Takara Bio Europe AB

Cellartis® iPS Cell to Hepatocyte Differentiation System User Manual

Cat. No. Y30055
(030218)

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I. Introduction

The Cellartis iPS Cell to Hepatocyte Differentiation System is a complete system for differentiation of human pluripotent stem (hPS) cells to hepatocytes via definitive endoderm (DE). The system includes three components: the Cellartis DEF-CS 100 Culture System, the Cellartis Definitive Endoderm Differentiation Kit, and the Cellartis Hepatocyte Differentiation Kit.

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 culture instructions listed below have been followed.

II. List of Components

Cellartis iPS Cell to Hepatocyte Differentiation System (Cat. No. Y30055)

- Cellartis DEF-CS 100 Culture System (Cat. No. Y30020; not sold separately. A larger 500 ml version of this product is sold as Cat. No. Y30010.)
  - 100 ml DEF-CS 100 Basal Medium
  - 800 µl DEF-CS COAT-1 (for 100 ml)
  - 300 µl DEF-CS GF-1 (for 100 ml)
  - 100 µl DEF-CS GF-2 (for 100 ml)
  - 40 µl DEF-CS GF-3 (for 100 ml)
- Cellartis Definitive Endoderm Differentiation Kit (Cat. No. Y30030; not sold separately)
  - 1 tube Definitive Endoderm Differentiation Coating (10 ml)
  - 1 bottle Definitive Endoderm Differentiation Day 0 (18 ml)
  - 1 bottle Definitive Endoderm Differentiation Day 1 (18 ml)
  - 1 bottle Definitive Endoderm Differentiation Day 2 (18 ml)
  - 1 bottle Definitive Endoderm Differentiation Day 3 (25 ml)
  - 1 bottle Definitive Endoderm Differentiation Day 4 (47 ml)
  - 1 bottle Definitive Endoderm Differentiation Day 6 (47 ml)
- 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 pluripotent stem cells, e.g.:
  - Cellartis Human ES Cell Line 121 (SA121) Kit (Cat No. Y00025)
  - Cellartis Human ES Cell Line 167 (SA167) Kit (Cat. No. Y00065)
  - Cellartis Human ES Cell Line 181 (SA181) Kit (Cat. No. Y00105)
  - Cellartis Human ES Cell Line 461 (SA461) Kit (Cat. No. Y00145)
  - Cellartis Human iPS Cell Line 7 (ChiPSC7) Kit (Cat. No. Y00275)
  - Cellartis Human iPS Cell Line 12 (ChiPSC12) Kit (Cat. No. Y00285)
  - Cellartis Human iPS Cell Line 18 (ChiPSC18) Kit (Cat. No. Y00305)
  - Cellartis Human iPS Cell Line 22 (ChiPSC22) Kit (Cat. No. Y00325)
- Fetal bovine serum (FBS) or KnockOut Serum Replacement (KO-SR)

- PBS Dulbecco's with Ca^{2+} & Mg^{2+} (D-PBS +/+)
- PBS Dulbecco's w/o Ca^{2+} & Mg^{2+} (D-PBS –/–)
- TrypLE Select Enzyme (1X), without phenol red
- 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.
- Cellartis Hepatocyte Maintenance Medium (Cat. No. Y30051)*
- 96-well plates, flat bottom, cell-culture treated
- 24-well plates, flat bottom, cell-culture treated
- Cell culture vessels, tissue-culture treated polystyrene surface
- General cell culture equipment used in cell culture laboratory

*If prolonged culture (more than 32 days from start of differentiation) is desired.

IV. General Considerations

The human pluripotent stem cell line of choice is transferred to the Cellartis DEF-CS culture system, adapted, and expanded before the start of differentiation to definitive endoderm (referred to as day 0). After seven days of differentiation, the DE cells are passaged and differentiation to hepatocytes continues. A schematic workflow is presented in Figure 1.

hPS cell lines previously grown in the Cellartis DEF-CS culture system can be cultured in the provided Cellartis DEF-CS 100 Culture System or Cellartis DEF-CS 500 Culture System according to the routines already established. To start the DE differentiation ≥3 x 10^6 cells are required and it is recommended to prepare cells in a T25 flask to have enough cells at start of differentiation.

For hPS cell lines previously grown in other culture systems, a recommended culture protocol for transferring hPS cells to DEF-CS culture system and upscaling of undifferentiated hPS cells to be used for DE differentiation, using Cellartis DEF-CS 100 Culture System, is described in Section V.

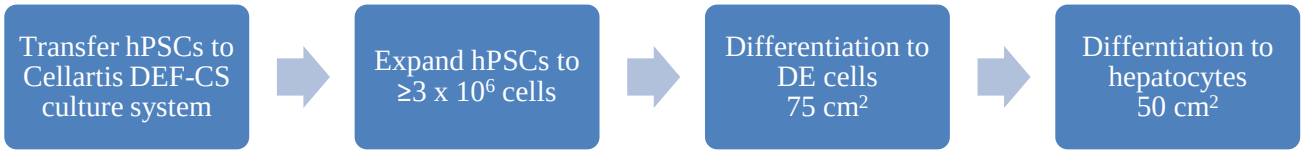

Figure 1. Schematic presentation of Cellartis hPS Cell to Hepatocyte Differentiation System. The indicated area corresponds to the culture area each kit provides reagent for.

A suggested schedule for differentiation of hPS cells to hepatocytes via definitive endoderm is presented in Appendix A.

NOTE: Always work under aseptic conditions.

A. Storage and Handling

1. Cellartis DEF-CS 100 Culture System

Store DEF-CS 100 Basal Medium and DEF-CS COAT-1 (for 100 ml) (COAT-1) at 2–8°C; shelf life specified on product label. The DEF-CS 100 Basal Medium formulation contains penicillin and streptomycin.

Store the DEF-CS additives; DEF-CS GF-1 (for 100 ml), DEF-CS GF-2 (for 100 ml), and DEF-CS GF-3 (for 100 ml) (GF-1, GF-2, and GF-3) at –20°C; shelf life specified on product label. At first use, thaw provided vials, and then gently mix and aliquot each component separately into appropriate volumes. Store aliquots at –20°C according to expiry date on original vial. Thawed vials may be stored at 2–8°C for up to one week. Do not re-freeze aliquots after thawing.

NOTE: All three DEF-CS additives (GF-1, GF-2, and GF-3) are used when thawing and passaging hPS cells. Only DEF-CS additives GF-1 and GF-2 are needed when changing medium on hPS cells.

2. Cellartis Definitive Endoderm Differentiation Kit

Store all components of the Cellartis Definitive Endoderm Differentiation Kit at –20°C; shelf life is specified on product label.

Use thawed Cellartis Definitive Endoderm Differentiation Coating and media – Cellartis Definitive Endoderm Differentiation Day 0, 1, 2, 3, 4, and 6 – on the same day that they are thawed. If thawed and stored at 2–8°C, leftover Cellartis Definitive Endoderm Differentiation Coating and media can be refrozen on the day of thawing.

Always discard warmed, leftover Cellartis Definitive Endoderm Differentiation Coating and media.

3. Cellartis Hepatocyte Differentiation Kit

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 VII.B
Hepatocyte Thawing and Seeding Medium (1)	Section VII.B
Hepatocyte Progenitor Medium (2)	Section VII.C
Hepatocyte Maturation Medium Base (3A)	Section VII.D
Hepatocyte Maturation Medium Supplement (3B)	Section VII.D
Hepatocyte Maintenance Medium (4)	Section VII.E

V. Transferring hPS Cells to the Cellartis DEF-CS Culture System and Upscaling of hPS Cells Prior to Start of DE Differentiation

A. General information

hPS cell lines that are maintained in Cellartis DEF-CS culture system should be passaged every three to four days, with daily medium changes. As a general rule, cells should be seeded at a density of 4.0–5.0 x 10^4 cells/cm^2, resulting in near confluency one day after passage (see Figure 3 in Section VIII). The cells are not ready to be passaged at this stage, but should be grown until they have the morphology displayed in Figure 4 and Figure 5 in Section VIII, representing a density of 1.5–3.0 x 10^5 cells/cm^2, on the day of passage. It is highly recommended to culture hPS cells in flask format for routine culture. A suggested weekly schedule is described in Table II. For detailed information on routine culture using the Cellartis DEF-CS culture system, see the Cellartis DEF-CS Culture System User Manual.

