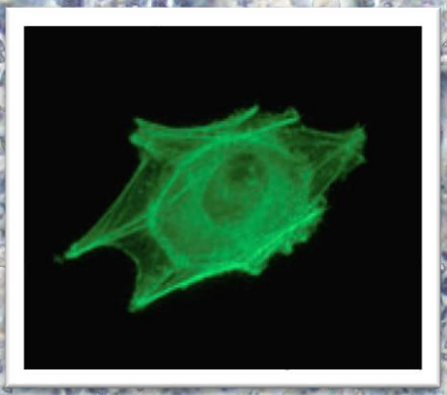
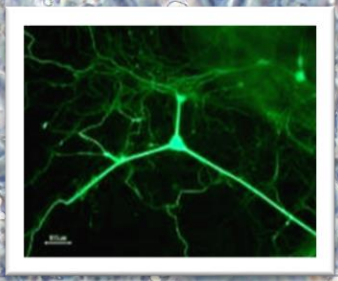
Stem Cell Application Protocols

ヒトiPS/ES細胞用培地

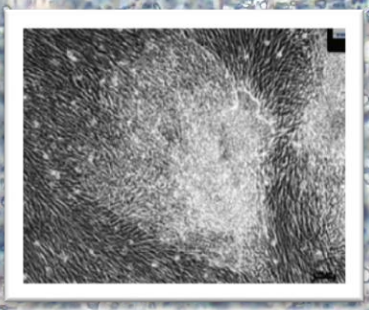
Cellartis®
DEF-CS™ 500 Culture System



Single-Cell Cloning



EB formation



Reprograming

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PRODUCTS

製品コード	製品名	容量	価格(税別)
Y30010	Cellartis® DEF-CS™ 500 Culture System	1 Kit	¥59,800

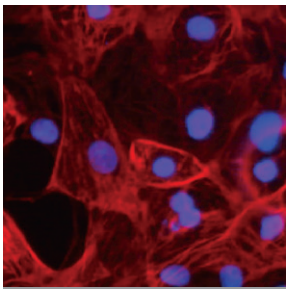
[キット内容]

Cellartis DEF-CS 500 Basal Medium	500 ml	・・・ 基本培地
Cellartis DEF-CS 500 COAT-1	4 ml	・・・ プレートコーティング剤 (製品コード Y30012)
Cellartis DEF-CS 500 Additives	1 Set	・・・ 培地添加剤 3種 (製品コード Y30016)

Clontech TaKaRa cellartis

Application

Stem Cell Application Protocols of Cellartis® DEF-CS™ 500 Culture System



Single-Cell Cloning and Expansion

With the Cellartis® DEF-CS™ 500 Culture System

Stem Cell Application Protocol

I. Introduction

Pluripotent stem cells can be seeded as single cells into a 96-well plate. This technique enables the expansion of single-cell clones, such as those edited by CRISPR/Cas9.

II. Materials Required

- Cellartis DEF-CS 500 Culture System (contains COAT-1, Basal Medium, GF-1, GF-2, and GF-3)
- 96-well plates, flat bottom, cell-culture treated
- 48-well plates, flat bottom, cell-culture treated
- TrypLE Select Enzyme (1X), w/o phenol red
- PBS Dulbecco's with Ca^{2+} & Mg^{2+} (D-PBS +/+)
- PBS Dulbecco's w/o Ca^{2+} & Mg^{2+} (D-PBS –/–)

III. Protocol

A. Single-Cell Seeding

NOTE: If cells have not previously been adapted to growing in DEF-CS 500, it is strongly recommended to transition cells by passaging five times in DEF-CS 500 prior to performing single-cell seeding experiments.

Coating of a 96-Well Plate

1. Dilute the required volume of DEF-CS COAT-1 in D-PBS +/+ before use. Make a 1: **5** dilution.
2. Mix the diluted DEF-CS COAT-1 solution gently and thoroughly by pipetting up and down.
3. Add the diluted DEF-CS COAT-1 solution to a 96-well plate for single-cell seeding (use 50 µl/well), making sure the entire surface of each well is covered.
4. Place the cell culture plate in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for a minimum of 3 hr.
5. Aspirate the DEF-CS COAT-1 solution from the cell culture plate just before use.

Preparing the Supplemented DEF-CS Medium

1. Prepare the appropriate volume of supplemented DEF-CS medium by adding DEF-CS GF-1 (dilute 1:333), GF-2 (dilute 1:1,000), and GF-3 (dilute 1:1,000) to DEF-CS Basal Medium.
2. Prepare fresh medium on the day of intended use and warm it to 37°C ± 1°C immediately before use. Discard any leftover warmed medium.

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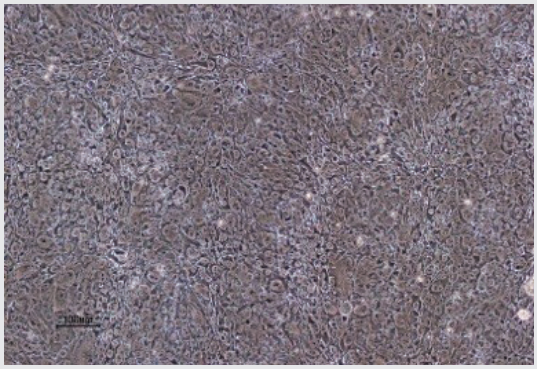
Single-Cell Seeding

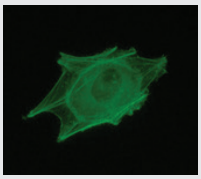
NOTE: To optimize survival rate and expansion potential during single-cell cloning, we recommend using cells that are in a proliferative state. We recommend starting with a confluent but not dense (not growth-arrested) culture, corresponding to a density of 0.8–1.5 x 10^5 cells/cm^2 (example image below). Furthermore, if cells have been manipulated (*i.e.*, by transfection or gene editing), it is highly recommended to let the cells recover for at least five days prior to conducting single-cell cloning.



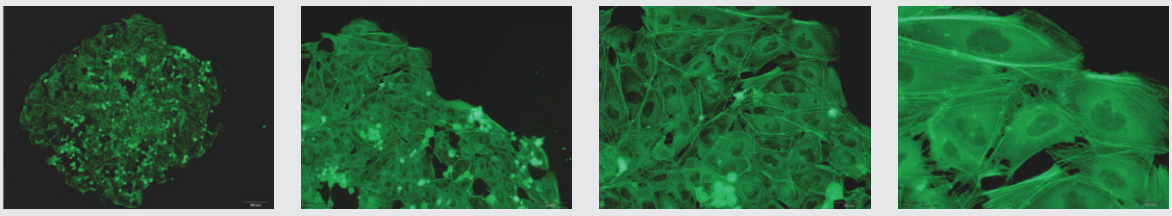
Cells with a density of 0.8–1.5 x 10^5 cells/cm^2.

1. Check cells under a phase contrast microscope; photo document as necessary.
2. Aspirate the medium from the cell culture flasks and wash the cell layer once with D-PBS –/–.
3. Add 20 µl/cm^2 of TrypLE Select to the cell culture flasks and incubate for 5 min, or until the cell layer has detached. Detachment can be aided by swirling the cell culture flask or by tapping the side of the cell culture flask firmly but gently.
4. Resuspend the cells in the supplemented DEF-CS medium and pipet up and down several times to ensure a single-cell suspension. (The cells will aggregate if left too long in TrypLE Select.)
5. Count the cells using a hemocytometer or a cell counter (optimized for hiPS cells).
6. Use your preferred method to single sort your cells: FACS, limiting dilution, or automated clone picking. Seed a single cell in 100 µl of supplemented DEF-CS medium per well.
7. Leave the plate in the incubator for 48 hr. After 48 hr, carefully add 100 µl of fresh, supplemented DEF-CS medium per well. There should now be 200 µl per well. **Do not discard any media.**

NOTE: When seeding single cells, cell characteristics will be different. Newly passaged single cells will spread out. However, when proliferating, the cell density increases, and the typical undifferentiated stem cell morphology (i.e., high nucleus to cytoplasm ratio, defined borders, and prominent nucleoli) appears.



