selected as it had displayed suitable stability in previous studies [8].

1.3 Design of the CRM project

Human CSF was collected from normal pressure hydrocephalus patients by continuous lumbar drainage, aliquoted and frozen at -80 °C. For the preparation of the certified reference material a selected number of CSF donations were thawed and combined in order to produce three materials with high, middle and low levels of $A\beta_{1-42}$.

For each mass concentration level, batches of 0.5 mL aliquots were prepared and stored frozen at -70 °C.

Based on the outcome of several processing feasibility studies and the known fact that $A\beta_{1-42}$ is very prone to adhesion to surfaces, assessment of the homogeneity of the processed material was crucial.

After verification of the suitable homogeneity of ERM-DA481/IFCC the other two materials were processed using the same processing technique.

The CRMs were characterised by liquid chromatography with mass spectrometry detection (LC-MS) using reference measurement procedures [5, 6]. The measurements were performed by expert laboratories that have previously shown their proficiency in a ring trial. The LC-MS measurements were calibrated using calibrators prepared from a common stock solution of $A\beta_{1-42}$. The calibrants were prepared, aliquoted and characterised by the JRC.

2 Participants

2.1 Project management and evaluation

European Commission, Joint Research Centre (JRC), Geel, BE (accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

2.2 Provision of raw materials

The Sahlgrenska Academy, University of Gothenburg, Mölndal, SE (accredited to ISO 15189, Swedish Board for Accreditation and Conformity Assessment, No. 1240)

2.3 Processing

European Commission, Joint Research Centre (JRC), Geel, BE (accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

2.4 Homogeneity study

Roche Diagnostics GmbH, Penzberg, DE

ADx NeuroSciences NV, Gent, BE

2.5 Stability study

European Commission, Joint Research Centre (JRC), Geel, BE (accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

ADx NeuroSciences NV, Gent, BE

2.6 Characterisation

European Commission, Joint Research Centre (JRC), Geel, BE

PPD Laboratories, Richmond, VA, US

Roche Diagnostics GmbH, Penzberg, DE

Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, US

The Sahlgrenska Academy, University of Gothenburg, Mölndal, SE (accredited to ISO 15189, Swedish Board for Accreditation and Conformity Assessment, No. 1240)

Waters Corporation, Milford, MA, US

3 Material processing and process control

3.1 Origin of the starting material

The raw material was human CSF provided by Sahlgrenska Academy at University of Gothenburg (Mölndal, SE). The CSF was collected from normal pressure hydrocephalus patients by continuous lumbar drainage from a catheter in a plastic bag over several hours at room temperature. After collection, the CSF was aliquoted into 50 mL polypropylene (PP) tubes and frozen individually at the Swedish neurological clinics where it was collected. The anonymised CSF donations were then sent on dry ice to the Salgrenska Academy at the University of Gothenburg where the $A\beta_{1-42}$ concentration of each donation was determined by ELISA (INNOTEST® Amyloid β(1-42), Fujirebio Europe N.V., Gent, Belgium). Each individual CSF donation was tested for anti-HIV1/HIV2 antibodies and found to be negative. Since the donors were under continuous medical surveillance, there is no particular risk that this donor population should have had any bacterial or viral infections of the central nervous system at the time of CSF collection.

3.2 Processing

For the processing of each CRM, a selected number of CSF donations were removed from the freezer and thawed in a water bath at room temperature. Based on the $A\beta_{1-42}$ concentrations of each of the individual donations, the three materials were produced by pooling together different donations with high, middle and low levels of $A\beta_{1-42}$. The pooling was done in three autoclaved 2 litre PP beakers and the CSF pools were stirred, using a 6 cm magnetic stir bar, for one hour at room temperature and 200 rpm. Each CSF pool was then divided between two autoclaved PP centrifuge bottles and centrifuged for ten minutes at 2000 x *g* at room temperature. The supernatant was transferred to a clean, autoclaved PP bottle (4 litre bottle for ERM-DA480/IFCC and ERM-DA482/IFCC and 2 litre bottle for ERM-DA481/IFCC) and stirred, using a 6 cm magnetic stir bar, for one hour at 200 rpm and room temperature. Then it was transferred to a cold room (4 ± 3 °C) where it remained overnight until further processing.

The CSF pools, were filled in 0.5 mL sterile microvials SCT-050-SS-L-X-S (where X depends on the cap colour) maximum recovery (Axygen Scientific, Inc., Union City, CA, USA) and closed with screw caps with O-rings using a filling machine (Dencore ApS, Jyllinge, DK) placed under a clean cell (Terra Universal, Inc., Fullerton, CA, USA). The CSF pool was stirred and kept on ice during filling. Each vial was filled with at least 0.5 mL of CSF. The vials were stored at (-70 ± 10) °C immediately after filling and labelling.

4 Homogeneity

A key requirement for any reference material aliquoted into units is equivalence between those units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty of the certified value, but it is not relevant if this variation between units is significant compared to the analytical variation. Consequently, ISO Guide 34 [1] requires reference material producers to quantify the between unit variation. This aspect is covered in between-unit homogeneity studies.

The within-unit inhomogeneity does not influence the uncertainty of the certified value when the minimum sample intake is respected, but determines the minimum size of an aliquot that is representative for the whole unit.

4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified values of the CRMs are valid for all vials of the material, within the stated uncertainties.

The number of vials selected corresponds to at least the cube root of the total number of vials produced. Seventy vials were selected for ERM-DA481/IFCC and 35 vials for ERM-DA480/IFCC and ERM-DA482/IFCC using a random stratified sampling scheme covering the whole batch for the between-unit homogeneity test. For this, the batch for ERM-DA481/IFCC was divided into 70 and the other two batches were divided into 35 groups (with a similar number of vials) and one vial was selected randomly from each group. Three independent samples were taken from each selected vial and analysed with a fully automated immunoassay (Roche Elecsys β-amyloid (1-42), Roche Diagnostics GmbH, Penzberg, DE). In addition to that, another 70 vials of ERM-DA481/IFCC and 35 vials of ERM-AD480/IFCC as well as ERM-DA482/IFCC were selected and analysed in the same manner to assess the homogeneity of $A\beta_{1\text{-}42}$ with a manual ELISA (EUROIMMUN beta-amyloid (1-42), EUROIMMUN AG, Lübeck, DE). Except for the measurements of ERM-DA481/IFCC using the automated analyser, all the measurements were performed under repeatability conditions, and in a randomised manner to be able to separate a potential analytical drift from a trend in the filling sequence.

Regression analyses were performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence. No trends in the filling sequence were observed at a 95 % confidence level, besides a trend for ERM-DA481/IFCC analysed with the fully automated immunoassay. For this last material it was confirmed with the EUROIMMUN ELISA that there was no trend in the filling sequence. Significant (95 % confidence level) trends in the analytical sequence were visible for both methods. The correction of biases, even if they are statistically not significant, was found to combine the smallest uncertainty with the highest probability to cover the true value [9]. Correction of trends is therefore expected to improve the sensitivity of the subsequent statistical analysis through a reduction in analytical variation without masking potential between-unit heterogeneities. As the analytical sequence and the unit numbers were not correlated, trends significant on at least a 95 % confidence level were corrected as shown below. The systematic decrease of $A\beta_{1\text{-}42}$ seen for the results of the fully automated immunoassay were corrected as shown in Equation 1 and the interplate variability of the ELISA was corrected as shown in Equation 2.

$$x_{i\_corr} = \frac{x_i \cdot \bar{x}_{replicate\_1}}{\bar{x}_{replicate\_i}}$$ **Equation 1**

x_i = individual result within the run

$\bar{x}_{replicate\_1}$ = average of results replicate run 1

$\bar{x}_{replicate\_i}$ = average of results replicate run with individual result *i*

$$x_{i\_corr} = \frac{x_i \cdot \bar{x}_{all\_plates}}{\bar{x}_{plate}}$$ **Equation 2**

x_i = individual result on the plate

$\bar{x}_{all\_plates}$ = average of results of all plates

$\bar{x}_{plate}$ = average of results of the plate with result *i*

The trend-corrected datasets were assessed for consistency using Grubbs outlier tests at a confidence level of 99 % on the individual results and on the unit means. No outlying individual results or outlying unit means were detected.

Quantification of between-unit inhomogeneity was undertaken by analysis of variance (ANOVA), which separates the between-unit variation (s_{bb}) from the within-unit variation (s_{wb}). The latter is equivalent to the method repeatability if the individual samples were representative for the whole vial.

