# Human Hepatocyte Culture

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## 1. Introduction

Primary culture of hepatocytes is an in vitro model widely used to investigate various aspects of liver physiology and pathology ***(1)***. In particular, such cultures have been extensively used for assessing the expression and function of drug-metabolizing enzymes including cytochromes P450 (CYP), drug metabolism, drug-drug interactions, and the mechanisms of cytotoxicity and genotoxicity. Most of these studies have been carried out with rodent hepatocytes. However, because of species-specificity in both the regulation and activity of drug-metabolizing enzymes, extrapolation from animals to humans is not generally possible. For this reason, several groups have developed human hepatocyte culture systems ***(2–8)***. The technique used to isolate human hepatocytes is based on the two-step collagenase perfusion first introduced by Berry and Friend ***(9)*** and modified by Seglen ***(10)***. Originally performed *in situ* for obtaining hepatocytes from the adult rat, this technique of perfusion has been adapted to the ex vivo treatment of human liver tissue. The aim of this chapter is to describe the authors' experience in the isolation of hepatocytes from human liver tissue and the preparation of short- and long-term cultures.

### *1.1. Human Liver Samples*

1. The use of human liver samples for hepatocyte preparation for scientific purposes has to be approved by National Ethics Committees or by other regulatory authorities.
2. Because of the extensive use of donor livers for transplantation, the availability of whole human liver has dramatically decreased in the last few years. Some donor livers are considered by surgeons to be unsuitable for transplantation (owing to a high level of steatosis or cholestasis for example); however, these are generally not suitable for hepatocyte isolation either (*see* **Note 1**).
3. Our main source of human liver tissue therefore consists of lobectomies or smaller fragments resected for medical purposes. In general, the pathologies requiring such resections include primary tumor, metastasis, adenoma, angioma, or hydatid cyst. This kind of sample has many advantages:
   a. as it would otherwise be discarded, the impact of ethical considerations is greatly reduced;
   b. it is generally used within 4 hour of removal (and in many occasions within 1 hour), the cold ischemia period thus being reduced with respect to whole donor livers;
   c. anatomo-pathological data are available;
   d. a sufficient amount of histologically normal tissue is generally available.

### *1.2. Requirements*

1. The quality of the perfusion is critical for hepatocyte isolation. To this end, several requirements concerning the liver sample itself must be fulfilled.

4. Minimum leakage (if any) must occur during perfusion. For this purpose, the sample must be encapsulated in Glisson's capsula on all areas except, obviously, the edge left by the surgeon. In the case of lobectomy resection, the part of the tissue encompassing the tumor(s) is dissected by the surgeon in the operation theatre and sent to anatomopathologists, while the remaining down-stream encapsulated part is used for hepatocyte preparation. (*see* **Note 2**).
5. There must be several veins apparent on the cut edge; these will be used for perfusion. In some cases, these will have been sutured by the surgeon during the operation to avoid excessive bleeding and must be reopened for perfusion (*see* **Note 3**).
6. The mass of the liver sample is another critical parameter. It should be between approx 50 and 400 g. The problem with small samples is the difficulty of finding good vascular entries to obtain satisfactory perfusion. The problem with samples that exceed 400 g is that the ratio of the mass of tissue to the mass of collagenase is too high. This results in a decrease in the yield and quality of hepatocytes. When a whole liver is used, it is better to split it into two or three smaller pieces each meeting the requirements of **items 2** and **3**. Before beginning of perfusion, fresh tissue samples are collected, stored in liquid nitrogen and used for comparisons in further studies.
7. When the tissue is collected it is placed in a sterile plastic bag, in the presence of a sufficient amount of 0.9 g/L NaCl to overlay it, and is transported on ice.

## 2. Materials

### *2.1. Buffers and Solutions*

Buffers and solutions are prepared in deionized water, sterilized by passing through 0.22-µm filters, and stored in refrigerated stoppered bottles. All products are purchased from Sigma except: Penicillin/streptomycin, fungizone and phosphate buffer saline (PBS) (Gibco InVitroGen), selenium acetate (Aldrich), glucagene (Novo Nordisk), epidermal growth factor (Peprotech), Phagosurf DD (Phagogene DEC).

1. HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) buffer: 10 m*M* HEPES, 136 m*M* NaCl, 5 m*M* KCl, 0.5% glucose, pH 7.6.
2. EGTA ([Ethylene-bis(oxyethylenenitrilo)]tetraacetic acid) solution: 0.5 m*M* EGTA in HEPES solution (see above).
3. Antibiotic solution: 10 000 U/mL penicillin, 10 mg/mL streptomycin. Add 10 mL/L to HEPES buffer and to the EGTA solution.
4. Fungizone solution: 250 µg/mL fungizone. Add 3 mL/L to HEPES buffer and EGTA solution. 70 m*M* $CaCl_2$ solution. Add to HEPES buffer for collagenase solution.
5. BSA-HEPES solution: dissolve 5 g of BSA (fraction V)/L of HEPES buffer. Supplement with antibiotics and fungizone.
6. Phagosurf DD (Phagogene DEC) 0.25 % solution in water: this product inactivates viruses including hepatitis B virus and HIV and other pathogenes, in less than 12 h.

### *2.2. Culture Media*

We use two different chemically and hormonally defined culture media for short (approximately 1 week) or long term (approximately 1 month) cultures. These media, first described by Isom and Georgoff ***(11)*** and Lanford et al. ***(12)*** for the culture of rat and monkey hepatocytes, respectively, have been adapted to human hepatocyte cultures in our laboratory ***(13, 14)***. The short-term culture medium is used for hepatocytes preparation and plating and for studies requiring only a few days (< 7), such as for example CYP induction in response to xenobiotics. Other investigations requiring longer culture time are carried out in the long-term culture medium. Note that the long-term culture medium is three times more expensive than the short-term culture medium.

*2.2.1. Short-Term Culture Medium*

1. Ham-F12 medium: Dissolve the amount of powdered medium required for 5 L in approximately 4.5 L of deionized water. Add 5.88 g $NaHCO_3$ and, after 15 min bubbling with a mixture of 95% $O_2$ and 5% $CO_2$, adjust to pH 7.4. Adjust volume to 5 L.
2. William's medium E: Dissolve the amount of powdered medium required for 5 L in approximately 4.5 L deionized water. Add 11 g $NaHCO_3$ and, after 15 min bubbling with a mixture of 95% $O_2$ and 5% $CO_2$, adjust to pH 7.4. Adjust volume to 5 L.
3. Combine Ham-F12 and William's E media and sterilize by passing through a 0.22-µm filter. The mixture is kept refrigerated in the dark in 1-L stoppered bottles. Ham-F12 and William's E media may also be purchased (but at a higher price) as liquid media.
4. The mix of additives (see *2.2.2.*) for complementing 25 L of Ham-F12/William's E medium is prepared by combining the three submixes, 1, 2, and 3, and 75 mL of sterile fungizone solution (total volume 925 mL) (*see* **Note 4**). The mix is aliquoted in 37 ml fractions and store at –80°C.
5. The final chemically and hormonally defined short-term culture medium is prepared just - before use by supplementing 1 L Ham-F12/William's E medium with 37 mL mix and 2 mL vitamin C solution.

*2.2.2. Submixes of additives for the short-term culture medium*

1. Submix 1: 8.75 g glutamine, 31.5 g glucose, $2.5 \times 10^6$ units penicillin and 2.5 g streptomycin are first dissolved in 500 mL water and the solution is sterilized through 0.22-µm filters.
2. Submix 2: 1.1 g sodium pyruvate, 1 mg dexamethasone (dissolved in 500 µL ethanol), and 1.25 g transferrin are dissolved in a final volume of 75 mL water.
3. Submix 3: 100 µL ethanolamine, 50 mg insulin (dissolved in 10 mL water containing 100 µL glacial acetic acid), 5 mg glucagon (dissolved in 10 mL water containing 100 µL of 1 *M* NaOH), and 37.5 mg linoleic acid are dissolved in a final volume of 25 mL water.
4. Vitamin C solution: 50 mg in 2 mL water. Sterilized by passing through a 0.22-µm filter. Prepare just before use.

