



Certificate of Analysis

Standard Reference Material[®] 971

Hormones in Frozen Human Serum

This Standard Reference Material (SRM) is intended primarily for use in evaluating the accuracy of procedures for the determination of the steroid hormones cortisol, progesterone, and testosterone, and thyroid hormones total thyroxine (T4), and total 3,3',5-triiodothyronine (T3) in human serum. It is also intended for use in validating working or secondary reference materials. A unit of SRM 971 consists of two materials: one from a pool of healthy, premenopausal adult females and one from a pool of healthy adult males. Both materials are unfortified. Each vial contains approximately 5.0 mL of human serum.

Certified Concentration Values: The certified mass fraction and amount-of-substance concentration values for cortisol (female and male sera), testosterone (female and male sera), progesterone (female serum only), total T4 (female and male sera), and total T3 (female and male sera) and their uncertainties are listed in Table 1. Values were determined using results from NIST reference measurement procedures based upon isotope dilution liquid chromatography tandem mass spectrometry (ID LC-MS/MS) [1-6], from the Physikalisch-Technische Bundesanstalt (PTB) in Braunschweig, Germany (cortisol and progesterone) using isotope dilution gas chromatography/mass spectrometry (ID-GC/MS), and from LGC Limited in Teddington, UK using ID LC-MS/MS (cortisol only). 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 [7]. The NIST methods have been listed in the Joint Committee for Traceability in Laboratory Medicine (JCTLM) database as reference measurement procedures of a higher order [8]. The certified concentrations apply only to serum thawed to room temperature, 20 °C to 25 °C (see “Instructions for Handling, Storage, and Use”).

Reference Values: The reference mass fraction and amount-of-substance concentration values for progesterone in the male serum are listed in Table 2. The reference concentration of progesterone in the male serum was determined from measurements at NIST using the ID LC-MS/MS-based reference measurement procedure [2]. Reference values are noncertified 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 [7] and are provided with associated uncertainties that may reflect only measurement repeatability, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods.

Expiration of Certification: The certification of **SRM 971** is valid, within the measurement uncertainty specified, until **01 January 2020**, 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.

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.

Support for the certification measurements of total T3 and total T4 in SRM 971 was provided in part by the National Institutes of Health, Office of Dietary Supplements (NIH-ODS). Technical consultation was provided by S.A. Wise, J.M. Betz, and P.M. Coates of NIH-ODS.

Coordination of the technical measurements leading to the certification of this SRM was performed by S.S.-C. Tai and M.J. Welch of the NIST Chemical Sciences Division and by K.W. Phinney of the NIST Biomolecular Measurement Division.

Carlos A. Gonzales, Chief
Chemical Sciences Division

Gaithersburg, MD 20899
Certificate Issue Date: 12 December 2016
Certificate Revision History on Last Page

Steven J. Choquette, Director
Office of Reference Materials

Acquisition of the material was performed by K.E. Sharpless of the NIST Office of Special Programs. Certification measurements at NIST were performed by S.S.-C. Tai. Additional measurements were performed at PTB by C. Gollub, K. Schild, R. Ohlendorf, and A. Henrion, and at LGC Limited by C. Mussell and G. O'Connor.

Statistical analysis was provided by J.H. Yen 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 WARNINGS TO USERS

SRM 971 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 FDA-licensed methods and found non-reactive/negative for hepatitis B surface antigen, hepatitis C virus, human immunodeficiency virus (HIV), and human immunodeficiency virus antigen 1. 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 Manual [9].

INSTRUCTIONS FOR HANDLING, STORAGE, AND USE

Vials of the SRM to be analyzed should be removed from the freezer and allowed to stand at room temperature (20 °C to 25 °C) until thawed. After the material is thawed, it should be used immediately. The material should be swirled gently to mix it (DO NOT CENTRIFUGE OR VORTEX MIX) before aliquots are withdrawn.

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 °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 °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.

Stability: The material is kept at -80 °C for long-term storage at NIST. Under these conditions, the hormones are expected to be stable. NIST will continue to monitor the stability of the hormones in this material and will notify purchasers of the material of any changes in the certified concentrations.

PREPARATION AND ANALYSIS⁽¹⁾

Source of Material: SRM 971 was prepared by Bioreclamation Inc. (Hicksville, NY). Off-clot serum was collected from healthy, adult donors, ages 21 years to 40 years (males) and 21 years to 39 years (premenopausal females), who were not taking prescription medications. The serum was processed according to Clinical Laboratory Standards Institute (CLSI) Publication C37-A [10] to reduce matrix effects.