Evaluation by ANOVA requires mean values per vial, which follow at least a unimodal distribution and results for each vial that follow unimodal distributions with approximately the same standard deviations. Too few data are available for the unit means to make a clear statement of the distribution based on statistical tests. Therefore, it was checked visually whether all individual data follow a unimodal distribution using histograms and normal probability plots. Minor deviations from unimodality of the individual values do not significantly affect the estimate of between-unit standard deviations. The results of all statistical evaluations are given in Table 1 and 2.

Table 1: Results of the statistical evaluation of the homogeneity studies of $A\beta_{1-42}$ using a fully automated immunoassay (Roche Elecsys β-amyloid (1-42), Roche Diagnostics GmbH)

$A\beta_{1-42}$	Trends (before correction)*		Outliers**		Distribution	
	Analytical sequence	Filling sequence	Individual results	Unit means	Individual results	Unit means
ERM-DA480/IFCC	yes	no	none	none	unimodal	unimodal
ERM-DA481/IFCC	yes	yes	none	none	unimodal	unimodal
ERM-DA482/IFCC	yes	no	none	none	unimodal	unimodal

* 95 % confidence level

** 99 % confidence level

Table 2: Results of the statistical evaluation of the homogeneity studies of $A\beta_{1-42}$ using an ELISA (EUROIMMUN beta-amyloid (1-42), EUROIMMUN AG)

$A\beta_{1-42}$	Trends (before correction)*		Outliers**		Distribution	
	Analytical sequence	Filling sequence	Individual results	Unit means	Individual results	Unit means
ERM-DA480/IFCC	yes	no	none	none	unimodal	unimodal
ERM-DA481/IFCC	no	no	none	none	unimodal	unimodal
ERM-DA482/IFCC	yes	no	none	none	unimodal	unimodal

* 95 % confidence level

** 99 % confidence level

Method repeatability ($s_{wb,rel}$), between-unit standard deviation ($s_{bb,rel}$) and $u^*_{bb,rel}$ were calculated as:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{x}} \qquad \textbf{Equation 3}$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{x}} \qquad \textbf{Equation 4}$$

$$u^*_{bb,rel} = \frac{\sqrt{\frac{MS_{within}}{n}}\sqrt[4]{\frac{2}{v_{MSwithin}}}}{\bar{x}} \qquad \textbf{Equation 5}$$

MS_{within}	mean of squares within-unit from an ANOVA
$MS_{between}$	mean of squares between-unit from an ANOVA
$\bar{x}$	mean of all results of the homogeneity study
n	mean number of replicates per unit
$v_{MSwithin}$	degrees of freedom of MS_{within}

It should be noted that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and are therefore subject to random fluctuations. Therefore, the mean square between groups ($MS_{between}$) can be smaller than the mean squares within groups (MS_{within}), resulting in negative arguments under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be lower than zero. In this case, u^*_{bb}, the maximum inhomogeneity that could be hidden by method repeatability, was calculated as described by Linsinger *et al.* [10]. u^*_{bb} is comparable to the limit of detection of an analytical method, yielding the maximum inhomogeneity that might be undetected by the given study setup.

The results of the evaluation of the between-unit variation are summarised in Table 3 and 4. The resulting values from the above equations were converted into relative uncertainties.

Table 3: Results of the homogeneity studies of $A\beta_{1-42}$ using a fully automated immunoassay

$A\beta_{1-42}$ in CSF	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]	$u_{bb,\ rel}$ [%]
ERM-DA480/IFCC	1.7	2.0	0.4	2.0
ERM-DA481/IFCC	3.5	1.1	0.7	1.1
ERM-DA482/IFCC	1.5	1.1	0.4	1.1

Table 4: Results of the homogeneity studies of $A\beta_{1-42}$ using an ELISA

$A\beta_{1-42}$ in CSF	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]	$u_{bb,\ rel}$ [%]
ERM-DA480/IFCC	5.1	1.3	1.2	1.3
ERM-DA481/IFCC	5.1	1.3	1.0	1.3
ERM-DA482/IFCC	5.2	1.5	1.2	1.5

The homogeneity study showed no outlying unit means or trends in the filling sequence. Therefore, the between-unit standard deviation can be used as estimate of u_{bb}. As u^*_{bb} sets the limits of the study to detect inhomogeneity, the larger value of s_{bb} and u^*_{bb} is adopted as uncertainty contribution to account for potential inhomogeneity.

4.2 Within-unit homogeneity and minimum sample intake

The within-unit homogeneity is closely correlated to the minimum sample intake. The minimum sample intake is the minimum amount of sample that is representative for the whole unit and thus should be used in an analysis. Using sample sizes equal or above the minimum sample intake guarantees the certified value within its stated uncertainty.

Homogeneity and stability experiments were performed using a 15 µL sample intake. This sample intake gives acceptable repeatability, demonstrating that the within-unit inhomogeneity no longer contributes to analytical variation at this sample intake.

5 Stability

Time and temperature were regarded as the most relevant influences on the stability of the materials. The influence of ultraviolet or visible light was minimised by storing the material in the dark and dispatching in boxes, thus removing any possibility of degradation by light. Therefore, only the influences of time and temperature needed to be investigated.

Stability testing is necessary to establish the conditions for storage (long-term stability) as well as the conditions for dispatch of the materials to the customers (short-term stability). During transport, especially in summer time, temperatures up to 60 °C can be reached and stability under these conditions must be demonstrated, if the samples are to be transported without any additional cooling.

The stability studies were carried out using an isochronous design [11]. In this approach, samples were stored for a particular length of time at different temperature conditions. Afterwards, the samples were moved to conditions where further degradation can be assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples were analysed simultaneously under repeatability conditions. Analysis of the material (after various exposure times and temperatures) under repeatability conditions greatly improves the sensitivity of the stability tests.

5.1 Short-term stability study

For the short-term stability study, samples were stored at -70 °C, -20 °C, 4 °C and 18 °C for 0, 1, 2 and 4 weeks (at each temperature). The reference temperature was set to -150 °C (stored above liquid nitrogen). Two vials per storage time were selected using a random stratified sampling scheme. From each vial, three samples were measured by ELISA (EUROIMMUN beta-amyloid (1-42), EUROIMMUN AG, Lübeck, DE). The measurements were performed under repeatability conditions, and a randomised sequence was used to differentiate any potential analytical drift from a trend over storage time.

The data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test on a confidence level of 99 %. No outlying individual results were found (Table 5).

In addition, the data were evaluated against storage time, and regression lines of mass concentration versus time were calculated, to test for potential increases/decrease of $A\beta_{1\text{-}42}$ mass concentration due to shipping conditions. The slopes of the regression lines were tested for statistical significance. None of the trends was statistically significant at a 95 % confidence level for -20 °C (these results are shown in Annex B), but some trends were detected at the other temperatures tested. The results of the statistical evaluation of the short-term stability are summarised in Table 5.

Table 5: Results of the short-term stability tests

$A\beta_{1\text{-}42}$ in CSF	Number of individual outlying results*				Significance of the trend**			
	-70 ºC	-20 ºC	4 °C	18 °C	-70 ºC	-20 ºC	4 °C	18 °C
ERM-DA480/IFCC	none	none	none	none	yes	no	no	yes
ERM-DA481/IFCC	none	none	none	none	no	no	yes	yes
ERM-DA482/IFCC	none	none	none	none	no	no	no	yes

* 99 % confidence level

** 95 % confidence level

No statistical outliers were detected for the analyte (99 % confidence level), and all the samples were retained for the estimation of u_{sts}.

A significant trend at -70 °C was found for ERM-DA480/IFCC, but since the long-term stability study results at -70 °C did not show a trend, the observation in the short-term stability study was regarded as a statistical artefact. Furthermore, no statistically significant trend was found for ERM-DA481/IFCC or ERM-DA482/IFCC at -70 °C or -20 °C. A significant trend at 4 °C was found for ERM-DA481/IFCC, but not for ERM-DA480/IFCC and ERM-DA482/IFCC. A significant trend at 18 °C was found for all three materials.

The material shall be shipped on dry ice to avoid freeze-thaw cycles.

5.2 Long-term stability study

For the long-term stability study, samples were stored at -70 °C and -20 °C for 0, 4, 8 and 12 months (at each temperature). The reference temperature was set to -150 °C (stored above liquid nitrogen). Two samples per storage time were selected using a random stratified sampling scheme. From each vial, three samples were measured by ELISA (EUROIMMUN beta-amyloid (1-42), EUROIMMUN AG, Lübeck, DE). The measurements were performed under repeatability conditions in a random sequence to be able to separate any potential analytical drift from a trend over storage time. For ERM-DA480/IFCC, one value was removed for technical reasons.

