National Institute of Standards and Technology
U.S. Department of Commerce

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Standard Reference Material® 971a
Hormones in Frozen Human Serum
CERTIFICATE OF ANALYSIS

Purpose: The certified values delivered by this Standard Reference Material (SRM) are intended for validating methods for determining testosterone and progesterone in human serum and similar materials.

Description: 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. Both materials are unfortified. Each vial contains approximately 2 mL of human serum.

Certified Values: 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]. Metrological traceability is to the International System of Units (SI) derived unit of mass fraction and converted to be expressed as nanomoles per liter and nanograms per deciliter. The certified values apply only to serum thawed to room temperature (20 °C to 25 °C; see "Storage" and "Use"). Table 1 certified values are reported on an as-received basis [2].

Table 1. Certified Concentration Values for SRM 971a for Female Level and Male Level[(a)]

Analyte	Level	Molar Concentration[(b)] (nmol/L)	Lower 95 % Confidence Interval (nmol/L)	Upper 95 % Confidence Interval (nmol/L)	Mass Concentration[(c)] (ng/dL)	Lower 95 % Confidence Interval (ng/dL)	Upper 95 % Confidence Interval (ng/dL)
Testosterone	Female	1.12 ± 0.01	1.10	1.14	32.31 ± 0.50	31.81	32.81
Progesterone	Female	8.36 ± 0.60	7.17	9.54	263 ± 19	231	296
Testosterone	Male	20.14 ± 0.16	19.83	20.44	580.8 ± 9.0	571.8	589.8
Progesterone	Male	0.134 ± 0.011	0.112	0.156	4.21 ± 0.35	3.61	4.80

[(a)] Each certified concentration value is the consensus value of results from an isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS) reference measurement procedure at NIST and ID-LC-MS/MS reference measurement procedure at the Centers for Disease Control and Prevention (CDC). The uncertainty provided with each value is an expanded uncertainty about the consensus 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)] Amount concentration values, nmol/L, are calculated from the mass concentration results, nanogram per deciliter, via multiplication by 10/M, where M is the molar mass, grams per mol, of the analyte. The molar masses used are: testosterone = 288.424 g/mol and progesterone = 314.461 g/mol. These molar masses have associated standard uncertainty u(M) = 0.011 g/mol for testosterone and 0.012 g/mol for progesterone.

[(c)] Mass concentrations were calculated from the mass fractions for each compound using the measured serum density of 1.0238 ± 0.0012 g/mL at 22.3 ºC for the female level and at 1.0252 ± 0.0012 g/mL at 21.6 ºC for the male level.

Non-Certified Values: Non-certified values are provided in Appendix A.

Period of Validity: The certified values delivered by **SRM 971a** are valid within the measurement uncertainty specified until **01 May 2029**. The certified values are nullified if the material is stored or used improperly, damaged, contaminated, or otherwise modified.

Maintenance of Certified Values: NIST will monitor this SRM over the period of its validity. If substantive technical changes occur that affect the certification, NIST will issue an amended certificate through the NIST SRM website (https://www.nist.gov/srm) and notify registered users. SRM users can register online from a link available on the NIST SRM website or fill out the user registration form that is supplied with the SRM. Registration will facilitate notification. Before making use of any of the values delivered by this material, users should verify they have the most recent version of this documentation, available through the NIST SRM website (https://www.nist.gov/srm).

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Safety: SRM 971a IS INTENDED FOR RESEARCH USE. This is a human-source material. Handle product as a biohazardous material potentially 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 Biosafety Level 2 as recommended by the Centers for Disease Control and Prevention/National Institutes of Health's Biosafety in Microbiological and Biomedical Laboratories [4] for human-derived blood products where the presence of infectious agent(s) may be unknown.

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

Storage: The serum is shipped frozen (on dry ice) and, upon receipt, should be stored frozen until ready for use. The material is kept at –80 °C for long-term storage at NIST. Under these conditions, analytes are expected to be stable. 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 analyte concentrations.

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 be mixed thoroughly by inverting and/or rolling (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 and Preparation: SRM 971a was prepared from serum collected by Bioreclamation IVT, now BioIVT (Westbury, NY). The serum materials are comprised from 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 non-smokers 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.

