



National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material[®] 1955

Homocysteine and Folate in Frozen Human Serum

This Standard Reference Material (SRM) is intended primarily for use in evaluating the accuracy of clinical procedures for the determination of homocysteine and folate (in various forms) in human serum. It is also intended for use in validating working or secondary reference materials. A unit of SRM 1955 consists of three bottles of frozen human serum, each of a different analyte concentration level. Each bottle contains 1 mL of human serum.

Certified Concentration Values: The certified concentrations and their expanded uncertainties for total homocysteine (tHCY) and 5-methyltetrahydrofolic acid (5MT) are listed in Table 1. The certified concentrations of tHCY were determined at NIST using a combination of higher-order reference measurement procedures based upon isotope dilution with gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS), and liquid chromatography/tandem mass spectrometry (LC/MS/MS) [1–3]. The certified concentrations for 5MT were determined by a combination of reference measurement procedures based upon isotope dilution LC/MS/MS performed at NIST [3,4] and the Centers for Disease Control and Prevention (CDC) [5]. The methods performed at NIST for tHCY and 5MT are recognized as approved higher order reference measurement procedures by the Joint Committee on Traceability in Laboratory Medicine (JCTLM) [6]. The certified concentrations apply only to serum thawed to room temperature, 20 °C to 25 °C (see “Instructions for Storage, Stability, and Use”).

Reference Concentration Values: The reference concentrations for folic acid (FA) and their expanded uncertainties are listed in Table 2. The reference concentrations were determined using LC/MS/MS-based methods at NIST and CDC. Because agreement among the methods for this low-level analyte does not meet NIST criteria for a certified value, the results are listed as reference values [7].

Information Values: CDC provided additional results for total folate, 5-formyltetrahydrofolic acid (5FT), tHCY, and vitamin B₁₂ (cobalamin). The values are listed as method-specific results in Table 3. For those analytes for which multiple methods were used, individual results are provided because the different methods may not be measuring the same entities. Information values cannot be used to establish metrological traceability.

Expiration of Certification: The certification of **SRM 1955** is valid, within the measurement uncertainty specified, until **31 December 2018**, provided the SRM is handled and stored in accordance with the instructions given in the certificate (see “Instructions for Storage, Stability, 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 the analyses at NIST were performed by M.J. Welch of the NIST Chemical Sciences Division.

Analytical measurements at NIST were performed by B.C. Nelson, M.B. Satterfield, and L.T. Sniegoski of the NIST Chemical Sciences Division. Measurements at CDC were performed by M. Zhang, Z. Fazili, S. Strider, and L. Jia under the direction of C. Pfeiffer.

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Certificate Revision History on Last Page

Statistical analysis was provided by A. Hornikova and N.F. Zhang 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 WARNING TO USERS

SRM 1955 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 was tested by an FDA-approved method and was found to be nonreactive for HbsAG, HCV, and HIV-1 antibodies. However, no known test method can offer complete assurance that hepatitis B virus, hepatitis C virus, 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 CDC/National Institutes of Health (NIH) Manual [8].

INSTRUCTIONS FOR STORAGE, STABILITY, AND USE

Storage: The serum is shipped frozen (on dry ice), and upon receipt, should be stored frozen until needed for use. A freezer temperature of $-20\text{ }^{\circ}\text{C}$ is acceptable for storage up to one week. If a longer storage time is anticipated, the material should be stored at or below $-50\text{ }^{\circ}\text{C}$. The SRM should not be exposed to sunlight or ultraviolet radiation. Storage of thawed material at room or refrigerator temperatures may result in changes in the analyte concentrations.

Stability: The material is kept at $-80\text{ }^{\circ}\text{C}$ for long-term storage at NIST. NIST will continue to monitor the stability of the analytes in this material and will notify purchasers of the material of any changes in the certified concentrations.

Use: Bottles of the SRM to be analyzed should be removed from the freezer and allowed to stand at room temperature until thawed. After the material is thawed to room temperature, it should be used **immediately**. The material should be swirled gently to mix it before aliquots are withdrawn.

SOURCE, PREPARATION, AND ANALYSIS⁽¹⁾

Source of Material: SRM 1955 Homocysteine and Folate in Frozen Human Serum was prepared by Aalto Scientific Ltd. (Carlsbad, CA).

Preparation of Material: The material was prepared from a human serum master pool. The analyte concentrations in the level 1 and level 2 materials were unfortified. The analyte concentrations in the level 3 material were fortified. The level 2 material is equivalent to the human serum master pool. The level 1 material was prepared by diluting the level 2 material with phosphate-buffered saline solution, pH 7.04. The level 3 material was prepared by adding appropriate quantities of homocysteine and 5-methyltetrahydrofolic acid to the level 2 material.

