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Reference Measurement Procedure for the Absolute Quantification of Albumin in Urine Using Isotope Dilution-Liquid Chromatography-Tandem Mass Spectrometry (ID-LC-MS/MS)

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Abstract

Urinary excretion of albumin is a major diagnostic and prognostic marker of kidney disease; therefore, accurate measurement of urine albumin is essential to clinical diagnosis. To support the accuracy and comparability of clinical urine albumin measurements, the National Institute of Standards and Technology (NIST) has partnered with the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Laboratory Working Group (LWG) 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. NIST has developed the foundational components of the reference measurement system for urine albumin, a series of higher-order reference materials and a reference measurement procedure (RMP), to establish a traceability scheme that will link clinical urine albumin to the International System of Units (SI). The NIST RMP for urine albumin is based on the detection and quantification of signature proteotypic (trypsin) albumin peptides via isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) and multiple reaction monitoring (MRM). The RMP incorporates an isotopically labeled (^{15}N) full-length recombinant human serum albumin (^{15}N-rHSA) material as the internal standard, which permits the absolute quantification of albumin in urine. Out of the 11 tryptic peptides selected to monitor the albumin in urine, 5 albumin peptides were used for the absolute quantification of albumin in urine. This publication outlines the NIST RMP for the absolute quantification of albumin in urine by ID-LC-MS/MS.

Keywords

Albumin, isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS), measurement uncertainty (MU), metrological traceability, multiple reaction monitoring (MRM), recombinant human serum albumin, reference measurement procedure (RMP), Standard Reference Material® (SRM), urine.

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Executive Summary

Decisions made by healthcare practitioners regarding disease diagnosis and management are heavily influenced by the validity of clinical laboratory results. It is crucial for healthcare practitioners to have access to accurate and consistent patient care information, which can only be achieved through standardization of clinical laboratory results. When clinical results are standardized, the clinical value is precise, equivalent, and not dependent on the method or laboratory used. This 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, different values may be obtained for the same clinical sample using different methods or clinical laboratories. This can have a significant impact on patient care, from the delivery of erroneous medical decisions to inflated healthcare costs. 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.

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 [1]. 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 [2,3]. 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.

Urinary excretion of albumin is a major diagnostic and prognostic marker of kidney disease; therefore, accurate clinical urine albumin results are essential for patient care decisions. 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) Laboratory Working Group (LWG) 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. NIST has developed a series of higher-order reference materials and a reference measurement procedure (RMP) to establish a metrological traceability framework that will link clinical urine albumin to the International System of Units (SI). The traceability scheme in Figure 1, illustrates how the NIST RMP is used to support metrological traceability of clinical urine albumin results.

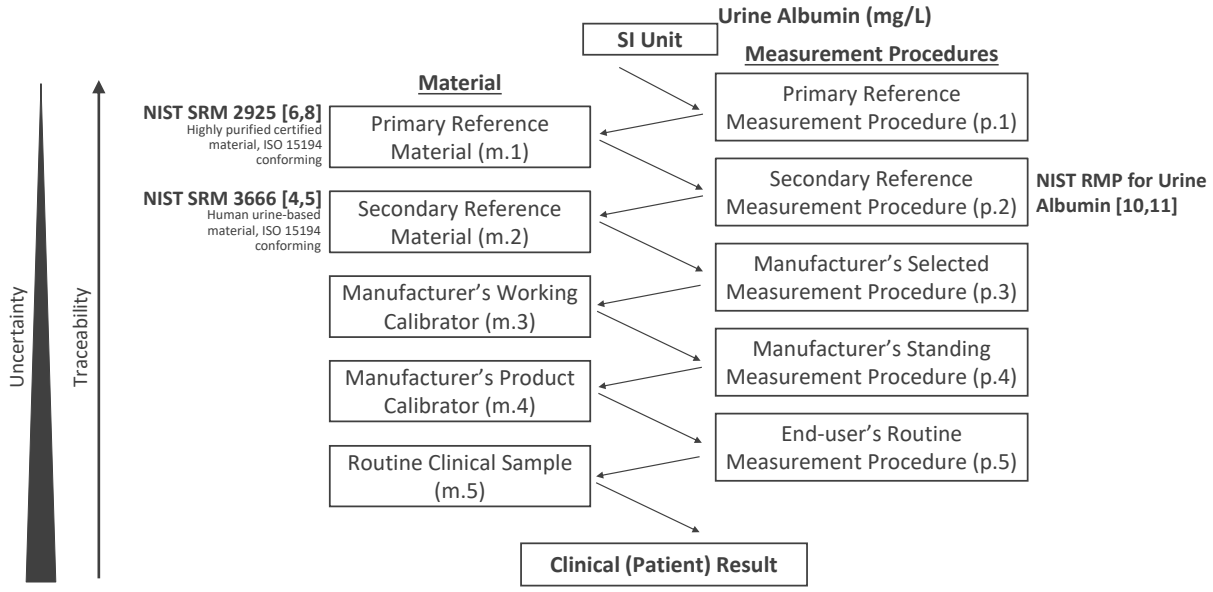

Fig. 1. Proposed reference measurement system (RMS) for traceability of clinical urine albumin results using NIST SRMs and the NIST RMP. Adapted from ISO 17511 [9].

The NIST RMP (Fig. 1, p.3) is intended for use in the value-assignment of albumin in NIST Standard Reference Material® (SRM) 3666 (Fig. 1, m.2), the matrix-based (human urine) secondary reference material [4,5]. Calibration of the RMP is SI traceable via the use of NIST SRM 2925 as the primary

calibrator [6]. (currently listed in JCTLM database – #C18RM1; m.2) NIST SRM 2925 serves as the primary reference material (Fig. 1, m.1) for the urine albumin reference measurement system (Fig. 1) and is currently listed in the Joint Committee for Traceability in Laboratory Medicine (JCTLM) database for reference materials [7,8]. NIST SRM 3666 is a four (4)-level (Level I to Level IV) human urine material intended for use as a secondary reference material (Fig. 1, m.2) to support the accuracy and comparability of clinical urine albumin and urine creatinine results used in clinical decisions for kidney disease [4,5].

The NIST RMP for urine albumin incorporates isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) using the multiple reaction monitoring (MRM) scan mode to detect and measure proteolytic albumin peptides in urine with high selectivity and sensitivity [10, 11]. To target full-length albumin and to reduce bias introduced by sample preparation and enzymatic digestion, an isotopically labeled (^{15}N) full-length recombinant human serum albumin (^{15}N-rHSA) that is chemically equivalent to the native albumin is used as the internal standard (IS). With the incorporation of the full-length ^{15}N-rHSA IS, 11 tryptic peptides that span the sequence of albumin are measured and 5 peptides are used for the absolute quantification of albumin in urine. Using a series of measurement equations, the consensus mean mass fraction and mass concentration values are determined for albumin in urine [11].