Table II. Weekly schedule for medium changes and passaging.

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Passage	Change medium	Change medium	Passage	Change medium	–	Change medium

Undifferentiated hPS cells maintained in other culture systems can be readily transferred to the Cellartis DEF-CS culture system. Fresh cultures can be transferred at passage (Section V.E) and cryopreserved cultures can be thawed directly into the Cellartis DEF-CS culture system (Section V.D). It takes between two and five passages to adapt a cell line to the Cellartis DEF-CS culture system. The cell density on the day after transfer/passage can vary considerably and the cells might also initially grow at a slightly slower rate. A suitable passage interval might therefore be between two and seven days for the first few passages. The cells are ready for passage when they have acquired the morphology displayed in Figure 4 and Figure 5 (Section VIII); however, if the cells are sparse after seven days in culture, a passage is still recommended. A suggested culture schedule for transfer to the Cellartis DEF-CS culture system is depicted in Table III.

When initially transferring hPS cells to this system, some cell characteristics might be different from what was observed in previously used culture systems:

- The Cellartis DEF-CS culture system utilizes single-cell suspension passaging. The cells grow in a homogeneous monolayer and do not form colonies.
- Newly passaged cells tend to spread out. The atypical spread morphology is especially distinguishable if the cells are sparse, as displayed in Figure 3 (Section VIII). Even though the cells have an atypical morphology they are still undifferentiated, see Figure 6 (Section VIII). When proliferating, the cells get dense and show the typical undifferentiated stem cell morphology (i.e., high nucleus to cytoplasm ratio, defined borders, and prominent nucleoli; see Figure 4 and Figure 5, Section VIII).

Table III. Suggested culture scheme during transfer and upscaling of hPS cells for start of DE differentiation.

	Recommended culture vessel	COAT-1 dilution	Seeding density (cells/cm^2)	Passage interval (days)
Passage 1 or after thawing	One well of a 12-well plate (or 24-well plate if cell numbers are limiting)	1:5	5–8 x 10^4	2–7
Passage 2	T12.5 flask (or 12-well plate if cell numbers are limiting)	1:5	5–6 x 10^4	3–6
Passage 3	T12.5 flask	1:20	5–6 x 10^4	3–5
Passage 4	T25 flask	1:20	4–5 x 10^4	3–4
Passage 5	T25 flask	1:20	4–5 x 10^4	3–4

B. Coating Cell Culture Vessels

1. Dilute the required volume of COAT-1 in D-PBS +/+ before use (Table III and Table IV).
2. Mix the diluted COAT-1 solution gently and thoroughly by pipetting up and down.
3. Add the appropriate volume of diluted COAT-1 solution to the cell culture vessels (Table IV), make sure the entire surface is covered.
4. Place the cell culture vessels for a minimum of 20 min in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity or 0.5–3 hr at room temperature (RT, 15–25°C).
5. Aspirate the COAT-1 solution from cell culture vessels just before seeding of the cells.

Table IV. Recommended volumes of COAT-1 for different cell culture vessels.

Format	24-well	12-well	6-well	T12.5 flask	T25 flask
COAT-1 solution (ml)	0.4	0.8	1.5	1.25	2.5

C. Preparing Cellartis DEF-CS Medium

1. Medium for Thawing or Passaging hPS Cells

1. Decontaminate the external surface of all additives and the medium bottle with an appropriate disinfectant and place in the biological safety cabinet.
2. Prepare the appropriate volume (Table V or Table VI) of "Cellartis DEF-CS medium for thawing or passaging" by adding DEF-CS GF-1 (dilute 1:333), GF-2 (dilute 1:1,000), and GF-3 (dilute 1:1,000) to Cellartis DEF-CS basal medium.
3. Prepare fresh medium on the day of intended use.

Table V. Recommended volumes for seeding of the cell suspension at thawing, for different cell culture vessels

Format	24-well	12-well	6-well	T12.5 flask	T25 flask
DEF-CS medium (ml)	1.0	2.0	4.0	4.0	7.0

Table VI. Recommended volumes for seeding of the cell suspension at passage, for different cell culture vessels

Format	24-well	12-well	6-well	T12.5 flask	T25 flask
DEF-CS medium (ml)	0.5	1.0	2.0	3.0	4.0

2. Medium for Maintenance of hPS Cells

1. Decontaminate the external surface of all additives and the medium bottle with an appropriate disinfectant and place into the biological safety cabinet.
2. Prepare the appropriate volume (Table VII) of "Cellartis DEF-CS medium for maintenance" by adding DEF-CS GF-1 (dilute 1:333) and GF-2 (dilute 1:1,000) to Cellartis DEF-CS basal medium
   NOTE: Do not add DEF-CS GF-3 to the maintenance medium.
3. Prepare fresh medium on the day of intended use.

Table VII. Recommended volumes of DEF-CS medium at medium change, for different cell culture vessels

Format	24-well	12-well	6-well	T12.5 flask	T25 flask
DEF-CS medium (ml)	1.0	2.0	4.0	4.0	7.0

D. Transferring Frozen hPS Cells into the Cellartis DEF-CS Culture System

Cryopreserved cells, cultured in other culture systems prior to freezing, can be thawed directly into the DEF-CS Culture System. It is recommended to thaw 1 x 10^6 hPS cells into one well of a 12-well plate.

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. Preparation

- Coat cell culture vessels as described in Section V.B. **Use COAT-1 at a 1:5 dilution.**
- Prepare the appropriate volume of Cellartis DEF-CS medium for thawing or passaging:
  - Two sterile centrifuge tubes with 4 ml and 1 ml for the thawing process (adjust the temperature to RT)
  - Appropriate volume for seeding, as described (Section V.C.1; warm to 37°C ± 1°C).
- Warm all other reagents to RT before use.

2. Thawing into the Cellartis DEF-CS culture system

1. Using forceps, transfer the vial directly from liquid nitrogen into a 37°C ± 1°C water bath. Thaw the vial by gently pushing it under the surface of the water. Do not submerge the cap of the vial in the water bath, as this could contaminate the cells.
2. Allow the vial to thaw until the cell suspension can be poured out of the vial. (It is okay if the suspension has a slushy consistency, as long as it can be poured out.)
3. Decontaminate the vial with an appropriate disinfectant.
4. Pour the entire contents of the vial into the sterile tube containing 4 ml supplemented Cellartis DEF-CS medium (RT).
5. Rinse the vial with 1 ml supplemented Cellartis DEF-CS medium, warmed to RT. Add to the cell suspension.
6. Centrifuge at 300 x g for 1 minute.
7. After centrifugation, aspirate the supernatant and gently resuspend the pellet in the appropriate volume of supplemented Cellartis DEF-CS medium for thawing or passaging; see Table V (37°C ± 1°C).
8. Aspirate the COAT-1 solution from the cell culture vessel.
9. Pipet the cell suspension into the cell culture vessel.
10. Ensure that the cells and medium are evenly distributed across the surface of the cell culture vessel, and place the cell culture vessel in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

E. Transferring Fresh hPS cells into the Cellartis DEF-CS Culture System

Fresh cultures maintained in other culture systems can be transferred on their scheduled passage day. It is recommended to dissociate and seed in the Cellartis DEF-CS culture system according to the protocol below. It is important to use a seeding density of 5.0–8.0 x 10^4 cells/cm^2.

The cells can also be dissociated according to the protocol of the previous culture system. In that case, they are to be seeded as single cells or aggregates using a 1:1 or 1:2 split ratio based on culture area.

