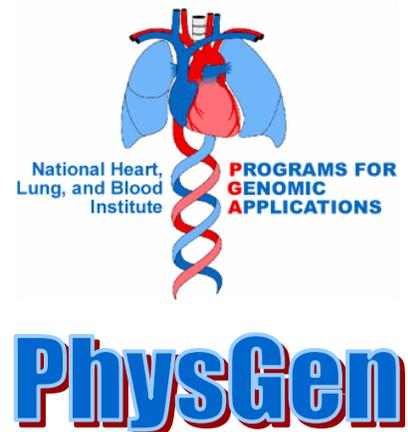


Respiration Phenotypes

High-throughput characterization of
chemoreceptor control under conditions of
hypercapnia and hypoxia
in inbred rats

Jaime Petersen and Amy Rider
with
Melinda Dwinell, Ph.D. and Hubert Forster, Ph.D.



I. Experimental setup for respiration studies

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

- 4 plethysmograph chambers
- gas tanks and regulators
- two computer recording stations
- Bayer Blood Gas analyzer

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

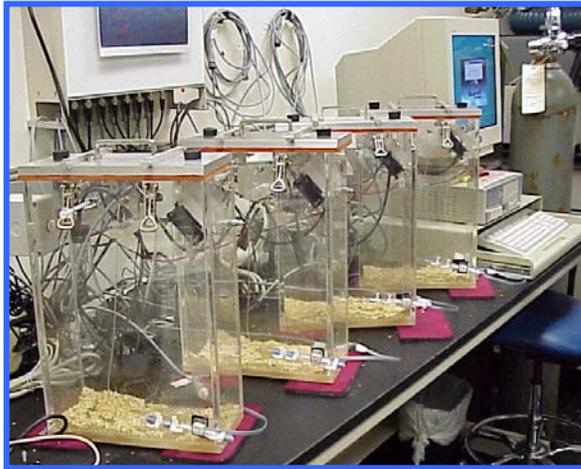


Figure 1A: Four plethysmographs are used to perform high-throughput respiration studies in conscious rats.

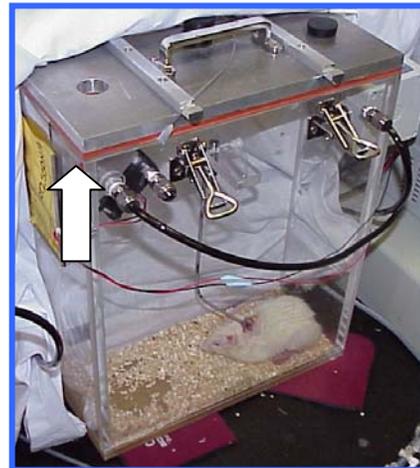


Figure 1B: Close-up view of plethysmograph indicating entry port for introduction of gases into chamber.

II. Experimental protocol for respiration 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.

- a. Using the micro-dissecting forceps, carefully part the tissues as you tunnel down toward the femoral artery. 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 that 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 forcep 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, 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.
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, and 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. Next, fill the cavity with antibiotic ointment (neomycin & polymyxin B sulfate, bacitracin zinc & hydrocortisone acetate).