Undifferentiated single cell—spread out and with a non-typical stem cell morphology.



View of an emerging single-cell colony (left), progressively zooming in to emphasize cell morphology.

B. Culture of Single-Cell Colonies

Changing Media on Single-Cell Colonies

Media change in the 96-well plate is recommended from day 4 after single-cell seeding and then **every other day.** If the medium turns yellow due to high metabolic activity, change the medium every day.

1. Prepare supplemented DEF-CS medium according to the directions in Section III.A, "Preparing the Supplemented DEF-CS Medium." Prepare at least 150 µl of medium per well.

   NOTE: Normally, GF-3 is only added at passage, and not at media change. However, for the first passage after single-cell seeding, use it for media changes.

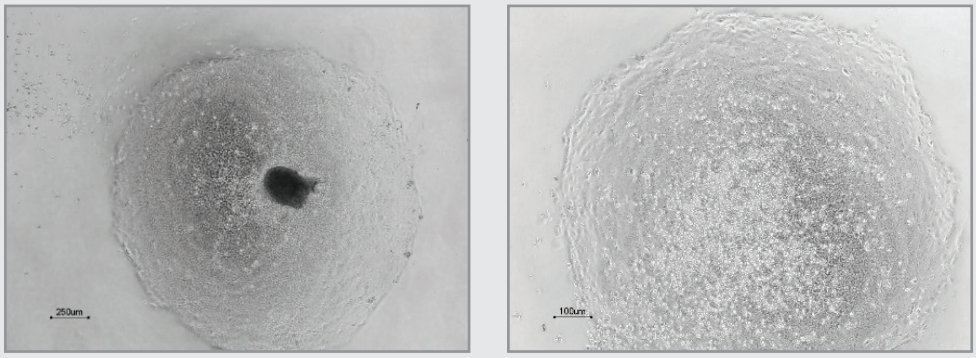
2. Check cells under the microscope; photo document as necessary.
3. Carefully withdraw 150 µl of medium and add 150 µl of newly warmed medium into each well of the plate. **Avoid flushing medium directly onto the cell layer.**
4. Place the cell culture plate in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

Coating of a 48-Well Plate

1. Dilute the required volume of DEF-CS COAT-1 in D-PBS +/+ before use. Make a 1: **5** dilution.
2. Mix the diluted DEF-CS COAT-1 solution gently and thoroughly by pipetting up and down.
3. Add the diluted DEF-CS COAT-1 solution to a 48-well plate (use 200 µl/well), making sure the entire surface of each well is covered.
4. Place the cell culture plate in the incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for a minimum of **3 hr.**
5. Aspirate the DEF-CS COAT-1 solution from the cell culture plate immediately before use.

Passaging Single-Cell Colonies

NOTE: The colonies will be ready to passage after 7–10 days.



Dense colonies, ready to transfer to larger wells. The cells have the typical undifferentiated stem cell morphology (*i.e.*, high nucleus to cytoplasm ratio, defined borders, and prominent nucleoli).

1. Prepare supplemented DEF-CS medium according to the directions in Section III.A, "Preparing the Supplemented DEF-CS Medium."
2. Check the cells under the microscope; photo document as necessary.
3. Aspirate the medium from the wells and wash the cell layer with D-PBS –/–.
4. Add 50 µl of TrypLE Select (room temperature) to the cells. Make sure the whole colony in the well is covered. Incubate for 5 min or until all of the cells have detached.

5. Resuspend the cells in 0.5 ml of pre-warmed supplemented DEF-CS medium. Transfer all of the cell suspension to a newly coated well in a 48-well plate.

   NOTE: To prevent cell loss, counting the cells at this stage is not recommended.

6. Tilt the dish 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.

C. Scaling Up

Coating of Culture Vessels for Scale-Up

1. Dilute the required volume of DEF-CS COAT-1 in D-PBS +/+ before use. Make a 1:**20** dilution.
2. Mix the diluted DEF-CS COAT-1 solution gently and thoroughly by pipetting up and down.
3. Add the diluted DEF-CS COAT-1 solution to the chosen culture vessels, making sure the entire surface of each well is covered (see table below).
4. Place the culture vessels for a minimum of 30 min in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for 0.5–3 hr at room temperature (15–25°C).
5. Aspirate DEF-CS COAT-1 solution from the culture vessels immediately before use.

Preparing Medium for Passaging During Scale-Up

1. Prepare the appropriate volume of supplemented DEF-CS medium by adding DEF-CS GF-1 (dilute 1:333), GF-2 (dilute 1:1,000), and GF-3 (dilute 1:1,000) to DEF-CS Basal Medium.
2. Prepare fresh medium on the day of intended use and warm it to 37°C ± 1°C immediately before use. Discard any leftover warmed medium.

Passaging During Scale-Up

1. Check the cells under the microscope; photo document as necessary.
2. Coat the appropriate number of wells (1 well per clonal population, in the appropriate format; see table below.)
3. Aspirate medium from one well at a time and gently wash the cell layer with D-PBS –/–.
4. Add the appropriate volume (see table below) of TrypLE Select (room temperature) to the cells. Make sure the entire culture surface in the well is covered. Incubate for 5 min or until the cells have detached.
5. Resuspend the cells in the appropriate volume (see table below) of pre-warmed supplemented DEF-CS medium. Transfer all of the cell suspension to a newly coated culture vessel.

   NOTE: To prevent cell loss, counting the cells at this stage is not recommended.

6. Tilt the dish backwards and forwards gently to ensure that the cell suspension is dispersed evenly over the surface, repeat the transfer process with the remaining wells, then place the vessels in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

Passage Number	Starting Format	Passage Interval	New Format	Volume of COAT-1	Volume of TrypLE Select	Volume of Supplemented Medium
2 → 3	1 well in 48-well plate	3–7 days	1 well in 24-well plate	400 µl/well of 24-well plate	50 µl/well of 48-well plate	1 ml/clone
3 → 4	1 well in 24-well plate	2–5 days	1 well in a 6-well plate	1.5 ml/well of 6-well plate	100 µl/well of 24-well plate	3 ml/clone
4 → 5	1 well in a 6-well plate	2–5 days	1 T25 flask	2.5 ml/T25 flask	300 µl/well of 6-well plate	5 ml/clone

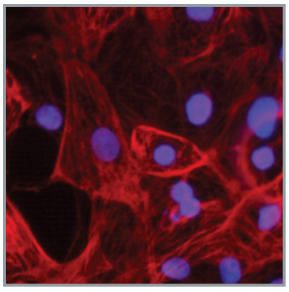
Changing Media During Scale-Up

Media change is recommended daily (except on the day of passage). The volume of medium should be determined using the table above. If the medium turns yellow due to high metabolic activity, increase the medium volume.

1. Prepare the appropriate volume of supplemented DEF-CS medium by adding DEF-CS GF-1 (dilute 1:333) and GF-2 (dilute 1:1,000) to DEF-CS Basal Medium.

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

2. Prepare fresh medium on the day of intended use and warm it to 37°C ± 1°C immediately before use. Discard any leftover warmed medium.
3. Check the cells under the microscope; photo document as necessary.
4. Carefully aspirate the media and pipet freshly prepared, warmed medium into the vessel. **Avoid flushing medium directly onto the cell layer or letting the surface dry.**
5. Place the culture vessel in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.
6. Continue to culture and passage cells until they are scaled up to a T25 vessel per seeded single cell. From here on, the lines should be cultured according to the user manual for the Cellartis DEF-CS 500 Culture System.