The long-term stability data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test at a confidence level of 99 %. One outlying individual result was found for ERM-DA482/IFCC (Table 6). As no technical reason for the outlier could be found, all data were retained for statistical analysis.

In addition, the data were plotted against storage time and linear regression lines of mass concentration versus time were calculated. The slopes of the regression lines were tested for statistical significance (loss/increase due to storage). No significant trend was detected at a 95 % confidence level.

The results of the long-term stability measurements at -70 °C are shown in Annex C. The results of the statistical evaluation of the long-term stability study are summarised in Table 6.

Table 6: Results of the long-term stability tests

$A\beta_{1-42}$ in CSF	Number of individual outlying results*		Significance of the trend**	
	-70 °C	-20 °C	-70 °C	-20 °C
ERM-DA480/IFCC	none	none	no	no
ERM-DA481/IFCC	none	none	no	no
ERM-DA482/IFCC	none	1	no	no

* 99 % confidence level

** 95 % confidence level

None of the trends was statistically significant on a 99 % confidence level for any of the temperatures. The material can therefore be stored at -70 °C.

5.3 Estimation of uncertainties

Due to the intrinsic variation of measurement results, no study can entirely rule out degradation of materials, even in the absence of statistically significant trends. It is therefore

necessary to quantify the potential degradation that could be hidden by the method repeatability, i.e. to estimate the uncertainty of stability. This means that, even under ideal conditions, the outcome of a stability study can only be that there is no detectable degradation within an uncertainty to be estimated.

The uncertainties of stability during dispatch and storage were estimated as described in [12]. In this approach, the uncertainty of the linear regression line with a slope of zero was calculated. The uncertainty contributions u_{sts} and u_{lts} were calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

$$u_{sts,rel} = \frac{s_{rel}}{\sqrt{\sum \left(t_i - \bar{t}\right)^2}} \cdot t_{tt}$$ **Equation 6**

$$u_{lts,rel} = \frac{s_{rel}}{\sqrt{\sum \left(t_i - \bar{t}\right)^2}} \cdot t_{sl}$$ **Equation 7**

s_{rel} relative standard deviation of all results of the stability study

t_i time elapsed at time point *i*

$\bar{t}$ mean of all t_i

t_{tt} chosen transport time (1 week at -20 ºC)

t_{sl} chosen shelf life (12 months at -70 ºC)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. This was estimated from the -20 °C studies. The uncertainty describes the possible change during a dispatch at -20 °C lasting for one week.
- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated from the -70 °C study. The uncertainty contribution describes the possible degradation during 12 months storage at -70 °C.

The results of these evaluations are summarised in Table 7.

Table 7: Uncertainties of stability during dispatch and storage. $u_{sts,rel}$ was calculated for a temperature of -20 °C and 1 week; $u_{lts,rel}$ was calculated for a storage temperature of -70 °C and 12 months

$A\beta_{1-42}$ in CSF	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
ERM-DA480/IFCC	0.7	3.0
ERM-DA481/IFCC	0.8	3.7
ERM-DA482/IFCC	0.6	3.1

Although two of the three materials did not show a significant degradation at 4 °C, transport on dry-ice is necessary. Freeze-thaw cycles should be avoided.

After the certification study, the materials will be included in the JRC's regular stability monitoring programme, to verify its further stability.

6 Characterisation

The material characterisation is the process of determining the property value of a reference material.

This was based on an interlaboratory comparison of expert laboratories, i.e. the mass concentration of each of the three materials was determined in different laboratories that applied different measurement procedures to demonstrate the absence of a measurement bias. Due to the nature of the analyte however, all participants used the same pre-analytical sample preparation method for the measurements. This approach aims at randomisation of laboratory bias, which reduces the combined uncertainty.

6.1 Calibration

6.1.1 Preparation of a $A\beta_{1-42}$ stock solution

Preparation of the $A\beta_{1-42}$ stock solution was based on a procedure adapted from Broersen *et al.* [13]. Recombinant human $A\beta_{1-42}$ peptides were purchased in form of lyophilised hexafluoroisopropanol (HFIP) films (rPeptide, Bogart, GA, USA) with a purity stated to be ≥97 %. The peptides were dissolved in HFIP (VWR International LLC, Leuven, BE) at a concentration of 1 mg/mL, vortex mixed for 30 seconds and dried with argon. Then the peptides were dissolved in dimethyl sulfoxide (G-Biosciences, St. Louis, MO, USA) at a concentration of 1 mg/mL and vortex mixed for 30 seconds. The peptide solution was injected on a HiTrap desalting column (GE Healthcare Europe GmbH, Diegem, BE) equilibrated with 20 % acetonitrile and 1 % ammonium hydroxide in water. Fractions were collected, pooled and diluted with 20 % acetonitrile and 1 % ammonium hydroxide. The peptide solution was filled in 0.5 mL PP vials (Sarstedt, Nümbrecht, DE) with each vial containing 0.1 mL of solution. The vials were immediately stored at (-70 ± 10) °C.

6.1.2 Value assignment of the stock solution

The value assignment of the stock solution was performed by IDMS using a TSQ Vantage MS (Themo Fisher Scientific, Waltham, MA, US), with SPE and LC conditions according to the reference measurement procedure [5, 6]. Six different $A\beta_{1-42}$ preparations were used for calibration of these measurements, producing six datasets for the stock solution. The values of these six aforementioned $A\beta_{1-42}$ preparations were determined by amino acid analysis [14]. The value for the stock solution (the mean of means of the six datasets) was converted from mass fraction to mass concentration using the density of the stock solution. This density was determined using a DMA 4500M Density Meter (Anton Paar, Graz, AT), and found to be 0.9641 g/cm^3, with an expanded uncertainty (k = 2) of 0.0001 g/cm^3 (based on repeatability only) which corresponds to an expanded relative uncertainty of 0.01 %.

The resulting mass concentration for the stock solution is given in Table 8.

Table 8: Assigned value and uncertainty of the $A\beta_{1-42}$ concentration in the pure peptide solution

Assigned value [mg/L]	$u_{cal,rel}$ [%]
68.87	3.54

6.2 Selection of participants

Five laboratories were selected based on criteria that comprised both technical competence and quality management aspects. Each participant was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field of $A\beta_{1-42}$ measurements in human CSF by submitting results from proficiency testing. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was obligatory. Where measurements are covered by the scope of accreditation, the accreditation number is stated in the list of participants (Section 2).

6.3 Study setup

Each laboratory received nine vials each of ERM-DA480/IFCC, ERM-DA481/IFCC and ERM-DA482/IFCC and was requested to provide 27 independent results per CRM, three per vial. The units for material characterisation were selected using a random stratified sampling scheme and covered the whole batch. The sample preparations and measurements had to be spread over at least three days to ensure intermediate precision conditions. Each laboratory also received six vials of $A\beta_{1-42}$ stock solution to perform an independent calibration for each day of analysis.

6.4 Methods used

The participating laboratories were using validated procedures (Annex D) that were based on the IFCC reference measurement procedures for the quantification of $A\beta_{1-42}$ in CSF [5, 6].

6.5 Evaluation of results

The characterisation study resulted in 5 datasets per material. All individual results of the participants are displayed in a graphical form in Annex E.

6.5.1 Technical evaluation

The obtained data were first checked for compliance with the requested analysis protocol and for their validity based on technical reasons. The following criteria were considered during the evaluation:

- appropriate validation of the measurement procedure
- compliance with the analysis protocol: sample preparations and measurements performed on three days

During this characterisation study all the data sets were retained.

6.5.2 Statistical evaluation

The datasets accepted based on technical reasons were tested for normality of dataset means using kurtosis/skewness tests and normal probability plots and were tested for outlying means using the Grubbs test and using the Cochran test for outlying standard deviations, (both at a 99 % confidence level). Standard deviations within (s_{within}) and between ($s_{between}$) laboratories were calculated using one-way ANOVA. The results of these evaluations are shown in Table 9.

Table 9: Statistical evaluation of the technically accepted datasets for each CRM. *p*: number of technically valid datasets

$A\beta_{1-42}$ in CSF	*p*	Outliers		Normally distributed	Statistical parameters			
		Means	Variances		Mean [µg/L]	*s* [µg/L]	$s_{between}$ [µg/L]	s_{within} [µg/L]
ERM-DA480/IFCC	5	none	none	yes	0.45	0.05	0.08	0.07
ERM-DA481/IFCC	5	1	1	yes	0.72	0.08	0.12	0.12
ERM-DA482/IFCC	5	1	1	yes	1.22	0.15	0.24	0.20

For ERM-DA480/IFCC, the laboratory means follow normal distributions. None of the data contains outlying means and variances. The datasets are therefore consistent and the mean of laboratory means is a good estimate of the true value. Standard deviations between laboratories are considerably larger than the standard deviation within laboratories, showing that confidence intervals of replicate measurements are unsuitable as estimate of measurement uncertainty.

