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Certification of Standard Reference Material® 3666

Albumin and Creatinine in Frozen Human Urine

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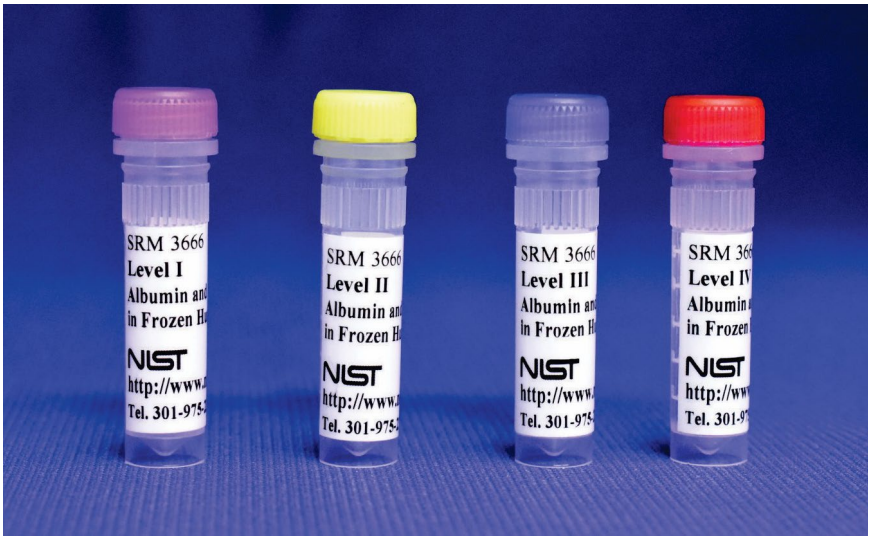

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Abstract

The National Institute of Standards and Technology (NIST) Standard Reference Material® (SRM®) 3666 Albumin and Creatinine in Frozen Human Urine delivers certified values for albumin and creatinine in human urine. The material is intended for 1) use in validating measurement procedures and 2) use in qualifying control materials produced in-house and analyzed using measurement methods for the determination of albumin, creatinine, or the albumin-to-creatinine ratio (ACR) in human urine. A unit of SRM 3666 consists of four (4) vials of frozen pooled human urine with four (4) difference levels (Level I to Level IV) of endogenous albumin determined using the NIST candidate reference measurement procedure (RMP) for albumin in urine [1]; and creatinine determined using the NIST RMP for creatinine in serum [2]. The certified values and associated uncertainties for albumin and creatinine were used to calculate the ACR for each level (Level I to Level IV). This publication documents the production, measurement processes, results, and statistical evaluations involved in the production and certification of SRM 3666.

Keywords

Albumin, Albumin-to-Creatinine Ratio (ACR), Creatinine, Human Urine, Reference Measurement Procedure (RMP), Standard Reference Material (SRM).

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EXECUTIVE SUMMARY

Patient care decisions made by healthcare practitioners for disease diagnosis and management, are influenced by the validity of clinical laboratory results. Uniformity of clinical laboratory results is essential for healthcare practitioners to provide accurate and consistent patient care. When clinical results are standardized, the clinical value is precise, equivalent, and independent of method or laboratory. Equivalent clinical results can be achieved by establishing metrological traceability of the results to higher-order reference materials and measurement procedures. However, when clinical results are not standardized, meaning a different value may be obtained for the same clinical sample using different methods or clinical laboratories, the entire spectrum of patient care can be affected, from the delivery of erroneous medical decisions to inflated healthcare costs. Therefore, metrological traceability is applied to establish a traceability framework that underpins the confidence and global comparability of clinical results used in the diagnosis and management of disease.

ACKNOWLEDGEMENTS

The authors thank Dr. Greg Miller and Dr. Lorin Bachmann of the Virginia Commonwealth University (VCU) for conducting the preliminary assessments using the routine clinical assays for albumin and creatinine and the International Federation of Clinical Chemistry (IFCC) Working Group for the Standardization of Albumin Assays in Urine (WG-SAU) Laboratory Working Group (in collaboration with the National Institute of Diabetes and Digestive and Kidney Diseases).

1. INTRODUCTION

Kidney disease is a major global public health issue, with chronic kidney disease (CKD) representing one of the most prominent causes of death worldwide (12th leading cause of death in 2017). It was estimated in 2017 that more than 10 % of the general global population was diagnosed with CKD, which totals to greater than 800 million individuals [3]. Based on data from the National Health and Nutrition Examination Survey (NHANES; 2017 to 2020), an estimated 15 % of adults in the United States (37 million individuals) have been diagnosed with CKD [4,5]. The public health and economic impact of CKD and other renal diseases has led to the need for the accurate detection of kidney disease biomarkers, such as urine albumin, for early diagnosis, evaluation of treatment efficacy, and disease management.

Urine albumin is a major diagnostic and prognostic biomarker of renal disease and is used for clinical decisions associated with renal therapy. Due to the clinical importance of urine albumin, accurate and precise measurement is key for the early detection of renal dysfunction and evaluation of treatment efficacy. Normal excretion of protein in urine is less than 150 mg/L per 24 h and normal urine is composed of approximately 10 mg/L of albumin [6]. Due to the abundance of albumin in human plasma and subsequent presence in human urine, albumin has become a key protein used in the assessment of urinary excretion of plasma proteins. Albumin is a globular protein produced in the liver and functions as a transport protein in plasma and a regulator of plasma oncotic pressure [7]. Mature, native albumin contains 585 amino acids (removal of signal sequence amino acids 1 to 24) arranged into three distinct domains (Fig. 1) [7]. Normal urine albumin levels range from 0 mg/L to 30 mg/L (normoalbuminuria); however, increased excretion of albumin in urine is divided into two groups: microalbuminuria (30 mg/L to 300 mg/L) and macroalbuminuria (>300 mg/L) [8]. To selectively measure albumin in urine, current clinical methodologies utilize affinity-based techniques, such as enzyme-linked immunosorbent assays (ELISAs) and immunoturbidity assays [9-10]. These affinity-based methods are routinely used in clinical laboratories to detect albumin in urine; however, there are distinct measurement challenges that affect the accuracy and precision of clinical results. Currently, no reference material for urine albumin exists to support clinical methods. Most clinical urine albumin results are traceable to ERM-DA470 (Institute for Reference Materials and Measurements; Geel, Belgium), a higher-order serum protein reference material with an assigned concentration value of 39.7 g/L (39,700 mg/L) for albumin, which is over 1000-fold higher than the clinical range for microalbuminuria (30 mg/L to 300 mg/L) [8, 11-13].

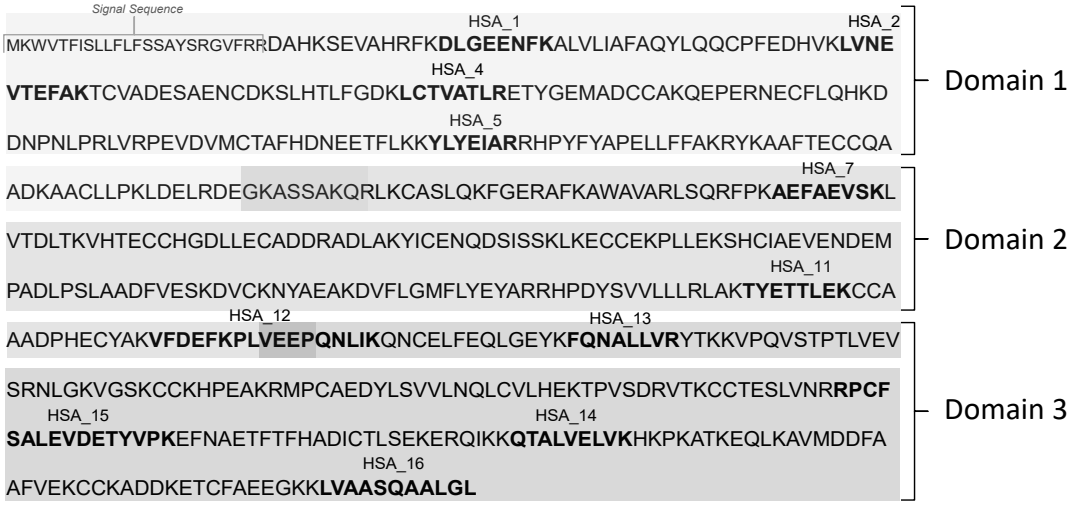

Fig. 1. Amino acid sequence of albumin (human serum albumin, HSA) illustrating the 23 MRM transitions used in NIST candidate RMP [1] in bold and the three (3) domains (I, II and III).

