

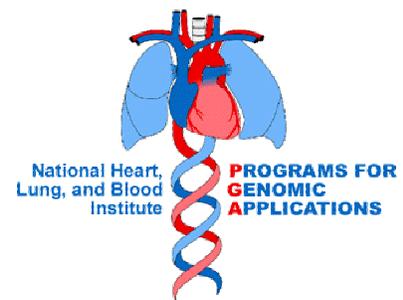
Renal and Cardiovascular Phenotypes

Protocol B

High-throughput assessment of salt-sensitivity and renal function in conscious rats

**Erica Liss and Angelo Piro
with**

**David Mattson, Ph.D., Richard J. Roman, Ph.D.,
and Allen W. Cowley, Jr., Ph.D.**



PhySGen

I. **Experimental setup for renal and cardiovascular studies in conscious rats (instrumentation and calibration procedures)**

Instrumentation and equipment used in setup [order information listed in section V]:

- Surgical station: thermostatically controlled surgical board, stereo-microscope, fiber-optic light, surgical instrument pack, recovery station with temperature controlled pads, HEPA filter hood area for preparation of animals for surgery. [Please see the Surgical Pack Protocol section where complete details are given related to the instruments, supplies, and equipment used in the surgical preparation]
- Chronic Monitoring Facility—recording rooms used for studies allow simultaneous collection of hemodynamic data from up to 64 animals. Specially designed cages permit collection of urine for analysis. [A CMF section will be added at a later date]
- Biochemical Core Lab- analysis of samples collected for electrolytes, protein, microalbuminuria, plasma renin activity, and creatinine is performed in the PGA Biochemistry Core Lab and the Physiology Biochemical Core Laboratory [See Biochemistry Protocol section for more complete description]

The following series of pictures depict the experimental set-up as used daily.



Figure 1: A close-up view of one of four surgical stations used in the preparation of the animals for chronic study.



Figure 2: Specialized recording rooms allow continuous on-line collection of hemodynamic data from 64 rats simultaneously

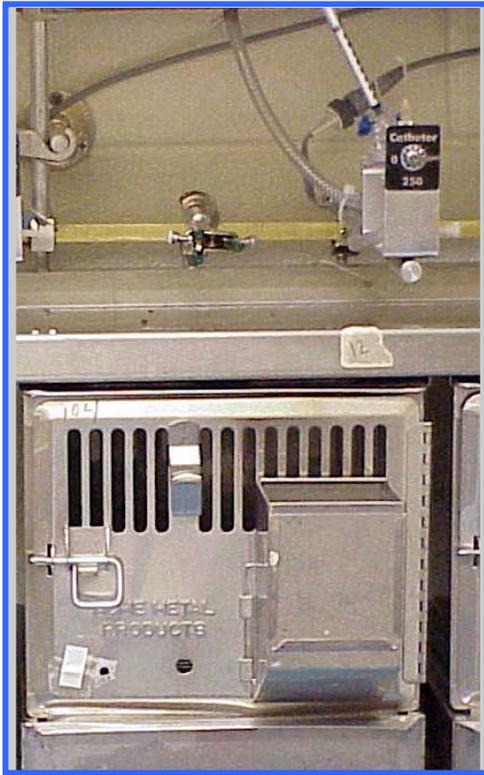


Figure 3: Close-up of transducer used for blood pressure measurements. Special valve permits calibration procedure to be performed on all transducers at once.

II. Experimental protocol for renal and cardiovascular studies.

A. Surgical preparation of animals for study.

1. Carefully don sterile gloves in the appropriate size. Once these gloves are on, *do not* touch any non-sterile surfaces! Doing so will compromise the sterile surgery and, consequently, the rat's health. If at any time you do accidentally touch a non-sterile area or find a hole in your glove, replace the glove immediately.
2. Arrange the sterile instruments so that they are organized and within easy reach, taking care that they at all times remain in the sterile field. Attach the 22-gauge adapter to the sterile 1cc syringe and fill with sterile saline. Use this to fill the microrenathane catheter you will implant during the surgery. Check again to insure the rat is adequately anesthetized before you make your incision!
3. **Implanting the catheter:** The femoral vein and artery run along the same path as the femur. The incision should be made in that area and close to, but not on, the abdominal wall. It is not advisable to make a large incision, as these seem to irritate the rats more readily, but the incision needs to be large enough to work comfortably (about 1 to 1.5 cm should be sufficient). Once the skin is open, look through the microscope to continue with the rest of the implantation.