For ERM-DA481/IFCC and for ERM-DA482/IFCC, the statistical evaluation flags laboratory L5 as outlier for the laboratory mean. However, it must be borne in mind that outlier tests do not take uncertainty information into consideration. A closer investigation revealed that the difference between the mean value of laboratory L5 and the other results is covered by the measurement uncertainty of laboratory L5. There is therefore no evidence that the results of laboratory L5 deviate from the other results.

For ERM-DA481/IFCC and for ERM-DA482/IFCC, statistical evaluation flags laboratory L3 as outlying variance. All laboratories used SPE followed by HPLC-MS, but laboratory L3 only had 2 replicates per sample. As all measurement data sets were found technically sound, all results were retained.

The uncertainty related to the characterisation was based on the standard error of the mean of laboratory means (Table 10).

Table 10: Uncertainty of characterisation

$A\beta_{1-42}$ in CSF	*p*	Mean [µg/L]	*s* [µg/L]	$u_{char,rel}$ [%]
ERM-DA480/IFCC	5	0.45	0.05	5.0
ERM-DA481/IFCC	5	0.72	0.08	5.1
ERM-DA482/IFCC	5	1.22	0.15	5.6

7 Value Assignment

Certified values were assigned.

Certified values are values that fulfil the highest standards of accuracy. Full uncertainty budgets in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [4] were established.

7.1 Certified values and their uncertainties

The unweighted mean of the means of the accepted datasets as shown in Table 9 was assigned as certified value for each parameter.

The assigned uncertainty consists of uncertainties relating to characterisation, u_{char} and to the calibrant used u_{cal} (Section 6), potential between-unit inhomogeneity, u_{bb} (Section 4.1), and potential degradation during transport, u_{sts}, and long-term storage, u_{lts} (Section 5). These different contributions were combined to estimate the relative expanded uncertainty of the certified value ($U_{CRM,\,rel}$) with a coverage factor k given as:

$$U_{CRM,rel} = k \cdot \sqrt{u_{bb,rel}^2 + u_{cal,rel}^2 + u_{sts,rel}^2 + u_{lts,rel}^2 + u_{char,rel}^2} \qquad \textbf{Equation 8}$$

- u_{char} was estimated as described in Section 6
- u_{cal} is the relative standard uncertainty on the pure peptide solution as described in Section 6
- u_{bb} was estimated as described in Section 4.1. For ERM-DA480/IFCC, u_{bb} was taken from the homogeneity study performed by ELISA and for ERM-DA481/IFCC and ERM-DA482/IFCC from the homogeneity study performed by the fully automated immunoassay.
- u_{sts} and u_{lts} were estimated as described in Section 5.3

The relevant number of degrees of freedom calculated using the Welch-Satterthwaite equation is larger than 30 for any of the three materials which justifies the use of a coverage factor k = 2 to expand the confidence level to about 95 %. After rounding, the certified values and their uncertainties are summarised in Table 11.

Table 11: Certified values and their uncertainties for ERM-DA480/IFCC, ERM-DA481/IFCC and ERM-DA482/IFCC

$A\beta_{1-42}$ in CSF	Certified value [µg/L]	$u_{char,rel}$ [%]	$u_{cal,rel}$ [%]	$u_{bb,rel}$ [%]	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]	U_{CRM} [1)] [µg/L]
ERM-DA480/IFCC	0.45	5.0	3.5	1.3	0.7	3.0	0.07
ERM-DA481/IFCC	0.72	5.1	3.5	1.1	0.8	3.7	0.11
ERM-DA482/IFCC	1.22	5.6	3.5	1.1	0.6	3.1	0.18

[1)] Expanded (k = 2) and rounded uncertainty.

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

The $A\beta_{1-42}$ peptide is a chemically clearly defined substance. However, the characterisation of the matrix materials ERM-DA480/IFCC, ERM-DA481/IFCC and ERM-DA482/IFCC was performed by using SPE, Liquid Chromatography and Mass Spectrometry (LC-MS) methods and therefore the measurand is defined as $A\beta_{1-42}$ peptide as prepared by SPE and measured by LC-MS methods used in the present procedure.

Quantity value

Only validated methods were used for the determination of the assigned values. The property value assigned to the common calibrant is traceable to the SI, as described in this report, and all relevant input parameters were calibrated. The individual results are therefore traceable to the SI, as it is also confirmed by the agreement among the technically accepted datasets. As the assigned values are combinations of agreeing results individually traceable to the SI, the assigned quantity values themselves are traceable to the SI as well.

8.2 Commutability

Many measurement procedures include one or more steps which select specific (or specific groups of) analytes from the sample for the subsequent whole measurement process. Often the complete identity of these 'intermediate analytes' is not fully known or taken into account. Therefore, it is difficult to mimic all analytically relevant properties of real samples within a CRM. The degree of equivalence in the analytical behaviour of real samples and a CRM with respect to various measurement procedures (methods) is summarised in a concept called 'commutability of a reference material'. There are various definitions that define this concept. For instance, the Clinical and Laboratory Standards Institute Guideline EP30-A [15] recommends the use of the following definition for the term *commutability*:

> "A property of a reference material, demonstrated by the equivalence of the mathematical relationships among the results of different measurement procedures for a reference material and for representative samples of the type intended to be measured."

The commutability of a CRM defines its fitness for use and is therefore a crucial characteristic when applying different measurement methods. When the commutability of a CRM is not established, the results from routinely used methods cannot be legitimately compared with the certified value to determine whether a bias does not exist in calibration, nor can the CRM be used as a calibrant. For instance, CRMs intended to be used to establish or verify metrological traceability of results obtained by routine clinical measurement procedures must be commutable for the routine clinical measurement procedures for which they are intended to be used.

The individual patient donations were pooled and tested in a commutability study before they were processed into the CRMs. The outcome of that study has been published elsewhere [7]. After processing, ERM-DA480/IFCC, ERM-DA481/IFCC and ERM-DA482/IFCC were tested in another commutability study and shown to be commutable for the combination of the following routine measurement procedures:

- EUROIMMUN beta-amyloid (1-42) (EUROIMMUN AG, Lübeck, DE)
- IBL Amyloid-beta (1-42) CSF ELISA (IBL International GmbH, Hamburg, DE)
- INNOTEST® β-AMYLOID (1-42) (Fujirebio Europe, N.V., Gent, BE)
- Lumipulse® G β-Amyloid (1-42) (Fujirebio Europe N.V., Gent, BE)

- V-PLEX® Aβ Peptide Panel 1 (6E10) (Meso Scale Discovery, LLC., Rockville, MD, US)
- Roche Elecsys β-amyloid (1-42) (Roche Diagnostics GmbH, Penzberg, DE)

If ERM-DA480/IFCC, ERM-DA481/IFCC and ERM-DA482/IFCC are used for the calibration of other $A\beta_{1-42}$ measurement procedures, the user should verify that the materials or their dilutions used are commutable (Annex F).

9 Instructions for use

9.1 Safety information

The usual laboratory safety measures apply. Do not discharge the waste into the drain. Each individual CSF donation was tested for anti-HIV1/HIV2 antibodies and found to be negative. Since the donors were under continuous medical surveillance, there is no particular risk that this donor population should have had any bacterial or viral infections of the central nervous system at the time of CSF collection. However, the product must be handled with adequate care as any material of human origin. It is intended for *in vitro* use only.

9.2 Storage conditions

The materials should be stored at -70 ± 10 °C in the dark.

Please note that the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially for opened vials.

9.3 Preparation and use of the material

The vials shall be thawed at room temperature. Avoid vortexing or inverting the vial in order to prevent contact between the solution and additional surface of the vial.

The user should be aware of the fact that $A\beta_{1\text{-}42}$ in CSF can aggregate and bind to surfaces, particularly to glass.

9.4 Minimum sample intake

The minimum sample intake for which within-vial homogeneity was proven is 15 µL. For smaller sample intakes the user needs to verify the within-vial homogeneity.

9.5 Use of the certified value

The purpose of these materials is either the calibration or the quality control of methods for the measurement of $A\beta_{1\text{-}42}$ in CSF. As any reference material, they can be used for establishing control charts or in validation studies.

Comparing an analytical result with the certified value

A result is unbiased if the combined standard uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1, www.erm-crm.org [16].

