Laboratory Procedure Manual

Analyte:	Urine Albumin
Matrix:	Urine
Method:	Liquid Chromatography Tandem Mass Spectrometry
As performed by:	University of Minnesota Advanced Research and Diagnostic Laboratory (ARDL) 1200 Washington Ave S, Suite 175 Minneapolis, MN 55415
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Important Information for Users

The Advanced Research and Diagnostic Laboratory (ARDL) periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table:

File Name	Variable Name	SAS Label
ALB_CR_L	URXUMA	Albumin, urine (µg/mL)
	URXUMS	Albumin, urine (mg/L)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Albumin is the most abundant plasma protein in healthy individuals (3.5–5 g/dL), trace amounts are normally found in urine from healthy individuals <30 mg/day. Human serum albumin is synthesized in the liver and serves many important physiologic roles in humans and maintains oncotic pressure. Albumin is also responsible for the bodily transport of various compounds such as, hormones, vitamins, and drugs. Increased renal elimination of serum albumin may be observed in chronic kidney disease (CKD).(1) Monitoring urinary albumin excretion provides diagnostic and prognostic information for both renal and cardiovascular outcomes.(2) Unfortunately, this marker displays concentration bias between clinical analyzer diagnostic vendors.(3) The concentration bias is likely attributed to assay calibration and assay specific differences between platforms.(4) Kidney Disease Improving Global Outcomes (KDIGO) has recommended clinical cutoffs for urinary albumin excretion. However, given the large biases observed between diagnostic vendors, patient misclassification is like to occur between health systems with various platforms. This illustrates the need for standardization of this important kidney disease biomarker.(5)

The liquid chromatography tandem mass spectrometry (LC-MS/MS) assay quantifies albumin concentrations in human urine following enzymatic digestion. This measurement procedure utilizes proteolysis with trypsin, targeting a peptide specific to human serum albumin. Calibrators were value-assigned using the National Institute of Standards and Technology (NIST) 2925 human albumin reference material.

2. SAFETY PRECAUTIONS

A. Follow the Laboratory Safety and General Laboratory Practice regulations from the College of American Pathologists (CAP), the Clinical Laboratory Improvement Amendments (CLIA), and Occupational Safety and Health Administration (OSHA). Observe Universal Blood and Body Substance Technique (UBBST) and the Centers for Disease Control (MMWR 36;2S;1987) precautions for prevention of HIV transmission in the health care setting.

B. Wear laboratory coats and disposable gloves when handling urine specimens. Cover the work surface with disposable, absorbent toweling. Place urine tubes, pipet tips, gloves, toweling, etc., and closed residual urine specimens into plastic bags and secure tightly. Urine can be discarded into regular trash. Clean the work surfaces with 0.5% bleach.

C. Recommend to laboratory personnel preforming the assay that they receive the HBV vaccine. Maintain records of vaccination or signed declination forms in the laboratory.

D. Label all reagents indicating the preparation date, expiration date, formula, lot number if applicable, hazards of the reagent, antidote of contact with hazard, and the initials of the technician.

E. Note the location for the Material Safety Data Sheets notebook for all chemicals used in the laboratory.

F. Perform annual Safety Training and Laboratory-Specific Safety Tour noting the location of the chemical spill kit (a solid absorbent material), the location of the fire extinguishers, alarms, fire blankets, eye wash, etc. Dial 911.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

ARDL utilizes a highly specialized Laboratory Information System (LIS) (StarLIMS, Abbott Informatics Corporation; Hollywood, FL, 33021-6755) for all lab functions. Major instrument platforms are interfaced directly to the LIS, allowing data to be electronically transferred directly to the main database. The system provides an extensive quality assurance package and data management tools. Numerous networked computer workstations are used in the laboratory for data management and transmission, and also include software for word and spreadsheet creation and manipulation, statistical analysis, report presentation, and electronic communication. All workstations are user password protected with job specific security access levels and have idle time out functionality. All systems are redundantly backed up on a real time basis.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

A. SPECIMEN COLLECTION PROCEDURE

1. Timed or random urine collections are obtained from study subjects as per study protocols. (For the NHANES, random urine specimens are collected at the survey Mobile Examination Centers (MEC).)
2. No special instructions such as fasting or special diets are requested.
3. The optimum specimen tube is a 3- to 5-mL screw-top cryogenic vial. Tubes are selected per study protocols.
4. 160 µL is used for sampling. Additional dead volume is necessary to allow complete sampling, typically 50 µL. The minimum acceptable volume is 500 µL, preferred volume is 1 mL.
5. Patients are prepared according to study protocols that are designed to ensure consistent specimen collection procedures for the variables that affect urine albumin excretion (such as exercise, time of day, water loading, and posture).
6. Female participants are requested to provide a specimen when they are not menstruating to avoid falsely elevated albumin levels.

7. Patient history may include conditions such as urinary tract infection, marked hyperglycemia, severe hypertension, or congestive heart failure, as the interpretation of urine albumin measurements may be confounded by these conditions.
8. Labeling and identification procedures are determined by study protocols. Specimens are identified numerically, not by name.
9. Preservation procedures are determined by study protocols. No chemical preservatives have been used. Temperature preservation is used according to freeze or refrigeration protocol.

B. SPECIMEN STORAGE PROCEDURE

1. Urine specimens arrive frozen (on dry ice) or fresh (2-8^{o}C) as per study protocols. (For the NHANES, MEC specimens are shipped on dry ice by overnight courier and refrigerated to thaw upon arrival.) Specimens can remain refrigerated up to 3 weeks until the completion of the analysis.
2. Temperature-sensitive equipment is available for specimen storage. This includes ultra-cold freezers at - 80^{o}C, refrigerator at 2-8 °C, and walk-in cold room at 2-8 °C. The university building facilities alarm system provides continuous electronic data on temperatures. Alarms prompt personnel when temperature exceeds set tolerance.
3. Analyzed specimens are returned to frozen storage at -80^{o}C. According to study protocols, specimens are retained for a designated period, then either discarded or returned as described by protocol.
4. Specimen stability at -80 °C for at least 1 year has been documented.
5. Specimens are discarded after permission by project director(s).

