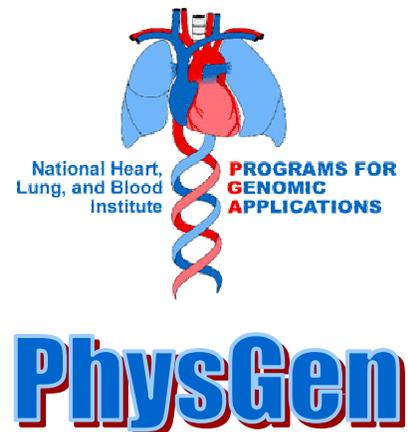


Cardiac Phenotypes

High-throughput characterization of the resistance in response to and cardiac function during myocardial ischemia in normoxic and chronically hypoxic rats

**William Hutchins and Jessica Laessig with
Mary Pat Kunert, RN, Ph.D. and John Baker, Ph.D.
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I. Experimental setup for isolated heart studies (instrumentation and calibration procedures)

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

- 6 Langendorff isolated heart perfusion set-ups with reservoirs and circulators
- Oxygen tanks
- Dissection station with fiber-optic light, microscope [Leica GZ6], surgical instruments and plexiglass dissection tray
- analytical balance for weighing heart
- Leica GZ6 microscope for assessment of infarct size



Figure 1A: View of 6 Langendorff perfusion set-ups used for high-throughput studies.

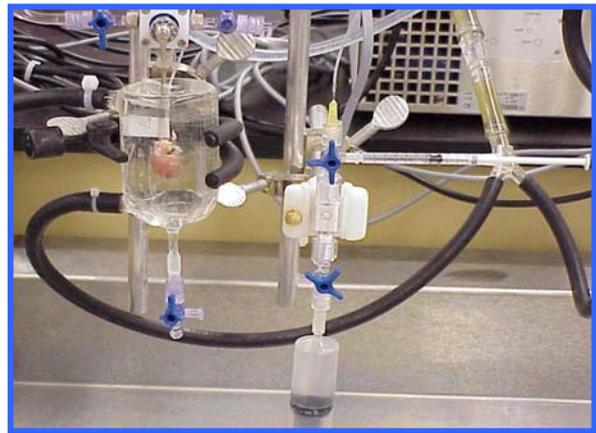


Figure 1B: Heart mounted in chamber at base of perfusion apparatus.

The isolated rat heart preparation is a retrograde perfusion method via the aorta as described by Langendorff. The apparatus used consists of a Langendorff thermostatted glass reservoir (950 cm x 24.5 cm with sintered filter disc) and a condensing tube. The perfusion fluid [bubbled with 95% O₂ and 5% CO₂ to maintain pH 7.0-7.4] flows through the large filtered reservoir and then through the thermostatted extension condenser unit to a stainless steel cannula to the aorta. The heart is contained in a water-jacketed chamber [figure 1B], which is designed to collect coronary effluent after it leaves the heart. The heart and perfusion fluids are enclosed in the glass reservoirs that maintain the myocardial temperature of 37°C using a temperature controlled circulator.

