Title: Human Urine alpha1-microglobulin (A1M) Protocol using the Siemens BNII Nephelometer


Version Number: 1.1

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</tr>
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</tr>
</tbody>
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Document History

<table>
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<tr>
<th>Date</th>
<th>Comment</th>
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Purpose

The Standard Operating Procedure (SOP) describes the process for the quantitative assessment of alpha-1-microglobulin (A1M) in human urine specimens on the SIEMENS BNII platform.

Introduction

The clinical relevance of the A1M assay relates to the identification of tubular proteinuria. A1M, also referred to as protein HC3, is a low-molecular weight glycoprotein (MW ~28,000), primarily produced by liver and filtered through glomeruli. As with other plasma proteins which are filtered through the glomeruli, reabsorption and catabolism take place in the proximal tubules. Detection of elevated urinary concentrations of A1M can be indicative of tubular damage as may occur in the context of nephritides, advanced diabetic nephropathy, after exposure to heavy metals or after administration of nephrotoxic medications or other kidney diseases (acute or chronic).

Sample Collection, Handling & Storage

Frozen urine samples received from investigators will be immediately stored at -80 °C. All the personnel handling the specimens will have undergone Environmental Health & Safety training at Brigham & Women’s Hospital. Protective gear including lab coats and gloves must be worn while working in the laboratory. The reagents necessary for the experiment (such as OWLA, OQLV, and OQLW) will be stored at 4°C fridge as instructed by the individual insert sheet.

Procedure

Location

Harvard Institutes of Medicine Room 550

Renal Division/ Department of Medicine/ Brigham and Women’s Hospital

Required equipment / reagents

- SEIMENS BNII instrument and connected Apple Macintosh computer
- BNII software
- N-Predilution Wells (SEIMENS REF # OVIC11)
- BioTek Precision XS™ (PRC3841M)
- Refrigerated Centrifuge (Eppendorf 5430R Rotor F-35-6-30)
- Vortex
- VIAFLO II Electronic pipettes
  - VIAFLO Electronic Pipets: single (Part # 4013), 8 (Part # 4624) and 12 (Part # 4632 and 4633) channels.
- Single Channel Manual Pipettes
  - Rainin Classic 0.5 μl-10 μl (catalog # PR-10)
  - Rainin Classic 10 μl-100 μl (catalog # PR-100)

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- Rainin Classic 100 μl-1000 μl (catalog # PR-1000)
- 37°C incubator (Thermolyine, Type 41900)

### SIEMENS BNII Reagents

- BN Additive for Wash solution (Ref OQKY61)
- N Diluent (Ref OUMT65)
- N Reaction buffer (Ref OUMS65)
- neodisher GK (Ref OQRK51)
- N α1-Microglobulin kit (Ref OWLA11)
- N Protein Standard UY (Ref OQLV11)
- N/T Protein Control LC (Ref OQLW15)

- MilliQ water (Type 1, reagent grade water) (MilliQ Academic, Cat # ZMQ600017)

### Buffer Preparation and Maintenance

<table>
<thead>
<tr>
<th>Name</th>
<th>REF #</th>
<th>Preparation</th>
<th>Stability once opened</th>
</tr>
</thead>
<tbody>
<tr>
<td>BN II Additive</td>
<td>OQKY61</td>
<td>Stock in 4°C refrigerator. One bottle of BN II Additive is for 5 L of Di-water.</td>
<td>7 d</td>
</tr>
<tr>
<td>N Diluent®</td>
<td>OUMT65</td>
<td>Stock at RT. Ready for use</td>
<td>6 w</td>
</tr>
<tr>
<td>N Reaction buffer®</td>
<td>OUMS65</td>
<td>Stock at RT. Ready for use</td>
<td>6 w</td>
</tr>
<tr>
<td>neodisher GK®</td>
<td>OQRK51</td>
<td>Stock in 4°C refrigerator. Dissolve 10g powder in 1L of warm water.</td>
<td>Immediately use</td>
</tr>
<tr>
<td>N α1-Microglobulin kit</td>
<td>OWLA11</td>
<td>Stock in 4°C refrigerator. Ready for use</td>
<td>4 w in fridge</td>
</tr>
<tr>
<td>N Protein Standard UY®</td>
<td>OQLV11</td>
<td>Stock in 4°C refrigerator. Add 0.5 mL of Di-water, mix gently and wait for 30 min</td>
<td>14 d in fridge</td>
</tr>
<tr>
<td>N/T Protein Control LC®</td>
<td>OQLW15</td>
<td>Stock in 4°C refrigerator. Add 1.0 mL of Di-water, mix gently and wait for 30 min</td>
<td>14 d in fridge. The control in the analyzer is valid for 4h</td>
</tr>
</tbody>
</table>

### Experimental Procedure

**Assay Protocol for the BNII System**

1. For a BNII System, reagents will be stored as described in the table above.

2. The assay protocols are given in the Instruction Manual and software of the instrument. All steps are performed automatically by the system.