To support the accuracy and comparability of clinical urine albumin measurements, NIST has partnered with the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and the International Federation of Clinical Chemistry (IFCC) Working Group for the Standardization of Albumin Assays in Urine (WG-SAU) to develop a reference measurement system for urine albumin. A reference measurement system, as defined in ISO 17511, is a "*measuring system accepted as fit for its intended purpose in assessing or establishing measurement trueness for quantity values obtained from other MPs for the measurand; comprised of: 1) a unit of measurement, 2) a definition of the measurand, 3) RMP(s), 4) RM(s), and 5) one or more laboratories providing reference measurement services*" [14]. The reference materials and measurement procedures in the urine albumin reference measurement system will create an unbroken chain that links routine clinical results to the International System of Units (SI) (Fig. 2) [14]. Figure 2 illustrates the reference materials and measurement procedure developed by NIST in the traceability hierarchy to support clinical urine albumin measurements [14]. NIST has developed a candidate RMP [1] for detection of albumin in urine and a series of higher-order reference materials, SRM 2925 Recombinant Human Serum Albumin Solution (Primary Reference Calibrator for Urine Albumin, Frozen) [15, 16] and SRM 3666 Albumin and Creatinine in Frozen Human Urine. The NIST candidate RMP is a targeted, multiplexed procedure that incorporates isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS), SRM 2925 (unlabeled calibrant), and a full-length isotopically labeled (^{15}N) internal standard (IS) for absolute quantification of albumin in urine [1]. The NIST candidate RMP and validation attributes are detailed in Ref. [1]. The candidate RMP is intended for use in the value-assignment of albumin in SRM 3666. SRM 2925 is a highly pure solution of recombinant human serum albumin (HSA) and is intended for use in the calibration of LC-MS/MS procedures for the determination of albumin in urine [15, 16]. SRM 2925 serves as a primary reference material for the urine albumin reference measurement system (Fig. 2) and is currently listed in the Joint Committee for Traceability in Laboratory Medicine (JCTLM) database for reference materials [17]. The JCTLM database is an internationally recognized database of higher-order reference materials for clinical applications and inclusion of SRM 2925 in the database is an initial step in establishing a reference measurement system for urine albumin to support global comparability of

clinical results [17]. SRM 3666 is a four (4)-level (Level I to Level IV) human urine material intended for use as a secondary reference material to support the accuracy and comparability of clinical urine albumin and urine creatinine results used in clinical decisions for kidney disease. The material is intended for 1) use in validating measurement procedures and 2) use in qualifying control materials produced in-house and analyzed using measurement methods for the determination of albumin, creatinine, or the albumin-to-creatinine ratio (ACR) in human urine by in vitro diagnostic (IVD) manufacturers or clinical laboratories. This document outlines the production, value-assignment, and statistical evaluations of SRM 3666.

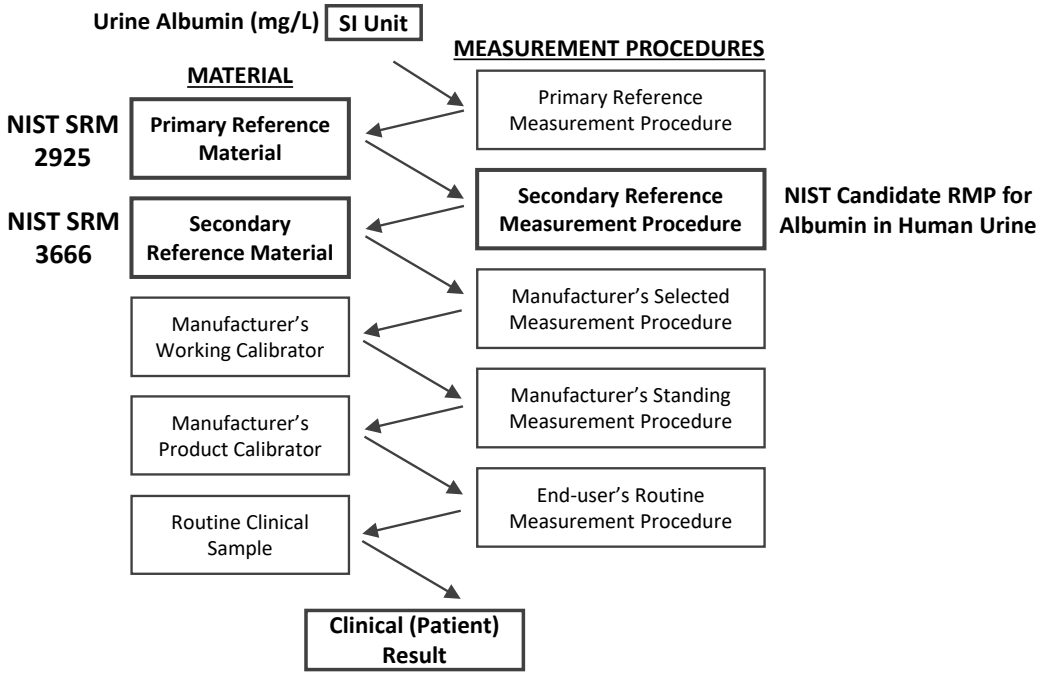

Fig. 2. The proposed full metrological traceability framework for clinical urine albumin results using NIST SRMs and candidate RMP [Adapted from ISO 17511, Ref. 14]

2. PRODUCTION OF SRM 3666

An open solicitation for quotations was issued for the acquisition and pooling/packaging of the human urine material used for preparation of SRM 3666 (Level I to Level IV). The following Sections summarize the scope for the material acquisition and pooling/packaging contracts.

2.1. Acquisition of Human Urine Material for SRM 3666

The material acquisition contract describes the requirements for collection of fresh, non-frozen single donor human urine specimens with target endogenous urine albumin levels within the concentration ranges and quantities outlined in Table 1. The material acquisition contract (Contract# SB134117SE0320) was awarded to Virginia Commonwealth University (VCU).

Table 1. Target endogenous albumin ranges and quantities each level (Level I to Level IV)

SRM 3666 Level	Target Endogenous Albumin Ranges (mg/L)	Vial Cap Color	Volume of Total Human Urine (mL)	Total Number of Specimen Donors	Volume of Urine per Specimen Donor (mL)
Level I	5 mg/L to 10 mg/L	Purple	2700	54	50
Level II	20 mg/L to 50 mg/L	Yellow	2700	54	50
Level III	60 mg/L to 180 mg/L	Blue	2700	54	50
Level IV	200 mg/L to 600 mg/L	Red	2700	54	50

The fresh, individually packaged single donor human urine specimens were stored at ≤ -70 ºC and the frozen specimens (in batches, with a minimum of 12 shipments) were shipped overnight on dry-ice to a NIST-specified institution for further processing (pooling and packaging). There were no donor specifications regarding age, gender, ethnicity, body mass index (BMI), or health status for the specimen collection process. All single donor urine specimens with a positive result for: urine nitrites, leukocyte esterase, or the presence of blood in urine using an FDA-approved urinalysis dipstick diagnostic assay were excluded. Through visual inspection, urine specimens with a color other than pale yellow, yellow, or amber were also excluded. The fresh urine specimens were stored in the original sterile collection containers at room (ambient) temperature (20 ºC to 25 ºC) for no more than 6 h after collection. Following collection, the urine specimen was centrifuged at 2000 × g_n for 10 min at low temperature (2 ºC to 5 ºC). The urine supernatant was transferred to one or more 50 mL sterile polypropylene containers and stored at ≤ -70 ºC. To ensure the urine specimen possessed endogenous urine albumin within the target clinical intervals listed in Table 1, a routine clinical assay for urine albumin was used to measure the endogenous urine albumin content in each urine specimen. No albumin was spiked-in nor were the high endogenous urine albumin levels diluted to achieve the target urine albumin concentrations. Each single donor urine specimen was labeled with the following information: a donor code, container number (if multiple containers per donor), and the urine albumin level (Level I to Level IV).