*2.2.3. Long-Term Culture Medium*

1. Dissolve one 5 L-doses powdered Williams E medium in approx 4 L deionized water. Add 11.9 g HEPES and 11 g $NaHCO_3$, and adjust to pH 7.2. Adjust volume to 5 L.
2. Sterilize by passing through a 0.22-µm filter and keep refrigerated in the dark in 500 mL stoppered bottles.
3. The mix of additives (see *2.2.4.*) for complementing 5 L of Williams E medium is prepared by adding in a final volume of 250 mL of Williams E medium: 10 mL BSA-linoleic-linolenic solution, 5 mL insulin solution (50mg), 1 mL transferin solution (25mg), 50µL selenium acetate solution (64 µg), 200 µL dexamethasone solution (0.2 mg), 100 µL liver growth factor solution (100 µg), 250 µL cAMP solution (12.25 mg), 500 µL prolactin solution (50 UI), 100 µL ethanolamine solution (0.3 µg), 5 mL glucagon solution (5 mg), 250 µL epidermal growth factor solution (250 µg), 50 ml glutamine solution (1.46 g), 50 ml penicilline/streptomycin solution (500,000 U and 500 mg, see 2.1.1). The mix is sterilized by passing through a 0.22-µm filter (PES, Nalgene). Add 15 ml sterile fungizone (see 2.1.1). The mix is aliquoted in 25 mL fractions and store at –80°C.
4. The final chemically and hormonally defined long-term culture medium is prepared just before use by supplementing 500 mL of Williams E medium with 25 mL of Mix.

*2.1.4. Additives for preparing the mix for the long-term culture medium*

1. BSA-linoleic-linolenic acid solution: dissolve 1 g BSA(fractionV) in a final volume of 10 mL PBS (phosphate buffer saline). Add 20µL linoleic acid and 20µL linolenic acid.
2. Insuline: 100 mg in 10 mL 1% acetic acid.
3. Transferin: 100 mg in 4 mL water.
4. Selenium acetate: 6.45 mg in 5 mL water.
5. Dexamethasone: 1 mg in 1 mL dimethylsulfoxide (DMSO).
6. Liver growth factor: 500 µg in 500 µL Williams E medium.
7. Cyclic AMP (N6,2'-O Dibutyryladenosine 3'-5' cyclic monophosphate): 49 mg in 1 mL water.
8. Prolactin (luteotropic hormone): 250 UI in 2.5 mL 10 mM chlorhydric acid.
9. Ethanolamine: 3 µL in 1 mL DMSO.
10. Glucagon: 5 mg in 5 mL water.
11. Epidermal growth factor: 500 µg in 500 µL water.
12. Glutamine solution: 200 mM from Sigma.

**2.3. Collagenase Solution**

1. Prepare 1 L of HEPES buffer supplemented with antibiotics, fungizone, and 10 mL of 70 m*M* $CaCl_2$ and divide it into two parts of 250 and 750 mL.
2. Dissolve 500 mg collagenase in the 250 mL aliquot of this buffer and sterilize by passing through 0.45- and 0.22-µm filters if necessary (*see* **Note 5**). Because of the cost of collagenase, this solution should be prepared only when perfusion of the tissue has been shown to proceed correctly (*see* **3.2., step 4**).
3. Add to the 750 mL aliquot of HEPES buffer. This solution of collagenase will be used to dissociate the liver tissue.

### 2.4. Cell Culture Materials

1. Type I collagen-coated dishes (60 or 100 mm diameter, or 6, 12 or 24 well plates) from Beckton Dickinson.
2. Fetal calf serum (FCS) tested for hepatocyte cultures.
3. Laminar-flow microbiology safety cabinet.
4. Nylon filter (250 mesh) sterilized by autoclaving.
5. Perfusion vessel (pyrex or stainless steel), rubber tubing (hoses), teflon terminal tip, and stoppers that can be sterilized by autoclaving.
6. Thermostated water bath for buffers and solutions. Heater for perfusion vessel.
7. Pump for tissue perfusion with flux adjustment between 10 and 500 mL/min.
8. Vacuum liquid-aspiration device (for removal of liquid waste).
9. Waste collectors for tissue, liquids (blood, perfusion effluents) and other solid materials (undigested tissue, gloves, Whatman paper, aluminum foil, and so on).
10. Decontamination reservoir (50 L) for dissection instruments, perfusion vessel, tubing, and other reusable materials.
11. Standard apparatus for cell culture: incubators, low speed centrifuge, optical microscope, rotary agitator, and so on.