1. Preparation

- Coat cell culture vessels as described in Section V.B. **Use COAT-1 at a 1:5 dilution.**
- Prepare the appropriate volume of supplemented Cellartis DEF-CS medium for thawing or passaging as described above (Section V.C.1) and warm it to 37°C ± 1°C before use.
    a. Medium for suspension of the cells prior to counting; see Table VIII.
    b. Medium for resuspension of the cell pellet after centrifugation; see Table VIII.
    c. Medium for seeding; see Table VI.
- Warm all other reagents to RT before use.

2. Passaging into the Cellartis DEF-CS Culture System

1. Check the cells under a microscope; photo document as necessary.
2. Aspirate the medium from the cell culture vessels and wash the cell layer once with D-PBS –/–.
3. Add TrypLE Select Enzyme to the cell culture vessels (for volumes, Table VIII) and incubate at 37°C ± 1°C for 5 min or until the cell layer has detached. Detachment can be aided by tapping the side of the cell culture vessel firmly but gently. It is not recommended to tilt or swirl the cell culture vessel.
4. Neutralize the TrypLE Select Enzyme by suspending the cells in the appropriate volume of Cellartis DEF-CS medium for thawing or passaging (see Table VIII) and pipet up and down several times to ensure a single-cell suspension. (The cells will aggregate if left too long in TrypLE Select Enzyme.)
5. Count the cells in a hemocytometer or in a cell counter (using optimized settings for hPS cells).
6. Centrifuge the cells at 200 x g for 2–5 min.
7. Resuspend the cell pellet in the appropriate volume of Cellartis DEF-CS medium for thawing or passaging (see Table VIII) and pipet up and down several times to ensure a single-cell suspension.
8. Aspirate the COAT-1 solution from the cell culture vessel.
9. Add the appropriate volume of cell suspension to the cell culture vessels to obtain the desired density (5–8 x 10^4 cells/cm^2). Adjust the medium volume (see Table VI).
10. Tilt the flask backwards and forwards gently to ensure that the cell suspension is dispersed evenly over the surface, then place in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

Table VIII. Recommended volumes of TrypLE Select Enzyme (1X) and Cellartis DEF-CS medium when passaging hPS cells from different cell culture vessels.

Culture vessel (cells to be passaged)	24-well	12-well	6-well	T12.5 flask	T25 flask
TrypLE Select Enzyme (1X) (ml)	0.1	0.2	0.3	0.3	0.5
DEF-CS medium used for suspension (before counting) (ml)	0.4	0.8	1.7	1.7	2
DEF-CS medium used for re-suspension (after centrifugation) (ml)	2	4	4	7	10

F. Passaging hPS Cell Lines in the Cellartis DEF-CS Culture System

1. Preparation

- Coat cell culture vessels as described (Section V.B). For the second passage in the Cellartis DEF-CS culture system, use COAT-1 at a 1:5 dilution, for the third passage onward use COAT-1 at a 1:20 dilution; see Table III.
- Prepare the appropriate volume of supplemented Cellartis DEF-CS medium for thawing or passaging as described (Section V.C.1) and warm it to 37°C ± 1°C before use.

- Warm all other reagents to RT before use.

2. Passaging

1. Check the cells under a microscope; photo document as necessary.
2. Aspirate the medium from the cell culture vessels and wash the cell layer once with D-PBS –/–.
3. Add TrypLE Select Enzyme to the cell culture vessels (for volumes, see Table VIII) and incubate at 37°C ± 1°C for 5 min or until the cell layer has detached (10 min maximum). Detachment can be aided by tapping the side of the cell culture vessels firmly but gently. It is not recommended to tilt or swirl the cell culture vessel.
4. Neutralize the TrypLE Select Enzyme by resuspending the cells in Cellartis DEF-CS medium for thawing or passaging (for volumes, see Table VIII) and pipet up and down several times to ensure a single cell suspension. (The cells will aggregate if left too long in TrypLE Select Enzyme.)
5. **OPTIONAL –** to remove TrypLE Select Enzyme, centrifuge at 200 x g for 2–5 min. There is no need to remove the TrypLE Select Enzyme after dissociation if the TrypLE Select Enzyme will be diluted at least 1:10 in the final cell suspension after seeding.
6. Count the cells in a hemocytometer or in a cell counter (using optimized settings for pluripotent stem cells).
7. Aspirate the COAT-1 solution from the cell culture vessel.
8. Add the appropriate volume of cell suspension to the newly coated cell culture vessels to obtain the selected density. Adjust the medium volume of Cellartis DEF-CS medium for thawing or passaging (see Table VI) for the culture vessel used.
9. Tilt the flask backwards and forwards gently to ensure that the cell suspension is dispersed evenly over the surface, and then place in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

G. Changing the Medium in the Cellartis DEF-CS Culture System

1. Preparation

Prepare the appropriate volume of Cellartis DEF-CS medium for maintenance as described above (Section V.C.2) and warm it to 37°C ± 1°C before use.

NOTE: Do not add Cellartis DEF-CS GF-3 to the Cellartis DEF-CS medium for maintenance.

2. Medium Change

1. Check cells under microscope; photo document as necessary.
2. Carefully aspirate the medium and pipet newly warmed medium into the cell culture flask. Avoid flushing medium directly onto the cell layer.
3. Place the cell culture flask in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

VI. Differentiation of hPS Cells to Definitive Endoderm Cells

A. General information

The Cellartis Definitive Endoderm Differentiation Kit is optimized for use with the Cellartis DEF-CS culture system. All procedures described in the manual are optimized for hPS cells cultured in the Cellartis DEF-CS culture system. If cells are maintained in culture systems other than the Cellartis DEF-CS culture system, the seeding density may have to be adjusted to get the cell densities shown in Figure 7, Section IX. If seeded too sparsely, the cells will die during differentiation, and if seeded too densely, the obtained DE cells may not be homogeneous.

The Cellartis Definitive Endoderm Differentiation Kit consists of six complete media and one ready-to-use coating solution, and it is optimized for differentiation in 2D culture. One kit is designed to differentiate hPS cells to DE cells on a total surface area of 75 cm^2. The smallest recommended format is a 6-well plate. The recommended culture schedule is presented in Appendix A.

This protocol for DE differentiation has generated DE cells from more than 25 human pluripotent stem cell lines, resulting in ≥80% SOX17 positive cells.

- Examples of cell morphology during differentiation are shown in Figure 7 and Figure 8. Morphology may vary depending on cell lines and differences in cell densities.
- It is possible to freeze the cells at day 7, for a recommended freezing protocol, see Appendix C.

B. Coating and Media Volumes

Coating and media volumes for different cell culture vessels are listed in Table IX. The smallest recommended format is a 6-well plate.

Table IX. Media Volumes for Cellartis Definitive Endoderm Differentiation Kit

Format	Coating	Days 0, 1 and 2	Day 3	Day 4 and 6
6-well plate	1 ml	2 ml	2.7 ml	5 ml
T25 flask	2.5 ml	5 ml	7 ml	13 ml
T75 flask	7.5 ml	15 ml	20 ml	40 ml

C. Day 0: Start Definitive Endoderm Differentiation

1. Preparation

- Thaw Definitive Endoderm Differentiation Coating and warm the appropriate volume (see Table IX) to RT.
- Thaw Cellartis Definitive Endoderm Differentiation Day 0 and warm the appropriate volume (see Table IX) to 37°C ± 1°C.

2. Coating Cell Culture Vessels

1. Add Definitive Endoderm Differentiation Coating solution to the cell culture vessels (0.1 ml/cm^2). Make sure the entire surface of each vessel is covered.
2. Incubate at RT for 30–120 min.
3. Aspirate the Definitive Endoderm Differentiation Coating solution from the cell culture vessels just before seeding.