Renal and Cardiovascular Protocol B
PhysGen

- a. Using the micro-dissecting forceps, carefully part the tissues as you tunnel down toward the femoral artery and vein. The actual implantation will occur very close to the abdominal wall, so work in that area. Avoid tearing the numerous small blood vessels in the connective tissue, the less disturbance caused the better. When you encounter a rather large 'knot' of the biggest vessels you have seen so far, you are near to your goal. The area used for implantation is located between this 'knot' and the abdominal wall. As you carefully part the clear connective tissue in this area, the 'knot' will move away and the straight section of femoral vein and artery often hidden by it, will be exposed. The vein is the larger, purple vessel, and the artery is the thinner white one.
- b. Next you will separate the artery from the vein. **IMPORTANT:** You must avoid disturbing the numerous nerves which run beside the artery. They appear white and thready. Once manipulated, the nerves do not recover, and will adversely effect the rat's ability to use the limb, to the point that the rat will mutilate it's own leg, not recognizing it as his own! For best results, work between the artery and vein to separate the two, and avoid touching the side near the nerves altogether.
- c. When the artery has been isolated, carefully and gently lift it with the forceps and draw three ties beneath it, taking care not to damage the vessel in the process. Use the microscope to arrange the ties so that one is at the distal end of the cleared vessel, one is in the center, and the third is at the proximal end. The tie at the distal end should be as close to the end of the cleared section of artery as possible. Tie this one in one (tight) knot. This will occlude the vessel. Attach a hemostat to the free ends of this tie so that a constant, but not excessive, tension is kept. With the tie at the proximal end, start a knot, draw it down until the opening is still large enough to allow the catheter to pass through, making sure it is as close to the abdominal wall as the cleared vessel will allow. Attach another hemostat to the free ends of this tie to achieve constant (but again, not excessive) tension to the vessel. Start a knot in the center tie, leaving it just as open as the previous one. Do not attach a hemostat to this center tie, but arrange it close to the top tie and leave the ends loose.
- d. At this point, you will need to measure the catheter's tip for a proper fit. Without allowing the catheter to touch the rat, determine the length the tip should be to reach from the vessel incision site to the point of, but not into, the aorta.
- e. You are now ready to make your cut into the artery. Check to make sure the top tie is effectively occluding the vessel! Trim the tip to that distance, creating a slight bevel on the very end. Take care that the bevel is not too sharp, as it will easily puncture the vessel wall during implantation. Recheck the entire catheter to insure it is completely filled with saline and there are no air bubbles present, and apply a catheter occluding forceps just beyond the syringe.
- f. Using the Vannas scissors carefully cut into the artery wall close to the distal tie, *but allowing for space to pass the catheter over the tie*. Needless to say,

Renal and Cardiovascular Protocol B
PhysGen

do not cut through the artery! It is better to have to cut twice in the same spot than to cut too much. Do not cut more than halfway through the vessel, especially when working with a Dahl rat. If, while you are making the cut, your field of view is suddenly filled with blood do not panic. Quickly put more tension on the upper tie by simply moving the hemostat back a little, effectively stopping the flow. Clean up the blood thoroughly, as it is an irritant to tissues outside of the vessels.

- g. With the artery incised, you are now ready to insert the catheter. Using the Dumont micro dissecting forceps, hold the tip of the catheter in one (allowing enough of the tip to protrude to begin the insertion into the artery) and with the other, carefully lift the top of the incision in the artery. Note: Avoid pulling excessively on this incision, as oftentimes it will enlarge and will eventually reach the upper tie making it extremely difficult, if not impossible, to successfully place the catheter. Always be aware that these forceps have sharp, pointed ends. Take care that they do not puncture the vessels or damage the nerves as you work.
- h. Holding the top lip of the incision, carefully insert the catheter tip into the opening. When enough of the tip has been implanted to allow it, release the lip of the incision and lightly grip the vessel around the catheter. This will allow you to hold the vessel in place as you continue to thread the catheter into the artery. Try to keep the artery parallel to the vein and as close to its natural position as possible. This will reduce the chance of punctures, and twisting of the vessel. When the tip has passed the middle tie and is near to, but not touching, the top tie, continue to hold the catheter inside the vessel while you use the other forceps to loosen the top tie. This can usually be accomplished by simply pulling down a little, moving the hemostat enough to allow the tie to be slack. Carefully pass the catheter tip through the top tie and continue advancing until the base of the catheter tip (where it joins the larger body of the catheter) rests at the incision in the artery.
- i. Move the center tie as close as possible to the catheter's joint, and use it to secure the vessel to the catheter tip at the base. Tie a double knot, but keep in mind that it is possible to occlude the catheter by tying too tightly! (Check the catheter after each knot is made to insure it is still working properly. If not, loosen the offending knot and retie.) Remove the hemostat from the top tie and tighten the knot between the center tie and the abdominal wall. Remove the hemostat from the bottom tie and secure this tie to the catheter *behind* the joint in the catheter, *on the larger tubing* (If this tie is not on the larger tubing, the catheter will easily pull out of the vessel). This knot can and should be tied rather tightly. Check the catheter once more to insure proper function then insert a 22-gauge plug into the end of the catheter. Trim all tie ends close to the knots, taking care to avoid cutting the knots in the process.
- j. Apply a small amount of Vet Bond to the site where the catheter joint meets the vessel. Take care that no Vet Bond touches any other surface. Vet Bond dries very quickly and is irritating to the rat is too much is used.
- k. Next, fill the cavity with antibiotic ointment (neomycin & polymyxin B sulfate, bacitracin zinc & hydrocortisone acetate).

