

Biochemistry Phenotypes

**High-throughput characterization of
indices of clinical chemistry and
hematology**

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I. Overview

A. Respiration:

The purpose of these phenotypes is to determine exercise-induced changes in arterial PO₂, PCO₂, and pH as measured by a blood gas analyzer [Bayer 840, see order information] in the Physiology PGA Biochemistry Lab. Samples are analyzed by Bayer's standard method.

Sample preparation and storage:

Blood is drawn into a 1ml lithium heparin syringe from indwelling arterial catheters in conscious animals and put immediately on ice. Blood gas measurements are made within 10-15 minutes of sample collection.

B. Cardiac:

The purpose of these phenotypes is to determine changes in lactate dehydrogenase (LDH) activity in response to global cardiac ischemia in rats that have been conditioned in hypoxic or normoxic conditions. Krebs' buffer is perfused through the heart and collected for pre-ischemia and post-ischemia LDH activity. Samples are analyzed by either the manual method (see pages 3-4) or the autoanalyzer method (see pages 5-7) in the Physiology Biochemistry Core Lab.

Sample preparation and storage:

10µl of 10% BSA is added to 990µl of effluent. The samples are then vortexed and stored immediately at -80°C until analysis.

C. Renal:

Conscious rats are fed a high salt diet (4.0% NaCl) for four weeks. An arterial blood sample is obtained and a 16-hour urine sample is collected. The animals are then weighed, a Lasix injection is administered to effect salt depletion, and the diets are changed to a low salt concentration (0.4% NaCl) [see the Renal protocol manual for more details]. A low salt arterial blood sample and a 16-hour urine sample are collected. The following phenotypes are measured in the Physiology Biochemistry Core Lab:

- Plasma Renin Activity – RIA (method on pages 7-14)
- Sodium and Potassium – flame photometry of serum and urine (method on pages 14-16)
- Creatinine – plasma and urine (autoanalyzer method pages 5-7)
- Total urine protein (autoanalyzer method on pages 5-7)
- Urine Microalbumin – microtiter plate, fluorescent assay (method on pages 16-18)
- Osmolality – freezing point depression osmometry. Samples are analyzed by Precision Systems, Inc. standard method [see order information]

Sample preparation and storage:

Urine: Collect in 2ml microtubes. Spin for 15 min at 5000 rpm/rcf. Clean urine is pulled and transferred to new barcode labeled tubes for storage at -80°C until use.

Blood: 80 μ l is collected in a 3ml (45 USP) lithium heparin vacutainer tube. Spin for 15 min at 5000 rpm/rcf at 4 °C. Plasma is pulled off and transferred to a new barcode labeled tube for storage at -80°C until use.

D. Lung:

Blood is drawn from carotid catheters in anesthetized rats following chronic exposure to hypoxia or normoxia. Samples are shipped to Marshfield Laboratories [see order information] for the small animal profile (ANP-15), which includes various blood chemistries: glucose, AST, ALT, alkaline phosphatase, total bilirubin, total cholesterol, total protein, albumin, urea nitrogen, creatinine, phosphorus, calcium, sodium, potassium, chloride, bicarbonate, and anion gap. Also, a complete blood count with differential: RBC, hemoglobin, hematocrit, MCV, MCH, MCHC, RDW, platelet, and WBC are performed.

Sample preparation and storage:

400 μ l is delivered into a purple EDTA tube and 600 μ l into a yellow serum separator tube. The EDTA tube should be inverted immediately and repeatedly to prevent clotting. Once collected, the EDTA tubes are spun for 15 min at 5000rpm/rcf at 4 °C. Samples are then shipped on an ice pack to Marshfield Laboratories for analysis. [Note: when collecting samples from rats conditioned in the hypoxia chamber, be sure that the full volume is withdrawn and delivered to the tubes. The hematocrit is significantly higher in hypoxic animals, which then delivers a lower volume of serum or plasma for biochemical measurements.]

II. Experimental Protocols

A. Respiration

Bayer blood gas analysis - see Bayer standard method.

B. Cardiac

1. Manual LDH Enzyme Activity Assay Protocol

I. Preparation

A. LDH Reagent A

1. Store in refrigerator [4°C]
2. Add 20ml of boxed water
3. Invert to mix
4. Stabilized for one day at room temperature (25°C) and then for four days in the refrigerator [4°C]
5. Write date and initials on the bottle when opened

B. LDH Reagent B

1. Store in refrigerator [4°C]
2. Add 5ml of boxed water
3. Invert to mix
4. Stabilized for one month in refrigerator and then for seven days at room temperature (25°)
5. Write date and initials on the bottle when opened

- II. Set up for Spectrophotometer
 - A. Turn on spectrophotometer (bottom right back corner)
 - B. Turn on screen
 - C. Allow system to warm up
 - D. Exit the first screen called 'Power Up Diagnostics'
 - E. Choose Kinetic/Time under Routine measure
 - F. Turn on both UV and VIS lamps (allow time to warm up)
 - G. Choose methods→ A:/LDH
 - H. Use an empty cuvette to set the blank
 - I. Set the number of test and samples accordingly on the screen
 - Autosampler
 - Number of samples
 - Sample alignment
 - J. Wait until lamps are on
 - K. Turn on the printer

