Takara Bio Europe AB

Cellartis® Hepatocyte Differentiation Kit User Manual

Cat. No. Y30050
(022818)

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I. Introduction

The Cellartis Hepatocyte Differentiation Kit contains complete media and ready-to-use coating for the differentiation of definitive endoderm (DE) cells into hepatocytes. This kit consists of one coating solution, four different media, and one supplement. All methods described in this user manual are for the differentiation of DE cells into hepatocytes in 2D monolayer cultures in 24- or 96-well plates. Other formats can be used, but optimization may be required. One kit is sufficient to differentiate DE cells into hepatocytes on a total culture area of 50 cm^2 and for a culture period of up to 25 days. For prolonged culture, up to at least day 35, it is recommended to use Cellartis Hepatocyte Maintenance Medium (Cat. No. Y30051).

The differentiated hepatocytes are ready to be used starting from day 14 after seeding or thawing of the DE cells. The usage window for applications that require high CYP activity is from day 16 onward. For optimal cell performance, it is recommended to change the medium the day before starting an assay.

This product should only be handled by persons who have been trained in laboratory techniques and should only be used in accordance with the principles of good cell culture practice. Takara Bio Europe AB recommends the use of media and reagents according to this manual. Takara Bio Europe AB cannot guarantee correct technical feedback on customer cultures unless the below culture instructions have been followed.

II. List of Components

- **Cellartis Hepatocyte Differentiation Kit (Cat. No. Y30050)**
  - 1 bottle Hepatocyte Thawing and Seeding Medium (1) (25 ml)
  - 1 bottle Hepatocyte Progenitor Medium (2) (50 ml)
  - 2 bottles Hepatocyte Maturation Medium Base (3A) (24 ml)
  - 1 tube Hepatocyte Maturation Medium Supplement (3B) (2.6 ml)
  - 3 bottles Hepatocyte Maintenance Medium (4) (50 ml)
  - 1 tube Hepatocyte Coating (7.5 ml)

III. Additional Materials Required

The following materials are required but not supplied:

- Human definitive endoderm cells.
  - Frozen DE cells:
    - Cellartis Definitive Endoderm Cells (from ChiPSC18) (Cat. No. Y10040)

    or
  - Kit for differentiation of pluripotent stem cells into DE cells:
    - Cellartis Definitive Endoderm Differentiation Kit with DEF-CS Culture System (Cat. No. Y30035)
- Fetal bovine serum (FBS)* or KnockOut Serum Replacement (KO-SR)*
- PBS Dulbecco's w/o Ca^{2+} & Mg^{2+} (D-PBS –/–)*
- TrypLE Select Enzyme (1X), without phenol red*
- Cellartis Hepatocyte Maintenance Medium (Cat. No. Y30051)**
- Equipment to check or control the temperature at 16–18°C, such as a water bath, a Biosafety Cabinet with Temp-Zone area or an infrared thermometer.
- 96-well plates, flat bottom, cell-culture treated
- 24-well plates, flat bottom, cell-culture treated
- General cell culture equipment used in cell culture laboratory

*If starting the hepatocyte differentiation from fresh DE cells derived with the Cellartis Definitive Endoderm Differentiation Kit with DEF-CS Culture System.

**If prolonged culture (more than 25 days after seeding the DE cells) is desired.

IV. General Considerations

A. Storage and Handling

Store all components of the Cellartis Hepatocyte Differentiation Kit at –20°C; shelf life is specified on the product label. For recommended thawing instructions see section referred to in Table I. Alternative thawing procedures can be found in Appendix B.

Table I. Thawing instructions and storage after thawing.

Component	Thawing instructions
Hepatocyte Coating	Section V
Hepatocyte Thawing and Seeding Medium (1)	Section V
Hepatocyte Progenitor Medium (2)	Section VI
Hepatocyte Maturation Medium Base (3A)	Section VII
Hepatocyte Maturation Medium Supplement (3B)	Section VII
Hepatocyte Maintenance Medium (4)	Section VIII

B. Starting Material

It is recommended to use Cellartis Definitive Endoderm Cells or DE cells derived using the Cellartis Definitive Endoderm Differentiation Kit as starting material.

