Takara Bio Europe AB

Cellartis® Definitive Endoderm Differentiation Kit with DEF-CS Culture System User Manual

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(030518)

Takara Bio Europe AB
A Takara Bio Company
Arvid Wallgrens backe 20, SE-413 46 Göteborg, Sweden
Europe Technical Support: tech-cellartis@takarabio.com

Cellartis Definitive Endoderm Differentiation Kit with DEF-CS Culture System User Manual

Table of Contents

Table of Figures

Table of Tables

Cellartis Definitive Endoderm Differentiation Kit with DEF-CS Culture System User Manual

I. Introduction

The Cellartis Definitive Endoderm Differentiation Kit with DEF-CS Culture System is used for the differentiation of human pluripotent stem (hPS) cells into definitive endoderm (DE) and includes two components: the Cellartis DEF-CS 100 Culture System and the Cellartis Definitive Endoderm Differentiation Kit. The Cellartis Definitive Endoderm Differentiation Kit is optimized for use with the Cellartis DEF-CS culture system, and the included Cellartis DEF-CS 100 Culture System is sufficient for transfer of your cell line if cultured in another cell culture system and upscaling of undifferentiated hPS cells prior to the start of DE differentiation. The DE cells obtained with this kit can be used for generating further differentiated cells of endodermal origin, such as hepatocytes and pancreatic endoderm.

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 Definitive Endoderm Differentiation Kit with DEF-CS Culture System (Cat. No. Y30035)

- 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)

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)
- 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

If applicable:

- Appropriate medium for suspending and/or freezing dissociated DE cells
- Cryovials, 1.5 ml
- Fetal bovine serum (FBS) or KnockOut Serum Replacement (KO-SR)
- DMSO
- Freezing container for slow freezing (rate of cooling close to –1°C/min)
- Trypan Blue Solution, 0.4%

IV. General Considerations

A. Storage and Handling

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.

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.

V. Culture of hPS Cells in the Cellartis DEF-CS Culture System

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 1). The cells are not ready to be passaged at this stage, but should be grown until they have the morphology displayed in Figure 2 and Figure 3, 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 I. For detailed information on routine culture using the Cellartis DEF-CS culture system, see the Cellartis DEF-CS Culture System User Manual.

Table I. Weekly schedule for medium changes and passaging.

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

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. In order to start the DE differentiation 3 x 10^6 cells are required and it is recommended to prepare cells in a T25 flask in order to have enough cells at start of differentiation.

For hPS cell lines previously grown in other culture systems, see Section VI for a recommended culture protocol using the Cellartis DEF-CS 100 Culture System to transfer hPS cells to the Cellartis DEF-CS culture system and upscale undifferentiated hPS cells for DE differentiation.

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

A. General information

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 VI.E) and cryopreserved cultures can be thawed directly into the Cellartis DEF-CS culture system (Section VII.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 3; 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 II.

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 4. 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 3.

Table II. 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 II and Table III).
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 III), 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 III. 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 IV or V) 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 IV. 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 V. 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 VI) 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 VI. 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 Cellartis DEF-CS culture system. It is recommended to thaw 1.0 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 above (Section 0). **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 above (Section VI.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 IV (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 above (Section VII.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 VI.C.1) and warm it to 37°C ± 1°C before use.
    - Medium for suspension of the cells prior to counting; see Table VII.
    - Medium for resuspension of the cell pellet after centrifugation.
    - Medium for seeding; see Table V.
- 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, see Table VII) 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 (Table VII) 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 (Table VII) 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 (Table V).
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 VII. Recommended volumes of TrypLE Select Enzyme (1X) and Cellartis DEF-CS medium for thawing or passaging for cell suspension 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 above (Section VII.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 II**.**
- Prepare the appropriate volume of supplemented Cellartis DEF-CS medium for thawing or passaging as described above (Section VI.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 VII) 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 VII) 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 (Table V) 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 VI.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.

VII. 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 5. 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 Table VIII.

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

- The RNA profile of two hPS cell lines during differentiation using the Cellartis Definitive Endoderm Differentiation Kit is shown in Section X.
- Examples of cell morphology during differentiation are shown in Section IX. Morphology may vary depending on cell lines and differences in cell densities.

Table VIII. Recommended Culture Schedule for Definitive Endoderm Differentiation.

