



Assay for Creatinine Clearance by HPLC

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Summary: This protocol describes the procedures for collecting, preparing and performing the AMDCC Creatinine Clearance assay via high performance liquid chromatography (HPLC)

Reagents and Materials:

Reagent/Material	Quantity Required	Vendor	Stock Number
Acidified acetonitrile (ACN)	See prep below		
Centrifuge (min 10,000 rpm)			
Speed Vac			
Nalgene Diuresis Cage		Nalgene	650-0322

Protocol:

Collection of timed mouse urines.

General procedure using the Nalgene Diuresis Cage (650-0322): a clean cage is prepared for use by lightly spraying the (1) metal mouse screen, (2) the mesh support grid and (3) the conical mouse funnel with silicone spray. Wipe off any excessive silicone and allow it to sit on bench to vent any fumes before assembling. This should be done periodically, but may last for months.

1. Assemble cage as described by instructions. Attach either the 15 ml fluid collector or 30 ml collector based on your experience with the specific strain, size and number of mice you will place in cage.
2. Place food in feeder and attach. Mice must have food overnight especially diabetic mice. Place autoclaved tap water (distilled water is also OK but deionized water should not be used) in water bottle and attach. In non-diabetic mice, sugar water (3-5% glucose or sucrose water can be used to insure complete collection (i.e. enough volume). For diabetic mice, they usually make enough urine without the need for sugar water. Check to make sure all connections are secure.
3. Place mice in cage, and secure top on cage. Make sure to label cage with mouse ID number.
4. Place 1.0 ml of water in the collection fluid collector (this provides some humidity in the cage).
5. Label a 15 ml plastic centrifuge tube and use this to cover the vent hole in the top of cage.
6. Collect over 24 hours (or any other known time interval).
7. After allotted time period, remove mice and record time.
8. Examine the inside bottom of cage to see if spots of dried urine are present. They will be easily apparent if present. Some mice, especially db/db and some stz induced diabetic mice have glucosuria, and spots of sticky urine do not flow down the conical. In this case, the collection tube is removed, and the bottom conical is removed and rinsed with a small volume of deionized or distilled water or urine from the collection tube. These rinses are combined with the urine in the collection tube. The volume is measured or weighed into a clean tared vessel. The volume is recorded to 3 significant figures.
9. Urine should be separated from any feces by centrifugation and aliquoted if necessary into labeled tubes then frozen.
10. Cages are cleaned with mild soap and water then rinsed thoroughly and dried before next use. If cages do not leave barrier room, we do not find it necessary to

autoclave them between collections. Autoclaving gradually deteriorates the plastic.

NOTE: Often feces contaminate urine even under the best conditions. This should not effect the volume to any major extent, but the urine must be separated from feces by centrifugation. We do not know how contact of urine with feces over 24 hrs effects creatinine levels in urine.

In facilities where room temperatures are below 70, some mice especially db/db have difficulties keeping warm. These individual mice will produce considerably less urine in metal mesh metabolic cage than they would in communal box/bedding cages. These mice can maintain body temperature much more effectively if in groups. For this reason, one might find that collecting urine from 2 mice is better.

Some add a disinfectant, dilute sulfuric acid or even mineral oil to the collection tube to provide bactericidal conditions or to stop evaporation. We have not found that to be necessary.

Preparation of plasma, serum and urine for creatinine measurement by HPLC

General -- Plasma and serum: Mix, and if necessary, squeeze any clot using wooden applicator stick, then centrifuge (minimum 3,000 rpm for 10 minutes).

Label enough tubes for protein precipitation. Prepare acidified acetonitrile (ACN) by adding 50 µl of HPLC grade glacial acetic acid to 10 ml of HPLC grade ACN.

For a 25 µl plasma/serum: Use this ratio of 4:1 (ACN to specimen – 100 µl ACN to 25 µl plasma) if less or more specimen is used keep the 4:1 ratio. (3:1 or 5:1 is also OK)

For urine, mix and centrifuge then just use 5µl. This may be adjusted upwards if very dilute urine is used. The ACN of 100 µl is also used. In reporting of final concentration the value is multiplied by 5. (i.e. 5µl of original urine taken up in 25 µl of mobile phase).