2.2. Pooling and Packaging of SRM 3666

The material pooling and packaging contract describe the requirements for pooling, processing, and vialing of the multi-level (Level I to Level IV) human urine material. The material pooling and packaging contract (Contract# SB134118SE0090) was awarded to Solomon Park. The frozen single donor human urine specimens were shipped to Solomon Park by VCU. Prior to processing, the frozen material was thawed at 20 ºC to 25 ºC (room temperature) for a maximum of 2 h and each level of SRM 3666 was processed separately. The thawed material was mixed with gentle inversion (10 cycles) and centrifuged at 2000 × g_n for 10 min at low temperature (2 ºC to 8 ºC). The single donor specimens were combined according to the composition specified by NIST to yield a total volume for each level of 2.7 L. The pooled bulk material was mixed (gentle) overnight (12 h to 18 h, not to exceed 18 h) at 2 ºC to 5 ºC to allow any precipitates or aggregates to form. After mixing, the pooled bulk material was placed on a sterile 0.2 µm polyvinylidene fluoride (PDVF; low-protein binding membrane filter) filtration device at 2 ºC to 5 ºC. Approximately 1 mL (± 0.05 mL) of filtered bulk pooled material was aliquotted into 1 mL sterile polypropylene screw cap vials at a temperature between 2 ºC to 5 ºC. A different vial cap color was used for each level (Level I to Level IV) to visually differentiate the four levels (Table 1). The frozen vials of SRM 3666 (Level I to Level IV) were packaged and shipped to NIST.

2.3. Preliminary Assessment of Packaged Human Urine Material

To evaluate the suitability of the packaged human urine material for use as SRM 3666, preliminary measurements of the albumin and creatinine content were conducted by NIST and VCU (through a collaboration). A total of twelve (12) vials of SRM 3666 were selected for the preliminary assessment, which represents three (3) vials selected from each level (Level I to Level IV) spanning the entire lot. The preliminary assessment sampling plan is outlined in Appendix A, Table A1. A minimum of two (2) technical replicates per vial were performed for each method.

To confirm the endogenous albumin levels for the packaged human urine material were within the target ranges (outlined in Table 1), the endogenous urine albumin content was assessed using a routine clinical microalbumin assay (Abbott Microalbumin Method) by VCU. The Abbott Microalbumin assay is a turbidimetric immunoassay that utilizes an anti-human albumin polyclonal antibody to detect albumin in human urine. The polyclonal antibody-albumin complex forms an insoluble aggregate that increases the turbidity of the solution. The degree of turbidity is proportional to the concentration of albumin in the urine specimen and is measured optically on the Abbott Architect c8000 instrument. The endogenous urine creatinine content was assessed via a routine clinical creatinine assay (Abbott Creatinine Method) by VCU. The Abbott Creatinine assay is a colorimetric method that applies the Jaffe reaction to determine creatinine content in human urine. Under alkaline conditions, creatinine reacts with picrate to form a creatinine-picrate complex. The level of complex formation is directly proportional to the concentration of creatinine in the sample. In addition to the routine clinical assays, the NIST candidate RMP for albumin in urine [1] and a modification of NIST RMP for creatinine in serum [2] were also used in the preliminary assessment of the packaged human urine material. The preliminary assessment results of the human urine material from the routine clinical assays (VCU) and the MS-based methods (NIST) are listed in Appendix A, Tables A2 and A3. Preliminary assessment of the endogenous albumin content of the human urine material supports the use of the material as SRM 3666.

3. CERTIFICATION MEASUREMENTS OF SRM 3666

3.1. Density of SRM 3666

Density values were needed for unit conversion (mass fraction to mass concentration) of the certified values and were determined by the Lang-Levy pipet method [18]. A 500 µL Lang-Levy pipet was calibrated with water at ambient room temperature (19 ºC). The water weighing was performed in four (4) replicates and the density of water at 19 ºC ($\rho_{H_2O,19\,℃}$) was determined using [19]:

$$\rho_{H_2O,19\,℃} = \frac{(999.84847+6.337563\times10^{-2}t-8.523829\times 10^{-3}t^2+6.943248\times 10^{-5}t^3-3.821216\times 10^{-7}t^4)}{1000}, \quad (1)$$

where t is the observed temperature (19 ºC) at which the water mass was measured. The volume (mL) of the pipet was determined from the water sample using:

$$V_{H_2O,19\,℃} = \frac{M_{H_2O,19\,℃}}{\rho_{H_2O,19\,℃}}, \quad (2)$$

where $M_{H_2O,19\,°C}$ is the mass of water at the observed temperature (19 ºC). The volume calibration measurements of the nominal 500 µL Lang-Levy pipet at 19 ºC are shown in Appendix B, Table B1. The volume at 19 ºC was determined to be 0.49976 mL ± 0.00092 mL (mean ± standard deviation).

To assess the density of SRM 3666, four (4) vials were randomly selected, thawed at room temperature (19 ºC), and pooled for each level (sampling plan listed in Appendix B, Table B2). The weighing procedure was repeated for each urine pool in replicate (4). The density results for SRM 3666 (Level I to Level IV) were calculated using:

$$\rho_{SRM\ 3666} = \frac{M_{SRM\ 3666,19\,°C}}{V_{H_2O,19\,°C}}. \quad (3)$$

The certified density values for SRM 3666 (Level I to Level IV) are shown in Table 2. Statistical analysis of the data for the determination of density for SRM 3666 was provided by the NIST Statistical Engineering Division (SED).

Table 2. Certified Human Urine Density of SRM 3666 at 19 °C

Material Level	Measurand	Density [a] (g/mL)
Level I	Human Urine Density at 19 °C	1.01519 ± 0.00058
Level II	Human Urine Density at 19 °C	1.01397 ± 0.00048
Level III	Human Urine Density at 19 °C	1.01784 ± 0.00058
Level IV	Human Urine Density at 19 °C	1.01549 ± 0.00070

[a]Values are expressed as $x \pm U(x)$, where x is the certified value and $U(x)$ is the expanded uncertainty of the certified value. The expanded uncertainty is calculated as $U(x) = ku_c$, where u_c is the combined uncertainty, and k is the coverage factor. For the certified values shown in Table 2, $k = 2$. The true value of the analyte is believed to lie within the interval $x \pm U(x)$. To propagate this uncertainty, treat the certified value as a normally distributed random variable with mean x and standard deviation $U(x)/2$.

3.2. Value-Assignment of Urine Albumin

The value assignment measurements for albumin in SRM 3666 Level I to Level IV detailed in this Section were performed using the NIST candidate RMP for albumin determination in human urine by ID-LC-MS/MS [1]. Certification of the albumin concentrations in SRM 3666 was performed at NIST and the statistical analysis of the data was provided by NIST SED.