Day 0	Coating: Definitive Endoderm Differentiation Coating (0.1 ml/cm^2) Cell Seeding: Definitive Endoderm Differentiation Day 0 (0.2 ml/cm^2)
Day 1	Medium change: Definitive Endoderm Differentiation Day 1 (0.2 ml/cm^2)
Day 2	Medium change: Definitive Endoderm Differentiation Day 2 (0.2 ml/cm^2)
Day 3	Medium change: Definitive Endoderm Differentiation Day 3 (~0.27 ml/cm^2)
Day 4	Medium change: Definitive Endoderm Differentiation Day 4 (~0.52 ml/cm^2)
Day 5	–
Day 6	Medium change: Definitive Endoderm Differentiation Day 6 (~0.52 ml/cm^2)

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 the Cellartis Definitive Endoderm Differentiation Kit.

Format	Coating	Days 0, 1, and 2	Day 3	Days 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 will 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 will 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), 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 above) 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).

I. Day 7: Differentiation to Downstream Lineages, or Perform Assays for Definitive Endoderm Formation

On day 7, the DE cells are ready to be differentiated to downstream linages, or to be assayed for the formation of definitive endoderm.

- If you wish to use the DE cells for differentiation into hepatocytes, we recommend using the Cellartis Hepatocyte Differentiation Kit (Cat. No. Y30050) according to its user manual, which contains methods for dissociating the DE cells and for further differentiation.
- If you wish to differentiate the DE cells into other lineages or if the DE cells are to be assayed, suggestions for DE cell dissociation and freezing are listed below.

J. Suggested Protocol for DE Cell Dissociation

1. Preparation

- Prepare an appropriate volume of 10% FBS or KO-SR in D-PBS –/–, or other appropriate medium depending on your application, and warm to 37°C ± 1°C.
- Warm the appropriate volumes of D-PBS –/– (0.1 ml/cm^2) and TrypLE Select Enzyme (0.1 ml/cm^2) to 37°C ± 1°C.

2. Dissociation

1. Remove the medium from the culture vessels with a pipette or vacuum pump.
2. Wash the cell culture vessels with warm D-PBS –/– (0.1 ml/cm^2).
3. Remove the D-PBS from the culture vessels with a pipette or vacuum pump.
4. Add the 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, 5% CO_2, and >90% humidity for 3–5 min, until the cells have detached.
6. Transfer the cell suspension to the required number of 50-ml tube(s).
7. Rinse the cell culture vessels with 10% FBS or KO-SR in D-PBS –/–, or other appropriate medium depending on your application (0.1 ml/cm^2), and transfer to the 50-ml tube(s) to achieve a 1:1 dilution of the cell suspension to stop the enzymatic reaction.
8. Centrifuge the cell suspension for 5 min at 300 x g at RT, discard the supernatant, and resuspend the cell pellet in medium appropriate for further applications.

K. Freezing of DE Cells

1. 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.

2. 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.