**Note:* (1) Principle: Pyruvate + NADH + H⁺ ↔ 2-Lactate + NAD⁺
(2) Run quality control before running samples

- III. Preparation of Samples/Quality Control Samples
 - A. Let reagents warm up at room temperature (25°)
 - B. Add 2.5ml LDH Reagent A to an empty cuvette
 - C. Add 0.1ml of the samples and incubate for one minute
 - D. Add 0.1ml LDH Reagent B, mix and wait thirty seconds
 - E. Read and record absorbance at 340nm
 - F. Continue incubation at 25°C and record absorbance at exactly 1, 2 and 3 minutes following the initial absorbance reading
 - G. Print the rates and raw data
 - H. Determine the mean absorbance change per minute ($\Delta A/\text{Min}$) and calculate the LDH activity by multiplying $\Delta A/\text{Min}$ with the factor:

$$\text{LDH Activity (U/L)} = \frac{\Delta A/\text{Min} \times \text{TV} \times 1000}{6.22 \times \text{LP} \times \text{SV}}$$

$\Delta A/\text{Min}$ = change in absorbance per minute at 340nm

TV = total volume

-Full Sample: 2.7ml

-Half Sample: 1.35ml

SV = sample volume

-Full Sample: 0.1ml

-Half Sample: 0.05ml

6.22 = millimolar absorptivity of NADH at 340nm

LP = light path (1 cm)

1000 = conversion of units per ml to units per liter

$\Delta A/\text{Min} \times 4340.836013 = \text{LDH Activity}$

- I. Take the mean of LDH activity and then compare to other data

2. Autoanalyzer Assay Protocol

I. Setting up Analyzer

- A. Turn on machine (bottom right back corner)
- B. Turn on the screen and monitor
- C. Allow the machine to warm up (temperature alert will light up)
- D. Turn on the printer (make sure there is enough paper)
- E. Align probe and test the wash bath for daily maintenance
 1. Main Screen → Diagnostics → Hardware Calibration → Align Probe
 2. Choose either:
 - a. Move to alignment position
 - Align probe with cross underneath it
 - Stroke probe from top to bottom with an alcohol or kimwipe to align
 - Press enter to repeat test to confirm proper alignment adjustments
 - b. Test alignment at wash bath
 - Put two drops of deionized water on the wash bath
 - Press enter
- F. Press the Esc key to exit after the test is completed

II. Loading the Standards

- A. Press the F12 key
- B. Choose reagents and then press enter
- C. Open chamber door
- D. Load reagents in machine slots
- E. Close chamber door tightly
- F. Press the F10 key
- G. Press the Esc key to exit

III. Calibration

- A. Set up test for calibration
 - Main Screen → Requisitions → Calibration
- B. Type in test code and press F10 (see Gemcal appear on screen)
- C. Press F10 again to accept
- D. Resume to the Main Screen and Load List
- E. Choose a number randomly and press enter (continue this process until the computer accepts the number given)
- F. Gemcal should appear on the Load List screen as well
- G. Press the F7 key to load segment
- H. Open chamber door to load segment then close door tightly
- I. Press F10 to accept

IV. Quality Control

- A. Set up test for quality control:
Main Screen→ Requisitions→ Quality Control
(Quality Control 1 (QC1) = Normal)
(Quality Control 2 (QC2) = Abnormal)
- B. Choose QC1 or QC2; one at a time
- C. Press the F5 key to delete results if wanted test is already shown. Press the F6 key to delete all tests shown to prepare own test list
- D. Press the F10 key to accept after each QC list is completed
- E. Press the Esc key to exit
- F. Prepare samples to load
- G. Return to the main screen and 'Load Lists'
- H. Choose a number randomly and press enter (continue this process until the computer accepts the number given)
- I. Press the F5 key to build the segment (this should give the lists of the chosen tests ordered)
- J. Press the F7 key to load the segment
- K. Open the chamber door, load the samples and then close the door tightly
- L. Press the F10 key to accept

V. Test Samples

- A. To load and test samples:
Main Screen→ Requisition→ Patient
- B. Choose a specific patient under the correct test and press enter (to add a new patient press INS under the patient screen)
- C. Press INS to insert a new test number under that patient
- D. Press the F10 key to accept; only to bring the cursor down to the bottom of the screen faster
- E. Enter the appropriate test (i.e. LDH)
- F. Press the F10 key to accept
- G. Repeat for each number of cups needed to test
- H. Press the Esc key to exit to the Main Screen
- I. Prepare samples to load = 0.07ml (70µl)
- J. Go to Main Screen and 'Load Lists'
- K. Enter a new number and press enter
- L. Press the F5 key to build the segment; list of tests ordered should appear on the screen
- M. Press the F7 key to load the segment/samples
- N. Open the lid, load the samples and then close the lid tightly
- O. Press the F10 key to accept

VI. Troubleshooting

- A. Press the F9 key to view the "Attention" alert
- B. Press the Esc key to exit
- C. If the machine needs more cuvettes press the F12 key to insert
-choose Inserter

