**Establishment of sandwich cultures of primary human hepatocytes**

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**Running head:** Sandwich cultures of human hepatocytes.

**Summary**

Primary hepatocytes and their adherent cultures are still considered as the golden standard in the field of liver-based *in vitro* modelling. However, they cope with progressive deterioration of their *in vivo*-like morphological and functional phenotype. Among the various strategies that are used to counteract this dedifferentiation process is the seeding and cultivation of freshly isolated or cryopreserved hepatocytes between 2 layers of extracellular matrix scaffolds. This so-called sandwich culture system allows to restore cell-extracellular matrix interactions and thereby to delay dedifferentiation. The practical set-up of the sandwich culture system of primary hepatocytes is described in the present chapter.

**Key Words**: primary human hepatocytes, sandwich culture, cryopreserved hepatocytes, dedifferentiation, cell-extracellular matrix interaction, long-term cultures, animal-free model.

# Introduction

*In vivo*, hepatocytes are highly polarized cells with apical and basolateral domains, the latter being in direct contact with a solid extracellular matrix (ECM) scaffold. The ECM plays a major role in the modulation of several cell functions, including morphogenesis and differentiation ***(1,2)***. The ECM consists of several compounds, such as laminin, fibronectin and collagen, which interact with both integrin and non-integrin receptors expressed by hepatocytes. This ligand-receptor binding activates critical hepatocyte signaling pathways responsible for regulating liver-specific gene expression ***(3,4)***. Based on this knowledge, the so-called sandwich culture technique has been developed as a strategy to maintain liver-specific morphology and functionality in primary hepatocyte cultures for extended periods of time ***(5,6)***. In this technique, freshly isolated or cryopreserved primary hepatocytes are seeded between 2 layers of ECM compounds. Collagen type I is the most common ECM compound used to set up sandwich culture systems ***(1)***. Other widely ECM scaffolds used for this purpose include extracts from murine Engelbreth-Holm Sward tumor, such as Geltrex™ and Matrigel™ ***(7)***. In the last few years, a plethora of synthetic polymers, such as poly (lacti-*co*-glycocholic) acid ***(8)***, polyurethane foam ***(9)*** and polyvinyl formal resin ***(10)***, have been proposed as substrata in sandwich cultures of primary hepatocytes. Sandwich-cultured hepatocytes are metabolically competent with proper localized drug transporters and a functional bile network. For this reason, they are often used when assessing hepatobiliary disposition of xenobiotics and drug transporter-based drug-drug interactions ***(11,12)***. Renewal of the ECM overlay in a sandwich configuration every 3-4 days was recently shown to extend the hepatocyte culture time up to 14 days. Hence, they represent suitable *in vitro* tools for long-term hepatotoxic hazard identification of xenobiotics ***(13)***. The sandwich culture method can be applied to both single cell culture dishes and multi-well plates, thus providing an opportune model for high-throughput screening ***(14)***. In this chapter, a detailed protocol for setting up sandwich cultures of cryopreserved primary human hepatocytes is described.

#  Materials

## Coating of multi-well plates

1. Laminar airflow cabinet.
2. Incubator (water jacketed, 37°C, humidified atmosphere of air containing 5% CO2).
3. Thermostated bath (37°C).
4. Sterile Pasteur pipettes and micropipettes.
5. Multichannel pipettes.
6. Sterile 15 mL Falcon tube.
7. Sterile 6-well, 24-well or 48-well multiwell plates.
8. 0.02 N glacial acetic acid solution: 0.2875 mL acetic acid solution (100%) in 250 mL sterile

ultrapure water. Pass through a 0.2 µm filter. Work in sterile conditions.

1. Collagen type I: dilute collagen type I stock solution with sterile 0.02 N

acetic acid solution to a concentration of 1.1 mg/mL. Prepare *ex tempore* (*see* **Note 1**).

10. Dulbecco’s Modified Eagle Medium (DMEM) with glucose (4.5 mg/mL). DMEM can be stored for 6 months at 4°C (*see* **Note 2**).

11. 10x DMEM: 10x concentrated DMEM with 3.7% sodium bicarbonate.

10x DMEM can be stored for 3 months at 4°C.

12. Phosphate buffered saline solution (PBS): 137 mM sodium chloride, 2.7 mM potassium

chloride, 10 mM disodium hydrogen phosphate and 1.8 mM potassium dihydrogen phosphate in sterile ultrapure water. Adjust to pH 7.4. Work in sterile conditions. PBS can be stored for 6 months at 4°C (*see* **Note 3**).