VIII. Images of Cellartis hPS Cells Maintained in the Cellartis DEF-CS Culture System

10X magnification

20X magnification

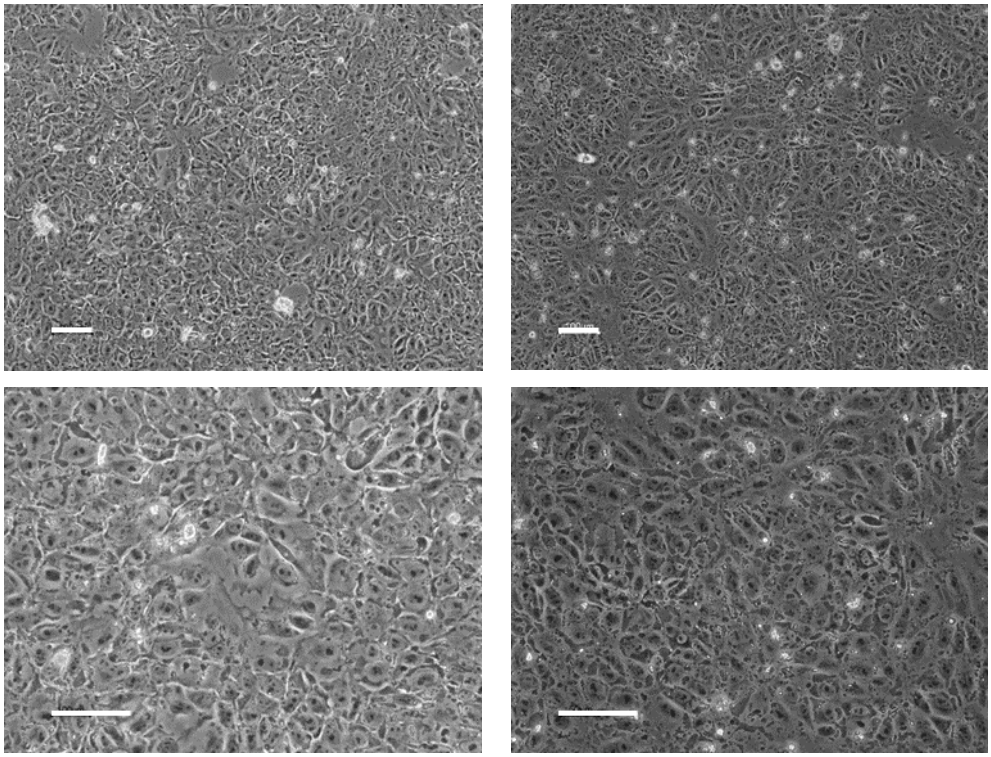

Figure 1. hPS cells in routine culture in the Cellartis DEF-CS culture system one day after seeding. Cell density is approximately 5 x 10^4 cells/cm^2. For all images, the scale bar is 100 µm.

10X magnification

20X magnification

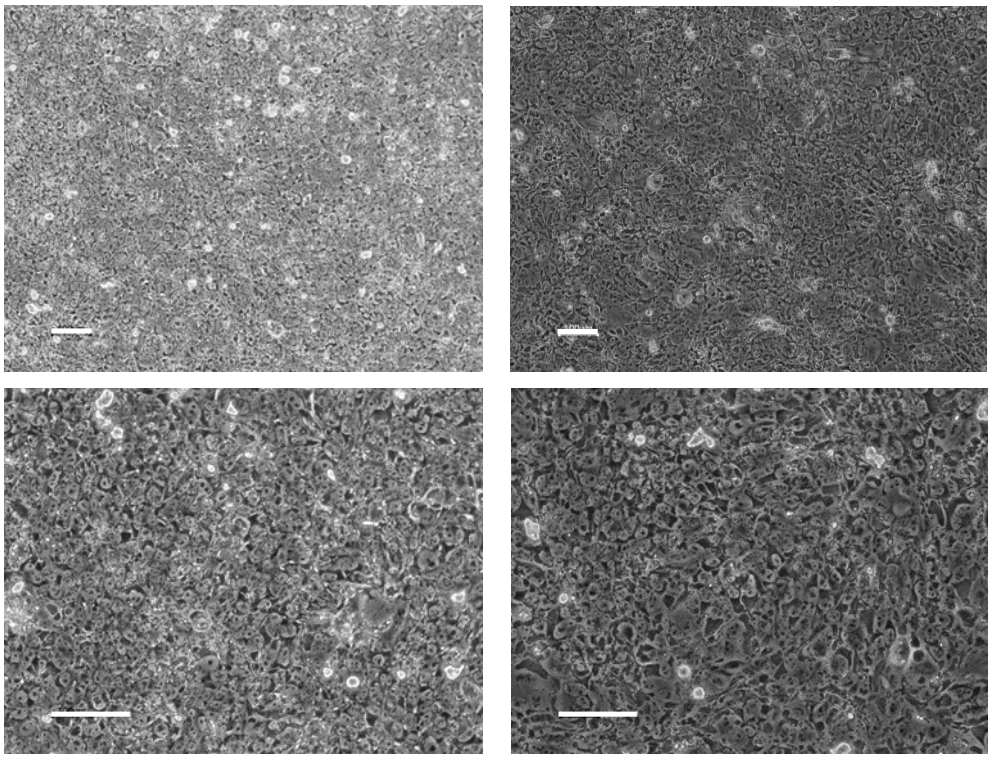

Figure 2. hPS cells in routine culture in the Cellartis DEF-CS culture system two days after seeding. Cell density approximately 1.5 x 10^5 cells/cm^2. For all images, the scale bar is 100 µm.

