



# National Institute of Standards & Technology

## Certificate of Analysis

### Standard Reference Material<sup>®</sup> 1949

#### Frozen Human Prenatal Serum

This Standard Reference Material (SRM) is intended primarily for use in validating analytical methods for the determination of specified constituents in maternal human serum. This SRM can also be used for quality assurance when assigning values to in-house control materials. A unit of SRM 1949 consists of two vials each of four levels, consisting of a base level (non-pregnant), and three pregnancy trimester levels (trimester 1, trimester 2 and trimester 3). Each vial contains approximately 1.8 mL of frozen human serum.

The development of SRM 1949 was a collaboration between the National Institute of Standards and Technology (NIST), the Centers for Disease Control and Prevention (CDC), National Centers for Environmental Health (Atlanta, GA), and the National Institutes of Health, Office of Dietary Supplements (NIH-ODS) (Bethesda, MD).

**Certified Concentration Values:** Certified concentration values for the iodine hormones total thyroxine, and total triiodothyronine, are 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 taken into account [1]. The certified concentrations were determined using higher-order reference measurement procedures [2] calibrated with high-purity chemical reference standards. The uncertainties are expanded uncertainties at the 95 % level of confidence [3].

**Reference Concentration Values:** Reference concentration values for copper, selenium, zinc, vitamin D binding protein (VDBP), 25(OH)D<sub>3</sub>, 25(OH)D<sub>2</sub> and 3-epi-25(OH)D<sub>3</sub> are provided in Table 2. The reference values are based on results obtained from measurements at NIST and from the collaborating external laboratory. Reference values are non-certified values that are the best estimate of the true value; however, the values do not meet NIST criteria for certification and are provided with associated uncertainties that may not include all sources of uncertainty [2].

**Information Values:** Information values for thyroglobulin, thyroid stimulating hormone (TSH), total reverse triiodothyronine, and serum density are provided in Table 3. An information value is considered to be a value that will be of use to the SRM user, but insufficient information is available to assess the uncertainty associated with the value or only a limited number of analyses were performed [2].

**Expiration of Certification:** The certification of **SRM 1949** is valid, within the measurement uncertainty specified, until **01 April 2027**, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see "Instructions for Handling, Storage, and Use"). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

Coordination of the technical measurements leading to the certification of this SRM was performed by S.E. Long and A.S.P. Boggs-Russell of the NIST Chemical Sciences Division and B.L. Catron-Kassim, formerly of NIST. Coordination of the collaborative production and measurements of the SRM at the CDC was performed by D.S. Tevis of the Inorganic and Radiation Analytical Toxicology Branch, Division of Laboratory Sciences, National Center for Environmental Health.

**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.

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Certificate Issue Date: 18 June 2019

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Analytical measurements were performed by A.S.P. Boggs-Russell, C.Q. Burdette, B.L. Catron, S.E. Long, and M.A. Nelson of the NIST Chemical Sciences Division, and L.E. Kilpatrick of the NIST Biomolecular Measurement Division. Analytical measurements at the CDC were performed by K. Darbonne, Z. Fultz, J.M. Jarrett, and K.L. Wallon.

Technical support for the development of this SRM was provided by the CDC, National Centers for Environmental Health, Division of Laboratory Sciences, under the direction of R.L. Jones and K.L. Caldwell.

Consultation on the statistical design of the experimental work and evaluation of the data were provided by J.H. Yen of the NIST Statistical Engineering Division.

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

## NOTICE AND WARNING TO USERS

SRM 1949 IS INTENDED FOR RESEARCH USE ONLY. THIS IS A HUMAN SOURCE MATERIAL. HANDLE PRODUCT AS A BIOHAZARDOUS MATERIAL CAPABLE OF TRANSMITTING INFECTIOUS DISEASE. The supplier has reported that each donor unit of plasma used in the preparation of this product was tested using FDA-licensed tests and found to be negative for human immunodeficiency virus (HIV), HIV-1 antigen, hepatitis B surface antigen, and hepatitis C. 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 Centers for Disease Control and Prevention/National Institutes of Health (NIH) Manual [4].

## INSTRUCTIONS FOR HANDLING, STORAGE, AND USE

**Storage:** The serum is shipped frozen (on dry ice) and, upon receipt, should be stored frozen until ready for use. A freezer temperature of  $-20\text{ }^{\circ}\text{C}$  is acceptable for storage for 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 hormone concentrations. The material is kept at  $-80\text{ }^{\circ}\text{C}$  for long-term storage at NIST. Under these conditions, the hormones are expected to be stable.

