National Institute of Standards and Technology
U.S. Department of Commerce

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Standard Reference Material® 3949
Folate Vitamers in Frozen Human Serum
CERTIFICATE OF ANALYSIS

Purpose: This Standard Reference Material (SRM) is intended for use in validating methods for determining folate vitamers in human serum, and for qualifying in-house control materials analyzed using those methods.

Description: A unit of SRM 3949 consists of one vial each of three materials: low (Level 1), medium (Level 3), and high (Level 2) folates. Each vial contains approximately 1.0 mL of frozen human serum.

Certified Values: Certified values are provided in Table 1. A NIST certified value is a value for which NIST has the highest confidence in that all known or suspected sources of bias have been evaluated [1,2]. These values are traceable to International System of Units (SI).

Table 1. Certified Values for Folate Vitamers in SRM 3949

	Mass Fraction[(a)] (ng/g)	Mass Concentration[(b)] (ng/mL)	Amount Concentration[(c)] (nmol/L)
Level 1			
Folic acid	0.43 ± 0.13	0.44 ± 0.14	1.00 ± 0.32
5-methyltetrahydrofolate	6.60 ± 0.97	6.75 ± 1.00	14.69 ± 2.18
Level 2			
Folic acid	2.91 ± 0.67	2.98 ± 0.68	6.75 ± 1.54
5-methyltetrahydrofolate	20.52 ± 1.83	21.00 ± 1.87	45.71 ± 4.07
Level 3			
Folic acid	2.01 ± 0.44	2.06 ± 0.45	4.67 ± 1.01
5-methyltetrahydrofolate	13.13 ± 1.69	13.45 ± 1.73	29.27 ± 3.77

[(a)] Values are expressed as $x \pm U_{95\%}(x)$, where x is the certified value and $U_{95\%}(x)$ is the expanded uncertainty of the certified value. The true value of the analyte is believed to lie within the interval $x \pm U_{95\%}(x)$ with 95 % confidence. To propagate this uncertainty, treat the certified value as a normally distributed random variable with mean x and standard deviation $U_{95\%}(x)/2$.

[(b)] Mass concentration values were calculated from mass fractions using measured serum densities: Level 1, 1.02332 ± 0.00033 g/mL; Level 2, 1.02376 ± 0.00031 g/mL; Level 3, 1.02435 ± 0.00047 g/mL.

[(c)] Amount concentration values, nmol/L, are calculated from mass concentration results, nanogram per milliliter, via multiplication by 1000/M, where M is the molar mass, grams per mole, of the analyte. These molar masses are: $M_{\text{folic acid}}$ = 441.40 g/mol, $M_{\text{5-methyltetrahydrofolate}}$ = 459.46 g/mol.

Non-Certified Values: Non-certified values are provided in Appendix A. Additional information is provided in Appendix B.

Period of Validity: The certified values delivered by **SRM 3949** are valid within the measurement uncertainty specified until **31 August 2028**. The certified values are nullified if the material is stored or used improperly, damaged, contaminated, or otherwise modified.

Maintenance of Certified Values: NIST will monitor this SRM over the period of its validity. If substantive technical changes occur that affect the certification, NIST will issue an amended certificate through the NIST SRM website (https://www.nist.gov/srm) and notify registered users. SRM users can register online from a link available on the NIST SRM website or fill out the user registration form that is supplied with the SRM. Registration will facilitate notification. Before making use of any of the values delivered by this material, users should verify they have the most recent version of this documentation, available through the NIST SRM website (https://www.nist.gov/srm).

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Certificate Revision History on Page 3

Safety: SRM 3949 IS INTENDED FOR RESEARCH USE. THIS IS A HUMAN SOURCE MATERIAL. HANDLE PRODUCT AS A BIOHAZARDOUS MATERIAL CAPABLE OF TRANSMITTING INFECTIOUS DISEASE. The supplier of this serum has reported that each donor unit of serum or plasma used in the preparation of this product has been tested by a FDA-approved method and found non-reactive/negative for hepatitis B surface antigen (HbsAg), human immunodeficiency virus (HIV) 1 and 2 antibodies, and hepatitis C virus (HCV). However, no known test method can offer complete assurance that hepatitis B virus, HCV, HIV, or other infectious agents are absent from this material. Accordingly, this human blood-based product should be handled at the Biosafety Level 2 or higher as recommended for any POTENTIALLY INFECTIOUS HUMAN SERUM OR BLOOD SPECIMEN in the Centers for Disease Control/National Institutes of Health Manual [3].

