Takara Bio Europe AB

Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium w/o antibiotics User Manual

Cat. Nos. Y30047
(041719)

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I. Introduction

Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium w/o antibiotics is a pre-clinical grade, chemically defined culture media that is free from human- and animal-derived components and is used for efficient expansion of undifferentiated human pluripotent stem (hPS) cells in 3D suspension culture.

All procedures described in the manual have been optimized for Cellartis hPS cell lines. If you wish to use Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium w/o antibiotics for other human pluripotent stem cells, please be aware that procedures and protocols may have to be adjusted.

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 DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium w/o antibiotics (Cat. No. Y30047)

- **Cellartis DEF-CS 500 Xeno-Free Basal Medium w/o antibiotics (Cat. No Y30046; not sold separately)**
- **Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Additives (Cat. No. Y30048)**
    - 500 µl DEF-CS Xeno-Free 3D Spheroid Additive 1 (1,000X)
    - 200 µl DEF-CS Xeno-Free 3D Spheroid Additive 2 (4,000X)
    - 400 µl DEF-CS Xeno-Free 3D Spheroid Additive 3 (500X)

III. Additional Materials Required

The following materials are required but not supplied:

- PBS Dulbecco's w/o Ca^{2+} & Mg^{2+} (D-PBS –/–)
- Accumax (Merck Millipore, Cat. No. SCR006)
- Cell culture vessels, tissue culture treated polystyrene surface, conical flasks or bioreactor
- General cell culture equipment used in cell culture laboratory

If applicable

- Gentamicin (Thermo Fisher, Cat. No. 15750)
- Versene Solution (Thermo Fisher, Cat. No. 15040) (for transfer from monolayer culture)

IV. Recommended Materials

The following materials are recommended but not supplied:

- 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)

V. General Considerations

A. Storage and Handling

Cellartis DEF-CS 500 Xeno-Free Basal Medium w/o antibiotics (Cat. No. Y30046) should be stored at 2–8°C; shelf life specified on product label.

Cellartis DEF-CS Xeno-Free 3D Spheroid Additives should be stored at –20°C; shelf life specified on product label. At first use, thaw provided vials, mix gently and aliquot into appropriate volumes. Store at –20°C according to expiry date on original vial. Thawed vials of DEF-CS Xeno-Free 3D Spheroid Additives 1 and 3 may be stored at 2–8˚C for up to one week. Thawed vials of DEF-CS Xeno-Free 3D Spheroid Additive 2 should be used the same day. Do not subject the additives to more than a single refreeze and thaw cycle.

NOTE: Cellartis DEF-CS Xeno-Free 3D Spheroid Additive 2 is light sensitive; therefore, avoid unnecessary exposure to light.

B. Antibiotics

If you do elect to use an antibiotic in your medium, we recommend using Gentamicin. Add 0.5 ml to 500 ml of Cellartis DEF-CS 500 Xeno-Free Basal Medium w/o antibiotics for a final concentration of 50 mg/l.

C. Cell culture unit

This protocol describes how to culture hPS cell lines in 3D spheroids in a dynamic system, using a conical flask on an orbital shaker placed in an incubator.

Table I. Guidelines for choice of culture unit (conical flask size).

Number of seeded cells	Conical flask size	Culture volume
1–4 x 10^6	25 ml	5–15 ml
3–8 x 10^6	100 ml	16–30 ml
7–20 x 10^6	250 ml	35–65 ml

For use of other cell culture vessels or bioreactors, optimization of the protocol will be necessary.

VI. Culturing of hPS Cells in Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium

A schematic picture of the transfer, maintenance and cryopreservation of hPS cell lines in Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium w/o antibiotics is shown in Figure 1.