3. Seeding Cells

1. Detach and dissociate the hPS cells into a single-cell suspension using TrypLE Select Enzyme.
2. Count the cells.
3. Transfer the appropriate number of cells to a 15-ml tube. You need enough cells to end up with a cell density of 4.0 x 10^4 cells/cm^2 in the cell culture vessel. (For a T75 flask, you need 4.0 x 10^4 cells/cm^2 x 75 cm^2 = 3.0 x 10^6 cells.)
4. Centrifuge the hPS cell suspension for 5 min at 200 x g at RT.
5. Remove the supernatant and resuspend the cells in Cellartis Definitive Endoderm Differentiation Day 0 and seed in the coated cell culture vessels. Resuspend to a cell concentration of 2.0 x 10^5 cells/ml and add 0.2 ml cell suspension/cm^2 to the culture vessel. (For a T75 flask; add 15 ml of cell suspension (2.0 x 10^5 cells/ml) to the T75 flask, which will result in a final cell density of 4.0 x 10^4 cells/cm^2).
6. Place the cell culture vessels in the incubator at 37°C ± 1°C, 5% CO_2, and ≥90% humidity.

D. Day 1: Change Medium

NOTE: Use nitrile gloves when preparing and changing medium, and discard any remaining medium in a closed container as hazardous waste.

1. Preparation

- Thaw Cellartis Definitive Endoderm Differentiation Day 1 and warm the appropriate volume (see Table IX) to 37°C ± 1°C.

2. Medium Change

1. Remove the medium from the culture vessels with a pipette or vacuum pump.
2. Add warm Definitive Endoderm Differentiation Day 1 according to the volumes stated in Table IX.
3. Return the cell culture vessels to the incubator (37°C ± 1°C, 5% CO_2, and ≥90% humidity).

E. Day 2: Change Medium

NOTE: Use nitrile gloves when preparing and changing medium, and discard any remaining medium in a closed container as hazardous waste.

1. Preparation

- Thaw Cellartis Definitive Endoderm Differentiation Day 2 and warm the appropriate volume (see Table IX) to 37°C ± 1°C.

2. Medium Change

1. Remove the medium from the culture vessels with a pipette or vacuum pump.
2. Add warm Definitive Endoderm Differentiation Day 2 according to the volumes stated in Table IX.
3. Return the cell culture vessels to the incubator (37°C ± 1°C, 5% CO_2, and ≥90% humidity).

F. Day 3: Change Medium

NOTE: Use nitrile gloves when changing medium, and discard the old medium in a closed container as hazardous waste.

1. Preparation

- Thaw Cellartis Definitive Endoderm Differentiation Day 3 and warm the appropriate volume (see Table IX) to 37°C ± 1°C.

2. Medium Change

1. Remove the medium from the culture vessels with a pipette or vacuum pump.
2. Add warm Definitive Endoderm Differentiation Day 3 according to the volumes stated in Table IX.
3. Return the cell culture vessels to the incubator (37°C ± 1°C, 5% CO_2, and ≥90% humidity).

G. Day 4: Change Medium

1. Preparation

- Thaw Cellartis Definitive Endoderm Differentiation Day 4 and warm the appropriate volume (see Table IX) to 37°C ± 1°C.

2. Medium Change

1. Remove the medium from the culture vessels with a pipette or vacuum pump.
2. Add warm Definitive Endoderm Differentiation Day 4 according to the volumes stated in Table IX.
3. Return the cell culture vessels to the incubator (37°C ± 1°C, 5% CO_2, and ≥90% humidity).

H. Day 6: Change Medium

1. Preparation

- Thaw Cellartis Definitive Endoderm Differentiation Day 6 and warm the appropriate volume (see Table IX) to 37°C ± 1°C.

2. Medium Change

1. Remove the medium from the culture vessels with a pipette or vacuum pump.
2. Add warm Definitive Endoderm Differentiation Day 6 according to the volumes stated in Table IX.
3. Return the cell culture vessels to the incubator (37°C ±1°C, 5% CO_2, and ≥90% humidity).

VII. Differentiation of Definitive Endoderm Cells to Hepatocytes

A. General information

The Cellartis Hepatocyte Differentiation Kit consists of four media, one supplement, and a ready-to-use coating compound for the differentiation of DE cells to hepatocytes in 2D culture. The Cellartis Hepatocyte Differentiation Kit is optimized for differentiating cells in 24- or 96-well plates**.** Other formats can be used, but optimization may be required. All methods described in this user manual are for differentiation of DE cells to hepatocytes in 2D monolayer cultures on a total area of 50 cm^2. For examples of cell morphology during differentiation, please refer to Figure 9, Section X. These images are just examples, and the morphology may vary depending on the cell line used and differences in cell density. For a suggested schedule for differentiating your cells into hepatocytes and handling of kit components, see Appendix B.

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.

B. Day 7: Dissociation and Seeding of Fresh Definitive Endoderm Cells

1. 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).
    i. Add the Hepatocyte Coating to the cell culture vessels (0.15 ml/cm^2). Make sure the entire surface is covered.
    ii. Incubate at RT for at least 30 min.
    iii. Remove excess Hepatocyte Coating from the cell culture vessels just before seeding. Do not let the surface dry out.
4. Warm TrypLE Select Enzyme (1X) and D-PBS –/– to 37°C ± 1°C.
5. Prepare an appropriate volume of 10% FBS or KO-SR in D-PBS–/– and warm to 37°C ± 1°C.

2. Dissociation and Seeding of DE Cells

1. Remove the media from the culture vessels with a pipette or vacuum pump.
2. Wash the DE cell cultures with warm D-PBS –/– (0.1 ml/cm^2).
3. Remove the D-PBS from the cell cultures with a pipette or vacuum pump.
4. Add the warm TrypLE Select Enzyme to the DE cell cultures (0.1 ml/cm^2).
5. 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.
6. Transfer the cell suspension to the a 50-ml tube.
7. 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.

8. 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.
9. Transfer a volume of the cell suspension corresponding to 6.5 x 10^6 cells to a centrifugation tube.
10. 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.
11. 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.
12. 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 Appendix A).

C. Days 9 and 11: Medium Changes

On days 9 and 11 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).

1. Preparations, Day 9

1. Thaw the bottle of Hepatocyte Progenitor Medium (2) at RT and transfer 25 ml to a sterile tube for use on day 9. Store the remaining medium at 2–8°C for use on day 11.

2. Medium Change, Days 9 and 11

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 VII.A 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.

D. Days 14 and 16: Medium Changes

On days 14 and 16 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 16 onward. Some components of the Hepatocyte Maturation Medium (used on day 14 and day 16) gelatinize on top of the cell layer, and this gelatinized matrix overlay **should not be disrupted or aspirated at any time** (see Figure 2).
- 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.

1. Preparations

Day 14:

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

Days 14 and 16 (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.

2. Medium Change on Day 14

1. Remove the medium from the cell culture vessels according to Section VII.A 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 15 – 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.

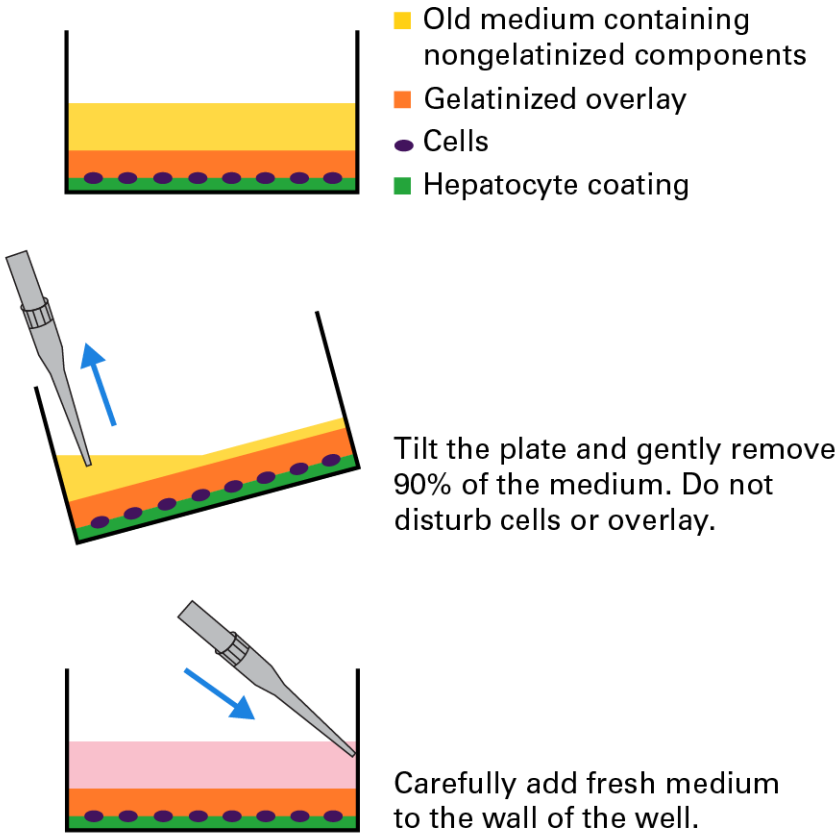

Figure 2. Schematic representation of a medium change from day 16 onward. Use a manual single- or multi-channel pipette for removing 90% of the old medium (shown in yellow) and replacing it with fresh medium (shown in pink). Take great care not to disturb the gelatinized overlay, shown here in orange.