When assessing the method performance, the measured values of the CRMs are compared with the certified values. The procedure is summarised here:

- Calculate the absolute difference between mean measured value and the certified value (Δ_{meas}).
- Combine the measurement uncertainty (u_{meas}) with the uncertainty of the certified value (u_{CRM}): $u_{\Delta} = \sqrt{u_{meas}^2 + u_{CRM}^2}$
- Calculate the expanded uncertainty (U_{Δ}) from the combined uncertainty (u_{Δ}) using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %
- If $\Delta_{meas} \leq U_{\Delta}$ then no significant difference exists between the measurement result and the certified value, at a confidence level of approximately 95 %.

10 Acknowledgments

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Annexes

Annex A: Results of the homogeneity measurements

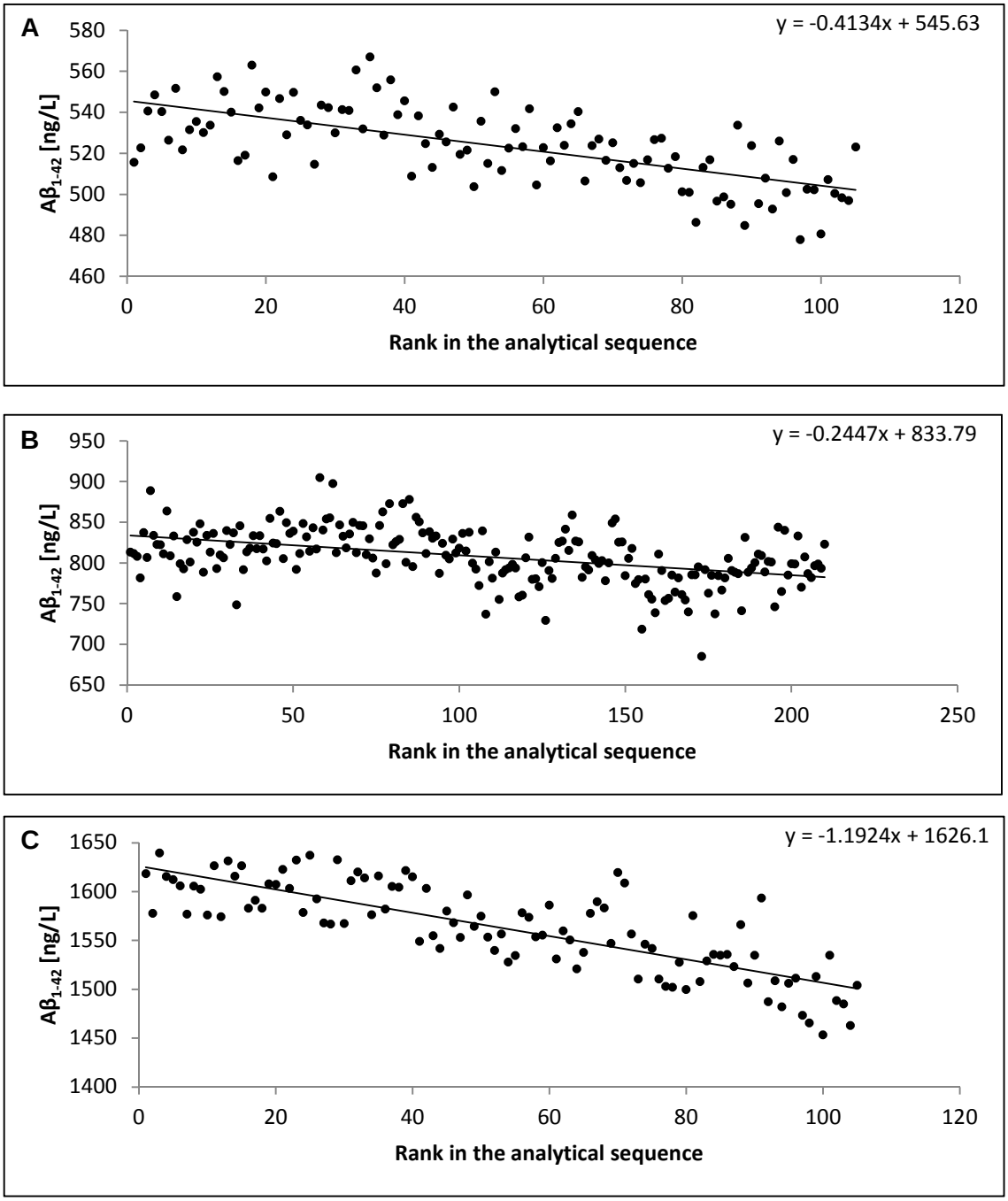


Figure A1: Regression of the results in the analytical sequence order for (A) ERM-DA480/IFCC, (B) ERM-DA481/IFCC and (C) ERM-DA482/IFCC as measured with a fully automated immunoassay.

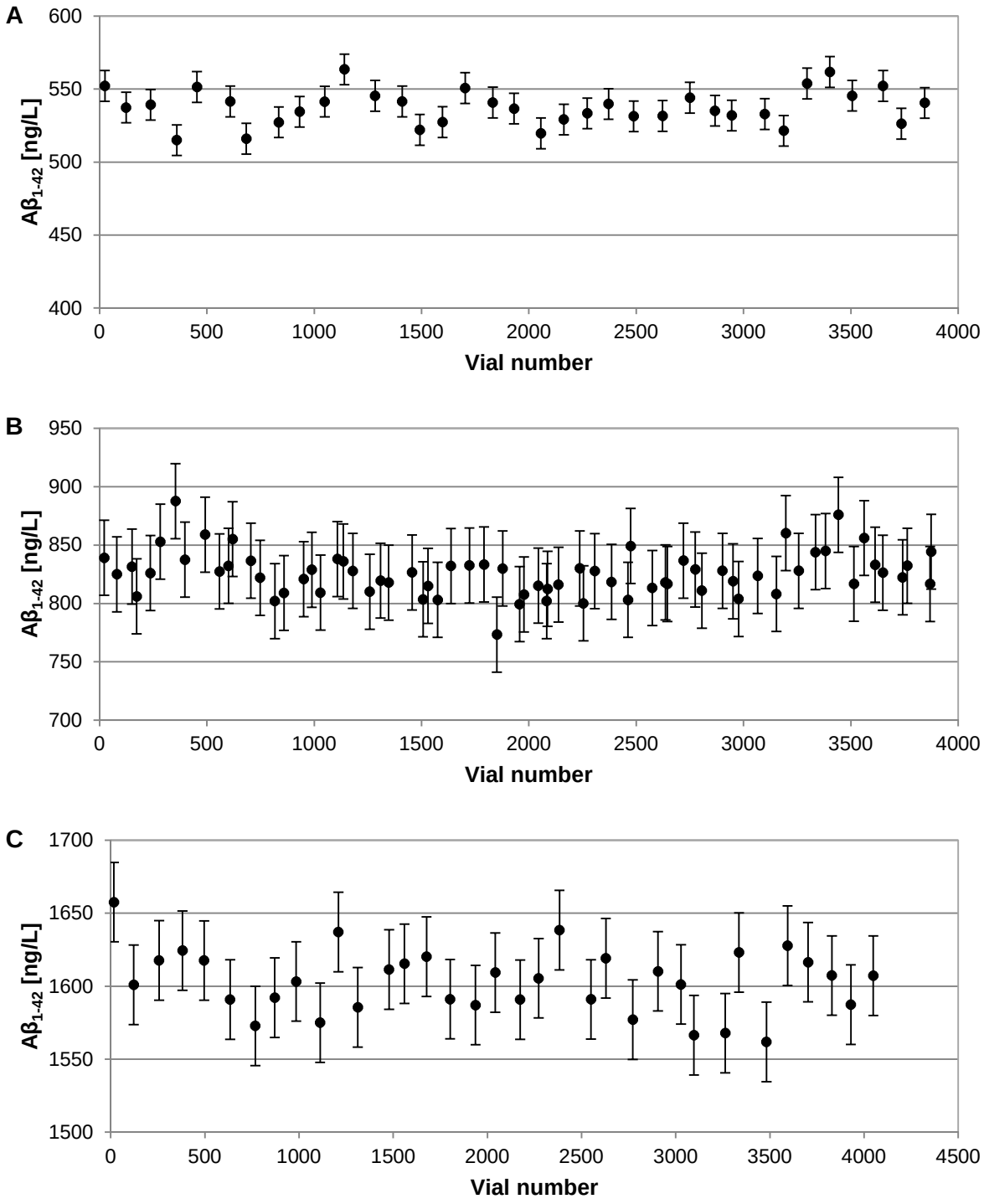
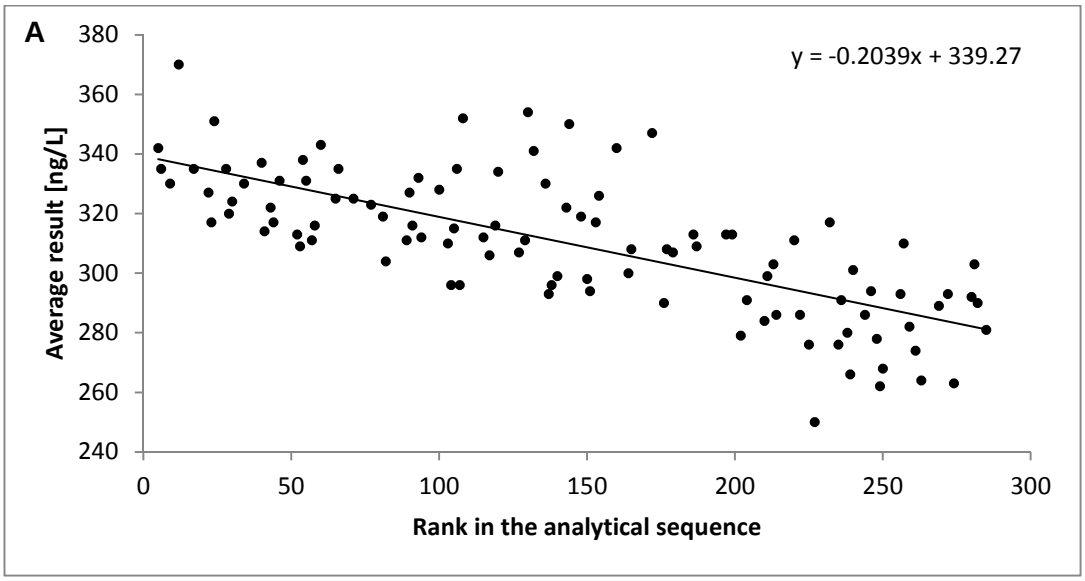