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 a 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-$^{13}C_3$.

Analytical Approach for Determination of Progesterone: The NIST reference measurement procedure for progesterone [8] involves spiking the serum with progesterone-$^{13}C_2$, equilibrating at room temperature under reduced laboratory lighting, then amending the pH to 9.8 ± 0.2 with 0.1 g/mL carbonate buffer (pH 9.8). Two rounds of hexane extraction were performed before drying the extract and reconstituting in methanol with 0.5 mL/L acetic acid. Samples were analyzed by ID-LC-MS/MS. Selected reaction monitoring was used with the following transitions: *m/z* 315.2 → *m/z* 97.2 (quantification) and *m/z* 315.2 → *m/z* 109.2 (confirmation) for progesterone, and

m/z 317.2 → *m/z* 97.2 (quantification) for progesterone-$^{13}C_2$. 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.

CDC measurements were conducted using a CDC Steroid Panel Method in serum by ID-LC-MS/MS with a progesterone-$^{13}C_3$ internal standard [9,10]. Progesterone was dissociated from carrier proteins by the addition of acetate buffer at pH 5.5 followed by a liquid-liquid extraction using ethyl acetate. Selected reaction monitoring was used with the following transitions: *m/z* 315 → *m/z* 97 (quantification) and *m/z* 315 → *m/z* 109 (confirmation).

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Certificate Revision History: 27 March 2023 (Updated storage conditions to remove short term storage temperature and add NIST storage temperature; editorial changes); **02 February 2022** (Addition of certified values for progesterone; addition of molar concentration and uncertainty values for testosterone; addition of non-certified values for 17-hydroxyprogesterone, androstenedione, dehydroepiandrosterone sulfate, 17β-estradiol, estrone, estrone sulfate, anti-Müllerian hormone, follicle stimulating hormone, luteinizing hormone, sex hormone binding globulin, thyroglobulin, and thyroglobulin antibodies; updated format; editorial changes); **16 August 2019** (Original certificate date).

Certain commercial equipment, instruments, or materials may be identified in this Certificate of Analysis 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.

Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the Office of Reference Materials 100 Bureau Drive, Stop 2300, Gaithersburg, MD 20899-2300; telephone (301) 975-2200; e-mail srminfo@nist.gov; or the Internet at https://www.nist.gov/srm.

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APPENDIX A

Non-Certified Values: Non-certified values are suitable for use in method development, method harmonization, and process control but do not provide metrological traceability to the International System of Units (SI) or other higher-order reference system. Non-certified values are provided below.

Table A1. Non-certified Molar Concentration Values of Steroids for SRM 971a Female Level[a]

Analyte	Molar Concentration[b]	Lower 95 % Confidence Interval	Upper 95 % Confidence Interval	Molar Concentration Units
17-Hydroxyprogesterone	2.640 ± 0.049	2.545	2.736	nmol/L
Androstenedione	2.829 ± 0.016	2.798	2.860	nmol/L
Dehydroepiandrosterone Sulfate	3.310 ± 0.076	3.161	3.459	µmol/L
17β-Estradiol	1073 ± 11	1051	1094	pmol/L
Estrone	680.2 ± 7.0	666.4	693.9	pmol/L
Estrone Sulfate	4139 ± 45	4050	4227	pmol/L

[a] Each concentration value is the mean value of results from an 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] Amount concentration values of nanomol per liter are calculated from the mass concentration results, nanogram per deciliter, via multiplication by 10/M, where M is the molar mass, gram per mol, of the analyte. Amount concentration values of picomol per liter are calculated from the mass concentration results, picogram per milliliter, via multiplication by 1000/M. Amount concentration values of micromol per liter are calculated from the mass concentration results, microgram per deciliter, via multiplication by 10/M. The molar masses used are: 17α-hydroxyprogesterone = 330.460 g/mol, androstenedione = 286.408 g/mol, dehydroepiandrosterone sulfate = 368.489 g/mol, 17β-estradiol = 272.381 g/mol, estrone = 270.365 g/mol, and estrone sulfate = 350.431 g/mol. These molar masses have associated standard uncertainty u(M) = 0.011 g/mol for androstenedione, estrone, and 17β-estradiol and u(M) = 0.012 for 17-hydroxyprogesterone, dehydroepiandrosterone sulfate, and estrone sulfate.