## 3. Methods

### *3.1. Safety Conditions*

1. Virological analysis of the patient from whom the liver sample has been resected must be carried out shortly before or at the time of the operation. The serologies include hepatitis A, B, and C viruses, and HIV. All laboratory staff should be vaccinated against hepatitis B virus and clearly informed of the possible risks of infection.
2. Even when the virological analysis is negative, all experimentation with human tissue samples must conform to the safety policies regarding the protection of staff and the containment standard of the equipment and of the laboratory rooms in which tissue processing, isolation of, and experimentation on, cell cultures are to be performed (European standard containment laboratory type L2).
3. In cases where donor tissue is infected with a hepatotrophic virus, isolation and culture must be performed in a containment laboratory type L3 (*see* **Note 6**).
4. All steps of hepatocyte isolation and culture are carried out in a laminar vertical-flow microbiology safety hood, to protect not only the staff from contamination, but also the liver sample. Staff must wear sterile gloves, glasses, masks, and disposable coats and boots.
5. All materials and liquid wastes must be decontaminated prior to discarding or resterilization by autoclaving (for recycled materials). Instruments and materials to be reused are decontaminated by immersion in Phagosurf DD 0.25 % solution (final concentration) for 24 h. Prepare 50 L of this solution in an appropriate reservoir shortly before hepatocyte isolation. Liquid wastes are stored in an appropriate reservoir in the presence of Phagosurf DD 0.25 % solution for 24 h. Other materials such as used culture dishes are decontamined by autoclaving before being discarded.

### *3.2. Perfusions*

1. Upon arrival in the laboratory, the liver sample is placed in the perfusion vessel and the edge is carefully examined in order to locate the various vein and artery sections that will be used for perfusion (*see* **Note 3**). The volumes indicated below for buffers and solutions are adequate for a sample of approximately 300 g; for smaller or larger samples, these should be modified accordingly.
2. All solutions and buffers are kept at 37°C, except for the albumin-HEPES solution used for hepatocyte washings. Do not oxygenate solutions and buffers before perfusion, because this will generate oxidative stress in the tissue.
3. The tissue is first washed with 1-2 L HEPES buffer supplemented with antibiotics and fungizone (**see 2.1.1.**), at a rate of approximately 1 mL/min/g of tissue with no recirculation. In this step, blood is washed away (if this has not previously been carried out with saline buffer in the operation theater), the tissue is warmed to 37°C, and it can be ensured that perfusion is proceeding correctly (*see* **Note 7**). During this and further perfusion steps, the cannula is inserted successively in all veins/arteries present on the edge for approximately 30 s each (one vein/artery at a time). Care must be taken not to injure the vein/artery section during this operation.
4. If perfusion is proceeding normally, preparation of collagenase solution should be started at this point (*see* **2.3.**).
5. The tissue is then perfused with 1 L of EGTA solution (*see* **2.1.1.**), supplemented under the same conditions as described above, with no recirculation.
6. The tissue is then perfused with 1 L supplemented HEPES buffer (*see* **2.1.1.**) to remove EGTA, under the same conditions as previously described. At the end of this step, the reservoir of the perfusion vessel is emptied and washed several times with water.
7. The tissue is then perfused with the collagenase solution under the conditions previously described, except that here the solution is recirculated and that the rate of perfusion is reduced to 100 mL/min. The duration of this step lasts for a maximum of 20 min. During this step, softening of the tissue gradually appears as well as marbling, indicating that dissociation is proceeding efficiently (*see* **Note 8**).

### *3.3. Hepatocyte Isolation and Washing*

1. At the end of the collagenase perfusion, the liver sample is transferred into a new stainless steel vessel, the Glisson's capsula is opened in several places. Tumor(s) or metastasis, if present, is (are) removed and sent to anatomopathologist (*see* **Note 2**).
2. The tissue is gently disrupted with scissors.
3. The homogenate is complemented with 1-2 L of BSA-HEPES buffer (see **2.1.1.**).
4. The homogenate is filtered through a nylon filter (250 mesh) and the filtrate is distributed into 150 mL centrifuge tubes. The filter is washed twice with approximately 200 mL of BSA-HEPES solution to collect the hepatocytes that are trapped in the undissociated tissue homogenate.
5. Tubes are centrifuged for 5 min at 50*g* and at room temperature.