- add cuvettes at an angle facing front (add only half way down)
 - remove and press the F10 key to accept
 - press the Esc key to exit
 - D. Press Control to override the temperature warning
- VII. Shutting Down the Analyzer
- A. To remove the reagents
 - B. Press the F12 key
 - C. Choose Reagents and press enter
 - D. Lift the lid, remove the reagents and recap
 - E. Press the F10 key to accept
 - F. Turn off the machine in the back right corner
 - G. Turn off the screen and monitor
 - H. Remove the data that was printed
 - I. Turn off the printer

C. Renal

1. Plasma Renin Activity determination

I. Summary of Methodology:

The measurement of plasma renin activity (measurement of generated angiotensin I) is one of the most frequently performed assays in the Biochemical Core Lab. The method used is a modification of the assay developed by Dr. Jean Sealey¹ who kindly provided us with antibody. The procedure using this antibody has been well documented for use in rat, dog and human plasma^(A-E) and has proven reliable and efficient in the ten+ years we have used it. This method uses radioimmunoassay to measure the rate of angiotensin I formation in plasma incubated at 5.7 pH. EDTA and PMSF are added to inhibit the destruction of the newly formed angiotensin I. We have designated this procedure "MACRO" and it is used for analyzing dog and human plasma samples in which available plasma volume is 0.5-1.0 ml. For the analysis of PRA in rat plasma, a modification of this procedure ("MICRO") has enabled us to measure PRA in 50 μ l of plasma. The "MICRO" method is the only method being performed currently; both the "MACRO" and the "MICRO" methods are described below. ***As of 8/98 all human, rat and mouse samples are run using the MICRO method, including a 0°C incubation for each.** Other species have not been validated with the MICRO method.

II. References:

- A. Sealey, J.E. and J.H. Laragh. How to do a plasma renin assay. Cardiovascular Medicine 2(11):1079-1092, 1977.
- B. Gallagher, J.F., J.H. Laragh, S.A. Atlas, and J.E. Sealey. Effect of trypsin or acid treatment on dog plasma renin activity measurement. Endocrinology 107:147-154, 1980.
- C. Laragh, J.H. and J.E. Sealey. Renin-sodium profiling: why, how and when in clinical practice. Cardiovascular Medicine 2(11):1053-1073, 1979.

- D. Sealey, J.E., C. Moon, J.H. Laragh, and S.A. Atlas. Plasma prorenin: in normal, hypertensive and anephric subjects and its effect in renin measurements. Circ. Res. 43 (Suppl.1):141-145, 1977.
- E. Atlas, S.A., J.E. Sealey, and J.H. Laragh. "Acid" and "Cryo" activated inactive plasma renin. Circ. Res. 43 (Suppl. 1):1128-1133, 1978.

III. Collection of Samples:

Whole blood (arterial for rats and dogs; venous for humans) is collected at **room temperature** [15% K₃EDTA tubes for dog and human and 0.3M Na₄EDTA (50 µl/ml) microfuge tubes for rats], and processed within 30 minutes. Blood is centrifuged at 2700 rpms for 15-20 minutes at 4°C for dog and rat and **room temperature** for human blood. The plasma is removed and frozen immediately at -80° C until ready to do the assay. (Cryoactivation of the prorenin in human blood will occur if handled below room temperature at this point.)

IV. Assay Set up - Standard Preparation:

We are currently using Peninsula # 7001 Angiotensin I standard (5 mg vial). Add 1 ml of Tris Buffer to the vial, now you have a "STOCK" of 5mg/ml. Aliquot at 10 µl in bullet tubes and store at -80° C.

- A. Take 1 bullet tube containing 10 µl of the 5 µg/µl stock solution and add 90 µl Tris Buffer directly to vial and mix thoroughly. Now the solution is 50 µg/100 µl.
- B. Transfer this 100 µl to a 50 ml centrifuge tube containing 49.9 mls of buffer. Rinse out the vial several times with part of the 49.9 mls to ensure that the standard is entirely removed from the vial. Now the concentration of the solution is 50 µg/50mls or 50,000ng/50mls or 1000ng/ml.
- C. Aliquot this 50 mls into 1.5 ml bullet tubes with 150 µl in each. Store these at -80° C. Remove one of these prior to each renin assay and do the following dilution.
- D. Add 1.15 mls of Tris Buffer to a 12 X 75 Sarstaedt tube and pipet 100 µl of the above solution (#3, 1000ng/ml). Vortex. This will be your 800 pg/10 µl standard. 1000 ng in 100 µl + 1.15 mls = 1000 ng/1.25 mls or 800 ng/ml which is the same as 80 pg/µl X 10 µl used in the assay. Pipet 500 µl of Tris Buffer into each of seven tubes labeled 400, 200, 100, 50, 25, 12.5 and 6.25.
- E. Pipet 500 µl of A (80 pg/µl) into the tube labeled 400, vortex, and continue this process until all standards have been serially diluted down to 6.25. See chart below:
 - 500 µl of A + 500 µl of buffer = 400 pg/10 µl (label B)
 - 500 µl of B + 500 µl of buffer = 200 pg/10 µl (label C)
 - 500 µl of C + 500 µl of buffer = 100 pg/10 µl (label D)

- 500 μ l of D + 500 μ l of buffer = 50 pg/10 μ l (label E)
 - 500 μ l of E + 500 μ l of buffer = 25 pg/10 μ l (label F)
 - 500 μ l of F + 500 μ l of buffer = 12.5 pg/10 μ l (label G)
 - 500 μ l of G + 500 μ l of buffer = 6.25 pg/10 μ l (label H)
- ** You will need these standards (starting from the A tube)
800-6.25 in triplicate (10 μ l) for an assay and they need to be made fresh for each assay.