Determination of Cortisol: The NIST reference measurement procedure [1] for cortisol involves spiking the serum with cortisol-*d*₃, acidifying the sample, isolating cortisol from the serum matrix using a solid-phase extraction cartridge (C18), further purifying cortisol by a liquid-liquid extraction, drying the sample, and reconstituting in water-methanol (59:41 by volume) containing 1 mL/L acetic acid. LC-MS/MS was performed using a C18 column and monitoring two transitions each for the unlabeled and labeled forms: m/z 363 → m/z 327 and m/z 363 → m/z 121 (unlabeled), and m/z 366 → m/z 330 and m/z 366 → m/z 121 (labeled). Calibration of the measurements was carried out by using SRM 921 Cortisol (Hydrocortisone). The PTB method for cortisol involved spiking the serum with cortisol-*d*₄, a liquid-liquid extraction, followed by a cleanup step using liquid chromatography. The cortisol was then derivatized in a two-step process, prior to analysis by GC/MS, monitoring m/z 605 and m/z 609. The LGC method involved spiking the serum with cortisol-*d*₃, a liquid-liquid extraction, and LC-MS/MS analysis using the transitions at m/z 363 → m/z 121 and m/z 366 → m/z 121 for the unlabeled and labeled forms, respectively [11].

⁽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.

Determination of Progesterone: The NIST reference measurement procedure for progesterone [2] involves spiking the serum with progesterone-¹³C₂, alkalizing the sample, performing a liquid-liquid extraction, drying the sample and reconstituting in methanol containing 0.5 mL/L acetic acid, followed by analysis using LC-MS/MS with a C18 column and using the transitions at m/z 315 → m/z 97 and m/z 317 → m/z 99 for the unlabeled and labeled forms, respectively. The PTB method involves spiking the serum with progesterone-¹³C₂, two liquid-liquid extractions, followed by derivatization. GC/MS measurements were performed using m/z 510 and m/z 512, for the unlabeled and labeled forms, respectively [12].

Determination of Testosterone: The NIST reference measurement procedure for testosterone [3] involves spiking the serum with testosterone-*d*₃, acidifying the sample, isolating testosterone from the serum matrix using a solid-phase extraction cartridge (C18), further purifying testosterone by a liquid-liquid extraction, drying the sample, and reconstituting in methanol containing 0.5 mL/L acetic acid. Analysis by LC-MS/MS was performed by monitoring the transitions at m/z 289 → m/z 97 and m/z 292 → m/z 97 for the unlabeled and labeled forms, respectively.

Determination of Total T3: The NIST reference measurement procedure for total T3 [4] involves spiking the serum with T3-¹³C₉, deproteinizing with acetone, isolating total T3 from the serum matrix using a solid-phase extraction cartridge (mixed-mode retention mechanisms of ion exchange and reversed-phase), drying the sample, and reconstituting in water/methanol (50:50 by volume) containing 20 mL/L acetic acid. Analysis by LC-MS/MS was performed by monitoring the transitions at m/z 652 → m/z 606 and m/z 661 → m/z 614 for the unlabeled and labeled forms, respectively.

Determination of Total T4: The NIST reference measurement procedure for total T4 [5-6] involves spiking the serum with T4-¹³C₆, acidifying the sample, deproteinizing with trichloroacetic acid, performing a liquid-liquid extraction, further purifying total T4 using a solid-phase extraction cartridge (mixed-mode retention mechanisms of ion exchange and reversed-phase), drying the sample, and reconstituting in water/methanol (50:50 by volume) containing 10 mL/L acetic acid. Analysis by LC-MS/MS was performed by monitoring the transitions at m/z 778 → m/z 732 and m/z 784 → m/z 738 for the unlabeled and labeled forms, respectively.

Homogeneity Analysis: Homogeneity was assessed at the time the certification analyses were performed. A stratified sampling plan was devised to test for homogeneity across the entire lot. No appreciable vial-to-vial differences were detected.

Commutability: The commutability of this material for routine clinical methods has not been evaluated. Plans are underway to conduct such a study and the certificate will be updated as appropriate. Based upon previous experience with reference materials for other analytes prepared following the CLSI C37a protocol, this material should be commutable to most clinical assays.