Renal and Cardiovascular Protocol B
PhysGen

ml/100 gm bwt s.c. When the rat has completely recovered from the effects of the Buprenex, he can be taken to his cage in the recording room. The rat must be conscious enough to drink from the water bottle upon return from the home cage.

6. **Daily assessment of animal health:** Throughout the remainder of the protocol, each rat must be observed for signs of illness or injury. First thing each morning, carefully examine each rat. Simply looking through the cage wire is not sufficient. Open the cage and watch the rat as he moves about. Note and record observations for each of the following:
- a. **Locomotion:** Is the rat moving about normally, using all four legs easily? If not, locomotion should be recorded as “abnormal”, and the reason for it listed.
 - b. **Posture:** A normal rat will move about the cage freely with no evidence of discomfort. A rat that is in pain or is ill will typically appear hunched, and be reluctant to stretch it’s body from that position even when prodded. Often a reluctance to lift the head or extend the rear limbs is present too. A rat that is found in this posture should be observed closely, and eliminated from the study if the condition deteriorates.
 - c. **Body condition:** Look closely at the rat’s body mass and hair coat. If a rat is too thin, his spinal column is readily evident. A rat’s coat is a direct reflection of his health. Healthy rats groom themselves constantly. Unhealthy rats do not. If the coat is scruffy, dirty or shedding excessively, the rat is not healthy and warrants close and frequent observation. Observe also the cage pan. Are the feces normal in appearance? Remember feces will change color with diet change. Black feces indicate that blood has been ingested. Look for urine. Bloody urine appears as a slight blood tinge in the bedding, and often times there will be blood-tinged urine around the genital vent. Diarrhea is cause for concern, as it can rapidly cause dehydration.
 - d. **Feet/legs:** To check the feet and legs, gently grasp the rat’s tail and lift it just until you are able to see the surgical incision. It should be clean and dry, with no discharge, redness or swelling, and no sutures missing (Missing sutures must be replaced, in the lab, under anesthesia and aseptic conditions). Pay special attention to the left rear leg. It should appear pink and healthy with full, or nearly full, range of motion.
 - e. **Eyes/nose:** Look at the eyes and nose for a reddish discharge surrounding them. This is often seen post-op, but should disappear the rat recovers and resumes grooming. Note such discharges.
- Eating, drinking:** These two observations are of most importance. The first thing an animal does when it does not feel well is to stop eating and drinking. The level in the rat’s water bottle and feeder should go down daily, Dahl S more so than the Brown Norway. High salt diet will naturally increase water intake, so be especially observant of water levels during that part of the protocol. If a rat does not seem to be eating enough, put a few pellets on the cage floor (except during 24 hour urine collection periods!) to encourage interest. If not enough water is being consumed, check the water bottle’s lickspout (while the bottle is in place on the cage) to insure it is working properly. Always attach water bottles so that they upright and are as low as

possible without actually touching the cage floor, and empty, rinse, and refill the bottles every other day to insure a clean fresh supply.