ANALYTICAL METHODS

Homocysteine Measurements at NIST: Homocysteine was measured using three isotope dilution mass spectrometry approaches [1–3,9]. For the LC/MS and most of the LC/MS/MS measurements, three independent sets were analyzed. A set consisted of two test portions per bottle from two bottles of each of the three levels. Each test portion was spiked with a known amount of the internal standard, homocysteine- d_4 . After reduction of the sample by addition of dithiothreitol (DTT), sample clean-up was performed using anion exchange solid-phase extraction. The solvent from the extraction was evaporated to $<50\text{ }\mu\text{L}$ and the residue was dissolved in 1.5 % (mass concentration) DTT in water. A commercial LC/MS/MS instrument with an electrospray ionization source and a triple quadrupole mass analyzer was used for the analysis. The LC separations were carried out using a commercial pentafluorophenyl column (25 cm \times 4.6 mm, 5 μm particle diameter) with an isocratic mobile phase at a flow rate of 0.5 mL/min. Electrospray ionization in the positive mode was used with selected ion monitoring to measure the $[\text{M} + \text{H}]^+$ ions at m/z 136 and m/z 140 for homocysteine and homocysteine- d_4 , respectively. Selected reaction monitoring of two transitions were carried out at m/z 136 \rightarrow m/z 90 and m/z 136 \rightarrow m/z 118 for homocysteine and m/z 140 \rightarrow m/z 94 and m/z 140 \rightarrow m/z 122 for homocysteine- d_4 on the same samples. Analyte concentrations were calculated by linear interpolation from calibration curves constructed independently for each set of samples. Three additional sets, each

⁽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.

consisting of two aliquots from one vial of each level, were prepared for LC/MS/MS measurements following NIST 5MT Method 3 [3] described below with measurements using the MS/MS conditions described above.

For measurements using GC/MS, three independent sets were analyzed. A set consisted of two test portions per bottle from two bottles of each of the three levels. Each test portion was spiked with a known amount of the internal standard, homocystine-*d*₈. DTT in sodium hydroxide solution was used to break disulfide bonds and release homocystine and homocystine-*d*₈, which were then isolated by absorption on anion exchange resin, followed by elution, concentration, derivatization with N-methyl-N-(tert-butyltrimethylsilyl)-trifluoroacetamide (MTBSTFA), and GC/MS with selective ion monitoring. The GC/MS measurements were performed using a mass selective detector with two different 30-meter capillary columns (one with a 5 % (mole fraction) phenylmethylpolysiloxane stationary phase, the other with 50 % phenylmethylpolysiloxane). Two different pairs of ions (*m/z* 420/424 and *m/z* 318/322) were monitored.

Homocysteine Measurements at CDC: CDC used two methods to measure homocysteine. These data were not used to calculate the certified values, but are presented to demonstrate the commutability of this SRM for routine methods for the determination of homocysteine. For the first method [10], total homocysteine (tHCY) was measured by isocratic liquid chromatography with fluorometric detection (LC-FD) at 385 nm excitation and 515 nm emission after reduction of protein-bound and oxidized thiols (disulfides and mixed disulfides) to free thiol with tris(2-carboxyethyl)phosphine (TCEP), protein precipitation with trichloroacetic acid, and fluorescent derivatization with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F). The separation of the obtained thiol-derivatives was performed isocratically within 7 min, with the use of an acetate mobile phase at pH 5.5. Cystamine was used as an internal standard. Quantitation was by peak area ratio (analyte to internal standard) and is based on a three-point standard curve in a serum matrix. For the second method [11], tHCY was measured by using an automated fluorescence polarization immunoassay (FPIA) from Abbott Diagnostics. In brief, DTT reduces homocysteine bound to albumin and to other small molecules, homocysteine, and mixed disulfides, to free thiol. S-adenosyl-homocysteine (SAH) hydrolase catalyzes conversion of homocysteine to SAH in the presence of added adenosine. In the subsequent steps, a specific monoclonal antibody and a fluoresceinated SAH analog tracer constitute the FPIA detection system. Serum tHCY concentrations were calculated by the Abbott AxSym using a machine-stored calibration curve.

Folate Measurements at NIST: For measurements of 5MT at NIST, three variations of LC/MS/MS-based methodology were used [3,4,9].