3.2.1. Materials

NIST SRM 2925 Recombinant Human Serum Albumin Solution (Primary Reference Calibrator for Urine Albumin) (Frozen) [2,3]; ^{15}N-labeled recombinant human serum albumin (*r*HSA) (Albumin Biosciences, Huntsville, AL); ammonium bicarbonate (AMBIC); Trypsin-Gold MS-grade (Promega, Madison, WI, USA); dithiothreitol (DTT, Pierce); iodoacetamide (IAM, Pierce); high-purity LC-MS grade water/0.1 % (volume fraction) formic acid and acetonitrile/0.1 % (volume fraction) formic acid (Honeywell Burdick and Jackson).

3.2.2. Certification Sampling Plan

A total of twelve (12) vials per level of SRM 3666 were randomly selected from the lot via a stratified random sampling scheme. See Appendix C, Table C1 for the certification sampling plan.

3.2.3. Certification Measurement Process

The NIST candidate RMP incorporates a full-length ^{15}N-labeled IS to selectively target and quantify full-length albumin in human urine with a high degree of accuracy and precision [1]. The MRM (multiple reaction monitoring) chromatographic and MRM retention time (RT) profiles (Appendix C, Fig. C1 and C2) of the SRM 3666 samples (unlabeled, endogenous albumin) and the IS (^{15}N-labeled *r*HSA) were consistent across all four levels (Level I to Level IV). Performance of the IS was also consistent across the 23 MRM transitions of each level (Level I to Level IV), as shown in the normalized IS peak area plot Appendix C, Fig. C3. Five (5) 3-point calibration curves, representing the 5 *qt*-MRM transitions, were generated using the concentration and peak area ratios of the calibration solutions for each level of SRM 3666 (Level I to Level IV) (Appendix C, Fig. C4).

Consensus means analysis of the albumin mass fraction (mg/g) results for the *qt*-MRM transitions was conducted (n = 160; 5 MRM transitions, twelve (12) vials and one (1) process replicate) for each level (Level I to Level IV) to determine the overall endogenous albumin content in each level of SRM 3666. The certified density values of SRM 3666 (Level I to Level IV) (Table 2) were used for the unit conversion from mass fraction (mg/g) to mass concentration (mg/L). The consensus mass fraction values ($\bar{\bar{X}}_{mg/g}$) and mass concentration values ($\bar{\bar{X}}_{mg/L}$) for albumin in SRM 3666 (Level I to Level IV) are shown in Table 3 [1, 20].

Table 3. Certified Values for Albumin in SRM 3666

Material Level	Mass Fraction (mg/g) (Mean ± Expanded Uncertainty[a])	Mass Concentration[b](g/mL) (Mean ± Expanded Uncertainty[a])
Level I	0.008 ± 0.001	8.28 ± 1.12
Level II	0.031 ± 0.003	31.11 ± 2.64
Level III	0.111 ± 0.011	112.77 ± 10.79
Level IV	0.355 ± 0.031	360.50 ± 31.11

[a]Values are expressed as $x \pm U(x)$, where x is the certified value and $U(x)$ is the expanded uncertainty of the certified value. The expanded uncertainty is calculated as $U(x) = ku_c$, where u_c is the combined uncertainty, and k is the coverage factor. For calculation of the combined uncertainty for albumin certified values see Ref. 3. For the certified values shown in Table 2, $k = 2$. The true value of the analyte is believed to lie within the interval $x \pm U(x)$. To propagate this uncertainty, treat the certified value as a normally distributed random variable with mean x and standard deviation $U(x)/2$.
[b]Mass concentration values were calculated from mass fraction values using the measured human urine density for each level, listed in Table 2.

The certified albumin mass concentration values for SRM 3666 are consistent with the expected target clinical ranges and the preliminary results obtained via the routine clinical assay and the NIST candidate RMP [1], as shown in Appendix C, Table C2.

3.3. Value-Assignment of Urine Creatinine

The value assignment measurements for creatinine in SRM 3666 detailed in this Section were performed using a modification of the NIST ID-LC-MS RMP for creatinine in serum [2]. Certification of the creatinine concentrations in SRM 3666 (Level I to Level IV) was performed at NIST and the statistical analysis of the data for the determination of creatinine in SRM 3666 was provided by NIST SED.

3.3.1. Materials

NIST SRM 914b Creatinine (mass purity of 99.9 % ± 0.1 %, Certificate of Analysis); stable isotope labeled IS material (*d3*-creatinine, Cayman Chemical Company); NIST SRM 3667 (quality control material); all calibration solutions, urine sample dilutions, and mobile phase were prepared using HPLC-grade water from Burdick & Jackson; HPLC-grade ammonium acetate (Fisher Scientific); and Hydrochloric (HCl) acid solution (2 mol/L, Fluka).

3.3.2. Certification Sampling Plan

See Appendix C, Table C1 for the certification sampling plan.

3.3.3. Certification Measurement Process

Calibration solutions, which contained known unlabeled:labeled creatinine mass ratios, were gravimetrically prepared. The IS solutions were added to all samples (SRM 3666 and SRM 3667) at the beginning of sample preparation process. The processed samples (calibration solutions, SRM 3667 quality control solutions, and SRM 3666 samples) were analyzed via liquid chromatography-mass spectrometry (LC-MS) and the unlabeled:labeled creatinine peak area ratios of the SRM 3667 and SRM 3666 samples were converted to mass ratios using data from the calibration curves generated from the calibration solutions. The mass ratios were then solved for the mass of the unlabeled creatinine, and the concentration of unlabeled creatinine in each sample was calculated.

Four (4) stock solutions of creatinine were gravimetrically prepared with mass fractions of ≈ 44 µg/g to 87 µg/g and the IS solution was gravimetrically prepared with an estimated mass fraction of ≈ 100 µg/g. From the stock solutions, working solutions were gravimetrically prepared, containing both unlabeled creatinine and IS. Calibration solution mixes were then diluted to a final volume of 1 mL with water, this resulted in calibrants containing ≈ 4 µg to 6 µg of creatinine and ≈ 5 µg of d_3-creatinine. Prior to processing, the SRM 3666 samples and quality control samples (SRM 3667) were thawed at room temperature (19 °C) for 1 h. The SRM 3667 control urine samples were prepared in duplicate from one vial on each of four days of analysis and the SRM 3666 urine samples were prepared in duplicate from twelve vials of each of four levels. The twelve (12) vials of SRM 3666 for each level were equally distributed over four separate preparation and analysis runs. All LC-MS analyses were performed on an Agilent 1200 Series LC system with an Agilent 6130 Quadrupole LC-MS. The column utilized was a Phenomenex (Torrance, CA) Luna C18(2), 25 cm × 4.6 mm, 5 µm particle. Selected ion monitoring (SIM) was used to detect creatinine at *m/z* 114 and d_3-creatinine at *m/z* 117.

An IS approach and relative response factor (RRF) were used for calibration according to the equation [2]:

$$RRF = \frac{(A_{Creatinine,Cal} \times M_{IS,Cal})}{(A_{IS,Cal} \times M_{Creatinine,Cal} \times P_{creatinine})}, \quad (4)$$

where $A_{Creatinine,Cal}$ represents the peak area for creatinine (unlabeled) for the calibration solution, $M_{IS,Cal}$ represents the mass of the IS in the calibration solution, $A_{IS,Cal}$ represents the peak area for the IS in the calibration solution, $M_{Creatinine,Cal}$ represents the mass of creatinine (unlabeled) in the calibration solution, and $P_{creatinine}$ represents purity correction factor for creatinine.