3. Medium Change on Day 16

1. Remove the medium from the cell culture vessels according to Figure 2 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 17 – 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.

E. Day 18 Onward: Medium Changes

From day 18 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 2).
- 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.

1. 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.

2. 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 Figure 2 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 21 (seeding of the undifferentiated hPS cells is day 0) and can be maintained until at least day 32 if handled according to these instructions. Applications that require high CYP activity should be performed from day 23 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.

VIII. Images of Cellartis Human iPS Cell Lines Maintained in the Cellartis DEF-CS Culture System

10X magnification

20X magnification

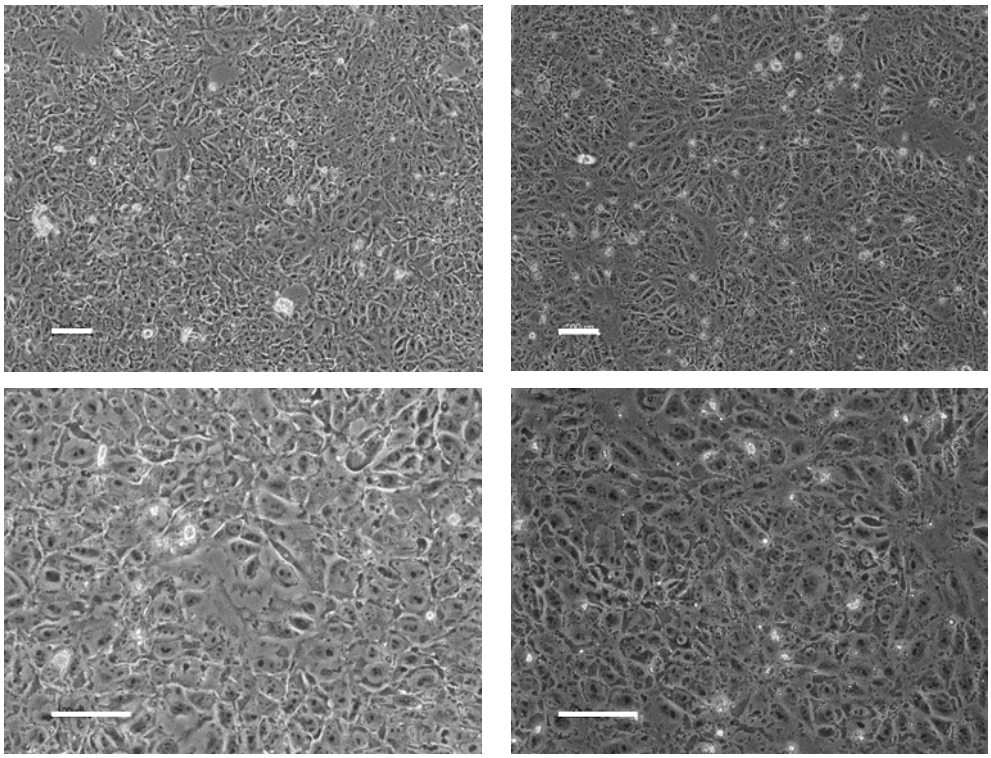

Figure 3. hPS cells in routine culture in the Cellartis DEF-CS culture system one day after seeding. Cell density is approximately 5 x 10^4 cells/cm^2. For all images, the scale bar is 100 µm.

10X magnification

20X magnification

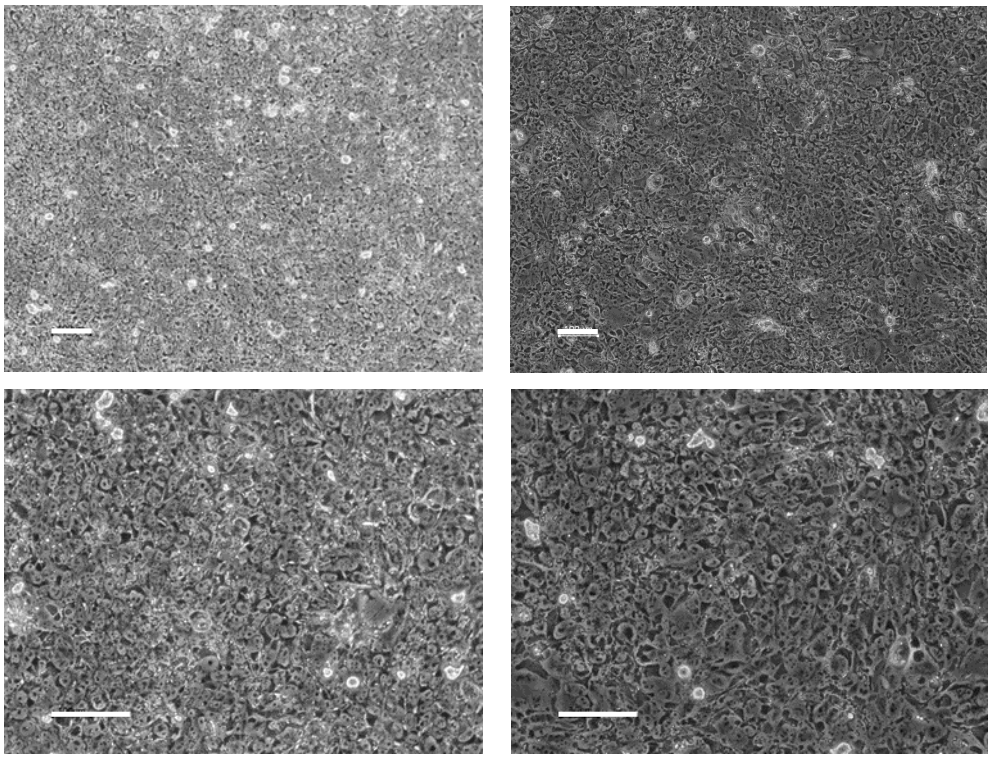

Figure 4. hPS cells in routine culture in the Cellartis DEF-CS culture system two days after seeding. Cell density approximately 1.5 x 10^5 cells/cm^2. For all images, the scale bar is 100 µm.