2. Warning & Safety

2.1. Chemical and Instrumentation

Chemical hazards will vary depending on the experiment and samples being analyzed. Read the SDS for any compound being used as a solvent prior to conducting the RMP. Take all the necessary precautions for these compounds. Care should be taken with operating a LC-MS system, general hazards and controls associated with the system include: high temperatures, electrical hazards, and high voltage. Personal protective equipment (PPE) is required for the RMP.

2.2. Human Urine Material

Handle urine samples as biohazardous material capable of transmitting infectious disease. For human-derived urine products where the presence of an infectious agent may be unknown, handle human urine-based products at Biosafety Level 2 as recommended by the Centers for Disease Control and Prevention's Biosafety in Microbiological and Biomedical Laboratories (6th edition) [12].

3. Scope

The objective of the RMP is to digest albumin and use ID-LC-MS/MS to detect and quantify the proteolytic products of albumin to determine the mass fraction ($\bar{X}_{mg/g}$) and mass concentration ($\bar{X}_{mg/L}$) values and associated uncertainties of albumin in urine. No detectable effects of the human urine matrix on measurement repeatability and precision compared to a buffer system (50 mmol/L ammonium bicarbonate) were previously observed [10]. Trypsin is used to generate the proteolytic albumin peptides (unlabeled and IS) and the mass ratio is determined from the peak area ratio of the unlabeled to labeled (IS) peak area for each MRM transition. The measurement interval for the RMP aligns with the clinical ranges for albumin in urine (5 mg/L to 500 mg/L).

4. Measurement Principle & Measurement Method

The RMP is a targeted multiplexed ID-LC-MS/MS assay that incorporates a full-length ^{15}N-rHSA IS for the absolute quantification of albumin in the urine. The procedure couples ID-LC–MS/MS with the multiple reaction monitoring (MRM) MS scan mode to selectively target signature tryptic albumin peptides. The ratio of unlabeled analyte to ^{15}N-labeled IS is used to generate a calibration curve for the determination of albumin in a urine sample.

5. Materials

A list of the materials and instrumentation used in the NIST RMP are listed in Table 1.
(NOTE: For reagents and equipment specified to a brand name, a different brand may be substituted if the required parameters listed in the protocol are met and fit-for-purpose.)

Table 1. List of materials and instrumentation for NIST RMP.

Item	Vendor
NIST SRM 2925 Recombinant Human Serum Albumin Solution (calibrator material) (listed in JCTLM database – C18RM1) [6,8]	NIST
NIST SRM 3666 Albumin and Creatinine in Frozen Human Urine (control material) [4,5]	NIST
Internal Standard: Full-length ^{15}N-recombinant Human Serum Albumin (^{15}N-*r*HSA) (≥ 99% label incorporation)	Albumin Biosciences
Trypsin-Gold MS-grade (Molecular Formula-$C_{35}H_{47}N_7O_{10}$; CAS: 9002-07-7)	Promega
Dithiothreitol (DTT) (No-Weigh Format, 7.7 mg DTT per microtube) (MW-154.24 g/mol; Molecular Formula-$C_4H_{10}O_2S_2$; CAS: 3483-12-3)	Pierce
Iodoacetamide (MW-184.96 g/mol; Molecular Formula-C_2H_4INO; CAS: 144-48-9)	Pierce
Water with 0.1 % (*volume fraction*) formic acid (high-purity LC-MS grade; Water CAS: 7732-18-5; Formic Acid CAS: 64-18-6)	Honeywell Burdick and Jackson
Acetonitrile (ACN) with 0.1 % (*volume fraction*) formic acid (ACN CAS: 75-05-8; Formic Acid CAS: 64-18-6)	Honeywell Burdick and Jackson
Autosampler vials (amber glass, screw cap)	Agilent
Caps for Autosampler Vials	Agilent
1.0 mL Tubes (polypropylene, sterile)	Eppendorf
6460 Triple Quadrupole Mass Spectrometer equipped with an Agilent 1290 Series LC system	Agilent
Zorbax 300 SB-C18 column (2.1 mm × 150 mm, 3.5 µm)	Agilent
Acetic Acid (glacial, ACS Reagent) (MW-60.05 g/mol; Molecular Formula-CH_3CO_2H; CAS: 64-19-7)	SIGMA
Balance	Mettler Toledo
SpeedVac (Vacuum Concentrator System)	
Incubator (For temperature stability at 97 °C, 60 °C, and 37 °C)	

6. Solutions

a. 1 mol/L Ammonium Bicarbonate (AMBIC) (total volume – 50 mL)
    a) Add 3.953 g of ammonium bicarbonate to 50 mL of water

b. 50 mmol/L AMBIC (total volume – 50 mL)
    a) Add 2.5 mL of 1 mol/L AMBIC to 47.5 mL of water

c. 200 mmol/L AMBIC (total volume – 15 mL)
    a) Add 3.0 mL of 1 mol/L AMBIC to 12 mL of water
d. 50 mmol/L Acetic Acid (total volume – 50 mL)
    a) Add 2.5 mL of 1 mol/L acetic acid to 47.5 mL of water
e. 500 mmol/L DTT (total volume – 100 µL) *(prepare fresh during digestion procedure)*
    a) Add 100 µL of 50 mmol/L AMBIC to 7.7 mg DTT
f. 375 mmol/L Iodoacetamide (total volume – 132 µL) *(prepare fresh during digestion procedure)*
    a) Add 132 µL of 200 mmol/L AMBIC to Iodoacetamide
g. 1 µg/µL Trypsin (total volume – 100 µL) *(prepare fresh during digestion procedure)*
    a) Add 100 µL of 50 mmol/L acetic acid to 100 µg of Trypsin

7. Sample Preparation Procedure

An illustration of the sample preparation procedure is shown in Figure 2.

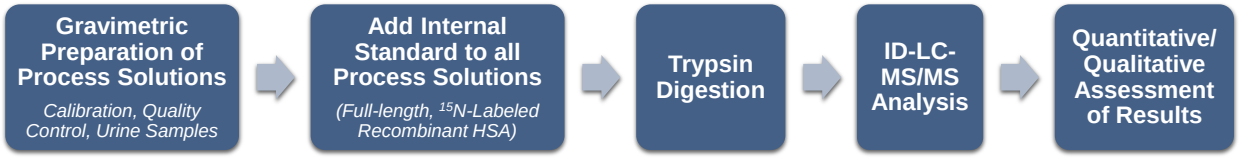

Fig. 2. Detailed measurement procedure for the NIST RMP for urine albumin [10,11].