V. Antibody:

We received 3 - 400 μ l aliquots of antibody #32 from the Sealey lab on 1-30-96. One tube was thawed and aliquoted into 3 - 100 μ l aliquots and stored at -80 °C. The 100 μ l that was left was diluted with 9.9 mls of Tris buffer to give a 1:100 dilution that was then aliquoted into 5 tubes with 2mls in each. One of these was aliquoted into small micro tubes with 50 μ l in each, these are the tubes that are thawed to add antibody to the tracer solution. The other 4 were left at 2 mls. The antibody is kept in the Core lab freezer at -30°C. Through several titer checks we have determined that the proper dilution factor is 0.22 to achieve ~50% binding. When making up the tracer solution, you will take the total number of mls made - 3 tubes (for the NSB's) and multiply this number by 0.22. This will give the correct number of μ ls of antibody to be added.

Example: 150mls made - 3 = 147 *0.22 = 32.3 μ l of antibody to be added.

VI. Tracer Dilution:

Store the tracer in the refrigerator until ready to dilute [Amersham catalog # IM176 I¹²⁵ Angiotensin I]. To dilute, add 200 μ l of sterile water to the vial and mix until all the powder is dissolved. Next add 1 ml of Tris buffer to the vial and mix again. When thoroughly mixed this solution should be clear. Aliquot the tracer into 0.5ml micro tubes with 75 μ l of trace in each. They should be labeled with the date and AI. When making trace with very fresh trace, start out with a small amount of trace in the desired amount of Tris buffer (you will take the number of tubes in your assay + 10 and this is the number of mls of diluted tracer needed) until you reach ~10,000 cpm / 1ml of trace. *Note - when the trace is 2-4 weeks old it will be about a 1:1000 dilution. For example, if you are making 100 mls of tracer solution you will need about 100 μ l of trace.

VII. Reagents:

All aqueous solutions are made fresh every 6 weeks with sterile distilled water. (NOTE: The PMSF is made fresh at each generation.) A label check is run at this time with a standard curve and pools to check all reagents before any samples are run.

A. **MICRO Generation Reagents**

1. Neomycin Sulfate (see below, B.1.) is diluted by taking 100 μ l of a 10% solution and adding 300 μ l of sterile water in a labeled 12 x 75 polypropylene tube. This should be discarded immediately after generation is complete. It is made fresh for each micro generation.
2. PMSF (see below, B.2.) must be diluted by taking 100 μ l of the 5% solution and adding 300 μ l of Ethanol in a micro tube. This should be labeled MICRO PMSF and discarded immediately after generation is complete. It is made fresh for each micro generation. You will add 1 μ l of this solution with a Rainin P2 to each 50 μ l plasma sample.
3. Micro Maleic Anhydride Acid Buffer (see below, B.3.) (0.55 M, Kodak 1226) 5.413 gm maleic anhydride + 100 mls sterile water. Stir for at least 15 minutes, until dissolved. Check that pH is \sim 1.25.
4. Micro Maleic and Neomycin Sulfate cocktail- will use 4 μ l per sample
Place Maleic Anhydride (0.55 M) on stirplate to mix well. Remove tube of Neomycin Sulfate from refrigerator; make sure it is room temperature before use. Mix 200 μ l of micro maleic and 200 μ l of micro Neomycin sulfate

B. **MACRO Generation reagents**-these are used as stock solutions for MICRO reagents

1. Neomycin Sulfate (Sigma #N-1876) 10% solution in sterile water. Weigh 0.5gm of Neomycin in a tared 13 x 100 polypropylene tube labelled Neomycin with the date, add 5 mls of sterile water, cap and mix thoroughly. This tube should be stored at 4°C, but be at room temperature during the generation process. You will add 5 μ l of this to each 500 μ l sample with an adjustable pipetman (P10).
2. PMSF (Sigma #P-7626). 5% solution in 95% ethanol (made fresh at each generation.) (The PMSF powder must be stored at room temperature and blanketed with argon after each use.) Weigh 0.25 gm PMSF into labelled, dated, tared polypropylene 13 x 100 tube, add 5 mls of 95% ethanol, cap and mix thoroughly. PMSF is **very toxic**, you should wear gloves while handling PMSF and do not breathe it in. This solution should be mixed until all the crystals are dissolved. This is the tube you will work from for generation. This tube should be stored at room temperature, if chilled it will crystallize.