Source: This SRM was developed after an appropriate human subjects research determination by NIST. Support for the development of SRM 3949 was provided in part by the National Institutes of Health (NIH) Office of Dietary Supplements (ODS).

Storage: Until required for use, SRM 3949 should be stored in the dark at a temperature below –60 °C.

Use: SRM 3949 is provided as a set of three vials of frozen serum that should be allowed to thaw at room temperature for at least 30 min under subdued light. The contents of a vial should then be gently mixed prior to removal of a test portion for analysis. Precautions should be taken to avoid exposure to strong ultraviolet (UV) light and direct sunlight. The certification only applies to the initial use, and the same results are not guaranteed if the remaining material is used at a later date.

Notice to Users: NIST strives to maintain the SRM inventory supply, but NIST cannot guarantee the continued or continuous supply of any specific SRM. Accordingly, NIST encourages the use of this SRM as a benchmark for the quality and accuracy of the user's in-house reference materials and working standards. As such, the SRM should be used to validate the more routinely used reference materials in a laboratory. Comparisons between the SRM and in-house reference materials or working measurement standards should take place at intervals appropriate to the conservation of the SRM and the stability of relevant in-house materials. For further guidance on how this approach can be implemented, see reference 4 or contact NIST by email at srms@nist.gov.

REFERENCES

[1] 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 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 July 2023).

[2] Thompson, A.; Taylor, B.N.; *Guide for the Use of the International System of Units (SI)*; NIST Special Publication 811; U.S. Government Printing Office: Washington, DC (2008); available at https://www.nist.gov/pml/pubs/sp811/index.cfm (accessed July 2023).

[3] CDC/NIH; *Biosafety in Microbiological and Biomedical Laboratories*, 6th ed.; HHS publication No. (CDC) 21-1112; Chosewood, L.C.; Wilson, D.E., Eds.; US Government Printing Office: Washington, D.C. (2020); available at https://www.cdc.gov/biosafety/publications/bmbl5/index.htm (accessed July 2023).

[4] Rukhin, A.L.; *Compatibility Verification of Certified Reference Materials And User Measurements*; Metrologia, Vol. 51, pp. 11–17 (2014).

[5] Fazili, Z.; Whitehead, Jr., R.; Paladugula, N.; Pfeiffer, C.M.; *A High-Throughput LC-MS/MS Method Suitable For Population Biomonitoring Measures Five Serum Folate Vitamers and One Oxidation Product*; Anal Bioanal Chem., Vol. 405, pp. 4549–4560 (2013).

[6] Fazili, Z.; Pfeiffer, C.M.; *Accounting For An Isobaric Interference Allows Correct Determination of Folate Vitamers in Serum by Isotope Dilution-liquid Chromatography-tandem Mass Spectrometry*; J. Nutr., Vol. 143, pp. 108–113 (2012).

[7] Fazili, Z.; Pfeiffer, C.M.; *Measurement of Folates in Serum and Conventionally Prepared Whole Blood Lysates: Application of an Automated 96-Well Plate Isotope-dilution Tandem Mass Spectrometry Method*; Clin. Chem., Vol. 50, pp. 2378–2381 (2004).

[8] Pfeiffer, C.M.; Fazili, Z.; McCoy, L.; Zhang, M.; Gunter, E.W.; *Determination of Folate Vitamers in Human Serum by Stable-Isotope-Dilution Tandem Mass Spectrometry and Comparison with Radioassay and Microbiologic Assay;* Clin. Chem., Vol. 50, pp.423–432 (2004).

Certificate Revision History: 11 July 2023 (Updated format; change of period of validity; editorial changes); **22 October 2018** (Original certificate date).

Certain commercial equipment, instruments, or materials may be identified in this Certificate of Analysis 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.

Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the Office of Reference Materials 100 Bureau Drive, Stop 2300, Gaithersburg, MD 20899-2300; telephone (301) 975-2200; e-mail srminfo@nist.gov; or the Internet at https://www.nist.gov/srm.

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APPENDIX A

Non-Certified Values: Non-certified values are suitable for use in method development, method harmonization, and process control but do not provide metrological traceability to the International System of Units (SI) or other higher-order reference system.