C. SPECIMEN HANDLING PROCEDURE

1. Handle all urine specimens as if they are capable of transmitting any infectious agent.
2. Return specimens to specified storage as soon as possible to avoid prolonged time at room temperature.
3. Transportation of urine specimens is on dry ice by FedEx. Laboratory personnel are trained on FedEx transport rules and also dry ice handling and other transport rules. Training is documented every 2 years.
4. The university's Advanced Research and Diagnostics Laboratory (ARDL) performs creatinine testing on the same urine specimens. The Creatinine Procedure Manual, the CLIA certificate, quality control data specific to NHANES, and Proficiency Testing records are filed in the Microalbumin Laboratory and signed by the laboratory medical director.
5. Hospital Specimen Receiving manages the transport of specimens between laboratories.
6. Test requests clearly describe the laboratory address and telephone number, the name of the medical director and contact personnel.

D. CRITERIA FOR SPECIMEN ACCEPTABILITY AND REJECTION

1. Corrupted specimen integrity, cracked or leaking tube, unreadable or missing label.
2. Visibly hematuric specimens.
3. Unacceptable temperatures occurred during transport or storage.
4. Sample spilled out. Tube or container empty.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

A. Equipment

1. ABSciex Qtrap 6500 coupled with Shimadzu LC-20XR liquid chromatography system with autosampler and column oven or suitable alternative. See 127191-47 LCMS-Operating Procedure and 127191-48 LCMS-Maintenance Procedure for details.
2. MultiTube Vortexer. Obtained from VWR; part number VWR-VX-2500 or suitable alternative. Speed range 1200 to 2400 RPM with speed accuracy within 25 RPM. Orbit of 3.6 mm. Timer Mechanical 0 to 60 seconds.
3. Digital Dry Baths/Block Heaters. Obtained from ThermoFisher Scientific; part number 88870003 or suitable alternative. Temperature Range Ambient +5° to 130°C (Ambient at 25°C). Temperature Accuracy ≤ ± 0.5°C. Temperature Uniformity ≤ ± 1°C.
4. Thermo Scientific, HERACELL VIOS 160i LK Incubator or suitable alternative. Thermal specification: The temperature deviation from the set value of 37 °C is ≤ ±0.3 °C.
5. Eppendorf Repeater Pipette. Manufactured by Eppendorf; obtained from Fisher Scientific; part number 14-287-150 or suitable alternative.
6. Ranin 20–200 µL Pipet Lite XLS (L-200XLS+) by Mettler-Toledo or suitable alternative.
    a. 20 µL: Accuracy ±0.5 µL (±2.5%), Precision ±0.2 µL (±1%)
    b. 100 µL: Accuracy ±0.8 µL (±0.8%), Precision ±0.25 µL (±0.25%)
    c. 200 µL: Accuracy ±1.6 µL (±0.8%), Precision ±0.3 µL (±0.15%)
7. Ranin 2.0–20.0 µL Pipet Lite XLS (L-20XLS+) by Mettler-Toledo or suitable alternative.
    a. 2 µL: Accuracy ±0.15 µL (±7.5%), Precision ±0.04 µL (±2%)
    b. 10 µL: Accuracy ±0.15 µL (±1.5%), Precision ±0.05 µL (±0.5%)
    c. 20 µL: Accuracy ±0.2 µL (±1%), Precision ±0.06 µL (±0.3%)

8. KIMAX® 8 mL Class A volumetric pipet or suitable alternative. Manufactured by KIMBLE, obtained from Neta Scientific, part number 37002-8 or suitable alternative. ± 0.01 mL tolerance.
9. KIMAX® 4 mL Class A volumetric pipet or suitable alternative. Manufactured by KIMBLE, obtained from Neta Scientific, part number 37002-4 or suitable alternative. ± 0.01 mL tolerance.
10. KIMAX® 2 mL Class A volumetric pipet or suitable alternative. Manufactured by KIMBLE, obtained from Neta Scientific, part number 37002-2 or suitable alternative. ± 0.01 mL tolerance.
11. KIMAX® 1 mL Class A volumetric pipet or suitable alternative. Manufactured by KIMBLE, obtained from Neta Scientific, part number 37002-1 or suitable alternative. ± 0.006 mL tolerance.
12. KIMAX® 10 mL Class A volumetric pipet or suitable alternative. Manufactured by KIMBLE, obtained from Neta Scientific, part number 37002-10 or suitable alternative. ± 0.02 mL tolerance.
13. KIMAX® 5 mL Class A volumetric pipet or suitable alternative. Manufactured by KIMBLE, obtained from Neta Scientific, part number 37002-5 or suitable alternative. ± 0.01 mL tolerance.
14. KIMAX® 6 mL Class A volumetric pipet or suitable alternative. Manufactured by KIMBLE, obtained from Neta Scientific, part number 37002-6 or suitable alternative. ± 0.01 mL tolerance.
15. 50 mL Fisherbrand™ Reusable Glass Class A Volumetric Flasks with Standard Taper Stopper or suitable alternative. Obtained from Fisher Scientific, part number 10-205B or suitable alternative. ± 0.05 mL tolerance.
16. 200 mL Fisherbrand™ Reusable Glass Class A Volumetric Flasks with Standard Taper Stopper or suitable alternative. Obtained from Fisher Scientific, part number FB400200 or suitable alternative. ± 0.1 mL tolerance.
17. Mettler Toledo MS104S/03 or suitable alternative. Technical specifications: The minimum accurately measurable weight is 16 mg, with an uncertainty of 1% (k=2). The maximum allowable load is 120 g. The acceptable accuracy for day-of-use maintenance ranges from 99.70% to 100.30%, verified using 1.0000 g and 5.0000 g weights. (Refer to document 127191-245 GEN – Balance Operation and Maintenance for details.)
18. Mettler Toledo XPR2 or suitable alternative. Technical specifications: The minimum accurately measurable weight is 0.1 mg, with an uncertainty of 1% (k=2). The maximum allowable load is 2.1 g. The acceptable accuracy for day-of-use maintenance ranges from 99.70% to 100.30%, verified using 0.0100 g and 1.0000 g weights. (Refer to document 127191-245 GEN – Balance Operation and Maintenance for details.)
19. Polyethylene Safety Can for Liquid Disposal, 5-Gallon. Manufactured by Justrite, obtained from Fisher Scientific, part number 14765 or suitable alternative.
20. Fisherbrand™ Sharps-A-Gator™ Sharps Containers. 1 gal. Obtained from Fisher Scientific, part number 14-827-63 or suitable alternative.
21. Hamilton Concept Fume hood or suitable alternative. See ‘127191-184 SAFE-Chemical Fume Hood’ for details. OSHA requires airflow into and within the hood