***NOTE For 250 μ l assay the Neomycin sulfate can be made at a 0.8 mg/ μ l concentration by adding 100 mg to 1.2 ml of sterile water. 3 μ l of this will give (3 x 0.8) a 0.24 mg concentration. This is the correct amount for incubation.

3. Maleic Anhydride Acid Buffer (Kodak #1226) Weigh out 2.709 gm and add 100 ml of sterile water, dissolve completely (by stirring for at least 15 minutes). Check the pH by pipetting a small amount onto pH paper, the pH should be ~1.25. Stir for 5 minutes before and during use, each time you use it. You will add 40 μ l to each 500 μ l sample (excluding human samples which get 50 μ l). This is added last and the sample is vortexed and placed on ice.

C. Assay Reagents

1. Tris Buffer

Trisma base	Sigma T1503	24.225 g
BSA	Sigma A4503	6.0 g
Lysozyme	ICN 100831	6.0 g
Phenylmercuric Acetate	Fisher 0-4195	0.06738 g
Neomycin Sulfate	Sigma N1876	4.0 g
Sterile dH ₂ O (box)	Fisher	2 liters

This is a temperature dependent buffer when measuring pH. Since this buffer is used cold, the pH must be adjusted cold with buffers that are cold also. Mix overnight in walk-in cold room until completely dissolved, mixture will be cloudy until pH is adjusted. Adjust pH with concentrated Acetic Acid to 7.5, filter into a 2-liter disposable, sterile flask.

2. Charcoal

For 100 mls, weigh out 0.4 gm of Dextran T-70 (Pharmacia #17-0280-01), add 50 mls of cold, sterile H₂O and stir until completely dissolved. Weigh 4 gms of Carbon, Decolorizing Neutral Norit (Fisher #C170-500), add gradually to beaker and add another 50 mls of cold, sterile H₂O. Stir for 10 - 15 minutes.

VIII. Procedure:

For the determination of renin activity, angiotensin I is generated under fixed conditions and the angiotensin I level is determined by radioimmunoassay. The generation and radioimmunoassay procedures are given below.

** Use conical polystyrene tubes (throughout the assay).*

A. Micro Generation of Samples

This procedure is used for 50 μ l plasma sample.

1. Turn on waterbath in room 536, it should be preset to 37°C, takes about 15 minutes to warm up.

2. Thaw samples in water at room temperature. Thaw as quickly as possible.
3. Label a renin worksheet with the samples that correspond to your generation numbers, placing pools at the beginning and end of samples. Run appropriate pools for the type of samples being generated.
4. Label 0.5ml microfuge bullet tubes starting from # 12 through the number needed for all samples. Each bullet tube must be labeled with the sample number as well as the assay number for future reference. Each sample must be >100 μ l and is incubated at 0°C (**ice bath in refrigerator**) for 3 hours and at 37° C for 3 hours. The values of the assay at 0°C incubation will be subtracted from the values of the assay obtained at 37° C. It is easiest if you label the same color bullet tube for both the 0°C incubation and the 37°C incubation. Both samples are pipetted and generated simultaneously. (i.e., #12 will be incubated in an ice bath (0°C) for 3 hours and #13 will be incubated at 37° C for 3 hours.)
5. Pipet samples and pools into generation tubes, 50 μ l each. All generation reagents are added to one sample at a time and sample is capped before moving on to next sample.
6. Add 1 μ l of micro PMSF to sample and 4 μ l cocktail. Use special Rainin shielded filter tips.
7. Vortex sample and place immediately on ice.
8. Repeat for all samples, until all samples have been generated.
9. When complete, all bullets are vortexed and microfuged (approximately 5 seconds at 10,000 rpms) in a chilled rotor to ensure all reagents are in the reaction mixture.
10. Place the samples for the three-hour 37°C incubation in the water bath. The samples for the 0°C incubation will remain on ice.
11. When the generation is complete, place the 37°C incubation in an ice bath. If the samples are not to be assayed immediately, place the samples incubated at 0°C and 37°C in the chest freezer (-30°C). On the assay day, thaw all samples in an ice bath.

B. **MACRO Generation Procedure**

** The same basic procedure is used except you will need to dilute Neomycin Sulfate and PMSF as directed on VII.B #1 and #2 (for micro) and you will use the 0.27M Maleic Anhydride Buffer. A 1.5 ml microfuge bullet tube is used instead of the 0.5 ml. The amounts added are:

500 μ l of sample
5 μ l of Neo. Sulfate
2 μ l of PMSF

40 μ l (dog) or 50 μ l (human) of Maleic

The sample is pipetted with a Finnepette P200 and the reagent amounts are added with a Rainin P2 or P10 pipet-plus Pipetman using filtered, shielded tips.

While your samples are in ice (after 3 hour incubation), dilute your standards and label assay tubes.