Stem Cell Application Protocol

Reprogramming peripheral blood mononuclear cells (PBMCs)

Using the Cellartis® DEF-CS™ 500 Culture System

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I. Introduction

Induced pluripotent stem (iPS) cells originate from adult cells that have been reprogrammed with key transcription factors to exhibit pluripotency. PBMCs are a popular source of adult cells for reprogramming due to routine blood collection methods and the presence of a wide variety of banked blood samples. Once the reprogramming factors have been delivered to PBMCs, the cells can be transferred to the DEF-CS system to maximize the number of emerging colonies and for the robust expansion into stable iPS cell lines.

This protocol has been developed using Sendai viruses for delivery of the transcription factors. Optimization may be necessary if using other delivery methods.

II. Materials required

- Cellartis DEF-CS 500 Culture System (Takara Bio, Cat. #Y30010) (includes COAT-1, Basal Medium, GF-1, GF-2, and GF-3)
- Stem Cell Cutting Tools (Vitrolife, Cat. # 14601)
- Transfer Pipettes (Vitrolife, Cat. # 14319)
- 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), no phenol red (Thermo Fisher Scientific, Cat. # 12563011)
- Cell culture vessels, tissue-culture-treated polystyrene surface

III. Preparing medium and coating cell culture vessels

A. Maintenance medium for human iPS cells

Prepare an appropriate volume of Cellartis DEF-CS Medium for Maintenance by adding GF-1 (dilute 1:333) and GF-2 (dilute 1:1,000) to Cellartis DEF-CS Basal Medium.

B. Passaging medium for human iPS cells

Prepare an appropriate volume of Cellartis DEF-CS Medium for Passaging by adding GF-1 (dilute 1:333), GF-2 (dilute 1:1,000), **and GF-3** (dilute 1:1,000) to Cellartis DEF-CS Basal Medium.

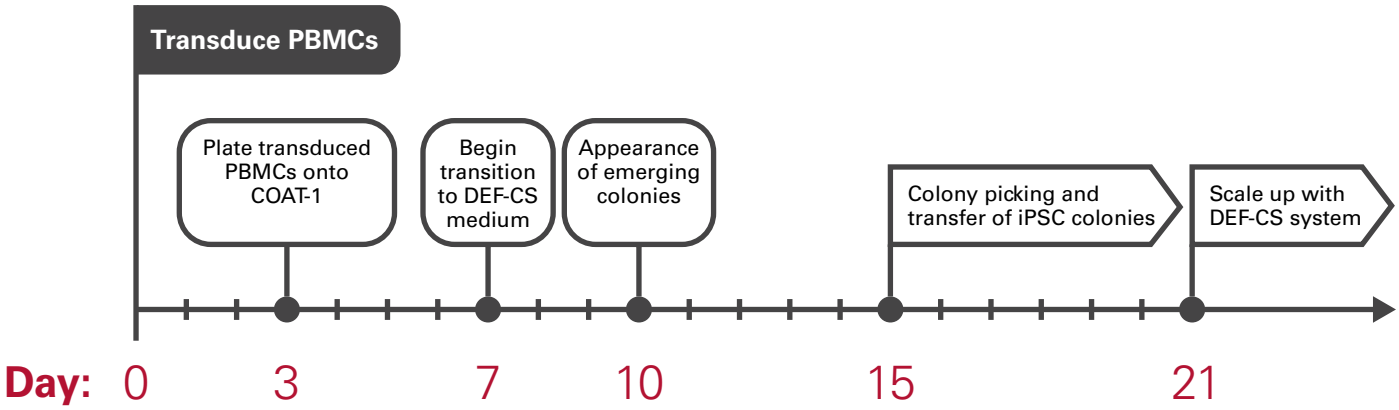
C. Coating cell culture vessels

1. Dilute the required volume of COAT-1 in D-PBS +/+ before use. Make a **1:5** dilution on Day 3, at colony picking, and for the first passage during scale-up. Use a 1:20 dilution for subsequent passages.
2. Mix the diluted COAT-1 solution gently and thoroughly by pipetting up and down.

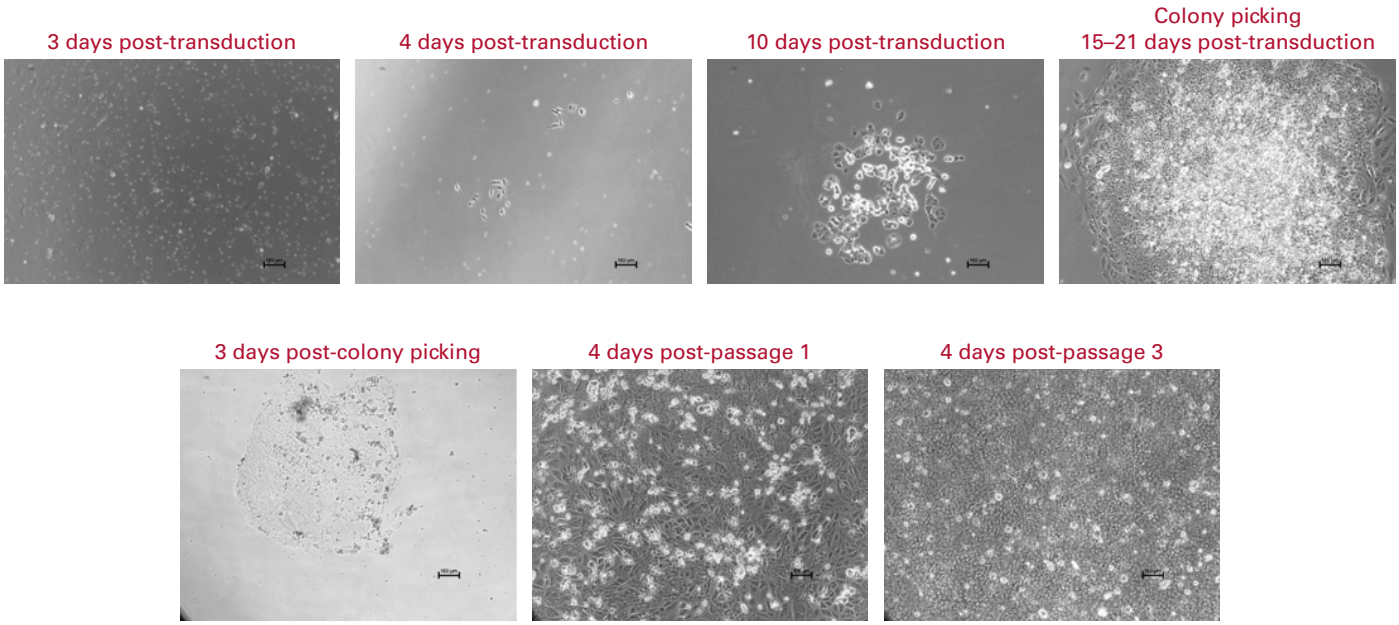
3. Add the appropriate volume of diluted COAT-1 solution to the cell culture vessel, making sure the entire surface is covered. Use the following volumes of COAT-1 solution: 50 µl/well of a 96-well plate, 200 µl/well of a 48-well plate, 400 µl/well of a 24-well plate, 800 µl/well of a 12-well plate, or 1.5 ml/well of a 6-well plate.
4. Place the cell culture vessel in an incubator for a minimum of 20 min at 37°C ± 1°C, 5% CO_2, and >90% humidity, or for 0.5–3 hr at room temperature (RT, 15–25°C).
5. Aspirate the COAT-1 solution from the cell culture vessel just before use.