6. The supernatant is discarded and the pellet, representing the hepatocytes, is gently resuspended in 200 mL of BSA-HEPES solution per tube by 5 successive up and down runs with a pipet (*see* **Note 9**).
7. **Steps 5** and **6**, are repeated twice. At the end of the last centrifugation, the yield of the preparation may be roughly estimated by measuring the volume of the pellet: 1 mL of pellet represents approximately $10^8$ cells. For more precise counting of cells, see next steps.
8. At the end of the last washing, the pellet is resuspended in an equal volume of BSA-HEPES solution and homogenized gently with a pipet as described in **step 6**.
9. 500 µL of hepatocyte suspension is dispersed in 9.5 mL of the short-term culture medium. 250 µL of this suspension are placed in a polystyrene tube and supplemented with 50 µL of a 1% Trypan blue solution. After 2 min at room temperature, a 10-µL aliquot of this suspension is placed in the compartment of a hemocytometer cell for counting.
10. Yield and viability of cells are classically evaluated by examination under a microscope using the Trypan-blue exclusion test. In our hands, the yield and viability are, on average, $7x10^6$ cells per gram of liver tissue and 85%, respectively (*see* **Note 10**).

### *3.4. Hepatocyte Plating and Culture*

1. After evaluation of yield and viability, an appropriate amount of short-term culture medium is complemented with FCS (5% in volume).
2. The hepatocyte suspension is diluted in this medium to 3.5 or $10x10^6$ viable cells/mL for plating in 60 or 100 mm diam culture dish, respectively.
3. Culture dishes are distributed on stainless steel trays (7 dishes of 100 mm or 18 dishes of 60 mm per tray) and 2 or 7 mL of culture medium are added per 60 or 100 mm dish, respectively (use adequate sizing for smaller wells).
4. Then, 1 mL of an appropriately diluted suspension of cells (3.5 or $10x10^6$ viable cells) is added per dish (use adequate sizing for smaller wells). This number of cells per dish corresponds approximately to a cell density of $12.5x10^4$ cells per $cm^2$ for a confluent monolayer (*see* **Note 11**). Care must be taken to rehomogenize cell suspension frequently by gentle circular agitation whilst distributing to the culture dish.
5. Cells are evenly distributed on the dish by gentle agitation (*see* **Note 11**).
6. Culture dishes are then placed in an incubator, in a humid atmosphere of air 5% $CO_2$ at 37°C.
7. After 4 h, the serum-supplemented medium is discarded and replaced with 3 or 8 mL of new serum-free medium per 60 or 100 mm dish, respectively (*see* **Note 12**).
8. The culture medium is then renewed every 24 hours for short-term cultures and every 48-72 hours for long-term cultures (*see* **Note 13**).

## 4. Notes

1. With high levels of steatosis or cholestasis, we generally found either poor yield, poor viability (<65%), and poor attachment of cells to culture dishes, or no dissociation at all.