### Specimens

Sample thawing: Sample tubes will be arranged in open tube racks with an empty space between each tube for better air circulation. Place these racks in a 30°C incubator until all samples are thawed (around 15 min, the tubes will still be cold after the samples are thawed). Vortex the tubes for 5 sec, centrifuge at 3000 rpm for 5 min at 4°C, keep on ice, and proceed for the analysis.
Urine samples are assayed in undiluted form. For the A1M test, the assay uses 20 µl of sample for each measurement, however the BNII instrument requires a volume of sample from 200 to 500 µl for handling the sample. The amount not used for the assay is available to retrieve after the assay is performed.

**Preparation of Standard**

Reconstitute N Protein Standard UY® (REF OQLV11) with 0.5 mL of distilled water. Mix by swirling carefully, avoiding foam formation and vigorous shaking. The product is ready for use after the content has dissolved to give a clear solution (usually takes 30 minutes). The reconstituted solution is ready to use on the analyzer. It is stable for 14 days if stored tightly closed at +2 to +8 °C directly after use. Standard solution must be discarded if it has developed turbidity or a sediment.

**Preparation of the Controls**

N/T Protein Control LC® (REF OQLW) is intended for use as an assayed accuracy and precision control for A1M. Reconstitute N/T Protein Control LC® with 1.0 mL of distilled water. Mix by swirling carefully, avoiding foam formation and vigorous shaking. The product is ready for use after the contents have dissolved to give a clear solution (usually takes 30 minutes). The reconstituted solution is stable for 14 days if stored tightly closed at +2 to +8 °C directly after use. Discard the control if any turbidity or sediment appears.

**Establishment of the Reference Curve**

Scan the barcode sheet that came along with the N Protein Standard UY kit which automatically registers the lot number and the amount of a1M present in the standard by the instrument. The reconstituted Standard UY bottle should be placed into rack 6-15. In the software, the procedures are following: Reference Curves → 29 a1MU in Assay section → Correct Lot # in Reagent Lots section → Measure.

Reference curves are constructed by multi-point calibration (Figure 1). Serial dilutions of N Protein Standard UY will be automatically prepared by the instrument using N Diluent®. The standard dilutions are to be used within four hours. The established reference curve is valid as long as the controls with corresponding method-depending target values, e.g. N/T Protein Control LC, are reproduced within their respective confidence range (For N/T protein Control LC, the range is 30.6 – 41.4 mg/L). If a different lot of antiserum is used, a new reference curve must be recorded.

**Control LC® Measurement**

Scan the barcode sheet of N/T Protein Control LC®, reconstitute the Control LC® following the previous instruction. The bottle should be put into rack 6-15. In the software, the procedures are following:

1. At the top of the menu, choose “Routine”, then “Request Controls”.

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2. Make sure the Lot number of Protein Control LC is correct, then select “X”, which will turn blue. Then click “Measure”.

3. Choose the current Lot number of a1-Microglobulin antiserum, then click “Measure”.

4. The “X” will turn to “?”, which means the instrument is in the measurement step.

Control LC® should be measured every time before the sample measurement. After the Control LC® measurement is done, immediately take Control LC® back to the fridge.
BioCon IDs Tracking

During the assay the BioCon ID is scanned at the time when the sample is loaded into the BNII Instrument (Figure 2). The BioCon IDs will be automatically sent to the Lab Journal in the BNII software.

Figure 1. Standard curve example. Assay: A1M

Figure 2. Sample BioCon ID scanning.
Sample Measurement

The samples should be inserted into the rack with barcode IDs facing left side toward the scanner. Push the tubes to the very bottom of the rack. If any of the sample tubes are not scanned properly in the tube rack, the sample loading window will pop up automatically after loading, which requires manual input. All the loaded samples will be ejected from the rack and shown in the “without request” section. Double click each sample and choose “29 a1MU” at the bottom left, select correct dilution factor (default 1:1), then click “Save & Close” and keep the sample rack back into the instrument, the sample will be accepted, and the measurement will start.