B. Calibration of pressure transducers and initiation of computer data acquisition system:

1. **Calibrating the Box.** Calibration should be done once a day everyday of recording. It should be the first thing you do if it has not already been done in the room where you are recording. Check the calendar to see if it has been done that day
 - Switch the toggle to the “cal” position on the box. This should give you a reading of zero. If it is not zero, adjust the knob marked “zero” until it does read zero.
 - Place the end of your mercury manometer in the hole next to the “cal” marking. Pump up your manometer to read 250mmHg. This should also be the reading on the box. If it is not, adjust the “gain” knob until it reads 250mmHg.
 - Release the pressure on the manometer and remove from the box. Switch the toggle back to the “run” position.
2. **Filling the Transducer.** The next step is to fill the transducers with saline so they are completely fluid filled and free of any air bubbles. This should be done at the beginning of your study and as needed. The transducers should be filled with saline only. They may be flushed (for aseptic purposes) once a week with 70% alcohol immediately followed by a saline flush. No heparin should pass through the transducer for any reason.
 - a. Take a 10cc syringe and fill it with sterile saline.
 - b. Remove transducer apparatus from the bar. Place syringe on the stopcock port between the transducer and the rat line (middle stopcock). Turn this stopcock so it is open to the transducer. Turn the apparatus so the transducer is in a vertical position with the transducer being at the top.
 - c. Turn the stopcock on the far end of the transducer (back stopcock) so that it is also open to the transducer. Apply pressure to the syringe to fill the transducer with the saline.
 - d. Close both stopcocks so they are off to the transducer.
 - e. Now invert the apparatus so the end with the rat line is on top.
 - f. Turn the middle stopcock so it is open to the rat line. Turn the front stopcock so it is open to “catheter”. Apply pressure to the syringe to fill end leading to the rat line, and then close the middle stopcock.
 - g. Place the apparatus back on the bar. Make sure there are no air bubbles anywhere in the transducer or rat line end. If there are, you must flush them out. You may need to have the apparatus in a horizontal position to fill the adapter and the rat line.
 - h. Repeat this for all the channels where you will be recording.
3. **Calibrating the Computer.** Computer calibration needs to be done each time you start a recording session. You cannot start a recording without calibrating each channel.

Renal and Cardiovascular Protocol B
PhysGen

- a. Turn all front stopcocks of the channels you will be using to “250” position.
 - b. Look at the computer screen. If all your channels are not configured for blood pressure measurement, then you need to configure them. If there is a “T” in each channel box, they are configured; if there is an “X” or “L”, then they are not configured for blood pressure measurement. To configure devices:
 - Go to “channels”
 - “Configure devices”
 - Click on each channel you will be using until a “T” appears in the box.
 - c. After configuring the devices, look on the screen again and make sure all the channels you will be using have a “T: in the box.
 - d. Go to “channels”, “Start Channels”. If this option is not available, it means the channels you will be using have a “T” in the box.
 - e. Go to “Channels”, “Start Channels”. If this option is not available, it means the system was shutdown and you need to restart it. To do this, you go to “System”, “Start system”. Then go back to “Channels”, “Start Channels”.
 - f. Select the type of data you would like to collect for each channel. If it is “MAP” data, you must also select the interval in which the computer will average your data (1-600 seconds).
 - g. Select “Start”. All boxes you are calibrating should appear white. Make sure all your stopcocks are turned to “250”, then select “go” for the high calibration. The channels you are calibrating should all turn yellow.
 - h. Next turn all your stopcocks to the “zero” position, then select “go” for the low calibration. All the boxes should turn green. If any boxes are red, you have a failed transducer and need to contact Research Services Core immediately.
 - i. If you would like a comment at the beginning of your file, just click on the number of the channel and a text box will appear for a comment.
 - j. Now you can either hook up your rats or select “done” to start your recording.
4. **Hooking Up the Animal**
This section will explain how to hook up the rat to the transducer. This should be done using aseptic techniques.
- a. Turn the front stopcock to “catheter”. Clamp the end of your catheter with a hemostat and remove the plug from the end.
 - b. Unclamp the hemostat and see if the catheter bleeds back. If not, place a 1cc syringe of 1:10 dilution of heparin:saline on the catheter end and flush in 0.05-0.1cc. Then remove syringe to see if it bleeds back. If it bleeds back, let 5-6 drops out to remove all the heparin from the line. Then refill the line with 0.1-0.2 cc (depending on your catheter dead space) of 1:10 heparin:saline and clamp off.
 - c. Place a syringe of 1:10 heparin:saline on the middle stopcock for flushing. Turn the stopcock open to the rat line. Make sure the front stopcock is turned to “catheter” and flush (from the middle stopcock) a small volume through to ensure the line is completely fluid filled. Then turn the middle stopcock off to the air.
 - d. Now insert the end of the rat line into your rat catheter line and unclamp your catheter.