should not be excessively turbulent; hood face velocity should be adequate, typically between 60-110 fpm.

B. Consumable Supplies

1. Auto sampler vials. Obtained from ChromTech; 100 per package, part number CTV-1103 or suitable alternative
2. Snap Top Cap with Slit Septum. Obtained from ChromTech; 100 per package; part number CTC-1370B or CTC-1370G or suitable alternative.
3. Volume reducing autosampler vial inserts, 250 µL glass insert with a bottom spring. Obtained from ChromTech; 100 per package; part number CTI-9425 or suitable alternative.
4. 1.5 mL microcentrifuge tubes. Manufactured by Sarstedt; obtained from UMarket; 500 per package; part number CX15497 or suitable alternative.
5. Eppendorf Combitips Advanced 0.1 mL tip. Manufactured by Eppendorf; obtained from Fisher; 100 per package; part number 0030089405 or suitable alternative.
    a. 1 µL: systematic error ± 0.08 µL (± 8%), random error ± 0.08 µL (± 8%)
    b. 2 µL: systematic error ± 0.032 µL (± 1.6%), random error ± 0.06 µL (± 3%)
    c. 10 µL: systematic error ± 0.12 µL (± 1.2%), random error ± 0.24 µL (± 2.4%)
    d. 20 µL: systematic error ± 0.2 µL (± 1%), random error ± 0.4 µL (± 2%)
6. Eppendorf Combitips Advanced 0.5 mL tip. Manufactured by Eppendorf; obtained from Fisher; 100 per package; part number 0030089421 or suitable alternative.
    a. 5 µL: systematic error ± 0.2 µL (± 4%), random error ± 0.4 µL (± 8%)
    b. 10 µL: systematic error ± 0.09 µL (± 0.9%), random error ± 0.15 µL (± 1.5%)
    c. 50 µL: systematic error ± 0.4 µL (± 0.8%), random error ± 0.4 µL (± 0.8%)
    d. 100 µL: systematic error ± 0.8 µL (± 0.8%), random error ± 0.6 µL (± 0.6%)
7. Eppendorf Combitips Advanced 2.5 mL tip. Manufactured by Eppendorf; obtained from Fisher; 100 per package; part number 0030089448 or suitable alternative.
    a. 25 µL: systematic error ± 0.5 µL (± 2%), random error ± 0.875 µL (± 3.5%)
    b. 250 µL: systematic error ± 2 µL (± 0.8%), random error ± 1.125 µL (± 0.45%)
    c. 1250 µL: systematic error ± 6.25 µL (± 0.5%), random error ± 3.75 µL (± 0.3%)
    d. 2500 µL: systematic error ± 12.5 µL (± 0.5%), random error ± 3.75 µL (± 0.15%)
8. Ranin LTS 20 µL Pipet Tips. Manufactured by Mettler-Toledo; part number 30389291 or suitable alternative.
9. Ranin LTS 250 µL Pipet Tips. Manufactured by Mettler-Toledo; part number 30389299 or suitable alternative.
10. Kinetex 2.6 µm C18 100 Å, LC Column 50 x 3 mm obtained from Phenomenex; part number 00B-4462-Y0 or suitable alternative.

11. Fisherbrand™ Reusable Glass Media Bottles with Cap. Manufactured by Fisher; part number FB8001000 or suitable alternative.
22. PYREX™ Economy Grade Cylinders with Double Metric Scales, Calibrated to Contain. Manufactured by Corning; obtained from Fisher; part number 08-562-5F or suitable alternative. ± 50 mL tolerance.

