National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 3949

Folate Vitamers in Frozen Human Serum

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. 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 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]. Metrological traceability is to the measurement units realized through purity determinations of the primary standards employed in the NIST methods.

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 g/mL; Level 2, 1.02376 g/mL; Level 3, 1.02435 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.

Expiration of Certification: The certification of **SRM 3949** is valid, within the measurement uncertainty specified, until **31 August 2023**, provided the SRM is handled and stored in accordance with the instructions given in this certificate. 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 the technical activities were performed by J. Camara of the NIST Chemical Sciences Division.

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Certificate Issue Date: 22 October 2018

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

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

NOTICE AND WARNINGS TO USERS

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].

INSTRUCTIONS FOR HANDLING, STORAGE, AND USE

Storage and Handling: 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. 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.

SOURCE, PREPARATION, AND ANALYSIS[(1)]

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).

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).

[(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.

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.

Non-Certified Values: Table 2 lists values that do not meet the NIST criteria for certification. Non-certified values were formerly known as Reference Values. Values are metrologically traceable to the measurement procedures as indicated in the footnotes.

Table 2. Estimated 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 g/mL; Level 2, 1.02376 g/mL; Level 3, 1.02435 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 $U_{95\%}(x)/2.16$.

REFERENCES

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

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[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).

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 https://www.nist.gov/srm.