Results

The data output will be provided automatically in units of mg/L. If the values are above the upper measurement range, the assay will be repeated automatically at a higher dilution of the sample. If the sample is below the detection range, it will not be repeated.
BWH Biomarkers Core validation of $\alpha$1-Microglobulin (Ref OWLA11)

Minimum sample volume required for Siemens BNII

The BNII instrument uses 20 $\mu$L sample volume per test (if further dilution is necessary, this amount will increase accordingly to the dilution required). However, the instrument defaults to require 500 $\mu$L of sample volume to perform the aspiration. Table 2 shows the procedure performed by the BWH Biomarkers Core using various volumes of sample. We concluded that 200 $\mu$L of urine sample volume is sufficient to measure A1M with precision (CV % <15). This allows us to conserve sample.

Table 1. Minimum sample volume handling assessment.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume Tested (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>500</td>
</tr>
<tr>
<td>LC Control®</td>
<td>37.2</td>
</tr>
<tr>
<td>QC Low GFR</td>
<td>244</td>
</tr>
<tr>
<td>Sample 1</td>
<td>211</td>
</tr>
<tr>
<td>Sample 2</td>
<td>17.6</td>
</tr>
<tr>
<td>Sample 3</td>
<td>22.5</td>
</tr>
<tr>
<td>Sample 4</td>
<td>126</td>
</tr>
<tr>
<td>Sample 5</td>
<td>41.4</td>
</tr>
</tbody>
</table>

nev, not enough volume

Table 2. BWH Laboratory test validation for A1M in urine using the SIEMENS BNII Nephelometer

<table>
<thead>
<tr>
<th>Assay Parameters</th>
<th>A1M Siemens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference Interval (mg/l)</td>
<td>0-12</td>
</tr>
<tr>
<td>Assay Range (mg/l)</td>
<td>LLQ = 5.63</td>
</tr>
<tr>
<td></td>
<td>ULQ = 180</td>
</tr>
<tr>
<td>Dilution Linearity</td>
<td>Linear across 1:5, 1:20.</td>
</tr>
<tr>
<td>Spike Recovery (%)</td>
<td>95-110%</td>
</tr>
<tr>
<td>Intra-assay Variability (Internal Control, n=10) (CV%)</td>
<td>LC Control® (37.07 mg/l) = 1.81</td>
</tr>
<tr>
<td>Inter-assay Variability (Internal Control, n=7) (CV%)</td>
<td>LC Control® (38.14 mg/l) = 4.10</td>
</tr>
<tr>
<td>Intra-assay Variability (QC urine sample, n=4) (CV%)</td>
<td>QC High GFR (57.40 mg/l) = 4.02</td>
</tr>
<tr>
<td></td>
<td>QC Low GFR (239.60 mg/l) = 1.2</td>
</tr>
<tr>
<td>Inter-assay Variability (QC urine sample, n=7) (CV%)</td>
<td>QC High GFR (56.70 mg/l) = 6.91</td>
</tr>
<tr>
<td></td>
<td>QC Low GFR (199.43 mg/l) = 5.42</td>
</tr>
<tr>
<td>Volume requirements</td>
<td>20 µl*</td>
</tr>
<tr>
<td>Time for the assay</td>
<td>40 samples in 20 minutes</td>
</tr>
<tr>
<td>High-throughput</td>
<td>Yes</td>
</tr>
<tr>
<td>Interference</td>
<td>Turbidity or particles in the sample. pH &lt;6</td>
</tr>
</tbody>
</table>

LLQ, Lower limit of quantification; ULQ, Upper limit of quantification; CV%, Coefficient of variation percentage.
* The minimum volume of sample for sample handling is 200 µL.

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Data Generation & Processing

Data Output

The BNII program creates a report of the results generating a file in pdf format. The report contains the BioCon ID, dilutions tested and the concentration of alpha-1-microglobulin in mg/L. The report is then manually exported to an excel sheet (Sheet 1).

Quality control

BNII Internal control

N/T Protein Control LC® is intended for use as inter laboratory quality control for assessment of precision and analytical bias in immunochemical determination of the protein. Control LC® is supplied as lyophilized powder. A1M concentration values in Control LC® varies with different lot number. Control LC will be analyzed every day. We will use control LC for assessing inter-assay variability.

QC controls

In addition to the above QC control, BWH Biomarkers Core use two additional urine samples as QC on daily basis to assess intra- and inter-assay variability using CKD samples. QC Control High is a urine sample from a CKD patient with eGFR higher than 60 ml/min/1.73m². QC control Low is a urine sample from a CKD patient with eGFR lower than 60 ml/min/1.73m². In both cases the basal concentrations of a1M have been previously tested securing both a medium and high levels of a1M.