Figure A2: Homogeneity data of $A\beta_{1-42}$ in (A) ERM-DA480/IFCC, (B) ERM-DA481/IFCC and (C) ERM-DA482/IFCC as measured with a fully automated immunoassay. Shown are the averages per vial number and their 95 % confidence interval based on the within-group standard deviation as derived from a one-way ANOVA of all data grouped by vial number after correction of the analysis trend.



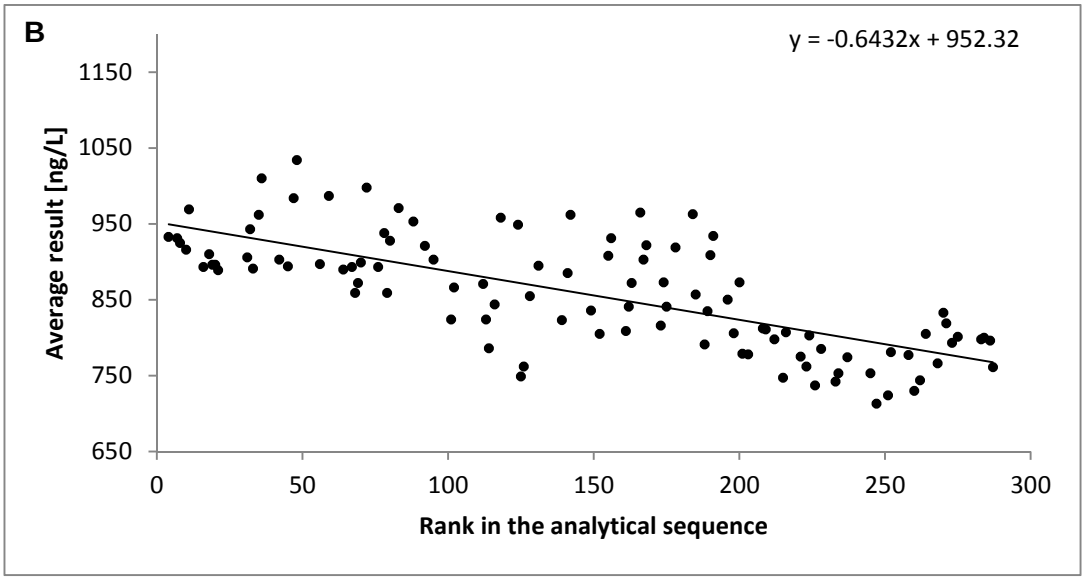


Figure A3: Regression of the results in the analytical sequence order for (A) ERM-DA480/IFCC and (B) ERM-DA482/IFCC as measured by ELISA.

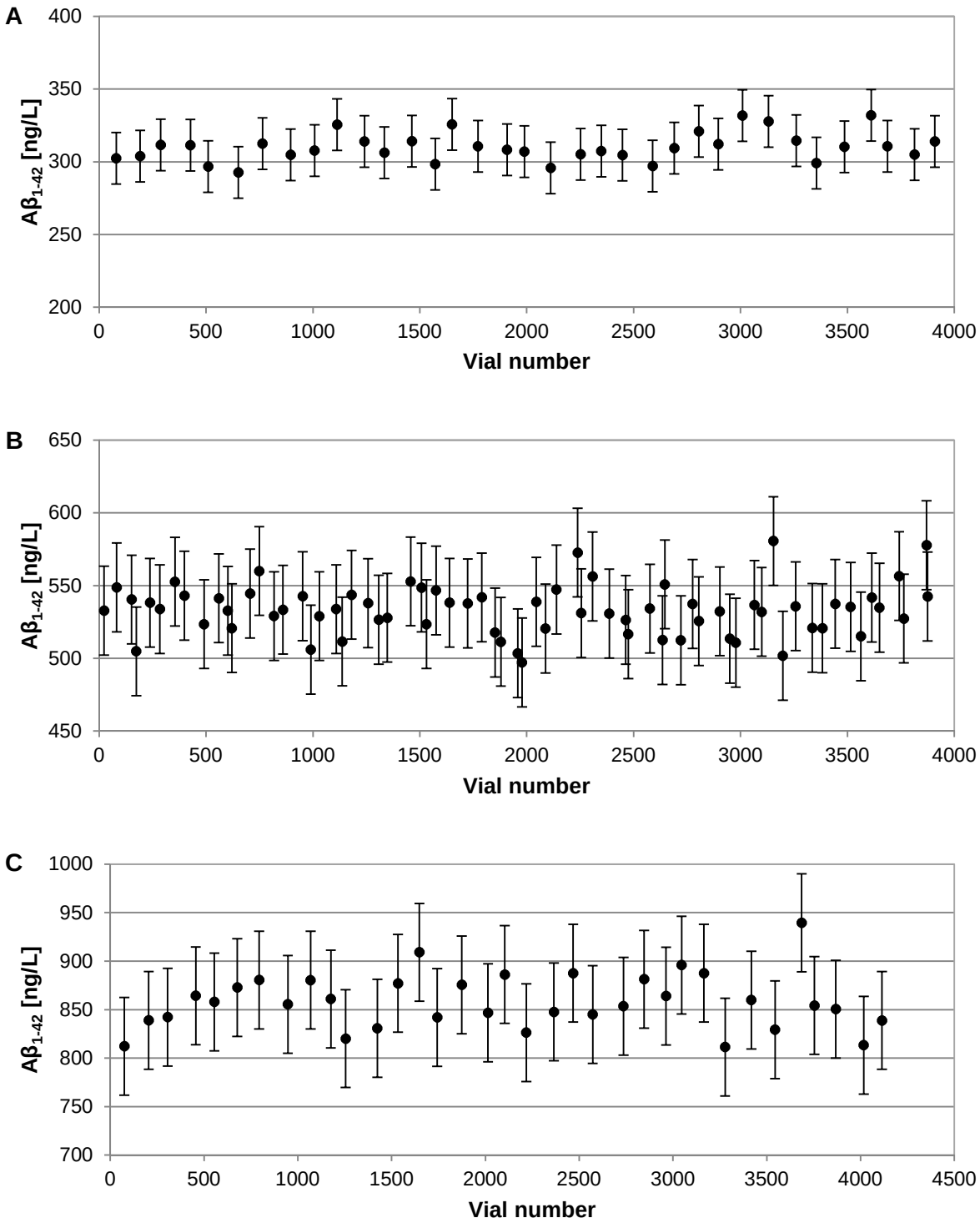


Figure A4: Homogeneity data of $A\beta_{1-42}$ in (A) ERM-DA480/IFCC, (B) ERM-DA481/IFCC and (C) ERM-DA482/IFCC as measured by ELISA. Shown are the averages per vial number and their 95 % confidence interval based on the within-group standard deviation as derived from a one-way ANOVA of all data grouped by vial number after correction of the analysis trend.