C. Reagents

1. Acetonitrile (CH_3CN). CAS# 75-05-8. HPLC Grade, ≥99.9% purity, Item# A998-4 (Fisher Scientific, Waltham, MA) or equivalent. Stable at room temperature for 5 years. Use in a fume hood. (See 127191.184 SAFE-Chemical Fume Hood)
2. Ammonium bicarbonate (NH_4HCO_3). IUPAC name: azanium;hydrogen carbonate. CAS# 1066-33-7. 99.5% BioUltra, Item# 09830-1KG (Sigma Aldrich, St. Louis, MO) or equivalent. Stable at room temperature for 10 years.
3. Formic acid (CH_2O_2). CAS# 64-18-6. 99% Synthesis, Item# RABF0950-500A (RICCA, Arlington, TX) or equivalent. Stable at room temperature for 5 years. Use in a fume hood. (See 127191.184 SAFE-Chemical Fume Hood)
4. Iodoacetamide (C_2H_4INO). CAS# 144-48-9. 99% BioUltra, Item# I1149-5G (Sigma Aldrich) or equivalent. Stable at 2-8 °C for 2 years.
5. Methanol (CH_3OH). IUPAC name: oxidane. CAS# 67-56-1. HPLC Grade, ≥99.9% purity, Item# A452SK-4 (Fisher Scientific) or equivalent. Stable at room temperature for 5 years.
6. Monobasic potassium phosphate (KH_2PO_4). IUPAC name: potassium;dihydrogen phosphate). CAS# 7778-77-0. 99% certified ACS, Item# P285-500 (Fisher Scientific) or equivalent. Stable at room temperature for 10 years.
7. Potassium phosphate dibasic (K_2HPO_4). IUPAC name: dipotassium;hydrogen phosphate. CAS# 7758-11-4. 98% certified ACS, Item# P288-500 (Fisher Scientific) or equivalent. Stable at room temperature for 10 years.
8. Recombinant Human Albumin, Albagen XL. 50 mg/mL, Sterile > 98%, highly monomeric recombinant human albumin. Sterile 5% solution in PBS containing 4mM Sodium Caprylate and 4mM Acetyltryptophan. Molecular Mass 66,357 Dalton. Isoelectric Point, PI 4.7. Isoelectric Point, PI 4.7. Isoelectric Point, PI 4.7. It is free from animal components, viruses and prions. Its chemical properties and structure are equivalent to Human Serum Albumin except for a single deletion at the N-terminus (Asp). Item# 9801 (Albumin Bioscience, Huntsville, AL) or equivalent. Stable at 2-8 °C for 2 years.
9. Recombinant Human Serum Albumin N15. Recombinant Human Albumin N15, Albagen, is manufactured using N15 as the nitrogen source, yielding albumin with more than 98% N15. It is free from animal components, viruses, and prions. Its chemical properties and structure are equivalent to Human Serum Albumin, except for a single deletion at the N-terminus (Asp), Item# 9805 (Albumin Bioscience) or equivalent. Stable at 2-8 °C for 2 years.
10. Recombinant Human Serum Albumin Solution. Certified Values 0.958 ± 0.022 g/L. Standard Reference Material 2925 (NIST, Gaithersburg, MD) or equivalent. **Safety:** SRM 2925 is a frozen aqueous solution of recombinant HSA, expressed in

Pichia pastoris yeast cells and should be handled according to applicable federal, state, and/or local regulations and according to policies and procedures of recipient's organization. Normal caution and care should be exercised during the material's handling and use. **Storage:** SRM 2925 is shipped frozen (on dry ice) and, upon receipt, should be stored frozen until ready for use. A freezer temperature of –20 °C is acceptable for storage for up to one week. If a longer storage time is anticipated, the material should be stored at or below –70 °C. The SRM should not be exposed to sunlight or ultraviolet radiation. Storage of thawed material at room (20 °C to 25 °C) or refrigerator (5 °C to 8 °C) temperatures may result in degradation or modification of the constituent protein.

11. Sodium chloride (ClNa). IUPAC name: sodium;chloride. CAS# 7647-14-5. 99.5% certified by BAM, Item# S7653 (Sigma Aldrich) or equivalent. Stable at room temperature for 10 years.
12. Sodium citrate tribasic dihydrate ($C_6H_5Na_3O_7.2H_2O$). IUPAC name: trisodium 2-hydroxypropane-1,2,3-tricarboxylate. CAS# 6132-04-3. 99% certified ACS, Item# S4641 (Sigma Aldrich) or equivalent. Stable at room temperature for 10 years.
13. Bond-Breaker™ Tris (2-carboxyethyl) phosphine (TCEP) Solution, Neutral pH: 5 mL, contains a pH neutralized and stabilized aqueous 0.5 M TCEP solution. Item# 77720 (Thermo Scientific, Waltham, MA) or equivalent. Stable at room temperature for 2 years. TCEP ($C_9H_{15}O_6P$). IUPAC name: 3-[bis(2-carboxyethyl)phosphanyl]propanoic acid. CAS# 5961-85-3.
14. Trypsin from bovine pancreas. TPCK Treated, essentially salt-free, lyophilized powder, ≥10,000 BAEE units/mg protein. % Protein (UV) 90 - 100. CAS# 9002-07-7. Item# T1426 (Sigma Aldrich) or equivalent. Stable at –20 °C for 10 years.
15. Urea (NH_2CONH_2). CAS# 57-13-6. Item# U15-500 (Fisher Scientific) or equivalent. Stable at room temperature for 10 years.
16. Water (H_2O). IUPAC name: oxidane. CAS# 7732-18-5. HPLC Grade, ≥99.9% purity, Item# W5-4 (Fisher Scientific) or equivalent. Stable at room temperature for 5 years.

D. Solution Preparation Procedures

1. Synthetic urine solution (40 mM sodium chloride, 10 mM potassium phosphate dibasic, 10 mM monobasic potassium phosphate, 20 mM sodium citrate tribasic dihydrate, and 200 mM urea)
   To prepare 1 L of synthetic urine solution, add 2.3 g of sodium chloride, 1.41 g potassium phosphate dibasic, 0.2 g monobasic potassium phosphate, 5.1 g sodium citrate tribasic dihydrate, and 12 g urea to 1L HPLC grade water. Stable for 1 year at room temperature.
2. 0.1 M Ammonium bicarbonate
   To prepare 1 L of 0.1 M Ammonium bicarbonate, add 7.906 g of Ammonium bicarbonate to 1 L HPLC grade water. Stable for 1 year stored at room temperature.
3. 50 mM Iodoacetamide
   To prepare 1 mL of 50 mM Iodoacetamide, add 9.24 mg to 1 mL HPLC grade water. Use immediately and discard after use. Always prepare fresh before each use, storing it in the dark at room temperature.
4. 200 mg/L working albumin internal standard (IS) solution
   Add 40 µL Albumin IS stock to 10 mL of 0.1 M Ammonium bicarbonate. Stable for 1 year stored at 4 °C.
5. Working trypsin solution
   Add 1 mg of trypsin from bovine pancreas in 1 mL 0.1M ammonium bicarbonate. Use immediately and discard after use. Always prepare fresh before each use, storing it in the dark at room temperature.
6. 40 mM Tris(2-carboxyethyl)phosphine (TCEP)
   Add 800 µL of TCEP to 10 mL 0.1 M Ammonium bicarbonate. Stable for 1 year stored at room temperature.
7. Aqueous Mobile Phase (A)
   Dilute 1 mL of formic acid to 1 L with HPLC grade water. Stable for 1 year at room temperature.
8. Organic Mobile Phase (B)
   Dilute 1 mL of formic acid to 1 L with HPLC grade acetonitrile. Store in flammable cabinet and use in fume hood unless connected to LC system. Stable for 1 year at room temperature.
9. Albumin intermediate stock 5000 mg/L
   Add 5 mL of Human Serum Albumin from Bioscience 9801 to 45 mL of HPLC grade water. Stable for 3 years at -80 °C.