- i. Using scissors as a spreader, tunnel a path for the trochar just below the skin, beginning at the top of the incision in the skin until the scissors can reach no further. Carefully thread the trochar from the incision to a location about 2 cm from the scapulas on the animal's back. While advancing the trochar, be sure to keep the tip of the bevel against the underside of the skin to avoid damaging muscles, vital organs, etc. When the site has been reached, leave the trochar in place and apply betadine to the site. Using scissors, create an opening large enough for the trochar, and later the button, to pass through. Leaving the trochar protruding from the opening at the shoulders and at the incision in the leg, prepare the catheter by wiping the entire exposed length with alcohol. Without letting the catheter touch anything else, carefully thread the catheter end into the trochar at the leg and advance it until it appears at the shoulders. At this point, observe the catheter within the leg as you continue slowly pulling it at the shoulders. Make sure the catheter in the leg does not twist or pull the artery, and leave a slight loop there so that it does not pull the artery as the catheter is later manipulated.
 - j. When the catheter is correctly in place, carefully remove the trochar by pulling gently on the end protruding at the shoulders. When the trochar is completely out, check the leg again to insure that the catheter did not move.
 - k. Now the leg incision can be closed, using Braunamide suture and interrupted stitches. Be sure to completely close the incision and cut ends as short as possible to minimize the opportunity for the rat to open the incision. Apply Betadine to the closed incision.
4. **Implanting the button:** Reposition the rat so that he is on his chest and facing you. Using scissors, enlarge the area under the skin surrounding the shoulder incision so that the button will rest upright and flat when the surgery is completed. Apply antibiotic ointment liberally to the area just enlarged with the scissors. Thread the catheter end through the button and bring the button to rest on the incision. Using forceps, work the outer edge of the button under the skin until all of the dacron is below the skin and lying flat. Tack the button into place with silk suture, passing the needle through the skin, then the dacron, then back out through the skin close to the initial entry site, and tie off. Do this in four evenly spaced sutures. Use Braunamide suture to close the skin incision, securing the button. Next, thread the catheter through the spring and carefully push the spring end into the button tubing until it *almost* touches the incision. Use a piece of tape to secure the other end of the spring to the overhead lamp, then apply Super Glue Gel to the very top of the button, continue applying the glue to about 1/2" of the spring above it. Allow it to dry thoroughly.
5. **Recovery:** Administer injectable antibiotic (Penicillin G) at 0.10cc/100 gm bwt I.M. to the right leg. Return rat to his recovery cage that is on the warming pad in the recovery area. Make sure the spring is protruding through the wire bar lid to prevent destruction of the catheter by the rat during recovery. Observe the rat frequently during recovery. Each time, manipulate the leg with the implanted catheter to aid collateral circulation and flexion of the limb. Massage the foot and "bicycle" the leg several times during recovery. When the rat is sternal and

moving about the cage, administer Buprenex 0.05cc/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:
- **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.
 - **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.
 - **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 oftentimes there will be blood-tinged urine around the genital vent. Diarrhea is cause for concern, as it can rapidly cause dehydration.
 - **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.
 - **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

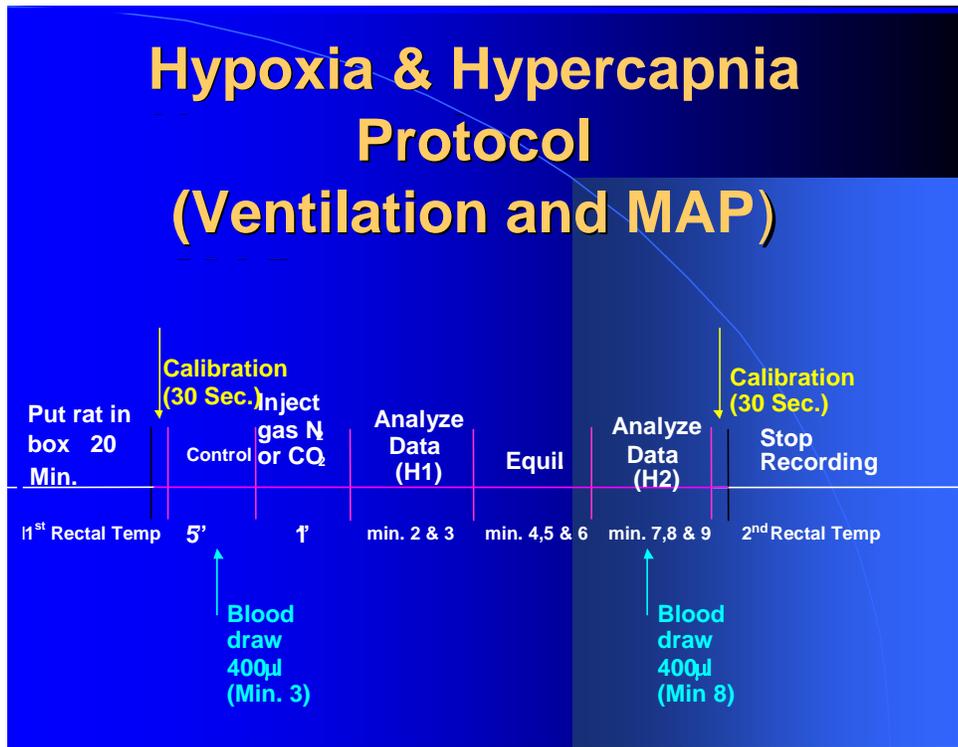
possible without actually touching the cage floor, and empty, rinse, and refill the bottles every other day to insure a clean fresh supply.