II. Experimental protocol for isolated heart studies

A. Preparation of equipment and instrumentation for beginning of experimental protocol.

1. Turn on computer, circulating heater pump [should be 37° C], and open both tanks to check gas level in the tank.

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2. Check the tubing and glassware for leaks and ensure that there are no air pockets throughout the perfusion system.
3. Turn the gas system on to the perfusion setup and begin delivery of the 95% O₂, 5% CO₂ mixture to the perfusion reservoirs and fill the system with 5 liters of Krebs Henseleit bicarbonate buffer via a constant head device placed on top of the perfusion setup to allow for flow of perfusate. The constant head device consists of a 2-Liter Boston or other suitable bottle, and a two-holed rubber stopper with metal tubing. The stopper should be placed tightly into the opening of the bottle, with the metal tubes offset about an inch apart in height. This will allow perfusate flow without the risk of overflowing the Langendorff glass reservoir. The fluid is poured through a 50 ml dispersion tube to permit constant flow into the system. Once the system is filled, a 30 ml syringe is used to evacuate any air bubbles by reverse flushing with the buffer from the 3-way stopcock located above the aortic cannula [see figure 2].
4. Calibration of pressure transducers and initiation of computer data acquisition system: Once the perfusion system has been cleared of all bubbles and trapped air, calibration can begin.
 - Turn on the WinDaq program and input all the correct settings for sample rate, compression, scroll/oscilloscope mode, etc.
 - Remove injecting syringe making sure the balloon stopcock is shut off and use a squeeze bottle to bring the meniscus to the edge of the stopcock. At this point, the transducer is sensing the lowest pressure (atmosphere) and the “low calibration” point is taken.
 - Attach the Veri-Cal (Pressure Transducer Tester, Utah Medical Products, Inc.) to pressurize the transducer to the highest pressure [~150 mmHg]
 - Release pressure from transducer and allow baseline pressure to return to atmosphere and then remove transducer tester.
 - To set the zero point for data collection, mount the intraventricular balloon at the approximate level of the heart to be tested and open the stopcock to the transducer and the balloon.
 - Completely deflate balloon and fill with approximately 0.05 to 0.1 ml of saline or just until slight finger depression of the balloon gives a pressure recording.
 - Once the above is accomplished, simply reset the low calibration setting to zero and you are ready for data collection.

B. Surgical removal of heart and preparation for mounting heart on perfusion apparatus.

1. Each rat delivered to the Cardiac Phenotyping station has had the transponder read at the time the rat is brought to the lab to verify the i.d. of the rat and its' corresponding group assignment and conditioning protocol [hypoxia conditioning in the hypoxic chamber or maintained in the holding room under normoxic conditions for 2 weeks prior to study]. The identification nomenclature has been described in an earlier section.
2. Data sheets for each animal are used which record the information from the animal i.d., date of study, verification of gender, body weight, and conditioning

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group [see Cardiac Worksheet, section IV]. Labels are made for each rat using the described nomenclature that will be placed on the perfusion bath, the samples collected for lactate dehydrogenase determination, and the tube for fixation of the heart for infarct size determination.

3. Rat is weighed and given an intraperitoneal injection of 0.4 ml of sodium heparin [400 IU/kg]. Following a 10-15 minute period to allow the anticoagulation effect to take place, sodium pentobarbital [50 mg/kg] is given i.p. to produce a deep anesthesia.
4. The anesthetized rat is positioned on the dissection board and secured using rubber bands that extend the arms and legs. The chest is opened with an incision along the left and right lateral aspect of the ribcage from the midline at the floating rib to the brachial plexus.
5. The diaphragm is cut and the anterior rib cage retracted cephalad to expose the heart and mediastinum. The pericardium is incised and the aorta located. The aorta, vena cava, and pulmonary vessels are cut.
6. The excised heart is transferred to a pre-weighed and tared weigh boat containing ice-cold (4°C) perfusion solution. The wet weight of the heart is then recorded.
7. After the heart is weighed, it is quickly transferred in the ice-cold perfusate from the dissection station to the Langendorff station.
8. The tap supplying the perfusion fluid is opened to allow the warm, oxygenated perfusate to flush through the aortic cannula. The tap is then partially closed to permit a slow drip from the end of the cannula.
9. Moving quickly, retrieve the heart from the cold perfusate using two iris forceps clamping one forcep to the wall of the aorta 2 mm distal from the severed end of the vessel and position the heart at the dripping cannula. Using the second forceps, clamp the aorta such that the aorta is slipped onto the stainless steel cannula.
10. Secure the aorta initially using a bulldog clamp. Then secure permanently using 4-0 silk suture. The pulmonary artery must then be incised to ensure adequate drainage while maintaining a perfusion pressure of 80 mmHg.
11. Once the heart is secured, the tap supplying the perfusion fluid to the heart is opened fully. **The transfer time following the removal of the heart to mounting and perfusion should be less than one minute to avoid inadvertent preconditioning of the heart.**
12. Begin recording data using Windaq acquisition with appropriate event markers.

