C-Reactive Protein
UME CRM 1008

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ABBREVIATIONS

CC-QM	Consultative Committee for the Amount of Substance: Metrology in Chemistry and Biology
CRM	Certified Reference Material
CRP	C-Reactive Protein
GUM	Guide to the Expression of Uncertainty in Measurement
ID	Isotope Dilution
ID-LC/MS-AAA	Amino amino acid analysis using isotope dilution liquid chromatography/ mass spectrometry
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
JCTLM	The Joint Committee for Traceability in Laboratory Medicine
LC-HRMS	Liquid Chromatography High-Resolution Mass Spectrometry
LTS	Long-Term Stability
$MS_{between}$	Between-groups mean square
MS_{within}	Within-group mean square
n	Number of replicates per unit
NIMJ	National Metrology Institute of Japan
NIST	National Institute of Standards and Technology (USA)
PCF	Propyl chloroformate
PCM	Post Certification Monitoring
PRS	Primary Reference Standard
RMP	Reference Measurement Procedure
RSD	Relative Standard Deviation
s	Standard deviation
s_{bb}	Between-units standard deviation
SEC-UV-HPLC	Size-exclusion chromatography/ultraviolet detection with high-performance liquid chromatography
SI	International System of Units
STS	Short-Term Stability
s_{wb}	Within-unit standard deviation
u_{bb}	Between-units heterogeneity-related standard uncertainty
u_{bb}^{*}	Standard uncertainty related to between-units heterogeneity that can be hidden by method reproducibility
u_{char}	Characterization standard uncertainty
$u_{char,rel}$	Characterization relative standard uncertainty
u_{lts}	Standard uncertainty related to long-term stability
$u_{lts,rel}$	Relative standard uncertainty related to long-term stability
u_{sts}	Standard uncertainty related to short-term stability
$u_{sts,rel}$	Relative standard uncertainty related to short-term stability
$v_{MS_{within}}$	MS_{within} within-group degrees of freedom
WHO	World Health Organization

ABSTRACT

This certified reference material (CRM) is designed for calibrating devices and procedures for the determination of C-reactive protein (CRP) in human serum. It can also be used for assigning values to calibrators and control materials. One unit of UME CRM 1008 consists of one vial containing approximately 1 mL of a recombinant CRP solution. The reference material production process includes stages of homogeneity, short-term stability, long-term stability, and characterization. The certification studies for UME CRM 1008 were conducted in accordance with the requirements of ISO 17034 standard and ISO Guide 35. Chemical measurements were performed in compliance with the requirements of ISO/IEC 17025. The uncertainties of the certified values were calculated in accordance with the JCGM 100 “Measurement Uncertainty Guide” (GUM). All stages of this reference material production project, including planning, coordination of activities, conducting experiments, and evaluating all obtained data, were carried out by TÜBİTAK UME experts using the institute’s infrastructure.

The certified value for the monomeric molality of CRP has been determined through amino acid analysis using isotope dilution liquid chromatography/high-resolution mass spectrometry (ID-LC/HRMS and verified using two available certified reference materials for CRP. The measured quantity is the total monomeric concentration of CRP, calculated using the determined amount of each amino acid and the known amino acid sequence for CRP. Metrological traceability is based on derived SI units for molality and is expressed in µmol/kg.

The certified value has been determined using a reference measurement procedure calibrated with high-purity amino acid standards.

INTRODUCTION

C-reactive protein (CRP) is a crucial human serum protein extensively utilized as a marker for inflammatory responses [1]. In response to inflammation, injury, or infection, it functions as a significant biomarker, increasing at least 100-fold [2]. Additionally, CRP is typically found in very low concentrations, below 1 mg/L, in normal human serum as an acute-phase protein [2]. Abnormal levels of CRP have been associated with cardiovascular and cerebrovascular diseases [3, 4]. Measurement of CRP poses challenges due to the lack of standardization in measurement methods and the diversity of reagents used, leading to significant deviations in measurement results [5]. The Joint Committee for Traceability in Laboratory Medicine (JCTLM) [6] currently lists the secondary certified reference material (CRM) ERM DA-474/IFCC. This CRM forms the basis for establishing metrological traceability in CRP measurement with end-user measurement procedures widely used in medical laboratories. The assigned value of ERM DA-470 is determined based on the international standard WHO 85/506, and this information is explained in the certification report. However, uncertainties persist regarding whether calibrators prepared with solutions based on WHO 85/506 accurately represent clinical samples [7]. Even widely accepted CRP measurement tests show significant discrepancies when using the IFCC CRP secondary reference material ERM-DA474/IFCC [8]. Furthermore, clinical test procedures for CRP measurements depend heavily on the device and procedure used, contributing to a lack of standardization in metrological procedures [9]. Establishing a reference measurement system for CRP measurements is crucial to address these issues and improve diagnostic accuracy. In the efforts to ensure metrological traceability for C-reactive protein (CRP) measurements, a consensus summary has recently been published by participants of the "Joint Committee Workshop on Traceability in Laboratory Medicine" [7]. The discussion emphasized that the metrological traceability framework of current CRP immunoassays is consistent with clinical sample results and meets clinical requirements. One desired goal from a metrological standpoint is to establish a calibration hierarchy comprising a primary substance reference material and a reference measurement procedure (RMP). Such a system ensures the standardization of measurement results and provides traceability for in vitro diagnostics. Primary reference materials can be single-component or mixed-component materials used to verify and calibrate primary reference methods defined by the CIPM Consultative Committee for the Amount of Substance (CC-QM). On the other hand, secondary reference materials are used to verify the calibration of serum-based protein standards during the development of CRP measurement methods and to provide control materials for national metrology institutes or clinical diagnostic laboratories. Currently, there are primary reference materials for CRP, such as CRM 6201-b produced by the National Institute of Metrology (NIMJ) in Japan and SRM 2924 produced by the National Institute of Standards and Technology (NIST) of the United States of America [11, 12]. When proposing a new candidate CRM or RMP in the database, JCTLM requires confirmation of the degree of equivalence to an existing CRM or RMP[13, 14]. However, there is a lack of documented pure primary CRM or RMP for the targeted measurement of CRP.