Annex B: Results of the short-term stability measurements

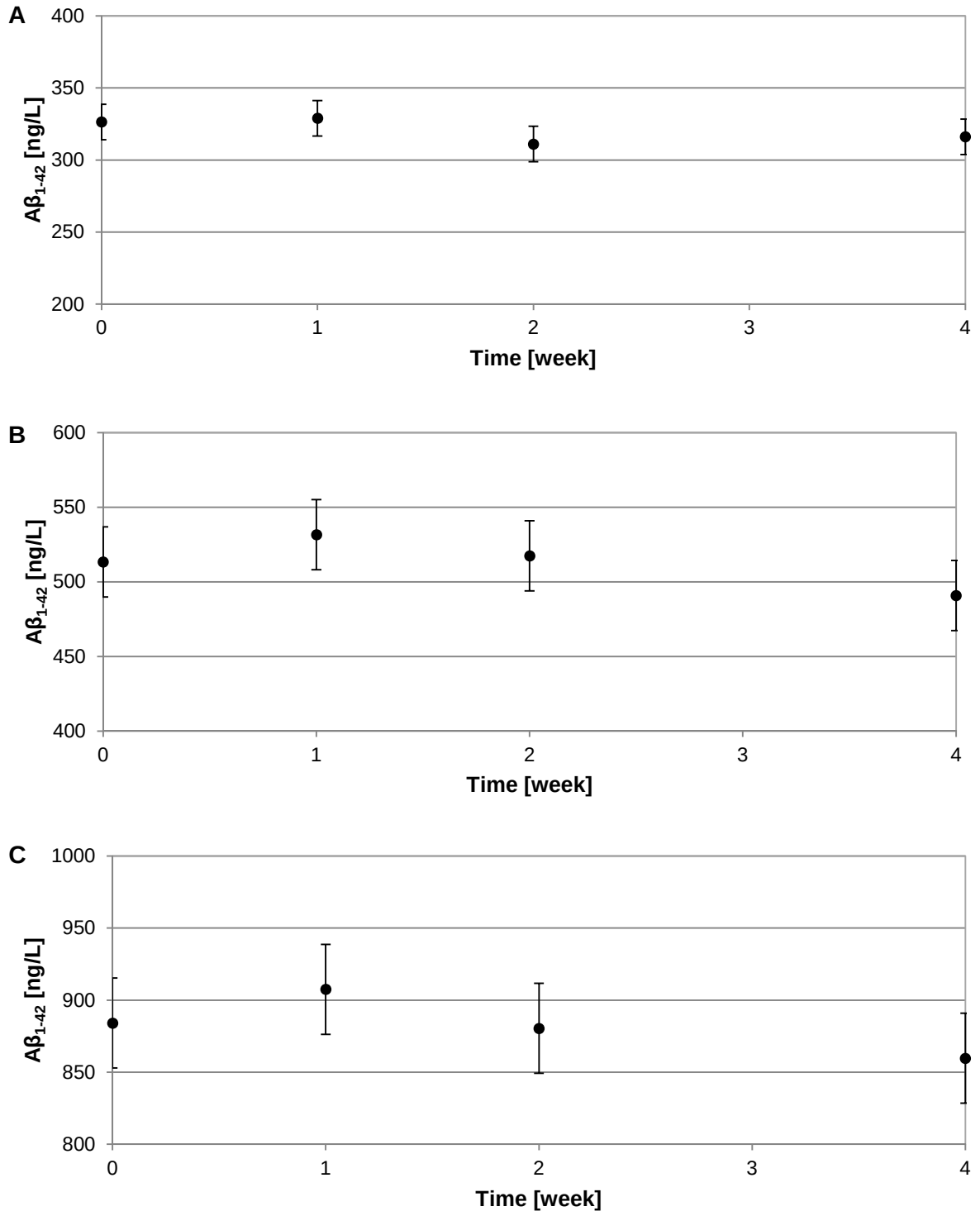


Figure B1: Isochronous short-term stability measurements of $A\beta_{1-42}$ (stored at -20 °C) in A) ERM-DA480/IFCC, B) ERM-DA481/IFCC and C) ERM-DA482/IFCC as measured by ELISA. Shown are the averages per time point and their 95 % confidence interval based on the within-group standard deviation as derived from a one-way ANOVA of all data grouped by time.

Annex C: Results of the long-term stability measurements

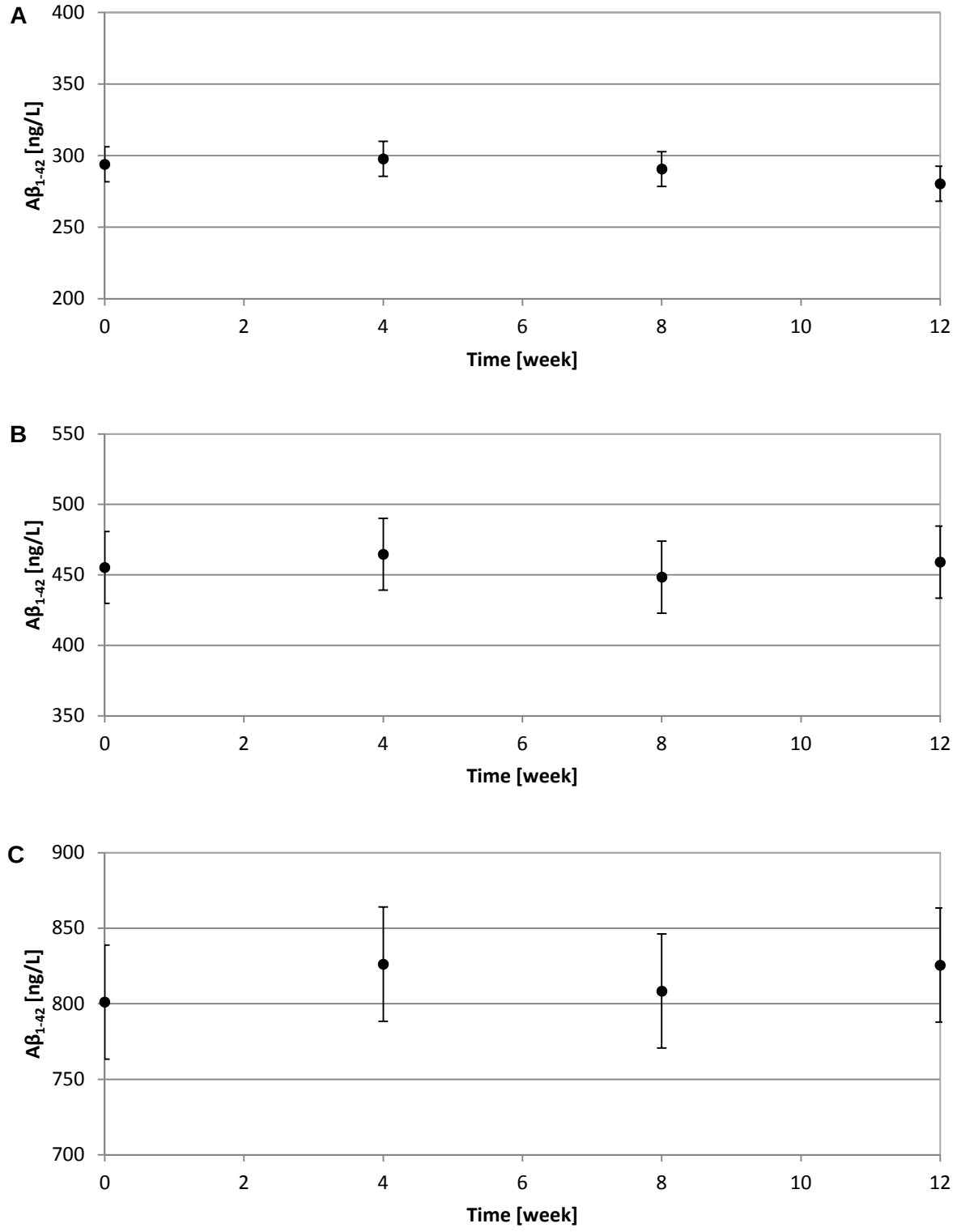


Figure C1: Isochronous long-term stability measurements of $A\beta_{1-42}$ (stored at -70 °C) in A) ERM-DA480/IFCC, B) ERM-DA481/IFCC and C) ERM-DA482/IFCC as measured by ELISA. Shown are the averages per time point and their 95 % confidence interval based on the within-group standard deviation as derived from a one-way ANOVA of all data grouped by time.