Table A2. Non-certified Mass Concentration Values of Steroids for SRM 971a Female Level[a]

Analyte	Mass Concentration[b]	Lower 95 % Confidence Interval	Upper 95 % Confidence Interval	Mass Concentration Units
17-Hydroxyprogesterone	87.25 ± 1.61	84.08	90.40	ng/dL
Androstenedione	81.03 ± 0.45	80.16	81.93	ng/dL
Dehydroepiandrosterone Sulfate	122.0 ± 2.8	116.3	127.6	µg/dL
17β-Estradiol	292.3 ± 3.0	286.3	298.5	pg/mL
Estrone	183.9 ± 1.9	180.2	187.5	pg/mL
Estrone Sulfate	1450 ± 16	1419	1483	pg/mL

[a] Each concentration value is the mean value of results from an 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 density of 1.0238 ± 0.0012 g/mL at 22.3 ºC.

Table A3. Non-certified Molar Concentration Values of Steroids for SRM 971a Male Level[a]

Analyte	Molar Concentration[b]	Lower 95 % Confidence Interval	Upper 95 % Confidence Interval	Molar Concentration Units
17-Hydroxyprogesterone	2.916 ± 0.042	2.833	2.998	nmol/L
Androstenedione	1.871 ± 0.021	1.829	1.913	nmol/L
Dehydroepiandrosterone Sulfate	5.019 ± 0.066	4.889	5.149	µmol/L
17β-Estradiol	126.8 ± 2.09	122.7	130.9	pmol/L
Estrone	125 ± 3	120	130	pmol/L
Estrone Sulfate	1805 ± 10	1785	1825	pmol/L

[a] Each non-certified concentration value is the mean value of results from an 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] Amount concentration values of nanomol per liter are calculated from the mass concentration results, nanogram per deciliter, via multiplication by 10/M, where M is the molar mass, gram per mol, of the analyte. Amount concentration values of picomol per liter are calculated from the mass concentration results, picogram per milliliter, via multiplication by 1000/M. Amount concentration values of micromol per liter are calculated from the mass concentration results, microgram per deciliter, via multiplication by 10/M. The molar masses used are: 17α-hydroxyprogesterone = 330.460 g/mol, androstenedione = 286.408 g/mol, dehydroepiandrosterone sulfate = 368.489 g/mol, 17β-estradiol = 272.381 g/mol, estrone = 270.365 g/mol, and estrone sulfate = 350.431 g/mol. These molar masses have associated standard uncertainty u(M) = 0.011 g/mol for androstenedione, estrone, and 17β-estradiol and u(M) = 0.012 g/mol for 17-hydroxyprogesterone, dehydroepiandrosterone sulfate, and estrone sulfate.

Table A4. Non-certified Mass Concentration Values of Steroids for SRM 971a Male Level[a]

Analyte	Mass Concentration[b]	Lower 95 % Confidence Interval	Upper 95 % Confidence Interval	Mass Concentration Units
17-Hydroxyprogesterone	96.35 ± 1.39	93.58	99.10	ng/dL
Androstenedione	53.59 ± 0.61	52.36	54.82	ng/dL
Dehydroepiandrosterone Sulfate	185.0 ± 2.5	180.0	189.7	µg/dL
17β-Estradiol	34.55 ± 0.57	33.41	35.67	pg/mL
Estrone	33.7 ± 0.7	32.2	35.2	pg/mL
Estrone Sulfate	632.5 ± 3.6	625.3	639.6	pg/mL

[a] Each non-certified concentration value is the mean value of results from an 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 density of 1.0252 ± 0.0012 g/mL at 21.6 ºC.