**Handling and Use:** SRM 1949 is provided as frozen serum that should be allowed to thaw at room temperature for at least 30 min under subdued light. After the material is equilibrated to room temperature, it should be used immediately. The contents of the vial should then be gently mixed prior to removal of a test portion for analysis. Precautions should be taken to avoid exposure to sunlight or ultraviolet radiation during handling and use.

## SOURCE, PREPARATION, AND ANALYSIS<sup>(1)</sup>

**Source and Preparation:** SRM 1949 was developed after an appropriate human subjects research determination by NIST. This SRM was prepared from maternal serum pools collected by Equitech-Bio Inc. (Kerrville, TX). The serum comprised four types: serum from donors in each of the three trimesters, and non-pregnant donors of reproductive age. Trimester collections were defined as follows: first trimester, 6 to 10 weeks pregnant, second trimester, 18 to 21 weeks pregnant, and third trimester, 32 to 35 weeks pregnant. The serum was shipped frozen on dry-ice to the CDC for processing where the pools were prepared in a Class 100 clean room. Materials expected to contact the serum, including containers, dispensers, and cryovials were pre-screened to ensure suitability for use in trace metals analysis. Serum (16 units,  $\sim 500\text{ mL}$  each, 4 units/pool) was thawed and homogenized on nutating mixers at  $4\text{ }^{\circ}\text{C}$ . The cold serum units were filtered through sterile gauze pads to remove suspended material and combined into the appropriate pools. The pools were then allowed to equilibrate to room temperature. Serum was then dispensed into cryovials using an automated dispenser system, with continuous homogenization of the serum using magnetic stirring plates. The cryovials were immediately capped and stored at approximately  $-70\text{ }^{\circ}\text{C}$ .

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<sup>(1)</sup> Certain commercial instruments, materials, or processes are identified in this report 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 instruments, materials, or processes identified are necessarily the best available for the purpose.

**Analytical Approach for Determination of Thyroxine, Triiodothyronine and Reverse Triiodothyronine (NIST):**

Total thyroxine ( $T_4$ ), total triiodothyronine ( $T_3$ ), and total reverse triiodothyronine ( $rT_3$ ) were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) using an analytical approach based on the NIST LC-MS/MS reference methods [5,6]. Samples of SRM 1949 were removed from storage at  $-80\text{ }^\circ\text{C}$  and equilibrated to room temperature for 2 h while protected from light. Sample aliquots of 0.5 g were gravimetrically spiked with a mixture of labeled internal standards ( $T_4$ - $^{13}\text{C}_6$ ,  $T_3$ - $^{13}\text{C}_6$ , and  $rT_3$ - $^{13}\text{C}_6$ ), diluted with 0.4 mL of deionized water, and allowed to equilibrate at room temperature for 1 h while protected from light. After equilibration, 70  $\mu\text{L}$  of 25 % ammonium hydroxide (volume fraction) was added to the sample. The alkaline sample was then extracted using solid phase extraction (SPE) Oasis MAX cartridges (Waters, Milford, MA), which had been pre-conditioned with 7 mL of methanol followed by 7 mL deionized water. The sample was loaded, the effluent collected, and reloaded to maximize analyte binding. The cartridge was then washed with 3 mL of 2 % ammonium hydroxide (volume fraction) in deionized water and 3 mL of methanol. The measurands were then eluted from the cartridge using 7 mL of methanol containing 0.05 % hydrochloric acid (volume fraction). The extract was evaporated to dryness under nitrogen at  $40\text{ }^\circ\text{C}$ , reconstituted in 100  $\mu\text{L}$  of methanol, water, and formic acid mixture (50:50:0.025, volume fraction) and transferred to a glass insert in an amber autosampler vial for injection onto the LC-MS/MS. An AB Sciex API 4000 LC-MS/MS system coupled to an Agilent 1100 Series LC was utilized for the analyses. Separation was conducted using an Agilent Eclipse C18 Plus column (2.1 mm x 150 mm, 5  $\mu\text{m}$  particle size). An isocratic mobile phase of methanol and deionized water (60:40 volume fraction) with 0.5 % formic acid was used at a flow rate of 200  $\mu\text{L}/\text{min}$  for 10 min. Electrospray ionization in positive mode was used for multiple reaction monitoring (MRM) of quantitative and qualitative transitions for each analyte. Samples were quantified using independent calibration curves for each analyte to remove the possibility of cross contamination. Calibrants were assayed for chemical mass purity using a quantitative  $^1\text{H}$  nuclear magnetic resonance spectroscopy ( $^1\text{H}$ -qNMR) procedure.