## Thawing of cryopreserved hepatocytes

1. Laminar airflow cabinet.
2. Thermostated bath (37°C).
3. Sterile Pasteur pipettes and micropipettes.
4. Sterile 50 mL Falcon tube.
5. Eppendorf tube.
6. Counting chamber.
7. Centrifuge.
8. Percoll.
9. 10x concentrated PBS: 1370 mM sodium chloride, 27 mM potassium chloride, 100 mM disodium hydrogen phosphate and 18 mM potassium dihydrogen phosphate in sterile ultrapure water. Adjust to pH 7.4. Work in sterile conditions. 10x concentrated PBS can be stored for 24 months at 4°C.

10. Isotonic Percoll: 90% Percoll and 10% 10x concentrated PBS. Work in sterile conditions.

11. Thawing medium: DMEM with 10% fetal bovine serum, 2 mMⅬ- glutamine, 1 µMdexamethasone, 100 µg/mL streptomycin, 100 IU/mL penicillin and 4 µg/mL insulin. Work in sterile conditions (*see* **Note 4**).

12. Seeding medium: Williams’E medium (WEM) with 10% fetal bovine serum, 2 mM Ⅼ- glutamine, 1 µM dexamethasone, 100 µg/mL streptomycin, 100 IU/mL penicillin and 4 µg/mL insulin. Work in sterile conditions (*see* **Note 5**).

13. Trypan blue.

## Culturing primary hepatocytes in collagen gel sandwich configuration

1. Laminar airflow cabinet.
2. Incubator (water jacketed, 37°C, humidified atmosphere of air containing 5% CO2).
3. Sterile Pasteur pipettes and micropipettes.
4. Multichannel pipettes.
5. Seedingmedium.
6. Culture medium: WEM medium with 2 mM Ⅼ- glutamine, 0.1 µM dexamethasone,

100 µg/mL streptomycin, 100 IU/mL penicillin and 0.1 µM Insulin-Transferrin-Selenium + ™ Premix. Work in sterile conditions.

1. Ice-cold Matrigel™ solution.

# Methods

## Coating of multiwell plates

1. Prepare collagentype I by diluting with 0.02 N glacial acetic acid solution to a

concentrationof 1.1 mg/mL. Work in sterile conditions and keep the solution on ice.

1. Place 10x concentrated DMEM, collagen type I (1.1 mg/mL) and a sterile 15 mL Falcon

tube on ice in a laminar airflow cabinet.

1. Mix 1 part of 10x DMEM with 10 parts of the collagen gel (1.1 mg/mL) in the sterile

Falcon tube.

1. Disperse 250, 500 or 1500 µL of this mixture on the 48-well, 24-well or 6-well multiwell

plates, respectively. Avoid air bubbles and make sure that the mixture evenly covers the complete surface of the wells.

1. Transfer the precoated multiwell plates to an incubator (37°C and 5% CO2) and incubate for

1.5 h.

1. Rinse 3 times with preheated PBS (37°C). Plates are immediately ready for use.

Under sterile conditions, precoated multiwell plates can be stored at 4°C for 1 week.

## Thawing of cryopreserved primary human hepatocytes

1. Add 16 mL isotonic Percoll and 25 mL thawing medium to a sterile 50 mL Falcon tube.

Heat the solution on the thermostated bath (37°C). Work in sterile conditions.

1. Remove the vial from the liquid nitrogen.
2. Release internal pressure by twisting the cap of the vial a quarter turn under the laminar

airflow cabinet. Close the cap again.

1. Immediately immerse the vial in the thermostated bath (37°C). Avoid submerging the vial

completely to prevent water entrance into the cap. Gently shake the vial while holding the tip of the vial for approximately 2 min until the ice crystals are melted.

1. Transfer the cryopreserved cell suspension into the Falcon tube with preheated thawing

medium and isotonic Percoll (37°C). Subsequently, rinse the vial with preheated thawing medium (37°) and add to the Falcon tube.

1. Dilute to 50 mL with preheated thawing medium (37°C). Gently invert the tube 2-3 times to

fully resuspend the cells.

1. Centrifugate for 20 min at 168x*g* at 15-25°C.
2. Carefully aspirate the supernatant and resuspend with 20 mL preheated thawing medium

(37°C).