E. Calibrators (Standards)

Calibrators Derived from SRM 2925 (Serial Dilution Preparation)

Calibrators are made using the dilution scheme shown in Table 1, a 200 mg/L standard will be prepared by using 0.209 mL of NIST SRM 2925 (certified values 0.958 ± 0.022 g/L) dispensed into an appropriate pre-weighed vessel, and recording the mass observed. This mass will be used as the "sample" mass. The corresponding volume of diluent was added to an approximate total volume of 1.0 mL. The mass was measured again, and used to calculate the true concentration of the high standard. This solution was serially diluted seven times using 0.500 mL of the preceding standard and 0.500 mL of diluent down to an expected concentration of 3.125 mg/L following Table 1. The mass was measured before and after each dilution, and the masses used to calculate the true concentration of each calibrator using the NIST Urine Albumin Calibrator Spreadsheet.

Table 1. Calibration Solution Construction Scheme NIST Material.

Calibraror	Concentration	Units	Final Volume	Units	Albumin Source	Source Volume	Units	Theoretical Mass	Units	Diluent Volume	Units	Theoretical Diluent Mass	Units
N9	200	mg/L	0.5	mL	SRM 2925	0.209	mL	0.2088	g	0.791	mL	0.7897	g
N8	100	mg/L	0.5	mL	N9	0.500	mL	0.4990	g	0.500	mL	0.4990	g
N7	50	mg/L	0.5	mL	N8	0.500	mL	0.4990	g	0.500	mL	0.4990	g
N6	25	mg/L	0.5	mL	N7	0.500	mL	0.4990	g	0.500	mL	0.4990	g
N5	12.5	mg/L	0.5	mL	N6	0.500	mL	0.4990	g	0.500	mL	0.4990	g
N4	6.25	mg/L	0.5	mL	N5	0.500	mL	0.4990	g	0.500	mL	0.4990	g
N3	3.125	mg/L	0.5	mL	N4	0.500	mL	0.4990	g	0.500	mL	0.4990	g

In house Human Serum Ablumin working calibrators

a. **200 mg/L**: Using a Class A volumetric pipet carefully transfer 8000 µL of the 5000 mg/L stock into a 200 mL Class A volumetric flask. Fill the flask to the mark with calibration diluent then invert to mix at least 10 times with the cap on. Make 1 mL aliquots. Stable for 5 years at -80 °C.
b. **100 mg/L**: Using a Class A volumetric pipet carefully transfer 4000 µL of the 5000 mg/L stock into a 200 mL Class A volumetric flask. Fill the flask to the mark with calibration diluent then invert to mix at least 10 times with the cap on. Make 1 mL aliquots. Stable for 5 years at -80 °C.
c. **50 mg/L**: Using a Class A volumetric pipet carefully transfer 2000 µL of the 5000 mg/L stock into a 200 mL Class A volumetric flask. Fill the flask to the mark with calibration diluent then invert to mix at least 10 times with the cap on. Make 1 mL aliquots. Stable for 5 years at -80 °C.
d. **25 mg/L**: Using a Class A volumetric pipet carefully transfer 1000 µL of the 5000 mg/L stock into a 200 mL Class A volumetric flask. Fill the flask to the mark with calibration diluent then invert to mix at least 10 times with the cap on. Make 1 mL aliquots. Stable for 5 years at -80 °C.
e. **10 mg/L**: Using a Class A volumetric pipet carefully transfer 10000 µL of the 200 mg/L stock into a 200 mL Class A volumetric flask. Fill the flask to the mark with calibration diluent then invert to mix at least 10 times with the cap on. Make 1 mL aliquots. Stable for 5 years at -80 °C.

f. **5 mg/L**: Using a Class A volumetric pipet carefully transfer 5000 µL of the 200 mg/L stock into a 200 mL Class A volumetric flask. Fill the flask to the mark with calibration diluent then invert to mix at least 10 times with the cap on. Make 1 mL aliquots. Stable for 5 years at -80 °C.

g. **3 mg/L**: Using a Class A volumetric pipet carefully transfer 6000 µL of the 100 mg/L stock into a 200 mL Class A volumetric flask. Fill the flask to the mark with calibration diluent then invert to mix at least 10 times with the cap on. Make 1 mL aliquots. Stable for 5 years at -80 °C.

Table 2. Calibration scheme of working calibrators using Bioscience 9801 stock solution. Refer to Table 6 for the value-assigned concentrations of working calibrators.

Calibrator Name	Standard Solution (mg/L)	Total Volume (mL)	Stock Solution added	Stock Volume added (mL)	Diluent added	Diluent Volume (mL)
Intermediate Stock	5000	50	50 mg/mL (Bioscience 9801)	5	Water	45
S7	200	200	5000 mg/L	8	Synthetic Urine*	192
S6	100	200	5000 mg/L	4	Synthetic Urine*	196
S5	50	200	5000 mg/L	2	Synthetic Urine*	198
S4	25	200	5000 mg/L	1	Synthetic Urine*	199
S3	10	200	200 mg/L	10	Synthetic Urine*	190
S2	5	200	200 mg/L	5	Synthetic Urine*	195
S1	3	200	100 mg/L	6	Synthetic Urine*	194

Value Assignment of In-House Calibrators Using SRM 2925 Calibration

Once calibrators are prepared from the SRM 2925 they are analyzed in quadruplicate on a same batch as the “in-house” prepared calibrators. The “in-house” calibrators will then be run as unknowns also in quadruplicate. Concentrations are expressed as the mean of the four independent preparations for both the “in-house” and SRM 2925 derived calibrators. This provided the concentration value assignment to be used moving forward for final validation of the albumin reference measurement procedure. This data will be entered in the Urine Albumin Value Assignment spreadsheet.