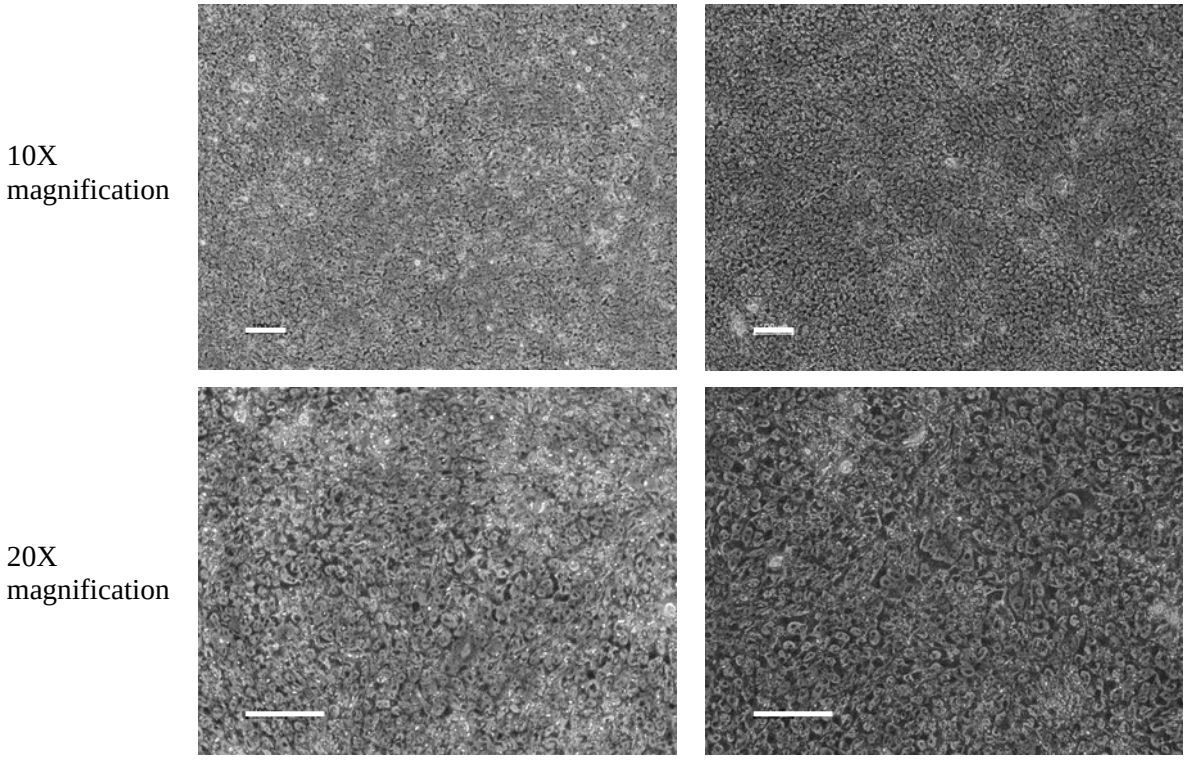

Figure 3. hPS cells in routine culture in the Cellartis DEF-CS culture system three to four days after seeding. Cell density >2 x 10^5 cells/cm^2. For all images, the scale bar is 100 µm.

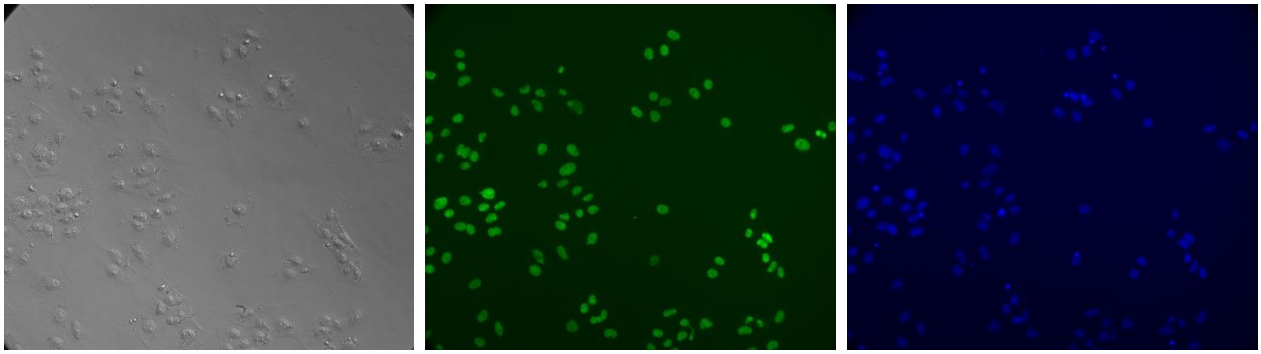

Figure 4. Morphology and pluripotency of a sparse hPS cell culture. Pluripotency is maintained, even in subconfluent cells grown in the Cellartis DEF-CS culture system. Imunocytochemical staining for the pluripotency marker Oct-4 reveals that over 99% of the cells are Oct-4 positive.