Renal and Cardiovascular Protocol B
PhysGen

- e. Check the computer screen to see the pulse wave and blood pressure measurement. If the signal is not good (i.e. pulse wave flat line or pulse pressure less than 10mmHg), you may need to flush, check for air bubbles, or determine that your catheter is failing.
- f. Repeat for all channels.

5. Checking the Data Acquisition

- a. Double-click on Network Neighborhood or create a shortcut on the lab computer.
- b. Then double click on cmf_room # you are using-east or west.
- c. Next double click on the Data folder.
- d. Double click on your file.
- e. Check data and current pulse pressure to see if your rat needs a flush.
- f. Close this file and check the next file.

- 6. Flushing the Animal:** After hooking up your animal or checking your data acquisition, you may find that the pulse pressure on your animal is dampened and therefore your animal needs a “flush”. Typically after the animal is hooked up, the flushing is done from the middle stopcock. This is done by opening this stopcock to the rat line and withdrawing approximately 0.1-0.2 cc and then flushing back in a slightly larger volume (0.2-0.3cc). Then turn the stopcock so the rat line is open to the transducer. If this does not work, you may want to flush from the rat catheter directly.

7. Stopping the Channels

- a. Select “Channels”, “Stop Channels”.
- b. Check the boxes of the channels you would like to stop recording on.
- c. Press “Stop”.

8. Unhooking the Animal

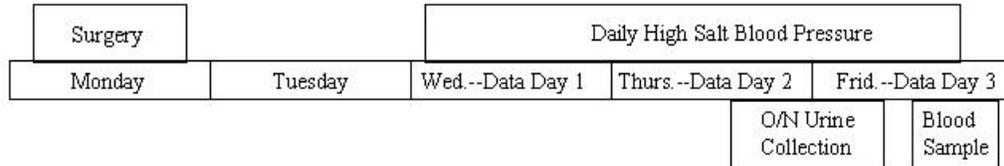
- a. Clamp off your catheter with a hemostat and disconnect the rat line from the catheter.
- b. Flush in a 1:1 heparin:saline solution (volume being the dead space of your catheter).
- c. Reinsert your catheter plug into the end of your catheter.

C. Experimental protocol: Conditioning, Surgical preparation, and study measurements

At weaning, the rats are kept on a 0.4% NaCl diet (Dyets). At approximately 10 weeks of age (17 days prior to surgery), the food is changed to an 8.0% NaCl diet (Dyets). At 12 weeks of age perform protocol listed below. **Note:** The FHH, the FHH/BN consomics, and some groups of BN rats will receive L-NAME (12.5 mg/l) in the drinking water when the diet is switched to high salt. These rats will be maintained on oral L-NAME continuously until the end of the experimental protocol (refer to the Physgen Production Schedule).

**Renal and Cardiovascular Protocol B
PhysGen**

Rats on High Salt Diet (8.0% NaCl)



Monday: Surgery: Rats are weighed and anesthetized with a cocktail of 7:2:1 mixture (by volume) of ketamine (stock concentration:100 mg/ml), rompun (stock: 20 mg/ml), and acepromazine (stock:10 mg/ml). The Dahl SS/Mcw parents are administered 0.04-0.06 ml/100kg and consomic rats are administered .03-.09cc/100g of the anesthetic cocktail. Using aseptic technique, catheters are implanted in the femoral artery and exteriorized at the shoulders, and passed through a spring. Antibiotic (0.1cc/ 100g bwt Penicillin G i.m.; 100 mg/ml stock concentration) and analgesic (0.05cc/100g bwt buprenex s.c.; 0.3 mg/ml stock) are administered, and the animals allowed to fully recover from anesthetic on a temperature controlled pad. Rats are placed in a “metabolic” cage in Room 106 with 8.0% NaCl chow and tap water *ad lib*. **Note:** The FHH, the FHH/BN consomics, and some groups of BN rats will receive L-NAME (12.5 mg/l) in the drinking water when the diet is switched to high salt. These rats will be maintained on oral L-NAME continuously until the end of the experimental protocol (refer to the Physgen Production Schedule).

Tuesday: Each rat will be exercised on the treadmill to improve collateral circulation in the catheterized leg. Each rat is exercised for 5 minutes at the lowest treadmill setting.