2. This requires good coordination with the surgeon and the anatomopathologist. In all cases, an identification number is ascribed to the liver tissue so that the anonymy of the patients (or donors) is fully respected. Only information on the gender, age, medical reasons for the surgical operation (liver lobectomies) or cause of death (liver donors) is communicated by the Hospital. The subjects cannot be identified, directly or through identifiers. Importantly, pathological examination of the surgical specimen must in no way be hindered by the procedure used to isolate hepatocytes.
3. The most convenient manner of proceeding here is to make a new edge (by cutting a 0.5- to 1-cm thick slice), parallel to the one left by the surgeon. This reveals various vascular entries.
4. Because of the numerous additives required to supplement the basal culture medium, it is preferable to prepare a concentrated solution of all the additives, in either one or several submixes.
5. We only use batches of collagenase with a specific activity greater than 400 U/mg. Some batches appear to be contaminated by micro-organisms. This is assessed by microsocopic examination of an aliquot of culture medium supplemented with 1 mg of collagenase after 96 hour incubation at 37°C under normal culture conditions. In such cases, the collagenase solution has to be sterilized just prior use (see **3.2**.) either directly by passing through a 0.22-µm filter or, to prevent the filter from becoming clogged, first through a 0.45-µm filter prior to a second filtration, through a 0.22-µm filter.
6. We have already prepared and cultured hepatocytes from a patient who was infected with hepatitis C virus. Yield, viability, and culture aspect were not significantly different from those observed with normal liver.
7. This is checked by touching the tissue upon perfusion and verifying the homogeneous warming of the liver sample. Should a part of the sample remain cold during this step, it means that the vein irrigating this area either has not been found (and it would be necessary to search for it to improve the yield of preparation) or it is obstructed by a clot. In the latter case, a reperfusion at a higher rate may be tried (care must be taken not to damage the tissue by excessive pressure).
8. It is important to monitor this step by direct touching of the tissue. Correct perfusion and dissociation is revealed by swelling of the various areas of the sample when moving from one vein to another.
9. The pellet is generally formed by one “tight” part in the bottom and one “soft” part above. The “soft” part contains damaged cells and erythrocytes that should be removed. This is performed by slowly tilting the tube. The “soft” pellet may represent up to 30% of material at the first washing step. Its volume gradually decreases thereafter to become negligible at the third washing. Removal of the “soft” pellet is critical in obtaining a preparation with a viability greater than 80%, and a high purity.
10. As a general rule, we have observed that the quicker the procedure for the isolation of hepatocytes, the better the quality of cells and subsequent cultures. Therefore, isolation must be performed as rapidly as possible. Indeed, here, time is quality! Nevertheless, the yield and viability are widely variable from one sample to another and range between 1 and $10x10^9$ cells, and 70 and 90%, respectively. The reasons for this variability are difficult to identify;

parameters such as duration of cold ischemia, health status of the liver, pathology, formation of intra-tissue clots, or quality of perfusion (number and state of vessels used for perfusing), could possibly be involved.

11. The hepatic differentiated phenotype is maintained in confluent but not in subconfluent cultures ***(15)***. Establishment of a homogeneous confluent-cell monolayer throughout the dish is therefore critical. Once the dishes present on one tray are supplemented with culture medium and cells, the tray is held horizontally and gently agitated from left to right (1 cycle/s for 10 s), and back and forth (1 cycle/s for 10 s) with a pause of 5 s in between. This allows the suspension to be spread homogeneously on the dish and avoids rotational movement of the suspension, which would result in preferential distribution of cells towards the periphery of the dish with low density in the center.
12. The culture medium must be aspirated and poured gently to avoid detachment of cells. Medium changes will be facilitated by placing the trays on an inclined plane.
13. Phenotype characterization. Several markers of hepatic phenotype have been investigated and shown to be maintained in our cultures for at least a week (short-term cultures, STC) and 5 weeks (long-term cultures, LTC). These include:

    a. the induction of CYP genes by prototypic inducers and expression of nuclear receptors involved in the control of induction (STC and LTC) ***(13, 14, 16–24)***;

    b. the oxidative metabolism of drugs and reconstitution of drug-drug interactions (STC and LTC) ***(25-27)***;

    c. the production of albumin (STC and LTC), α1-antitrypsin, fibrinogen and plasminogen (LTC) ***(14)***

    d. the production of apolipoproteins, ApoA, ApoB, lpa (STC and LTC) ***(14)***;

    e. the production of acute phase proteins (ceruloplasmin, fibrinogen, ferritin) in response to cytokines Il-1, Il-6, tumor necrosis factor (STC) ***(28)***;

    f. the production of blood coagulation factors (LTC) ***(29)***

    g. expression of C/EBP factors (LTC) ***(30)***

    h. the mitotic response of cells to specific hepatotrophic factors including epidermal growth factor, transforming growth factor a, hepatocyte growth factor and serum from patients with liver failure (STC, under low cell density) ***(31–33)***;

    i. in vitro infection by hepatitis C and D viruses (LTC) ***(34-36)***.

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