Table A1. Non-Certified Mass Fraction Values for Folate Vitamers in SRM 3949

	Mass Fraction[a] (ng/g)	Mass Concentration[b] (ng/mL)	Amount Concentration[c] (nmol/L)
Level 1			
Tetrahydrofolate	0.50 ± 0.08[d]	0.51 ± 0.08	1.14 ± 0.18
MeFox	0.73 ± 0.36[e]	0.75 ± 0.37	1.58 ± 0.78
Total folate			17.0 ± 0.4[f]
Level 2			
Tetrahydrofolate	0.67 ± 0.49[e]	0.68 ± 0.50	1.53 ± 1.12
MeFox	0.90 ± 0.18[e]	0.93 ± 0.19	1.96 ± 0.40
Total folate			56.0 ± 0.8[f]
Level 3			
Tetrahydrofolate	0.61 ± 0.43[e]	0.62 ± 0.44	1.39 ± 0.99
5-formyltetrahydrofolate	3.39 ± 1.86[e]	3.47 ± 1.90	7.33 ± 4.01
MeFox	1.02 ± 0.26[e]	1.05 ± 0.26	2.22 ± 0.55
Total folate			41.8 ± 0.5[f]

[a] Values are expressed as $x \pm U_{95\%}(x)$, where x is the estimated value and $U_{95\%}(x)$ is the expanded uncertainty of the value. As measured by the specific method(s), the true value of the analyte is believed to lie within the interval $x \pm U_{95\%}(x)$ with about a 95 % confidence. To propagate this uncertainty, treat the value as a normally distributed random variable with mean x and standard deviation $U_{95\%}(x)/2$.

[b] Mass concentration values were calculated from mass fractions using measured serum densities: Level 1, 1.02332 ± 0.00033 g/mL; Level 2, 1.02376 ± 0.00031 g/mL; Level 3, 1.02435 ± 0.00047 g/mL.

[c] Amount concentration values, nmol/L, are calculated from mass concentration results, nanogram per milliliter, via multiplication by 1000/M, where M is the molar mass, grams per mole, of the analyte. These molar masses are: $M_{\text{tetrahydrofolate}}$ = 445.43 g/mol, $M_{\text{5-formyltetrahydrofolate}}$ = 473.44 g/mol, M_{MeFox} = 473.44 g/mol.

[d] Value is based on results of NIST ID-LC-MS/MS Method 1.

[e] Values are based on the combination of results from NIST ID-LC-MS/MS Method 1 and results of ID-LC-MS/MS provided by the Centers for Disease Control and Prevention (CDC).

[f] Values are based on results of ID-LC-MS/MS provided by the CDC. Values are expressed as $x \pm U_{95\%}(x)$, where x is the estimated value and $U_{95\%}(x)$ is the expanded uncertainty of the value. As measured by the specific method, the true value of the analyte is believed to lie within the interval $x \pm U_{95\%}(x)$ with about a 95 % confidence. To propagate this uncertainty, treat the value as a normally distributed random variable with mean x and standard deviation $U_{95\%}(x)/2.16$.

Period of Validity: The non-certified values are valid within the measurement uncertainty specified until **31 August 2028**. The value assignments are nullified if the material is stored or used improperly, damaged, contaminated, or otherwise modified.

Maintenance of Non-Certified Values: NIST will monitor this material to the end of its period of validity. If substantive technical changes occur that affect the non-certified values during this period, NIST will update this Appendix and notify registered users. SRM users can register online from a link available on the NIST SRM website or fill out the user registration form that is supplied with the SRM. Registration will facilitate notification. Before making use of any of the values delivered by this material, users should verify they have the most recent version of this documentation, available through the NIST SRM website (https://www.nist.gov/srm).