C. Medium Changes

The medium changes should be performed with great care and the least disturbance to the cells. The following are general considerations for medium changes:

IMPORTANT:

- Using the Hepatocyte Maturation Medium (on day 7 and day 9) will result in a gelatinous layer of partly solidified medium components. This gelatinized matrix overlay **should not be disrupted or aspirated at any time**. Add fresh medium to the wall of the cell culture vessel, not directly onto the cell layer.
- Always use manual pipetting (never use a vacuum pump) to remove roughly 90% of the medium from each well. Perform the medium change on 4–12 wells at a time so the cells do not dry.
- 0.5 ml medium/cm^2 is used throughout the differentiation procedure.
- It is possible to temporarily aspirate the medium prior to photo imaging. Try to take the images within 1–2 minutes after the medium is aspirated. Then immediately add medium back to the well.
- Always discard any leftover warmed medium.

D. Suggested Schedule for the Cellartis Hepatocyte Differentiation Kit.

For a suggested schedule for differentiating your cells into hepatocytes and for information on handling of kit components, see Appendix C.

V. Day 0: Seeding of the Definitive Endoderm Cells

Hepatocyte Thawing and Seeding Medium (1) and Hepatocyte Coating are used for thawing and seeding of Cellartis Definitive Endoderm Cells (from ChiPSC18) or re-seeding the DE cells derived with Cellartis DE differentiation kit.

- DE cells derived using the Cellartis Definitive Endoderm Differentiation Kit can be dissociated at day seven after the start of differentiation and used as starting material for hepatic differentiation.
- One vial of Cellartis Definitive Endoderm Cells (from ChiPSC18) containing 6 x 10^6 viable cells is enough for 50 cm^2 of culture area (i.e., 24 wells in a 24-well plate or 150 wells in 96-well plates).

NOTE: If using other sources of frozen DE cells, the seeding density might need to be optimized. The cells should be seeded with at least 1.25–1.50 x 10^5 cells/cm^2. For example, use a cell concentration of 2.5–3.0 x 10^5 cells/ml and seed 0.5 ml cell suspension/cm^2.

A. Preparation

1. Thaw Hepatocyte Thawing and Seeding Medium at 37°C ± 1°C on the day of use.
2. Thaw Hepatocyte Coating at 2–8°C on the day of use.
3. Coat the cell culture vessels of your choice. One tube of Hepatocyte Coating is enough for 50 cm^2 of culture area (24 wells in a 24-well plate or 150 wells in 96-well plates).
    a. Add the Hepatocyte Coating to the cell culture vessels (0.15 ml/cm^2). Make sure the entire surface is covered.
    b. Incubate at RT for at least 30 min.
4. Remove excess Hepatocyte Coating from the cell culture vessels just before seeding. Do not let the surface dry out.

If starting with frozen Cellartis Definitive Endoderm Cells (from ChiPSC18), go to Step B.

If starting with fresh DE cells derived using the Cellartis DE differentiation kit, go to Step C.

B. Thawing of Frozen Cellartis Definitive Endoderm Cells

NOTE—FOR YOUR PROTECTION: Wear a protective face mask and protective gloves. Use forceps when handling a frozen vial. Never hold the vial in your hand as the cryovial may explode due to rapid temperature changes.