The RRF was averaged from duplicate injections of each of four (4) independently prepared calibration solutions over a narrow-bracketed mass ratio range (0.8 to 1.2 mass ratio, creatinine: d_3-creatinine). The RRF was applied to samples to determine the mass fraction of creatinine according to the equation [2]:

$$Mass\ Fraction\ \left(\frac{\mu g}{g}\right) = \frac{(A_{Creatinine,S} \times M_{IS,S})}{(A_{IS,S} \times M_S \times RRF)}. \quad (5)$$

where $A_{Creatinine,S}$ represents the peak area for creatinine (unlabeled) in the sample, $M_{IS,S}$ represents the mass of the IS in the sample, $A_{IS,S}$ represents the peak area for the IS in the sample, and M_S represents the mass of creatinine (unlabeled) in the sample.

The mean RRF values for the LC-MS runs were 1.142 (set 1), 1.354 (set 2), 1.154 (set 3), and 1.145 (set 4). Example chromatograms of SRM 3666 Level I, Level II, Level III, and Level IV are shown in Appendix D, Fig. D1-D4, respectively.

The overall ID-LC-MS creatinine values (consensus value ± expanded uncertainty; purity corrected for the known mass % purity of SRM 914b) for Level I, Level II, Level III, and Level IV are listed in Table 4.

Table 4. Certified Values for Creatinine in SRM 3666

Material Level	Mass Fraction (µg/g) (Mean ± Expanded Uncertainty[a])	Mass Concentration[b] (mg/dL) (Mean ± Expanded Uncertainty[a])
Level I	1178.96 ± 25.05	119.69 ± 2.63
Level II	1209.58 ± 26.28	122.65 ± 2.73
Level III	1249.58 ± 26.37	127.19 ± 2.78
Level IV	1289.01 ± 27.71	130.90 ± 2.96

[a]Values are expressed as $x \pm U(x)$, where x is the certified value and $U(x)$ is the expanded uncertainty of the certified value. The expanded uncertainty is calculated as $U(x) = ku_c$, where u_c is the combined uncertainty, and k is the coverage factor. For the certified values shown in Table 2, $k = 2.201$. The true value of the analyte is believed to lie within the interval $x \pm U(x)$. To propagate this uncertainty, treat the certified value as a normally distributed random variable with mean x and standard deviation $U(x)/2.201$.
[b]Mass concentration values were calculated from mass fraction values using the measured human urine density for each level, listed in Table 2.

The creatinine values (consensus ± expanded uncertainty) measured by the NIST ID-LC-MS RMP [2] are consistent with the preliminary assessment values provided by a routine clinical assay (Jaffe, VCU) (Appendix D, Table D1).

3.4. Value-Assignment of Albumin-to-Creatinine Ratio (ACR)

The value assignment results for albumin-to-creatinine ratio (ACR) in SRM 3666 (Level I to Level IV) detailed in this Section was determined from the certified mass concentration values and associated uncertainties of both albumin (Table 3) and creatinine (Table 4). Statistical analysis of the data for the determination of ACR in SRM 3666 was provided by NIST SED.

3.4.1. Certification Measurement Process

The ACR certified value for each level was determined from a ratio of the certified mass concentration values for albumin to creatinine using the following:

$$ACR = \frac{A}{C}, \tag{6}$$

where A represents the certified mass concentration value for albumin and C represents the certified mass concentration value for creatinine.

To determine the uncertainty of the ACR certified value, the propagation of uncertainty (also called propagation of error) was applied. Propagation of uncertainty is a technique for determining the approximate uncertainty of a function of two or more variables where the uncertainties of the individual variables are known [22-25]. Propagation of uncertainty formulas have been determined for a number of commonly used functions, including the ratio of two variables [22-25]. The certified values for albumin and creatinine were determined using separate measurement procedures and samples. Therefore, they can be reasonably considered independent and the covariance terms in the propagation of uncertainty formula can be ignored. The resulting propagation of uncertainty formula used to determine the standard uncertainty of the certified ACR value is:

$$u_{ACR} = \left|\frac{A}{C}\right| \times \sqrt{\left(\frac{u_A}{A}\right)^2 + \left(\frac{u_C}{C}\right)^2}. \tag{7}$$

where A represents the certified value for albumin (mass concentration, mg/L), u_A represents the combined standard uncertainty for albumin, C represents the certified value for creatinine (mass concentration, mg/dL) , and u_C represents the combined standard uncertainty for creatinine.

The ACR (mg/g) certified values and associated standard uncertainty and expanded uncertainty ($k = 2$) for SRM 3666 (Level I to Level IV) are listed in Table 5.

Table 5. Certified Values for ACR in SRM 3666

Material Level	Value[b] (mg/g) (Mean ± Expanded Uncertainty[a])
Level I	6.92 ± 0.95
Level II	25.36 ± 2.22
Level III	88.66 ± 8.70
Level IV	275.40 ± 24.57

[a]Values are expressed as $x \pm U(x)$, where x is the certified value and $U(x)$ is the expanded uncertainty of the certified value. The expanded uncertainty is calculated as $U(x) = ku_c$, where u_c is the combined uncertainty, and k is the coverage factor. For the certified values shown in Table 2, $k = 2$. The true value of the analyte is believed to lie within the interval $x \pm U(x)$. To propagate this uncertainty, treat the certified value as a normally distributed random variable with mean x and standard deviation $U(x)/2$.
[b]The albumin-to-creatinine ratio (ACR) is based on the combination of the certified values for albumin (Table 3) and creatinine (Table 4).

4. HOMOGENEITY OF SRM 3666

Homogeneity of the material was assessed prior to the certification analyses. A stratified sampling plan was devised to test for homogeneity across the lot. There was no apparent trend in the data when plotted against the sequence in which the vials were filled.

5. STABILITY OF SRM 3666

Short-term stability was assessed following the certification analyses. A random sampling scheme was used to examine the degree of degradation of albumin associated with the potential temperature conditions encountered during shipment from NIST to the end-user. There was no apparent trend in the data, which suggests that routine shipping temperature conditions will not affect the integrity of albumin over a 7-day period.

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Appendix A. SRM 3666 – Preliminary Assessment

This section contains supplemental information to support the preliminary assessment of albumin and creatinine in SRM 3666 (Level I to Level IV).

Table A1. Sampling scheme for preliminary assessment of albumin and creatinine in SRM 3666. The vial selection was the same for each level (Level I to Level IV)

Method	Box #	Box Vial #	Lot Vial #
NIST candidate RMP Albumin and NIST RMP for Creatinine	1	10	10
	5	72	400
	11	30	850
	16	70	1300
	20	42	1600
	30	40	2418
	31	40	2500
Abott Albumin/ Abott Creatinine	1	50	50
	15	46	1180
	31	30	2460

Table A2. Preliminary assessment results of albumin in the packaged human urine material for use as SRM 3666 via the NIST candidate RMP and a routine clinical immunoassay.

SRM 3666 Level	Target Endogenous Urine Albumin Content	Abbott Architect Microalbumin Immunoassay (mg/L), n = 6 (VCU) (Mean ± Standard Deviation)	NIST Candidate RMP (ID-LC-MS/MS) (mg/L), n = 160 (Mean ± Standard Deviation)
Level I	5 mg/L to 10 mg/L	8.1 ± 0.2	9.1 ± 0.6
Level II	20 mg/L to 50 mg/L	34.4 ± 0.1	34.9 ± 1.5
Level III	60 mg/L to 180 mg/L	100.1 ± 0.9	105.6 ± 5.2
Level IV	200 mg/L to 600 mg/L	354.9 ± 3.5	344.2 ± 19.3

Table A3. Preliminary assessment results of creatinine in the packaged human urine material for use as SRM 3666 via the modified NIST RMP and a routine clinical assay (Jaffe).