F. Controls

Quality Controls (QC) are prepared from pooled human urine specimens from laboratory employee volunteers. QC pools will be constructed such that the levels of albumin and correspond to three approximate levels.

Approximately 300 mL of human urine was pooled and then divided it into three 100 mL Class A volumetric flask and labeled them low, medium and high. We spike the 5000 mg/L albumin intermediate stock as Table 3. We then measured them on Roche Cobas to verify the albumin concentrations. The three levels of QCs are stable for five years at -80 °C.

Table 3. Urine QC construction scheme.

QC Name	Final Concentration (mg/L)	Total Volume (mL)	Stock Concentration added (mg/L)	Stock Volume Used (mL)	Diluent added	Diluent Volume (mL)
Low	30	100	5000	0.562	Pooled Human Urine	99.4
Med	80	100	5000	1.563	Pooled Human Urine	98.4
High	150	100	5000	2.963	Pooled Human Urine	97.0

Note: final albumin concentrations will fluctuate depending on endogenous concentrations of pooled human urine.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

Calibration curve

a. The calibration curve consists of 7 points ranging from 3.00 to 200 mg/L.
b. The lowest standard should be within ± 15% of the expected value.
c. All other standards should be within ± 10% of the expected value.
d. Acceptable curves will have an R^2 value of 0.980 or greater when calculated with 1/x weighting.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

Specimen Preparation Procedure

- The operating room temperature should be maintained between 20°C and 22°C.
- Each batch comprises 7 working calibrators, a blank (containing IS but no sample), a double blank (without IS or sample), 3 levels of urine QC, and urine samples.
- Run order: blank, double blank, low standard – high standard, blank, double blank, QC Low, half the patient samples, QC Med, other half of patient samples, QC High.

1. Incubate calibrators, QCs, and urine specimens at 37°C for 30 min in HERACELL VIOS 160i LK Incubator.
2. Print two sets of labels featuring both IDs and scannable barcodes using Bartender software and label one set of 1.5 mL microcentrifuge vials and another set of 2 mL autosampler vials. Place 250 µL volume reducing inserts in to appropriately labeled 2 mL glass auto sampler vials.
3. Mix reagents, calibrators, QCs, and urine samples by 10 inversions prior to preparation.
4. In each 1.5 mL microcentrifuge vial, combine below components. These mixtures will be referred to as "samples" in subsequent steps.
    a. 135 µL of 0.1 M ammonium bicarbonate solution using a repeater pipette.
    b. 40 µL from each calibrator, QC, and urine sample to appropriate tube, 40 µL 0.1M ammonium bicarbonate solution for blank, and 60 µL 0.1M ammonium bicarbonate solution for double blank using a L-200XLS+ pipette.

   c. 20 µL of the working internal standard solution to each tube except for the double blank using a repeater pipette.
   d. 10 µL of 40 mM TCEP in 0.1 M ammonium bicarbonate solution using a repeater pipette.
5. Vortex samples to mix using a MultiTube vortexer.
6. Incubate samples at 60 °C for 1 hr using a Digital Dry Bath/Block Heater.
7. Add 10 µL of 50 mM iodoacetamide solution to samples using a repeater pipette.
8. Vortex samples to mix using a MultiTube vortexer.
9. Incubate samples at room temperature for 30 minutes at dark.
10. Add 5 µL of working trypsin solution to samples using a repeater pipette.
11. Vortex samples to mix using a MultiTube vortexer.
12. Incubate samples at 37 °C for 1 hour using a Digital Dry Bath/Block Heater.
13. Add 2 µL of concentrated formic acid to samples using a repeater pipette under chemical fume hood.
14. Vortex samples to mix using a MultiTube vortexer.
15. Transfer approximately 200 µL of each sample from the 1.5 mL microcentrifuge vial to the volume-reducing insert in the corresponding 2 mL autosampler vial using a Rainin L-200XLS+ pipette. Dispose of the microcentrifuge tubes in a biohazard bag.
16. Close each autosampler vial with a snap top cap.
17. Place all autosampler vials in order of blank, double blank, standards (low to high), blank, double blank, QC low, half the samples, QC medium, the other half of the samples, and QC high on the autosampler tray and submit for analysis.
18. Once results are verified, dispose of autosampler vials in a biohazard bag.

Dilution of Samples with Albumin Concentrations above 200 mg/L

The confirmed analytical measurement range of the assay is 3.00 - 200 mg/L. Dilute any specimens exceeding 200 mg/L using HPLC grade water until an appropriate concentration is reached.

   a. To perform a 1:2 dilution, pipette 100 µL of the patient sample into 100 µL of HPLC Grade Water. Mix by vortexing 3 seconds, perform the assay, and multiply the result by a factor of 2.
   b. To perform a 1:10 dilution, pipette 20 µL of the patient sample into 180 µL of HPLC Grade Water. Mix by vortexing 3 seconds, perform the assay, and multiply the result by a factor of 10.
   c. To perform a 1:50 dilution, pipette 20 µL of the patient sample into 980 µL of HPLC Grade Water. Mix by vortexing 3 seconds, perform the assay, and multiply the result by a factor of 50.
19.