**Analytical Approach for Determination of Vitamin D Binding Protein by LC-MS (NIST):** Serum samples were processed using a tryptic digestion. A protein-denaturing mixture was prepared by adding 100  $\mu\text{L}$  trifluoroethanol (TFE), 44  $\mu\text{L}$  of 50 mmol/L tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (dissolved in 50 mmol/L Tris and adjusted to pH 8.0 using 5 mol/L NaOH), and 80  $\mu\text{L}$  of 50 mmol/L Tris buffer. Samples were prepared with 11  $\mu\text{L}$  of SRM 1949 serum in low binding tubes (Eppendorf, Hauppauge, NY) and 11  $\mu\text{L}$  of the denaturing solution. Iodoacetamide (2  $\mu\text{L}$  of 300 mmol/L) was added to each sample (25 mmol/L final concentration). Samples were then vortexed and incubated in the dark for 45 min. Samples were diluted with the addition of Tris buffer such that the concentration of TFE was  $<5\%$  by volume. Trypsin was prepared in 10 mmol/L acetic acid (17.5 units/ $\mu\text{L}$ ) and 20  $\mu\text{L}$  (350 units) and then added to each sample vial. Samples were digested at  $37\text{ }^\circ\text{C}$  for 19 h. The digestions were stopped by adding trifluoroacetic acid at a concentration of 0.5 % by volume. A 30  $\mu\text{L}$  aliquot was removed from each digested sample, and an appropriate amount of the labeled peptide mixture was added. Peptides TSALSAK and VLEPTLK were used for total VDBP quantification, by multiple reaction monitoring (MRM). The two transitions with the greatest intensity for each peptide were selected. LC MRM analyses of SRM 1949 digests were performed using a Discover BIO Wide C18 column (2.1 mm x 150 mm, 3  $\mu\text{m}$ , Sigma Aldrich, St. Louis, MO) and guard column of the same packing material (2.1 mm x 2 cm, 3  $\mu\text{m}$ ). The column was heated to  $40\text{ }^\circ\text{C}$  and coupled to an Agilent 6490 triple quadrupole. Calibrants (5  $\mu\text{L}$ ), and digested samples (3  $\mu\text{L}$ , approximately 1 pmol of each) were injected in triplicate. Peptides were eluted at 250  $\mu\text{L}/\text{min}$  with the following gradient: 2 % to 30 % B over 40 min., 30 % to 90 % B over 5 min., 90 % B to 2 % B over 1 min., and 2 % B for the last 20 min. Solvent A was 0.1 % formic acid in water (by volume) and solvent B was 0.1 % formic acid in acetonitrile (by volume).

**Analytical Approach for Determination of 25(OH) $D_3$ , 25(OH) $D_2$ , and 3-epi-25(OH) $D_3$  by LC-MS/MS (NIST):**

Serum (0.75 g) was spiked with an appropriate internal standard solution [25(OH) $D_3$ - $^{13}\text{C}_5$ , 25(OH) $D_2$ - $^{13}\text{C}_3$ , or 3-epi-25(OH) $D_3$ -d3]. After equilibration at room temperature for 1 h, the pH of each sample was adjusted to  $\text{pH } 9.8 \pm 0.2$  with carbonate buffer. Analytes were extracted twice from the serum matrix with a mixture of hexane and ethyl acetate. The combined extracts were dried under nitrogen at  $45\text{ }^\circ\text{C}$ , and the residues were reconstituted with methanol for LC-MS/MS analysis. Extracts were analyzed on a SCIEX QTRAP 6500+ LC-MS/MS system using an Ascentis Express F5 column under isocratic conditions with water:methanol mobile phases. APCI in the positive-ion mode and multiple reaction monitoring (MRM) mode were used. The following transitions were monitored:  $m/z$  401  $\rightarrow$   $m/z$  386 for 25(OH) $D_3$  and 3-epi-25(OH) $D_3$ ;  $m/z$  404  $\rightarrow$   $m/z$  386 for 3-epi-25(OH) $D_3$ -d3;  $m/z$  406  $\rightarrow$   $m/z$  388 for 25(OH) $D_3$ - $^{13}\text{C}_5$ ;  $m/z$  413  $\rightarrow$   $m/z$  395 for 25(OH) $D_2$ ; and  $m/z$  416  $\rightarrow$   $m/z$  398 for 25(OH) $D_2$ - $^{13}\text{C}_3$ .

