Takara Bio Europe AB

Cellartis® iPSC Single-Cell Cloning DEF-CS™ Culture Media Kit

Cat. No. Y30021
(030619)

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

United States/Canada	Asia Pacific	Europe	Japan
800.662.2566	+1.650.919.7300	+33.(0)1.3904.6880	+81.(0)77.565.6999

Table of Contents

Table of Figures

Table of Tables

I. Introduction

The Cellartis iPSC Single-Cell Cloning DEF-CS Culture Media Kit is a complete system for efficient generation and expansion of clonal populations from human induced pluripotent stem cells (iPSCs) in a feeder-free and defined environment. Establishment of a clonal population is inefficient, challenging, and time consuming; often, it results in cell death or premature differentiation. The DEF-CS culture system, a monolayer-based culture system, bypasses the challenges of colony-based culture by allowing single-cell passaging and promoting survival and further expansion of plated single cells (Figure 1).

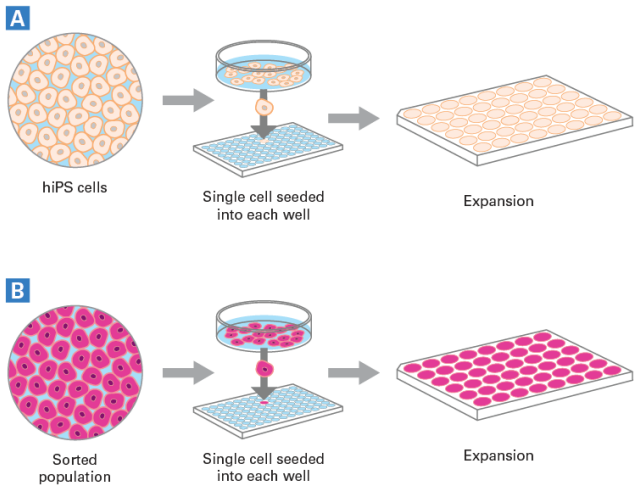

Figure 1. Workflow for the generation of clonal cell lines. Human induced pluripotent stem (hiPS) cells can be cultured and clonally expanded using the Cellartis iPSC Single-Cell Cloning DEF-CS Culture Media Kit. Initially, hiPS cells are adapted to the DEF-CS culture system, which maintains cells as a karyotypically stable and pluripotent monolayer. Using FACS or limiting dilution, cells can be individually seeded into wells of a 96-well plate (**Panel A**) and expanded into clonal lines. If desired, cells can be first sorted by flow cytometry, then seeded into wells of a 96-well plate, and finally expanded into clonal lines (**Panel B**).

All procedures described in this manual are optimized for Cellartis human iPS cell lines. If you wish to use the Cellartis iPSC Single-Cell Cloning DEF-CS Culture Media Kit for other human iPS cell lines or for Cellartis iPS cells grown in another system, please be aware that these cell lines will need to be adapted to the DEF-CS culture system before proceeding to single-cell culture (see Section IV.B).

II. List of Components

The Cellartis iPSC Single-Cell Cloning DEF-CS Culture Media Kit (Cat. No. Y30021) contains:

- 500 ml Cellartis DEF-CS 500 Basal Medium (Cat. No. Y30011; not sold separately)
- 2 x 800 µl Cellartis iPSC Single-Cell Cloning DEF-CS COAT-1 (Cat. No. Y30018; not sold separately)
- Cellartis iPSC Single-Cell Cloning DEF-CS Additives (Cat. No. Y30019; not sold separately)
    - 2 x 750 µl DEF-CS GF-1
    - 500 µl DEF-CS GF-2
    - 500 µl DEF-CS GF-3

III. Additional Materials Required

The following tissue culture materials are required but not supplied:

- 96-well plates, flat bottom, cell-culture treated (Corning, Cat. No. 3595 or equivalent)
- 48-well plates, flat bottom, cell-culture treated (Corning, Cat. No. 3548 or equivalent)
- TrypLE Select Enzyme (1X), no phenol red (Thermo Fisher Scientific, Cat. No. 12563011)
- PBS Dulbecco's with Ca^{2+} & Mg^{2+} (D-PBS +/+) (Sigma, Cat. No. D8662 or equivalent)
- PBS Dulbecco's w/o Ca^{2+} & Mg^{2+} (D-PBS –/–) (Sigma, Cat. No. D8537 or equivalent)

The Cellartis DEF-CS 500 Culture System (Takara Bio, Cat. No. Y30010) is recommended for maintaining hiPS cell lines, gene editing, adapting cells to the DEF-CS single-cell cloning system, and scaling up clonal lines.