IX. Images of hPS Cells Differentiated Using the Cellartis Definitive Endoderm Differentiation Kit

Figure 5 and Figure 6 show examples of morphology on days 1–7 for two hPS cell lines differentiated using the Cellartis Definitive Endoderm Differentiation Kit. Morphology may vary depending on the cell line used and differences in cell density.

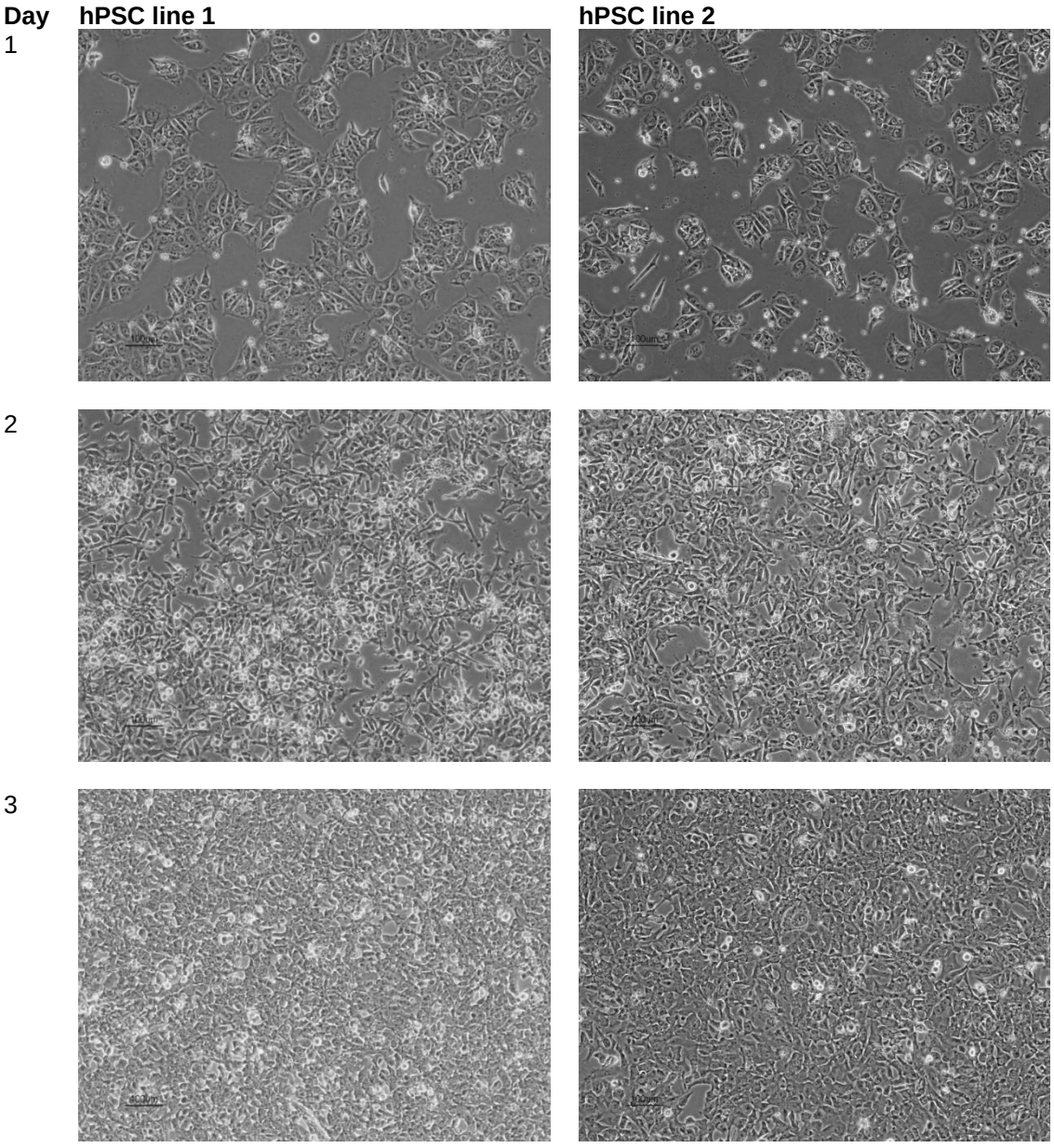

Figure 5. Day 1–3 morphology of two hPS cell lines differentiated using the Cellartis Definitive Endoderm Differentiation Kit. For all images, the scale bar is 100 µm.

