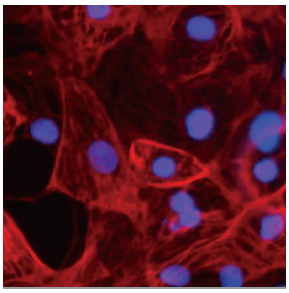


Single-Cell Cloning and Expansion

that's GOOD science!®

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 1:10 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

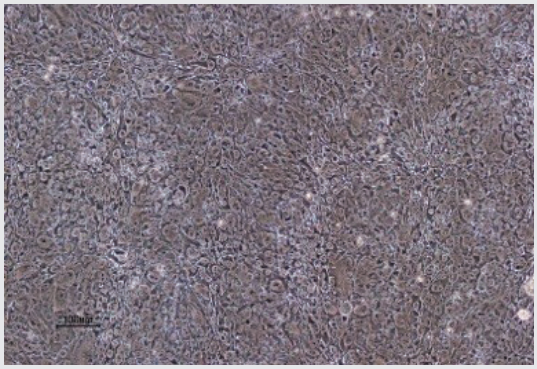
1. Prepare the appropriate volume of supplemented DEF-CS medium by adding DEF-CS GF-1 (dilute 1:333), GF-2 (dilute 1:1,000), and GF-3 (dilute 1:1,000) to DEF-CS Basal Medium.
2. Prepare fresh medium on the day of intended use and warm it to 37°C ± 1°C immediately before use. Discard any leftover warmed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

TaKaRa Clontech

Clontech Laboratories, Inc. • A Takara Bio Company
1290 Terra Bella Avenue, Mountain View, CA 94303, USA • Technical Support: tech@clontech.com
United States/Canada: +1.800.662.2566 • Asia Pacific: +1.650.919.7300 • Europe: +33.(0)1.3904.6880 • Japan: +81.(0)77.565.6999

Single-Cell Seeding

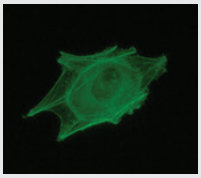
NOTE: To optimize survival rate and expansion potential during single-cell cloning, we recommend using cells that are in a proliferative state. We recommend starting with a confluent but not dense (not growth-arrested) culture, corresponding to a density of 0.8–1.5 x 10^5 cells/cm^2 (example image below). 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.



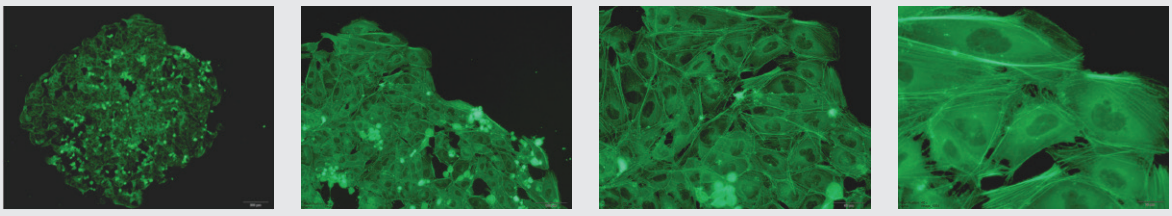
Cells with a density of 0.8–1.5 x 10^5 cells/cm^2.

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.

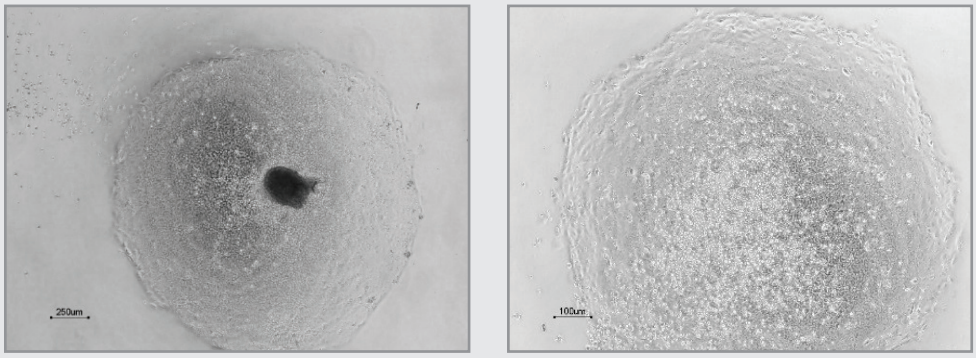
2. Check cells under the microscope; photo document as necessary.
3. Carefully withdraw 150 µl of medium and add 150 µl of newly warmed medium into each well of the plate. **Avoid flushing medium directly onto the cell layer.**
4. Place the cell culture plate in an incubator at 37°C ± 1°C, 5% CO_2, and >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 1:10 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 1:10 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.