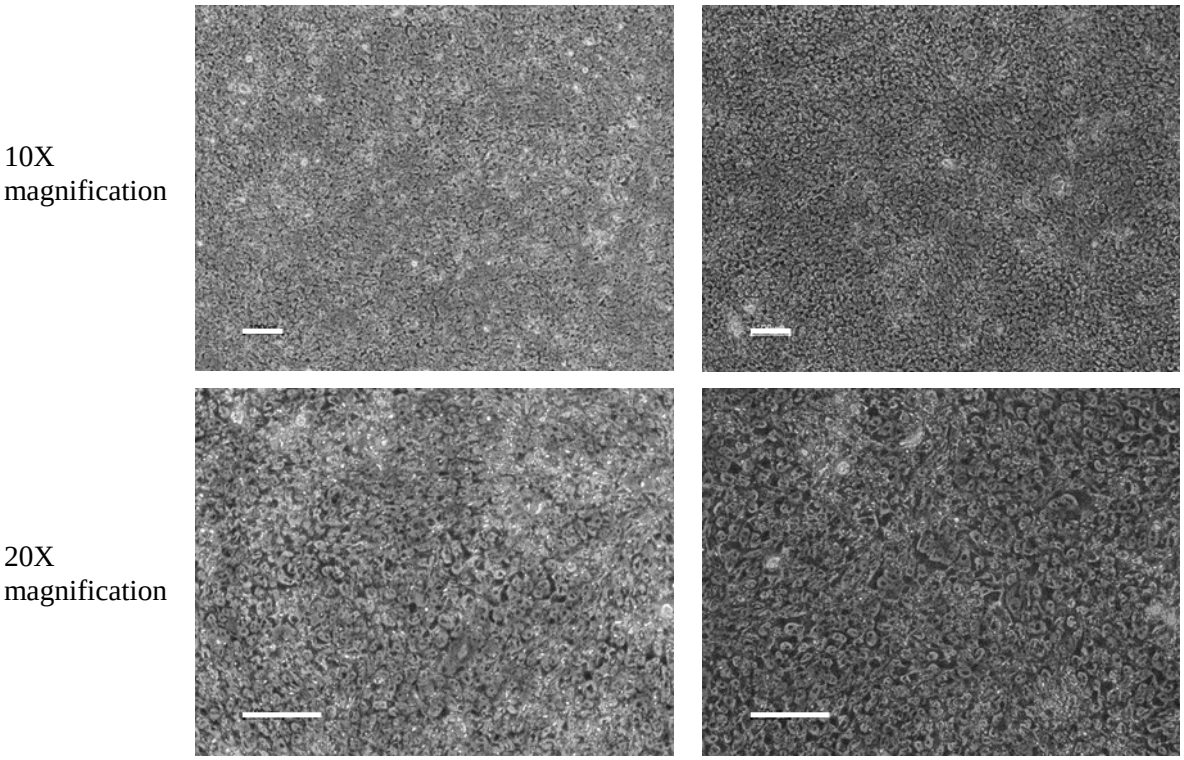

Figure 5. hPS cells in routine culture in the Cellartis DEF-CS culture system three to four days after seeding. Cell density >2 x 10^5 cells/cm^2. For all images, the scale bar is 100 µm.

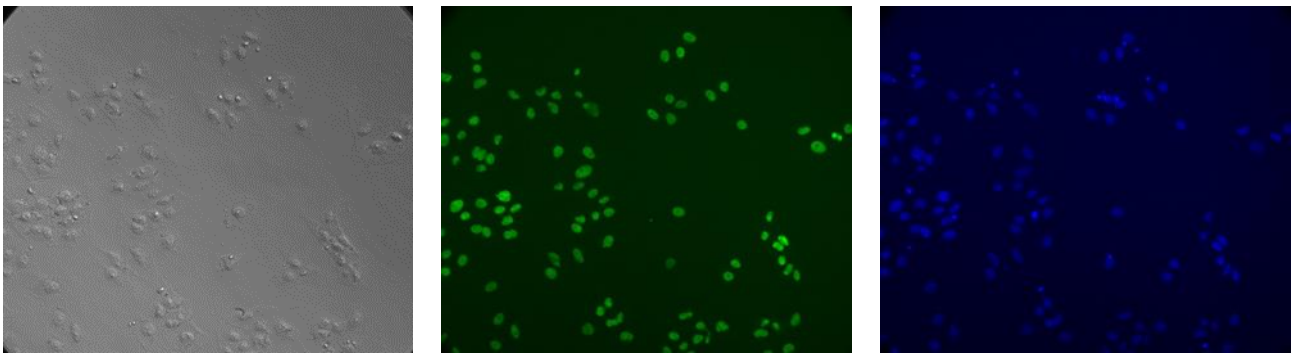

Figure 6. Sparse hPS Cells. In order to demonstrate the pluripotency of spread out cells (i.e., not exhibiting the traditional stem cell morphology), cells were seeded very sparse. Imunocytochemical staining for the pluripotency marker Oct-4 reveals that over 99% of the cells are positive for Oct-4.

IX. Images of Human iPS Cells Differentiated Using the Cellartis iPS Cell to Hepatocyte Differentiation System

Figure 7 and Figure 8 show examples of morphology on days one to seven, for two hPS cell lines. Morphology may vary depending on the cell line used and differences in cell density.

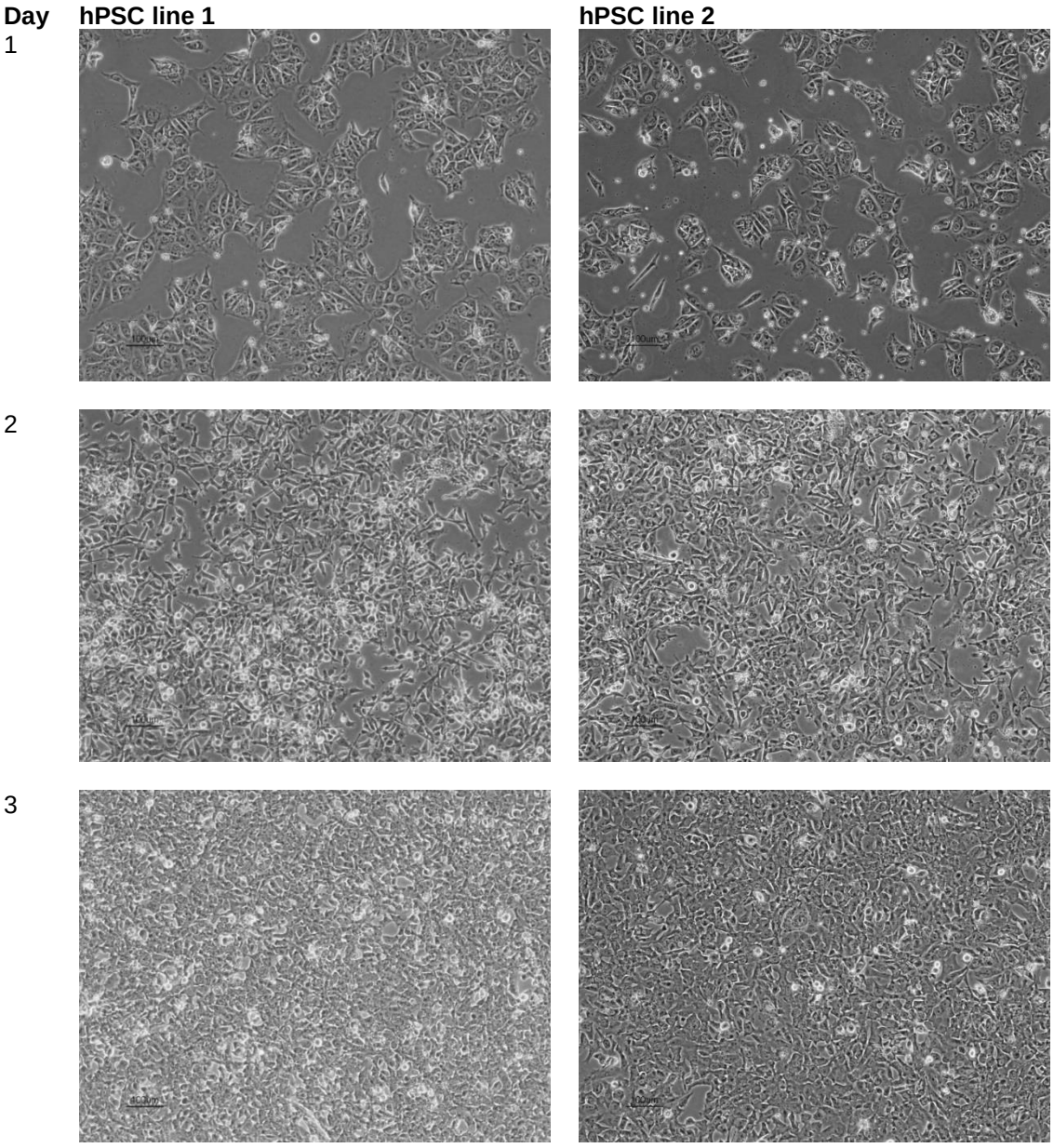

Figure 7. Day 1–3 morphology of two hPS cell lines differentiated using the Cellartis iPS Cell to Hepatocyte Differentiation System. For all images, the scale bar is 100 µm.