7.1. Preparation of Process Samples

1. Calibration Material: The NIST SRM 2925 is used to prepare the calibration solutions for the NIST RMP [6,8]. *(Use specifications outlined in NIST certificate of analysis for the usage and storage of NIST SRM 2925)*
2. Calibration Solutions: Gravimetrically prepare calibration solutions using NIST SRM 2925 and 50 mmol/L AMBIC within the analytical range (5 mg/L to 500 mg/L) of RMP. Add the volume of NIST SRM 2925 and 50 mmol/L AMBIC to the tube and record the mass of each volume (total volume of 150 µL).
    a. Two-curve or Three-curve calibration system that is concentration dependent
        i. Curve #1: approx. 5 mg/L to 50 mg/L
        ii. Curve #2: approx. 50 mg/L to 150 mg/L
        iii. Curve #3: approx. 150 mg/L to 500 mg/L
3. Urine Specimen and Quality Control Material (NIST SRM 3666 or in-house material): Gravimetrically prepare the urine specimen. Add 150 µL of urine specimen to the tube and record the mass. *(Use vendor specifications for the usage and storage of Quality Control Materials; Use specifications outlined in NIST certificate of analysis for the usage and storage of NIST SRM 3666)*
4. Blank Sample: Gravimetrically prepare blank sample by adding 150 µL of 50 mmol/L AMBIC to tube and record mass.
5. Internal Standard: Gravimetrically add the same amount (approx. 5 µg to 8 µg of protein) of internal standard to each calibration solution, quality control material, blank sample, and urine specimen.

6. Solubilization: Store Process Samples (calibration solutions, blank sample, quality control material, urine specimens) at 5 °C overnight protected from light. *(Do not store Process Samples at 5 °C for more than 24 h)*
7. After overnight incubation, equilibrate Process Samples to room temperature (approx. 25 °C) for 1 h prior to trypsin digestion.

7.2. In-Solution Trypsin Digestion

1. Record the start date and time for the trypsin digestion procedure
2. Incubate Process Samples at 97 °C for 10 min
3. Cool Process Samples to room temperature (approx. 25 °C) for 2 min
4. Prepare 500 mmol/L DTT stock
5. Add 500 mmol/L DTT stock to a final concentration of 5 mmol/L DTT to each Process Sample
6. Incubate Process Samples at 60 °C for 30 min
7. Prepare 375 mmol/L Iodoacetamide stock
8. Add 375 mmol/L Iodoacetamide stock to a final concentration of 15 mmol/L Iodoacetamide to each Process Sample
9. Incubate Process Samples at room temperature (approx. 25 °C) in dark for 30 min
10. Prepare 1 µg/µL Trypsin Solution
11. An approximate 1:30 mass ratio of trypsin-to-total protein is used for digestion
12. Incubate Process Samples at 37 °C for 24 h (record digestion start and end time)
13. Add 60 µL of 0.1 % (*volume fraction*) formic acid in water to quench the digestion reaction
14. Incubate Analytical Samples (digested Process Samples) at 37 °C in dark for 45 min
15. Concentrate Analytical Samples in SpeedVac (no heat) to dryness *(Concentrated Analytical Samples may be stored at -80 °C until ready for analysis.)*
16. Reconstitute the Analytical Samples in 100 µL of 0.1 % (*volume fraction*) formic acid in water

8. LC-MS System

Analysis of the digested Analytical Samples is performed on an Agilent 6460 triple quadrupole mass spectrometer in positive ion mode equipped with an Agilent 1290 Series LC system utilizing an Agilent Zorbax 300 SB-C18 column (2.1 mm × 150 mm, 3.5 µm). The column temperature is maintained at 45 °C, and the peptides are loaded onto the column at a flow rate of 200 µL/min in 97 % (*volume fraction*) mobile phase A (water with 1 mL/L formic acid) and 3 % (*volume fraction*) mobile phase B (ACN with 1 mL/L formic acid). The LC gradient is outlined in Table 2 [10,11].

Table 2. LC gradient for NIST RMP.

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Flowrate (mL/min)
0.0	97 %	3 %	0.2
2.0	97 %	3 %	0.2
4.0	90 %	10 %	0.2
14.0	80 %	20 %	0.2
24.0	70 %	30 %	0.2
26.0	30 %	70 %	0.2
27.0	3 %	97 %	0.2
32.0	3 %	97 %	0.2
34.0	97 %	3 %	0.2
45.0	97 %	3 %	0.2

General mass spectrometric conditions: gas temperature of 300 °C; gas flow of 7 L/min; nebulizer of 20 psi (1.4×10^5 Pa); sheath gas temperature of 300 °C; sheath gas flow of 6 L/min; capillary voltage of 4000 V; and a nozzle voltage of 1500 V. The MRM segment for the MS method is outlined in Table 3 [10,11]. The segment time for the MS analysis is dependent upon the analytical column type and the LC type and configuration.

Table 3. MRM segments for tandem MS analysis.

MRM Segment Number	Segment Time (min)	Scan Mode	Divert Valve Status	Store Data (Y/N)
1	0.0	MS2 Scan	To Waste	N
2	4.0	MRM	To MS	Y
3	9.5	MRM	To MS	Y
4	12.5	MRM	To MS	Y
5	15.7	MRM	To MS	Y
6	18.5	MRM	To MS	Y
7	21.0	MRM	To MS	Y
8	29.0	MS2 Scan	To Waste	N

Analysis of the Analytical Samples (calibration samples, quality control samples, human urine samples) is conducted in a randomized sequence with replicate measurements (4 technical replicates) to reduce the influence of systematic bias on the output measurements. The four (4) technical replicates for each Analytical Sample are not acquired in sequential order in the same analytical run. Instead, the replicate measurements are acquired independently in 4 separate analytical runs across different days for a total of 4 technical replicates per Analytical Sample. Randomization of the sample acquisition order and separation of the 4 technical replicates minimizes systematic error associated with LC-MS/MS analysis of the samples. An example sample analysis sequence is shown in Appendix B. The MRM transitions (11 peptides with 2 or 3 MRM transitions per peptide) are listed in Table 4 [10,11].

Table 4. MRM transition list for the NIST RMP.

Peptide Type – Qt or Qa[a]	MRM Transition Number	Peptide	Precursor m/z	Product m/z	IS[b] Precursor m/z	IS[b] Product m/z	MS MRM Segment
Qt	**Transition 3**	**TYETTLEK**	**492.75**	**720.30**	**497.23**	**727.40**	**2**
Qt	**Transition 7**	**VFDEFKPLVEEPQNLIK**	**682.37**	**900.00**	**689.35**	**909.50**	**7**
Qt	**Transition 9**	**FQNALLVR**	**480.78**	**685.40**	**487.27**	**695.40**	**4**
Qt	**Transition 18**	**LCTVATLR**	**467.26**	**660.40**	**472.75**	**669.40**	**3**
Qt	**Transition 20**	**YLYEIAR**	**464.25**	**651.30**	**469.24**	**659.30**	**4**
Qa	Transition 1	DLGEENFK	476.22	723.30	481.21	731.30	3
Qa	Transition 2	DLGEENFK	476.22	229.07	481.21	231.07	3
Qa	Transition 4	TYETTLEK	492.75	265.10	497.23	279.20	2
Qa	Transition 5	VFDEFKPLVEEPQNLIK	682.37	970.50	689.35	981.50	7
Qa	Transition 6	VFDEFKPLVEEPQNLIK	682.37	712.40	689.35	721.37	7
Qa	Transition 8	FQNALLVR	480.78	276.09	487.27	279.09	4
Qa	Transition 10	QTALVELVK	500.81	488.27	506.29	493.25	5
Qa	Transition 11	QTALVELVK	500.81	587.30	506.29	593.40	5
Qa	Transition 12	RPCFSALEVDETYVPK	637.65	961.50	644.3	972.40	5
Qa	Transition 13	RPCFSALEVDETYVPK	637.65	244.17	644.3	247.16	5