Intra-assay Variability

- We will ensure that biomarker levels obtained using the assay on control specimens (QC High and QC Low) are within mean ± 2SD limit using a Levey-Jennings control chart.
- If both samples are within mean ± 2SD limit, we will accept the data. If one of the two control values are outside mean ± 2SD, we will employ the Westgard 2 rules to determine if the analysis run results can be accepted.
- If neither of the assayed control results is within mean ± 2SD limits, then we will follow the Westgard three-quality control rule and not accept the data.

Quality Control for each sample

The BNII Siemens Instrument is an automatic, easy-to-use, reliable nephelometric analyzer where duplicate measurements of the same sample are not usually recommended. This recommendation, however, does not consider variability introduced by matrix effects of CKD urine samples. When a sample has a higher concentration of the A1M protein range for current dilution ratio (table 3), the machine will automatically re-measure the sample at higher dilution. For instance, if the concentration of a1M is more than 180 mg/L in neat sample, then the machine will automatically dilute the sample to 1:5 dilution.

Table 3. Protein Concentration Range for different Dilution Ratio

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### Quality Control troubleshooting procedure

If the controls fail the Westgard quality control procedure, then we will employ the following procedure to resolve the issue:

1. Contact the Lab Director
2. Rerun controls Control LC, QC High and QC Low again.
3. If the quality control values pass the above described Westgard rules proceed with sample testing.
4. If the quality control failed again, make fresh wash solution and re-calibrate the instrument using new standard and new LC Control.
5. If the quality control indicates that the Westgard rules are passed, proceed with testing
6. If QC fails again, recalibrate the assay (use new standard lot) and make fresh Diluent N and Reaction Buffer.
7. If after calibration the QC determinations have passed Westgard criteria, proceed with testing and document correction
8. If Westgard criteria were not met after preparation of fresh reagents, do not proceed with the analysis and contact the Laboratory Director.

### Data Storage and Reporting

After the analysis has passed the quality control test, analyte values along with their sample IDs will be copied and pasted into a Master Data sheet that contains a compendium of all the measurements for a specific BioCon project. To compile the data in a Master Data sheet results file, we will use the excel built-up function VLOOKUP, to automatically match the BioCon IDs for merging the data.

The master data sheet will contain the following information:

1. Sample BioCon ID
2. Date of the assay
3. Platform and the Instrument used for the assay
4. Concentration of biomarker (mg/l)
5. Levey-Jennings plots of LC Control, QC High and Low Control samples (these plots will be updated, and cumulative plots will be reported with each data submission).

The data will be shared internally via Dropbox across the BWH Biomarker team.

### BNII Instrument’s Maintenance

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<table>
<thead>
<tr>
<th>Frequency</th>
<th>Tasks</th>
</tr>
</thead>
</table>
| Daily     | • Check syringes, tubing and syringe valves for leaks, bubbles, crimps, cracks, contamination, and crystallization.  
• Replace used dilution strips.  
• Check liquid levels of reaction buffer, diluent, and washing solution, and replace as needed.  
• Empty waste container. |
| Weekly    | • Do **Syringes Washing**, Clean **Dispensing Probe** and **Cuvette Rinsing** procedures every week for the long-term maintenance. All functions can be found in “System-User service” section. DI-water is preferred for the long-term shut-down maintenance.  
• Check syringes, tubing, and syringe valves for contamination and crystallization.  
• Clean instrument surface and rack lanes with 70% ethanol solution.  
• Clean and inspect dispensing probes.  
• Back up Data folder to USB. |
| Monthly   | • Decontaminate system with Neodisher GK.  
• Replace wash filter.  
• Replace and clean cuvettes.  
• Replace wash solution container.  
• Clean level sensors with moist, lint-free cloth.  
• Clean barcode scanner with 70% ethanol solution.  
• Clean the mouse. |
| Yearly    | • Replace the syringes. |

**Brief protocol for BNII System**

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System Startup
1. Execute Daily Maintenance, turn on the analyzer and computer.
2. Turn on the BNII software, wait for ~15 min for the automatic initialization.
3. Dilution strips window will automatically open after the initialization. Click on Change dilution strips button, then click Left new and Right new buttons, click OK.
4. Make sure the Information Display Area show “analyzer is ready” in the system status field.

New Control or Standard – Lot No. Entry
1. (Automatic Entry) Scan the barcode documents in the control and standard kits, the information window will automatically show up, continue scan and the window will automatically close after all lines have been entered.
2. (Manual Entry) If the barcode document is not in the kits, select Calibration in Menu Bar, then Control Lots or Standard Lots, double click on the appropriate control or standard, enter the last two digits of the lot number, check that the desired units are displayed. Convert assigned values from the insert sheet units to the displayed units. Enter a value for the first assay, click Continue to proceed to the next assay, Click Save.