SRM 3666 Level	Abbott Architect Creatinine Assay - Jaffe (mg/dL), n = 6 (VCU) (Mean ± Standard Deviation)	NIST RMP (ID-LC-MS) (mg/dL), n = 12 (Mean ± Standard Deviation)
Level I	116.7 ± 0.4	116.0 ± 0.3
Level II	118.4 ± 0.6	118.0 ± 0.8
Level III	123.0 ± 0.7	124.8 ± 0.6
Level IV	127.4 ± 0.4	129.0 ± 0.4

Appendix B. SRM 3666 – Density Determination

This section contains supplemental information to support density determination for SRM 3666 (Level I to Level IV).

Table B1. Calibration of Lang-Levy Pipet Volume with Water.

Water Sample	Mass (g)	Density at 19 °C (g/mL)	Volume at 19 °C (mL)	Mean Volume at 19 °C (mL) (n = 4) (Mean ± Standard Deviation)	%CV
Water-1	0.50029	0.9984	0.50109	0.49976 ± 0.00092	0.18
Water-2	0.49816	0.9984	0.49896		
Water-3	0.49875	0.9984	0.49955		
Water-4	0.49865	0.9984	0.49945		

Table B2. Sampling scheme for density (19 °C) determination of SRM 3666 (Level I to Level IV).

SRM 3666 Level	Sample Label	Box #	Vial #
Level I	**1**	3	17
	2	16	77
	3	22	36
	4	28	10
Level II	**1**	6	38
	2	9	81
	3	26	22
	4	29	27
Level III	**1**	6	7
	2	13	19
	3	19	72
	4	27	57
Level IV	**1**	2	60
	2	15	27
	3	23	11
	4	24	14

Appendix C. SRM 3666 – Certification of Albumin

This section contains supplemental information to support the certification of albumin in SRM 3666 (Level I to Level IV).

Table C1. Sampling scheme for certification of endogenous albumin and creatinine in SRM 3666. The vial selection was the same for each level (Level I to Level IV).

Measurand	Box #	Vial 1 Box Location #	Vial #1 Lot Number	Vial 1 Box Location #2	Vial #2 Lot Number
Albumin	**5**	10	334	22	346
	11	53	863	80	890
	14	6	1059	56	1109
	19	21	1479	64	1522
	25	31	1975	74	2018
	30	17	2366	46	2395
Creatinine	**5**	11	335	23	347
	11	54	864	79	889
	14	7	1060	55	1108
	19	20	1478	65	1523
	25	32	1976	76	2020
	30	16	2365	45	2396

Table C2. Comparison of albumin results for the preliminary assessment and the material certification.

SRM 3666 Level	Target Endogenous Urine Albumin Content	Certified Mass Concentration of Albumin (mg/L) (NIST ID-LC-MS/MS Method), n = 280 (Mean ± Standard Deviation)	NIST Candidate RMP (ID-LC-MS/MS) (mg/L), n = 160 (Mean ± Standard Deviation)	Abbott Architect Microalbumin Immunoassay (mg/L), n = 6 (VCU) (Mean ± Standard Deviation)
Level I	5 mg/L to 10 mg/L	8.28 ± 0.56	9.1 ± 0.6	8.1 ± 0.2
Level II	20 mg/L to 50 mg/L	31.11 ± 1.32	34.9 ± 1.5	34.4 ± 0.1
Level III	60 mg/L to 180 mg/L	112.77 ± 5.39	105.6 ± 5.2	100.1 ± 0.9
Level IV	200 mg/L to 600 mg/L	360.5 ± 15.56	344.2 ± 19.3	354.9 ± 3.5

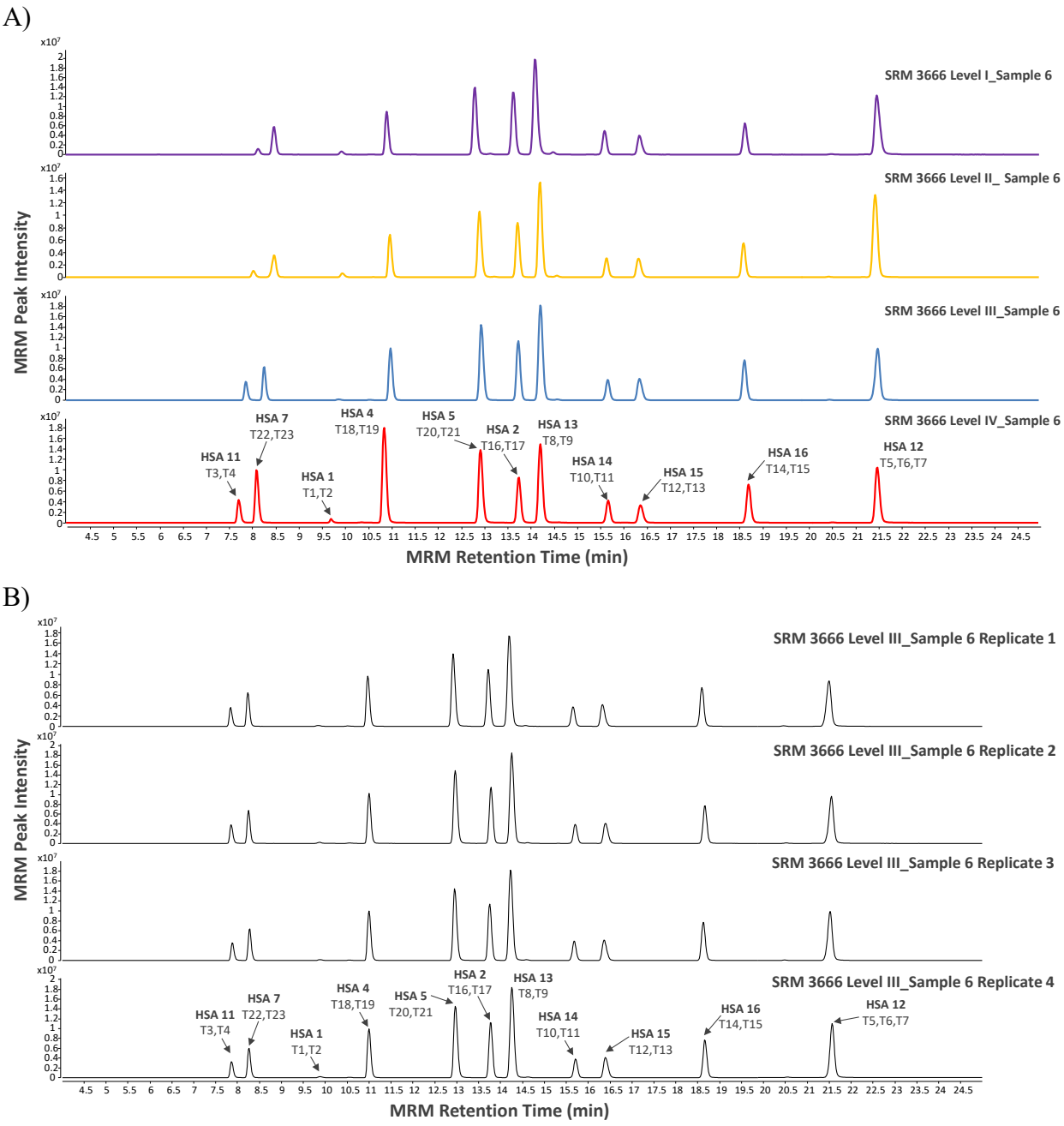

Fig. C1. Plot of MRM chromatograms containing the 11 MRM peptides for SRM 3666 selected Sample #6 (Box# 14, Vial# 1109), each level (Level I to Level IV) (A) and four technical replicates of Level III (B).