Peptide Type – Qt or Qa[a]	MRM Transition Number	Peptide	Precursor m/z	Product m/z	IS[b] Precursor m/z	IS[b] Product m/z	MS MRM Segment
Qa	Transition 14	LVAASQAALGL	507.30	189.08	513.29	191.08	6
Qa	Transition 15	LVAASQAALGL	507.30	712.40	513.29	721.40	6
Qa	Transition 16	LVNEVTEFAK	575.31	937.40	581.29	947.40	4
Qa	Transition 17	LVNEVTEFAK	575.31	694.40	581.29	701.40	4
Qa	Transition 19	LCTVATLR	467.26	274.12	472.75	276.12	3
Qa	Transition 21	YLYEIAR	464.25	277.20	469.24	279.10	4
Qa	Transition 22	AEFAEVSK	440.72	201.05	445.21	203.04	2
Qa	Transition 23	AEFAEVSK	440.72	680.32	445.21	687.30	2

[a] The "Peptide Type" differentiates the Quantitative (Qt) and Qualitative (Qa) MRM Transitions. The five Qt-MRM Transitions are in bold

[b] IS represents the internal standard (^{15}N-rHSA)

Figure 3 is a representative chromatographic profile of the 23 MRM transitions for both the unlabeled analyte (NIST SRM 2925) and IS (^{15}N-labeled rHSA) using the conditions and parameters listed in this document. The retention time (RT) for the unlabeled (NIST SRM 2925) MRM transitions overlaps with the RT for the corresponding IS (^{15}N-labeled rHSA) MRM transition (Fig. 3, qt-MRM Transitions labeled in bold with an asterisk*).

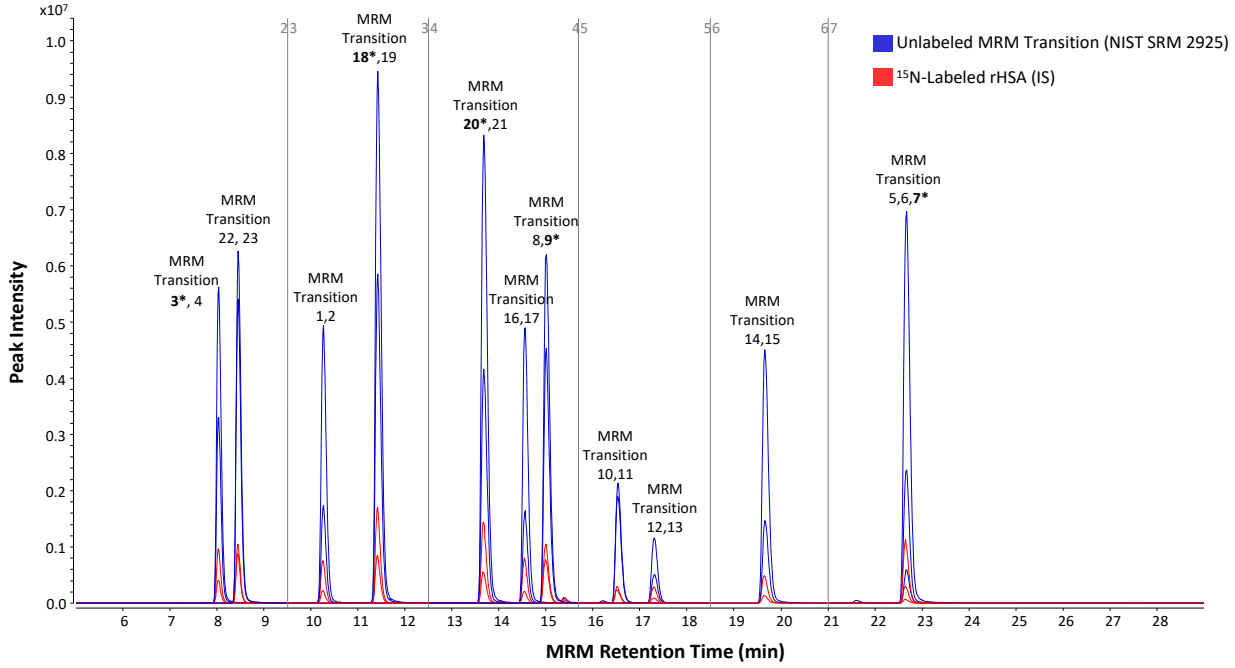

Fig. 3. Chromatographic profile of unlabeled (NIST SRM 2925) and labeled (IS) MRM Transitions

9. Quantitative Method

9.1. Integration of MRM Transitions

The Agilent MassHunter Quantitative Analysis software (Version B.10.00 or higher) is used to automatically integrate the MRM chromatographic peaks for both the unlabeled albumin and IS MRM transitions. Following automatic integration, manually confirm all automatic peak integrations. If manual correction of the automatic integration is required, record the corrected peak integration. A total of 46 measurements (11 peptides with 2 or 3 MRM transitions per peptide for both unlabeled

and ^{15}N-labeled albumin) are collected for the 23 MRM transitions in each sample. Following integration, the instrument data (peak area, peak intensity, retention time) is imported from MassHunter into Microsoft Excel for manual quantitative assessment of the raw data. For the quantitative evaluation, the unlabeled peak area and IS peak area are used to calculate the Peak Area Ratio (PAR) for each transition and sample. The peak intensity and retention time data are used to monitor the repeatability of the MRM measurements and the performance of the LC-MS system.

9.2. Calibration Curve

Linear regression analysis (concentration ratio vs. PAR) of the calibration solutions is used to generate the calibration curve (2-level or 3-level calibration system) for each MRM transition (qt-MRM and qa-MRM transitions). An example 2-level calibration system is presented in Fig. 4. The linear equation and coefficient of determination (R^2 value) for each calibration curve are determined using Microsoft Excel.

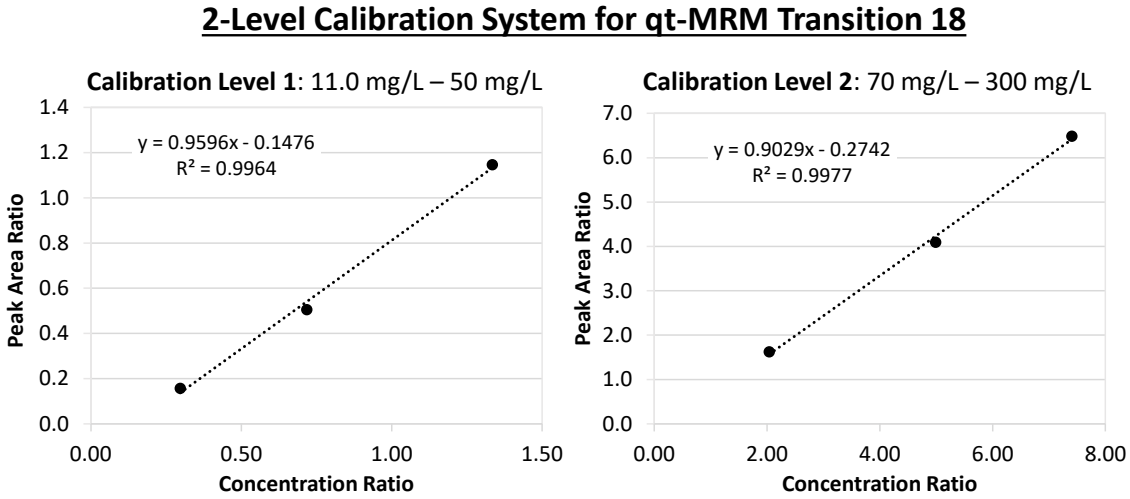

Fig. 4. Two-level calibration system for qt-MRM Transition 18.