Interpretation of Results

1. Integration
    a. Integration of peaks is performed using the MultiQuant software, a companion to the Analyst software used to control the LC-MS/MS system.
    b. Albumin is quantified using LVNEVTEFAK y8 peptide transition.
    c. N^{15} labeled human serum albumin is used as the internal standard for human serum albumin transitions.
2. Calibration curve
    a. The calibration curve consists of 7 points ranging from 3 to 200 mg/L. Enter the concentrations under 'actual concentration' in the MultiQuant software. A minimum of 6 calibration points is required.
    b. The lowest standard should be within ± 15% of the expected value.
    c. All other standards should be within ± 10% of the expected value.
    d. Acceptable curves will have an R^2 value of 0.980 or greater when calculated using linear regression with 1/x weighting.
3. Internal standard
    a. Highlight the internal standard area column for each analyte. MultiQuant software metric plot function is used to visually inspect the internal standard peak area to check for ± 50% deviation from the mean.
    b. Internal standards should be consistent within each batch. If trending deviations occur, check assay and verify quality control results. Repeat if necessary.
    c. If internal standards are indicating a downward trend throughout the assay, test for charging.
    d. If one specimen's internal standard is outside ± 50% from the mean, that specimen should be reinjected. If the sample still fails to meet the IS recovery criteria after reinjection, it should be repeated with a new calibration curve. If the IS recovery remains outside the acceptable range and the repeated results are within 20% of the original, report the repeated results.
4. Retention time
    a. Highlight the retention time column for each analyte and graph using the metric plot.
    b. All retention times for a given analyte should be stable within each batch. Check any outliers to verify the correct peak is assigned: retention time should be within ± 0.4 minutes of mean calibrator retention time.
5. Ion ratio
    a. An ion ratio is assigned from each calibration curve to catch potential interferences in samples.
    b. The ion ratio will flag if it is outside the acceptable limits of 20%.
6. High urine Albumin results

a. Urine albumin samples greater than 200 mg/L when run at a x10 dilution (actual value is greater than 2,000 mg/L) need to be repeated with a x20 (or greater, if required) dilution. Samples injected after the high concentration sample should be reinjected to ensure that no carryover occurred.
b. Diluted values should be multiplied by dilution factor in the resulting MultiQuant file.

9. REPORTABLE RANGE OF RESULTS

The confirmed analytical measurement range of the assay is 3.00-200 mg/L. Dilute any specimens exceeding this limit using HPLC Grade Water until an appropriate concentration is reached.

Table 4. Analytical measurement range and reportable range of results.

Analytical Measurement Range	3.00 mg/L to 200 mg/L
Reportable Range	3.00 mg/L to 10,000 mg/L

10. QUALITY CONTROL (QC) PROCEDURE

All levels of quality control are analyzed once on each batch of samples. Results are verified for acceptability prior to reporting specimen values. All controls are compared to a 21-day inter-assay established mean and standard deviation by plotting on the albumin QC Tracking sheet inside the Mass Spec folder on the shared drive (S:). All values are recorded and plotted on the spreadsheet, Concentration values within ± 2SD are considered acceptable. Values within ± 3SD may be accepted based on tech notes in the QC tracking spreadsheet. Values outside ± 3SD are unacceptable and are repeated following the procedure below. A new mean and standard deviation will be established for each new lot of quality controls.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If calibrators and QC values are outside of specified ranges, do the following, in order, until result is acceptable:

1. Reinject the calibrator and or QC sample.
2. Perform the last dilution step again with the saved supernatant and inject on the instrument.
3. Repeat the analysis using fresh calibrator and QC material.

4. Repeat the entire batch, including standards and patient specimens.
5. Bring issue to Laboratory Director.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Limit of Detection (Signal to Noise of 3)	0.02 mg/L
Limit of Quantitation (%CV < 20%)	3.00 mg/L
Intra-assay %CV (LVNEVTEFAK y8) (20 within-day replicates at a urine concentration of 32.5 mg/L)	3.44%
Inter-assay %CV (LVNEVTEFAK y8) (20 between-day replicates at a urine concentration of 31.2 mg/L)	3.12%
Inter-assay %CV (LVNEVTEFAK y8) (20 between-day replicates at a calibrator concentration of 3.00 mg/L)	3.51%

No interference was observed in measurement procedure due to hemoglobin or IgG content.

13. REFERENCE RANGES (NORMAL VALUES)

2nd morning urine
Adults: <20 mg albumin/g creatinine
Children (3-5 years): <20 mg/L albumin <37 mg albumin/g creatinine

24-hour urine: <20 mg/L <30 mg/24 h (0.456 µmol/24 h)

CSF (>4 years): 10-30 mg/L

• Advanced Research and Diagnostic Laboratory ranges:

All ages and genders: <30 mg albumin/g creatinine

14. CRITICAL CALL RESULTS ("PANIC VALUES")

There are no “panic values” which indicate immediate medical intervention for unusual urine albumin values. Study-specific critical values are defined with a code as determined by the study coordinators. Nephropathy can be defined by increased urine albumin values when measured by urine albumin excretion rate or urine albumin/creatinine ratio.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens are stored at 2-8^{o}C during preparation of testing. Specimens may reach room temperature during the following procedures: pipetting, micro-centrifugation, and during the Micromedic procedure. A critical temperature during the testing procedure is the 37^{o}C

incubation. When incubation ends, a buffer is introduced to quench the reaction and to begin the centrifugation/washing procedure.

16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

a. We do not have an alternative method for performing the test if the test system fails.
b. Manual pipettes can be substituted for the automatic dispensers.
c. Return specimens to -80°C if the test system is out of operation.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Results are downloaded from StarLIMS. See separate procedure "NHANES LIS Workflow" from University of Minnesota/ARDL for additional detail. The results spreadsheet is sent electronically by the ARDL LIS contact person to Westat.
There is no critical result for this procedure.

18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

A. Specimen Receipt

Shipments for NHANES generally will arrive on Tuesdays and/or Wednesdays and Fridays. These shipments are recorded on the Log of Quality Assurance located in the ARDL Share Drive. The specimen barcode numbers in the boxes are checked against the manifests. The receipt date is written on top of the boxes. The frozen samples are placed in the designated -70C freezer and the refrigerated samples are placed in the designated 2-8C refrigerator until analysis. The manifests are filed in a binder labeled NHANES Shipping Manifests located in the receiving area. The shipping box is returned back to the study.

B. Quality Assurance Log

A Quality Assurance Specimen Receipt and Specimen Return Log is maintained by laboratory staff. The following parameters are tracked: NHANES shipper I.D., NHANES Container I.D., Vial #, Date Received, Specimen Receipt Conditions, Number of Specimens Received, 2.5% QC Repeats, Total Number of Specimens, 21 Day Due Date, Analysis Date, Date Results Sent, Number of Days For Result Return, Thaw Date (if applicable), Return To Freezer Date, Number of Days at Refrigerated Temperature, 1 Year Discard or Return Date, and Biorepository receipt number.

