National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 927f

Bovine Serum Albumin (7 % Solution)

(Total Protein Standard)

This Standard Reference Material (SRM) is intended primarily for use in the standardization of procedures employed in clinical analyses for total serum protein, for critical evaluation of daily working standards used in these procedures, and as a reference standard for assays of total protein by colorimetric methods. This SRM is a solution (mass fraction approximately 7 %) of known protein concentration and purity. The total protein content of this SRM was determined using the biuret reference method [1] that is recommended for use in standardizing laboratory-prepared protein solutions and "normal" serum pools. Such standardized "normal" sera could then be used to calibrate refractometers or other instruments for serum protein estimations. SRM 927f may also be used for other procedures, such as gel diffusion, amino acid analysis, electrophoresis, nitrogen assays, or other tests that require well-characterized protein for calibration or evaluation. A unit of SRM 927f consists of 10 ampoules, each containing approximately 2.2 mL of solution. Measurements of the bovine serum albumin (BSA) concentration were made using two independent methods including amino acid analysis and the biuret method. The results from the two approaches are reported as follows: 1) certified BSA concentration by amino acid analysis and 2) reference BSA concentration by the biuret method.

Certified Concentration Value: The certified concentration value for BSA as determined by amino acid analyses is provided in Table 1. 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 analyzed [2]. The certified value for BSA concentration is based upon the results from isotope dilution liquid chromatography/tandem mass spectrometry (ID-LC/MS/MS) [3].

Reference Values: The reference BSA concentration determined using the biuret method is provided in Table 2. The biuret reference method [1] was employed to determine the BSA concentration in SRM 927f using SRM 927e as an external standard. Reference values are non-certified values that are the best estimate of the true values based on available data; however, the values do not meet the NIST criteria for certification and are 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 [2]. Reference values are provided in Table 3 for additional properties including density and relative average molecular mass as determined using electrospray ionization mass spectrometry.

Expiration of Certification: The certification of **SRM 927f** is valid, within the measurement uncertainty specified, until **30 June 2026**, provided the SRM is handled and stored in accordance with instructions given in this certificate (see "Instructions for Storage and Use"). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

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.

Overall direction and coordination of technical measurements leading to the certification were performed by E.L. Kilpatrick of the NIST Biomolecular Measurement Division.

Statistical analysis was provided by N.F. Zhang of the NIST Statistical Engineering Division.

Michael J. Tarlov, Chief
Biomolecular Measurement Division

Gaithersburg, MD 20899
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Certificate Revision History on Last Page

Steven J. Choquette, Director
Office of Reference Materials

Acquisition of the material was performed by K.W. Phinney. Certification measurements were performed by M.S. Lowenthal (amino acid analysis, intact molar mass) and A.B. Green (biuret analysis, density) of the NIST Biomolecular Measurement Division.

Support aspects involved in the issuance of this SRM were coordinated through the NIST Office of Reference Materials.

NOTICE AND WARNINGS TO USERS

Warning: SRM 927f IS INTENDED FOR RESEARCH USE. The blood used in the preparation of SRM 927f Bovine Serum Albumin (7 % Solution) was collected from cattle sourced in the United States. Only blood from cattle/carcasses that have passed ante-mortem and post-mortem U.S. Department of Agriculture (USDA) Food Safety Inspection Service (FSIS) inspection was used. This material has not come from cattle that have been in a herd in which a case of Bovine Spongiform Encephalopathy (BSE) has appeared, and the product does not contain, and is not derived from, specified risk material as defined in Commission Decision 97/534/EC. No bovine blood was used from tuberculosis and/or brucellosis reactors. No bovine blood was used from animals subject to emergency slaughter or identified as U.S. Suspect. There were no additives to the pooled serum prior to protein purification.

INSTRUCTIONS FOR STORAGE AND USE

Storage: This SRM is supplied to the user in sealed ampoules. The SRM should be stored in a refrigerator at a temperature between 2 °C and 8 °C. The ampoules should not be frozen because of possible breakage of ampoules during the thawing process.

Instructions for Use: Once an ampoule is opened, the solution should be used promptly. Any unused solution in opened ampoules should be discarded.

Preparation of Dilutions: Protein solutions of lower concentration may be prepared by transferring the appropriate aliquot to a volumetric flask and diluting to volume. Diluents are not furnished with the SRM; an aqueous sodium chloride diluent, such as a solution having a concentration of 0.15 mol/L, may be used. Caution should be taken when performing dilutions greater than 100-fold due to the potential for protein loss (possibly due to protein absorption on laboratory equipment).

Inappropriate Uses: This SRM is not intended to be used as a standard for dye-binding tests as the dye-binding characteristics of BSA will likely be dissimilar to other proteins being assayed. Additionally, this SRM is not intended for checking pre-calibrated refractometers, for immunochemical methods, or as an additive for bilirubin standardization.