Specific

1. Transfer 25.0 µl of plasma (or 5 µl of urine) to each tube containing 100 µl of ACN; vortex about 15 seconds to mix, extracting the creatinine into the ACN. Let sit for about 15 minutes in the minus 20 freezer. This aids in precipitation and freezes the aqueous ppt. avoiding carryover and undissolved particles.
2. Centrifuge (minimum 10,000 rpm @ 6° for 10 minutes) at 4 degrees.

3. Transfer the supernatant to new clean labeled tube (1.5 or 0.5 ml Starstadt tubes work well).
4. The volume should be very close to 125 μ l. Careful not to transfer any of the ppt. (button) which should be on the wall or bottom of the tube.
5. Evaporate the ACN to dryness using the Speed Vac, Centrivap or stream of dry nitrogen. Mild heat may be used if necessary in Speed Vac. This should take about 30 minutes.
6. Remove the tubes and reconstitute to 25.0 μ l with filtered Mobile Phase (Solvent A). Mix to dissolve the residue containing creatinine using the pipettor.
7. Transfer the liquid containing the creatinine to the special Perkin Elmer tubes for autosampler. Cap with special slit caps and centrifuge (@2,000-3,000 rpm for 10 minutes). Use swinging bucket rotor to get any debris to go to the bottom. Carefully remove autosampler vials from centrifuge and transfer to the Peltier tray. Examine each tube to insure that the liquid is touching the bottom (the V) of the tube.
8. Program the autosampler making sure the vials correspond to the numbers and the ID of the specimens. Check twice; they sometimes change.
9. With each run, a banked quality control (QC) sample must be run. The QC is a previously assayed pooled mouse plasma that has been aliquoted and stored in a minus 20 freezer.

Assay for creatinine by High Performance Liquid Chromatography

(HPLC):

All reagents are HPLC grade. All aqueous buffers are filtered through 0.22 micron filter. Tubing is PEEK 0.005 mm tubing with hand-tight connectors.

- Mobile phase: 5mM sodium acetate adjusted to pH 4.1 ± 0.1 with glacial acetic acid (final solvent strength is about 15 mM after acetic acid is added. Mobile phase was degassed.
- Column: Zorbax SCX, -- strong cation exchange, (Aglient, Wilmington, DE), 50 mm x 2.1 mm, 5 μ particle size). Five micron in-line filter and SCX guard column placed in front of analytical column.

- Temperature and flow rate: Column run at $45 \pm 0.5^\circ \text{C}$. at a flow of 0.30 ± 0.02 ml/min. Backpressure was around 900 psi. Runtime was 10 minutes.
- Detection: UV (deuterium source) at 225nm (flow cell volume is 12 μl).
- Injection volume: three microliters (3 μl) from auto sampler. Temperature of autosampler tray was $18 \pm 0.5^\circ \text{C}$.
- Identification and quantitation: Identification by comparison of retention time to pure standard of creatinine. Average retention time was 3.654 ± 0.022 minutes. Quantitation was achieved by external standard ranging from 0.003 to 1.000 mg/dl by serial dilutions using a weighted $\frac{1}{x^2}$ regression regression line (8-10 points). May also use Excel, Prism or any other software to calculate a regression line and compute the unknowns.

Calculation of creatinine clearance:
$$\frac{U[\text{Cr}] * [\text{Volume}]}{P[\text{Cr}] * [\text{Time}]}$$

Reagent Preparation: *(This area may have several different preparations with the table of contents below.)*

[Acidified Acetonitrile \(ACN\)](#)

Acidified acetonitrile (ACN):

Reagents and Materials

Reagent/Material	Quantity Required	Vendor	Stock Number
Acetonitrile (HPLC grade)	10 ml		
Glacial Acetic Acid (HPLC grade)	50 μl		

Procedure

Prepare acidified acetonitrile (ACN) by adding 50 μl of HPLC grade glacial acetic acid to 10 ml of HPLC grade ACN.