1. Centrifugate for 5 min at 100x*g* at 15-25°C.

10. Carefully aspirate the supernatant and resuspend in 3 mL preheated seeding medium (37°C) (*see* **Note 6**).

11. Pipet 10 µL of the diluted cell suspension into an Eppendorf tube and add 10 µL of trypan blue. Add 10 µL to the cell counter and determine the number of cells and cell viability. The viability should be at least 85%.

12. Add the required volume of preheated seeding medium (37°C) to dilute the cell suspension (**Table 1**).

## Culturing primary hepatocytes in a sandwich configuration **(Fig. 1)**

1. Add 250, 500 and 1500 µL per well of the diluted cell suspension to the collagen coated

48-well, 24-well and 6-well multiwell plates, respectively (*see* **Note 7**).

1. Gently agitate the multiwell plates in a back-and-forth and side-to-side manner to assure

homogenous cell distribution.

1. Transfer the dishes to an incubator (37°C and 5% CO2).
2. Incubate for 24 h to allow the cells to attach.
3. Prepare the ice-cold Matrigel™ overlay by diluting the commercial solution with cold

seeding medium (4°C) to a concentration of 0.25 mg/mL on ice. Use precooled tips (4°C) (*see* **Note 8**).

1. Shake the multiwell plates to resuspend unadherent cells. Aspirate the seeding medium.
2. Overlay the cells with 250, 500 and 1500 µL per well of ice-cold Matrigel™ working

solution onto the plated cells in 48-well, 24-well and 6-well multiwell plates, respectively.

1. Incubate for 24 h in an incubator (37°C and 5% CO2).
2. Refresh the cells every day with preheated culture medium (37°C) (*see* **Note 9, Note 10** and

**Note 11**).

# Notes

1. Collagen type I can be purchased from a commercial source or can be freshly isolated from

rat tails. For the latter, usually 9 rat tails are used and a collagen concentration up to 3 mg/mL is obtained ***(2)***.

1. Consider using phenol-red free medium DMEM and WEM when the aim of the experiment

is measuring fluorescence. Also, keep this in mind when choosing the appropriate Matrigel™, which can contain either phenol-red or phenol-red free DMEM. Besides phenol-red containing media, it should be noted that collagen has an auto-fluorescence property, which can cause an additional background fluorescence.

1. Sterility of the self-produced media is assured by doing a contamination test using

thioglycolate medium. As such, 1 mL of the prepared medium is added to 25 mL of autoclaved thioglycolate followed by incubation at 37°C for 2 days. This mixture is further investigated for any growth.

1. Collagen gel cultures are susceptible to fungal and bacterial infections. To avoid

contaminations, a mixture of broad-spectrum and narrow-spectrum antibiotics is routinely

added to the cell culture medium. Differences in the composition and concentration of these antibiotics may, however, result in interlaboratory differences.

1. Although adding an extra layer of ECM has the advantage of long-term expression of liver

specific genes, soluble factors, including insulin and glucocorticoids, are still required for maintaining the *in vivo*-like hepatic phenotype ***(4,16)***. Other additives commonly used, such as serum ***(17)*** and L-proline ***(18)***, can be omitted from collagen gel cultures.

1. It is recommended to resuspend the cell pellet in a small volume. Care should be taken to

avoid clot formation. Thereafter, the solution can be further diluted.

1. Freshly isolated hepatocytes can also be used for setting up sandwich culture systems.

However, the lack of suitable liver tissue for *in vitro* modelling purposes is a ubiquitous problem. For this reason, primary hepatocytes freshly isolated from species other than human, such as rodents, dogs and monkeys, are sometimes used. When doing so interspecies differences must be taken into account ***(10,19)***.

1. Thawing Matrigel™ on wet ice takes about 8 h. Therefore, thawing overnight is

advised. During preparation of the Matrigel™ working solution, prechilled pipettes (4°C) are recommended to avoid polymerization. As the solution is delicate, mixing should be done by carefully inverting the tube 3 times. Besides Matrigel™, other natural and synthetic matrices can be used as an overlay. However, based on own research results, Matrigel™ together with a first layer of collagen type I are preferred for embedding the human hepatocytes.

1. Sandwich cultures require daily cell culture medium changes due to accumulation of toxic

metabolites.

10. When aspirating cell culture medium, care should be taken to avoid damaging or accidentally removing the overlay.

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