SOURCE, PREPARATION, AND ANALYSIS[(1)]

Source and Preparation: SRM 927f was prepared and packaged by Equitech-Bio, Inc. (Kerrville, TX). The source bovine serum was produced in the U.S. at establishments registered with the USDA and is intended only for manufacture into products for use in in vitro diagnostic, research, and further manufacturing or technical purposes. The BSA for this SRM was diluted from 30 % BSA commercial stock (Equitech-Bio, Inc., Kerrville, TX) to approximately 7 % using 0.02 mol/L sodium chloride, and the pH adjusted to 6.5 to 6.8 with sodium hydroxide. The material was sterilized by membrane filtration, placed into sterile glass argon-flushed ampoules and flame sealed.

Analysis: All analyses in the value assignment of SRM 927f were performed at NIST.

Measurement of BSA 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) [3]. Underivatized samples of SRM 927f and SRM 927e (as a control) were diluted and then combined with stable isotope-labeled analogs of phenylalanine, proline, isoleucine, leucine, and valine followed by hydrolysis with liquid-phase hydrochloric acid (HCl) for 48 h at approximately 130 ºC in a sealed pressure vessel. 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 reversed-phase analytical column with embedded acidic ion-pairing groups. Measurements were performed on a triple quadrupole mass spectrometer, monitoring specific transitions for each amino acid. The measurements were calibrated using solutions prepared from

[(1)]Certain commercial equipment, instruments, or materials are identified in this certificate to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

commercially available amino acid certified reference materials with known purity and uncertainty. Data were collected for phenylalanine, proline, isoleucine, leucine and valine. Based upon the known amino acid sequence for BSA, the mass fraction of BSA was calculated from the mass fractions determined for each of the amino acids using the appropriate molar masses and molar stoichiometries. BSA concentration was determined as the product of the BSA mass fraction multiplied by the density of SRM 927f (described below) with correction to a gram per liter basis.

Measurement of BSA Concentration (Biuret): The reference BSA concentration was measured using the biuret reference method for total serum protein [1]. The measurements involve a direct comparison between the current SRM 927f and previously issued SRM 927e and were performed by spectrophotometry.

Additional Analyses: Density measurement was performed gravimetrically [4]. Relative average molecular mass was determined using liquid chromatography/mass spectrometry (LC/MS). Measurements were performed on a time-of-flight mass spectrometer operated in the positive ion mode via LC using a commercial C18 column and adding 0.1 mL/L trifluoroacetic acid as an ion-pairing agent. Reversed phase gradient elution was performed by increasing the mobile phase acetonitrile content from 200 mL/L to 950 mL/L (balance water). The relative average molecular masses of the four major forms of BSA found in SRM 927f are shown in Table 3 in decreasing order of abundance. The previous issues of this material, SRM 927e and SRM 927d, had a similar range of molecular masses.

Homogeneity Analysis: The 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 ampoules. There was no apparent trend in the data when plotted against the sequence in which the ampoules were prepared.

Certified Value: The measurand is the total concentration of bovine serum albumin. Metrological traceability is to the International System of Units (SI) derived unit for mass concentration (expressed as gram per liter). The uncertainty provided with the measured BSA certified concentration value is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence, consistent with the ISO/JCGM Guide [5]. 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 for this analyte [5]. For the certified value shown below, $k = 2$.

Table 1. Certified Bovine Serum Albumin Concentration by Amino Acid Analysis

BSA Concentration: 83.2 g/L ± 0.7 g/L

Reference Value: The reference value in Table 2 is based specifically on the biuret reference method. The measurand is the bovine serum albumin concentration as determined using the biuret method. Metrological traceability is to the SI derived units for mass concentration (expressed as gram per liter). The uncertainty provided with the reference value in Table 2 is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence; it incorporates Type B uncertainty components related to the analyses, consistent with the ISO/JCGM Guide and with its Supplement 1 [5,6]. 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 for this analyte [5]. For the reference value shown in Table 2, $k = 2$.

Table 2. Reference Bovine Serum Albumin Concentration by the Biuret Method

BSA Concentration: 87.6 g/L ± 2.7 g/L

Additional Reference Values: The reference values are based on the method used for each measurand as described above. Metrological traceability of the density value is to the SI units for gram per milliliter. The uncertainty provided with each reference value in Table 3 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 for each analyte [6]. For the reference values shown in Table 3, $k = 2$.

Table 3. Additional Reference Values for Properties of SRM 927f

Density[a] (g/mL)
1.02181 ± 0.0005

Relative Average Molecular Mass of the Major Molecular Forms of BSA[b] (unitless)
66 432
66 453
66 550
66 597

[a] The uncertainty of the solution density was combined with the uncertainty of the amino acid analysis in determining the expanded uncertainty of the BSA concentration.
[b] Decreasing order of abundance.

Additional Information: The theoretical relative average molecular mass of BSA was calculated to be 66 398.1. The calculation of the average relative molecular mass of BSA is based on the reported amino acid sequence [7].

