Hepatocyte Isolation System

Most traditional methods published for isolating hepatocytes use crude and partially purified enzyme preparations including various types of collagenase and other proteases. More recently the use of better characterized preparations of collagenase such as Types 1 and 4 (CLS-1, 4) have provided better results. All crude collagenase preparations can contain lot-variable contaminating proteases, esterases and other enzymes requiring researchers to pre-screen several lots of enzymes and/or continually modify isolation parameters and protocols.

The Biopurechem's Hepatocyte Isolation System has been developed to provide researchers with a reliable, convenient, and consistent hepatocyte cell isolation system. By using the pre-optimized combination of enzymes contained in this kit, it is possible to minimize the lot-to-lot variation and improve the quality of the isolated hepatocytes. In addition, Biopurechem's use-tests each lot by isolating hepatocytes from adult rat to assure performance, reliability, and consistent yield of viable cells.

The method is based on that described by Berry, M.N., modified by Seglen, P.O. (Methods in Cell Biology, vol XIII, David M. Prescott ed., Academic Press, 1976; Chapter 4, "Preparation of Isolated Rat Liver Cells", pp 29-83), and further optimized in conjunction with several researchers.

Stability/Storage: The reagents are stable at ambient temperatures for the periods of time expected in normal shipping procedures, but the package should be refrigerated upon arrival. Contents may be stored at 2-8°C for 4-6 months before use. Store at 2-8°C.

Package Contents The package contains sufficient materials for five separate adult rat liver perfusions. For larger or smaller tissue applications, prepare proportionate volumes of reagents at each step and combine them in the same ratio as described in the protocol.

Vial #1: 10X CMF-HBSS Concentrate, 1 bottle, 500ml Sterile calcium- and magnesium-free Hank's Balanced Salt Solution (CMF-HBSS). The solution is used for washing and perfusing the liver prior to the addition of the dissociating enzyme solution.

Vial #2: Collagenase-Elastase Enzyme Vial, 5 Vials Biopurechem's collagenase (Code: CLS-1) and elastase (Code: ESL), filtered through 0.22µm pore size membrane, and lyophilized. Before use, reconstitute with the L-15/MOPS solution and swirl gently to dissolve contents as directed in the following procedure. Store unreconstituted vials at 2–8°C.

Vial #3: 1,000 Units DNase I each, 5 Vials Biopurechem's DNase I (Code: D), filtered through 0.22µm pore size membrane, and lyophilized. Before use, reconstitute with L-15/MOPS solution and swirl gently to dissolve contents as directed in the following procedure. Store unreconstituted vials at 2–8°C.

Vial #4: 0.15M MOPS, pH 7.5, 1 bottle, 75ml 0.15M MOPS, pH 7.5 buffer concentrate, used to buffer the reconstituted Leibovitz L-15 media.

Vial #5: 7.5% Sodium Bicarbonate ($NaHCO_3$), 1 bottle, 100ml 7.5% Sodium bicarbonate concentrate, used to buffer the diluted CMF-HBSS.

Pouch, containing Leibovitz L-15 Media Powder, 1 x 1L Reconstitute entire contents of pouch by cutting open top of envelope and pouring contents into beaker containing approximately 800ml of cell culture grade water. Rinse pouch 2 - 3 times with an additional 100ml water. Bring total volume to 1000ml and filter through a 0.22-micron pore size membrane.

Required for Perfusion Isolation but not Included:

- Equipment and tools for animal anesthesia and surgery

- A perfusion apparatus with a bubble trap suitable for liver perfusion at 10-30ml/min, 37°C. The tubing to be inserted into the portal vein is thin-walled with an inner diameter of 0.35-0.45mm

Note: Measure the dead volume of the perfusion circuit

- A low-speed centrifuge suitable for sedimentation of hepatocytes

- Labware for cell sedimentation, and culture or incubation including sterile 150 X 25mm culture plates

- A means to count or estimate the yield of cells

- A means to sterile-filter solutions, if desired

- Cell culture media and supplies, if needed

- Sterile cell culture grade water

- Concentrated antibiotics: penicillin, streptomycin, Fungazone, etc. for culture, if needed.

