Protocol for use no. 12:
upcyte® Hepatocytes – ready-to-use

upcyte technologies

(v1.9 – April 26th 2021)

When breaking the sealed bag of upcyte® cells you are explicitly accepting the terms of the limited use label license provided with the purchase of the cells. IT IS STRICTLY PROHIBITED TO EXPAND THE CELLS.

Unless indicated otherwise, upcyte technologies products and services are for research purpose only. Do not use for diagnostic or therapeutic applications.

Introduction

This PFU describes how to culture upcyte® Hepatocytes as 2D monolayers with and without a Matrigel™ overlay for endpoint measurements. Before seeding the cells for the experiment, an initial sub-culture is performed to increase recovery after cryopreservation. Please note that upcyte® hepatocytes are adherent cells and not suitable for studies in suspension.

Required products for upcyte® Hepatocyte culture

upcyte® Hepatocytes ($5 \cdot 10^6$ frozen cells)

Each vial contains ~$5x10^6$ frozen upcyte® Hepatocytes from which at least 70% recovery is expected after thawing. The protocol includes one sub-culture step. **THE CELLS ARE NOT FOR FURTHER EXPANSION**.

Storage: upcyte® Hepatocytes should be stored in liquid or vapour phase nitrogen.

Shelf life: 2 years after receipt.

Hepatocyte Thawing Medium (50 ml)

A ready-to-use formulation for thawing upcyte® Hepatocytes. No additional supplements are required.

Storage: Store Thawing Medium protected from light at 2−8°C.

Shelf life: The expiration date is indicated on the respective label.

Hepatocyte High Performance Medium HPM (500 ml)

The Hepatocyte High Performance Medium (HPM) is designed for the optimal culture and endpoint measurement of upcyte® Hepatocytes. In order to obtain Hepatocyte HPM, add the entire contents of supplement A and L-glutamine to the basal medium.

Storage: Store basal medium and fully supplemented medium protected from light at 2−8°C. Store Supplement A at -20°C. The expiration date is indicated on the respective label.

Shelf life: The shelf life of the fully supplemented media is 6 weeks. Once fully supplemented, do not freeze the media.

Note: Our Media do not contain antibiotics. Add antibiotics only if it is necessary for your experiments.

Our upcyte® Hepatocyte Starter Kits consists of 1 vial of upcyte® Hepatocytes, 50 ml Hepatocyte Thawing Medium and 500 ml Hepatocyte High Performance Medium.

Additional products not supplied by upcyte technologies GmbH:

- PBS without Ca^{2+} or Mg^{2+}
- Trypsin-EDTA (0.05% Trypsin/0.02% EDTA)
- Foetal bovine serum (FBS) for quenching trypsin activity
- Optional: Matrigel™
- Collagen coated (type I) culture vessels
  Coated culture vessels can either be bought (e.g. from Corning, Bedford, MA, USA) or self-prepared. For coating, dilute collagen type I (e.g. Sigma-Aldrich, C3867) with 20 mM acetic acid to a final concentration of 50 µg/ml. Add 0.1 ml/cm² of the diluted collagen solution to the culture dishes and incubate for 1 h at RT. Wash the plate twice with PBS and use directly or air dry before storing at 4°C for max of one week.

Culture protocol

Day 1: Thawing and sub-culture of cryopreserved upcyte® Hepatocytes (e.g. Monday)

1. Pre-warm 50 ml Hepatocyte Thawing Medium to RT.
2. Carefully remove the cryovial from the storage tank. This should only take seconds; longer times will decrease the cell yield.
3. Thaw cells in a 37°C water bath until only a small chunk of ice is left. Do not shake the vial.
4. Spray the vial and the tube containing the thawing medium with 70% ethanol, wipe and transfer to a laminar flow-hood.
5. Transfer the now completely thawed cell suspension from the cryovial into 50 ml Hepatocyte Thawing Medium by gently pouring the cells into the medium.
6. Using a 1 ml pipette, transfer 1 ml of the thawing medium back to the cryovial and pour the contents back into the 50 ml tube. Repeat this process twice to completely remove the cells from the cryovial.
7. Pellet the cells by centrifuging at **90×g for 5 min** at RT. **Important note:** Higher *g*-forces may reduce cell recovery.
8. Aspirate the supernatant without disturbing the pellet. Leave approximately 200-400 µl medium on top of the cells.
9. Gently loosen and re-suspend the cells without adding any extra medium by agitating and rotating the tube. Do not vortex or shake the cells as this will compromise cell survival.
10. Add an appropriate volume of pre-warmed HPM Medium to the pellet (approximately 1ml per million cells) and resuspend the cells. Avoid pipetting the cells up and down.
11. Determine the viable cell number by using a hemocytometer or a cell counter.
12. Dilute upcyte® Hepatocytes in HPM and seed at ~10,000 cells/cm² in **collagen coated** cell culture flasks (e.g. T175) or appropriate cell culture dishes. One vial containing 5 million cells is usually sufficient for 3x T175 flasks.
13. Incubate the cells at 95% humidity, 37°C and 5% CO_2.