1. Transfer, as quickly as possible, the frozen vials from liquid nitrogen to a 37°C ± 1°C water bath using forceps.
2. Thaw the cells by gently pushing the vial under the surface of the water, without swirling the vial. **Do not submerge the cap of the vial in the water bath as this could contaminate the cells.**
3. After approximately 1 min, check if the cell suspension is thawed by carefully turning the vial upside down.
4. Once thawed, decontaminate the external surface of the vial with an appropriate disinfectant, place the vial into the biological safety cabinet, and pour the cell suspension into 24 ml of 37°C ± 1°C Hepatocyte Thawing and Seeding Medium (1). Rinse the vial with 1 ml of 37°C ± 1°C Hepatocyte Thawing and Seeding Medium (1) and add to the cell suspension.
5. Mix the cell suspension by inverting the tube with cell suspension carefully approximately three times. Thawed Cellartis Definitive Endoderm Cells (from ChiPSC18) are fragile. Avoid using a pipette for mixing the cell suspension.
6. Remove the Hepatocyte Coating from the cell culture vessels just prior to seeding the cells.
7. Using a pipette, seed 0.5 ml cell suspension per cm^2. Mix the cell suspension in between by swirling or inverting the tube.
8. Place the cell culture vessels in an incubator at 37°C ± 1°C, 5% CO_2, and ≥90% humidity and leave undisturbed overnight. The first medium change is performed on day two after seeding (see Section VI).

C. Dissociation and Seeding of Fresh Definitive Endoderm Cells

1. Warm TrypLE Select Enzyme (1X) and D-PBS –/– to 37°C ± 1°C.
2. Prepare an appropriate volume of 10% FBS or KO-SR in D-PBS–/– and warm to 37°C ± 1°C.
3. Remove the media from the culture vessels with a pipette or vacuum pump.
4. Wash the DE cell cultures with warm D-PBS –/– (0.1 ml/cm^2).
5. Remove the D-PBS from the cell cultures with a pipette or vacuum pump.
6. Add the warm TrypLE Select Enzyme to the DE cell cultures (0.1 ml/cm^2).
7. Incubate the cell cultures in the incubator at 37°C ± 1°C, 5% CO2, and >90% humidity for 3–5 minutes, until the cells have detached.
8. Transfer the cell suspension to the a 50-ml tube.
9. Rinse the cell culture vessels with 10% FBS or KO-SR in D-PBS –/– (0.1 ml/cm^2) using a pipette and transfer to the 50-ml tube to achieve a 1:1 dilution of the cell suspension.
10. Count the viable cells using a hemocytometer. Take 50 µl of the cell suspension, add 5 µl trypan blue, count the cells, and multiply the counted viable cells by 1.1 to get the concentration of viable cells.
11. Transfer a volume of the cell suspension corresponding to 6.5 x 10^6 cells to a centrifugation tube.

12. Centrifuge the cell suspension at 300 x g for 5 min at RT and resuspend the cell pellet in 25 ml Hepatocyte Thawing and Seeding Medium (1) to achieve a concentration of 2.6 x 10^5 cells/ml.
13. Seed 0.5 ml cell suspension per cm^2, corresponding to 1.2–1.3 x 10^5 cells/cm^2. It is crucial to use the correct seeding density to achieve successful hepatic differentiation.
14. Place the cell culture vessels in an incubator at 37°C ± 1°C, 5% CO_2, and ≥90% humidity and leave untouched overnight. The first medium change is performed on day two after seeding (see Section VI).

VI. Days 2 and 4: Medium Changes

On days two and four of differentiation, Hepatocyte Progenitor Medium (2) is used for medium changes. Thawed Hepatocyte Progenitor Medium (2) can be stored for up to two weeks at 2–8°C. Do not refreeze the thawed Hepatocyte Progenitor Medium (2).

A. Preparations, Day 2

1. Thaw the bottle of Hepatocyte Progenitor Medium (2) at RT and transfer 25 ml to a sterile tube for use on day 2. Store the remaining medium at 2–8°C for use on day 4.

B. Medium Change, Days 2 and 4

1. Warm 25 ml of Hepatocyte Progenitor Medium (2) to 37°C ± 1°C.
2. Aspirate the medium from the cell culture vessels using a manual pipette according to Section IV.C and discard.
3. Carefully add warm Hepatocyte Progenitor Medium (2) to the cell culture plate, at 0.5 ml/cm^2.
4. Place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and ≥90% humidity.