- Surgical thread, silk, size 000

- Heparin (optional)

For Cell Quantitation and Viability Assessment:

- Improved Neubauer hemocytometer

- Counter

- Pasteur pipet or micropipettor

- Microscope (10X), preferably inverted phase-contrast

- Standard 10ml serological pipets

Note: The following procedure presumes previous experience in liver digestion and cell isolation. For those not experienced, refer to the publication by Seglen referenced above, or to Alpini et al. entitled "Recent Advances in the Isolation of Liver Cells" published in Hepatology (1994) 20:494-514. Perfusion of the liver while still in the peritoneal cavity is described in "Isolated Hepatocytes Preparation, Properties and Application", by Berry, M.N., Edwards, A.M. and Barritt, GJ; RH Burdon and PH Van Knippenberg, eds., Elsevier, Amsterdam, New York, Oxford, Chapter 2, (1991).

I. Preliminary Steps for Digestion of 1 Liver

The volumes specified in the following protocol are suitable for perfusion volumes of approximately 80-100ml. Proportional adjustments may be necessary for different perfusion systems.

Note: Sterile techniques, glassware and plasticware should be used. The use of a sterile hood is also recommended to avoid culture contamination.

Prepare:

Vial #1, 10X CMF-HBSS: Dilute 100ml of the 10X CMF-HBSS with 850ml of sterile water and add 4.7ml of 7.5% Sodium Bicarbonate (Vial #5, NaHCO3) in a sterile 1L bottle. Adjust pH if necessary to 7.4. Bring (QS) to a total volume of 1L with sterile water. If sterile water is not available, mix ingredients and sterile (0.22u) filter. Makes a total of 5L.

• Leibovitz L-15 Media, 1 x 1L: Reconstitute entire contents of pouch by cutting open top of envelope and pouring contents into beaker containing 800ml of cell culture grade water. Rinse pouch 2 - 3 times with an additional 100ml water. Bring total volume to 1000ml and filter through a 0.22-micron pore size membrane.

• Enzyme Buffer Solution: Combine 13.3ml of MOPS concentrate with 10ml sterile water and 76.7ml of L-15 in a sterile 100ml bottle. Transfer sufficient L-15/MOPS into one each of Vial #2 and into one Vial #3 to dissolve the contents, mix gently to completely dissolve and transfer the enzymes back to the 100ml bottle. The collagenase, elastase and DNase concentrations will be approximately 225U/ml, 0.3U/ml and 10U/ml, respectively.

• Flush the sterile perfusion apparatus with CMF-HBSS, eliminating all air from the system except that in a bubble trap.

• Place the 150 x 25mm or equivalent Petri dish close to the perfusion apparatus to receive the perfused liver.

II. Perfusion and Digestion of Adult Rat Liver

The following steps should be performed in a laminar flow hood or safety cabinet. In particular, the digested liver should be processed under sterile conditions unless acute incubations will terminate the procedures.

1. Pretreatment of the rat with heparin is helpful. Inject i.p. about 20 minutes before perfusion, or into a vein (Seglen suggests the iliolumbar vein) after opening the abdomen. Use from 100-200U/100g body weight.

2. Anesthetize a rat, 200-400g weight, and position it for dissection. Install sufficient padding under the rat to hold the blood and initial perfusate. Place the rat on its back, tape down the legs, sterilize the abdomen with an iodine solution or 70% ethanol, and open the abdomen to

expose the liver. Move the intestines to the left side of the abdomen (to the right as you look down with the rat's head away from you) exposing the hepatic portal vein.

3. Using a pair of fine, curved forceps, place a segment of 000 surgical thread underneath and around the portal vein just above (toward the head) the intersection of the portal vein and the final mesenteric vein close to the liver. Tie a loose half-square or equivalent knot around the vein. Locate the vena cava so it can be opened for drainage just before the portal vein (vena porta) is cannulated.

