National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 2924

C-Reactive Protein Solution

This Standard Reference Material (SRM) is primarily intended for use in calibrating procedures and devices for the determination of C-reactive protein (CRP) in human serum. It can also be used for value-assignment of calibrators and control materials. A unit of SRM 2924 consists of three vials, each containing approximately 1 mL of a solution of recombinant CRP.

Certified Concentration Value: The certified value for monomeric concentration of CRP was determined through amino acid analysis using isotope dilution liquid chromatography/tandem mass spectrometry (ID-LC/MS/MS) [1] with verification using an existing, commercially available certified reference material for CRP. The measurand is the total monomeric concentration of CRP calculated using the amount-of-substance determined for each of the amino acids and the known amino acid sequence for CRP. Metrological traceability is to the SI derived units for molar concentration (expressed as micromoles per liter).

Certified CRP concentration: 20.6 µmol/L ± 1.2 µmol/L $k = 2.15$

A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account [2]. The certified concentration was determined using higher-order reference measurement procedures [3] calibrated with high-purity amino acid standards. The uncertainty provided for the value is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence. The expanded uncertainty is calculated as $U = ku_c$, where u_c is the combined uncertainty, and k is a coverage factor corresponding to approximately 95 % confidence [4].

Reference Density, Relative Average Mass and Concentration Values: The reference values for density, relative average molecular mass and reference CRP concentration (expressed in terms of grams per liter) are shown in Table 1. The reference values for density and relative average molecular mass were determined by the Lang-Levy pipet method and mass spectrometry, respectively. The reference concentration, expressed in terms of grams per liter, was calculated using the relative average mass and the certified CRP concentration value above.

NIST reference values are non-certified values that represent the best estimate of the true values based on available data. All known or suspected sources of bias have not been fully investigated and are therefore provided with associated uncertainties that may reflect only measurement precision, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods. The uncertainty provided with the reference value is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence. The expanded uncertainty is calculated as $U = ku_c$, where u_c is the combined uncertainty, and k is a coverage factor corresponding to approximately 95 % confidence [4].

Expiration of Certification: The certification of **SRM 2924** is valid, within the measurement uncertainty specified, until **30 March 2022**, provided the SRM is handled and stored in accordance with instructions given in this certificate (see "Instructions for Storage and Use"). This certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

Overall direction and coordination of technical measurements leading to the certification were performed by E.L. Kilpatrick of the NIST Biomolecular Measurement Division. Additional technical guidance was provided by M.S. Lowenthal and K.W. Phinney of the Biomolecular Measurement Division.

Statistical analysis was provided by J.H. Yen of the NIST Statistical Engineering Division.

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Certificate Issue Date: 10 July 2017

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Acquisition of the material was performed by E.L. Kilpatrick. Certification measurements were performed by E.L. Kilpatrick, L.E. Kilpatrick of the Biomolecular Measurement Division and L.T. Sniegoski of the Chemical Sciences Division.

Support aspects involved with the certification and issuance of the SRM were coordinated through the NIST Office of Reference Materials.

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet or register online) will facilitate notification.

NOTICE AND WARNING TO USERS

Warning: SRM 2924 IS INTENDED FOR RESEARCH USE ONLY.

INSTRUCTIONS FOR STORAGE AND USE

Storage: The SRM is shipped frozen on dry ice in polypropylene vials. Upon receipt, material should be stored frozen below –50 °C until ready for use in the original unopened vial.

Use: Vials of the SRM to be analyzed should be removed from the freezer and allowed to stand at room temperature (20 °C to 25 °C) until thawed. After the material is thawed, it may be gently mixed and then centrifuged briefly (1000 g_n – 5 min) to clear material from the cap threads. Unused material may be stored at 4 °C for up to one week.

SOURCE, PREPARATION, AND ANALYSIS

Source and Preparation: The recombinant human CRP was procured from OYC Americas (Andover, MA) in a buffer composed of 20 mmol/L tris buffer (pH 7.5), 140 mmol/L sodium chloride, 2 mmol/L calcium chloride and 0.05 % (m/v) sodium azide packaged in a 2 mL polypropylene vial. The recombinant CRP was expressed in *Escherichia coli* and purified by affinity binding to 6-aminohexanoic acid as described in Tanaka, et al [5]. A total of 1200 vials packed into twelve boxes were received at NIST at 4 °C. The material was frozen and kept at –80 °C following preliminary investigation showing no effects of a freeze/thaw cycle on concentration or structure.