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APPENDIX B

NIST Analytical Approach for Determination of Folates (NIST ID-LC-MS/MS Method 1): For simultaneous measurement of folic acid, 5-methyltetrahydrofolate, tetrahydrofolate, 5-formyltetrahydrofolate, and MeFox**,** an isotope-dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS) method was used to measure folate forms in SRM 3949 [5]. The method quantifies folic acid, 5-methyltetrahydrofolate, tetrahydrofolate, 5-formyltetrahydrofolate, 5,10-methenyltetrahydrofolate, and the pyrazino-s-triazine derivative of 4-α-hydroxy-5-methyltetrahydrofolate (MeFox). To prepare samples for analysis, serum (275 µL) was mixed with ammonium formate buffer and an internal standard mixture that contained $^{13}C_5$-labeled folate forms. Sample clean-up was performed using 100 mg phenyl sorbent solid phase extraction (SPE) cartridges. Samples were eluted from the SPE cartridges with an organic elution solvent containing both ascorbic acid and acetic acid and analyzed by LC-MS/MS in positive ion mode using electrospray ionization coupled to a LC system. Chromatographic separation was achieved using a C_8 analytical column with an isocratic mobile phase and a total run time of 10 min. Quantitation was performed by peak area ratio (analyte to internal standard) and based on a 6-point aqueous calibration curve where calibrants were carried through all sample preparation steps. For certified values, the purity of neat 5-methyltetrahydrofolate calibrant material was determined at NIST using LC-absorbance and the purity of neat folic acid calibrant material was determined at NIST using quantitative protein nuclear magnetic resonance spectroscopy (^{1}H-qNMR).

Second NIST Analytical Approach for Determination of Folic Acid (NIST ID-LC-MS/MS Method 2): As a second approach to the measurement of folic acid, a variation of ID-LC-MS/MS was used to measure only folic acid in SRM 3949. To prepare samples for analysis, serum (300 µL) was mixed with ammonium formate buffer, and an internal standard mixture that contained $^{13}C_5$-labeled folic acid. Sample clean-up was performed using 100 mg phenyl sorbent SPE cartridges. Samples were eluted from the SPE cartridges with an organic elution solvent containing acetic acid and analyzed by LC-MS/MS (different system than NIST ID-LC-MS/MS Method 1 approach for folates) in positive ion mode using electrospray ionization coupled to a LC system. Chromatographic separation was achieved using a C_{18} analytical column with a gradient mobile phase and a total run time of 20 min. Quantitation was performed by peak area ratio (analyte to internal standard) and based on a bracketed, matching aqueous calibration approach in which each calibrant was carried through all sample preparation steps. Purity of neat calibrant material was determined at NIST using ^{1}H-qNMR.

Analyses by the Centers for Disease Control and Prevention: An ID-LC-MS/MS method was used to measure folate forms in SRM 3949 [5–8]. The method quantifies folic acid, 5-methyltetrahydrofolate, tetrahydrofolate, 5-formyltetrahydrofolate, 5,10-methenyltetrahydrofolate, and the pyrazino-s-triazine derivative of 4-α-hydroxy-5-methyltetrahydrofolate (MeFox). To prepare samples for analysis, serum (150 µL) was mixed with ammonium formate buffer and an internal standard mixture that contained $^{13}C_5$-labeled folate forms. Sample clean-up was performed using a 50 mg phenyl SPE 96-well plate and an automated 96-probe SPE system. Samples were eluted from the SPE plate with an organic elution solvent containing ascorbic acid and acetic acid and analyzed by LC-MS/MS in positive ion mode using electrospray ionization coupled to a LC system. Chromatographic separation was achieved using a C_8 analytical column with an isocratic mobile phase and a total run time of 7 min. Quantitation was performed by peak area ratio (analyte to internal standard) and based on a 5-point aqueous calibration curve where calibrants were carried through all sample preparation steps.

Homogeneity Assessment: The homogeneity of all folates was assessed at NIST using the methods and test portions described above and found no significant variance.

Value Assignment: Value assignment of the concentration of folic acid in SRM 3949 Level 1 was based on the combination of results provided from NIST ID-LC-MS/MS Method 2 and from ID-LC-MS/MS at the CDC. Value assignment of folic acid in SRM 3949 Level 2 and Level 3 was based on the combination of results from NIST ID-LC-MS/MS Method 1, NIST ID-LC-MS/MS Method 2, and ID-LC-MS/MS at the CDC. Value assignment of the concentrations of 5-methyltetrahydrofolate, tetrahydrofolate (except for Level 1), 5-formyltetrahydrofolate, and MeFox are based on the results from NIST ID-LC-MS/MS Method 1 and ID-LC-MS/MS at the CDC. The tetrahydrofolate value assignment of Level 1 was based on the results of one analytical method at NIST ID-LC-MS/MS Level 1. Value assignment of total folate was based on results from ID-LC-MS/MS at the CDC.

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