Calibration
1. Load reagents and insert racks following the table below:

<table>
<thead>
<tr>
<th>Name</th>
<th>Rack #</th>
<th>Lane #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagents</td>
<td>No. 20</td>
<td>1 to 5</td>
</tr>
<tr>
<td>Supplemental reagents</td>
<td>No. 20</td>
<td>3 to 5</td>
</tr>
<tr>
<td>Standards and controls</td>
<td>No. 10</td>
<td>6 to 15</td>
</tr>
<tr>
<td>Samples</td>
<td>No. 01 (plasma samples)</td>
<td>6 to 15</td>
</tr>
<tr>
<td></td>
<td>No. 05 (urine samples)</td>
<td></td>
</tr>
</tbody>
</table>

2. Put the loaded racks in. The analyzer shows missing or insufficient reagents.
3. New Lot Number. A window opens automatically to advise a calibration curve is needed.
4. Click OK. The Reference curves window opens automatically.
5. Click on appropriate assay, then choose the appropriate lot #, click on Measure.
6. After the measurement, Click on Reference curves Icon.

Processing Controls
1. From the menu bar, select Routine.
2. Select Request Controls.
3. In the control request screen, click in the cells for the appropriate controls and assays.
4. When all cells have been highlighted, click the Measure button. The “X” will become “?”.
5. Load the appropriate reagents and controls, click on the Control Journal icon to review the control results.

Processing Samples
1. Sample Loading
• Load samples and reagents in corresponding racks with barcode labels facing left. Insert each rack until it clicks. The sample barcode labels will be scanned by the barcode reader and will be sent to the Lab journal in the order in which the samples were loaded.

• From the menu bar of the Info dialog, select **Routine**.

• Select **Enter job list** and type the sample ID in the appropriate field. Select individual assays and/or profiles by clicking in the corresponding fields. Click on **Save** to continue entering data or **Save & Close** if finished.

Select “Sample” section by mouse, then scan the barcode if applicable, or input the sample ID manually.

• The Sample ID will be shown in “Sample” section, then select “29 a1MU” in “Assays” section. The default dilution ratio is neat. If needed, change the dilution ratio manually by clicking “Dilution” button. Click “Save” for next sample, or click “Save & Close” when finish inputting the sample ID.
• The saved sample ID will be shown in the “missing” section, meaning the instrument is waiting for the sample loading.

• Click on the Loading icon on the top of the menu (the first icon).

• Find the correct Rack ID that is used to load sample. The rack ID for plasma sample starts with 01, whereas the rack ID for urine sample starts with 05.
- Select the first well, click “Autoload”, then the information of ten samples will be autoloaded into the rack by the previous sequence of scanning. Different racks can be used to load sample, but make sure the Rack ID is correct. Eventually all the sample information should be loaded into rack, and the “New samples only” window in the “Show: “ field should be empty.

- Close the “Loading” window and insert the sample rack. The saved samples will move to “loaded” section, meaning the instrument is now running the test. When test is done, the results can be found in “finished” section.
Changing Samples to Controls

If QC statistics is needed, the sample can be changed into a control by following procedure:

1. Select the sample ID, open the Action pop-up menu.
2. Select the Change sample to control function, by clicking the previous defined control, select the assignment to the required probe and confirm this with Assign.

Querying the results

1. Click the Lab journal button to view the results.
2. To release the results manually, highlight the relevant results and click the Release button in the toolbar.

BN II System Shutdown Procedure

1. Make sure that all results have been printed or released to host computer.
2. Delete the Lab journal.
3. Select BNII, Quit BNII from the menu bar.
4. Click on Perform in the Shift Change screen.
5. Click on Close in the “Result documentation” screen. “Quit the Program” screen appears automatically.
   Do not click on Cancel prematurely button, allow the software to close automatically, and the desktop will be displayed.

BN II Data Folder Backup

1. Quit the BN II program as instructed.
2. On the desktop, Macintosh HD → Documents → BN II folder → Data folder → select Runtime folder and drag it to the Trash.
3. Insert a USB drive, duplicate Data folder and drag the copy to the USB drive. If there is already a Data copy in the USB drive, choose Replace All.
Technical Support

Technical Service (call it first): 800-664-3822
Katherine (technician): 508-648-6129
Dan Pierce (Siemens Healthineers): 860-488-8193