C. ***Placement of the intraventricular balloon***

1. The installation of the intraventricular balloon is critical to this preparation since it permits the monitoring of the heart pressure throughout the experimental protocol.
2. As soon as the heart has been successfully perfused, a latex balloon filled with boiled and degassed saline is inserted into the left ventricle via the mitral valve through an incision in the left atrium. This balloon has been previously prepared for insertion as described in the legend of figure 2. The balloon position is adjusted until the peak of the intraventricular pressure wave is ≥ 100 mmHg.

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The balloon is then securely taped to the aortic cannula using surgical tape preventing herniation through the mitral valve.

3. Attach the balloon to the pressure transducer via a fluid-filled catheter. End diastolic pressure is set to 5 mmHg using a 1 cc syringe attached to the transducer system and kept constant throughout the experiment. The developed pressure (systolic minus diastolic pressure) is measured during steady-state levels of function in the protocol.

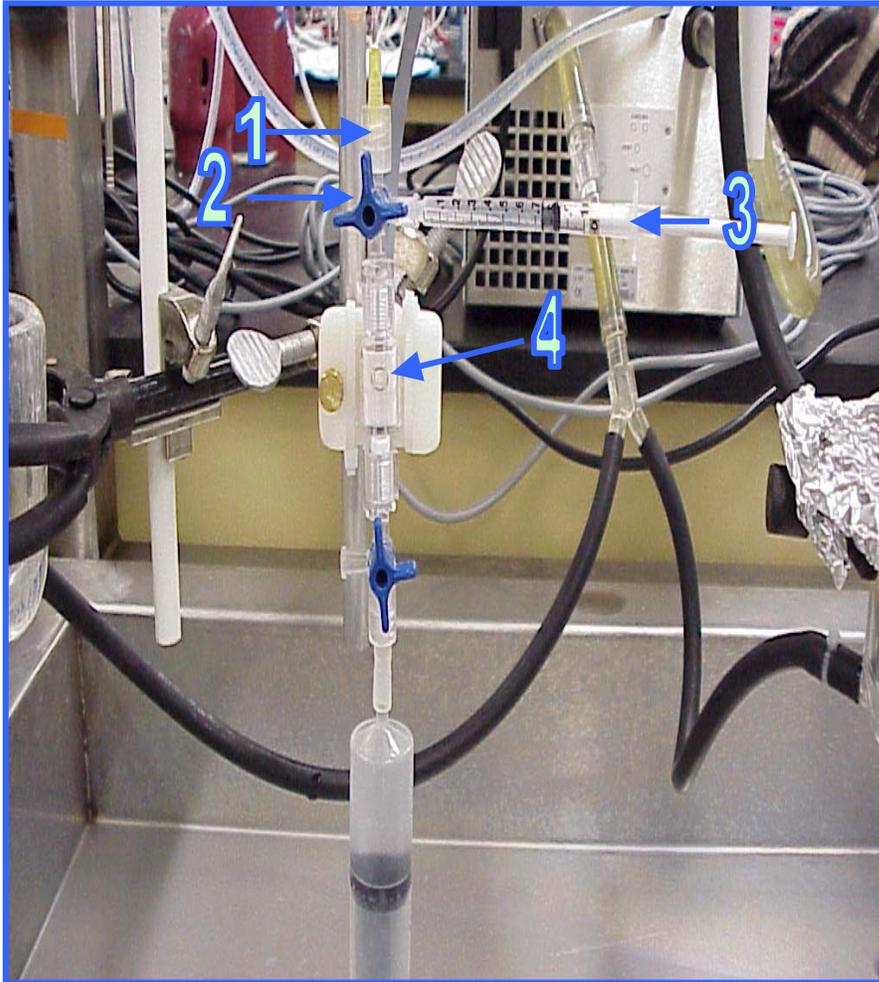


Figure 2: Close up photo of setup for preparation of balloon. Stub adaptor [1]; 3-way stop cock [2]; 1 ml syringe [3]; transducer [4];