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        10         20    25   30         40         50         60
MKWVTFISLL LLFSSAYSRG VFRRDTHKSE IAHRFKDLGE EHFKGLVLIA FSQYLQQCPF
        70         80         90        100        110        120
DEHVKLVNEL TEFAKTCVAD ESHAGCEKSL HTLFGDELCK VASLRETYGD MADCCEKQEP
       130        140        150        160        170        180
ERNECFLSHK DDSPDLPKLK PDPNTLCDEF KADEKKFWGK YLYEIARRHP YFYAPELLYY
       190        200        210        220        230        240
ANKYNGVFQE CCQAEDKGAC LLPKIETMRE KVLASSARQR LRCASIQKFG ERALKAWSVA
       250        260        270        280        290        300
RLSQKFPKAE FVEVTKLVTD LTKVHKECCH GDLLECADDR ADLAKYICDN QDTISSKLKE
       310        320        330        340        350        360
CCDKPLLEKS HCIAEVEKDA IPENLPPLTA DFAEDKDVCK NYQEAKDAFL GSFLYEYSRR
       370        380        390        400        410        420
HPEYAVSVLL RLAKEYEATL EECCAKDDPH ACYSTVFDKL KHLVDEPQNL IKQNCDQFEK
       430        440        450        460        470        480
LGEYGFQNAL IVRYTRKVPQ VSTPTLVEVS RSLGKVGTRC CTKPESERMP CTEDYLSLIL
       490        500        510        520        530        540
NRLCVLHEKT PVSEKVTKCC TESLVNRRPC FSALTPDETY VPKAFDEKLF TFHADICTLP
       550        560        570        580        590        600
DTEKQIKKQT ALVELLKHKP KATEEQLKTV MENFVAFVDK CCAADDKEAC FAVEGPKLVV
    607
STQTALA
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The sequence of the mature protein is not expected to include the signal peptide which is removed as part of normal post-translational processing. Therefore, the sequence of circulating BSA includes only amino acids from position 25 to 607 as indicated by the non-underlined text above. BSA is also reported to contain 17 disulfide bonds [7] at the following cysteine residue pairs: 77-86, 99-115, 114-125, 147-192, 191-200, 223-269, 268-276, 288-302, 301-312, 339-384, 383-392, 415-461, 460-471, 484-500, 499-510, 537-582, and 581-590.

REFERENCES

[1] Doumas, B.T.; Bayse, D.D.; Carter, R.J.; Peters, T., Jr.; Schaffer, R.; *A Candidate Reference Method for Determination of Total Protein in Serum. I. Development and Validation*; Clin. Chem., Vol. 27, pp. 1642–1650 (1981).

[2] Beauchamp, C.R.; Camara, J.E.; Carney, J.; Choquette, S.J.; Cole, K.D.; DeRose, P.C.; Duewer, D.L.; Epstein, M.S.; Kline, M.C.; Lippa, K.A.; Lucon, E.; Molloy, J.; Nelson, M.A.; Phinney, K.W.; Polakoski, M.; Possolo, A.; Sander, L.C.; Schiel, J.E.; Sharpless, K.E.; Toman, B.; Winchester, M.R.; Windover, D.; *Metrological Tools for the Reference Materials and Reference Instruments of the NIST Material Measurement Laboratory*; NIST Special Publication (NIST SP) 260-136, 2021 edition; U.S. Government Printing Office: Washington, DC (2021); available at https://nvlpubs.nist.gov/nistpubs/SpecialPublications/NIST.SP.260-136-2021.pdf (accessed Oct 2021)

[3] Bunk D.M.; Lowenthal M.S; Isotope Dilution Liquid Chromatography-Tandem Mass Spectrometry for Quantitative Amino Acid Analysis; In *Amino Acid Analysis. Methods and Protocols;* part of the *Methods in Molecular Biology* book series, Vol 2030; Alterman, M.A., Ed.; Humana: New York, NY, pp. 143–151 (2019).

[4] Sniegoski, L.T.; Moody, J.R.; *Determination of Serum and Blood Densities*; Anal. Chem., Vol 51, pp. 1577–1578 (1979).

[5] JCGM 100:2008; *Evaluation of Measurement Data — Guide to the Expression of Uncertainty in Measurement* (GUM 1995 with Minor Corrections); Joint Committee for Guides in Metrology (JCGM) (2008); available at https://www.bipm.org/en/publications/guides (accessed Oct 2021); see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*; NIST Technical Note 1297; U.S. Government Printing Office: Washington, DC (1994); available at https://www.nist.gov/pml/nist-technical-note-1297 (accessed Oct 2021).

[6] JCGM 101:2008; *Evaluation of Measurement Data — Supplement 1 to the "Guide to the Expression of Uncertainty in Measurement" — Propagation of Distributions Using a Monte Carlo Method*; JCGM (2008); available at https://www.bipm.org/en/publications/guides (accessed Oct 2021).

[7] The UniProt Consortium; *UniProt Database* (2021); available at https://www.uniprot.org (accessed Oct 2021).

Certificate Revision History: 27 October 2021 (Amended uncertainty value for biuret protein concentration; editorial changes); 23 June 2021 (Original certificate date).

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; e-mail srminfo@nist.gov; or via the Internet at https://www.nist.gov/srm.