Table 1. Certified Concentrations and Certified Mass Fractions for SRM 971

	Female		Male	
	Concentration ^(a) (nmol/L)	Mass Fraction ^(b) (ng/g)	Concentration ^(a) (nmol/L)	Mass Fraction ^(b) (ng/g)
Cortisol ^(c)	250.1 ± 5.8	88.5 ± 2.1	296.8 ± 4.1	105.0 ± 1.5
Progesterone ^(c)	6.20 ± 0.22	1.903 ± 0.068		
Testosterone ^(d)	0.961 ± 0.022	0.271 ± 0.006	22.31 ± 0.51	6.279 ± 0.143
Total T3 ^(d)	1.571 ± 0.081	0.999 ± 0.051	1.511 ± 0.078	0.960 ± 0.049
Total T4 ^(d)	91.0 ± 2.8	69.1 ± 2.1	84.6 ± 2.6	64.2 ± 2.0

^(a) Molar concentrations were calculated from the mass fractions using the relative molecular masses for each compound and the measured serum densities for the two serum pools, which were 1.0241 g/mL (female) and 1.0247 g/mL (male). The measurand is the total concentration of each analyte listed in Table 1. Metrological traceability is to the SI derived units for amount-of-substance concentration, expressed as nanomoles per liter.

^(b) The measurand is the total mass fraction of each analyte listed in Table 1. Metrological traceability is to the SI derived units for mass fraction, expressed as nanograms per gram.

^(c) The uncertainties in the certified values are calculated as $U = ku_c$, where u_c is the combined standard uncertainty calculated according to the ISO/JCGM Guide [13], using a random effect model for combining results from multiple methods [14], and $k = 2$ is the coverage factor. The values of u_c are intended to represent, at the level of one standard deviation, the uncertainties in mean concentration. The expanded uncertainty, $U = ku_c$, is defined as an interval estimated to have a level of confidence of at least 95 %.

^(d) The uncertainties in the certified values are calculated as $U = ku_c$, where u_c is the combined standard uncertainty calculated according to the ISO/JCGM Guide [13], and $k = 2$ is the coverage factor. The values of u_c are intended to represent, at the level of one standard deviation, the uncertainties in mean concentration. The expanded uncertainty, $U = ku_c$, is defined as an interval estimated to have a level of confidence of at least 95 %.

Table 2. Reference Concentration and Mass Fraction Values for Progesterone in Male Serum in SRM 971

Male	Concentration ^(a) (nmol/L)	Mass Fraction ^(b) (ng/g)
	0.131 ± 0.020	0.0403 ± 0.0062

^(a) The molar concentration was calculated from the mass fraction using the relative molecular mass of progesterone and the measured serum density (1.0247 g/mL). The measurand is the concentration of progesterone listed in Table 2 as determined by the method described above (see *Determination of Progesterone*). Metrological traceability is to the SI derived units for amount-of-substance concentration, expressed as nanomoles per liter.

^(b) The uncertainty in the reference value is calculated as $U = ku_c$, where u_c is the combined standard uncertainty calculated according to the ISO/JCGM Guide [13], and $k = 2$ is the coverage factor. The values of u_c are intended to represent, at the level of one standard deviation, the uncertainties in mean concentration. The expanded uncertainty, $U = ku_c$, is defined as an interval estimated to have a level of confidence of at least 95 %. The measurand is the concentration of progesterone listed in Table 2 as determined by the method described above (see *Determination of Progesterone*). Metrological traceability is to the SI derived units for mass fraction, expressed as nanograms per gram.