9.3. Calculation of Albumin Content

A full description of the equations used to calculate the albumin content is presented in Ref. 11. The mass fraction value ($X_{t_1 mg/g}$) for each MRM transition is derived from [11]:

$$X_{t_1 mg/g} = \frac{PAR_t - B_{0,t}}{\mathrm{B}_{1,t}} \times \left(\frac{\mathrm{m}_U}{\frac{\mathrm{m}_{IS}}{d_{IS}} \times C_{IS}} \times 100 \right). \quad (1)$$

Table 5. Description of variables for Equation 1.

Variable	Description
$X_{t_1 mg/g}$	Mass fraction value of an MRM transition
PAR_t	t_1-MRM transition PAR (unlabeled t_1-MRM transition in urine sample to IS t_1-MRM transition, ^{15}N-labeled IS)
$B_{0,t}$	y-intercept of the linear calibration curve for the t_1-MRM transition
$\mathrm{B}_{1,t}$	Slope of the linear calibration curve for the t_1-MRM transition
m_U	Mass of the unknown urine sample

m_{IS}	Mass of the IS
d_{IS}	Density of the IS
C_{IS}	Mass concentration of the IS
m_U	Mass of the unknown urine sample

The consensus mass concentration value ($\bar{\bar{X}}_{mg/L}$) is derived from [11]:

$$\bar{\bar{X}}_{mg/L} = \bar{\bar{X}}_{mg/g} \times (d_{urine} \times 1000). \quad (2)$$

Table 6. Description of variables for Equation 2.

Variable	Description
$\bar{\bar{X}}_{mg/L}$	Consensus mass concentration value
$\bar{\bar{X}}_{mg/g}$	Consensus mass fraction value
d_{IS}	Density of the IS

9.4. Calculation of Combined Uncertainty

A full description of the equations used to calculate the combined uncertainty for $\bar{\bar{X}}_{mg/L}$ and $\bar{\bar{X}}_{mg/L}$ is outlined in Ref. 11. The uncertainty is concentration-dependent, the higher the albumin concentration, the higher the combined uncertainty. The combined uncertainty for the $\bar{\bar{X}}_{mg/g}$ is derived from [11]:

$$u_{mg/g} = \sqrt{u^2{}_{Type\ A} + u^2{}_{Type\ B}}\,. \quad (3)$$

Table 7. Description of variables for Equation 3.

Variable	Description
$u_{Type\ A}$	Type A uncertainty of $\bar{\bar{X}}_{mg/g}$ determined via the DSL-HHD or DSL-bootstrap methods
$u_{Type\ B}$	Combined Type B uncertainties [11]

The combined uncertainty for the $\bar{\bar{X}}_{mg/L}$ is derived from [11]:

$$u_{mg/L} = \sqrt{d^2 u^2{}_{mg/g} + \bar{\bar{X}}_{mg/g} u^2{}_d}\,. \quad (4)$$

Table 8. Description of variables for Equation 4.

Variable	Description
$u_{mg/L}$	$\bar{\bar{X}}_{mg/L}$ combined uncertainty
d_{urine}	Density of the urine material
u_d	Density uncertainty

9.5. Calculation of Expanded Uncertainty

The $u_{mg/g}$ and $u_{mg/L}$ values are expressed as expanded uncertainties (U), which are calculated using [11]:

$$U_{mg/g} = k \times u_{mg/g} \quad \text{or} \quad U_{mg/L} = k \times u_{mg/L}. \quad (5)$$

Table 9. Description of variables for Equation 5.

Variable	Description
$U_{mg/g}$	Expanded uncertainty for $u_{mg/g}$
$u_{mg/g}$	$\overline{\overline{X}}_{mg/g}$ combined uncertainty
$U_{mg/L}$	Expanded uncertainty for $u_{mg/L}$
$u_{mg/L}$	$\overline{\overline{X}}_{mg/L}$ combined uncertainty
k	Coverage factor (k = 2)

10. Summary

The NIST RMP is a targeted, multiplexed procedure that applies quantitative proteomics techniques for the absolute quantification of albumin in urine. The RMP incorporates a full-length IS (^{15}N-labeled rHSA) that allows the detection of multiple signature peptides (11 peptides) that span the complete amino acid sequence of albumin. By including multiple peptides, both quantitative and qualitative information about albumin in the urine sample is observed. Applying the MS-based MRM technique offers a high degree of analytical specificity and sensitivity for the detection of albumin in urine. The triple quadrupole mass spectrometer selectively separates the precursor/product ion pair of each target peptide from the bulk digestion products; therefore, the urine sample can be analyzed with minimal sample preparation. As a result of the selective nature of the MRM method, the sensitivity of the assay is enhanced, which enables the detection of urine albumin at the lower normoalbuminuria range (LLOQ is 5.0 mg/L) with high precision and accuracy. The dynamic range of the multiplexed assay traverses all three albuminuria stages (5 mg/L to 500 mg/L), and this supports the usage of the assay for early detection of microalbuminuria (30 mg/L to 300 mg/L) and the observation of renal dysfunction progression from normoalbuminuria (0 mg/L to 30 mg/L) to macroalbuminuria (> 300 mg/L).

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Appendix A. List of Symbols, Abbreviations, and Acronyms

ACN	Acetonitrile
AMBIC	Ammonium Bicarbonate
CKD	Chronic Kidney Disease
DTT	Dithiothreitol
HSA	Human Serum Albumin
ID-LC-MS/MS	Isotope Dilution-Liquid Chromatography-Tandem Mass Spectrometry
IFCC	International Federation of Clinical Chemistry
IFCC WG-SAU	IFCC Working Group for the Standardization of Albumin Assays in Urine
IS	Internal Standard
JCTLM	Joint Committee for Traceability in Laboratory Medicine
LLOQ	Lower limit of Quantification
MRM	Multiple Reaction Monitoring
MU	Measurement Uncertainty
NHANES	National Health and Nutrition Examination Survey
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
NIDDK LWG	NIDDK Laboratory Working Group
NIST	National Institute of Standards and Technology
PAR	Peak Area Ratio
PPE	Personal Protective Equipment
Qt	Quantitative
Qa	Qualitative
RM	Reference Material
RMP	Reference Measurement System
SRM	Standard Reference Material®

Appendix B. Example Acquisition Sequence

The example acquisition sequences represent an experimental design consisting of 5 Calibration solutions, 2 Quality Control (QC) samples, NIST SRM 3666 Level II (3 process replicates or 3 aliquots of a single vial), and 12 Clinical Human Urine samples (3 process replicates per sample). In the example, the 4 technical replicates for each sample are acquired in separate analytical runs conducted on different days. For each analytical run, the sample acquisition order is randomized.