C. Specimen Ordering and Preparation for Analysis

Electronic files for all NHANES specimens are sent via email from Westat, Inc to the NHANES contact person shortly before they are to be received. These files include the

Sample ID, Analyte Type, Slot No, Sample Collection Date, Sample Comment, Age Grouping, Astro ID, Receipt Date, Analysis Date, Run Number, Tech ID, Analyte Result, Result Comment, Adjusted Result, QC Repeat, LOD, Change Reason, and Change Reason Other. The first seven columns are protected and cannot be altered. The files are saved on the laboratory's common S drive in the respective NHANES folder. After analysis, the contact person returns the completed files via their website to Westat, Inc.
The NHANES spreadsheets are used to set up pending batches for batch accession upload in the Laboratory Information system (StarLIMs). New labels are generated out of the Laboratory Information System (StarLIMs). The new bar-coded labels are attached to a carrier tube.

D. Specimen Storage

The temperatures for all freezers and refrigerators are monitored 24 hours a day/ 7 days a week. If the temperature for any unit falls outside the allowable range, action is taken to resolve the problem. If the temperature cannot be corrected, the contents are moved to a different unit.

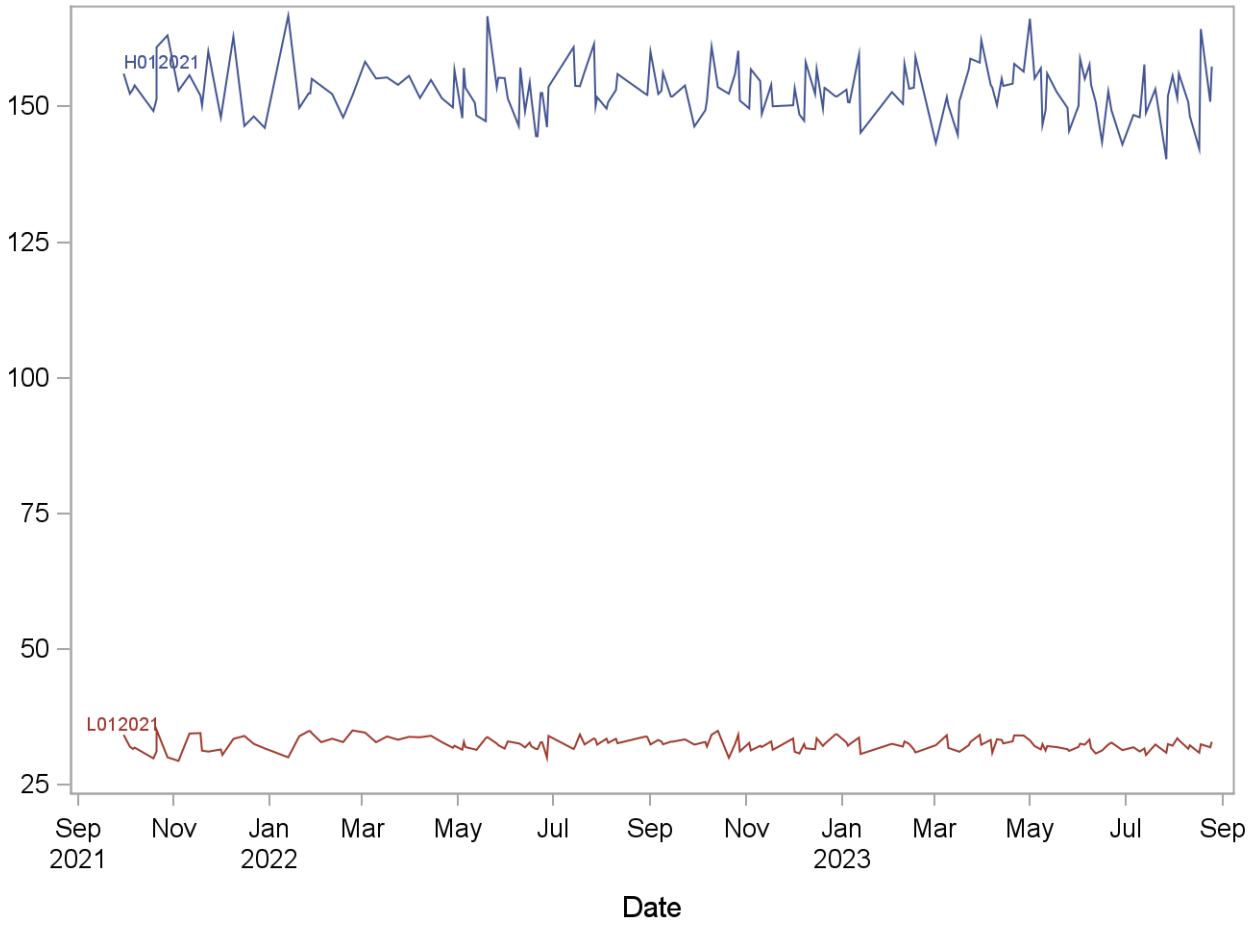
E. Specimen Handling and Return

On the day of analysis, the specimens are selected and thawed by the Cobas 8000 operator. The samples are thawed at 37C and mixed before centrifuging. The samples remain at room temperature until testing is completed. After analysis and the QC repeats have been run, the specimens are refrozen. After 1 year, the specimen vials that are designated for return are shipped to SriSai Biopharmaceuticals in Frederick, MD. These specimens are shipped on dry ice via FedEX

19. SUMMARY STATISTICS AND QC GRAPHS

August 2021-August 2023 Summary Statistics and QC Chart
URXUMA (Albumin, urine (ug/mL))

Lot	N	Start Date	End Date	MEAN	Standard Deviation	Coefficient of Variation
H012021	170	30SEP21	25AUG23	152.920	4.706	3.1
L012021	170	30SEP21	25AUG23	32.492	1.163	3.6



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