1. Remove one 150 μ l aliquot of AI (1000ng/ml) standard from the -30°C freezer and dilute serially according to previous standard directions.
2. Label 12 x 75 conical, polystyrene tubes in triplicate for standard curve (tubes #1 - 11), and duplicate for samples (#12-?).
3. Place an empty rack in another ice bath and as you pipet standards and samples place into rack. The assay tubes should be cold also, placing them in a freezer to chill.
4. Place tubes #1-3 into rack (these do not get any standard, they are nonspecific binding, total and B₀ (zero binding)), pipet 10 μ l of standards (starting with 6.25) in triplicate, changing tips, in #4-11. Vortex and spin the generation tubes again.
5. Pipet 10 μ l of samples into corresponding tubes in duplicate, changing the tip for each new sample.
6. When all standards and samples have been pipetted and are being kept cold, you will make up trace/antibody mixture. Refer to previously described "tracer dilution" procedure. When tracer is ~ 10,000 cpm/1ml, pipet 1 ml in triplicate into the tubes for #1.
7. Subtract 3 from your total volume of tracer and follow the directions previously listed for "antibody dilution". When the appropriate volume of Ab is determined, add that volume to the tracer solution and mix for 5 minutes before adding 1 ml to each assay tube (except #1's).
8. Cover each assay rack with parafilm and vortex, store in 4° C refrigerator overnight (~18 hours).
9. Store generated sample rack in chest freezer (-30 °C) with label tape (date and assay#) in case any samples need to be reassayed.

C. **Termination: This is for both Micro and Macro method**

After ~ 18 hours at 4°C, remove assay racks from refrigerator.

1. Make up Charcoal solution as previously described (you may cut the recipe in half if your assay is small).

2. After spinning the tubes for 20 minutes, add 250 μ l to each tube (except #2s, these get 250 μ l of Tris Buffer) with a repeater pipet (200-1000 μ l); you have to cut the tip so the charcoal suspension does not clog up the pipet tip
3. After adding charcoal solution to all tubes, parafilm again and vortex racks.
4. Centrifuge all tubes at 2700 rpm's for 20 minutes.
5. While centrifuging, label a duplicate set of tubes to pour samples into.
6. Pour over samples and count supernatant for 2 minutes in gamma counter.
7. Calculate cpm's using STATLIA program (see STATLIA protocol) for PRA'S and obtain the ng Al/ml concentrations. The B₅₀ for this assay should be between 95-135. Plug ng Al/ml numbers into the renin template in EXCEL and get the Ng Al/ml/hr final numbers to report out.

*** Note -- if a sample is too high and is off the standard curve, you can repeat the assay with 5 or 2.5 μ l of generated sample and multiply the end number by 2 or 4. If a sample is too low it must be regenerated for 15 hours and then re-assayed.*

Chart 1.

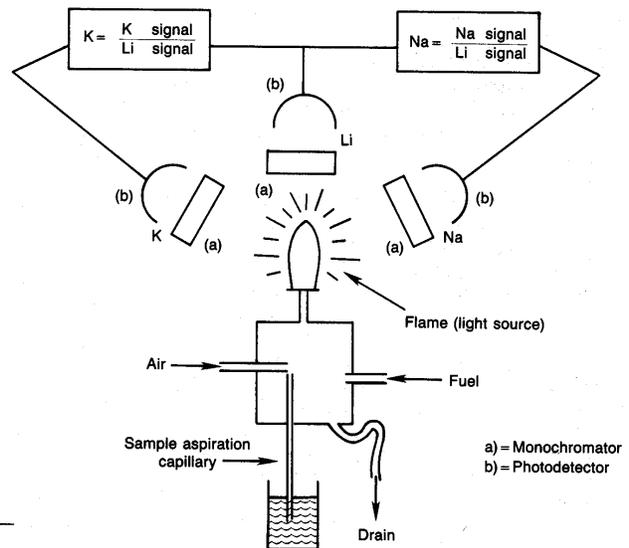
ID	TUBE#	VOL	TRACE	TRACE + AB	CHARCOAL	TRIS
NSB	1	-	1ML	-	250 μ l	-
TOTAL	2	-	-	1ML	-	250 μ l
B0	3	-	-	1ML	250 μ l	-
STDS	4 TO 11	10 μ l	-	1ML	250 μ l	-
SMPLS	12+	10 μ l	-	1ML	250 μ l	-

2. Sodium and Potassium Determination (Flame)

- I. Introduction: Plasma and urine electrolytes are important to the MCW Physiology Department because of their role in controlling body and cellular fluid volume in relation to blood pressure. Changes in electrolyte balance are useful in evaluating overall kidney function and plays a role in the renin-angiotensin system and aldosterone regulation. Sodium (Na⁺) and Potassium (K⁺) concentrations are determined by either Ion Selective Electrode (ISE) or Flame Emission Spectrometry.
- II. Methodology: ISE technology is the most commonly used method to measure Na⁺ and K⁺, and is a very reliable method for most clinical analysis. The linearity of ISE technology can be limited in the concentration ranges of some of our samples. We choose to use the flame (IL943) because it is more accurate and precise over

a larger range of sample matrixes and conditions. Samples can be diluted without assay linearity being affected.

III. Principle: A 1:100 dilution of sample (plasma or urine) is aspirated into a flame. The flame atomizes the sample and the atoms absorb energy from the flame creating an excited electron state. As the atoms return to ground state they emit light energy of a characteristic wavelength. This



light is detected through wavelength filters (monochromator) and converted to an electronic signal (photodetector) proportional to the concentration of atoms in the sample. Cesium (Cs) is used in the dilution buffer as an internal standard to account for any fluctuations in air or flame. Concentration is calculated by comparing a ratio of standard (calibrator) emission to the ratio of emission from the internal standard, which should remain constant.