**Analytical Approach for Determination of Trace Metals (CDC):** Reference mass concentration values for copper, selenium and zinc are the means of results from the CDC using inductively coupled plasma-mass spectrometry (ICP-MS). Fifteen replicates were analyzed for each level of SRM 1949. Each serum replicate was diluted 1:30 with diluent (10  $\mu\text{g}/\text{L}$  Ga, as an internal standard, in 2 % volume fraction nitric acid, 5 % volume fraction ethyl alcohol and 0.01 % volume fraction Triton<sup>TM</sup> X-100 in high-purity de-ionized water), and then analyzed by introducing an aerosol of this dilution to an ELAN Dynamic Reaction Cell (DRC) II ICP-MS instrument (PerkinElmer, Waltham, MA) using an SC4-DX (Elemental Scientific Inc., Omaha, NE) autosampler. External, matrix-matched, weighted linear

calibration using SI-traceable standards (High-Purity Standards, Charleston, SC) was used to quantify the total elemental concentrations. All elements were measured in dynamic reaction cell mode (using ammonia gas) to eliminate polyatomic interferences.

**Analytical Approach for Determination of Thyroglobulin and Thyroid Stimulating Hormone (NIST):** Information values for thyroglobulin and TSH were determined at NIST using commercial enzyme linked immunoassay (ELISA) kits (thyroglobulin: Abcam, Cambridge, UK; TSH: Abcam, Cambridge, UK and Biomatik, Cambridge, Ontario, Canada). Samples of SRM 1949 were brought to room temperature, gently mixed, buffered, and analyzed according to kit specifications. Following application of sample aliquots to the plate wells, an orbital shaker was used to incubate the samples at room temperature, and the plate was then read at 450 nm absorbance using a BioTek® Synergy HT microplate reader (Winooski, VT).

**Homogeneity Assessment:** The homogeneity of the trace metals was assessed at the CDC using the methods described above. Based on the repeatability data for fifteen replicate measurements, the material does not show significant heterogeneity with respect to trace elements across all the pool levels. Additional assessment of the homogeneity of the iodine hormones was made during the value assignment measurements at NIST. Again, no significant heterogeneity was indicated.

**Certified Value Assignment:** Each certified value listed in Table 1 is the mean of measurements from a single method. The uncertainty of each value incorporates the standard error of that mean with additional components of uncertainty (Type B components related to the analysis), consistent with the ISO/JCGM Guide [3]. The uncertainty provided with each 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 standard uncertainty, and  $k$  is a coverage factor corresponding to approximately 95 % confidence [3]. For expression of units appropriate to the measurement community, the values have been converted, where necessary, from a mass fraction basis to a mass concentration basis using the measured serum density. The value can be converted back to a mass fraction basis if needed, by dividing by the listed density value (Table 3). The measurands are the total concentrations of the analytes listed in Table 1. Metrological traceability is to the SI derived units of mass concentration, expressed as micrograms per deciliter or nanograms per deciliter.

Table 1. Certified Values for SRM 1949<sup>(a)</sup>

Level	Total Thyroxine (T <sub>4</sub> ) (µg/dL)	Coverage Factor, $k$	Total Triiodothyronine (T <sub>3</sub> ) (ng/dL)	Coverage Factor, $k$
Non-pregnant	6.74 ± 0.12	2.36	101.4 ± 6.4	2.57
First Trimester	9.82 ± 0.17	2.45	147.6 ± 6.6	2.45
Second Trimester	11.14 ± 0.19	2.45	176.0 ± 10.0	2.57
Third Trimester	11.07 ± 0.19	2.78	176.7 ± 10.1	2.57

<sup>(a)</sup> LC-MS/MS at NIST

**Reference Value Assignment:** Each reference value listed in Table 2 is the mean of measurements from a single method. The uncertainty of each value incorporates the standard error of that mean with additional components of uncertainty (Type B components related to the analysis), consistent with the ISO/JCGM Guide [3]. The uncertainty provided with each 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 standard uncertainty, and  $k$  is a coverage factor corresponding to approximately 95 % confidence [3]. For expression of units appropriate to the measurement community, the values have been converted, where necessary, from a mass fraction basis to a mass concentration basis using the measured serum density. The value can be converted back to a mass fraction basis if needed, by dividing by the listed density value (Table 3). The measurands are the concentrations of the analytes listed in Table 2, as determined by the methods indicated. Metrological traceability is to the SI derived units of mass concentration or amount of substance concentration, expressed as nanograms per deciliter, micrograms per deciliter, nanograms per milliliter, micrograms per milliliter or micromoles per kilogram.