IV. Protocol Overview

A



B



Protocol for the reprogramming of PBMCs into iPS cells generates colonies that show typical monolayer growth when passaged in the Cellartis DEF-CS 500 Culture System. **Panel A.** Suggested schedule for reprogramming of PBMCs in the DEF-CS system. **Panel B.** Representative photos of the cells during reprogramming and after transfer into the DEF-CS system.

V. Protocol

A. Transduce PBMCs (Day 0)

Deliver the reprogramming factors to your PBMCs using your method of choice.

B. Plate transduced PBMCs onto coated 6-well plates (Day 3)

On Day 3 after transduction, the transduced PBMCs should be transferred to 6-well plates coated with COAT-1. *Do not switch to Cellartis DEF-CS medium at this time.* Continue culturing the transduced PBMCs using the PBMC medium specific to your transduction method of choice.

NOTE: Change pipette tips between wells to prevent cross-contamination.

Preparation

Warm the PBMC medium to 37°C ± 1°C. Coat the appropriate number of wells in the 6-well plate(s) with COAT-1 diluted 1:5.

Passaging

1. Transfer the cell suspension from each well to a separate sterile centrifuge tube.
2. Rinse each well with 500 µl of the PBMC medium and transfer to the corresponding tube.
3. Centrifuge the cells at 200*g* for 10 min.
4. Discard the supernatants and resuspend each cell pellet in 500 µl of the PBMC medium.
5. Count the cells in a hemocytometer or a cell counter (optimized for the specific cell type).
6. Seed 2.5–5 x 10^4 cells/well in 6-well plate(s) coated with COAT-1. Use 2 ml of PBMC medium/well. We recommend using two different seeding densities.

C. PBMC media changes (Days 4 and 6)

For media changes on Days 4 and 6, *do not switch to Cellartis DEF-CS medium. Continue using PBMC medium as on Day 3 (above).*

NOTE: Change pipette tips between wells to prevent cross-contamination.

Preparation

Warm the PBMC medium (1 ml/well of a 6-well plate) to 37°C ± 1°C.

Media change (50% of the volume)

1. Carefully discard 1 ml of media per well.
2. Carefully add 1 ml of PBMC medium per well.
3. Place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

D. Begin the transition to DEF-CS medium (Day 7)

Preparation

Prepare the appropriate volume of Cellartis DEF-CS Medium for Maintenance (1 ml/well of a 6-well plate) and warm it to 37°C ± 1°C before use.

Media change (50% of the volume)

1. Carefully discard 1 ml of PBMC medium per well.
2. Carefully add 1 ml of Cellartis DEF-CS Medium for Maintenance per well.
3. Place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for 24 hr.

E. Complete the transition to DEF-CS medium (Day 8)

Preparation

Prepare the appropriate volume of Cellartis DEF-CS Medium for Maintenance (2 ml/well of a 6-well plate) and warm it to 37°C ± 1°C before use.

Media change (100% of the volume) *24 hr ± 2 hr after performing the media change on Day 7*

1. Carefully discard all of the media in the wells.
2. Carefully add 2 ml of Cellartis DEF-CS Medium for Maintenance per well.
3. Place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

F. Daily DEF-CS media changes (Days 9–21)

Preparation

Prepare the appropriate volume of Cellartis DEF-CS Medium for Maintenance (2 ml/well of a 6-well plate) and warm it to 37°C ± 1°C before use.

Media change (100% of the volume)

1. Examine the cells under a microscope and check for colonies; photo document as necessary.
2. Carefully discard all of the media in the wells.
3. Carefully add 2 ml of Cellartis DEF-CS Medium for Maintenance per well.
4. Place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

G. Colony picking (during the time span of Days 15–21 post-transduction)

When colonies are 1.5–3 mm in diameter, they are ready to be transferred/picked.

Preparation

Prepare the appropriate volume of Cellartis DEF-CS Medium for Passaging (250 µl/well of a 48-well plate) and warm it to 37°C ± 1°C before use. Coat the appropriate number of wells (one well for each colony to be picked) with 200 µl/well of COAT-1 solution, diluted 1:5.

How to pick colonies

1. Aspirate the COAT-1 solution from the cell culture vessel and add 250 µl of Cellartis DEF-CS Medium for Passaging per well.
2. Try to keep the plate at 37°C ± 1°C.
3. Working under a dissection microscope, use a fresh Stem Cell Cutting Tool to microdissect a colony into 2–4 pieces.
4. Use a fresh Transfer Pipette to transfer each piece into a separate well of the 48-well plate.
5. Repeat Steps 3 and 4 until the desired number of colonies has been picked.
6. Place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for 48–54 hr. Do not move the plate during this time.

VI. Scale-up using the Cellartis DEF-CS 500 Culture System

A. Changing media during scale-up

Prepare the appropriate volume of Cellartis DEF-CS Medium for Maintenance and warm to 37°C ± 1°C immediately before use.

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

B. Passaging during scale-up

Prepare the appropriate volume of Cellartis DEF-CS Medium for Passaging and warm to 37°C ± 1°C immediately before use. Coat the appropriate number of wells with COAT-1 (1 well per clonal population, in the appropriate format; see **Table 1** below). As a general rule, the area covered by the cells at passage should not be less than 20% of the area of the destination vessel. Passage single and/or small colonies to a new 48-well plate. If a larger area in the well is covered by cells, passage to a 24-well plate.

1. Check the cells under a microscope; photo document as necessary.
2. Aspirate the media from the cell culture vessel and gently wash the cell layer with D-PBS –/–.
3. Add the appropriate volume (Table 1) of TrypLE Select (room temperature) to the cells. Make sure that the entire surface of the well is covered. Incubate for 5 min or until the cells have detached.
4. Resuspend the cells in the appropriate volume (Table 1) of pre-warmed Cellartis DEF-CS Medium for Passaging and transfer all cells from a well to a newly coated well.

   NOTE: Counting the cells is not recommended.

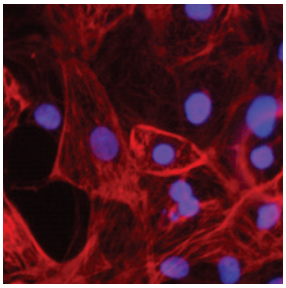
5. Immediately after plating, hold each cell culture vessel in one hand and mix gently using a figure-eight motion, which distributes the cells evenly over the surface. Place in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

NOTE: When the cells have been scaled up to one T-25 flask per clone, the lines should be cultured according to the Cellartis DEF-CS 500 Culture System User Manual.

Table 1: Suggested schedule for scaling up reprogrammed clones.

Passage number	Starting format	Passage interval*	New format	Dilution of COAT-1	Volume of diluted COAT-1	Volume of TrypLE Select	Volume of Cellartis DEF-CS Medium for Passaging
Colony picking	Colony in 1 well in 48-well plate	5–10 days	1 well in 48-well plate	1:5	200 µl/well in 48-well plate	50 µl/well in 48-well plate	250 µl/clone
1 (start scale-up)	1 well in 48-well plate	2–5 days	1 well in 24-well plate	1:5	400 µl/well in 24-well plate	50 µl/well in 48-well plate	1 ml/clone
2	1 well in 24-well plate	2–5 days	1 well in 12-well plate	1:20	800 µl/well in 12-well plate	100 µl/well in 24-well plate	2 ml/clone
3	1 well in 12-well plate	2–5 days	1 well in 6-well plate	1:20	1.5 ml/well in 6-well plate	200 µl/well in 12-well plate	3 ml/clone
4	1 well in 6-well plate	2–5 days	1 T-25 flask	1:20	2.5 ml per T-25 flask	300 µl/well in 6-well plate	5 ml/clone

* If a clone grows fast and the culture is very dense at passaging, it is possible to expedite the scale-up by skipping some vessels—i.e., passaging from a well of a 24-well plate directly into a well of a 6-well plate.