In this study, the National Metrology Institute of Turkey (TÜBİTAK UME) has developed a certified primary reference material for CRP [15]. The aim of the study is to contribute to establishing traceability in clinical measurements and ensuring consistent and reliable results. Two measurement methods were used in the study: amino acid analysis using isotope dilution liquid chromatography/high-resolution mass spectrometry (ID-LC/HRMS) and size-exclusion chromatography/ultraviolet detection with high-performance liquid chromatography (SEC-UV-HPLC). ID-LC/MS-AAA is a preferred technique for determining protein quantity, providing traceable results compatible with the International System of

Units (SI) [16,17]. The vapor-phase hydrolysis method was preferred for amino acid analysis due to its optimized hydrolysis process and the purity and efficiency of the obtained samples. While the ID-LC/MS-AAA method was used for characterization and value assignment, SEC-HPLC was performed for stability assessment and homogeneity analysis. Statistical analyses, including ANOVA, were conducted, and uncertainty contributions for the experimental process were determined to create an uncertainty budget [16]. The certified molality was determined using higher-order reference measurement procedure calibrated with highly purified amino acid standards. Certification studies for UME CRM 1008 were conducted in accordance with the requirements of ISO 17034 [18] and ISO Guide 35 [19]. Chemical measurements were performed in compliance with the requirements of ISO/IEC 17025 [20]. The uncertainty of the certified value has been estimated in accordance with the JCGM 100 Measurement Uncertainty Guide (GUM) [24].

PARTICIPANTS

Certified reference material production involves material processing, homogeneity, stability, and characterization studies, and information about the organization involved in all project activities is provided in Table 1.

Table 1. The organization participating in the production and certification processes

Job Description	Laboratory/Institution
Material Processing	TÜBİTAK UME (National Metrology Institute) Gebze - Kocaeli, Türkiye
Homogeneity Study	
Stability Studies	
Characterization Study	
Project Management and Data Evaluation	

MATERIAL PROCESSING

Material Source and Preparation

Recombinant human CRP was purchased from OYC Europe in a 1.25 L bottle containing a buffer solution with 20 mmol/L Tris (pH 7.5), 140 mmol/L sodium chloride, 2 mmol/L calcium chloride, and 0.05% (w/v) sodium azide. The recombinant CRP was produced in Escherichia coli and purified by binding to 6-aminohexanoic acid, as described by Tanaka et al. [22]. The 1250 mL of recombinant CRP was divided into 1 mL aliquots. These aliquots were then placed into 1250 pre-labeled 2 mL polypropylene CRM vials in the order corresponding to the listing of the samples. After preparation at +4 °C, the material was stored at -80 °C.

HOMOGENEITY

The between units homogeneity study was conducted to assess whether CRP is within the specified values across all units. The homogeneity study for UME CRM 1008 production was determined based on a random stratified sample selection program (TRaNS) for 12 units. This ensures the selected samples representation of the total number of produced units. Homogeneity analyses were conducted under repeatability conditions by measuring three sub-samples from each unit. Validated SEC-UV-HPLC method, was used for homogeneity analysis. Measurements were randomly ordered to detect possible trends related to filling and analysis sequence during the measurements.

Statistical evaluation of molality values was performed using one-way analysis of variance (ANOVA). Prior to the statistical evaluation with ANOVA, data distributions were examined. For this purpose, histograms were used to check for a single-peaked distribution of results among units. Additionally, the obtained data showed a normal distribution when subjected to the Shapiro-Wilk test. These statistical tests were conducted by the validated Microsoft Excel® templates developed by TÜBİTAK UME.

Measurement results were statistically evaluated for the presence of outliers and trends related to analytical measurement and/or filling sequence. The results are presented in Table 2. No trend was detected based on filling or analysis sequence. When one-sided and two-sided Grubb's tests were applied, no outliers were found.

Table 2. Statistical evaluation of homogeneity test results

Parameter	Trend related to filling order?		Outliers?		Distribution?
	Measurement Related	Filling Related	All data	Unit Averages	Normal/Unimodal
CRP	No	No	No	No	Yes / Yes

ANOVA is applied using the following Equation 1 and Equation 2, respectively, for calculating within-unit (s_{wb}) and between-unit (s_{bb}) standard deviations:

$$s_{wb} = \sqrt{MS_{within}} \tag{1}$$

MS_{within} : Mean square of within-group variance
s_{wb} : Equivalent to the method's standard deviation as long as the sub-samples adequately represent the entire unit

$$s_{bb} = \sqrt{\frac{MS_{between} - MS_{within}}{n}} \tag{2}$$

$MS_{between}$: Between-group variance's mean square
n : Number of repetitions per unit

In situations where the method repeatability may not be good enough to detect the homogeneity of the material or due to fluctuations that may have occurred randomly during the measurement, $MS_{between}$ may be found to be smaller than MS_{within}. In these cases, since s_{bb} cannot be calculated, the highest heterogeneity uncertainty, including method repeatability, is calculated using u_{bb}^{*} Equation 3.

$$u_{bb}^{*} = \frac{s_{wb}}{\sqrt{n}} \sqrt[4]{\frac{2}{\nu_{MS_{within}}}} \quad (3)$$

$\nu_{MS_{within}}$: The degrees of freedom for the MS_{within} value

The results obtained from the homogeneity study are presented in Table 3. In the calculations performed by applying ANOVA, the value for s_{bb} or u_{bb}^{*} is taken as the homogeneity uncertainty component (u_{bb}), with the larger of the two being considered.