REFERENCES

- [1] Tai, S.S-C; Welch, M.J.; *Development and Evaluation of a Candidate Reference Method for the Determination of Total Cortisol in Human Serum using Isotope Dilution Liquid Chromatography/Mass Spectrometry and Isotope Dilution Liquid Chromatography/Tandem Mass Spectrometry*; Anal. Chem., Vol. 76, pp. 1008–1014 (2004).
- [2] Tai, S.S-C.; Xu, B.; Welch, M.J.; *Development and Evaluation of a Candidate Reference Measurement Procedure for the Determination of Progesterone in Human Serum using Isotope Dilution Liquid Chromatography/Tandem Mass Spectrometry*; Anal. Chem., Vol. 78, pp. 6628–6633 (2006).
- [3] Tai, S.S-C.; Xu, B.; Welch, M.J.; Phinney, K.W.; *Development and Evaluation of a Candidate Reference Measurement Procedure for the Determination of Testosterone in Human Serum using Isotope Dilution Liquid Chromatography/Tandem Mass Spectrometry*; Anal. Bioanal. Chem., Vol. 388, pp. 1087–1094 (2007).
- [4] Tai, S.-C; Bunk, D.M.; White, E.V; Welch, M.J.; *Development and Evaluation of a Reference Measurement Procedure for the Determination of Total 3,3',5-Triiodothyronine in Human Serum Using Isotope-Dilution Liquid Chromatography-Tandem Mass Spectrometry*; Anal. Chem., Vol. 76, pp. 5092–5096 (2004).
- [5] Tai, S.-C; Sniegoski, L.T.; Welch, M.J.; *Candidate Reference Method for Total Thyroxine in Human Serum: Use of Isotope-Dilution Liquid Chromatography-Mass Spectrometry with Electrospray Ionization*; Clin. Chem., Vol. 48, pp. 637–642 (2002).
- [6] Thienpont, L.; Van Uyfanghe, K.; Marriot, J.; Stokes, P.; Siekmann, L.; Kessler, A.; Bunk, D.; Tai, S.; *Metrological Traceability of Total Thyroxine Measurements in Human Serum. Efforts to Establish a Network of Reference Measurement Laboratories*; Clin. Chem., Vol. 51, pp. 161–168 (2005).
- [7] May, W.; Parris, R.; Beck II, C.; Fassett, J.; Greenberg, R.; Guenther, F.; Kramer, G.; Wise, S.; Gills, T.; Colbert, J.; Gettings, R.; MacDonald, B.; *Definition of Terms and Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements*; NIST Special Publication 260-136 (2000); available at <http://www.nist.gov/srm/publications.cfm> (accessed Dec 2016).
- [8] Joint Committee for Traceability in Laboratory Medicine (JCTLM) database available at <http://www.bipm.org/jctlm/> (accessed Dec 2016).
- [9] CDC/NIH; *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed.; HHS publication No. (CDC) 21-1112; Chosewood, L.C.; Wilson, D.E., Eds.; US Government Printing Office: Washington, D.C. (2009); available at <http://www.cdc.gov/biosafety/publications/bmb15/index.htm> (accessed Dec 2016).
- [10] *Preparation and Validation of Commutable Frozen Human Serum Pools as Secondary Reference Materials for Cholesterol Measurement Procedures*; NCCLS Publication C37-A, Clinical Laboratory Standards Institute: Wayne, PA (2000).
- [11] Jung, P.G.; Kim, B.; Park, S.-R.; So, H.-Y.; Shi, L.H.; Kim, Y.; *Determination of Serum Cortisol Using Isotope Dilution-Liquid Chromatography-Mass Spectrometry as a Candidate Reference Method*; Anal. Bioanal. Chem., Vol. 380, pp. 782–788 (2004).
- [12] Thienpont, L.M.; De Brabandere, V.I.; Stöckl, D.; and De Leenheer, D.P.; *Use of Cyclodextrins for Prepurification of Progesterone and Testosterone from Human Serum Prior to Determination with Isotope Dilution Gas Chromatography/Mass Spectrometry*; Anal. Chem., Vol. 66, pp. 4116–4119 (1994).
- [13] 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 (2008); available at http://www.bipm.org/utlis/common/documents/jcgm/JCGM_100_2008_E.pdf (accessed Dec 2016); 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 <http://www.nist.gov/pml/pubs/index.cfm> (accessed Dec 2016).
- [14] Rukhin, A.L.; *Weighted Means Statistics in Interlaboratory Studies*; Metrologia, Vol. 46, pp. 323–331 (2009).

Certificate Revision History: **12 December 2016** (Editorial changes); **20 October 2016** (Addition of certified values for total T4 and total T3; editorial changes); **24 November 2015** (Editorial changes); **19 February 2015** (Change of expiration date; editorial changes); **29 Feb 2011** (Addition of certified values for testosterone; update of certified and reference values for cortisol and progesterone; change of expiration date; editorial changes); **19 December 2008** (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; fax (301) 948-3730; e-mail srminfo@nist.gov; or via the Internet at <http://www.nist.gov/srm>.