Stem Cell Application Protocol

Reprogramming fibroblasts

Using the Cellartis® DEF-CS™ 500 Culture System

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I. Introduction

Induced pluripotent stem (iPS) cells originate from adult cells that have been reprogrammed with key transcription factors to exhibit pluripotency. Fibroblasts are a popular source of adult cells for reprogramming. Once the reprogramming factors have been delivered to fibroblasts, the cells can be transferred to the DEF-CS system to maximize the number of emerging colonies and for robust expansion into stable iPS cell lines.

This protocol has been developed using Sendai viruses for delivery of the transcription factors. Optimization may be necessary if using other delivery methods.

II. Materials required

- Cellartis DEF-CS 500 Culture System (Takara Bio, Cat. #Y30010) (includes COAT-1, Basal Medium, GF-1, GF-2, and GF-3)
- Stem Cell Cutting Tools (Vitrolife, Cat. #14601)
- Transfer Pipettes (Vitrolife, Cat. #14319)
- 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), no phenol red (Thermo Fisher Scientific, Cat. #12563011)
- Cell culture vessels, tissue-culture-treated polystyrene surface

III. Preparing medium and coating cell culture vessels

Detailed information about media preparation is available in the Cellartis DEF-CS 500 Culture System User Manual.

A. Maintenance medium for human iPS cells

Prepare an appropriate volume of Cellartis DEF-CS Medium for Maintenance by adding GF-1 (dilute 1:333) and GF-2 (dilute 1:1,000) to Cellartis DEF-CS Basal Medium.

B. Passaging medium for human iPS cells

Prepare an appropriate volume of Cellartis DEF-CS Medium for Passaging by adding GF-1 (dilute 1:333), GF-2 (dilute 1:1,000), **and GF-3** (dilute 1:1,000) to Cellartis DEF-CS Basal Medium.

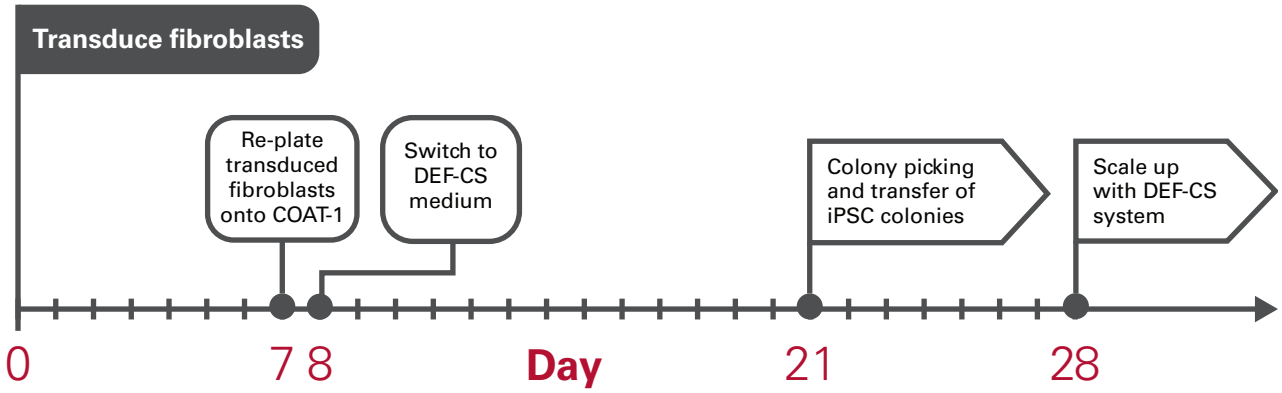
C. Coating cell culture vessels

1. Dilute the required volume of COAT-1 in D-PBS +/+ before use. Make a **1:5** dilution at Day 7, at colony picking, and for the first passage during scale-up. Use a **1:20** dilution for subsequent passages.
2. Mix the diluted COAT-1 solution gently and thoroughly by pipetting up and down.

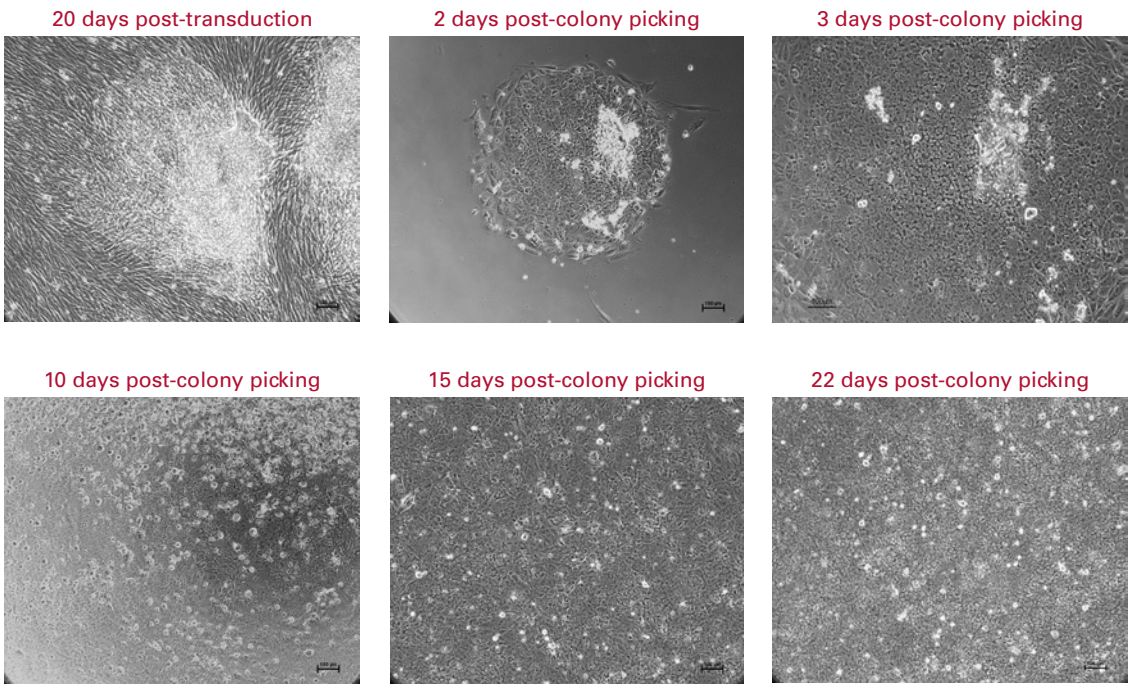
3. Add the appropriate volume of diluted COAT-1 solution to the cell culture vessel, making sure the entire surface is covered. Use the following volumes of COAT-1 solution: 50 µl/well of a 96-well plate, 200 µl/well of a 48-well plate, 400 µl/well of a 24-well plate, 800 µl/well of a 12-well plate, or 1.5 ml/well of a 6-well plate.
4. Place the cell culture vessel in an incubator for a minimum of 20 min at 37°C ± 1°C, 5% CO_2, and >90% humidity, or for 0.5–3 hr at room temperature (RT, 15–25°C).
5. Aspirate the COAT-1 solution from the cell culture vessel just before use.

IV. Protocol overview

A



B



Protocol for the reprogramming of fibroblasts into iPS cells generates colonies that show typical monolayer growth when passaged in the Cellartis DEF-CS 500 Culture System. Panel A. Suggested schedule for reprogramming of fibroblasts in the DEF-CS system. **Panel B.** Representative photos of the cells during reprogramming and after transfer into the DEF-CS system.

V. Protocol

A. Transduce fibroblasts (Day 0)

Deliver the reprogramming factors to your fibroblasts using your method of choice.