Technical Replicate 1 (Run 1)

Sample Name	Inj Vol (uL)	Sample Position	Data File
Clean1	1.0	P1-A1	Clean1
Blank1	1.0	P1-A1	Blank1
UA Sample – 3.1	2.6	P1-C8	UA Sample – 3.1_Run 1
UA Sample – 7.2	2.4	P1-E3	UA Sample – 7.2_Run 1
Level 2_Calibrant 1	3.8	P1-B1	Level 2_Calibrant 1_Run 1
UA Sample – 12.1	2.2	P1-F8	UA Sample – 12.1_Run 1
UA Sample – 11.1	2.3	P1-F5	UA Sample – 11.1_Run 1
UA Sample – 1.3	2.8	P1-C4	UA Sample – 1.3_Run 1
UA Sample – 8.3	2.3	P1-E7	UA Sample – 8.3_Run 1
UA Sample – 10.1	2.3	P1-F2	UA Sample – 10.1_Run 1
UA Sample – 6.1	2.5	P1-D8	UA Sample – 6.1_Run 1
Level 2_Calibrant 4	2.2	P1-B4	Level 2_Calibrant 4_Run 1
UA Sample – 9.2	2.3	P1-E9	UA Sample – 9.2_Run 1
Level 2_QC 1	3.2	P1-B6	Level 2_QC 1_Run 1
UA Sample – 2.1	2.6	P1-C5	UA Sample – 2.1_Run 1
UA Sample – 9.3	2.4	P1-F1	UA Sample – 9.3_Run 1
UA Sample – 12.3	2.2	P1-A9	UA Sample – 12.3_Run 1
UA Sample – 5.1	2.6	P1-D5	UA Sample – 5.1_Run 1
UA Sample – 4.2	2.6	P1-D3	UA Sample – 4.2_Run 1
UA Sample – 10.2	2.3	P1-F3	UA Sample – 10.2_Run 1
SRM 3666 Level II_2	2.3	P1-B9	SRM 3666 Level II_2_Run 1
UA Sample – 3.3	2.6	P1-D1	UA Sample – 3.3_Run 1
UA Sample – 12.2	2.3	P1-F9	UA Sample – 12.2_Run 1
UA Sample – 5.3	2.6	P1-F6	UA Sample – 5.3_Run 1
UA Sample – 11.2	2.3	P1-F6	UA Sample – 11.2_Run 1
UA Sample – 6.2	2.5	P1-D9	UA Sample – 6.2_Run 1
Level 2_Calibrant 5	2.0	P1-B5	Level 2_Calibrant 5_Run 1
UA Sample – 9.1	2.3	P1-E8	UA Sample – 9.1_Run 1
UA Sample – 8.2	2.3	P1-E6	UA Sample – 8.2_Run 1
UA Sample – 7.3	2.4	P1-E4	UA Sample – 7.3_Run 1
UA Sample – 11.3	2.3	P1-F7	UA Sample – 11.3_Run 1
SRM 3666 Level II_3	2.3	P1-C1	SRM 3666 Level II_3_Run 1
UA Sample – 8.1	2.3	P1-E5	UA Sample – 8.1_Run 1
UA Sample – 7.1	2.5	P1-E2	UA Sample – 7.1_Run 1
UA Sample – 1.2	2.8	P1-C3	UA Sample – 1.2_Run 1
UA Sample – 2.3	2.6	P1-C7	UA Sample – 2.3_Run 1
Level 2_QC 2	2.4	P1-B7	Level 2_QC 2_Run 1
UA Sample – 6.3	2.5	P1-E1	UA Sample – 6.3_Run 1
SRM 3666 Level II_1	2.3	P1-B8	SRM 3666 Level II_1_Run 1
UA Sample – 5.2	2.6	P1-D6	UA Sample – 5.2_Run 1
UA Sample – 10.3	2.3	P1-F4	UA Sample – 10.3_Run 1
Level 2_Calibrant 2	2.7	P1-B2	Level 2_Calibrant 2_Run 1
UA Sample – 1.1	2.8	P1-C2	UA Sample – 1.1_Run 1
Level 2_Calibrant 3	2.5	P1-B3	Level 2_Calibrant 3_Run 1
UA Sample – 3.2	2.6	P1-C9	UA Sample – 3.2_Run 1
UA Sample – 2.2	2.6	P1-C6	UA Sample – 2.2_Run 1
UA Sample – 4.3	2.6	P1-D4	UA Sample – 4.3_Run 1
UA Sample – 4.1	2.5	P1-D2	UA Sample – 4.1_Run 1
Clean2	1.0	P1-A1	Clean2
Blank2	1.0	P1-A1	Blank2

Technical Replicate 2 (Run 2)