VII. Days 7 and 9: Medium Changes

On days 7 and 9 of differentiation, Hepatocyte Maturation Medium is prepared by adding Hepatocyte Maturation Medium Supplement (3B) to Hepatocyte Maturation Medium Base (3A). Hepatocyte Maturation Medium Supplement (3B) can be stored for up to three days at 2–8°C. Do not refreeze the thawed Hepatocyte Maturation Medium Supplement (3B).

NOTES:

- Be very careful when changing the medium from day 9 onward. Some components of the Hepatocyte Maturation Medium (used on day 7 and day 9) gelatinize on top of the cell layer, and this gelatinized matrix overlay **should not be disrupted or aspirated at any time** (Figure 1).
- Hepatocyte Maturation Medium Base (3A) is light sensitive; avoid unnecessary exposure to light.
- Hepatocyte Maturation Medium Base (3A) contains DMSO. Therefore, use nitrile gloves when preparing and changing medium and discard old medium in a closed container as hazardous waste.

A. Preparations

Day 7:

1. Thaw Hepatocyte Maturation Medium Supplement (3B) at 2–8°C and keep cold.

Days 7 and 9 (day of use):

1. Place one bottle of Hepatocyte Maturation Medium Base (3A) at 16–18°C or at RT. If placed at RT, it is important to check the temperature and use the medium when it reaches 16–18°C. This can be done either by measuring the temperature with an infrared thermometer or by placing the bottle of Hepatocyte Maturation Medium Base (3A) in a water bath at <15°C and measure the temperature of the water bath with a regular thermometer.
2. Add 1.3 ml of Maturation Medium Supplement (3B) (at 2–8°C) to the bottle of Hepatocyte Maturation Medium Base (3A) (at 16–18°C) to make the complete Hepatocyte Maturation Medium.
3. Mix gently and use immediately.

B. Medium Change on Day 7

1. Remove the medium from the cell culture vessels according to Section IV.C and discard.
2. Carefully add warm Hepatocyte Maturation Medium (at 16–18°C) to the cell culture plate, at 0.5 ml/cm^2.

3. Place the cells in an incubator at 37°C ±1 °C, 5% CO_2, and ≥90% humidity.
4. Leave the cells undisturbed in the incubator for 48 hr. **Do not move the cells on day 8 – not even to look at them in the microscope.** If the cells are moved, there is a great risk that the overlay will form unevenly, and this will have a significant negative effect on differentiation.