Analytical Approach for Determination of Additional Steroid Hormones: CDC measurements were conducted for 17α-hydroxyprogesterone, androstenedione, progesterone, estrone, 17β-estradiol, estrone sulfate, and dehydroepiandrosterone 3-sulfate using the CDC Steroid Panel Method in serum by ID LC-MS/MS [9,10]. Progesterone measurements were used for certification (see above). Internal standards for each analyte were 17α-hydroxyprogesterone-$^{13}C_3$, androstenedione-$^{13}C_3$, estrone-$^{13}C_3$, 17β-estradiol-$^{13}C_3$, estrone sulfate-$^{13}C_6$, and dehydroepiandrosterone sulfate-d_5. Steroids were dissociated from carrier proteins by the addition of acetate buffer at pH 5.5. A series of liquid-liquid extractions (LLE) were then used to extract different hormone classes. First, progestogens were isolated using ethyl acetate. Second, androgens and estrogens were isolated using ethyl acetate and hexane. Last, conjugated steroids were isolated using butanol. Cleanup was then conducted to deprotonate and remove phospholipids using another LLE. Selected reaction monitoring was conducted with the following transitions: *m/z* 331 → *m/z* 97 (quantification) and *m/z* 331 → *m/z* 109 (confirmation) for 17-hydroxyprogesterone, *m/z* 287 → *m/z* 97 (quantification) and *m/z* 287 → *m/z* 109 (confirmation) for androstenedione, *m/z* 269 → *m/z* 145 (quantification) and *m/z* 269 → *m/z* 143 (confirmation) for estrone, *m/z* 271 → *m/z* 145 (quantification) and *m/z* 271 → *m/z* 183 (confirmation) for 17β-estradiol,

m/z 349 → *m/z* 269 (quantification) and *m/z* 349 → *m/z* 145 (confirmation) for estrone sulfate, and *m/z* 367 → *m/z* 80 (quantification) and *m/z* 367 → *m/z* 97 (confirmation) for dehydroepiandrosterone 3-sulfate. A C18 guard column with a Phenyl-hexyl column were used for separation. The column oven was set to 40 ºC. A gradient with a flow of 250 µL/min transitioned the mobile phases (A) methanol:ethanol:water (0.2 mM ammonium formate) and (B) methanol from 10 % to 86 %.

Table A5. Non-certified Mass Concentration Values of Peptide Hormones and Proteins for SRM 971a Female Level

Analyte	Mass Concentration[b]	Lower 95 % Confidence Interval	Upper 95 % Confidence Interval	Units[c]
Anti-Müllerian Hormone[a]	2.74 ± 0.04	2.66	2.81	ng/mL
Follicle Stimulating Hormone[a]	7.30 ± 0.07	7.17	7.43	mIU/mL
Luteinizing Hormone[a]	10.58 ± 0.07	10.45	10.72	mIU/mL
Sex Hormone Binding Globulin[a]	87.98 ± 1.47	85.07	90.98	nmol/L
Thyroglobulin[b]	18.8 ± 0.3	18.2	19.4	ng/mL
Thyroglobulin Antibody[a]	6.0 ± 0.3	5.4	6.6	IU/mL

[a] Each concentration value is the mean value of results from an enzyme-linked immunosorbent assay (ELISA) measurement procedure at CDC or Mayo Clinic. 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] The thyroglobulin concentration value is the mean value of results from an LC-MS/MS measurement procedure at the Mayo Clinic. 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.

[c] International Unit (IU).

Table A6. Non-certified Mass Concentration Values of Peptide Hormones and Proteins for SRM 971a Male Level

Analyte	Mass Concentration	Lower 95 % Confidence Interval	Upper 95 % Confidence Interval	Units[d]
Anti-Müllerian Hormone[a]	5.79 ± 0.09	5.62	5.97	ng/mL
Follicle Stimulating Hormone[a]	5.13 ± 0.07	5.00	5.26	mIU/mL
Luteinizing Hormone[a]	6.73 ± 0.08	6.57	6.89	mIU/mL
Sex Hormone Binding Globulin[a]	42.47 ± 0.88	40.7	44.22	nmol/L
Thyroglobulin[b]	22.4 ± 0.2	22.0	22.8	ng/mL
Thyroglobulin Antibody[a]	ND[c]	-	-	IU/mL

[a] Each concentration value is the mean value of results from an ELISA measurement procedure at CDC or Mayo Clinic. 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] The thyroglobulin concentration value is the mean value of results from an LC-MS/MS measurement procedure at the Mayo Clinic. 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.

[c] Not detectable.

[d] International Unit (IU).