Table 3. Results of the homogeneity study

Parameter	$s_{wb,rel}$ (%)	$s_{bb,rel}$ (%)	$u_{bb,rel}^{*}$ (%)	$u_{bb,rel}$ (%)
CRP	0.85	1.1	0.24	1.1

The graphs corresponding to the data obtained from homogeneity tests are presented in Annex 1.

STABILITY

Stability studies were conducted to simulate environmental conditions that might occur during the transportation of certified reference material to the end user (short-term stability, STS) and to mimic storage conditions in a laboratory environment (long-term stability, LTS). The selection of 18 units for short-term stability testing and 22 units for long-term stability testing was determined based on the random stratified sampling principle using the TRaNS software. The stability test measurements were performed using the validated SEC-UV-HPLC method.

For short-term stability studies, temperatures of +4 °C and -20 °C were chosen, with durations of 1, 2, 3 and 4 weeks. Two units for each time interval at each temperature were placed in the respective test chambers. Two units were designated as reference points and directly placed at the reference temperature of -80 °C. At the end of each test period, two units from both temperature environments were transferred to the reference temperature. After completing the four-week test period, all units transferred to the reference temperature were analyzed simultaneously *(isochronous*) with the units designated as reference.

For long-term stability studies, temperatures of +4 °C and -20 °C were chosen, with durations of 1, 3, 6, 9 and 12 months. Two units for each time interval at each temperature were placed in the respective test chambers. Two units were designated as reference points and directly placed at the reference temperature of -80 °C. At the end of each test period, two units from both temperature environments were transferred to the reference temperature. After completing the 12-month test period, all units transferred to the reference temperature were analyzed simultaneously *(isochronous*) with the units designated as reference.

For both stability tests, measurements were analyzed using an analytical measurement sequence created by randomly arranging the test period and filling sequence to distinguish possible trends related to filling or test duration. Statistical calculations of the obtained data were performed using validated templates prepared by experts from TÜBİTAK UME using Microsoft Excel®.

Results of Short-Term Stability Study

The measurement results isochronously obtained in the short-term stability study were initially grouped according to the time points, and evaluations were made for each time point. These evaluations were separately conducted for both temperatures.

The measurement values obtained for each duration were examined for outliers at 95% and 99% confidence levels using Grubb's test to determine their compatibility with other values in that temperature group. Outliers were identified in the +4 °C test for unit no: 509 (t = 3 weeks) in the first parallel measurement and in the -20 °C test for unit no: 113 (t = 2 weeks) in the second parallel measurement. Since outliers were observed in only one parallel measurement each, they were not included in the evaluation.

In the evaluation of short-term stability data, graphs of the calculated values for each time point were plotted over time. The relationship between variables was examined through regression analysis to determine if there was any significant change in concentration values over time. Linear graphs were drawn for CRP, and the slopes of these trends were tested for significance at the 95% confidence level (α = 0.05) using a t-test (two-tailed tcrit value). The results of the short-term stability tests are presented in Table 4 and Graph 2. Uncertainty calculations for short-term stability were performed considering the uncertainty associated with this trend and the two weeks of exposure time using Equation 4 [23].

$$u_{sts,rel} = \frac{RSD}{\sqrt{\sum(t_i - \bar{t}\,)^2}} \times t \qquad (4)$$

RSD : Relative Standard Deviation of points on the regression line

t_i : Time point for each parallel

$\bar{t}$: Mean of all time points

t : Maximum time foreseen for transfer: 2 weeks

Table 4. Short-term stability test results and uncertainty values for a duration of 2 weeks

Parameter	Number of outliers at 95% Confidence Level		Is the slope different from zero at 95% Confidence Level?		$u_{sts,rel}$ (%) for 2 weeks	
	+4 °C	-20 °C	+4 °C	-20 °C	+4 °C	-20 °C
CRP	1	1	Yes	No	0.29*	**0.14**

* Slope found significant at 95% confidence level, and u_{sts} was calculated considering the decomposition; (u_{sts} = Slope of Regression Curve / √3)

Based on the assessment, it has been observed that the parameter to be certified in the produced certified reference material is stable for a duration of 2 weeks at -20 °C. As a result of this study, it has been concluded that the samples can be delivered to the end user at a temperature of -20 °C or below, provided that the temperature does not exceed -20 °C and the duration does not exceed 2 weeks. The uncertainty provided in the certificate has been calculated taking into account a transfer temperature of -20 °C and a transfer time of 2 weeks.

Results of Long-Term Stability Study

The shelf life of the produced CRM is determined based on the results of long-term stability studies. Graphs of repeated measurement results at each time point are provided in Annex 3. The error bars at each time point were calculated as the standard deviation of the two results obtained for each of the two units.

Within the obtained data, a review was conducted to determine the presence of outliers at 95% and 99% confidence levels using both one-sided and two-sided Grubb's tests. No outliers were found at either test temperature.