Method 1: For each sample, a 500 μ L aliquot of SRM 1955 was spiked with [¹³C₅]-5MT stock standard, immediately diluted with an equal volume of an aqueous solution containing (mass concentrations) 10 % ascorbic acid and 1 % ethylenediaminetetracetic acid (EDTA) and allowed to equilibrate on ice for \geq 15 min. 5MT was extracted from samples using C₁₈ SPE cartridges: methanol, water, and 1 % ascorbic acid/1 % EDTA solution were used for conditioning, 95+5 water+methanol solution was used for washing and 1 % ascorbic acid/1 % formic acid in 50+50 water+methanol was used for analyte elution. A commercial LC/MS/MS instrument with an electrospray ionization source and a triple quadrupole mass analyzer was used for extract analysis. LC separations were carried out using a gradient of formic acid in water and methanol on a phenylpropyl LC column (3.9 mm \times 150 mm, 4 μ m particle diameter). LC/MS/MS detection and quantification of 5MT and [¹³C₅]-5MT were conducted in multiple reaction monitoring (MRM) mode. MRM transitions for each analyte were individually optimized for protonated analyte molecules [M + H]⁺ and stable, protonated fragments. The relevant MRM mass transitions were *m/z* 460 \rightarrow *m/z* 313 for 5MT and *m/z* 465 \rightarrow *m/z* 313 for [¹³C₅]-5MT. Analyte concentrations were calculated by linear interpolation from calibration curves constructed independently for each set of samples.

Method 2: Test portions were spiked and equilibrated as with Method 1. Proteins were precipitated with metaphosphoric acid and the remaining supernatant was neutralized by the addition of a solution containing 0.4 mol/L K₂HPO₄ and 0.5 mol/L NaOH. 5MT was extracted from samples using solid-phase affinity extraction (SPAEC) columns: a 1 % ascorbic acid (mass concentration) and 0.1 mol/L glycine-HCl solution (pH 3.0), water, and 50 mmol/L potassium phosphate buffer (pH 7.4) were used for conditioning, water was used for washing, and the ascorbic acid/glycine-HCl solution was used for analyte elution. LC/MS/MS was performed as in Method 1.

Method 3: For the analysis of 5MT and FA, sample preparation and instrumentation were as described for Method 1. LC separations were carried out using a gradient of formic acid in water and methanol on a C₁₈LC column (4.6 mm × 150 mm, 5 μm particle diameter). LC/MS/MS detection and quantification of 5MT/[¹³C₅]-5MT and FA/[¹³C₅]-FA was conducted in multiple reaction monitoring (MRM) mode. MRM transitions for each analyte were individually optimized for protonated analyte molecules [M + H]⁺ and stable, protonated fragments. MRM mass transitions: *m/z* 460 → *m/z* 313 for 5MT, *m/z* 465 → *m/z* 313 for [¹³C₅]-5MT, *m/z* 442 → *m/z* 295 for FA and *m/z* 445 → *m/z* 295 for [¹³C₅]-FA. Analyte concentrations were calculated by linear interpolation from calibration curves constructed independently for each set of samples.

Folate and Vitamin B₁₂ Measurements at CDC: For the measurements of folates (5MT, FA, and 5FT) at CDC using LC/MS/MS [5], the target analytes were quantitatively isolated from 275 μL of the SRM 1955 serum using a phenyl solid-phase extraction cartridge, then detected and quantified in stabilized serum extracts by positive-ion electrospray ionization LC/MS/MS using an isocratic mobile phase of acetic acid in organic solvent on a C₈ analytical column. ¹³C-labeled folates were used as internal standards. For the CDC microbiological assay for total folates [12], using a 96-well plate microtiter method, diluted SRM test portions were added to an assay medium containing all of the nutrients except folic acid necessary for growth of *Lactobacillus casei* (*L. casei*, NCIB 10463). The assay medium was then inoculated with *L. casei*, and the microtiter plate was incubated for 42 h at 37 °C. Because the growth of *L. casei* is proportional to the amount of total folate present in the serum sample, the folate concentration was quantified by measuring the turbidity of the inoculated assay medium at 590 nm in a micro plate reader. For the CDC radioassay for total folates and vitamin B₁₂, a radio-protein binding assay was used. Test portions of the SRM were combined with the tracers ¹²⁵I-folate and ⁵⁷Co-vitamin B₁₂. The mixture was boiled to inactivate endogenous folate-binding proteins and to convert the various forms of vitamin B₁₂ to cyanocobalamin. After cooling, the mixture was combined with immobilized affinity-purified porcine intrinsic factor and folate-binding proteins, incubated for 1 h at room temperature, and finally centrifuged and decanted. The endogenous and labeled folate and B₁₂ compete for the limited number of binding sites on the basis of their relative concentrations and were concentrated in the bottom of the tube in the form of a pellet. The unbound folate and vitamin B₁₂ in the supernatant were discarded. The radioactivity associated with the pellet was counted. Standard curves were prepared by using the pre-calibrated folate/vitamin B₁₂ standards in a human serum albumin base. The concentration of the folate and vitamin B₁₂ in the SRM was calculated from the standard curve.

