National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 971a

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 hormone, testosterone, in human serum. A unit of SRM 971a consists of two vials each of two materials: one from a pool of healthy, premenopausal adult females and one from a pool of healthy adult males. Donors were not taking medications that would alter hormonal concentrations such as, but not limited to birth control, steroidal pain relievers, or thyroid medication. Both materials are unfortified. Each vial contains approximately 2 mL of human serum.

The development of SRM 971a was a collaboration between the National Institute of Standards and Technology (NIST) and the Centers for Disease Control and Prevention (CDC).

Certified Concentration Values: Certified mass fraction and mass concentration values for testosterone and the uncertainties 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 values were determined using higher-order reference measurement procedures [2] calibrated with high-purity chemical reference standards in combination with higher-order measurement procedures conducted by the CDC; the uncertainties are expanded uncertainties at the 95 % level of confidence [3]. The measurands are the rational quantities of testosterone reported in Table 1. Metrological traceability is to the SI-derived unit of mass fraction expressed as nanograms per gram. The certified values apply only to serum thawed to room temperature, 20 °C to 25 °C (see "Instructions for Handling, Storage, and Use").

Expiration of Certification: The certification of **SRM 971a** is valid, within the measurement uncertainty specified, until **01 May 2029**, 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.

Coordination of the technical measurements leading to the certification of this SRM was performed by A.S.P. Boggs of the NIST Chemical Sciences Division. Coordination of the collaborative measurements of the SRM at CDC was performed by H.W. Vesper of the CDC Hormone Reference Laboratory, Clinical Chemistry Branch, Division of Laboratory Sciences, the National Center for Environmental Health.

Technical support for the development of this SRM was provided by J.R. Kucklick and K.A. Lippa of the NIST Chemical Sciences Division.

Analytical measurements were performed by A.S.P. Boggs, M.A. Nelson, K.R. Huncik, S.E. Long, and J. Camara of the NIST Chemical Sciences Division. Analytical measurements at the CDC were performed by H.W. Vesper and K. Poynter.

Consultation on the statistical design of the experimental work, and evaluation of the data were provided by B. Toman of the NIST Statistical Engineering Division.

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Certificate Issue Date: 16 August 2019

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

NOTICE AND WARNING TO USERS

SRM 971a IS INTENDED FOR LABORATORY 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 serum used in the preparation of this product was tested by 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 °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.

Handling and Use: SRM 971a 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 thawed, it should be used immediately. The contents of the vial should then be gently swirled to mix (DO NOT CENTRIFUGE OR VORTEX MIX) before aliquots are withdrawn. Precautions should be taken to avoid exposure to strong UV light and direct sunlight.

SOURCE, PREPARATION, AND ANALYSIS[(1)]

Source and Preparation: SRM 971a was prepared from serum collected by Bioreclamation IVT, now BioIVT (Westbury, NY). The serum materials comprised of 50 male and 50 female donors. The male samples were required to originate from non-smokers between the ages of 21 and 40. The female samples were required to originate from premenopausal nonsmokers between the ages of 21 and 40. Donors were excluded if they were taking over the counter or prescription medications that would alter hormone concentrations such as steroidal pain relievers, birth control, or thyroid medications. All serum was collected according to the CLSI C37-A guideline "Preparation and Validation of Commutable Frozen Human Serum Pools as Secondary Reference Materials for Cholesterol Measurement Procedures" [5]. Briefly, serum was collected off clot with no additives in an ice bath and bottled. Whole blood was centrifuged within 5 minutes of collection, allowed to clot overnight at 4 °C, centrifuged, then the clear serum was aseptically transferred to a sterile container. Serum was tested for clotting factors and the process repeated if tested positive. The materials were shipped frozen on dry-ice to Solomon Park (Burian, WA) for pooling and bottling. The frozen serum was thawed at 4 °C to 8 °C on a shaker system. Serum was then passed through sterile cheese cloth to remove any larger particles, mixed overnight, and then filtered through 0.22 µm spiral filters. Aliquoting was conducted using a peristaltic pump dispenser into vials which were on cooled pads. Nitrogen was introduced into the vials as they were being stoppered with the Teflon stoppers. Once the entire pool was aliquoted, boxes were frozen at -70 °C.

This SRM was developed after an appropriate human subjects research determination by NIST.

[(1)] 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 Testosterone: The NIST reference measurement procedure for testosterone [6] 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. Samples were analyzed by isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS). Selected reaction monitoring was used with following transitions: *m/z* 289.0 → *m/z* 97.0 (quantification) and *m/z* 289.0 → *m/z* 109.0 (confirmation) for testosterone, and *m/z* 292.0 → *m/z* 97.0 (quantification) and *m/z* 292.0 → *m/z* 109.0 (confirmation) for testosterone-d_3. Chemical purity characterizations of calibrants with metrological traceability to SI units were assessed using a quantitative ^{1}H nuclear magnetic resonance spectroscopy (^{1}H-qNMR) procedure.