Long-term stability is one of the four parameters contributing to the total uncertainty budget. The uncertainty value arising from the long-term stability of the certified reference material was calculated using Equation 5 [23].

$$u_{lts,rel} = \frac{RSD}{\sqrt{\sum(t_i - \bar{t}\,)^2}} \times t \qquad (5)$$

RSD : Relative standard deviation of points on the regression line

t_i : Time point for each parallel

$\bar{t}$: Mean of all time points

t : The determined shelf life for storage at the specified temperature: 12 months

The calculated results are presented in Table 5, and the graphs are provided in Annex 3. The shelf life for stability has been determined to be 12 months for the contribution to the total uncertainty. To ensure stability beyond the determined shelf life, periodic reassessments will be conducted based on post certification monitoring (PCM) results at specific intervals.

Table 5. Long-term stability test results and uncertainty values for a duration of 12 months

Parameter	Number of outliers at 95% Confidence Level		Is the slope different from zero at 95% Confidence Level?		$u_{lts,rel}$ (%) for 12 months	
	+4 °C	-20 °C	+4 °C	-20 °C	+4 °C	-20 °C
CRP	0	0	Yes	Yes	0.97*	**0.37***

* Slope found significant at 95% confidence level, and u_{lts} was calculated considering the decay of the regression curve (u_{lts} = Slope of Regression Curve / $\sqrt{3}$)

In-Use Stability Study Results (Freeze/Thaw Cycles)

For UME CRM 1008, in addition to long and short-term stability studies, an in-use stability study has been conducted. In-use stability studies were conducted in the form of 0, 1, 2, 3 and 4 freeze/thaw cycles. For each cycle, measurements were performed using 2 units. Each unit was subjected to a 4-hour freeze/thaw process for a total of 4 cycles.

After completing the freeze/thaw cycles, all samples were analyzed isochronously. Graphs of repeated measurement results for each cycle are presented in Annex 4. The error bars at each time point were calculated as the standard deviation of the two results obtained for each of the two units. The relative standard deviation (RSD) of 20 measurement results was calculated as 0.50% through the analysis. Additionally, the analysis results were compared with the results obtained in homogeneity studies, and these data were subjected to an F-test. As a result, no significant difference was found between the two measurement sets. These results indicate that the material is stable when exposed to four freeze/thaw cycles.

CHARACTERIZATION

ISO 17034 standard [18] allows for various designs in the characterization study. One of these designs is the characterization study conducted using a reference method in a single laboratory. In this production process, the certified value for the monomeric concentration of CRP was determined through amino acid analysis using isotope dilution liquid chromatography/high-resolution mass spectrometry (ID-LC/HRMS). Details regarding the measurement method are provided in Annex 5.

The characterization study was conducted on randomly selected 10 units using the TRaNS program. In this context, 5 samples were taken on the 1st and 2nd days, and three parallel measurements were performed on each sample. Additionally, on the 3rd day, a 4th parallel measurement was conducted on all 10 units. The measurements were carried out using a validated method.

NIST SRM 2924 and NIMJ CRM 6201-c solutions were used for the purpose of verifying the method in this study. Table 6 presents the summary statistics of the measurements conducted on these reference materials. The results of this comparison show that the molarity values obtained using the same measurement method as the characterization measurement method for UME CRM 1008 are consistent with the certified values of the reference materials.

Table 6. Standards used for method validation and measurement statistics

	Certificate Molality[a]		Measured Molality ($n = 6$)Summary Statistics[b]			
CRM	**Value**	U_{95}	**Value**	**SD**	u_c[c]	U_{95}[d]
NIMJ 6201-c	40.2	1.7	39.8	0.6	0.9	1.9
NIST SRM 2924	20.6	1.2	20.4	0.4	0.5	1.0

[a] Certificate value and the %95 confidence interval uncertainty listed in CRM certificates.

[b] Measured value and %95 confidence interval uncertainty. Analyses were conducted using isotope dilution amino acid analysis.

[c] Standard measurement uncertainty, u_c

[d] Expanded uncertainty for %95 confidence interval (k=2).

The uncertainty calculations for amino acid analysis have been carried out in accordance with the "Guide to the Expression of Uncertainty in Measurements (GUM)" [24] and the "EURACHEM/CITAC Guide Quantifying Uncertainty in Analytical Measurement" [25] documents.

PROPERTY OF VALUE AND ASSIGNMENT OF UNCERTAINTY

The assignment of the certified property value and its uncertainty was carried out by considering data obtained from the characterization study, along with the associated uncertainty values, as well as contributions from homogeneity and stability tests. The uncertainty value for the property value has been calculated by combining contributions from characterization, homogeneity, short-term, and long-term stability uncertainties, as given in Equation 6.

$$u_{CRM} = \sqrt{u_{char}^2 + u_{bb}^2 + u_{sts}^2 + u_{lts}^2} \quad (6)$$

Here, u_{CRM} represents the combined standard uncertainty for the property value. This value is transformed into the expanded uncertainty for the certified value by multiplying it with the coverage factor k, as shown in Equation 7.

$$U_{CRM} = k \times u_{CRM} \quad (7)$$

The uncertainty value given along with the certified value is expanded by multiplying it with the coverage factor k = 2, corresponding to a 95% confidence level. The certified value and the associated uncertainty are provided in Table 7. The relative and combined results of contributions from homogeneity, short-term, and long-term stability, as well as uncertainty from value assignment, are shown in Table 8.

Table 7. Certified Value and its Uncertainty

Parameter	Molality (µmol/kg)	U_{CRM} (*k* = 2) (µmol/kg)	Relative U_{CRM} (*k* = 2) (%)
CRP	43.2	2.2	5.1

Table 8. Contributions of Uncertainty Components to Expanded Uncertainty, U_{CRM}

Parameter	u_{char} (%)	u_{bb} (%)	u_{lts} (%)	u_{sts} (%)
CRP	2.3	1.1	0.37	0.14

INFORMATIVE VALUE

The value and uncertainty have been calculated from the molality values, using the material's density at 25 ºC (1.0047 g/cm³) and the average molecular weight (23028.2 g/mol) obtained through mass spectrometry. The calculated values are provided in Table 9.