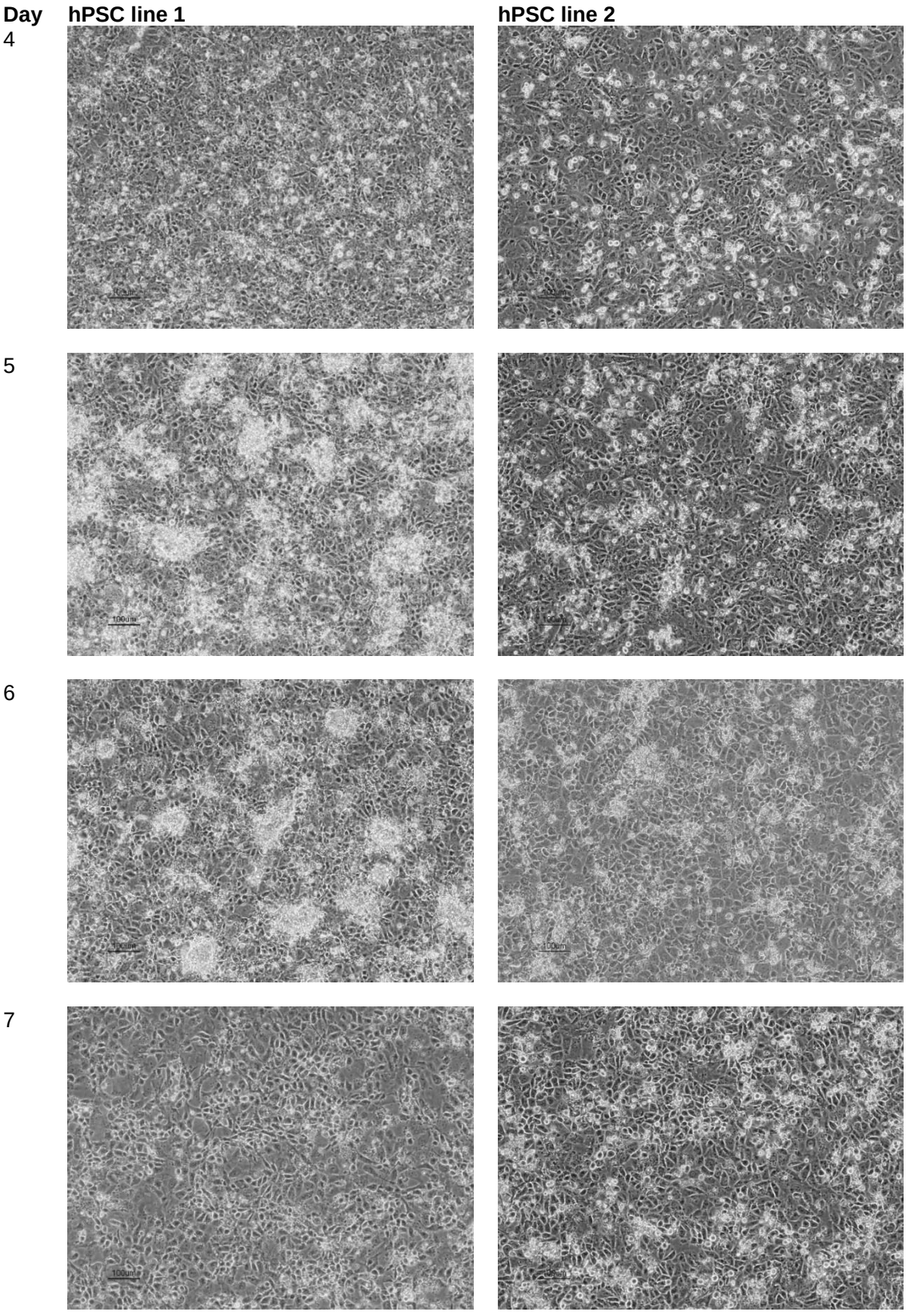

Figure 6. Day 4–7 morphology of two hPS cell lines differentiated using the Cellartis Definitive Endoderm Differentiation Kit. For all images, the scale bar is 100 µm.

X. RNA Profile of hPS Cells Differentiated Using the Cellartis Definitive Endoderm Differentiation Kit

Figure 7 and Figure 8 show examples of mRNA expression profiles from two different hPS cell lines, both differentiated using the Cellartis Definitive Endoderm Differentiation Kit. The mRNA levels were analyzed using RT-qPCR and the graphs represent relative expression levels compared to expression at Day 0. Similar patterns have been seen in other hPS cell lines, but levels may vary from cell line to cell line.