The CDC measurement method for testosterone [7] involves spiking the serum with testosterone-$^{13}C_3$, acidifying the sample, isolating testosterone with two liquid-liquid extractions, drying the sample, and reconstituting in water:acetonitrile mixture (90:10, volume fraction) with 0.1 % formic acid (volume fraction). Selected reaction monitoring was used with following transitions: *m/z* 289.3 → *m/z* 97.0 (quantification) and *m/z* 289.3 → *m/z* 109.0 (confirmation) for testosterone, and *m/z* 292.3 → *m/z* 100.0 (quantification) and *m/z* 292.3 → *m/z* 112.0 (confirmation) for testosterone-$^{13}C_3$.

Homogeneity Assessment: Homogeneity was assessed at the time the certification analyses were performed. A stratified random sampling plan was devised to test for homogeneity of testosterone across the entire lot. Based on the repeatability data for ten replicate measurements from ten independent vials, the material does not show significant heterogeneity with respect to testosterone across all the pool levels.

Value Assignment: Each certified value listed in Table 1 is the consensus mean of measurements from NIST and the CDC. For expression of units appropriate to the clinical measurement community, the values have been converted from a mass fraction basis to a mass concentration basis using the measured serum density. The mean of densities measured by NIST using an oscillation frequency density meter was 1.0252 g/mL at 21.6 ºC for the male material and 1.0238 g/mL at 22.3 ºC for the female material. Densities were measured on each aliquot by CDC. It is recommended that each laboratory conduct a density measurement according to their laboratory conditions to improve accuracy.

Table 1. Certified Mass Fraction and Mass Concentration Values for SRM 971a[(a)]

	Female		Male	
	Mass Fraction (ng/g)	Mass Concentration [(b)] (ng/dL)	Mass Fraction (ng/g)	Mass Concentration [(b)] (ng/dL)
Testosterone	0.316 ± 0.006	32.31 ± 0.50	5.667 ± 0.066	580.8 ± 9.0

[(a)] Each certified concentration value is the consensus mean of results from an ID-LC-MS/MS reference measurement procedure at NIST and ID-LC-MS/MS reference measurement procedure at CDC. The uncertainty provided with each value is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence, consistent with the ISO/JCGM Guide [3]. The expanded uncertainty is calculated as $U = ku_c$, where u_c is the combined uncertainty that incorporates within-method uncertainty and Type B uncertainty components related to the analysis, and k is the coverage factor corresponding to approximately 95 % confidence for each analyte.

[(b)] Mass concentrations were calculated from the mass fractions for each compound using the measured serum densities.

REFERENCES

[1] 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 https://www.nist.gov/sites/default/files/documents/srm/SP260-136.PDF (accessed Aug 2019).

[2] ISO 15193:2009; *In Vitro Diagnostic Medical Devices - Measurement of Quantities in Samples of Biological Origin - Requirements for Content and Presentation of Reference Measurement Procedures*; International Organization for Standardization (ISO) (2009); available at https://www.iso.org/standard/42021.html (accessed Aug 2019).

[3] 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 (JCGM) (2008); available at https://www.bipm.org/utils/common/documents/jcgm/JCGM_100_2008_E.pdf (accessed Aug 2019); 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 https://www.nist.gov/pml/pubs/index.cfm (accessed Aug 2019).

[4] CDC/NIH; *Biosafety in Microbiological and Biomedical Laboratories, 5th ed*.; Richardson, J.; Barkley, W.E.; Richmond, J.; McKinney, R.W., Eds.; U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention and National Institutes of Health; US Government Printing Office: Washington, D.C. (2007); available at https://www.cdc.gov/biosafety/publications/index.htm (accessed Aug 2019).

[5] CLSI Publication C37-A; *Preparation and Validation of Commutable Frozen Human Serum Pools as Secondary Reference Materials for Cholesterol Measurement Procedures*; Clinical Laboratory Standards Institute: Wayne, PA (1999).

[6] 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).

[7] Botelho, J. C.; Shacklady, C.; Cooper, H. C.; Tai, S.S.C.; Van Uytfanghe, K.; Thienpont, L.M.; Vesper, H.W.; *Isotope-Dilution Liquid Chromatography-Tandem Mass Spectrometry Candidate Reference Method for Total Testosterone in Human Serum*. Clin. Chem., Vol. 59, pp. 372-380 (2013).

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.