IV. Operation:

- A. Turn power on (lower left side, rear, toggle switch)
- B. Open air valve (blue, in wall, handle "in-line" with pipe is on)
- C. Open propane gas valve (turn knob on top of cylinder clockwise)
- D. Push flame button-wait 3 min for temp stabilization, "autocal" light will flash when ready. Busy sign will appear.
- E. Press "printer" to enable the on-board printer
- F. Select sample mode by pushing serum, urine or lithium (this choice determines what concentration calibrators is used)
- G. Press "autosample" button
- H. Place appropriate cal solution (approximately 200-300 μ l) in micro cup in cal position of tray and appropriate zero solution in cup in zero position (see chart). Cal solution will be present on every run, and additional cal cup is placed between cups 16 and 17 on the wheel if there are more than 16 samples.

matrix	autocal solution	zero solution
serum (Na/K)	140/5/1	dH ₂ O
urine (Na/K)	100/100	dH ₂ O
lithium	140/5/1	140/5

- I. Press the autocal key. Flame will now perform a calibration cycle to set the linearity of the Na/K for either serum or urine, as selected with the mode buttons. *Repeat this at least twice without samples.*
- J. Place some pool or standard samples in the tray following the cal solutions and press auto cal again.
- K. Check that the values of the controls are within 1 standard deviation from the accepted norm. If not, re-calibrate and notify core lab personnel.
- L. Load samples (approximately 70µl) and press auto cal.
- M. Shut down:
 1. Place aspirator needle in pre-dilute position.
 2. Aspirate 25 mls of a 1:50 dilution of a commercially prepared rinse solution (IL cat 33110), this takes approx. 15 min.
 3. Close propane valve
 4. Close air valve-blue handle **not** the regulator
 5. Turn power off
 6. Check waste bottle, empty when half full

3. Creatinine and Total Urine Protein Determination

These determinations are only performed on high salt urine samples by the autoanalyzer [see Cardiac autoanalyzer method, page 5].

4. Urine Microalbumin Determination

- I. Principle: Albumin Blue 580 [Molecular Probes cat# A-6663] is an anionic dye that binds strongly and specifically to albumin, giving a red fluorescent signal. Other dyes bind specifically to albumin but the lability of the reagent solutions limited their practical use. The stability, specificity and low cost of Albumin Blue 580 make it a practical alternative to immunoassays for the determination of serum and urine microalbumin. A standard curve is prepared from human or rat albumin and standards and samples are mixed with a working solution of Albumin Blue 580 in buffer and isopropanol in a microplate format. The plate is read immediately on the FL600 (exc 590/20 emm 645/40). Sample values are read directly from non-linear standard curve. The binding rate of AB 580 is species specific; care **MUST** be taken to use species specific standards. The assay is useful in the 2-150 mg/L range for human urine and 0.033-1 mg/ml for rat urine by this method.

Ranges: Human urine 1.25-80 mg/L
Rat urine 0.033-1.0 mg/ml

II. Procedure- Prepare diluted standards from stock solution.

Rat (Sigma cat#-A-4538)

	conc (mg/ml)	stock	vol	diluent vol
A	1	25 mg/ml	80 ul	1.92 mls
B	0.5	A	1ml	1ml
C	0.25	B	1ml	1ml
D	0.125	C	1ml	1ml
E	0.0625	D	1ml	1ml
F	0.0313	E	1ml	1ml

Human (Sigma cat# A-8763)

	conc (mg/L)	stock	vol	diluent vol
A	80.00	mg/L	160 ul	1.84 mls
B	40.00	A	1ml	1ml
C	20.00	B	1ml	1ml
D	10.00	C	1ml	1ml
E	5.00	D	1ml	1ml
F	2.50	E	1ml	1ml
G	1.25	F	1ml	1ml

(use the **bold** lettered standards (ABCEFGH) in assay)

III. Reagents:

A. Standard Diluent: 500 ml

chemical	vendor	cat #	amt (g)	FW	final conc (mM)
KH ₂ PO ₄	Sigma		2.7	136.1	39.68
K ₂ HPO ₄	Sigma		0.9	174.2	10.33
Na ₂ EDTA	Sigma		0.5	372.2	2.69
NaCl	Sigma		4.5	58.44	154.00
IgG-human	Sigma	G-4386	0.05		0.00

B. MOPS buffer: 1 L

chemical	vendor	cat #	amt (g)	FW	final conc (mM)
MOPS free acid	Sigma	M-1254	3.0	209.3	14.33
MOPS Na salt	Sigma	M-9381	9.0	231.2	77.85
Na ₂ EDTA	Sigma	E-4884	1.0	372.2	5.37
NaCl	Sigma		12.0	58.44	410.68

- mix well, q.s. to 900 ml (pH should be 7.4 ± 0.2)
- add 100ml 2-propanol (isopropanol)