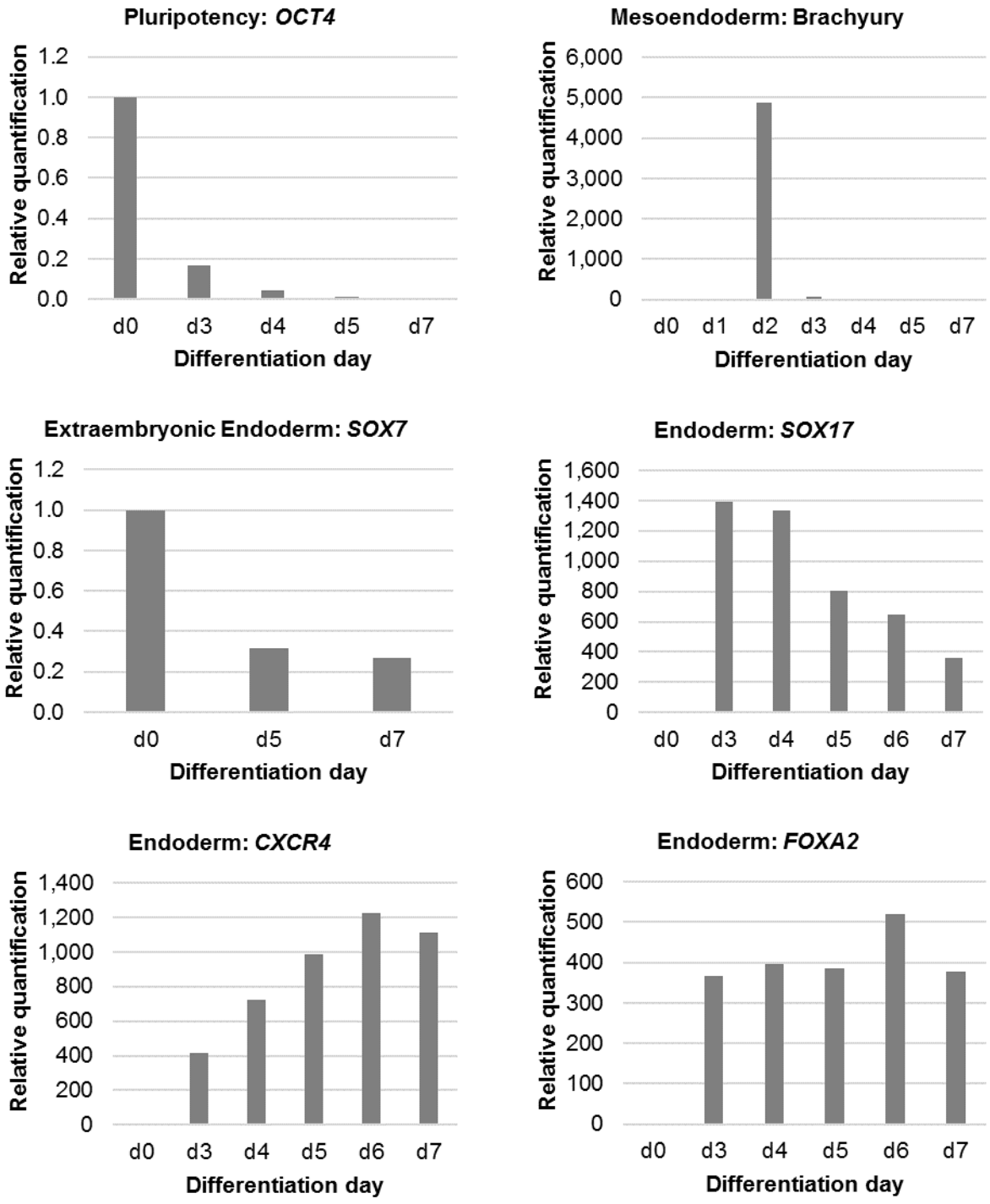

Figure 7. RNA profile of hPS cell line 1 differentiated using the Cellartis Definitive Endoderm Differentiation Kit. The graphs represent relative expression levels compared to expression at day 0.