4. Turn on the perfusion pump containing plain CMF-HBSS with a flow rate 10-15ml/min so that the tubing or cannula can be inserted into the portal vein. The bath temperature is adjusted so that the perfusate temperature is 37°C. Cut a nick in the vena cava near the right kidney to lower the blood pressure, and then with fine surgical scissors cut a nick in the portal vein (partially through) about 5mm below (towards the tail) the knotted thread. Insert the tubing into the portal vein towards the liver and only several millimeters past the loose knot. The liver should clear of blood. Tie the surgical threads tightly around the portal vein and tubing. Cut the vena cava through and increase the perfusate flow rate to 20-25ml/min. Note: Establishment of an effective perfusion that flushes the entire vasculature is essential to the success of the digestion.

5. Remove the liver from the animal with great care; do not rush. Place the liver onto a mesh stage in such a manner that it can be perfused in a recirculating fashion. The initial CMF-HBSS perfusate, however, goes to waste.

6. After 7-10 min of CMF-HBSS perfusion, switch to perfusion with the Enzyme Buffer Solution (L-15 digestion medium containing the enzymes). Start recirculation after one system-dead-volume of the remaining CMF-HBSS has gone to waste.

7. Perfuse the liver with the digestion mixture until it swells fully (but not prematurely) and the liver is fully digested, about 20-30 minutes. Note: Halt the perfusion immediately by stopping the pump and removing the liver if the portal vein breaks or if the surface of the liver shows signs of disintegration when touched with forceps or a blunt object.

8. At the end of the perfusion, stop the pump, gently place the liver in the 150ml or equivalent culture dish and remove the perfusion tube. Transfer the culture dish to a sterile hood if not already in one and add approximately 150ml of fresh CMF-HBSS to the dish.

9. In the culture dish, gently pull off the lobular capsule membranes with forceps or dog comb (recommended by Seglen) and rake out the cells. Remove the large central tree of connective and vascular tissue, and any undigested tissue or connective tissue.

10. Gently agitate the dish to disperse the cells. Place the dish at an angle by propping one side on the lid. Allow clumps or connective tissue to settle for a minute or so, then remove the dispersed cells from the top of the buffer at the deepest part of the plate, i.e., close to the lower edge, and transfer the cell suspension to 50ml sterile tubes.

11. Centrifuge for three minutes at low speed (just rapidly enough for loose cell pellets, e.g. (100 x g) at room temperature.

12. Add more CMF-HBSS to the culture dish and repeat the process to increase the yield of cells. Repeat as long as clean cells can be removed.

13. As soon as cells are sedimented, add fresh CMF-HBSS, suspend the cells by inverting the capped tubes, and re-centrifuge as above. Repeat process once more to remove traces of the digestive enzymes from the cells. Discard the supernatant(s) and transfer cells to culture medium or buffered medium in a second 100mm or 150mm culture dish. The yield of cells from a good digestion of a liver of a 300gm rat is approximately 4-5ml of packed volume after gentle sedimentation in a centrifuge.

Culture of Hepatocytes (Optional)

Although application specific, hepatocytes have been cultured in a number of media including DMEM, Leibovitz's L15, modified Chee's medium, Williams E medium, RPMI and Waymouth's MB 752/1. In general, media are supplemented with numerous factors in order to maintain a differentiated state. Among these are EGF, insulin, glucagon, dexamethasone, selenite, nicotinamide and hepatocyte growth factor (Chen et al., 1998). Specialized media for hepatocytes may be purchased commercially from other suppliers. In order to successfully plate hepatocytes, culture ware is generally coated with a matrix such as collagen, laminin, or some type of commercial matrix.

Advances in the culture of hepatocytes include the use of three-dimensional matrices (gels) of collagen or MatrigelTM. Chen et al. (1998) plated hepatocytes on MatrigelTM (TMBecton Dickinson, Bedford MA) and after several weeks removed the cells and replated them on a collagen gel. After 24hr, a second layer of collagen gel was added. Alternatively, cells may be directly plated in a collagen gel and maintained as a three-dimensional culture.

A review discusses the effects of culture variables on human hepatocytes (Le Cluyse, 2001), and is likely applicable to culture of hepatocytes from other species. The same author has reviewed optimal conditions for the culture of rat hepatocytes (Le Cluyse et al., 1996).