Table 9. Informative value

Parameter	Mass Concentration	Uncertainty	Unit
CRP	0.999	0.051	g/L

TRACEABILITY

The traceability of the certified reference material has been ensured by using primary reference standards, which were determined and certified for purity by NIMJ. The primary reference standards used for traceability in the certification studies are provided in Table 10.

Table 10. Primary reference standards used as a source of traceability in certification studies

Parameter	Name	Purity ± Uncertainty (*k* = 2) (%)	Traceability
Proline	NIMJ 6016-a	99.9 ± 0.2	SI
Valine	NIMJ 6015-a	99.8 ± 0.2	SI
Leucine	NIMJ 6012-a	99.9 ± 0.2	SI
Phenylalanine	NIMJ 6014-a	99.9 ± 0.2	SI
Alanine	NIMJ 6011-a	99.9 ± 0.2	SI

INSTUCTIONS FOR USE

This CRM is intended to be used as a calibration standard for CRP measurement procedures and instruments. It can also be utilized for assigning values to other calibration standards and quality control materials. Additionally, the material can be used to control the accuracy and validate the validity of analytical methods in amino acid analysis. When used in immunological tests for determining or assigning the quantity of CRP in human serum, commutability should be verified.

Storage Conditions

The CRM is shipped in a polypropylene vial in a frozen state. Upon receipt, the material should be stored in its original unopened vial at -20 °C or lower. TÜBİTAK UME cannot be held responsible for changes in the material due to non-compliance with the reported storage conditions and usage instructions.

Safety Precautions

The material is a recombinant human CRP protein in aqueous solution and is not classified as a hazardous substance. It is suitable for in vitro use only and has been produced solely for laboratory use. General laboratory precautions should be applied during the storage and use of the material. The use and disposal of the material are recommended according to the existing safety regulations.

Handling of the Material

The CRM sample to be analyzed should be removed from the freezer and kept at room temperature (20 ± 3) °C until completely thawed. After thawing, the material can be gently mixed, and then a brief centrifugation process (1000 g, 1 min) can be performed to transfer the solution from the vial cap and walls to the vial base. The dissolved material can be stored at +4 °C for up to one week. It has been determined that four freeze/thaw cycles have no impact on the molarity and concentration values of the material. The material can be safely dispatched where the temperature does not exceed -20 °C and the transportation period of 2 weeks.

Minimum Sample Intake

In certification studies, **a minimum of 10 µL** of sample was used for each measurement. The minimum sample volume should be determined by the end user based on their measurement capability, taking into consideration the impact on the uncertainty of the prepared working solution for the study.

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REVISION HISTORY

Date	Remarks
20.02.2024	First issue.