Day 2: Medium change (e.g. Tuesday)

14. Refresh the HPM the next day.
15. Culture the cells for additional 2-3 days until reaching 70-80% confluence. Cells are subsequently passaged using trypsin. Do not expand the cells further.

Day 4/5: Seeding into multiwell plates (depending on the cell density, e.g. Thursday or Friday)

16. Pre-warm PBS, trypsin/EDTA and Hepatocyte High Performance Medium to 37°C. Prepare the needed amount of HPM + 10%FBS (see 22) for stopping the trypsin activity.
17. Carefully aspirate the culture supernatant.
18. Wash the plate once with 100 µl PBS/cm^2.
19. Add 50 µl/cm^2 trypsin/EDTA.
20. Incubate for 3-4 min at 37°C until most of the cells are rounded up and detached. Avoid incubating the cells for more than 7 min.
21. Gently tap the cell culture vessel to detach remaining adherent cells.
22. Stop the trypsin activity by adding twice the volume of HPM containing 10% FBS.
23. Rinse the surface with the cell suspension.
24. Transfer the complete suspension to a tube and centrifuge at **90xg for 5 min** at RT.
25. Resuspend the cells in an appropriate volume of High Performance Medium (~ 3 ml per T175 flask).
26. Determine the number of viable cells. The cells should have doubled their number at this point.
27. Seed the cells at 150,000 cells/cm^2 in High Performance Medium in collagen type I coated plates. Full confluence is crucial for proper differentiation and full functionality of upcyte® hepatocytes. If cells are seeded on Thursday, we recommend an additional medium change and a Matrigel overlay on Friday.

Day 5: Matrigel™ overlay for sandwich configuration (e.g. Friday)

A Matrigel™ overlay improves many hepatic functions such as CYP enzyme activities. Matrigel™ is available from commercial suppliers (e.g. Corning, REF 354234). Before starting the overlay, Matrigel™ needs to be thawed at 2-8°C overnight and, together with PBS, medium and pipette tips, kept refrigerated/on ice. In general, we recommend an overlay for any type of metabolic measurement.

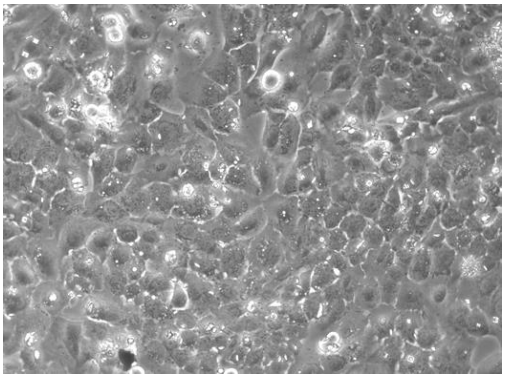
- After seeding, incubate the cells for at least 6 hours until the cells are attached. Do not move the plate during the attachment phase.
- Dilute the Matrigel™ to 0.25 mg/ml using HPM and keep the mixture on ice.
- Aspirate the supernatant and wash the cells once with ice-cold PBS.
- Gently overlay the cells with diluted, ice-cold Matrigel™ (250 µl/cm^2).

Day 8: Start performing your assay of choice (e.g. Monday)

upcyte® Hepatocytes may be used for various applications, including metabolism and cytotoxicity testing. Therefore, the medium is replaced with fresh HPM with the respective solvent, test compounds or inducers for the cytotoxicity assays and phenotypic characterization.

