

# PagLab Protocols

Laboratory of Dr. Pagliassotti  
Department of Food Science and Human Nutrition  
Colorado State University  
[www.paglab.com](http://www.paglab.com)

## Liver perfusion and isolation of hepatocytes

Solutions needed (enough for 2 rats)

### Perfusion Buffer

100 ml 10X Ringers  
2.092g NaHCO<sub>3</sub>  
1.998g Glucose  
0.55g Sodium Pyruvate

Pull 300 ml into round bottom flask with neck and 2 side arms; place in water bath at 37°C, gas with 95%O<sub>2</sub>/5%CO<sub>2</sub> for 15 min; pH to 7.4 using 1M HCL; replace into water bath and continue gassing.

### Wash Buffer with 1% gelatin

100 ml 10X Ringers  
2.092g NaHCO<sub>3</sub>  
1.998g Glucose  
0.55g Sodium Pyruvate  
0.366g CaCl<sub>2</sub>/2H<sub>2</sub>O for fed rats or 0.450g for fasted rats  
10g Gelatin (Fisher 0143-17-9)

\*\* Add gelatin while on stirring. Place in warm water bath (43°C) to melt gelatin and pH to 7.4 with 1M HCL. Leave at room temperature.

### Incubation medium with 1% gelatin

25 ml 10X Ringers  
0.0705g CaCl<sub>2</sub>/2H<sub>2</sub>O  
0.523g NaHCO<sub>3</sub>  
2.5g Gelatin (make as described above but place in water bath at 37C following adjustment of pH)  
take up to 250 ml with ddH<sub>2</sub>O

### **Procedure.**

1. Recirculate perfusion buffer making sure that perfusate temperature is 37°C when it is delivered to liver (our water bath temp is set at 43°C).
2. Anesthetize rat and make vertical incision up to xyphoid. Cut at right angle from initial incision along bottom of rib cage on both sides. Do not puncture liver or diaphragm. Make small incision at joint of hindlimbs. Fold flaps of skin

- outside to expose the abdominal cavity. Move intestines to right, and expose vena cava (VC) and hepatic portal vein (PV).
3. Isolate descending VC above renal vein and place loose ligature. Do not occlude blood flow. Wrap ends of suture with clips and place to side of animal for easy access later.
  4. Isolate hepatic PV, and place two loose ligatures around vessel. Place ends of ligatures in an easily accessible place.
  5. Using forceps, grasp tissue around PV and insert Angiocath (20 gauge, 1.16 in, Becton Dickinson) past the most proximal ligature. Another person is needed to hold catheter tip in place while the needle is removed. Once needle is removed, check for backflow of blood and advance tip to the branching of the PV. Second person should then tighten the two loose sutures previously placed around PV. This must be done delicately without pulling up on sutures.
  6. Insert tubing that will deliver perfusate, tape down and immediately cut descending VC. Double tie the ligatures on the PV.
  7. Cut through diaphragm and cut along right side of sternum to top of rib cage. Make cut to the left to expose lungs, ascending VC and heart.
  8. Isolate ascending VC and place a loose ligature around it. Using forceps, grasp right atrium and cut small hole and insert a 14 gauge catheter (Beckon Dickinson, #2876) through right atrium into ascending VC. Catheter should be positioned so that tip is just above the top of the liver. Tighten ligature to secure catheter.
  9. Tighten ligature previously placed in step 3 above. At this point perfusate should be diverted to ascending VC catheter. Insert tubing into this catheter and allow perfusate to drain into a waste vessel until clear. Once clear place tip of tubing into collection vial to recirculate.
  10. After ~5-10 mins add 45mg for fed rat, 60mg for fasted rat of collagenase (type 1, ~200 U/mg, Worthington Biochemical Corp. #4196) to perfusate using a 10ml syringe with tubing attached. Start timer. \*Note: quantity of collagenase will vary depending on enzyme activity. In most cases several lots required testing prior to initiating studies.
  11. Perfusion continues until integrity of liver disrupted (liver will begin to look pale and mushy, usually 15-18 min).

### **Isolation and suspension of hepatocytes.**

Remove liver and place into weigh boat with ~60 ml of wash buffer. Remove liver capsule with forceps. Pour into 100 ml beaker and using hemostats shake liver to release hepatocytes into wash buffer.

Pour suspended cells through gauze and polypropylene funnel (1 cm diameter stem) into clean 250 ml polypropylene Erlenmeyer flask and place into 37C water bath under 95/5 (oxygen/carbon dioxide), shaking at 55-80 rpm. Leave for 10 min.

Remove cells from water bath and filter through mesh (90  $\mu$ m for fasted rat; 105  $\mu$ m for fed rat) into clean 250 ml Erlenmeyer flask.

Pour filtered hepatocytes (~ 10ml) into each of four 50 ml tubes and bring each tube up to 45 ml with wash buffer.

Centrifuge for 2 min at room temp at 450-500 rpm (2min includes getting up to speed and back to 0).

Decant off wash buffer from each tube watching for white layer of dead cells to go into waste.

Resuspend hepatocytes with 10 ml wash buffer and gently shake to get pelleted cells into suspension. Bring all tubes up to 35 ml with wash buffer. Centrifuge as above and decant as above. Repeat this step.

After 3<sup>rd</sup> wash, resuspend first of the four tubes with 10 ml incubation buffer, shaking gently to get into suspension. Decant this tubes contents into the second tube and continue this procedure. Wash tubes with ~10 ml incubation buffer and combine into final tube.

Filter suspended cells through mesh and into a clean 250 ml Erlenmeyer flask.

Swirl cells and remove 250 µl into a pre-weighed 1.5 ml eppendorf tube. Do this in duplicate. Place the flask into a 37C water bath and gas. Start timer and allow cells to recover for 25 mins. Following this time period cells are ready to study.

Spin eppendorf tubes at 9000 rpm for 1min. Get rid of fluid and spin again. Remove any remaining fluid with a small syringe. Be sure sides of tube are dry. Weigh tubes.

#### **Calculation of cell concentration and yield:**

Weight of 250 µl of cells x 4 = grams of cells/ml (Density 1)

We typically perform studies with 30 mg of cells/ml incubation buffer. So you need to calculate how much incubation to add to achieve this concentration.

Remove cells from gas, filter through mesh into 100 ml graduated cylinder. Record volume (volume 1). Calculate volume of incubation buffer to achieve 30 mg/ml:

Volume 1 \* density 1 = unknown volume \* 0.030 g/ml

Pour diluted cells into clean 250 ml flask, swirl and remove 250 µl of cells into empty 1.5 ml eppendorf tube. Place rest of cells back in water bath under gas. Add 250 µl of trypan blue to 250 µl of cells and mix by flicking tube.

Remove ~10 µl onto hepatometer under cover slip and place under microscope to determine viability. Viability is determined by % of cells that exclude the dye. We usually determine this in a sample of 200 or more cells. We require >90% exclusion.

Attachment Period (4h): RPMI, 11 mM glucose, 10<sup>-7</sup> M Insulin, 10<sup>-7</sup> M Dex. If we are not doing transfections, we coat plates with matrigel.

Overnight incubation: RPMI, 8 mM glucose, 10<sup>-8</sup> M insulin, 10<sup>-7</sup> M Dex.