ANNEXES

ANNEX 1. Graph for Homogeneity Study

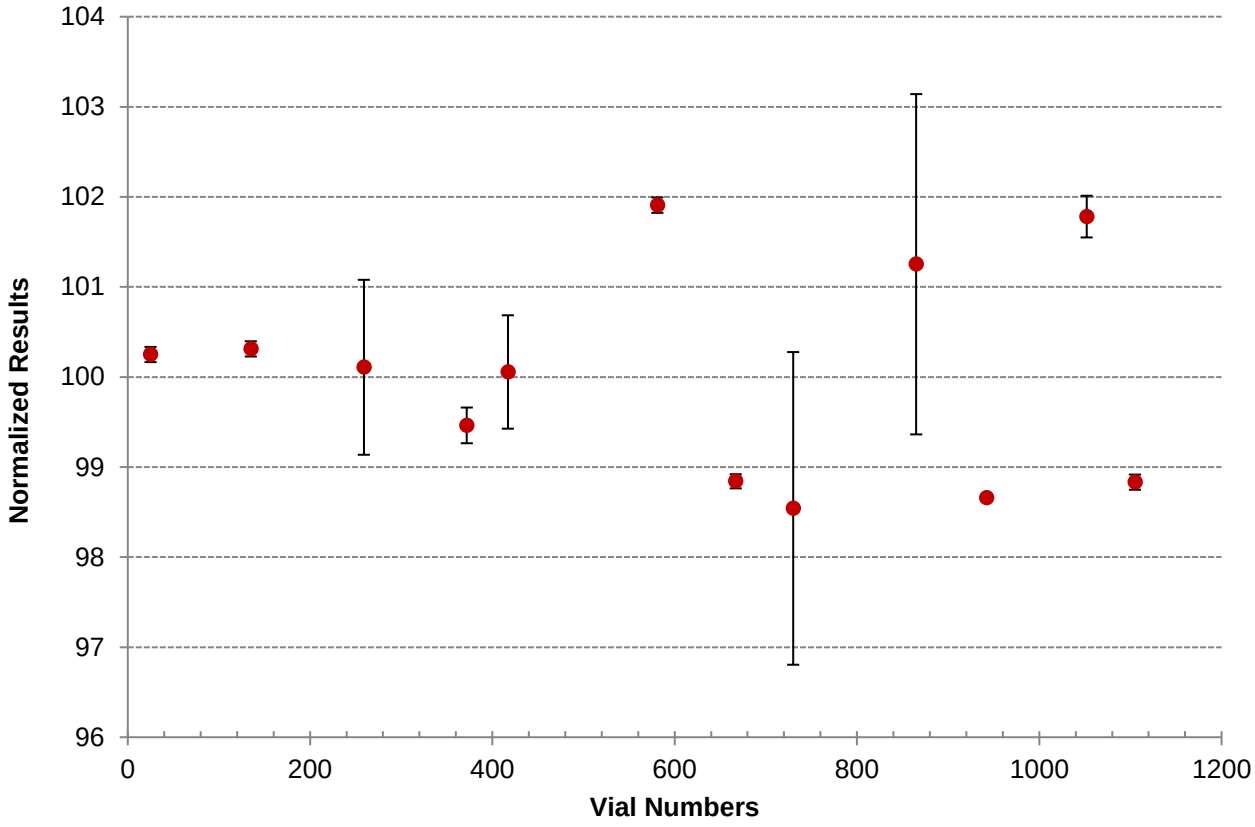

Figure A1.1. CRP graph for homogeneity

ANNEX 2. Graphs for Short Term Stability Studies

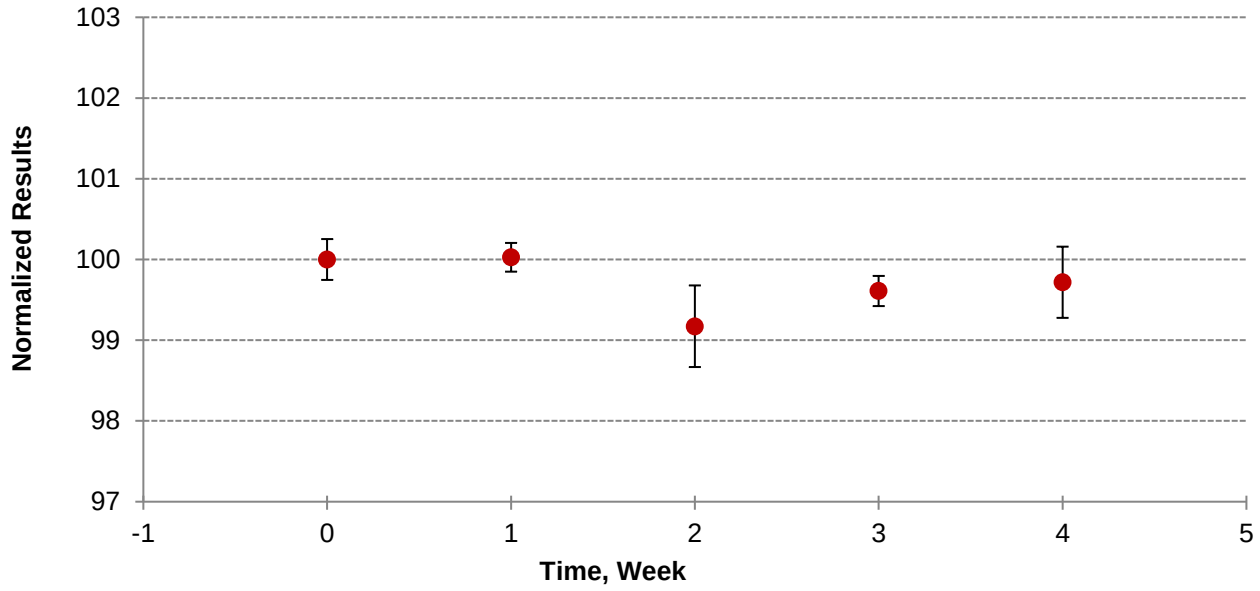

Figure A2.1. CRP graph for short term stability at -20 °C

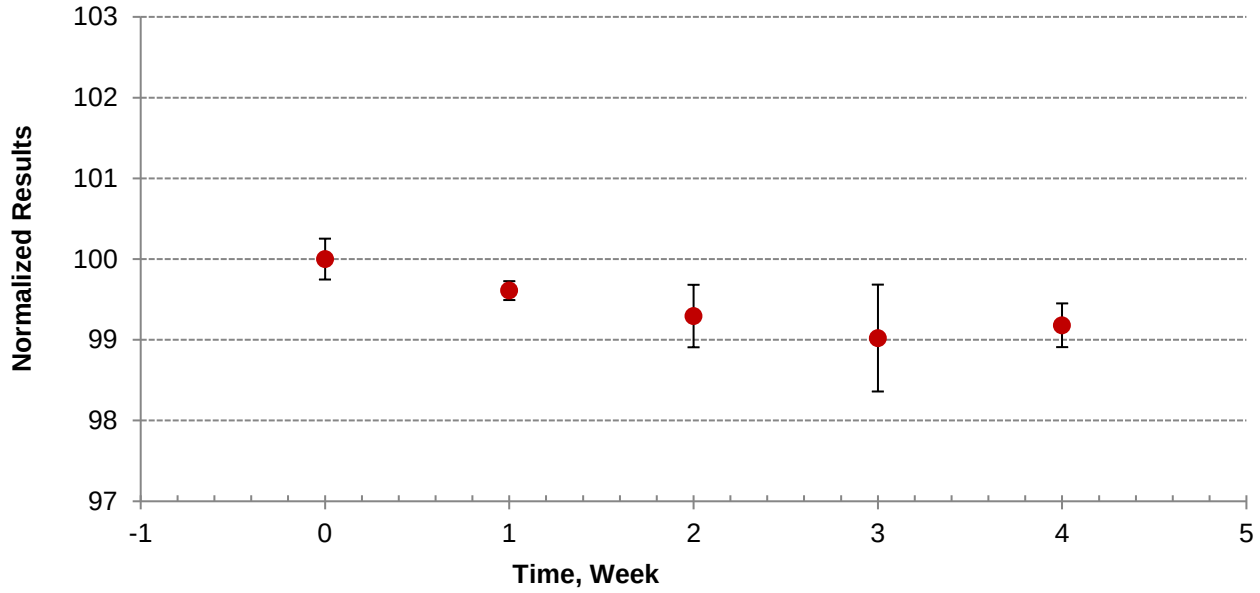

Figure A2.2. CRP graph for short term stability at +4 °C

ANNEX 3. Graphs for Long Term Stability Studies

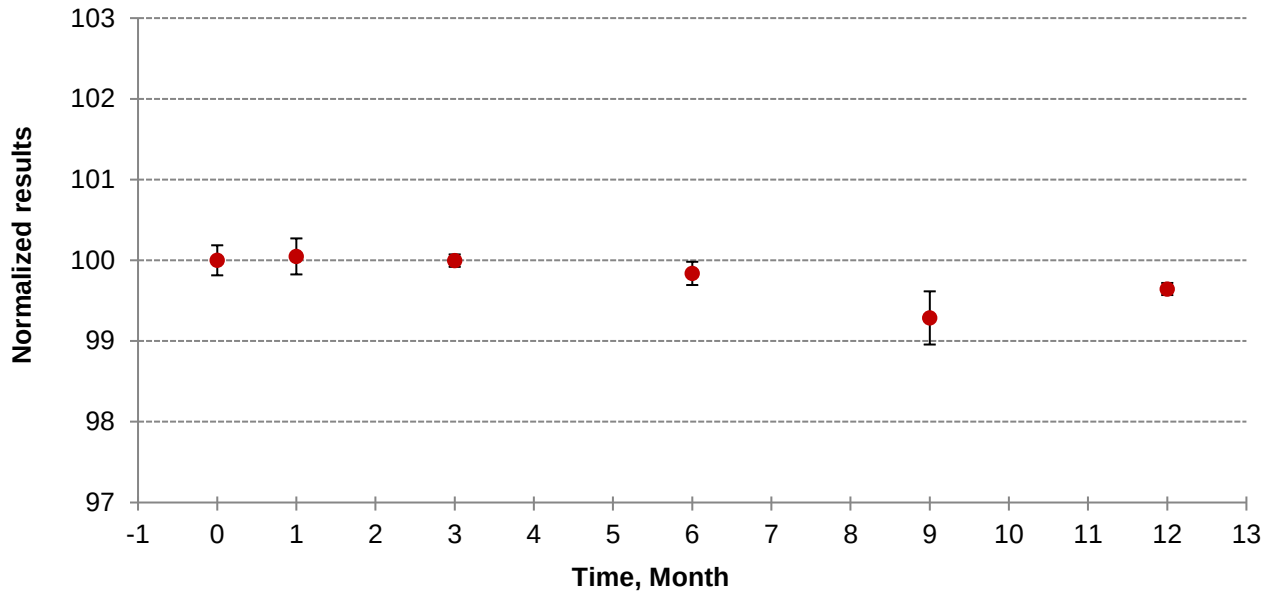

Figure A3.1. CRP graph for long term stability at -20 °C

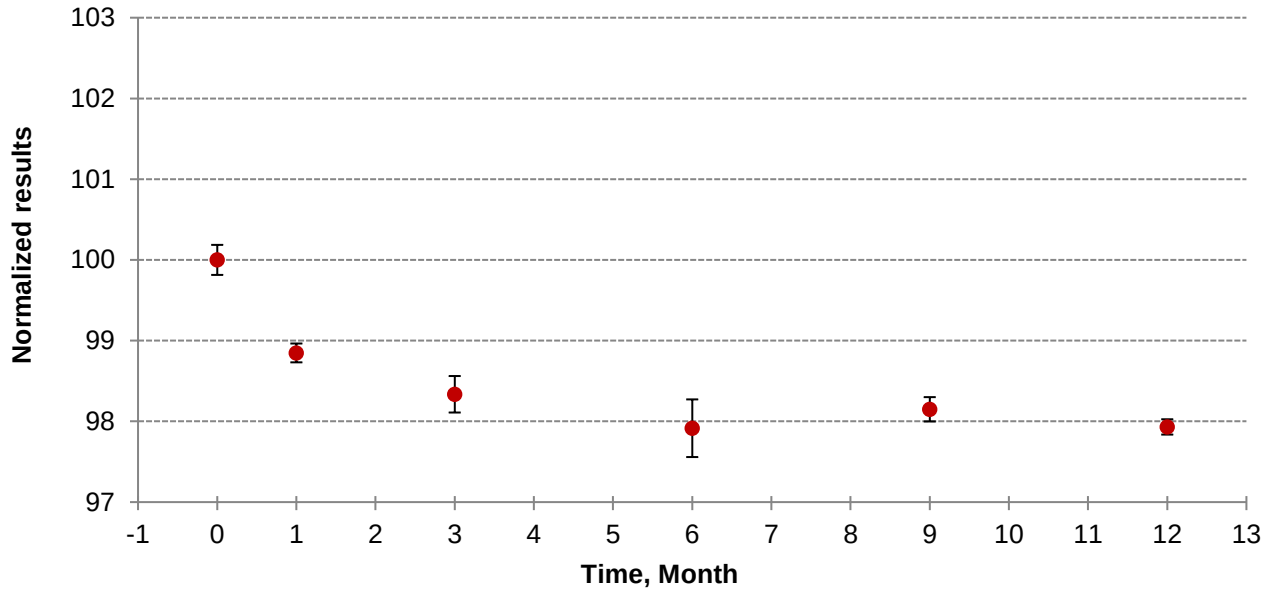

Figure A3.2. CRP graph for long term stability at +4 °C

ANNEX 4. In-Use Stability Study Graph

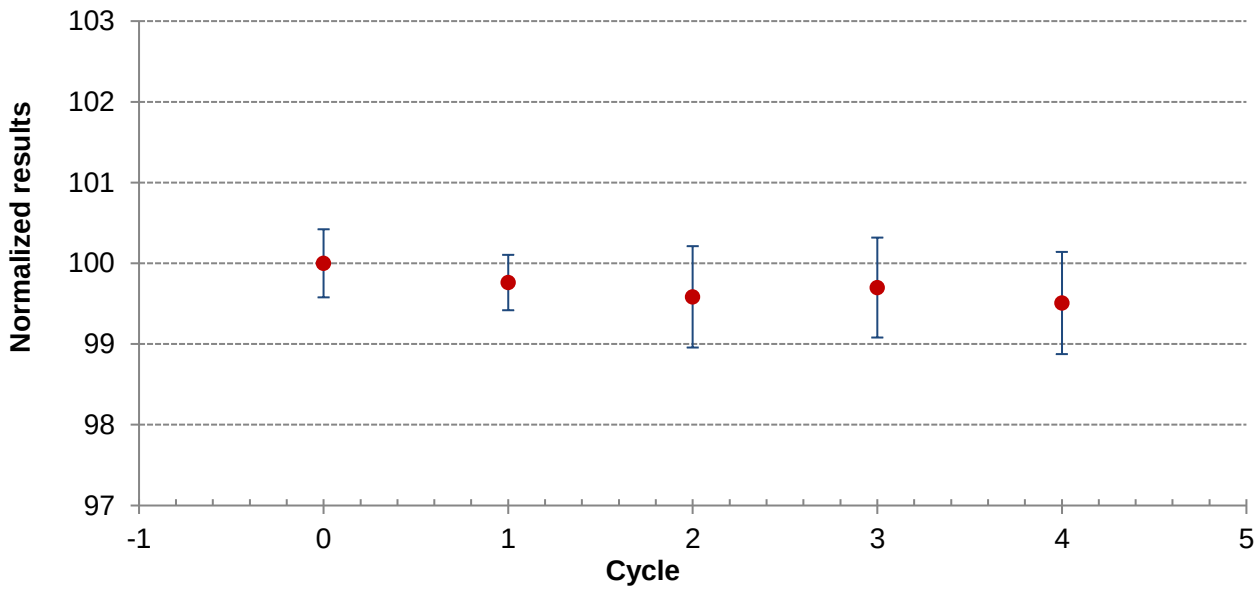

Figure A4.1. CRP stability graph for freeze/thaw cycles

ANNEX 5. Details of the Measurement Method

Short Description of the Characterization Measurement Method

For peptide impurity determination, a 20 µmol/L CRP solution is prepared gravimetrically in a 0.1 mol/L HCl solution. Standard calibration solutions containing 1.25 µM, 2.5 µM, 5 µM, 10 µM, 20 µM and 40 µM equivalents of C - reactive protein, each containing Proline, Valine, Phenylalanine, Leucine, and Alanine amino acids, are prepared. An isotopic standard solution is