Wednesday-Friday: Daily Arterial Blood Pressure Measurements: Daily measurement of systolic, mean and diastolic blood pressure and heart rate will be made from 9:00-12:00 on Wednesday, Thursday, and Friday. Data will be obtained at 320 Hz and averaged at 1 minute intervals.

Thursday p.m. Urine Collection: At the end of the day at 4 p.m., a 16 hour urine collection will be made. Metabolic funnels will be installed on each cage following siliconizing (careful to drain off any excess silicone). Graduated cylinders will be used for the collections. Each cylinder will be labeled with the rat i.d. **Note the time that the collection begins. This is important for the calculation of the urine flow.**

Renal and Cardiovascular Protocol B
PhysGen

Friday a.m. Remove urine cylinders **carefully noting the time of removal.** Measure the volume of urine and record the volume and the time of collection. Take aliquot of urine for measurement of protein, microalbumin, creatinine, and electrolytes. The tubes must be labeled with the rat i.d. (NOT the channel number), and the date of collection.

Friday p.m.: Blood Sample: Following the daily measurement of arterial blood pressure, collect whole blood for the measurement of plasma renin activity, creatinine, and hematocrit. The catheter should be cleared of saline by allowing the catheter to bleed back with 4-5 drops of blood indicating that the catheter has been cleared of all saline. A 22 g. needle attached to a heparinized 3 cc. syringe. You should not be pulling on the syringe; it should be filling from the catheter. Remove the needle, and evacuate 0.8 ml of the syringe contents into a 3.0 ml green top tube containing lithium heparin [invert to mix-gently]. The remaining 0.250 goes into a separate tube with the appropriate amount of EDTA for the determination of plasma renin activity. Draw 2-65 ul hematocrit tubes from the catheter end before clearing the line with 0.1 cc of normal saline followed by 0.2 cc of a 1:1 heparin:saline solution. Trim the end of the catheter line and insert a catheter plug that has been stored in 70% ETOH.. Collected samples should be processed immediately [no longer than 30 minutes].

Samples for analysis:

- 1) High salt urine protein
- 2) High salt urine microalbumin
- 3) High salt urine sodium
- 4) High salt urine potassium
- 5) High salt urine creatinine
- 6) High salt plasma renin activity
- 7) High salt hematocrit
- 8) High salt plasma creatinine

III. SOLUTIONS

A. Rat Anesthesia Protocol for Chronic Animal Preparation

Stock solutions:

Ketamine 100 mg/ml

Xylazine 20 mg/ml

Acepromazine 10 mg/ml

Use the following protocol:

Mixture: 7:2:1 “Working stock” mix in one bottle at this ratio of ketamine:xylazine:ace

Give into the muscle of the right leg

- Sprague Dawley, Brown Norway, Fawn Hooded rats give 0.06-0.08 cc/100g
- Dahl S (MCW strain) and Consomics of this strain give 0.04 cc/100

This will keep the rats out for 30 mins. If a supplement is needed, then give a 100 gm volume (0.04 for Dahl S and 0.06 for SD, BN, or FHH). The i.m. route should slow the absorption enough to give you a good induction with a longer down time.

IV. Order Information

- A. Harvard infusion pump
Harvard Apparatus, Inc.
84 October Hill Rd.
Hollisston, MA 01746-1371
1-800-272-2775

AA55-5920 - Infusion pump 22, only with 6/10 multi syringe rack.
24-00-067 – Anti-siphon bar
24-00-67 – Retainer Syringe
5009-099 – Spring Clip
5100-012 – Thumb screw

- B. Dacron buttons
Instech Laboratories
5209 Militia Hill Rd.
Plymouth Meeting, PA 19462-1216
1-800-443-4227

DC95B/Bulk – Dacron Buttons Bulk (100)

- C. Springs
McMaster Carr Supply Co.
PO Box 4355
Chicago, IL. 60680
630-833-0300

9665K42 – Springs (5 pkg), Type 302 SS Continuous Lgth. Extension spring,
20" lg., 1/8" od., .020" wire

V. Diet

Renal protocol animals are fed Dyets low salt (0.4% NaCl) chow, order #113755, from weaning. At 17 days prior to tissue collection they are put on Dyets Inc. high salt (8.0% NaCl) chow, order # 100078. L-NAME (N-omega-Nitro-L-Arginine Methyl Ester Hydrochloride) is given in the drinking water at 12.5 mg/L for 17 days prior to the tissue collection for the high salt + L-NAME protocol animals

Please see <http://www.teklad.com/index.htm> and <http://www.dyets.com/dietcom.htm> for more information.