Protocol for use No. 12:
upcyte® Hepatocytes

upcyte technologies

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Description of protocol

This PFU describes how to culture upcyte® Hepatocytes as 2D monolayers with and without a Matrigel overlay for endpoint measurement. upcyte® Hepatocytes can be directly seeded without an initial sub-culture of the cells. For some applications a pre-culture is necessary to allow the cells to recover from the cryopreservation process and possible debris.

Required products for upcyte® Hepatocyte cultures

- **upcyte® Hepatocytes (cryopreserved)**

Each vial contains ~$5x10^6$ upcyte® Hepatocytes (upcyte; "upregulated hepatocytes") frozen per vial from which at least 50% recovery is expected after thawing.
Storage: upcyte® Hepatocytes should be stored in liquid or vapour phase nitrogen. They should not be stored at -70°C for longer than one week.

- **Hepatocyte Thawing Medium, 50 mL**

This is ready-to-use for thawing upcyte® Hepatocytes. No additional supplements are required.
Storage: Store Thawing Medium protected from light at 2–8°C.
Shelf Life: The shelf life of the Thawing Medium is 3 months.

- **Hepatocyte High Performance Medium (Endpoint Medium), 500mL**

The Hepatocyte High Performance Medium is designed for the optimal culture and endpoint measurement of upcyte® Hepatocytes. In order to obtain Hepatocyte High Performance Medium add the entire contents of supplement A and L-Glutamine to the basal medium. Due to the manufacturing process, the medium may appear opaque but this does not affect the performance of the cells.
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 label of the basal medium as well as on the supplement label.
Shelf Life: The shelf life of the fully supplemented media and the Thawing Medium is 6 weeks. Once fully supplemented do not freeze the upcyte® Hepatocyte Media. Add antibiotics if preferred and necessary for your experiments.

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

- **Collagen coated (Type I) culture vessels**

These are not provided by upcyte technologies. 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 0.02M acetic acid to a final concentration of 50µg/mL. Add 0.1mL/cm^2 of the diluted collagen solution to the culture dishes and incubate for 1h at RT. Wash the plate twice with PBS and use directly or air dry before storing at 4°C.

Additionally products not supplied by upcyte technologies GmbH:

- **Foetal bovine serum (FBS) or Trypsin Neutralization Solution (TNS)**
- **PBS without Calcium or Magnesium**
- **Trypsin/EDTA (0.05% /0.02% EDTA).**
- **Matrigel™**

A Matrigel overlay step improves some of the hepatic functionalities such metabolism and clearance activities in long-term cultures. Matrigel™ is available from commercial suppliers (e.g. Corning, Bedford, MA, USA, REF 354234). **Note:** Matrigel™ is stored frozen. It can to be thawed overnight in a refrigerator. Keep pipette tips and medium for dilution of Matrigel™ in a refrigerator before use.

Protocol for culture preparation

Note: Media can be supplemented with antibiotics if desired.

Step A: Thawing of cryopreserved upcyte® Hepatocytes (Day 1, e.g. Monday)

1. Pre-warm 50mL Hepatocyte Thawing Medium to 37°C.
2. Carefully remove the cryovial from the storage tank. **Note:** This should only take **seconds** (longer times will decrease the cell yield).
3. Thaw cells in a 37°C water bath until all the ice has completely disappeared. Do not shake the vial, or take it out of the water during thawing, as this will damage the cells.
4. Spray the vial and the tube containing 50mL of thawing medium with 70% ethanol and transfer to a laminar flow-hood.
5. Transfer the thawed cell suspension (1mL) from the cryovial into 50mL thawing medium by gently pouring the cells into the medium.
6. Using a 1mL pipette, transfer 1mL of the thawing medium back to the cryovial and pour the contents back into the 50mL 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. **Note:** higher *g*-forces can reduce cell recovery significantly.
8. Aspirate the supernatant without disrupting the pellet. Leave approximately 200 - 400µL 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. **Note:** Do not vortex or shake the cells as this will reduce cell survival.
10. Add an appropriate volume of appropriate pre-warmed culture medium to the pellet (approximately 1mL per million cells thawed) and re-suspend the cells. **Note:** avoid pipetting the cells up and down.
11. Determine cell number by e.g. using Trypan blue exclusion or a cell counter.

Step B: Seeding of upcyte® Hepatocytes (Day 1, e.g. Monday)

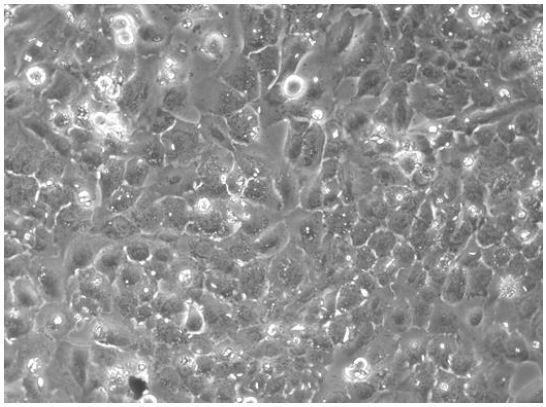
12. Dilute upcyte® Hepatocytes in pre-warmed, fully supplemented High Performance Medium to a density of 0.15×10^6 cells/mL and seed at 20,000-30,000 cells / cm^2 in collagen type I coated culture vessels. For sub-culture, seed the cells at ~10,000vcells / cm^2 in a collagen coated flask (e.g. T175) or dish and proceed with Step C. If the endpoint is to be performed in the same vessels in which the cells have been plated proceed to Step D. One cryovial of upcyte® Hepatocytes should be sufficient for two plates of various formats (e.g. 96-well, 48-well, 24-well plates).