Respiration Protocol Timeline

	Monday	Tuesday	Wednesday	Thursday	Friday
Week 1 Pre-Surgical Conditioning				[11 week old rats]	Weigh and Separate into Individual Cages
Week 2 Surgery & Adaptation	<u>Surgery</u> Catheter Implantation in Femoral Artery	<u>Surgery</u> Catheter Implantation in Femoral Artery	Box Adaptation (20 min)	Box Adaptation with N ₂	Box Adaptation with CO ₂
Week 3 Studies	Box Adaptation with N ₂	Hypoxia Study	Hyper- capnia Study		

B. Adaptation of animals for study in plethysmographs

1. Check the arterial catheter of the rat before putting into the chamber. Catheter should bleed back then connect to the swivel.
2. On the first day of adaptation place rat into plethysmograph chamber and connect arterial line to blood pressure transducer.
3. Rat is left to adapt in the chamber with chamber lid open. Repeat until all four chambers have animals in place.
4. Leave animals in chambers for 10 minutes, then at the 10th minute place lid on chamber and close down for 10 more minutes.
5. This adaptation conditioning should be performed each day for the four days before the plethysmograph studies.
6. On the second day of adaptation, the lid is closed down and at the 10th minute of adaptation, 4.5 L of nitrogen gas is injected into the top of the plethysmograph through the designated port in the lid [see diagram in Figure 1B].
7. On the third day of adaptation, the lid is closed down and at the 10th minute of adaptation 650 ml of CO₂ gas is injected into the top of the plethysmograph through the designated port in the lid.
8. The fourth day of adaptation is followed just like the second day of adaptation.



D. Experimental protocol for determination of ventilatory response to acute hypoxia.

- 1. Preparation of experimental station prior to introduction of rat into chamber.** Put ½ inch of clean bedding material into bottom of chamber and turn chamber fans on. Connect and fill pressure transducers. Prepare saline syringes for flushing of lines. Prepare blood collections syringes [1 ml syringe containing a lithium heparin pellet]. Prepare single channel swivels and fill with saline.
- 2. Preparation of rat for hypoxia study.** Check the arterial catheter before putting rat into chamber. The catheter should bleed back to indicate it is patent. If it does not bleed back, flush with a small volume of saline and check to see that it bleeds back then connect catheter to single channel swivel.
 - Take first rectal temperature
 - Place rat into chamber and connect arterial line to blood pressure transducer
 - Leave rat in chamber with chamber lid for approximately 20 minutes while calibrating the blood pressure transducer
 - Calibrate transducer to 0 and 200 mmHg and create a file with the filename = month-day-rat i.d.-strain [i.e. 0405F011SSBN16] and collect in this file some of the 0-200 calibration data.
- 3. Initial calibration.** Close the chamber lid and tighten so there are no leaks. Begin 30-second calibration of chamber pressure sensor using a 1 ml syringe

- inserted into the port on the side of the chamber [0 and 0.3 ml repeated several times to give a calibrated “breath” on the recording].
4. **Control period.** Time is set and started for a 5 min period. At 3 minutes of the control period an arterial blood sample is withdrawn
 - Withdraw 0.8 ml volume from arterial catheter (mixed saline & blood)
 - Withdraw 0.4 ml from the catheter with the syringe containing lithium heparin pellet; mix syringe contents gently and place on ice.
 - Give back to the rat the 0.8 ml removed before the sample; flush with 0.2 ml of saline
 - Blood gases and pH are measured by the PGA Biochemical Core Laboratory within 30 minutes of collection.
 5. **Introduce gas mixture.** Fill 5-liter syringe with 4.5 liters of 100% nitrogen and introduce into the chamber through the designated port on the top of the plethysmograph. Set the timer for 1 minute. At the end of 1 minute, timer is reset to 2 minutes to begin period H1 [hypoxia 1].
 6. **Hypoxic period 1.** Collect ventilation (V_E in ml/min), tidal volume (V_T ; ml) and frequency (f ; ventilation rate in breaths/minute) as well as mean arterial pressure (MAP in mmHg) and heart rate (HR in beats/minute) for 2 consecutive minutes.
 7. **Hypoxic period 2.** Collect ventilation (V_E in ml/min), tidal volume (V_T ; ml) and frequency (f ; ventilation rate in breaths/minute) as well as mean arterial pressure (MAP in mmHg) and heart rate (HR in beats/minute) for 3 consecutive minutes. A blood sample is withdrawn after minute 1 for measurement of arterial blood gases and pH as described above for the control sample.
 8. **Final calibration.** The calibration procedure for the chamber pressure sensor is repeated. The chamber is opened and the rat is disconnected from the transducer and rectal temperature measured for the second time. The rat is disconnected from the swivel and the arterial line filled with 0.2 ml of a 1:1 heparin/saline mix and returned to the transport cage to be returned to the home cage.