Sample Name	Inj Vol (uL)	Sample Position	Data File
Clean1	1.0	P1-A1	Clean1
Blank1	1.0	P1-A1	Blank1
SRM 3666 Level II_1	2.3	P1-B8	SRM 3666 Level II_1_Run 2
UA Sample – 1.1	2.8	P1-C2	UA Sample – 1.1_Run 2
UA Sample – 11.1	2.3	P1-F5	UA Sample – 11.1_Run 2
Level 2_QC 2	2.4	P1-B7	Level 2_QC 2_Run 2
UA Sample – 9.1	2.3	P1-E8	UA Sample – 9.1_Run 2
UA Sample – 12.2	2.3	P1-F9	UA Sample – 12.2_Run 2
Level 2_Calibrant 4	2.2	P1-B4	Level 2_Calibrant 4_Run 2
UA Sample – 8.1	2.3	P1-E5	UA Sample – 8.1_Run 2
UA Sample – 4.1	2.5	P1-D2	UA Sample – 4.1_Run 2
Level 2_Calibrant 1	3.8	P1-B1	Level 2_Calibrant 1_Run 2
UA Sample – 7.3	2.4	P1-E4	UA Sample – 7.3_Run 2
UA Sample – 6.2	2.5	P1-D9	UA Sample – 6.2_Run 2
SRM 3666 Level II_2	2.3	P1-B9	SRM 3666 Level II_2_Run 2
UA Sample – 3.2	2.6	P1-C9	UA Sample – 3.2_Run 2
UA Sample – 10.3	2.3	P1-F4	UA Sample – 10.3_Run 2
UA Sample – 2.1	2.6	P1-C5	UA Sample – 2.1_Run 2
UA Sample – 3.3	2.6	P1-D1	UA Sample – 3.3_Run 2
UA Sample – 11.2	2.3	P1-F6	UA Sample – 11.2_Run 2
UA Sample – 7.2	2.4	P1-E3	UA Sample – 7.2_Run 2
SRM 3666 Level II_3	2.3	P1-C1	SRM 3666 Level II_3_Run 2
UA Sample – 6.3	2.5	P1-E1	UA Sample – 6.3_Run 2
UA Sample – 1.3	2.8	P1-C4	UA Sample – 1.3_Run 2
UA Sample – 5.2	2.6	P1-D6	UA Sample – 5.2_Run 2
UA Sample – 4.2	2.6	P1-D3	UA Sample – 4.2_Run 2
UA Sample – 1.2	2.8	P1-C3	UA Sample – 1.2_Run 2
UA Sample – 12.1	2.2	P1-F8	UA Sample – 12.1_Run 2
UA Sample – 2.3	2.6	P1-C7	UA Sample – 2.3_Run 2
Level 2_Calibrant 2	2.7	P1-B2	Level 2_Calibrant 2_Run 2
UA Sample – 2.2	2.6	P1-C6	UA Sample – 2.2_Run 2
UA Sample – 12.3	2.2	P1-A9	UA Sample – 12.3_Run 2
UA Sample – 9.2	2.3	P1-E9	UA Sample – 9.2_Run 2
UA Sample – 4.3	2.6	P1-D4	UA Sample – 4.3_Run 2
UA Sample – 8.3	2.3	P1-E7	UA Sample – 8.3_Run 2
Level 2_Calibrant 3	2.5	P1-B3	Level 2_Calibrant 3_Run 2
UA Sample – 5.1	2.6	P1-D5	UA Sample – 5.1_Run 2
UA Sample – 10.2	2.3	P1-F3	UA Sample – 10.2_Run 2
UA Sample – 3.1	2.6	P1-C8	UA Sample – 3.1_Run 2
UA Sample – 5.3	2.6	P1-D7	UA Sample – 5.3_Run 2
Level 2_QC 1	3.2	P1-B6	Level 2_QC 1_Run 2
UA Sample – 7.1	2.5	P1-E2	UA Sample – 7.1_Run 2
UA Sample – 11.3	2.3	P1-F7	UA Sample – 11.3_Run 2
UA Sample – 9.3	2.4	P1-F1	UA Sample – 9.3_Run 2
UA Sample – 8.2	2.3	P1-E6	UA Sample – 8.2_Run 2
UA Sample – 10.1	2.3	P1-F2	UA Sample – 10.1_Run 2
UA Sample – 6.1	2.5	P1-D8	UA Sample – 6.1_Run 2
Level 2_Calibrant 5	2.0	P1-B5	Level 2_Calibrant 5_Run 2
Clean2	1.0	P1-A1	Clean2
Blank2	1.0	P1-A1	Blank2

Technical Replicate 3 (Run 3)

Sample Name	Inj Vol (uL)	Sample Position	Data File
Clean1	1.0	P1-A1	Clean1
Blank1	1.0	P1-A1	Blank1
UA Sample – 4.3	2.6	P1-D4	UA Sample – 4.3_Run 3
UA Sample – 5.3	2.6	P1-D7	UA Sample – 5.3_Run 3
UA Sample – 6.2	2.5	P1-D9	UA Sample – 6.2_Run 3
UA Sample – 9.1	2.3	P1-E8	UA Sample – 9.1_Run 3
SRM 3666 Level II_2	2.3	P1-B9	SRM 3666 Level II_2_Run 3
UA Sample – 1.1	2.8	P1-C2	UA Sample – 1.1_Run 3
UA Sample – 10.2	2.3	P1-F3	UA Sample – 10.2_Run 3
UA Sample – 9.2	2.3	P1-E9	UA Sample – 9.2_Run 3
Level 2_QC 1	3.2	P1-B6	Level 2_QC 1_Run 3
UA Sample – 10.1	2.3	P1-F2	UA Sample – 10.1_Run 3
UA Sample – 12.1	2.2	P1-F8	UA Sample – 12.1_Run 3
UA Sample – 9.3	2.4	P1-F1	UA Sample – 9.3_Run 3
UA Sample – 1.3	2.8	P1-C4	UA Sample – 1.3_Run 3
UA Sample – 2.1	2.6	P1-C5	UA Sample – 2.1_Run 3
UA Sample – 6.3	2.5	P1-E1	UA Sample – 6.3_Run 3
UA Sample – 1.2	2.8	P1-C3	UA Sample – 1.2_Run 3
Level 2_Calibrant 4	2.2	P1-B4	Level 2_Calibrant 4_Run 3
UA Sample – 10.3	2.3	P1-F4	UA Sample – 10.3_Run 3
UA Sample – 5.2	2.6	P1-D6	UA Sample – 5.2_Run 3
UA Sample – 12.3	2.2	P1-A9	UA Sample – 12.3_Run 3
UA Sample – 2.2	2.6	P1-C6	UA Sample – 2.2_Run 3
SRM 3666 Level II_3	2.3	P1-C1	SRM 3666 Level II_3_Run 3
UA Sample – 11.3	2.3	P1-F7	UA Sample – 11.3_Run 3
Level 2_QC 2	2.4	P1-B7	Level 2_QC 2_Run 3
Level 2_Calibrant 1	3.8	P1-B1	Level 2_Calibrant 1_Run 3
UA Sample – 11.2	2.3	P1-F6	UA Sample – 11.2_Run 3
UA Sample – 7.3	2.4	P1-E4	UA Sample – 7.3_Run 3
UA Sample – 3.3	2.6	P1-D1	UA Sample – 3.3_Run 3
Level 2_Calibrant 2	2.7	P1-B2	Level 2_Calibrant 2_Run 3
UA Sample – 3.2	2.6	P1-C9	UA Sample – 3.2_Run 3
UA Sample – 8.1	2.3	P1-E5	UA Sample – 8.1_Run 3
Level 2_Calibrant 3	2.5	P1-B3	Level 2_Calibrant 3_Run 3
UA Sample – 6.1	2.5	P1-D8	UA Sample – 6.1_Run 3
UA Sample – 3.1	2.6	P1-C8	UA Sample – 3.1_Run 3
UA Sample – 12.2	2.3	P1-F9	UA Sample – 12.2_Run 3
UA Sample – 4.2	2.6	P1-D3	UA Sample – 4.2_Run 3
UA Sample – 7.1	2.5	P1-E2	UA Sample – 7.1_Run 3
SRM 3666 Level II_1	2.3	P1-B8	SRM 3666 Level II_1_Run 3
UA Sample – 11.1	2.3	P1-F5	UA Sample – 11.1_Run 3
UA Sample – 8.2	2.3	P1-E6	UA Sample – 8.2_Run 3
UA Sample – 5.1	2.6	P1-D5	UA Sample – 5.1_Run 3
UA Sample – 7.2	2.4	P1-E3	UA Sample – 7.2_Run 3
UA Sample – 4.1	2.5	P1-D2	UA Sample – 4.1_Run 3
Level 2_Calibrant 5	2.0	P1-B5	Level 2_Calibrant 5_Run 3
UA Sample – 2.3	2.6	P1-C7	UA Sample – 2.3_Run 3
UA Sample – 8.3	2.3	P1-E7	UA Sample – 8.3_Run 3
Clean2	1.0	P1-A1	Clean2
Blank2	1.0	P1-A1	Blank2