Analytical Approach for Determination of Peptide Hormones and Hormone Related Proteins: Measurements for anti-Müllerian hormone (AMH), follicle stimulating hormone (FSH), luteinizing hormone (LH), and sex hormone binding globulin (SHBG), were performed by the CDC using automated enzyme-linked immunosorbent assays (ELISA) using a Roche Cobas e411 per the manufacturer's instructions. The methods were validated in accordance with Clinical Laboratory Improvement Amendments (CLIA) and CDC Division of Laboratory Sciences policies and procedures. Methods were standardized against the 1st World Health Organization (WHO) International Standard for SHBG from the National Institute for Biological Standards and Control (NIBSC) code: 95/560, and the 2nd WHO International Standard for LH, Human, Pituitary, NIBSC code: 80/552. For FSH, the method has been standardized against the Enzymun-Test FSH method which was standardized against the 2nd International Reference Preparation WHO reference material 78/549. AMH was standardized against the Beckman Coulter AMH Gen II ELISA (unmodified version without predilution). For in-run quality assessment, a number of quality control steps were implemented because no certified reference materials exist for SHBG, AMH, FSH, and LH. The instrument was calibrated for each reagent kit four times throughout the study. Instrument manufacturer QCs, three-level in-house bench QCs, and a low/high blind QC material were run for each analyte alongside the SRM 971a samples. Prior to running the study samples, College of American Pathologists (CAP) survey samples were run for SHBG.

Analytical Approach for Determination of Thyroglobulin: Non-certified values for thyroglobulin were determined at the Mayo Clinic using LC-MS/MS. Six aliquots were analyzed using the method described by Netzel et al. (cited method Tg-MS-1) [11] with slight changes. High molecular weight proteins (> 80 kDa) were precipitated with ammonium sulfate. Pellets were re-suspended, reduced and alkylated. Stable isotope-labeled (^{13}C) IS peptide was added, and specimens were trypsin-digested for 16 h. Antibody coated mass spectrometric immunoassay disposable automation research tips (Thermo Fisher Scientific) were added to capture the proteotypic Tg peptide (FSPDDSAGASALLR), which was assayed post elution by multiple reaction monitoring (MRM) LC-MS/MS (AB SCIEX 6500).

Analytical Approach for Determination of Thyroglobulin Antibodies: Non-certified values for Tg antibodies were determined at the Mayo Clinic using FDA approved automated immunoassays on a Beckman Access (Beckman Coulter) per the manufacturer's instructions. Three aliquots were analyzed using the method described by Netzel et al. [12]. The Tg antibody assays were standardized against the WHO Anti-Thyroglobulin Serum, Human, NIBSC code: 65/093 international reference reagent.

Period of Validity: The non-certified values are valid within the measurement uncertainty specified until **01 May 2029.** The value assignments are nullified if the material is stored or used improperly, damaged, contaminated, or otherwise modified.

Maintenance of Non-Certified Values: NIST will monitor this material to the end of its period of validity. If substantive technical changes occur that affect the non-certified values during this period, NIST will update this Appendix. Before making use of any of the values delivered by this material, users should verify they have the most recent version of this documentation, available through the NIST SRM website (https://www.nist.gov/srm).

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APPENDIX B

Laboratories Contributing Data to the Value Assignment of SRM 971a

Coordination of the collaborative measurements of the SRM at the Centers for Disease Control and Prevention (CDC) was performed by H.W. Vesper. Analytical measurements at the CDC were performed by H.W. Vesper, K. Poynter, U. Danilenko, C. Ulmer, A. Doty, L. Duke, and P. Kim of the Hormone Reference Laboratory, Clinical Chemistry Branch, Division of Laboratory Sciences, the National Center for Environmental Health (Atlanta, GA).

Coordination of the collaborative measurements of the SRM at the Mayo Clinic was performed by R. Singh and S. Grebe. Analytical measurements at the Mayo Clinic were performed by J. Kemp of the Department of Medicine, Division of Endocrinology (Rochester, MN).

Analytical measurements were conducted by A.S.P. Boggs, M.A. Nelson, K.R. Huncik, and J. Camara of the NIST Chemical Sciences Division, and S.E. Long, formerly of NIST.

Statistical analysis was performed by T. Blaza of the NIST Statistical Engineering Division.

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