E. Experimental protocol for determination of ventilatory response to acute hypercapnia.

1. **Preparation of experimental station prior to introduction of rat into chamber.** Put $\frac{1}{2}$ inch of clean bedding material into bottom of chamber and turn chamber fans on. Connect and fill pressure transducers. Prepare saline syringes for flushing of lines. Prepare blood collections syringes [1 ml syringe containing a lithium heparin pellet]. Prepare single channel swivels and fill with saline.
2. **Preparation of rat for hypercapnia study.** Check the arterial catheter before putting rat into chamber. The catheter should bleed back to indicate it is patent. If it does not bleed back, flush with a small volume of saline and check to see that it bleeds back then connect catheter to single channel swivel.
 - Take first rectal temperature.
 - Place rat into chamber and connect arterial line to blood pressure transducer.

- Leave rat in chamber with chamber lid for approximately 20 minutes while calibrating the blood pressure transducer.
 - Calibrate transducer to 0 and 200 mmHg and create a file with the filename = month-day-rat i.d.-strain [*i.e.* 0405F011SSBN16] and collect in this file some of the 0-200 calibration data.
3. **Initial calibration.** Close the chamber lid and tighten so there are no leaks. Begin 30-second calibration of chamber pressure sensor using a 1 ml syringe inserted into the port on the side of the chamber [0 and 0.3 ml repeated several times to give a calibrated “breath” on the recording].
 - **Control period.** Time is set and started for a 5 min period.
 4. **Introduce gas mixture.** Fill 1-liter syringe with 650 ml of CO₂ and introduce into the chamber through the designated port on the top of the plethysmograph. Set the timer for 1 minute. At the end of 1-minute, timer is reset to 2 minutes to begin period H1 [hypercapnia1].
 5. **Hypercapnia period 1.** Collect ventilation (VE in ml/min), tidal volume (VT; ml) and frequency (f; ventilation rate in breaths/minute) as well as mean arterial pressure (MAP in mmHg) and heart rate (HR in beats/minute) for 2 consecutive minutes.
 6. **Hypercapnia period 2.** Collect ventilation (VE in ml/min), tidal volume (VT; ml) and frequency (f; ventilation rate in breaths/minute) as well as mean arterial pressure (MAP in mmHg) and heart rate (HR in beats/minute) for 3 consecutive minutes.
 7. **Final calibration.** The calibration procedure for the chamber pressure sensor is repeated. The chamber is opened and the rat is disconnected from the transducer and rectal temperature measured for the second time. The rat is disconnected from the swivel and the arterial line filled with 0.2 ml of a 1:1 heparin/saline mix and returned to the transport cage to be returned to the home cage.

III. Solutions

Heparin Solution

Heparin used is 1,000 units/ml Medico Mart [catalog #00641-2450-45]. Mix equal parts heparin and normal saline [from sterile source] in a syringe and use for studies on that day.

IV. Worksheets

Following are worksheets for the respiration protocol.