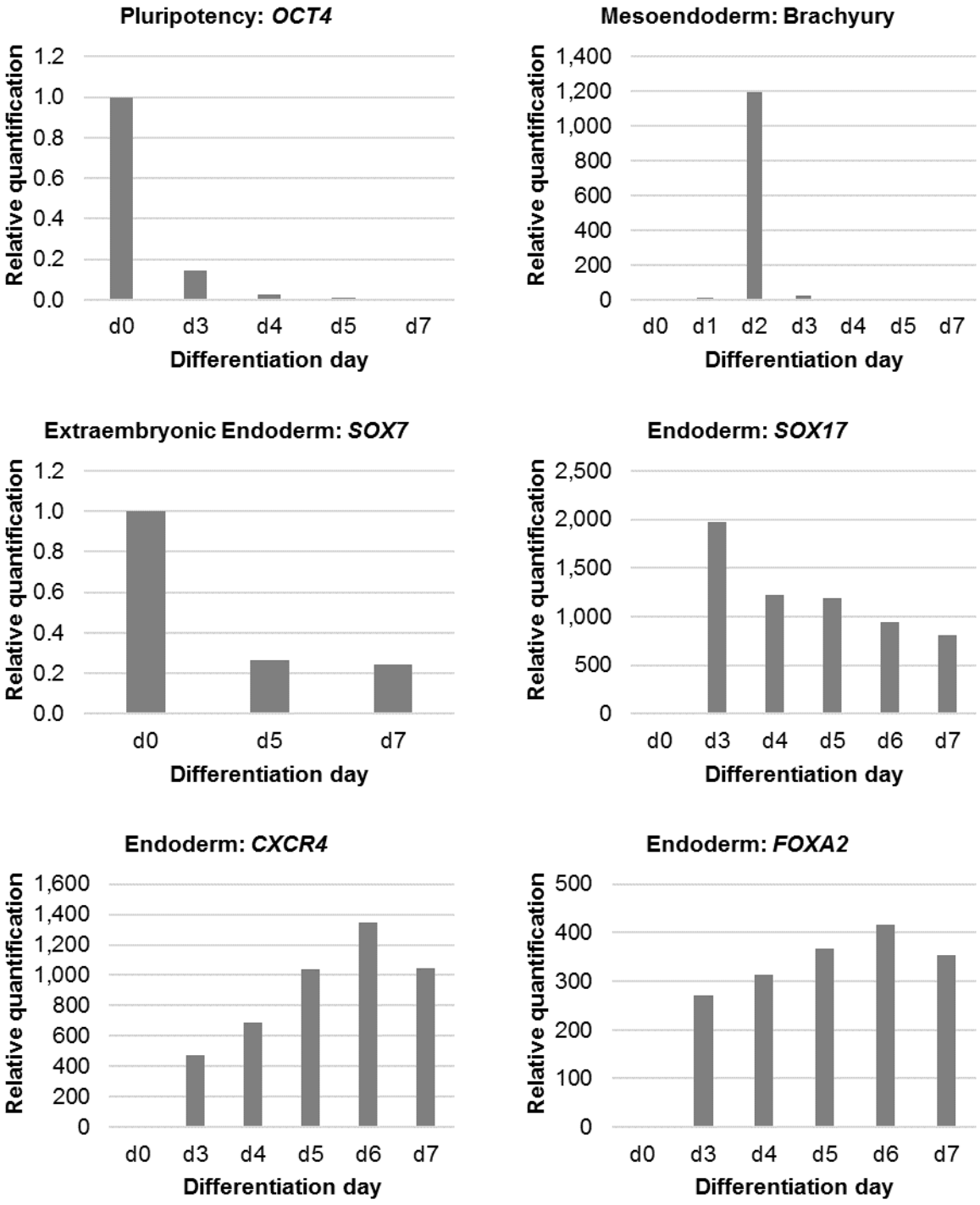

Figure 8. RNA profile of hPS cell line 2 differentiated using the Cellartis Definitive Endoderm Differentiation Kit. The graphs represent relative expression levels compared to expression at day 0.

Appendix A. Troubleshooting Guide Cellartis DEF-CS Culture System

Table X. Troubleshooting Guide for transfer to the 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 B. Troubleshooting Guide for the Cellartis Definitive Endoderm Differentiation Kit

Table XI. Troubleshooting Guide for the 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).

Contact Us	
Customer Service/Ordering	**Technical Support**
tel: +33.(0)1.3904.6880	tel: +46.(0)31.758.0900
fax: +33.(0)1.3904.6870	fax: +46.(0)31.758.0910
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e-mail: orders@takarabio.com	e-mail: tech-cellartis@takarabio.com

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