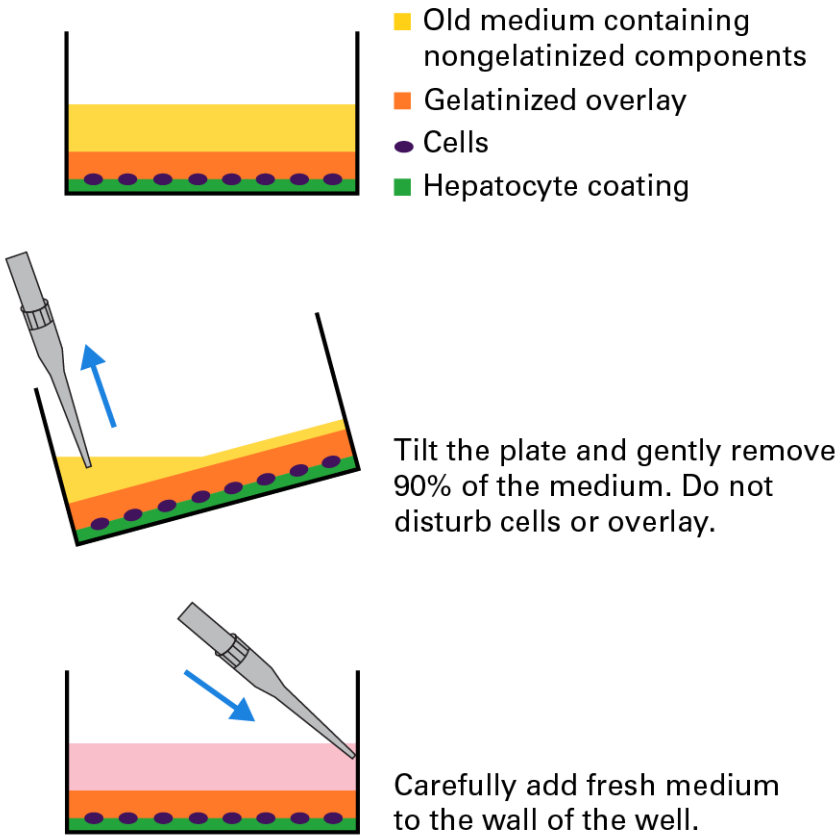


Figure 1. Schematic representation of a medium change from day 9 onward. Use a manual single- or multi-channel pipette for removing 90% of the old medium (shown in yellow) and replacing it with fresh medium (shown in pink). Take great care not to disturb the gelatinized overlay, shown here in orange.

C. Medium Change on Day 9

1. Remove the medium from the cell culture vessels according to Section IV.C and discard.
2. Carefully and slowly add the Hepatocyte Maturation Medium (at 16–18°C) to the wall of the cell culture vessel. To avoid damaging the overlay, do not add the medium dropwise or at the center of the well. Use 0.5 ml/cm^2.
3. Place the cells in an incubator at 37°C ± 1 °C, 5% CO_2, and ≥90% humidity.
4. Leave the cells undisturbed in the incubator for 48 hr. **Do not move the cells on day 10 – not even to look at them in the microscope.** If the cells are moved, there is a great risk that the overlay will form unevenly, and this will have a significant negative effect on differentiation.

VIII. Day 11 Onward: Medium Changes

From day 11 onward, Hepatocyte Maintenance Medium (4) is used for medium changes. The recommended days for medium changes are Mondays, Wednesdays, and Fridays. No medium change is necessary during the weekend. Thawed Hepatocyte Maintenance Medium (4) should be stored at 2–8°C and used within three days. Do not refreeze the Hepatocyte Maintenance Medium (4).

NOTES:

- Be careful when the medium is changed; the gelatinized matrix overlay **should not be disrupted or aspirated at any time** (see Figure 1).
- Hepatocyte Maintenance Medium (4) is light sensitive, therefore avoid unnecessary exposure to light.
- Hepatocyte Maintenance Medium (4) contains DMSO. Therefore, use nitrile gloves when preparing and changing the medium and discard old medium in a closed container as hazardous waste.

A. Preparations

1. Thaw a bottle of Hepatocyte Maintenance Medium (4) at RT and transfer 25 ml to a sterile tube for use at the first medium change. Store the remaining medium at 2–8°C for use at the following medium change.

B. Medium Change

1. Warm 25 ml of Hepatocyte Maintenance Medium to 37°C ± 1°C.
2. Remove the medium from the cell culture vessels according to Section VII.B and discard, making sure not to disrupt or aspirate the gelatinized matrix overlay.
3. Carefully add Hepatocyte Maintenance Medium to the cell culture plate at 0.5 ml/cm^2.
4. Place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and ≥90% humidity.

The hepatocytes are ready to be used in your preferred application from day 14 (seeding of the DE cells is day 0) and can be maintained until at least day 25 if handled according to these instructions. Applications that require high CYP activity should be performed from day 16 onward. For optimal results, change the medium the day before starting an assay.

NOTE: The gelatinized matrix overlay contains components that might influence protein measurements and immunocytochemistry imaging.

Appendix A. Troubleshooting Guide

Table II. Troubleshooting Guide.

Problem	Possible Explanation	Solution
The cells die.	All medium has been aspirated at medium changes (i.e., the cells have been left dry too long).	Leave approximately 10% of the medium on the cells at every medium change.
The cells are not confluent.	The maintenance medium was not prewarmed to 37°C before medium change.	Always prewarm the medium to 37°C at medium changes on day 11 onward.
The cells are not confluent.	The DE cells were seeded too sparsely at day 0.	Use at least 1.25 x 10^5 cells/cm^2. If using frozen DE cells (other than Cellartis definitive endoderm cells), try a higher seeding density.
The gelatinized matrix overlay is detaching.	The gelatinized layer has been disrupted at medium changes.	Always change the medium very carefully with a manual pipette. Never use a vacuum pump for medium changes.
Precipitation is observed when mixing Hepatocyte Maturation Medium Base (3A) and supplement (3B).	The temperature of Hepatocyte Maturation Medium Base (3A) was too high when the supplement (3B) was added.	Closely monitor the temperature of the 3A medium (16–18°C) before adding the 3B supplement (2–8°C). Perform the medium change **immediately** after mixing the two components.