IV. General Considerations

A. Storage and Handling

Cellartis DEF-CS 500 Basal Medium and Cellartis iPSC Single-Cell Cloning DEF-CS COAT-1 should be stored at 2–8°C; shelf life is specified on the product label. The Cellartis DEF-CS 500 Basal Medium formulation contains penicillin and streptomycin.

Cellartis iPSC Single-Cell Cloning DEF-CS Additives (DEF-CS GF-1, DEF-CS GF-2, and DEF-CS GF-3) should be stored at –20°C; shelf life is specified on the product label. At first use, thaw provided vials, mix each vial gently, and aliquot each component separately into appropriate volumes. Store at –20°C until the expiration date on the original vial. Thawed vials may be stored at 2–8°C for up to one week. Do not re-freeze aliquots after thawing.

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.

B. Transferring Human iPS Cells to the DEF-CS Culture System

It is strongly recommended to transfer cells from other systems to the Cellartis DEF-CS 500 Culture System (Cat. No. Y30010) before single-cell cloning with the Cellartis iPSC Single-Cell Cloning DEF-CS Culture Media Kit. Human iPS cells maintained in other culture systems can be readily transferred: fresh cultures can be transferred at passage and cryopreserved cultures can be thawed directly into the Cellartis DEF-CS 500 Culture System. Cells should be passaged at least five times in this system prior to performing single-cell cloning experiments using the Cellartis iPSC Single-Cell Cloning DEF-CS Culture Media Kit.

Expected Morphology of Human iPS Cells in the DEF-CS System

DEF-CS technology uses enzyme-based passaging in conjunction with a specific coating to promote single-cell survival, rapid expansion, and easier passaging. When transferring iPS cells to this system, you will notice that some cell characteristics differ from those of iPS cells cultured in your previous system. In contrast to commonly used colony-based culture systems, the DEF-CS culture system yields a monolayer of evenly spaced cells. Newly passaged cells grown in the DEF-CS culture system tend to spread out; however, as cells proliferate, the culture gets denser, and cells display the typical undifferentiated stem cell morphology (i.e., high nucleus to cytoplasm ratio, defined borders, and prominent nucleoli).

V. Workflow

Single-cell isolation and further expansion to form a clonal cell line requires specific culture conditions enabled by the Cellartis iPSC Single-Cell Cloning DEF-CS Culture Media Kit. Table 1 describes a schedule of all media changes (volume and composition) necessary to create clonal lines in 24-well plates that are ready for culture with the Cellartis DEF-CS 500 Culture System.

Table 1. Media volume guide for single-cell seeding and expansion.

Plate type	Day	Additives used	Volume of medium (µl per well)	Total volume (µl per well)
96-well plate	1	GF-1, GF-2, and GF-3	100	100
	3		100*	200
	5		150	200
	(6)		150	200
	7		150	200
	(8)		150	200
	9**		150	200
48-well plate	Passaging	GF-1, GF-2, and GF-3	500	500
	Medium Change	GF-1 and GF-2	500	500
24-well plate	Transition to Cellartis DEF-CS 500 Culture System			

*Add medium; do not replace.

**Use the same volumes for subsequent days until the cells are ready for passaging.

( ) Media change is only necessary if media is yellow due to high metabolic activity.

VI. Single-Cell Seeding into a 96-Well Plate

NOTE: If the cells used for the generation of the clonal cell line 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.

To optimize survival rate and expansion potential during single-cell seeding, use cells that are in an early proliferative state. We recommend starting with a *confluent but not dense* culture, corresponding to a density of 0.8–1.5 x 10^5 cells/cm^2 (Figure 2).