B. Daily media changes (Days 1, 2, 3, 4, 5, and 6)

Prepare the appropriate volume of fibroblast medium (2 ml/well of a 6-well plate) and warm it to 37°C ± 1°C before use. *On Day 1, you can perform the media change 24 hr ± 2 hr post-transduction.*

NOTE: Change pipette tips between wells to prevent cross-contamination.

Media change (100% of the volume)

1. Carefully discard all of the media in the wells.
2. Carefully add 2 ml of fibroblast medium per well.
3. Place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

C. Plate transduced fibroblasts onto coated 6-well plates (Day 7)

On Day 7 post-transduction, the transduced fibroblasts should be transferred to 6-well plates coated with COAT-1. *Do not switch to Cellartis DEF-CS medium at this time. Plate the transduced fibroblasts using the fibroblast medium specific to your transduction method of choice.*

Preparation

Warm the fibroblast medium to 37°C ± 1°C. Coat the appropriate number of wells in the 6-well plate(s) with COAT-1 diluted 1:5.

1. Check the cells under a microscope; photo document as necessary.
2. Aspirate all of the media from the cell culture vessel and gently wash the cell layer with D-PBS –/–.
3. Add 500 µl/well (of a 6-well plate) of TrypLE Select (room temperature) to the cells. Make sure that the entire surface of the well is covered. Incubate for 3 min, or until the cells have detached.
4. Resuspend the cells in 2 ml/well (of a 6-well plate) of pre-warmed fibroblast medium and transfer all cells from each well to a single centrifuge tube.
5. Centrifuge the cells at 200*g* for 4 min.
6. Carefully aspirate the media and resuspend the cells in an appropriate amount of fibroblast medium.
7. Count the cells and seed them at 0.2–1.0 x 10^4 cells/cm^2 into 6-well plate(s) coated with COAT-1. Use 2 ml of fibroblast medium per well. We recommend using two different seeding densities.
8. Immediately after plating, hold each cell culture vessel in one hand and mix gently using a figure-eight motion, which distributes the cells evenly over the surface. Place in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

D. Transition to DEF-CS medium (Day 8)

Preparation

Prepare the appropriate volume of Cellartis DEF-CS Medium for Maintenance (2 ml/well of a 6-well plate) and warm it to 37°C ± 1°C before use.

NOTE: Change pipette tips between wells to prevent cross-contamination.

Media change (100% of the volume)

1. Carefully discard all of the medium in the wells.
2. Carefully add 2 ml of Cellartis DEF-CS Medium for Maintenance per well.
3. Place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

E. Daily DEF-CS media changes (Days 9–28)

Preparation

Prepare the appropriate volume of Cellartis DEF-CS Medium for Maintenance (2 ml/well of a 6-well plate) and warm it to 37°C ± 1°C before use.

Media change (100% of the volume)

1. Examine the cells under a microscope and check for colonies; photo document as necessary.
2. Carefully discard all of the medium in the wells.
3. Carefully add 2 ml of Cellartis DEF-CS Medium for Maintenance to each well.
4. Place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

F. Colony picking (during the time span of Days 21–28 post-transduction)

When colonies are 1.5–3 mm in diameter, they are ready to be transferred/picked.

Preparation

Prepare the appropriate volume of Cellartis DEF-CS Medium for Passaging (250 µl/well of a 48-well plate) and warm it to 37°C ± 1°C before use. Coat the appropriate number of wells (one well for each colony to be picked) with 200 µl/well of COAT-1 solution, diluted 1:5.

How to pick colonies

1. Aspirate the COAT-1 solution from the cell culture vessel and add 250 µl of Cellartis DEF-CS Medium for Passaging per well.
2. Try to keep the plate at 37°C ± 1°C.
3. Working under a dissection microscope, use a fresh Stem Cell Cutting Tool to microdissect a colony into 2–4 pieces.
4. Use a fresh Transfer Pipette to transfer each piece into a separate well of the 48-well plate.
5. Repeat Steps 3 and 4 until the desired number of colonies has been picked.
6. Place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for 48–54 hr. Do not move the plate during this time.

VI. Scale-up using the Cellartis DEF-CS 500 Culture System

A. Changing media during scale-up

Prepare the appropriate volume of Cellartis DEF-CS Medium for Maintenance and warm to 37°C ± 1°C immediately before use.

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

B. Passaging during scale-up

Prepare the appropriate volume of Cellartis DEF-CS Medium for Passaging and warm to 37°C ± 1°C immediately before use. Coat the appropriate number of wells with COAT-1 (1 well per clonal population, in the appropriate format; see **Table I** below). As a general rule, the area covered by the cells at passage should not be less than 20% of the area of the destination vessel. Passage single and/or small colonies to a new 48-well plate. If a larger area in the well is covered by cells, passage to a 24-well plate.

1. Check the cells under a microscope; photo document as necessary.
2. Aspirate the media from the cell culture vessel and gently wash the cell layer with D-PBS –/–.

3. Add the appropriate volume (Table I) of TrypLE Select (room temperature) to the wells. Make sure that the entire surface of the well is covered. Incubate for 5 min or until the cells have detached.
4. Resuspend the cells in the appropriate volume (Table I) of pre-warmed Cellartis DEF-CS Medium for Passaging and transfer all cells from a well to a newly coated well.

   NOTE: Counting the cells is not recommended.

5. Immediately after plating, hold each cell culture vessel in one hand and mix gently using a figure-eight motion, which distributes the cells evenly over the surface. Place in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

NOTE: When the cells have been scaled up to one T-25 flask per clone, the lines should be cultured according to the Cellartis DEF-CS 500 Culture System User Manual.

Table I: Suggested schedule for scaling up reprogrammed clones.

Starting format	Passage interval*	New format	Dilution of COAT-1	Volume of diluted COAT-1	Volume of TrypLE Select	Volume of Cellartis DEF-CS Medium for Passaging
Picked colony in 1 well in 48-well plate	5–10 days	1 well in 48-well plate	1:5	200 µl/well in 48-well plate	50 µl/well in 48-well plate	250 µl/clone
1 well in 48-well plate	2–5 days	1 well in 24-well plate	1:5	400 µl/well in 24-well plate	50 µl/well in 48-well plate	1 ml/clone
1 well in 24-well plate	2–5 days	1 well in 12-well plate	1:20	800 µl/well in 12-well plate	100 µl/well in 24-well plate	2 ml/clone
1 well in 12-well plate	2–5 days	1 well in 6-well plate	1:20	1.5 ml/well in 6-well plate	200 µl/well in 12-well plate	3 ml/clone
1 well in 6-well plate	2–5 days	1 T-25 flask	1:20	2.5 ml per T-25 flask	300 µl/well in 6-well plate	5 ml/clone

*If a clone grows fast and the culture is very dense at passaging, it is possible to expedite the scale-up by skipping some vessels—i.e., passaging from a well of a 24-well plate directly into a well of a 6-well plate.

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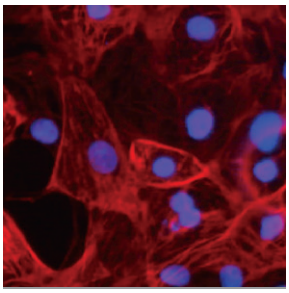


Stem Cell Application Protocol

Spin Embryoid Body Formation and Confirmation of Pluripotency

With the Cellartis® DEF-CS™ 500 Culture System

that's GOOD science!

I. Introduction

Embryoid bodies (EBs) are three-dimensional aggregates comprised of human pluripotent stem cells (hPSCs). hPSCs within EBs undergo differentiation and cell specification along the three germ layers, which are commonly used as an assessment of the initial hPSCs' pluripotency. The protocol for formation of spin EBs can also be used as a basis for the development of protocols for directed differentiation.