Culture the cells for 5 days at full confluence before performing your endpoint measurements.

Cells can be maintained over a period of at least 18 days after plating. Change the medium every 2-3 days when maintaining the cells for longer periods.



Example of the morphology of upcyte® Hepatocytes at confluence

Examples for endpoint measurements

Cytotoxicity testing

Remove the medium and replace with HPM containing either vehicle control or test compound (Monday, Day 8). We recommend incubating treated cells for 72-96 h for short-term toxicity testing. For longer-term toxicity, change the medium every 2-3 days up to 3 weeks. After treatment determine the viability of cells using standard endpoint measurements (e.g. ATP, LDH, MTT/MTS).
For additional information, see **PFU No. 4**.

Metabolism testing

Induction: Refresh the medium with HPM containing test compounds or controls daily. When analysing CYP expression levels after induction, we recommend a treatment period of 48h-72h for RNA levels and 72h for protein levels/enzyme activity (e.g. day 8-10, endpoint day 10).
Inhibition: If you are performing a CYP1A2 inhibition assay, 50 µM Omeprazole should be included in the HPM at the day of the Matrigel™ overlay (day 5) to pre-induce the activitiy. All other CYP inhibition assays can be done without additional pre-induction. Perform your endpoint on day 10.
Clearance: For testing low metabolized compounds, cells can survive 5 days with a media change. Analyze the production of metabolites using appropriate analytical methods such as HPLC or LC-MS/MS.

For additional information, see **PFU No. 5** (induction) & **PFU No. 8** (inhibition).

Co-cultures

Our upcyte® Liver Sinusoidal Endothelial Cells (LSECs) are suitable for co-culture studies with upcyte® hepatocytes. Please ask for our Technical Advice TA01.

If you have concerns about protein binding on our media, contact us.

Product information

Product	Supplements/Components	Product number
upcyte® Hepatocytes (cryopreserved)	• cryopreserved vial, 5 Mill Cells (1 ml)	CHE002
Hepatocyte High Performance Medium	• Basal medium (500 ml) • Supplement A (5 ml) • L-glutamine (5 ml)	MHE003
Hepatocyte Thawing Medium	• ready-to-use (50 ml)	MHE001
upcyte® Hepatocyte Starter Kit	• cryopreserved vial, 5 Mill Cells (1 ml) • Hepatocyte Thawing Medium (50 ml) • High Performance Medium (500 ml)	KHE001

Purchaser Notification

Limited Use Label License (upcyte technologies)

This cellular product (upcyte® cells) generated using the upcyte® technology is provided under an intellectual property license from upcyte technologies GmbH. The transfer of the upcyte® cells is conditioned on the buyer using the purchased product solely in research conducted by the buyer, and the buyer must not use, sell or otherwise transfer this product, its components or any data generated with the product
(a) for diagnostic, therapeutic or prophylactic purposes; or
(b) for resale; or
(c) for the production of therapeutic, diagnostic, prophylactic or any other products; or
(d) to provide a service to deliver information or materials to a third party.
This intellectual property license is timely limited and granted to the buyer for 24 months after delivery of the upcyte® cells. After this time the buyer has to destroy remaining upcyte® cells and populations thereof or obtain a follow-up license. For information on purchasing a license to this upcyte technologies product for other purposes, for an extended time frame and/or for commercial purposes, contact upcyte technologies GmbH, Osterfeldstrasse 12-14, 22529 Hamburg, Germany or info@upcyte.com.

During the generation of these upcyte® cells a product licensed from Life Technologies Corporation containing the following Limited Use Label License was used:

Limited Use Label License (Life Technologies Corporation)

This product is provided under an intellectual property license from Life Technologies Corporation. The transfer of this product is conditioned on the buyer using the purchased product solely in research conducted by the buyer, and the buyer must not use, sell or otherwise transfer this product or its components for (a) diagnostic, therapeutic or prophylactic purposes; or (b) resale, whether or not resold for use in research or (c) or for the commercial production of therapeutic, diagnostic, or prophylactic products or other products not intended for research use; or (d) to provide a service to deliver information or materials to a customer other than for research purposes of such customer. For information on purchasing a license to this product for purposes other than research, contact Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, CA 92008 USA or outlicensing@lifetech.com.