Appendix B. Instructions for Thawing Kit Components

Table III. Thawing Instructions for Cellartis Hepatocyte Differentiation Kit Components.

Component	Recommended thawing condition	Alternative thawing condition
Hepatocyte Coating	2–8°C on the day of use	2–8°C overnight
Hepatocyte Thawing and Seeding Medium (1)	37°C ± 1°C on the day of use	RT on the day of use or 2–8°C overnight
Hepatocyte Progenitor Medium (2)	RT on the day of use	2–8°C overnight
Hepatocyte Maturation Medium Base (3A)	RT on the day of use	2–8°C overnight
Hepatocyte Maturation Medium Supplement (3B)	2–8°C on the day of use	2–8°C overnight
Hepatocyte Maintenance Medium (4)	RT on the day of use	2–8°C overnight

Appendix C. Suggested Schedule for the Cellartis Hepatocyte Differentiation Kit

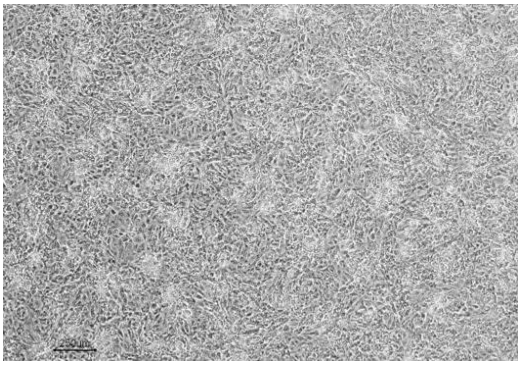
Table IV. Suggested Culture Schedule for the Cellartis Hepatocyte Differentiation Kit.

Day	Suggested Day	Action	Described in Section	Handling of Kit Components
0	Monday	Seeding of DE cells	V	Thaw Hepatocyte Coating at 2–8°C. Coat cell culture vessel(s). Thaw Hepatocyte Thawing and Seeding Medium (1) at 37°C.
1	Tuesday			
2	Wednesday	Medium change (MC) with progenitor medium	VI	Thaw Hepatocyte Progenitor Medium (2) at RT. Warm 25 ml to 37°C and store the remaining medium at 2–8°C until day 4.
3	Thursday			
4	Friday	MC with progenitor medium	VI	Warm 25 ml of Hepatocyte Progenitor Medium (2) to 37°C.
5	Saturday			
6	Sunday			
7	Monday	MC with maturation medium	VII	Thaw one bottle of Hepatocyte Maturation Medium Base (3A) at RT. Thaw Hepatocyte Maturation Medium Supplement (3B) at 2–8°C; keep cold. Prepare the complete Hepatocyte Maturation Medium by adding 1.3 ml of 3B to 24 ml of 3A.
8	Tuesday	**Do not disturb the cells**		
9	Wednesday	MC with maturation medium	VII	Thaw one bottle of Hepatocyte Maturation Medium Base (3A) at RT. Use the remaining Hepatocyte Maturation Medium Supplement (3B) to prepare the complete Hepatocyte Maturation Medium (4).
10	Thursday	**Do not disturb the cells**		
11	Friday	MC with maintenance medium	VIII	Thaw one bottle of Hepatocyte Maintenance Medium (4) at RT. Warm 25 ml to 37°C and store the remaining medium at 2–8°C until day 14.
12	Saturday			
13	Sunday			
14	Monday	MC with maintenance medium	VIII	Warm 25 ml of Hepatocyte Maintenance Medium (4) to 37°C.
15	Tuesday			
16	Wednesday	MC with maintenance medium	VIII	Thaw one bottle of Hepatocyte Maintenance Medium (4) at RT. Warm 25 ml to 37°C and store the remaining medium at 2–8°C until day 18.
17	Thursday			
18	Friday	MC with maintenance medium	VIII	Warm 25 ml of Hepatocyte Maintenance Medium (4) to 37°C.
19	Saturday			
20	Sunday			
21	Monday	MC with maintenance medium	VIII	Thaw one bottle of Hepatocyte Maintenance Medium (4) at RT. Warm 25 ml to 37°C and store the second aliquot at 2–8°C until day 23.
22	Tuesday			
23	Wednesday	MC with maintenance medium	VIII	Warm 25 ml of Hepatocyte Maintenance Medium (4) to 37°C.