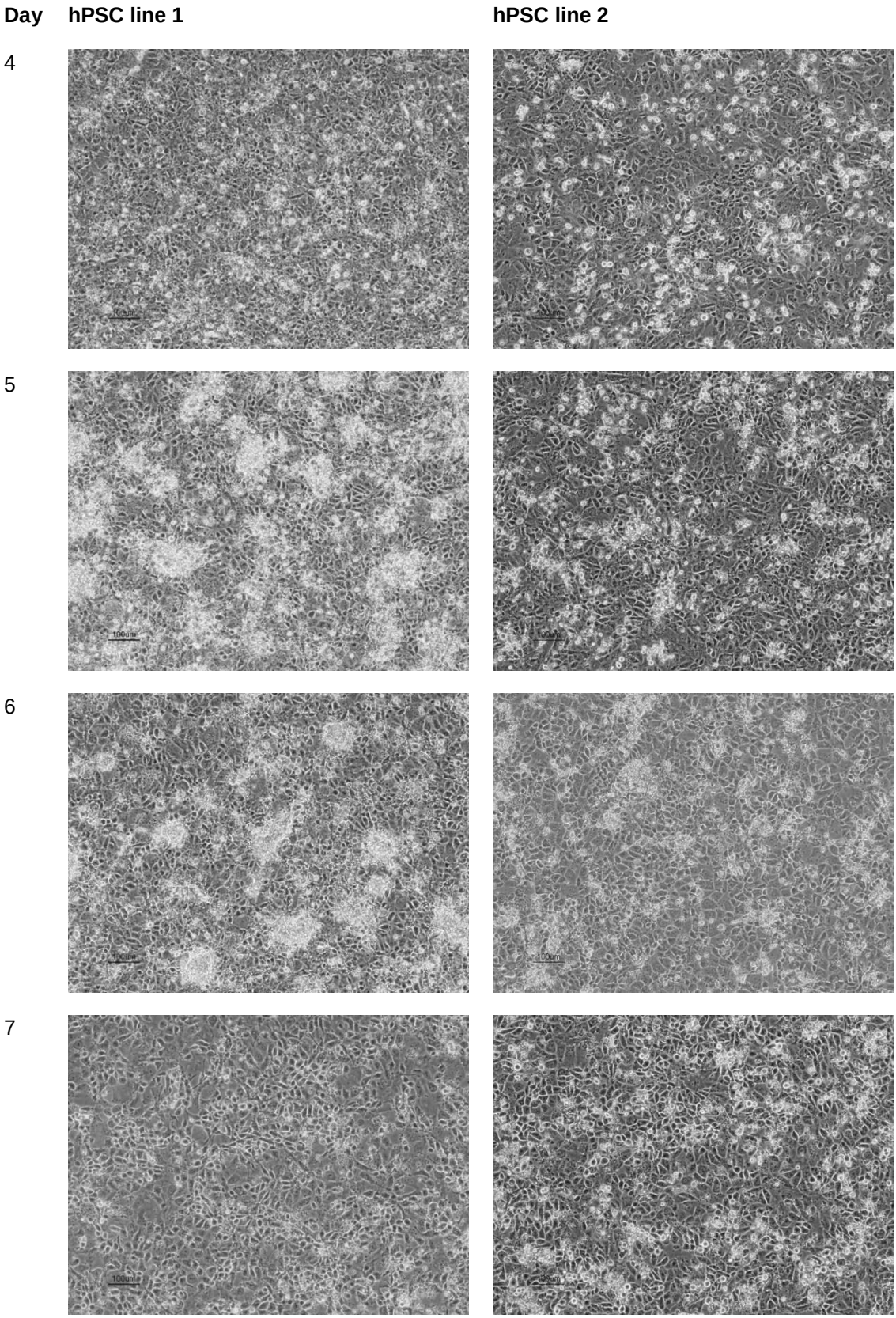

Figure 8. Day 4–7 morphology of two hPS cell lines differentiated using the Cellartis iPS Cell to Hepatocyte Differentiation System. For all images, the scale bar is 100 µm.

X. Images of Cells Differentiated using the Cellartis iPS Cell to Hepatocyte Differentiation System

Figure 9 shows the morphology of the cells as they are differentiating into hepatocytes. Please note that morphology may vary between experiments performed with different cell lines.

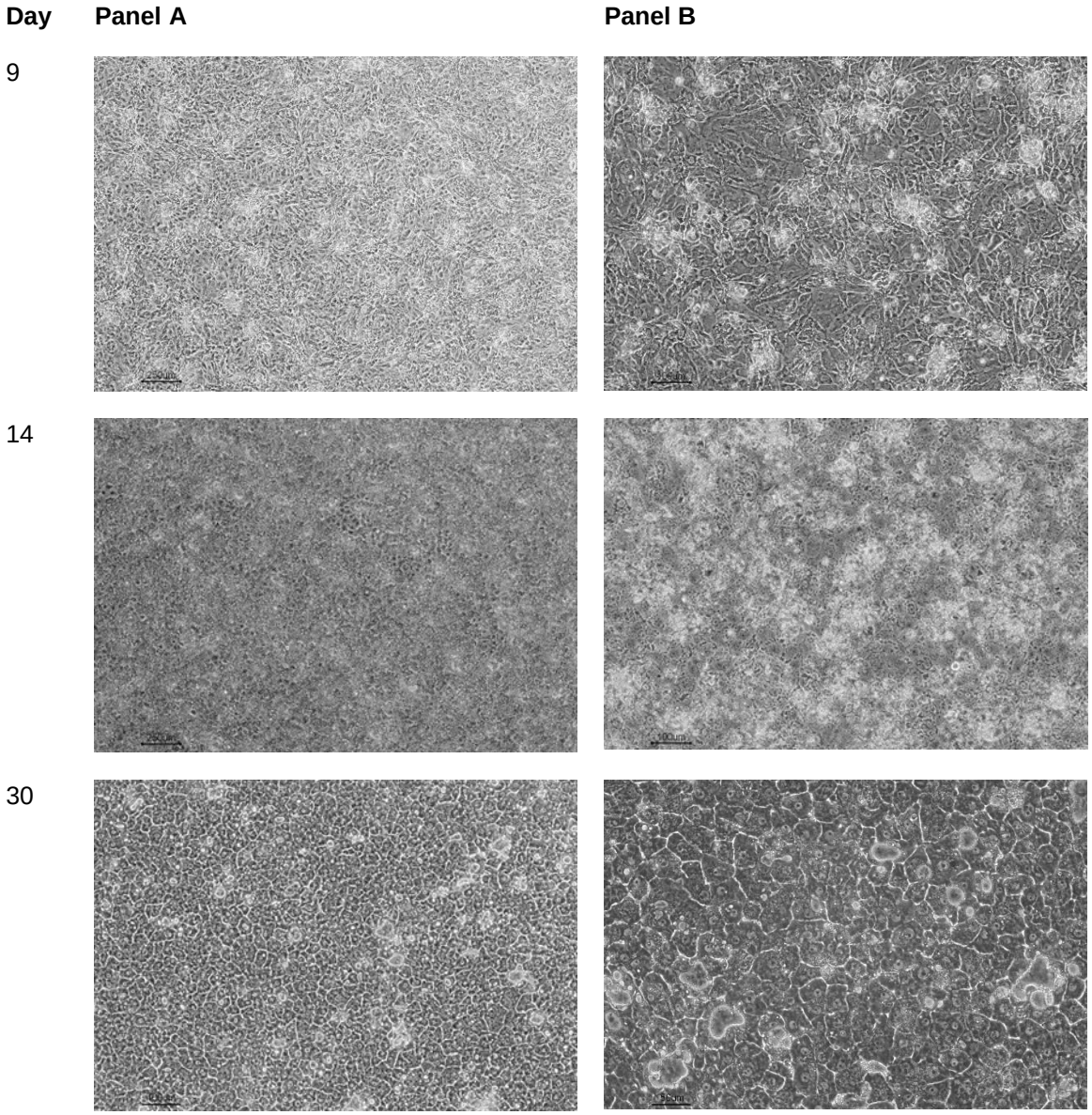

Figure 9. Day 9–30 morphology of hPS cells differentiated using the Cellartis iPS Cell to Hepatocyte Differentiation System. Scale bar corresponds to 250 µm **(Panel A)** and 100 µm **(Panel B).**