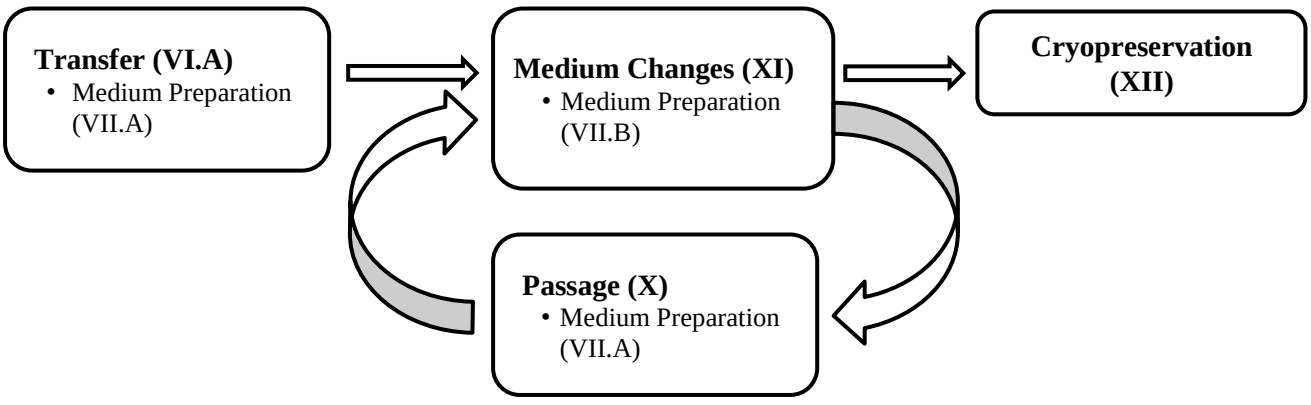

Figure 1. Schematic presentation of the Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium w/o antibiotics work flow. Corresponding sections of this user manual are referenced in brackets.

All hPS cell lines that are maintained in Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium w/o antibiotics should be passaged every 4–5 days for best results. For the first passage from 2D culture into suspension culture, passage at day 7. When the cell density is sparse, you can change the medium every other day; however, it is always important to change medium the day before passage. It is recommended that the cells are grown to a confluence of 1–2 x 10^6 cells/ml. Do not exceed 3 x 10^6 cells/ml unless the bioreactor can handle oxygen control and continuous media change (i.e. perfusion system).

A. Transfer of hPS Cells from 2D Culture to 3D Culture

We recommend transferring your cells to the Cellartis DEF-CS 500 Xeno-Free Culture Medium w/o antibiotics (Cat. No. Y30045) and culturing them in 2D monolayer culture for at least 1–3 passages before transferring to the 3D spheroid culture system. For transfer from 2D culture to 3D culture see section IX.

NOTE: Transferring cells from other culture systems directly into the 3D spheroid culture system is not recommended. However, for some cell lines and culture systems it might be possible to transfer directly into suspension culture. Keep the cell concentration as described for passage (Section X).

VII. Preparing Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium

Table II. Guidelines for media volumes at passage and maintenance.

Seeded number of cells	Seeding volume (Day 0)	Medium change, Day 2	Possible medium change, Day 3	Medium change, day before passage	Total volume from seed to harvest	Number of cells at harvest
2 x 10^6	5–10 ml	10–12 ml	10–12 ml	10-12 ml	36–48 ml	~10-15 x 10^6
5 x 10^6	12–25 ml	25–30 ml	25–30 ml	25–30 ml	90–120 ml	~20–30 x 10^6
10 x 10^6	25–50 ml	50–60 ml	50–60 ml	50–60 ml	180–240 ml	~40–60 x 10^6

A. Medium for Thawing or Passaging hPS Cells

1. Decontaminate the external surfaces of all additives and the medium bottle with an appropriate disinfectant and place into the biological safety cabinet.
2. Prepare the appropriate volume of Cellartis DEF-CS xeno-free 3D spheroid culture medium for thawing or passaging by adding DEF-CS Xeno-Free 3D Spheroid Additive 1 (dilute 1:1,000), Additive 2 (dilute 1:4,000), and Additive 3 (dilute 1:500) to prewarmed Cellartis DEF-CS 500 Xeno-Free Basal Medium w/o antibiotics (37°C ± 1°C).
3. Prepare fresh medium on the day of use. Discard any leftover warm medium.