Appendix D. Expected Morphology

These representative images show the morphology of the cells as they are differentiating into hepatocytes. Please note that morphology may vary between experiments performed with different cell lines.

A



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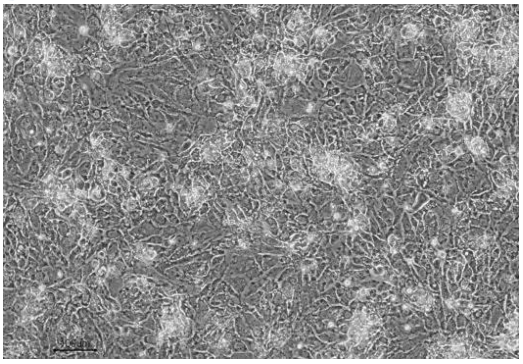
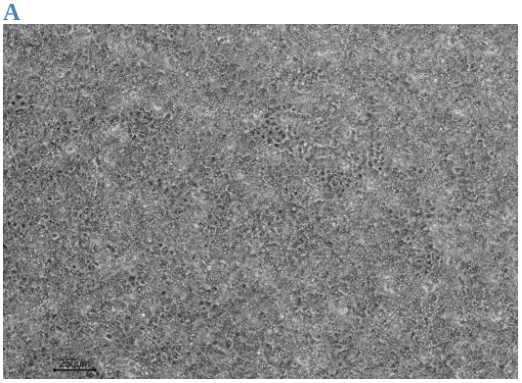


Figure 2. Day 2 morphology of a human iPS cell line differentiated using the Cellartis Hepatocyte Differentiation Kit. Scale bar corresponds to 250 µm **(Panel A)** and 100 µm **(Panel B).**

A



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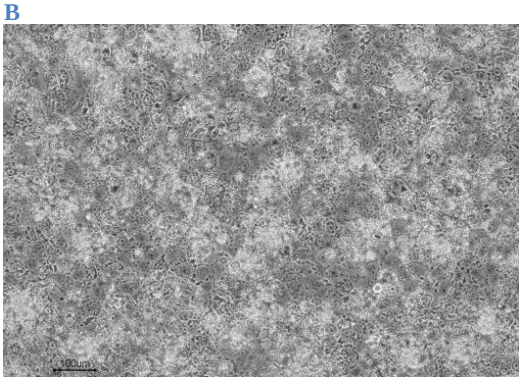
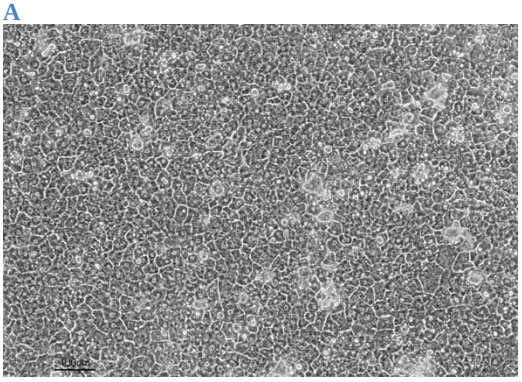


Figure 3. Day 7 morphology of a human iPS cell line differentiated using the Cellartis Hepatocyte Differentiation Kit. Scale bar corresponds to 250 µm **(Panel A)** and 100 µm **(Panel B).**