II. Materials Required

- 24-well plates, cell culture treated, flat bottom
- 96-well plates, untreated, V bottom
- Advanced RPMI 1640 (with glucose, sodium pyruvate, and non-essential amino acids; without L-glutamine and HEPES)
- B-27 Supplement (50X), serum free
- DEF-CS COAT-1
- GlutaMAX-I (100X; 200 mM)
- PBS Dulbecco's with Ca^{2+} & Mg^{2+} (D-PBS +/+)
- PBS Dulbecco's w/o Ca^{2+} & Mg^{2+} (D-PBS –/–)
- Penicillin-streptomycin (PEST; 10,000 units/ml of penicillin and 10,000 µg/ml of streptomycin)
- TrypLE Select Enzyme (1X), w/o phenol red
- Y27632

III. Protocol

A. Medium Preparation

Preparing the *In Vitro* Differentiation (IVD) Medium

Prepare the IVD medium by adding 10 ml B-27 Supplement (50X), 5 ml GlutaMAX-I (100X), and 5 ml PEST to 500 ml of Advanced RPMI 1640. Mix the solution properly and carefully. The medium expires one month after the date of preparation.

Preparing the Seeding Medium

Prepare the seeding medium by adding Y27632 (to a final concentration of 5 µM) to the IVD medium. Prepare fresh medium on the day of intended use.

B. Formation of Spin EBs

1. Warm the seeding medium to 37°C ± 1°C and all other reagents to room temperature (RT, 15–25°C) before use.
2. Wash **one** T25 flask containing confluent hPSCs with 5 ml D-PBS –/–.

   NOTE: The entirety of this flask will be used for spin EB formation. Other flasks or banked cells of equivalent passage number from the original line should be set aside if spin EB formation is being used to assess the pluripotency of that line.

3. Add 500 µl of TrypLE Select (20 µl/cm^2) and place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for 5 min, or until cells start to detach.
4. Add 5 ml of seeding medium and dissociate the cells, pipetting up and down until a single-cell suspension is achieved.
5. Count the cells to determine the initial cell concentration.
6. Make a cell suspension of 20 ml seeding medium containing 2.5 x 10^5 cells/ml.
7. Seed 200 µl of the final cell suspension into each well of a 96-well plate (untreated, V bottom), generating a seeding density of 5 x 10^4 cells/well.
8. Centrifuge at 400*g* at RT for 5 min.
9. Place the 96-well plate in the incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity and let it sit undisturbed for 7 days to form EBs.

   NOTE: Throughout this process, the cells are undergoing spontaneous differentiation. During the incubation period, directed differentiation protocols can be optimized and applied. Alternatively, if pluripotency is to be assessed, continue with Section III.C.

C. Spontaneous Differentiation of Spin EBs

Coating of the Cell Culture Plate

1. Dilute the required volume of DEF-CS COAT-1 in D-PBS +/+ before use. Make a 1:20 dilution.
2. Mix the diluted DEF-CS COAT-1 solution gently and thoroughly by pipetting up and down.
3. Add the appropriate volume of diluted DEF-CS COAT-1 solution to a 24-well plate (use 0.2 ml/cm^2), making sure the entire culture surface of each well is covered.
4. Place the plate for a minimum of 20 min in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for 0.5–3 hr at RT.
5. Aspirate DEF-CS COAT-1 solution from the plate immediately before use.

Transferring of Spin EBs

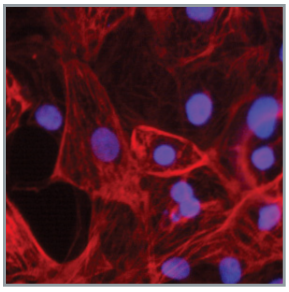
1. Warm IVD medium to 37°C ± 1°C.
2. Add 1.5 ml of fresh, warm IVD medium to each well of the coated 24-well plate.
3. Carefully detach and transfer the EBs using a pipette. Transfer 5–7 EBs to each well of the 24-well plate.
4. Place the 24-well plate in the incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity and let it sit undisturbed for 4 days.

Exchanging the Media

NOTE: Complete a 100% media change every 2–3 days.

1. Warm IVD medium to 37°C ± 1°C.
2. Completely exchange the media in each well of the 24-well plate (1.5 ml/well).

   NOTE: 18–21 days after the start of spin EB formation, the cells are ready to be analyzed for the presence of specialized cells along the three germ layers. The number of days needed depends on the cell line.



Stem Cell Application Protocol

Transfer of Human iPS Cells from Other Feeder-Free Culture Systems

that's GOOD science!

To the Cellartis® DEF-CS™ 500 Culture System

I. Introduction

Undifferentiated human iPS cells maintained in other feeder-free culture systems can be readily transferred to the DEF-CS Culture System. Cryopreserved human iPS cells can be thawed directly using the DEF-CS Culture System. Fresh cultures should be transferred on days when they would normally be passaged.

II. Materials Required

- Cellartis DEF-CS 500 Culture System (includes COAT-1, Basal Medium, GF-1, GF-2, and GF-3)
- TrypLE Select Enzyme (1X), w/o phenol red
- PBS Dulbecco's with Ca^{2+} & Mg^{2+} (D-PBS +/+)
- PBS Dulbecco's w/o Ca^{2+} & Mg^{2+} (D-PBS –/–)
- Cell culture vessels, tissue culture-treated polystyrene surface

III. Protocol

A. Transferring Fresh or Frozen Cultures to the DEF-CS System

Coating of Cell Culture Vessels

1. Dilute the required volume of DEF-CS COAT-1 in D-PBS +/+ before use. Make a 1: **5** dilution.
2. Mix the diluted DEF-CS COAT-1 solution gently and thoroughly by pipetting up and down.
3. Add an appropriate volume of diluted DEF-CS COAT-1 solution to a cell culture vessel (use 0.1 ml/cm^2), making sure that the entire surface is covered.
4. Incubate the cell culture vessel at 37°C ± 1°C, 5% CO_2, and >90% humidity for a minimum of 20 min, or at room temperature (15–25°C) for 0.5–3 hr.
5. Aspirate the DEF-CS COAT-1 solution from the cell culture vessel immediately before use.

Preparing Supplemented DEF-CS Medium

1. Prepare the appropriate volume of supplemented DEF-CS medium by adding DEF-CS GF-1 (dilute 1:333), GF-2 (dilute 1:1,000), and GF-3 (dilute 1:1,000) to DEF-CS Basal Medium.
2. Prepare fresh medium on the day of intended use and warm it to 37°C ± 1°C immediately before use. Discard any leftover warm medium.
3. Warm all other necessary reagents to room temperature (15–25°C) before use.

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Transferring Fresh hiPS Cells from Other Feeder-Free, Monolayer Culture Systems

NOTE: Fresh cultures should be transferred on days when they would normally be passaged. It is important to count the cells and use the recommended seeding density of 5–7 x 10^4 cells/cm^2.

1. Check cells under a microscope; photo document as necessary.
2. Aspirate the medium from the cell culture vessel and wash the cell layer once with D-PBS –/–.
3. Add 20 µl/cm^2 of TrypLE Select Enzyme (1X) to the cell culture vessel and incubate for 5 min, or until the cell layer has detached. Detachment can be aided by swirling the cell culture vessel or by tapping the side of the cell culture vessel firmly but gently.
4. Resuspend the cells in pre-warmed, supplemented DEF-CS medium (40 µl/cm^2) 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. Centrifuge the cells at 200*g* for 2–5 min.
6. Count the cells in a hemocytometer or in a cell counter that has been optimized for the cell type.
7. Add the appropriate volumes of cell suspension and additional medium (if necessary) to the newly coated cell culture vessel to obtain 5.0–7.0 x 10^4 cells/cm^2. The seeding volume of supplemented DEF-CS medium should be 0.15–0.25 ml/cm^2.
8. Tilt the vessel 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.