- Attach stub adaptor to one of the openings of the stopcock and thread the catheter tubing onto the stub adaptor needle.
- Attach the 1 ml syringe to a second port of the stopcock.
- Submerge in saline and draw the liquid into the apparatus using the syringe making sure no air is present anywhere in the system.
- Submerge one of the Biomedix balloons in saline and work the air out of it and then insert the catheter tubing into the

balloon and secure the position with two 4-0 silk ties.

- The catheter should be inserted $\frac{3}{4}$ of the way into the balloon.
- The third port of the stopcock is then attached to the transducer.

D. Experimental protocol:

1. Equilibration period: Perfuse the heart for 30 minutes in the Langendorff mode. Measure the coronary flow by collecting the effluent that spills over and drips

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from the heart chamber into a graduated cylinder **for 1-minute every 5 minutes**. This is done to assess the hemodynamic stability of the preparation. Once steady state for left ventricular pressure (mmHg) has been reached, the pre-ischemic phenotypes can be recorded.

2. Measurement of pre-ischemic enzyme leakage and coronary flow rate. At the beginning of the last 6-minute period of coronary perfusion begin a five minute collection of coronary effluent in a 250 ml flask. Record the volume collected [in liters] and mix the contents of the cylinder by gentle inversion. Draw up 990 μ l of the effluent and mix with 10 μ l of 1% BSA solution [to stabilize the enzyme activity during freezing]. Samples should be vortexed and frozen at -80°C .

Calculation of pre-ischemic coronary flow rate:

[Volume coronary effluent collected in 5 minutes (ml)]/wet wgt. heart (g)

Pre-ischemic enzyme leakage:

[Enzyme activity (IU/L)* volume coronary effluent collected in 5 minutes (L)]/wet weight of the heart (g)

3. **Creation of global ischemia:** Turn the stopcock tap supplying the perfusate to the heart 180 degrees in either direction to stop flow to the myocardium to produce global no-flow ischemia. Maintain the heart at the required 37°C throughout the 25-minute period of global ischemia.
4. **Measurement of ischemic phenotypes:** WinDaq will be used to measure time to onset of contracture, peak contracture pressure and time to peak contracture pressure during the 25-minute period of global ischemia. Contracture development during ischemia is defined as an increase in intracavity pressure of 4 mmHg above end-diastolic values.
5. **Reperfusion of the heart:** During the 24th minute of ischemia, drain the heart chamber by turning the stopcock open to the interior of the heart chamber. This removes any enzymes that might have accumulated after leaking out of the heart into the perfusate during ischemia. Reperfuse the heart after the 25- minute period of global ischemia by returning the valve supplying the perfusate to its original position. Collect the entire coronary reperfusate during the first 90 minutes of reperfusion, excluding the reperfusate remaining in the heart chamber. This will be done with a 1000ml beaker with ice surrounding it.
6. **Measurement of post-ischemic phenotypes:** Record the volume of the entire coronary effluent collected during the first 90 minutes of reperfusion as noted above. Mix the collected effluent by gentle inversion and pipette 990 μ l into the prepared tube containing 10 μ l of 1% BSA. Vortex the tubes and place in the -80°C freezer for later determination of lactate dehydrogenase activity

Calculation of post-ischemic coronary flow rate:

[Volume of coronary effluent collected in 90 minutes (ml)]/wet wgt. heart (g)

Post-ischemic enzyme leakage:

[Enzyme activity (IU/L)* volume coronary effluent collected in the 90-95th minute of reperfusion(L)]/wet weight of the heart (g)