Annex D: Summary of methods used in the characterisation study

Table D1: Summary of methods used in the characterisation study. Method information is given as reported by laboratory

Laboratory code	CSF volume [µL]	LC system	LC mobile phase	Column	MS
L01	100	ACQUITY UPLC (Waters)	A-0.1 % NH_4OH B-ACN:MeOH:TFE (70:25:5)	BEH300 C18, 1.7 µm, 2.1x150mm	Waters Xevo-TQS
L02	250	ACQUITY Classic UPLC	A-0.3% NH_4OH B-ACN:0.3% NH_4OH (90:10)	ACQUITY UPLC BEH C18 300Å, 2.1 x150 mm 1.7 µm PST column	Waters Xevo-TQS
L03	200	Dionex (Thermo Scientific) Ultimate 3000	A-5 % ACN, 0.3 % NH_4OH B-96 % ACN, 0.1 % NH_4OH	Dionex (Thermo Scientific) Proswift RP-4H 1x250	Thermo Scientific Q Exactive
L04	200	Dionex (Thermo Scientific) Ultimate 3000	A-5 % ACN, 0.075 % NH_4OH B-95 % ACN, 0.025 % NH_4OH	Dionex (Thermo Scientific) Proswift RP-4H 1x250	Thermo Scientific Q Exactive
L05	100	Three Waters Binary Solvent Manager (BSM)	A-0.3 % NH_4OH B-ACN:H_2O:TFE (90:5:5)	ACQUITY UPLC BEH300 C18, 2.1 mm x 150 mm, 1.7 µm, Waters	Waters Xevo-TQS

Annex E: Results of the characterisation measurements

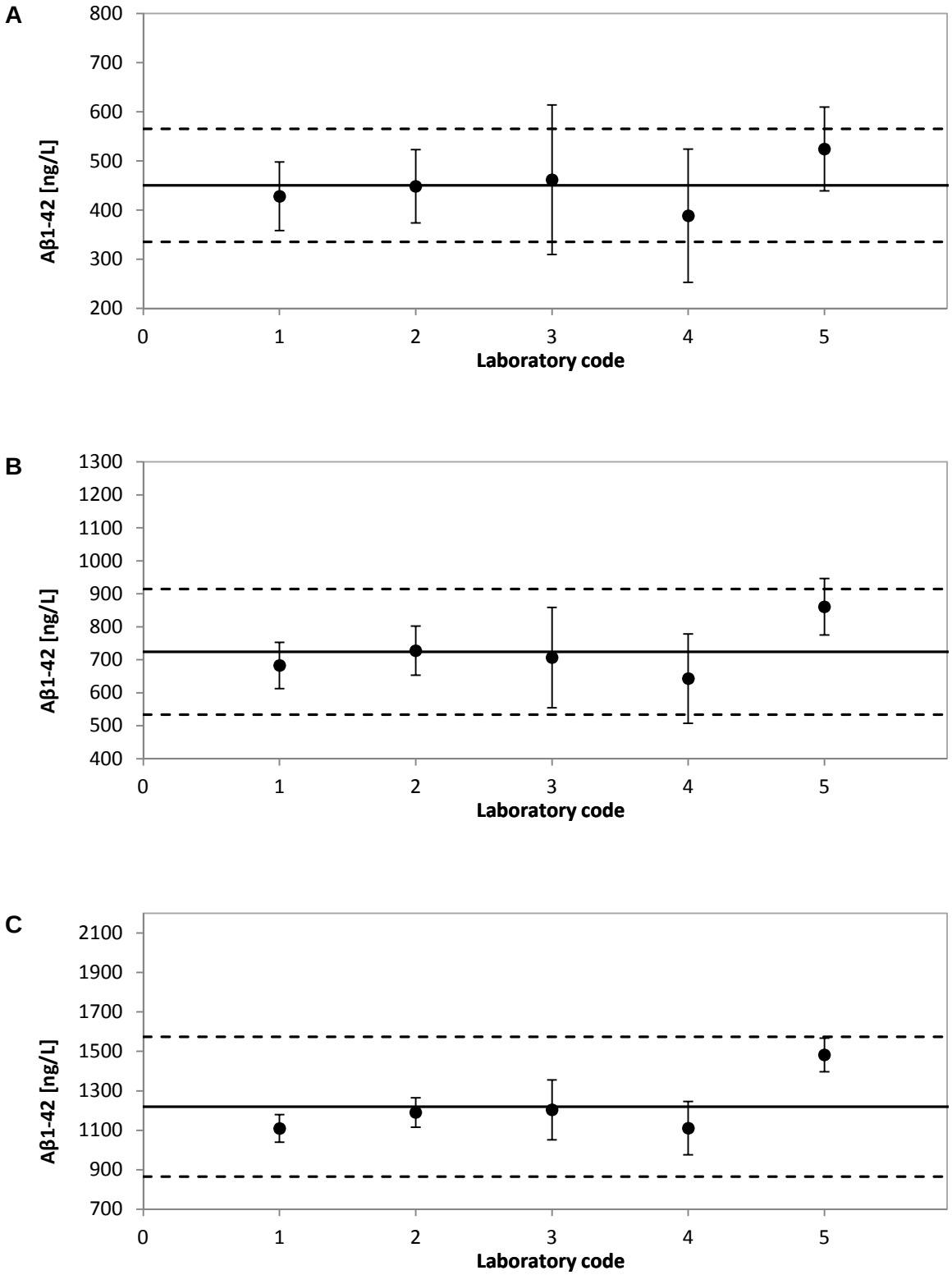


Figure E1: Graph showing average $A\beta_{1\text{-}42}$ concentrations in CSF in A) ERM-DA480/IFCC, B) ERM-DA481/IFCC and C) ERM-DA482/IFCC as measured with the reference measurement procedures. The bars represent the laboratory means ± 2s. The full line represents the mean of the means and the dotted lines represent the mean of the means ± 2s.

Annex F: Commutability

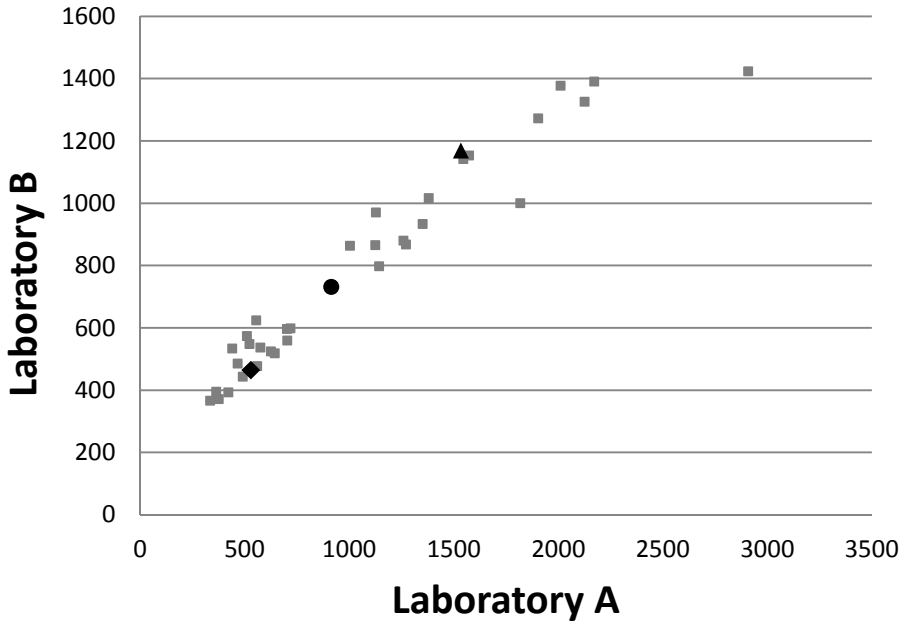


Figure F1: An example of results from commutability study for $A\beta_{1-42}$ in CSF. Plotted are the measurement results for clinical samples (in grey) and RM samples (in black). Patient samples are shown as (■), ERM-DA480/IFCC as (♦), ERM-DA481/IFCC as (●) and ERM-DA482/IFCC as (▲).

Table F1: Summary of methods used in the commutability study.

Measurement Procedure	Company/Institute
EUROIMMUN beta-amyloid (1-42)	EUROIMMUN AG
IBL Amyloid-beta (1-42) CSF ELISA	IBL International GmbH
INNOTEST® β-AMYLOID$_{(1-42)}$	Fujirebio Europe N.V.
Lumipulse® G β-Amyloid$_{(1-42)}$	Fujirebio Europe N.V.
V-PLEX® Aβ Peptide Panel 1 (6E10)	Meso Scale Discovery, LLC.
Elecsys β-amyloid (1-42)	Roche Diagnostics GmbH
LC-MS RMP1	University of Gothenburg

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