PGA Respiratory Protocol

Date: _____

Coat Color: color1

ID Tag#: _____

Strain: strain1

Gender: M or F

Event	Date	Check \checkmark	Comments:
Weighing			
Surgery			
Box (20 min)			
Box (10/10 min) with 650ml of N ₂			
Box (10/10 min) with 650ml of CO ₂			
Hypoxia Study			
Hypercapnia Study			

ADDITIONAL COMMENTS _____

Catheter Implant Surgery

Date: _____ Coat Color: color1

ID Tag#: _____ Strain: strain1

Anesthetist: _____ Gender: M or F

Surgeon: _____ Body Weight: _____ grams

Anesthesia: I.M. Injection Rt. Leg = 7:2:1 ratio, KETAMINE (100mg/ml Vial) & XYLAZINE (20mg/ml Vial)
& ACEPROMAZINE (10mg/ml) = 0.07cc/100gm bwt

Dose 1: _____ cc @ Time _____

Dose 2: Time _____ Dose 3: Time _____

Start time: _____ Finish time: _____ Conscious: _____

SURGERY COMMENTS _____

Penicillin (300,000units per mL) = 0.1cc/100gm I.M. = Amount _____ cc (given pre-surgical)

Buprenex (0.3mg in 1mL) = 0.03cc/100gm S.Q. = Amount _____ cc @ Time _____

Post Surgical Assessment

ID #: _____

Date: _____

ANIMAL HEALTH ASSESSMENT

<i>Event</i>	<i>(+) OR (-)</i>	<i>Comments</i>
Drinking / Eating	/	
Grooming		
Eyes / Nose	/	
Locomotion / Feet	/	

ADDITIONAL COMMENTS _____

Hypoxia Study

Date: _____

Coat Color: color1

ID Tag#: _____

Strain: strain1

Box Number: _____

Scientist: _____

File Name: _____

Rectal Temp #1 & #2: _____ / _____

GOOD / POOR / 0

MAP

2 / 1 / 0

BLOOD DRAWS

ADDITIONAL COMMENTS _____

Hypercapnia Study

Date: _____ Coat Color: color1
ID Tag#: _____ Strain: strain1
Box Number: _____ Scientist: _____
File Name: _____ Rectal Temp #1 & #2: _____ / _____
GOOD / POOR / 0 MAP

ADDITIONAL COMMENTS _____

Post Experimental Status

Sacrificed: _____ Tissues Taken: ear
ADDITIONAL COMMENTS _____

V. Order information

A. Syringes for introducing gas into chamber:

SGE Inc.

2007 Kraemer Lane

Austin, TX 78745-4095

512-837-7190

Nan Hallmark

PNS009092 – 5L, Jumbo syringe (Minimum Order 2)

PNS009920 - 1L

B. Blood Gas instrument:

Bayer CorpDiagnostics Division

115 Norwood Park

Norwood, MA 02062

Bill Gazda/Tom Rogers

1-800-255-3232

116769 – Gas Calibration W/Reg

119281 – Blood Gas Analyzer

473385000 – 7.3 solution blood gas analyzer

473386000 – 6.8 solution blood gas analyzer

473387000 – Wash blood gas analyzer

570096000 – Cal G/L Reagent blood gas analyzer

473120000 – Slope blood gas analyzer

473389000 – Cleaning 1 & 2 blood gas analyzer

111399000 – Bar code reader blood gas analyzer

478533000 – PH fill solution blood gas analyzer

478822000 – Reference fill solution blood gas analyzer

Fisher Scientific

Midwest Distribution Center

4500 Turnberry Drive

Hanover Park, IL 60103

1-800-766-7000

072728 – Blood Gas Controls (30 Pkg)

VWR Scientific Inc.

PO Box 66929

Chicago, IL 60666

1-800-932-5000

BD329-651 – (Pkg) U-100 ML Syringe with 25 5/8” needle (100 per Pkg)

C. Plethysmograph chambers were designed and built by the PhysGen Research Services Component [see Figures 2A and 2B]. Additional information is available upon request.