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7. **Infarct size:** Continue to reperfuse the heart for an additional 1 hour and 30 minutes.
 - The heart is then stained intravascularly by perfusing for 10 minutes with 1% 2,2,5 triphenyl tetrazolium chloride solution [Sigma Chemical Co.; catalog #T-8877] in phosphate buffer, pH 7.4, heated to 37°C.
 - The hearts are then removed from the perfusion apparatus after being stained and sliced across the long axis of the left ventricle from apex to base in 2 mm thick transverse sections using the rat heart matrixer.
 - Infarct areas will be enhanced by storage in a 10% buffered formalin solution for 24 hours before final measurement.
 - The areas of unstained viable and red stained necrotic tissue are separated using a surgical blade. The volume of red stained, infarcted tissue is then weighed in a pre-tared buffer filled weigh boat. Infarct volume is expressed as a percentage of the total left ventricular volume infarcted for each heart.
8. **Calculation of derived variables:** Several of the phenotypes obtained are derived by the following calculations using measurements described above.
 - Post-ischemic recovery of heart rate = post-ischemic value/pre-ischemic value x 100%
 - Post-ischemic recovery of coronary flow rate = post-ischemic value/pre-ischemic value x 100%
 - Post-ischemic recovery of systolic pressure = post-ischemic value/pre-ischemic value x 100%
 - Post-ischemic recovery of developed pressure = post-ischemic value/pre-ischemic value x 100%
9. **Clean-up of perfusion apparatus:** Drain the perfusate from the perfusion apparatus and flush the rig with boiling water heated in a 5 L Erlenmeyer flask. This is the most hazardous step in the protocol. Be sure that you have protective eye shields and insulated gloves.

Timeline:

Genomics Component
Animal Production

6 week old rat

NORMOXIC CONDITIONING
Conditioning Prot

HYPOXIC CONDITIONING

Anesthesia and Heart Excision	30 min perfusion	25 min Global Ischemia	40 min Reperfusion	2 hours, 20 min Reperfusion
	Pre-Ischemic		Post-Ischemic	

- Heart is placed in ice-cold
- Krebs-Henseleit bicarbonate
- buffer
- Heart is weighed in grams (phenotype)

PRE ISCHEMIC Phenotypes measured:

- heart rate
- coronary flow rate
- left ventricular diastolic pressure
- left ventricular systolic pressure
- left ventricular developed pressure
- enzyme leakage (lactate dehydrogenase)

POST ISCHEMIC Phenotypes measured:

- heart rate
- coronary flow rate
- left ventricular diastolic pressure
- left ventricular systolic pressure
- left ventricular developed pressure
- enzyme leakage (lactate dehydrogenase)

ISCHEMIC Phenotypes measured:

- time to onset of contracture
- peak contracture
- time to peak contracture

Infarct size determined in fixed tissue (phenotype).

III. Solutions

A. Bicarbonate Buffer Preparation

1. Weigh out the following chemicals into a 5L volumetric flask.

NaCl	34.63g
NaHCO ₃	10.50g
KCl	1.49g
MgSO ₄ •7H ₂ O	1.47g
KH ₂ PO ₄	0.80g
Glucose	9.91g

2. Add deionized water until flask is two thirds full. Swirl flask to dissolve the contents.
3. Gently gas with 95% O₂, 5% CO₂ for 20 minutes. Ensure no lumps remain in the flask.
4. Weigh out 1.32 g CaCl₂•2H₂O and add to the flask.
5. Fill the flask to the 5L mark with deionized water.
6. Place cap on and mix thoroughly by inversion.
7. Filter solution through a fresh 5.0 μm filter.
8. Prepare fresh solutions daily.