Table 2. Reference Values for SRM 1949

Constituent	Units	Non-pregnant Concentration	Coverage Factor, <i>k</i>	First Trimester Concentration	Coverage Factor, <i>k</i>
Copper (Cu) <sup>(a)</sup>	µg/dL	128.5 ± 1.9	2.06	174.1 ± 2.3	2.05
Selenium (Se) <sup>(a)</sup>	µg/L	109.3 ± 1.3	2.00	105.1 ± 1.9	2.03
Zinc (Zn) <sup>(a)</sup>	µg/dL	78.8 ± 3.2	2.13	105.7 ± 3.1	2.12
25(OH)D <sub>3</sub> <sup>(b)</sup>	ng/mL	24.98 ± 0.28	2.57	26.01 ± 0.22	2.57
3-epi-25(OH)D <sub>3</sub> <sup>(b)</sup>	ng/mL	1.32 ± 0.06	2.57	1.20 ± 0.05	2.57
VDBP <sup>(b)</sup>	µg/mL	211.5 ± 2.8	2.05	286.7 ± 3.8	2.06
VDBP <sup>(b)</sup>	µmol/kg	4.01 ± 0.05	2.23	5.43 ± 0.06	2.23
Constituent	Units	Second Trimester Concentration	Coverage Factor, <i>k</i>	Third Trimester Concentration	Coverage Factor, <i>k</i>
Cu <sup>(a)</sup>	µg/dL	214.2 ± 2.5	2.02	232.4 ± 3.6	2.07
Se <sup>(a)</sup>	µg/L	112.7 ± 1.5	2.00	98.9 ± 2.2	2.06
Zn <sup>(a)</sup>	µg/dL	101.1 ± 3.7	2.13	81.7 ± 2.3	2.12
25(OH)D <sub>3</sub> <sup>(b)</sup>	ng/mL	30.00 ± 0.50	2.57	29.43 ± 0.41	2.57
25(OH)D <sub>2</sub> <sup>(b)</sup>	ng/mL	0.514 ± 0.037	2.57	0.897 ± 0.057	2.57
3-epi-25(OH)D <sub>3</sub> <sup>(b)</sup>	ng/mL	1.87 ± 0.07	2.57	1.87 ± 0.04	2.57
VDBP <sup>(b)</sup>	µg/mL	349.7 ± 4.3	2.03	383.4 ± 5.1	2.06
VDBP <sup>(b)</sup>	µmol/kg	6.64 ± 0.07	2.20	7.28 ± 0.08	2.23

<sup>(a)</sup> Dynamic reaction cell ICP-MS at CDC<sup>(b)</sup> LC-MS/MS at NIST

**Information Value Assignment:** Each information value listed in Table 3 for thyroglobulin, total reverse triiodothyronine and serum density is the mean of measurements from a single method. Each information value listed in Table 3 for thyroid stimulating hormone is the mean of measurements from two methods. Information values cannot be used to establish metrological traceability.

Table 3. Information Values for SRM 1949

Level	Non-pregnant	First Trimester	Second Trimester	Third Trimester	Units
Thyroglobulin (Tg) <sup>(a)</sup>	25.3	111	165	205	µg/L
Thyroid Stimulating Hormone (TSH) <sup>(a)</sup>	3.6	1.4	1.1	1.1	mIU/L
Total Reverse Triiodothyronine (rT3) <sup>(b)</sup>	19.2	30.6	33.9	35.6	ng/dL
Serum Density <sup>(c)</sup>	1.0233	1.0234	1.0223	1.0217	g/mL

<sup>(a)</sup> Enzyme linked immunosorbent assay at NIST

<sup>(b)</sup> LC-MS/MS at NIST

<sup>(c)</sup> Oscillation frequency density meter at NIST (23 °C)

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*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](mailto:srminfo@nist.gov); or via the Internet at <https://www.nist.gov/srm>.*