- C. Dye stock (1 mM): This is made in advance and stored at -20°C
1. Dissolve Albumin Blue 580 (Molecular Probes cat# A-6663) to concentration ~ 30 mg/L in isopropanol.
 2. Dilute 1:10 with isopropanol and check the absorbance at 580nm.
 3. If $A_{580} = 1.0 \pm 0.05$ (blanked against isopropanol), use the crude stock as dye stock, otherwise adjust with dye or isopropanol until $A_{580} = 1.0 \pm 0.05$.
 4. Published data says dye stock should be stable at 4°C for 2 years, but current practice is storage at -20°C .
- D. Working Reagent:
1. Make a $2 \mu\text{M}$ working reagent from AB 580 assay buffer and crude AB 580 isopropanol stock.
 2. If you add 1ml of the stock AB 580 to 49 mls of AB 580 MOPS buffer this will be sufficient for two 96 well plates.

IV. Assay:

- A. Sample and standard volumes:
- Rat: $20 \mu\text{l}$ sample may need to dilute samples 1:5 or 1:10
 - Human: $40 \mu\text{l}$ standards and samples
- B. Pipette standards in triplicate onto 96 well opaque plate. Pipette samples in duplicate. Using multichannel pipettor, add $200 \mu\text{l}$ $1 \mu\text{M}$ AB580 to each well. Shake gently to mix, read at 590ex, 640em. Plate should be read within 5 minutes because the buffer will begin to evaporate.

5. Osmolality

See Precision Systems, Inc. standard method.

D. **Lung**

Samples are shipped on an ice pack to Marshfield Laboratories for analysis on a daily basis.

III. Timeline

A. Week 1.

1. Monday: Label samples for the next two weeks, enter Marshfield data. *Lung, Cardiac Protocols*
2. Tuesday: Renal – High Salt & Low Salt Urine and Blood Na/K. *Lung, Cardiac Protocols*
3. Wednesday: Renal – High Salt & Low Salt Urine and Blood Creatinine. *Lung, Cardiac Protocols*
4. Thursday: Data processing. *Lung, cardiac, renal – microalbumin and total protein, High Salt urine*
5. Friday: Cardiac – Lactate Dehydrogenase Activity

B. Week 2.

1. Monday: Lung – Blood Gas Analysis. Renal – Osmolality
Lung Cardiac Respiratory Protocols
2. Tuesday: Renal – High Salt Microalbumin & Total Protein.
Lung Cardiac Protocols
3. Wednesday: Renal – Extract plasma from blood, -80°C storage Renal
– High Salt Urine & Blood Creatinine, Na/K, PRA (bl)
Lung, Cardiac Protocols
4. Thursday: Lung – Blood Gas Analysis. Cardiac – Lactate
Dehydrogenase.
Lung, Cardiac, Respiratory Protocols
5. Friday: Lung – Blood Gas Analysis. Renal – Extract plasma from
blood, -80°C storage
Respiratory, Renal – Low Salt Urine and Blood

IV. Order Information

A. Blood biochemistry analysis

Marshfield Laboratories
Veterinary Diagnostic Services
1000 North Oak Avenue
Marshfield, WI 54449-5795
1-800-222-5835

Small animal profile (ANP-15)

B. Blood gas instrument

Bayer Corp
Diagnostics Division
115 Norwood Park
Norwood, MA 02062
1-800-255-3232

116769 – Gas Callibration W/Reg
119281 – Blood Gas Analyzer
473385000 – 7.3 solution blood gas analyze
473386000 – 6.8 solution blood gas analyze
473387000 – Wash blood gas analyze
570096000 – Cal G/L Reagent blood gas analyze
473120000 – Slope blood gas analyze
473389000 – Cleaning 1 & 2 blood gas analyze
111399000 – Bar code reader blood gas analyze
478533000 – pH fill solution blood gas analyze
478822000 – Reference fill solution blood gas analyze

C. Autoanalyzer

Schiapparelli Biosystems Inc.

Alfa Wassermann
368 Passaic Avenue
Fairfield, NJ 07004
(800) 220-4488 or (973) 882-8630
Customer Service Fax: (973) 227-0998
Sales/Service Fax: (973) 276-0383
www.sbiosys.com
e-mail: info@biosys.com

Nexct Analyzer
Catalog # 402444

D. Osmometer

Precision systems, Inc
60 Union Avenue
Sudbury, Massachusetts 01776
Telephone: 617-443-8912

Automatic High Sensitivity Micro Osmette
Catalog/Model # 5004

Instruction Manual
Catalog # 2094

E. Flame photometer

Instrumentation Laboratory
113 Hartwell Avenue
Lexington, MA 02421
(800) 955-9525
Fax: (781) 861-6135

Model # IL943

F. Microtiter plate fluorescent assay

Bio-Tek

Highland Park, Box 998

Winooski, Vermont 05404-0998

(800) 655-4040

(800) 451-5172

(800) 24-BIOTEK (Service)

(800) 655-4107 (Technical Assistance Center)

Fax: (802) 655-7941 (Sales)

(802) 655-3399 (Service)

<http://www.biotek.com>

e-mail: sales@biotek.com

Microplate Fluorescence Reader

Model # FL600