B. Preparation of 1% Triphenyl Tetrazolium Chloride Stain

1. Weigh out 0.25g of 2,3,5-Triphenyl Tetrazolium Chloride
2. Add to 360 ml of deionized water
3. Add 40 ml of K₂HPO₄
4. Refrigerate until use

C. 0.1M Potassium Phosphate Buffer

1. Weigh out 13.6g KH₂PO₄ and dissolve into 1L-deionized water
2. Using stir plate, adjust pH to 7.4 by dissolving potassium hydroxide pellets
3. Store in refrigerator with an expiration of 1 month from date and time of preparation

IV. Diet

Animals are fed Teklad low salt (0.4% NaCl) chow, order # 3075S, from weaning through the end of the studies. Please see <http://www.teklad.com/index.htm> for more information.

IV. Worksheet

**Cardiac Protocol
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Isolated Heart Worksheet	Cardiac Protocol		
Date	Channel #1	Channel #2	Channel #3
Rat ID (SexID#Strain Ex:M005SS)			
Body Weight (g)			
Heart Wet Weight (g)			
Amount of Nembutal (ml) [50mg/ml]			
Hypoxic or Normoxic			
Preischemic Phenotypes			
Steady state adjustment for EDPpressure: (5 +/- 1 mmHg)			
Coronary effluent rate at 10 minutes (ml)			
Coronary effluent rate at 15 minutes (ml)			
Coronary effluent rate at 20 minutes (ml)			
Coronary effluent volume at 24-29 minutes (ml)			
Preischemic LDH mean			
Preischemic Coronary Flow Rate (ml/min/g)			
Postischemic Phenotypes			
Coronary Effluent volume at 0-90 min (ml)			
Coronary Effluent volume at 40-45 mins (ml)			
Postischemic LDH mean			
Postischemic Coronary Flow Rate (ml/min/g)			
Infarct Size			
Mass of Stained Heart (g)			
Mass of Left And Right Ventricle (g)			
Mass of Left Ventricle (g)			
Contracture Phenotypes			
Time to Contracture (sec)			
Peak Contracture Pressure (mmHg)			
Time to Peak Contracture (sec)			
Ave LV Pressures and Heart Rates			
Pre-ischemic LV SysPressure (mmHg)			
Pre-ischemic LV DiaPressure (mmHg)			
Pre-ischemic LV DevPressure (mmHg)			
Pre-ischemic LV Heart Rate (BPM)			
Post-ischemic LV SysPressure (mmHg)			
Post-ischemic LV DiaPressure (mmHg)			
Post-ischemic LV DevPressure (mmHg)			

V. Order Information

A. Intraventricular balloons
BioMedix
2 West Avenue
Pinner
United Kingdom, HA5 5BY
Keith Rookledge
Tel: 011 44 20 8866 7017
Email: 100407,1233@compuserve.com
IVB 57 – Intraventricular balloon

B. Biomedix perfusion apparatus
BioMedix
2 West Avenue
Pinner
United Kingdom, HA5 5BY
Keith Rookledge
Tel: 011 44 20 8866 7017
Email: 100407,1233@compuserve.com

Langendorf Apparratus for Rat Perfusion Parts List

- B 1003 – Gassing stick
- B 1004 – Reservoir with automatic air flow
- B 1005 – Extension unit
- B 1006 – Bubble trap
- B 1008 – Heart Chamber
- B 1009 – Gas Manifold with six outlets
- B 1010 – Gas Humidifier
- B 1100 – Aortic Cannulae with four side arms
- B 1110 – Set of Bungs with cannulae for bubble trap
- B 1111 – Reservoir Clamps
- B 1112 – Extension Unit Clamps
- B 1112 – Bubble trap clamps
- B 1111 – Heart chamber Clamps
- B 1113 – Cross Connectors
- B 1114 – Connecting Tubing, 30m
- B 1115 - 2.0 mm silicone, 20m
- B 1116 – 4.5 mm tubing, 10m
- B 1117 – Pack connectors

VI. Data Acquisition Instructions

See attached Appendix