A



B

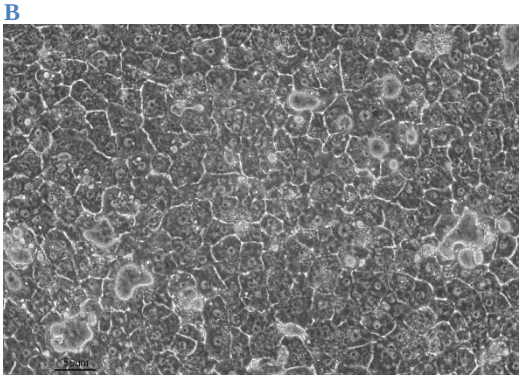


Figure 4. Day 23 morphology of a human iPS cell line differentiated using the Cellartis Hepatocyte Differentiation Kit. Scale bar corresponds to 100 µm **(Panel A)** and 50 µm **(Panel B).**

Appendix E. CYP Activity Assay

After 16 days in culture (seeding DE cells is day 0), the differentiated hepatocytes can be used in Cytochrome P450 (CYP) activity assays. Samples can be analyzed using LC/MS to measure the formation of the following specific metabolites: acetaminophen (CYP1A), 4-OH-Diclofenac (CYP2C9), 4-OH-Mephenytoin (CYP2C19), OH-Bufuralol (CYP2D6), and 1-OH-Midazolam (CYP3A).

A. Additional Material Required

- Williams' Medium E (WME), w/o L-glutamine or phenol red (Sigma-Aldrich, Cat. No. W1878)
- 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI)
- HEPES Solution, 1 M
- L-Glutamine Solution, 200 mM
- Penicillin/Streptomycin (PEST) (10,000 units/ml of penicillin and 10,000 µg/ml of streptomycin)
- Probe Substrate Cocktail:
    - Phenacetin (Sigma-Aldrich, Cat. No. 77440)
    - Bupropion (Sigma-Aldrich, Cat. No. B102)
    - Mephenytoin (Santa Cruz, Cat. No. sc-200975A)
    - Diclofenac (Sigma-Aldrich, Cat. No. D6899)
    - Bufuralol (Becton Dickinson, Cat. No. 451034)
    - Midazolam (Larodan, Cat. No. MID-111-HC)

B. Preparation

- Prepare a stock solution for the CYP substrate cocktail. In the assay, use the final concentrations listed in Table 3.
- Prepare medium to be used as washing medium and as basal medium for the CYP activity medium by adding 0.1% PEST to WME medium.
- Warm the appropriate volume of WME (0.1% PEST) for washing to 37°C ± 1°C.
- Prepare and warm CYP activity assay medium: WME (0.1% PEST), 25 mM HEPES, and 2 mM L-Glutamine. Add the CYP substrate cocktail just prior to use.

Table V. CYP Substrate Cocktail.

CYP	Substrate	Final Assay Concentration
1A	Phenacetin	10 µM
2B6	Bupropion	10 µM
2C19	Mephenytoin	50 µM
2C9	Diclofenac	10 µM
2D6	Bufuralol	10 µM
3A	Midazolam	5 µM

C. Activity Assay

1. Wash the differentiated hepatocytes twice very gently with 0.5 ml/cm^2 warm WME (0.1% PEST).
2. Add 110 µl/cm^2 warm CYP activity assay medium supplemented with CYP substrates to the cells.
3. Incubate for 2 hr at 37°C ± 1°C, 5% CO_2, and ≥90% humidity.
4. Collect 100 µl supernatant and store at –80°C until LC/MS analysis.
5. It is recommended that the cells are fixed after treatment and stained with DAPI. Using a fluorescence microscope, count the number of nuclei per field of view, then calculate the number of nuclei per well.
6. Normalize the metabolite concentrations measured by LC/MS to the number of nuclei per well and the assay duration (120 min).

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This document has been reviewed and approved by the Takara Bio Europe AB Quality Assurance Department.