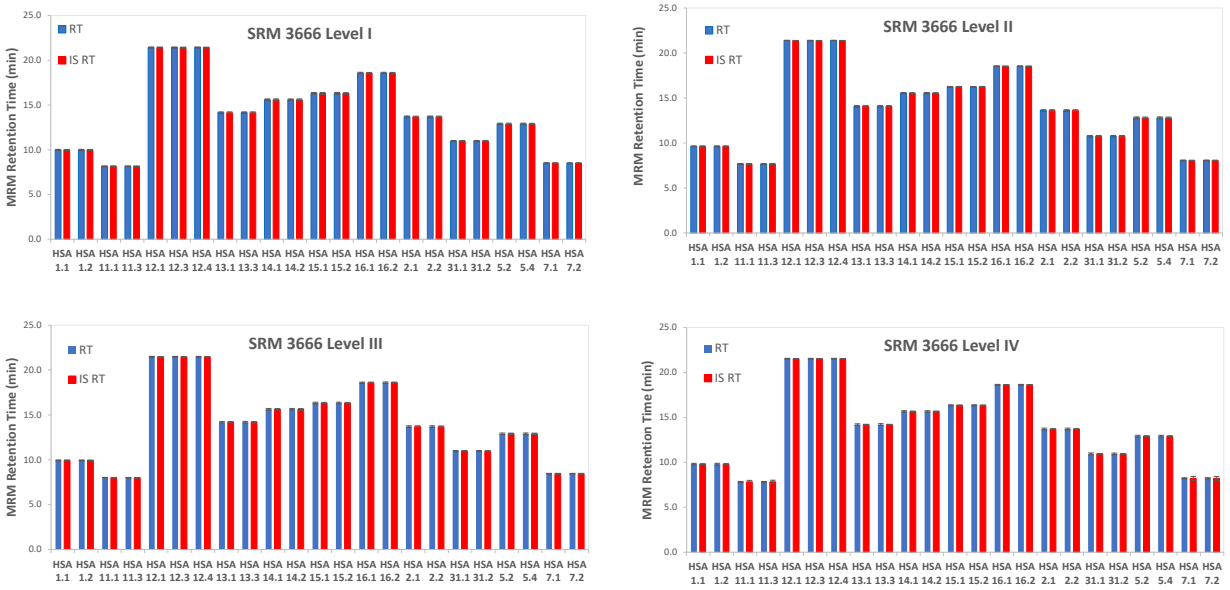

Fig. C2. MRM Retention Time (RT) profile (23 MRM Transitions) for endogenous albumin (unlabeled) and ^{15}N-Labeled recombinant HSA (IS) in SRM 3666 (Level I to Level IV). The error bars represent the standard error of the MRM RT results observed for each MRM transition. (n = 56 for each MRM transition)

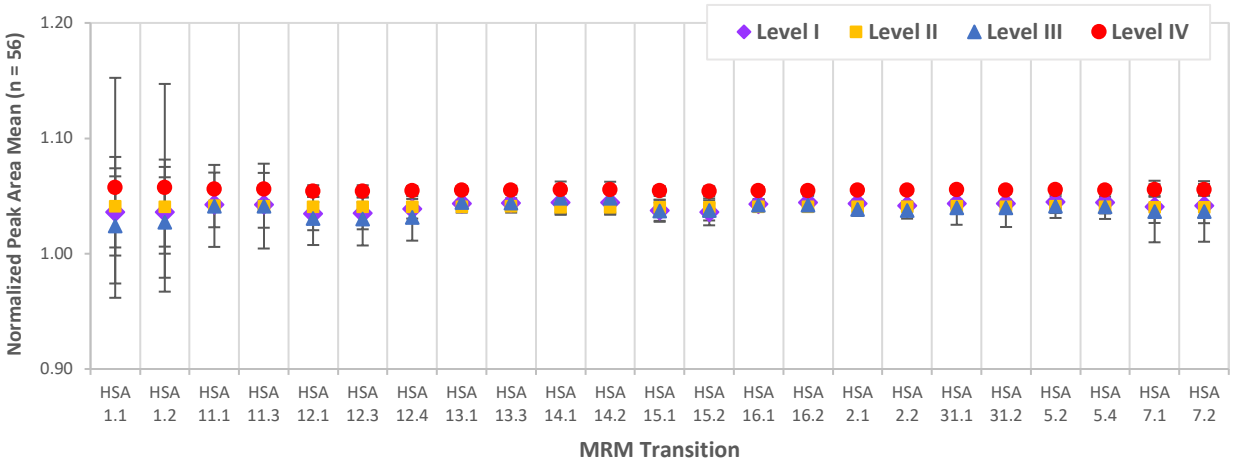

Fig. C3. Response plot of the normalized MRM peak area mean of the IS (^{15}N-labeled recombinant HSA) for each level of SRM 3666 (Level I to Level IV). The error bars represent the standard error of the MRM peak area results observed for each MRM transition (n = 56 for each MRM transition). (Level I- Purple, Level II - Yellow, Level III - Blue, and Level IV - Red)

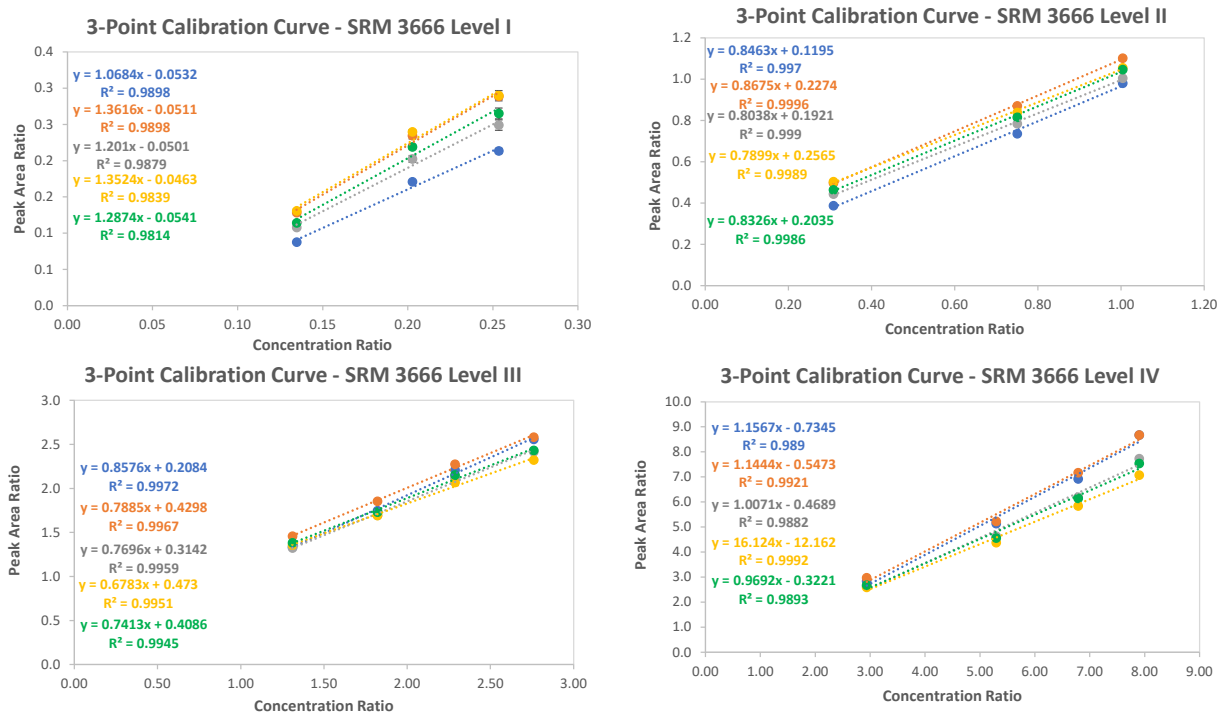

Fig. C4. Three (3)-point calibration curves for the five (5) quantitative MRM (*qt*-MRM) transitions for each level of SRM 3666 (HSA 11.1- Blue, HSA 12.4- Orange, HSA 13.3- Gray, HSA 31.1- Yellow, HSA 5.2- Green).