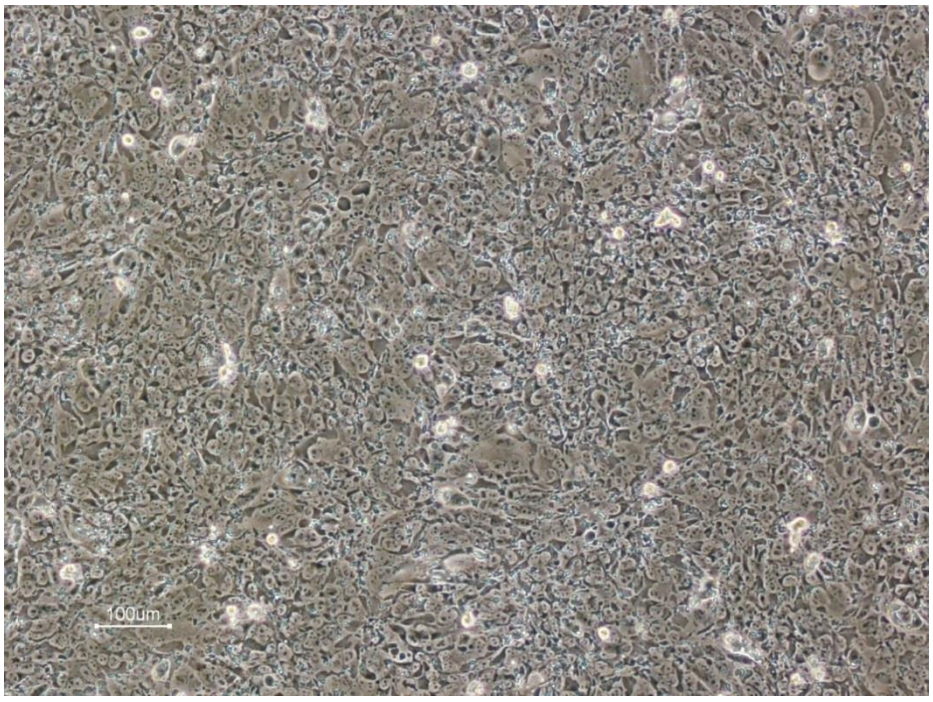

Figure 2. Recommended density of starting culture used for single-cell cloning. Representative image shows the morphology of cells with a density of 1.5 x 10^5 cells/cm^2 at 10X magnification. Scale bar = 100 microns.

A. Coating of a 96-Well Plate

1. Dilute the required volume of Cellartis iPSC Single-Cell Cloning DEF-CS COAT-1 (SSC-COAT-1) in D-PBS +/+ prior to use. Make a 1:10 dilution. Calculate the amount of diluted coating solution required depending on the number of wells to be used: 50 µl of diluted coating solution per well (see Table 2 for guidance).

Table 2. Preparation of coating solution for a 96-well plate.

Number of wells (96-well plate)	Volume of diluted coating solution (µl)	Volume of SSC-COAT-1 (µl)	Volume of D-PBS +/+ (µl)
1	50	5	45
2	100	10	90
96	4,800	480	4,320
n	50 x n	(50 x n)/10	(50 x n) – (volume of SSC-COAT-1)

2. Mix the diluted SSC-COAT-1 solution gently and thoroughly by pipetting up and down.
3. Add the diluted SSC-COAT-1 solution to a 96-well plate for single-cell seeding, 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 diluted SSC-COAT-1 solution from the cell culture plate just before use.

B. Preparing DEF-CS Medium for Single-Cell Seeding

Prepare the appropriate volume of "DEF-CS medium for single-cell seeding" 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. 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.

It is important to prepare enough DEF-CS medium for single-cell seeding to 1) neutralize the TrypLE Select Enzyme (1X) used to dissociate cells from the initial culture vessel (a 1:10 dilution) and 2) seed the detached cells using 100 µl/well into a 96-well plate. Use Table 3 as a guide to ensure there is sufficient medium for dissociation and seeding.

Table 3. Preparation of medium for dissociation and single-cell seeding.

Cell Dissociation

Plate type	TrypLE Select Enzyme (1X) (µl per well)	Supplemented DEF-CS medium (µl per well)
48 wells	18	200
24 wells	38	400
12 wells	76	800
6 wells	190	2,000
10 cm	1,100	12,000

Single-Cell Seeding

Plate type	DEF-CS medium for single-cell seeding (µl per well)
96 wells	100

C. Single-Cell Seeding

Seeding of Single Cells (Day 1)

1. Check cells under a phase contrast microscope; photo document as necessary.
2. Aspirate the medium from the culture vessel and wash the cell layer once with D-PBS –/–.
3. Add TrypLE Select Enzyme (1X) to the culture vessel, using an amount indicated in Table 3. Place the vessel in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for 5 min, or until the cell layer has detached. Detachment can be aided by tapping the side of the vessel firmly but gently. It is not recommended to tilt or swirl the cell culture vessel.