Step C: Sub-culture of upcyte® Hepatocytes (Day 1-5, passage step e.g. on Friday)

13. Culture cells until they reach 70-80% confluence. Refresh the High Performance Medium every 2-3 days. Cells are then ready to be passaged using Trypsin.
14. Pre-warm Hepatocyte Medium to RT.
15. Aspirate the supernatant from the cultures.
16. Wash the plate once with PBS. We recommend using ~100µl PBS/cm^2.
17. Wash the plate once with 1x Trypsin (0.05% /0.02% EDTA) - we recommend using ~100µl/cm^2.
18. Add 1x Trypsin/EDTA (~100µl/cm^2).
19. Incubate for 3 - 4 min at 37°C until most of the cells are rounded up and detached. If required, the cells can be incubated for longer periods up to 10 min.
20. Gently tap the cell culture vessel to detach the cells.
21. To stop the Trypsin activity, add x2 volume (200µl/cm^2) of High Performance Medium containing 10% foetal bovine serum or Trypsin Neutralization Solution.
22. Rinse the remaining attached cells with the cell suspension using a pipette.
23. Transfer the complete suspension to a tube and centrifuge at 90*xg* for 5 min at RT.
24. Seed the cells at 150,000 cells/cm^2 in High Performance Medium in collagen type I coated culture vessels.
25. Either include a Matrigel™ overlay step on day 5 to obtain a sandwich culture (Step E) or proceed to Step F for conventional monolayer culture.
26. The cells are then cultured for 3 days (over the weekend) for used in endpoint assays on the Monday.

Step D: Pre-Culture directly in the well plate (Day 1-5)

13. Perform a medium change on Day 2 and 4.
14. On day 5 (e.g. Friday) the cells should have reached full confluence.
15. Either include a Matrigel overlay step on day 5 if sandwich culture format is required (Step E) or proceed to Step F for conventional monolayer culture.
16. The cells are then cultured for 3 days (over the weekend) for used in endpoint assays on the Monday.



Example of the morphology of upcyte® Hepatocytes at confluence.

Note: If you are performing a CYP1A2 inhibition assay 50µM Omeprazole should be included in the medium at this step to pre-induce the expression of this enzyme. All other CYP inhibition assays can be done without pre-induction.

Note: Before starting the overlay, Matrigel™ needs to be thawed at 2-8°C overnight and, together with PBS, Medium and pipette tips, kept refrigerated or kept on ice.

Step E: Matrigel™ overlay for sandwich culture (Day 5, e.g. Friday)

27. Incubate cells under standard culture conditions (at 95% humidity, 37°C and 5% CO_2) until the cells have attached. This takes at least 4 hours. During this time the plate should not be moved.
28. Dilute the Matrigel™ to 0.25 mg/ml using supplemented High Performance Medium and keep the mixture on ice.
29. Aspirate supernatant and wash cells once with ice-cold D-PBS.
30. Gently overlay the cells with ice-cold 0.25 mg/mL Matrigel™ in Medium (250µl/cm^2),
31. Culture the cells under standard culture conditions for 3 days (without a medium change) before starting an assay.
32. Cells can be maintained over a period of at least 18 days after plating.

Step F: Monolayer culture (Starting on Day 5 or 8)

Test compounds can be added on Day 5 but they can also be maintained over the weekend for endpoint initiation on the following Monday.

27. Refresh the medium on Day 5 (with or without compound) and then culture the cells under standard culture conditions for 3 days (without a medium change).
28. Cells can be maintained over period of at least ? days after plating.

Examples for endpoint measurements (Day 7)

upcyte® hepatocytes can be used for various applications as e.g. metabolism and cytotoxicity testing. Therefore, the medium is replaced with fresh High Performance Medium with the respective solvent, test compounds or inducers for the cytotoxicity assays and phenotypic characterization. The incubations can start on a Monday after the cells have been prepared the previous week.

Cytotoxicity testing

Described briefly: remove the medium and replace with Hepatocyte High Performance Medium containing either vehicle control or test compound. We recommend incubating treated cells for 72-96h for short-term toxicity testing. For longer-term toxicity tests, use an Matrigel overlay. After treatment determine the viability of cells using standard endpoint measurements (e.g. ATP, LDH, MTT/MTS). **PFU No. 4**

Metabolism testing

Described briefly: refresh the medium with High Performance Medium containing either vehicle/positive control, or test compound daily for 3 consecutive days. Include solvent controls. On the fourth day (e.g. Thursday), the cells can be used in metabolism assays: Aspirate the medium and wash the cells twice with PBS (containing Ca^{2+} and Mg^{2+}). Incubate the cells with the desired test compound for hours (e.g. 0.5 - 2h, depending on the CYP) or days. For testing low metabolized compounds e.g. using clearance assay perform matrigel overlay for long-term culture. Analyze the production of metabolites using appropriate analytical methods e.g. HPLC, LC-MS/MS.

Product information

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

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