Appendix A. Suggested Schedule for the Cellartis iPS Cell to Hepatocyte Differentiation System

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

Day	Suggested Day	Action	Described in Section	Handling of Kit Components
0	Monday	Seeding of undifferentiated hPS cells	VI.C	Thaw Definitive Endoderm Differentiation Coating at RT. Coat cell culture vessel(s). Thaw Definitive Endoderm Differentiation Day 0 at 37°C.
1	Tuesday	Medium change (MC) with Definitive Endoderm Differentiation Day 2	VI.D	Thaw Definitive Endoderm Differentiation Day 1 at 37°C.
2	Wednesday	MC with Definitive Endoderm Differentiation Day 3	VI.E	Thaw Definitive Endoderm Differentiation Day 2 at 37°C
3	Thursday	MC with Definitive Endoderm Differentiation Day 4	0	Thaw Definitive Endoderm Differentiation Day 3 at 37°C
4	Friday	MC with Definitive Endoderm Differentiation Day 6	VI.G	Thaw Definitive Endoderm Differentiation Day 4 at 37°C
5	Saturday			
6	Sunday	MC with Definitive Endoderm Differentiation Day 6	VI.H	Thaw Definitive Endoderm Differentiation Day 6 at 37°C
7	Monday	Seeding of DE cells	VII.B	Thaw Hepatocyte Coating at 2–8°C. Coat cell culture vessel(s). Thaw Hepatocyte Thawing and Seeding Medium (1) at 37°C.
8	Tuesday			
9	Wednesday	MC with progenitor medium	VII.C	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.
10	Thursday			
11	Friday	MC with progenitor medium	VII.C	Warm 25 ml of Hepatocyte Progenitor Medium (2) to 37°C.
12	Saturday			
13	Sunday			
14	Monday	MC with maturation medium	VII.D	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.
15	Tuesday	**Do not disturb the cells**		
16	Wednesday	MC with maturation medium	VII.D	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.
17	Thursday	**Do not disturb the cells**		
18	Friday	MC with maintenance medium	VII.E	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 21.
19	Saturday			
20	Sunday			
21	Monday	MC with maintenance medium	VII.E	Warm 25 ml of Hepatocyte Maintenance Medium (4) to 37°C.
22	Tuesday			
23	Wednesday	MC with maintenance medium	VII.E	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 25.

24	Thursday			
25	Friday	MC with maintenance medium	VII.E	Warm 25 ml of Hepatocyte Maintenance Medium (4) to 37°C.
26	Saturday			
27	Sunday			
28	Monday	MC with maintenance medium	VII.E	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 30.
29	Tuesday			
30	Wednesday	MC with maintenance medium	VII.E	Warm 25 ml of Hepatocyte Maintenance Medium (4) to 37°C.

Appendix B. Thawing Instructions of Kit Components

Table XI. Thawing Instructions of 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. Freezing of Definitive Endoderm cells

Day 7 after start of differentiaton, the definitive endoderm cells can be harvested and cryopreserved as a single cell suspension.

A. Additional Material Required

- Cryo vials, 1.5 ml
- Fetal bovine serum (FBS) or KnockOut Serum Replacement (KO-SR)
- Freezing container for slow freezing (rate of cooling close to –1°C/minute)
- Trypan Blue Solution, 0.4%

B. Preparation

- Prepare an appropriate volume of 10% FBS or KO-SR in D-PBS –/– and warm to 37°C.
- Warm an appropriate volume of TrypLE Select Enzyme and D-PBS –/– to 37°C.
- Prepare the appropriate volume of freezing solution by adding 10% DMSO to FBS or KO-SR and cool to 2–8°C.
- Prepare the freezing container with cryovials and cool to 2–8°C.

C. Dissociation and Freezing

1. Aspirate the medium in the culture vessels using a pipette or vacuum pump.
2. Wash the cell culture vessels with D-PBS –/– (0.1 ml/cm^2).
3. Remove the D-PBS from the culture vessels using a pipette or vacuum pump.
4. Add warm TrypLE Select Enzyme to the cell culture vessels (0.1 ml/cm^2).
5. Incubate the cell culture vessels in the incubator at 37°C ± 1°C and 5% CO_2, and >90% humidity for 3–5 min, until the cells have detached.
6. Pipette the cell suspension 3–5 times and transfer the cell suspension to a 50-ml tube.
7. Rinse the cell culture vessels with 10% FBS or KO-SR in D-PBS –/– (0.1 ml/cm^2) and transfer to the 50-ml tube to achieve a 1:1 dilution of the cell suspension to stop the enzymatical reaction.
8. Count the viable cells by adding 5 µl Trypan Blue solution to a 50-µl sample of the cell suspension. Count the viable cells using a hemocytometer.
9. Centrifuge the cell suspension at 300 x g for 5 min at RT.
10. Discard the supernatant and flick the tube to loosen the cell pellet.
11. Add the chilled freezing solution to a final concentration of 7 x 10^6 viable cells/ml. Flick the tubes if clumps are observed.
12. Immediately transfer the cell suspension to cryovials, 1 ml per vial, and place the vials in the freezing container.
13. Place the freezing container directly in the freezer at ≤–65°C.
14. After 24 hr, transfer the vials into liquid nitrogen (≤–150°C) for long term storage.

Appendix D. Troubleshooting Guide Cellartis DEF-CS Culture System

Table XII. Troubleshooting Guide for transfer to Cellartis DEF-CS Culture System

Problem	Possible Explanation	Solution
Cells do not detach at passage	TrypLE Select Enzyme is too cold.	Make sure TrypLE Select Enzyme is at room temperature before use.
Cells do not detach at passage	Cell layer was washed with D-PBS +/+ prior addition of TrypLE Select Enzyme.	Make sure to use D-PBS *without* Mg^{2+} and Ca^{2+}.
Cells do not detach at passage	Cell density too low at passage.	Cells are normally easier to detach at higher densities.
Cells do not adhere at passage or thawing	DEF-CS COAT-1 has been diluted in D-PBS –/–.	Make sure to use D-PBS with Mg^{2+} and Ca^{2+}.
Cells do not adhere at passage or thawing	Too short incubation with DEF-CS COAT-1.	Prolong the incubation time with DEF-CS COAT-1.

Appendix E. Troubleshooting Guide Cellartis Definitive Endoderm Differentiation Kit

Table XIII. Troubleshooting Guide Cellartis Definitive Endoderm Differentiation Kit

Problem	Possible Explanation	Solution
Cells die during DE differentiation.	Cells are seeded too sparsely.	Increase seeding density at start of DE differentiation (referred to as day 0).
Differentiation to DE cells is not efficient; low yield of DE cells.	Cells are seeded too densely.	Decrease seeding density at start of DE differentiation (day 0).

Appendix F. Troubleshooting Guide Cellartis Hepatocyte Differentiation Kit

Table XIV. Troubleshooting Guide Cellartis Hepatocyte Differentiation Kit

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 18 onward.
The cells are not confluent.	The DE cells were seeded too sparsely at day 7.	Use at least 1.25 x 10^5 cells/cm^2.
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 G. CYP Activity Assay

After 23 days in culture (seeding undifferentiated hPS 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 XV.
- 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 XV. 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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