   NOTE: If starting from multiple samples in the same plate, please make sure to replace the culture vessel lid after removing a sample from a well, then gently tap the side of the vessel. This redistributes the dissociation enzyme and minimizes the risk of the other samples drying out.
4. Resuspend the cells in DEF-CS medium for single-cell seeding (using the volume indicated in Table 3) and pipet up and down several times to ensure a single-cell suspension. (The cells will aggregate if left too long in TrypLE enzyme.)
5. Use your preferred method to isolate single cells: FACS or limiting dilution. For limiting dilution, we recommend using a final dilution of 0.5 cells per well.
6. Place the plate in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity and leave the plate undisturbed for 48 hr. Continue culturing according to Table 1.

Adding Media to Wells Containing Single Cells (Day 3)

Without discarding any medium, carefully add 100 µl of fresh, DEF-CS medium for single cell seeding per well. (See Table 1 for guidelines.) There should now be a total of 200 µl per well.

VII. Culturing Single-Cell Colonies

After single-cell cloning, cells will proliferate into emerging colonies (Figure 3) that will be passaged for further expansion during scale-up.

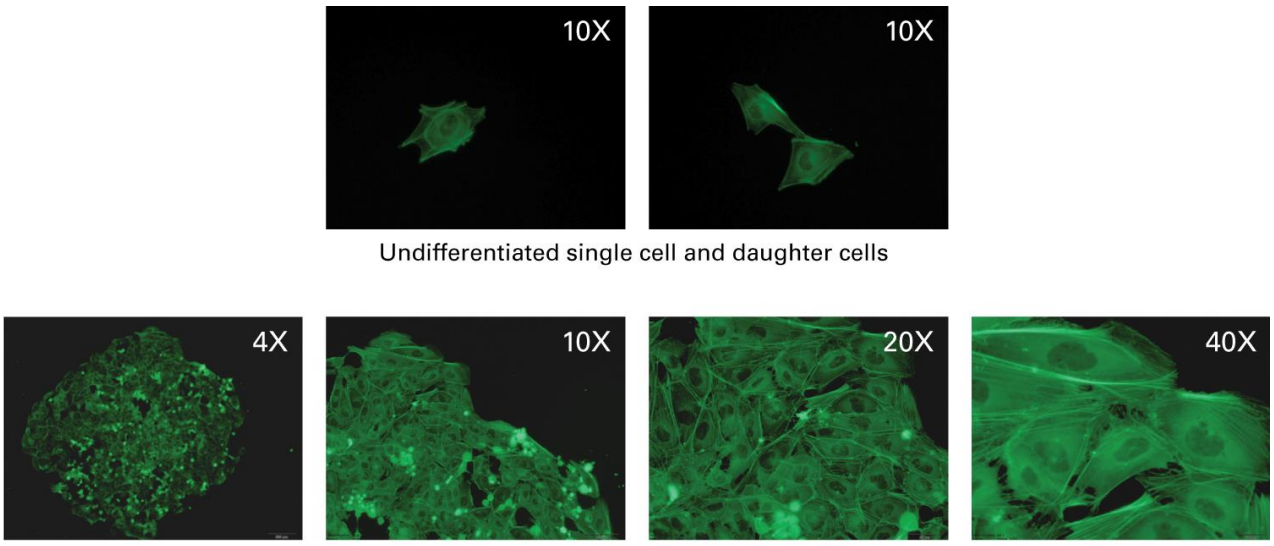

Figure 3. Single cell seeded in one well generates an emerging colony. Representative images of GFP-actin iPS cells taken 24 hr (top) or two weeks (bottom) after single-cell cloning.

A. Changing Media on Single-Cell Colonies in the 96-Well Plate

NOTE: Media change in the 96-well plate is recommended on Day 5 post-seeding and then every other day (see Table 1 for guidance). If the medium turns yellow due to high metabolic activity, change the media every day.

Preparing Medium for Media Change on Seeded Single Cells

1. Prepare 150 µl of DEF-CS medium for media change on seeded single cells per well 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 according to Table 1.
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.

Changing Media

1. Check cells under the microscope; photo document as necessary.
2. Carefully discard 150 µl of the medium from each well and add 150 µl of newly warmed medium into each well of the plate. It is recommended to always use manual pipetting (not a vacuum pump). **Avoid flushing medium directly onto the cell layer.**
3. Place the cell culture plate in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

NOTE: The colonies will be ready to passage from the 96-well plate to a 48-well plate after 8–14 days, depending on the generation time of the specific iPS cell line (Figure 4).