Technical Replicate 4 (Run 4)

Sample Name	Inj Vol (uL)	Sample Position	Data File
Clean1	1.0	P1-A1	Clean1
Blank1	1.0	P1-A1	Blank1
Level 2_Calibrant 5	2.0	P1-B5	Level 2_Calibrant 5_Run 4
UA Sample – 4.2	2.6	P1-D3	UA Sample – 4.2_Run 4
UA Sample – 10.3	2.3	P1-F4	UA Sample – 10.3_Run 4
UA Sample – 8.2	2.3	P1-E6	UA Sample – 8.2_Run 4
UA Sample – 1.3	2.8	P1-C4	UA Sample – 1.3_Run 4
UA Sample – 11.3	2.3	P1-F7	UA Sample – 11.3_Run 4
Level 2_QC 1	3.2	P1-B6	Level 2_QC 1_Run 4
UA Sample – 6.1	2.5	P1-D8	UA Sample – 6.1_Run 4
UA Sample – 11.2	2.3	P1-F6	UA Sample – 11.2_Run 4
UA Sample – 2.2	2.6	P1-C6	UA Sample – 2.2_Run 4
SRM 3666 Level II_1	2.3	P1-B8	SRM 3666 Level II_1_Run 4
UA Sample – 3.2	2.6	P1-C9	UA Sample – 3.2_Run 4
UA Sample – 8.1	2.3	P1-E5	UA Sample – 8.1_Run 4
Level 2_Calibrant 3	2.5	P1-B3	Level 2_Calibrant 3_Run 4
UA Sample – 12.3	2.2	P1-A9	UA Sample – 12.3_Run 4
UA Sample – 7.1	2.5	P1-E2	UA Sample – 7.1_Run 4
UA Sample – 1.1	2.8	P1-C2	UA Sample – 1.1_Run 4
UA Sample – 4.3	2.6	P1-D4	UA Sample – 4.3_Run 4
Level 2_Calibrant 4	2.2	P1-B4	Level 2_Calibrant 4_Run 4
UA Sample – 7.2	2.4	P1-E3	UA Sample – 7.2_Run 4
UA Sample – 9.1	2.3	P1-E8	UA Sample – 9.1_Run 4
UA Sample – 1.2	2.8	P1-C3	UA Sample – 1.2_Run 4
UA Sample – 5.1	2.6	P1-D5	UA Sample – 5.1_Run 4
UA Sample – 6.2	2.5	P1-D9	UA Sample – 6.2_Run 4
UA Sample – 5.3	2.6	P1-D7	UA Sample – 5.3_Run 4
SRM 3666 Level II_3	2.3	P1-C1	SRM 3666 Level II_3_Run 4
UA Sample – 3.1	2.6	P1-C8	UA Sample – 3.1_Run 4
UA Sample – 2.1	2.6	P1-C5	UA Sample – 2.1_Run 4
UA Sample – 3.3	2.6	P1-D1	UA Sample – 3.3_Run 4
UA Sample – 9.2	2.3	P1-E9	UA Sample – 9.2_Run 4
Level 2_QC 2	2.4	P1-B7	Level 2_QC 2_Run 4
UA Sample – 6.3	2.5	P1-E1	UA Sample – 6.3_Run 4
UA Sample – 12.2	2.3	P1-F9	UA Sample – 12.2_Run 4
UA Sample – 12.1	2.2	P1-F8	UA Sample – 12.1_Run 4
UA Sample – 10.2	2.3	P1-F3	UA Sample – 10.2_Run 4
Level 2_Calibrant 2	2.7	P1-B2	Level 2_Calibrant 2_Run 4
UA Sample – 4.1	2.5	P1-D2	UA Sample – 4.1_Run 4
UA Sample – 5.2	2.6	P1-D6	UA Sample – 5.2_Run 4
UA Sample – 9.3	2.4	P1-F1	UA Sample – 9.3_Run 4
Level 2_Calibrant 1	3.8	P1-B1	Level 2_Calibrant 1_Run 4
UA Sample – 2.3	2.6	P1-C7	UA Sample – 2.3_Run 4
UA Sample – 7.3	2.4	P1-E4	UA Sample – 7.3_Run 4
SRM 3666 Level II_2	2.3	P1-B9	SRM 3666 Level II_2_Run 4
UA Sample – 11.1	2.3	P1-F5	UA Sample – 11.1_Run 4
UA Sample – 8.3	2.3	P1-E7	UA Sample – 8.3_Run 4
UA Sample – 10.1	2.3	P1-F2	UA Sample – 10.1_Run 4
Clean2	1.0	P1-A1	Clean2
Blank2	1.0	P1-A1	Blank2

Appendix C. Data Report Template

Reference Measurement Procedure (RMP)	NIST RMP for the Absolute Quantification of Albumin in Urine Using Isotope Dilution-Liquid Chromatography-Tandem Mass Spectrometry (ID-LC-MS/MS)
Date(s) of Sample Collection (MM.DD.YY)	
Date(s) of Sample Processing (MM.DD.YY)	
Date(s) of Analysis (MM.DD.YY)	
Analyst Name	
Calibration System	☐ Two(2)-Level Calibration System ☐ Three(3)-Level Calibration System

Sample Results: Mass Fraction ($\overline{\overline{X}}_{mg/g}$) (units: mg/g)

Sample Name	Mass Fraction Value (mg/g), ($\overline{\overline{X}}_{mg/g}$)	Combined Uncertainty - Mass Fraction (mg/g), $u_{mg/g}$	Coverage factor (k)	Expanded Uncertainty - Mass Fraction (mg/g), $U_{mg/g}$

Sample Results: Mass Concentration ($\overline{\overline{X}}_{mg/L}$) (units: mg/L)

Sample Name	Mass Concentration Value (mg/L), ($\overline{\overline{X}}_{mg/L}$)	Combined Uncertainty - Mass Concentration (mg/L), $u_{mg/L}$	Coverage factor (k)	Expanded Uncertainty - Mass Concentration (mg/L), $U_{mg/L}$

Experimental Observations

Unusual properties of Samples	
Unusual features of the RMP or modifications	

Appendix D. Change Log

Corrections made in the errata update do not alter existing work or introduce substantive technical information; rather, they are intended to remove ambiguity and improve interpretation of the work.

Page Number	Update Description
3	Updated measurement interval of the RMP: 5 mg/L to 500 mg/L
4	Table 1: Added a detailed description of table entries and added four new entries
5	Section 7: Added sample storage details for NIST SRM 2925, NIST SRM 3666, Process Samples, and Analytical Samples
7	Section 8: Added a detailed description of the analytical run sequence
10	Section 9.4: Added statement - “The uncertainty is concentration-dependent, the higher the albumin concentration, the higher the combined uncertainty.”
13	Appendix A: Added “LLOQ – Lower Limit of Quantification”
14	Added “Appendix B: Example Acquisition Sequence”
15	Added “Experimental Observations” section to Appendix C