Respiration Protocol
PhysGen

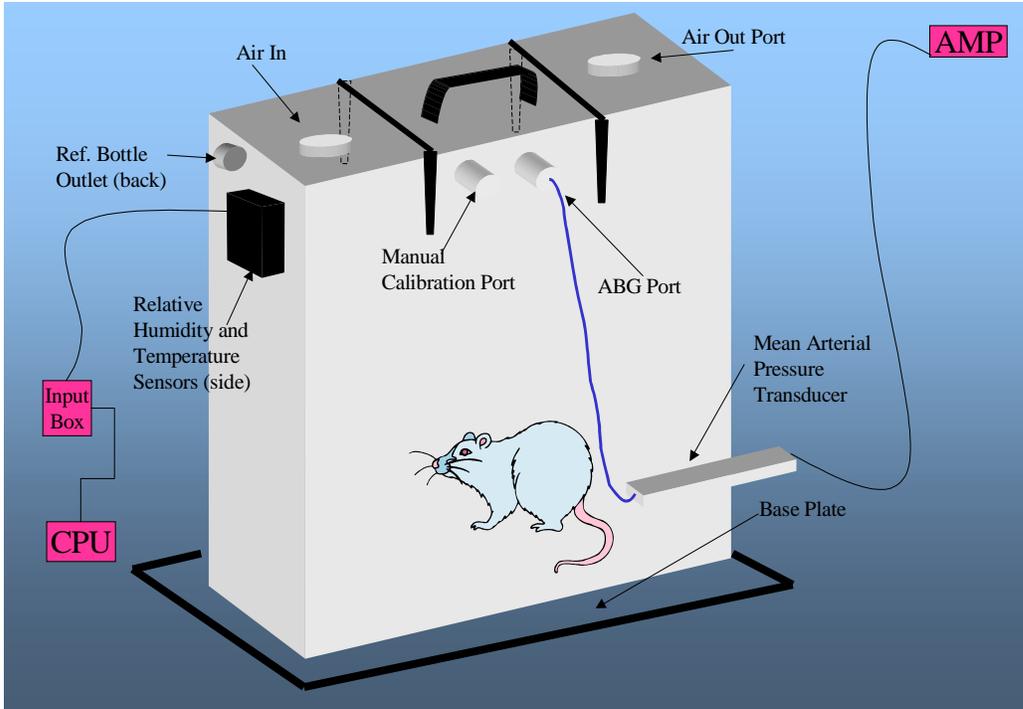


Figure 2A. Plethysmograph design showing the front of the chamber

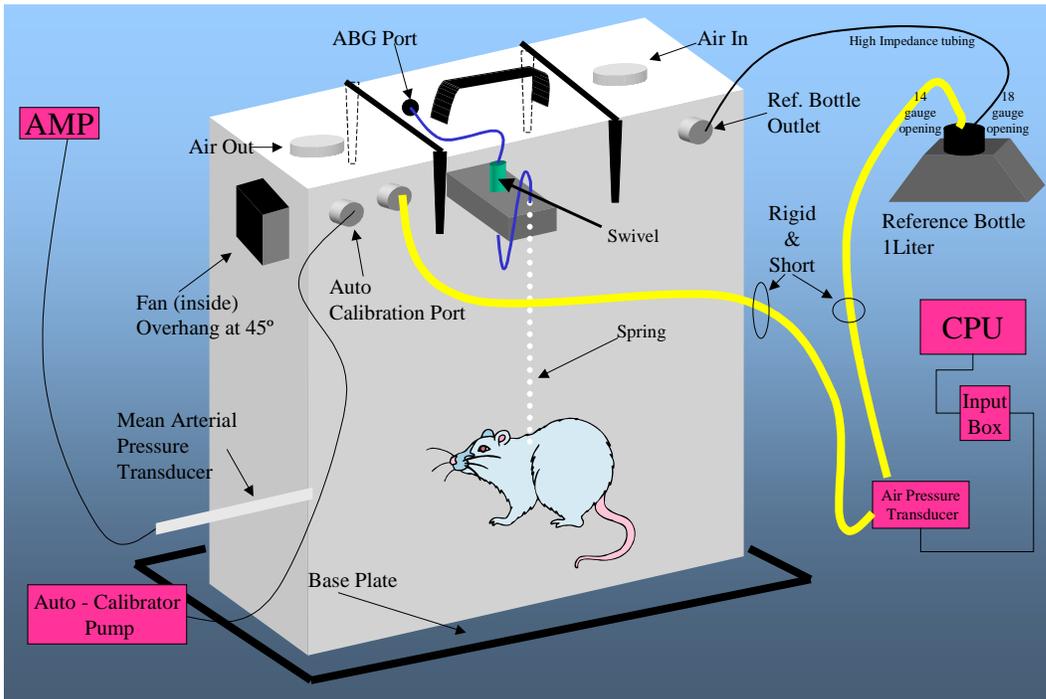


Figure 2B. Plethysmograph design showing the back of the chamber.