Transferring Cryopreserved hiPS Cells from Other Feeder-Free, Monolayer Culture Systems

NOTE: We recommend a seeding density at thawing of 1.5–2.5 x 10^5 cells/cm^2.

1. Thaw the cells according to your preferred protocol.
2. Transfer the cells to a newly coated cell culture vessel with pre-warmed, supplemented DEF-CS medium.
3. Tilt the vessel backwards and forwards gently to ensure that the cell suspension is dispersed evenly over the surface, then gently place in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

B. Scaling Up

NOTE: The single-cell passaging method employed by the DEF-CS Culture System causes iPS cells to initially assume a distinct morphology and sparser distribution relative to cells cultured using colony-based passaging methods. However, as the cells proliferate and form denser populations, morphologies commonly associated with undifferentiated stem cells (e.g., high nucleus-to-cytoplasm ratio, clearly defined borders, and prominent nucleoli) emerge.

- It may take 2–5 passages to adapt a cell line to the DEF-CS Culture System. Newly transferred cells might initially grow at a slightly slower rate. A suitable passage interval might therefore be between 3 and 7 days for the first few passages.
- Use a 1: **5** dilution of DEF-CS COAT-1:D-PBS +/+ for the first few passages to provide extra support during the adaptation process.
- To prevent cell loss during scale-up, we recommend *not* counting cells at passage when the total number of cells is quite low.
- If the hiPS cells were sparsely seeded or thawed in aggregates, they will grow as colonies on COAT-1. As a general rule, when passaging hiPS cells that are growing as colonies, the area covered by the cells at passage should not be less than 20% of the area of the destination vessel.
- For passages involving cells growing in a homogeneous monolayer (normal DEF-CS Culture System characteristics), cells are ready for passage when they have acquired the morphology displayed in Figures 3 and 4 in the Cellartis DEF-CS 500 Culture System User Manual. However, if cells remain sparsely distributed after seven days in culture, a passage is still recommended. The area of the destination vessel should be 3–6 times the area of the current vessel.
- Once cells have been scaled up to a T-25 flask, they should be cultured according to the Cellartis DEF-CS 500 Culture System User Manual.

Cellartis DEF-CS 500 Culture Sytstemの注目アプリケーション

Cas9/sgRNA RNPによる遺伝子ノックアウトiPS細胞クローンの取得

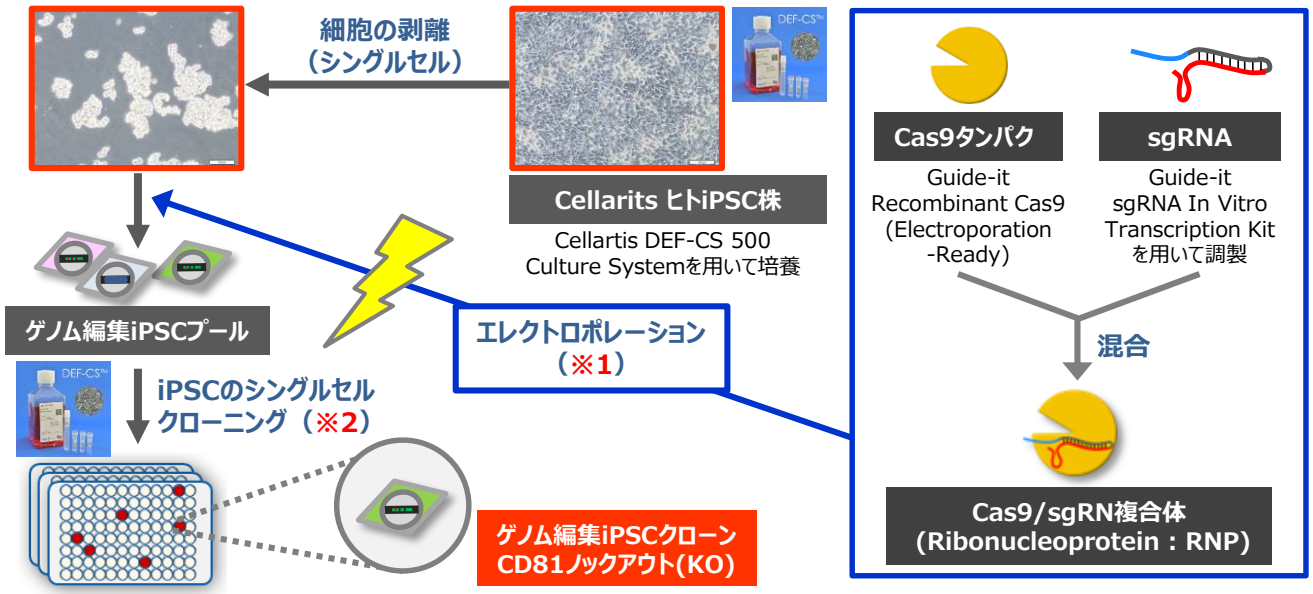

図1. Guide-it Recombinant Cas9とDEF-CS Culture Systemによるゲノム編集iPS細胞クローン取得フロー

※1: プロトコール詳細は、Guide-it Recombinant Cas9 (Electroporation-Ready)の説明書をご覧下さい。
※2: プロトコール詳細は、本誌p1 - 5をご覧下さい。

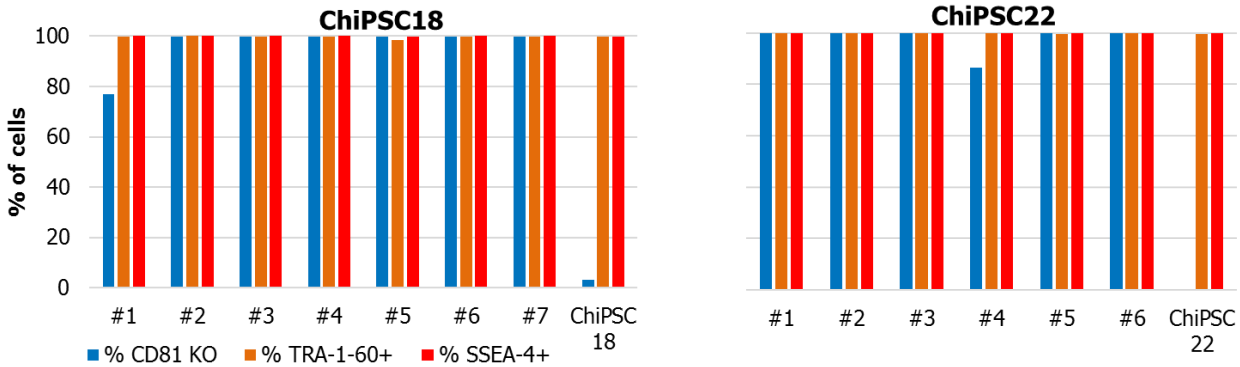

図2. DEF-CS培地によるシングルセルクローニングで取得したCD81 KO iPS細胞クローンのFACS解析結果

図1のフローにて取得したヒトCellarits iPS細胞株ChiPSC18、22由来のCD81ノックアウト(KO)株のCD81，TRA-1-60，SSEA-4発現量をFACS解析で確認したところ、何れのクローンでも高い未分化マーカー発現が確認された。
※ChiPSC18-#1, ChiPSC22-#4は、片アリルのみのCD81 KO株。

PRODUCTS

製品コード	製品名	容量	価格(税別)
632641	Guide-it™ Recombinant Cas9 (Electroporation-Ready)	100 µg	¥59,800
632635	Guide-it™ sgRNA In Vitro Transcription Kit	50回	¥150,000

2017年02月作成

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