Appendix D. SRM 3666 – Certification of Creatinine

This section contains supplemental information to support the certification of creatinine in SRM 3666 (Level I to Level IV).

Table D1. Comparison of creatinine results for the preliminary assessment and the material certification.

SRM 3666 Level	Certified Mass Concentration of Creatinine (mg/dL) (NIST ID-LC-MS RMP), n = 12 (Mean ± Standard Deviation)	NIST RMP (ID-LC-MS) (mg/dL), n = 12 (Mean ± Standard Deviation)	Abbott Architect Creatinine-Jaffe (mg/dL), n = 6 (VCU) (Mean ± Standard Deviation)
Level I	119.7 ± 0.5	116.0 ± 0.3	116.7 ± 0.4
Level II	122.7 ± 0.8	118.0 ± 0.8	118.4 ± 0.6
Level III	127.2 ± 0.8	124.8 ± 0.6	123.0 ± 0.7
Level IV	130.9 ± 0.6	129.0 ± 0.4	127.4 ± 0.4

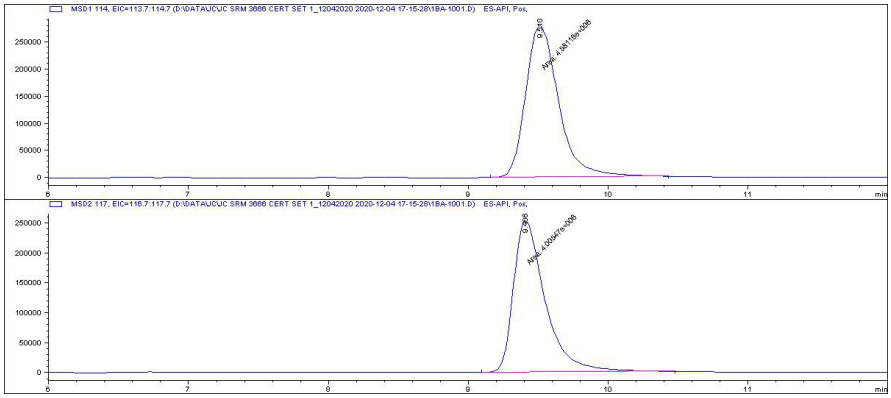

Fig. D1. Example LC-MS chromatogram of SRM 3666 Level I, box 11, vial 79, preparation 2, injection 1 (Set 1) displaying creatinine (top) and *d3*-creatinine (bottom)

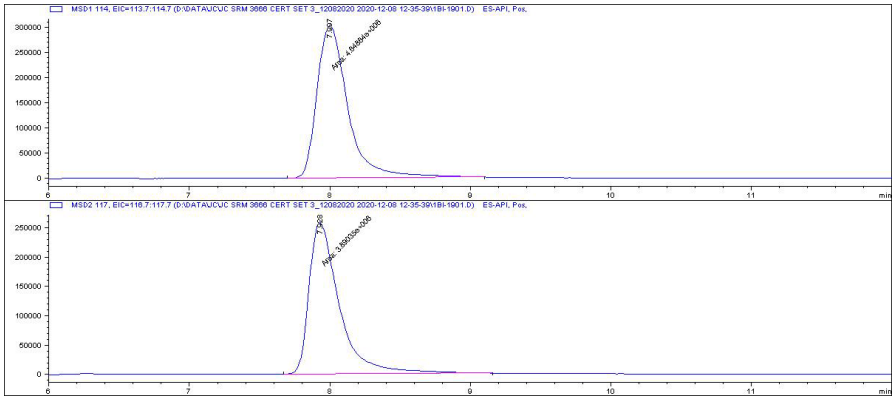

Fig. D2. Example LC-MS chromatogram of SRM 3666 Level II, box 30, vial 16, preparation 2, injection 1 (Set 3) displaying creatinine (top) and *d3*-creatinine (bottom)

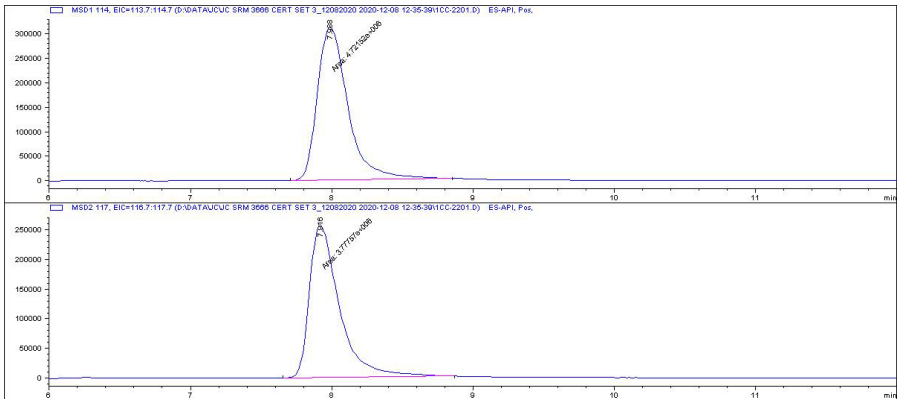

Fig. D3. Example LC-MS chromatogram of SRM 3666 Level III, box 14, vial 55, preparation 1, injection 1 (Set 3) displaying creatinine (top) and *d3*-creatinine (bottom)

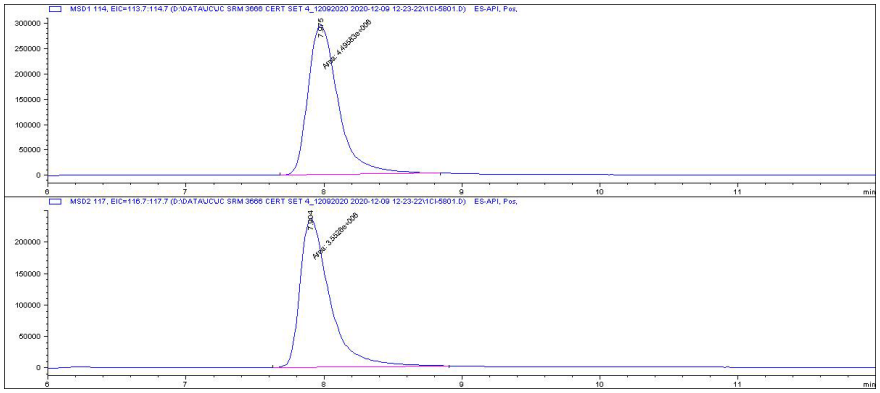

Fig. D4. Example LC-MS chromatogram of SRM 3666 Level IV, box 19, vial 65, preparation 1, injection 2 (Set 4) displaying creatinine (top) and *d3*-creatinine (bottom)

Appendix E. List of Symbols, Abbreviations, and Acronyms

ACR	albumin-to-creatinine ratio
AMBIC	ammonium bicarbonate
CKD	chronic kidney disease
COA	certificate of analysis
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
HCl	hydrochloric
HSA	human serum albumin
IAM	iodoacetamide
ID-LC-MS/MS	isotope dilution-liquid chromatography-tandem mass spectrometry
IFCC	International Federation of Clinical Chemistry
IVD	in vitro diagnostic
JCTLM	Joint Committee for Traceability in Laboratory Medicine
LC-MS	liquid chromatography-mass spectrometry
MRM	multiple reaction monitoring
NHANES	National Health and Nutrition Examination Survey
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
NIST	National Institute of Standards and Technology
PDVF	polyvinylidene fluoride
qt-MRM	quantitative MRM transition
*r*HSA	recombinant human serum albumin
RMP	reference measurement procedure
RRF	relative response factor
SED	NIST Statistical Engineering Division
SIM	selected ion monitoring
SRM	Standard Reference Material®
VCU	Virginia Commonwealth University
WG-SAU	IFCC Working Group for the Standardization of Albumin Assays in Urine