



The Creatinine Companion Protocol assay by Exocell Inc.

Version: 1.1

Replaced by version: 1.0

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IMPORTANT: **Use with Nephrat® (#NR002), Aluwell™ (#1004), Albuwell M™. (#1011)

Summary: This assay is designed for companion use with Exocell's immunospecific ELISAs for urine albumin excretion, allowing expression of results as µg albumin per mg creatinine in the urine. Adaptation of creatinine measurement to microtiter wells enables convenient, simultaneous determination of albumin and creatinine concentrations in the same specimen, using analogous microtiter plate formats. Normalization of albumin excretion in relation to creatinine facilitates classification according to defined ranges of microalbuminuria, the established marker of the early diabetic renal dysfunction. The urine albumin-to-creatinine ration in a random urine specimen is an accepted alternative to cumbersome 24 urine collections in the detection and monitoring of microalbuminuria.

The procedure is an adaptation of the alkaline picrate method and entails determination of the differential absorbance in a sample before and after the additions of acid to correct for color generation due to interfering substances..

Reagents and Materials:

Reagent/Material	Quantity Required	Vendor	Stock Number
Creatinine Companion	1 kit	Exocell, Inc.	#1012

Protocol:

Urine Collection Protocols

Spot Urine

Between 8:00 and 10:00 a.m., the mouse is picked up and induced to urinate into a 1.5 ml microfuge tube. If this is not successful, or not enough is collected, attempts on successive days can be combined (urine should be stored at 4o C between attempts).

Another way to collect spot urine is to place the mouse in a special cage (no food or water) the size of a 96-well plate. I have a custom made apparatus with compartments for eight mice. The mice stand on a 96-well plate for 2 hours. Urine is collected from the various wells the mouse urinates in, taking care to avoid any urine contaminated by feces.

24-hour

Mice are weighed before placement into Techniplast 24-hr urine collection cages. Mice remain in the cages for 72 hours. The urine is collected and mice are weighed after each 24 hour period. Urine is stored at 4o C until analysis. Food and water are replenished as necessary. The sample from the third 24-hour period is the preferred sample for albumin and creatinine measurement.

In general, if the sample is stored at 4o C, then analysis will occur within 7 days. If longer stored is required, the sample can be frozen at -60o C for two months.

Kit Contents:

- 96 well microtiter plate (2)
- Picrate Reagent (2X 10ml)
- 1 N NaOH
- Acid Reagent (2X 12ml)
- Standards (1,3, and 10 mg/dL)

NOTE: U of M is currently using control Standards of 10, 7.5, 5, 3, 2.5, and 1 mg/dl. We purchase Creatinine Standards from Sigma and create our own standards based on the kit protocol. We find the additional standard values helpful

Procedure:

1. Reagent Preparation: Add 2.0 ml of 1 N NaOH to a bottle of 10 ml Picrate Reagent. This is a working solution of alkaline picrate (picrate working solution), and must be used immediately after preparation.
2. Sample Dilution: For human samples, prepare 1:20 dilution in distilled water in disposable microfuge tubes. For animal samples, appropriate dilution will depend on method of collection. If washing of collection apparatus is employed, further dilution may not be required. Otherwise, a 1:20 dilution is acceptable.

3. Add 20 μ l of water to wells A1 and A2. These are control blanks.
4. Add 20 μ l of Creatinine Standard, 10 mg/dl to wells A3 and A4.
5. Add 20 μ l of Creatinine Standard 3 mg/dl to wells A5 and A6.
6. Add 20 μ l of Creatinine Standard 1 mg/dl to wells A7 and A8.
7. Add a 20 aliquot of diluted sample to wells A9 and A10.
8. Continue the addition of sample aliquots to the rest of the plate.
9. Add 100 μ l picrate working solution to each of the wells.
10. Let stand for 10 minutes on the bench top.
11. Determine the absorbance of the wells on a plate reader set at approximately 500 nm.
Well A1 serves as "Blank".
12. Add 100 μ l of Acid Reagent to each of the wells.
13. Let stand for 5 minutes.
14. Measure absorbance as described above.

Data Analysis:

1. Calculate the data absorbance: $A_{\text{delta}} = A_{\text{alkaline picrate}} - A_{\text{alkaline picrate+acid}}$
2. Determine the least squares regression line using delta absorbance versus creatinine concentration for each standard. Do not include blank.
3. Determine the concentration of diluted samples by substituting the respective delta absorbance appropriately.
4. Multiply these values by the reciprocal of the dilution factor to obtain concentration (mg/dL) in undiluted samples.

NOTE: U of M uses diluted mouse urine samples of 1:5 and 1:10 with good success