Analysis: All analyses in the value assignment of SRM 2924 were performed at NIST.

Measurement of CRP concentration by amino acid analysis (ID-LC/MS-MS): The amino acid analysis method involved isotope dilution liquid chromatography-tandem mass spectrometry (ID-LC/MS-MS) [1]. Samples of SRM 2924 were combined with isotope-labeled analogs of phenylalanine, proline, isoleucine, leucine, and valine and were hydrolyzed with vapor-phase hydrochloric acid (HCl) for 48 h at approximately 118 °C in sealed vessels. After hydrolysis, the samples were lyophilized and then reconstituted with 0.1 mL/L formic acid in water. Amino acids were separated using gradient-elution mixed-mode chromatography on a reverse-phase analytical column with embedded acidic ion-pairing groups. Measurements were performed on a triple quadrupole mass spectrometer, monitoring a specific transition for each amino acid. The measurements were calibrated using amino acid standards whose purity was assessed at NIST. Based upon the known amino acid sequence for CRP [6], the concentration of CRP was calculated as the mean of the concentrations determined for CRP by each of the amino acids. Analysis of SRM 2924 was performed in four independent groups containing six replicates on four separate days. Confirmation of the analysis was obtained by use of a commercially available certified reference material, NMIJ CRM6201-b, C-reactive protein solution [7], which was run in duplicate on each day of analysis. The mean value of the CRM6201-b concentration was 38.9 µmole/kg ± 0.26 µmole/kg (standard deviation) and was within the certified 95 % confidence range reported as 38.4 µmole/kg to 41.6 µmole/kg for CRM6201-b thus verifying the amino acid concentration analysis method for SRM 2924.

Homogeneity Analysis: The concentration homogeneity assessment was made at the time the certification analyses were performed. A stratified sampling plan was devised to test for homogeneity across the lot of vials. Differences were statistically identified depending from which box the sample was taken and the day of analysis. However, the magnitude of the effects on concentration were small and have been included in the overall uncertainty of the mean.

Structural Heterogeneity: Heterogeneity was assessed using size exclusion chromatography monitoring fluorescent response (excitation – 295 nm / emission – 350 nm). The use of known molecular mass standards established the presence of a principle peak (>99.5 % abundance) at the expected retention time for the pentameric form of CRP indicating heterogeneity was negligible.

Reference Analyses: Density measurements were performed gravimetrically using the Lang-Levy pipet method [8]. Metrological traceability of the density value is to the SI units for grams per milliliter. Relative average molecular mass of the monomeric form of CRP was determined using liquid chromatography/mass spectrometry (LC-MS). Measurements were performed on a high-resolution, accurate mass ion trap mass analyzer operated in positive ion mode and coupled to LC with commercial C_{18} column. The relative average molecular mass was obtained by deconvolution of the intact protein multi-charge envelope. The reference CRP concentration value was calculated from the Certified CRP concentration using the relative average molecular mass values. Metrological traceability of the concentration value is to the SI derived units for mass concentration (expressed as grams per liter). The theoretical molecular mass of CRP was 23 027.8 based on the reported amino acid sequence and post-translational modifications (amino-terminus pyroglutamation and a single disulfide bond) [6] as calculated using the program NIST Mass and Fragment Calculator [9, 10].

Table 1. Additional Reference Values for Properties of SRM 2924

Property	Reference Value			Coverage Factor, *k*
Reference Density (21.7 °C):	1.0050 g/mL	±	0.0003 g/mL	2.57
Reference CRP Relative Average Molecular Mass: (dimensionless)	23 028.0	±	0.3	2.20
Reference CRP concentration:	0.474 g/L	±	0.028 g/L	2.15

REFERENCES

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[10] Program available for downloading at: https://www.nist.gov/services-resources/software/nist-mass-and-fragment-calculator-software (accessed July 2017).

Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; fax (301) 948-3730; e-mail srminfo@nist.gov*; or via the Internet at http://www.nist.gov/srm.*