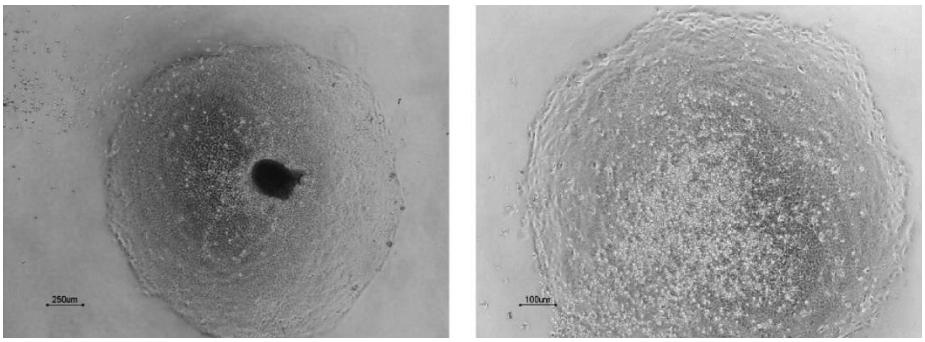

Figure 4. Clonal colonies, ready for transfer to larger wells and scale-up. The cells have the typical undifferentiated stem cell morphology (i.e., high nucleus-to-cytoplasm ratio, defined borders, and prominent nucleoli).

B. Passaging Cells from the 96-Well Plate to a 48-Well Plate

Coating a 48-Well Plate

1. Dilute the required volume of SSC-COAT-1 in D-PBS +/+ prior to use. Make a 1:10 dilution. Calculate the amount of diluted coating solution required depending on the number of wells to be used: 200 µl of diluted coating solution per well (see Table 4 for guidance).

Table 4. Preparation of coating solution for a 48-well plate.

Number of wells (48-well plate)	Volume of diluted coating solution (µl)	Volume of SSC-COAT-1 (µl)	Volume of D-PBS +/+ (µl)
1	200	20	180
2	400	40	360
48	9,600	960	8,640
n	200 x n	(200 x n)/10	(200 x n) – (volume of SSC-COAT-1)

2. Mix the diluted SSC-COAT-1 solution gently and thoroughly by pipetting up and down.
3. Add the diluted SSC-COAT-1 solution to a 48-well plate (using 200 µl/well), making sure the entire surface of each well is covered.

4. Place the plate in the incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for a minimum of 3 hr.
5. Aspirate the diluted SSC-COAT-1 solution from the 48-well plate immediately before use.

Preparing Medium for Passaging

1. Prepare the appropriate volume of "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 DEF-CS Basal Medium according to Table 1. The volume of medium needed for each well of the 48-well plate is 500 µl. Calculate the amount of medium needed depending on the number of clonal lines to be expanded.
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

1. Check the cells under the microscope; photo document as necessary.
2. Aspirate the media from the wells and wash the cell layer with D-PBS –/–.
3. Add 50 µl per well of room-temperature TrypLE Select Enzyme (1X) to the cells. Make sure the whole colony in the well is covered. Place the plate in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity, and incubate for 5 min or until all cells have detached.
4. Resuspend the cells in 500 µl per well of pre-warmed DEF-CS medium for thawing of passaging. 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.
5. Tilt the dish backwards and forwards gently to ensure that the cell suspension is dispersed evenly over the surface, then place it in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

VIII. Scaling up from the 48-Well Plate

Once the cells have been passaged into a 48-well plate, DEF-CS GF-3 is no longer needed in the growth medium used when changing the media. Prepare "DEF-CS medium for maintenance" by adding DEF-CS GF-1 (dilute 1:333) and GF-2 (dilute 1:1,000) to DEF-CS Basal Medium. When the cells are ready to be scaled up to 24-well plate, they can be cultured with the Cellartis DEF-CS 500 Culture System (Cat. No. Y30010).

Contact Us	
Customer Service/Ordering	**Technical Support**
tel: +33.(0)1.3904.6880	tel: +33.(0)1.3904.6880
fax: +33.(0)1.3904.6870	fax: +33.(0)1.3904.6870
web: www.takarabio.com	web: www.takarabio.com
e-mail: ordersEU@takarabio.com	e-mail: techEU@takarabio.com

Notice to Purchaser