Table 1. Certified Concentration Values^(a) for Total Homocysteine and 5-Methyltetrahydrofolic Acid in SRM 1955

	Homocysteine ^(b)	
	(μmol/L)	(μg/mL)
Level I	3.98 ± 0.18	0.538 ± 0.024
Level II	8.85 ± 0.60	1.196 ± 0.082
Level III	17.7 ± 1.1	2.39 ± 0.15
	5-Methyltetrahydrofolic Acid ^(c)	
	(nmol/L)	(ng/mL)
Level I	4.26 ± 0.25	1.96 ± 0.12
Level II	9.73 ± 0.24	4.47 ± 0.11
Level III	37.1 ± 1.4	17.03 ± 0.64

^(a) Each certified value is the weighted mean from multiple methods. A Bayesian approach [13] was used to combine the data from the multiple methods to determine the combined standard uncertainties, *u_c*, according to the ISO/JCGM Guide [14]. The expanded uncertainty, *U*, in the value is calculated as *U* = *ku_c* with coverage factor, *k* = 2.

^(b) The measurand is the total concentration of homocysteine reported in Table 1. Metrological traceability is to the SI derived unit for amount-of-substance concentration (expressed as micromoles per liters) and mass concentration (expressed as micrograms per milliliter).

^(c) The measurand is the total 5-methyltetrahydrofolic acid concentration at each level reported in Table 1. Metrological traceability is to the SI derived unit for amount-of-substance concentration (expressed as nanomoles per liters) and mass concentration (expressed as nanograms per milliliter).

Table 2. Reference Concentration Values^(a) for Folic Acid in SRM 1955

	(nmol/L)	(ng/mL)
Level I	0.49 ± 0.17	0.215 ± 0.075
Level II	1.05 ± 0.16	0.463 ± 0.071
Level III	1.07 ± 0.24	0.47 ± 0.11

^(a) Each reference value is the weighted mean from multiple methods. A Bayesian approach [13] was used to combine the data from the multiple methods to determine the combined standard uncertainties, u_c , according to the ISO/JCGM Guide [14]. The expanded uncertainty, U , in the value is calculated as $U = ku_c$ with coverage factor, $k = 2$. The measurand is the total folic acid concentration at each level reported in Table 2 based on the methods indicated. Metrological traceability is to the SI derived unit for amount-of-substance concentration (expressed as nanomoles per liter) and mass concentration (expressed as nanograms per milliliter).

Table 3. Method-Specific Information Concentration Values^(a) for Total Folate, 5-Formyltetrahydrofolic Acid, Total Homocysteine^(b), and Vitamin B₁₂ in SRM 1955

Level I			
Analyte	Method	Concentration ^(a)	Units
Total Folate	LC/MS/MS	6.0 ± 0.4	nmol/L
Total Folate	Microbiological	5.6 ± 1.2	nmol/L
Total Folate	Radioassay	4.5 ± 0.4	nmol/L
5-FT	LC/MS/MS	1.3 ± 0.4	nmol/L
Homocysteine	FPIA	4.2 ± 0.3	µmol/L
Homocysteine	LC-FD	3.5 ± 0.3	µmol/L
Vitamin B ₁₂	Radioassay	0.16 ± 0.01	nmol/L
Level II			
Analyte	Method	Concentration	Units
Total Folate	LC/MS/MS	13 ± 1	nmol/L
Total Folate	Microbiological	14 ± 3	nmol/L
Total Folate	Radioassay	10 ± 1	nmol/L
5-FT	LC/MS/MS	2.3 ± 0.8	nmol/L
Homocysteine	FPIA	8.6 ± 0.4	µmol/L
Homocysteine	LC-FD	8.2 ± 0.6	µmol/L
Vitamin B ₁₂	Radioassay	0.36 ± 0.05	nmol/L
Level III			
Analyte	Method	Concentration	Units
Total Folate	LC/MS/MS	41 ± 2	nmol/L
Total Folate	Microbiological	44 ± 11	nmol/L
Total Folate	Radioassay	25 ± 3	nmol/L
5-FT	LC/MS/MS	3.6 ± 1.3	nmol/L
Homocysteine	FPIA	17 ± 1	µmol/L
Homocysteine	LC-FD	17 ± 1	µmol/L
Vitamin B ₁₂	Radioassay	0.35 ± 0.05	nmol/L

^(a) Sufficient information was not available for a complete evaluation of uncertainty for the individual method results. The uncertainties are ± 2 times the standard deviation of the mean of the measurements.

^(b) See Table 1 for the certified values for homocysteine. Data used to generate the information values for homocysteine provided in this table were not included in the certified values.

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Certificate Revision History: **31 December 2015** (Editorial changes); **26 July 2013** (Extension of certification period; editorial changes); **11 November 2010** (Update of Table 3 Information Values; extension of certification period; editorial changes); **30 April 2008** (Editorial changes and update of the expiration date); **06 October 2005** (Original certification 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; fax (301) 948-3730; e-mail srminfo@nist.gov; or via the Internet at <http://www.nist.gov/srm>.