prepared from isotopic Proline, Valine, Phenylalanine, Leucine, and Alanine amino acids equivalent to 20 µmol/L peptide. By mixing the isotopic standard solution with sample and calibration solutions, sample and standard compositions are prepared gravimetrically. Sample and standard compositions are transferred to glass hydrolysis tubes and evaporated under vacuum in the gas phase hydrolysis device (ELDEX™). The dried samples are hydrolyzed at 130 °C for 24 hours with 6 mol/L "constant-boiling HCl™" containing 1% phenol. After hydrolysis, the samples are dissolved in 200 µL of 100 mmol/L HCl and derivatized with propyl chloroformate. The prepared working standards are analyzed using Dionex Ultimate 3000 UPLC and Orbitrap Q-Exactive HF-X HR-MS. After analysis, concentrations of each analyte are entered into the device's software to create an external calibration curve. Mass fractions of (Proline, Valine, Phenylalanine, Leucine, and Alanine) in peptide samples are calculated using calibration curves created with working standard solutions. The dilution factor values calculated gravimetrically for peptide samples are also entered as data into the software. Mass fractions in the peptide molecule are calculated using the software of the device.

LC-MS (Liquid Chromatography–Mass Spectrometry) Analysis

The prepared working standards were analyzed using the LC-HR-MS system consisting of Dionex Ultimate 3000 UPLC and Thermo Q-Exactive HF-X Orbitrap instruments. The experiments were conducted using a Phenomenex EZ:faast 4µ AAA column (250 mm × 2 mm i.d). The mobile phase consisted of A: 50:50 methanol:H_2O containing 10 mmol/L ammonium formate and B: methanol containing 10 mmol/L ammonium formate. The LC gradient program is provided in Table A5.1.

The column temperature was set to 30 °C, and the injection volume was adjusted to 10 µL. Mass spectrometry analysis was performed in Full MS mode following PCF derivatization. MS parameters for amino acids are presented in Table A5.2.

Table A5.1. LC Gradient Program for Amino acid Analysis

Time (min:s)	Mobile Phase (%A)	Mobile Phase (%B)	Flow Rate (µL/min)
0:00	65	35	250
12:00	45	55	250
12:10	0	100	250
17:00	0	100	250
17:10	65	35	250
22:00	65	35	250

Table A5.2. Mass Spectrometer (MS) Parameters

Scan	Full MS
Scan Begin	100 m/z
Scan End	500 m/z
Polarity	Positive
AGC Target	3E6
Max Inject Time	200 ms
Sheath Gas Flow	45
Sweep Gas Flow	2
Spray Voltage	3.50 kV
Capillary Temperature	250 °C
Aux Gas Temperature	300 °C