B. Medium for Maintenance of hPS Cells

1. Decontaminate the external surfaces of additives and the medium bottle with an appropriate disinfectant and place into the biological safety cabinet.
2. Prepare the appropriate volume of Cellartis DEF-CS xeno-free 3D spheroid culture medium for maintenance by adding DEF-CS Xeno-Free 3D Spheroid Additive 1 (dilute 1:1,000) and Additive 2 (1:4,000 dilution) to prewarmed Cellartis DEF-CS 500 Xeno-Free Basal Medium w/o antibiotics (37°C ± 1°C). Do not add DEF-CS Xeno-Free 3D Spheroid Additive 3 to maintenance medium.
3. Prepare fresh medium on the day of use. Discard any leftover warm medium.

VIII. Thawing hPS Cells Lines

We recommend thawing your cells into traditional monolayer culture using the Cellartis DEF-CS 500 Xeno-Free Culture Medium w/o antibiotics (Cat. No. Y30045) and culture them in monolayer culture for at least 1–3 passages before transferring to a 3D spheroid culture system. For transfer from culture in Cellartis DEF-CS 500 Xeno-Free Culture Medium w/o antibiotics (Cat. No. Y30045) (in 2D) to Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium, see section IX .

NOTE: Thawing directly into the 3D spheroid culture system is not recommended. However, it may be possible to seed some cell lines directly into suspension culture. Keep the cell concentration as described for passage (see section X).

IX. Transferring hPS Cell Lines from 2D Culture to 3D Culture

We recommend transferring your cells to the Cellartis DEF-CS 500 Xeno-Free Culture Medium w/o antibiotics (Cat. No. Y30045) and culturing them in 2D monolayer culture for at least 1–3 passages before transferring to the 3D spheroid culture system. For transfer from culture in Cellartis DEF-CS 500 Xeno-Free Culture Medium w/o antibiotics (Cat. No. Y30045) (in 2D) to Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium, see below.

A. Preparation

Prepare the appropriate volume of Cellartis DEF-CS xeno-free 3D spheroid culture medium for thawing or passaging, as described in Section VII.A and warm to 37°C ± 1°C. Warm all other reagents to RT.

B. Transfer from Cellartis DEF-CS Xeno-Free Culture Medium

1. Check cells under microscope; photo document as necessary.
2. Aspirate medium from cell culture vessel and wash the cell layer once with D-PBS (–/–).
3. Add 0.1 ml/cm^2 of Versene to the cell culture vessel and incubate for 20 minutes or until the cells round up. Detachment should be aided by beating the side of the cell culture vessel firmly or by hitting the short side of the culture vessel against the bench 3–5 times. Avoid flushing the cell layer, if possible.
4. Dilute the cells in Cellartis DEF-CS xeno-free 3D spheroid culture medium for thawing or passaging (1:1 dilution) and pipette up and down several times to ensure a single cell suspension is achieved.
5. Centrifuge the cells at 300*g* for 2–5 minutes.
6. Resuspend the cells in the Cellartis DEF-CS xeno-free 3D spheroid culture medium for thawing or passaging.
7. Count the cells in a haemocytometer or in a cell counter (optimized for the cell type).
8. Add the appropriate volume of cell suspension and medium to the cell culture flask to obtain the selected cell concentration. (See Table II.)
9. Place the cell culture flask on an orbital shaker at a speed of 60–100 rpm. Place the shaker in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity. If necessary, optimize the speed for the specific shaker used.

X. Passaging hPS Cells Lines

As a general rule, cells should be seeded at a density of 2–4 x 10^5 cells/ml. Use 2 x 10^5 cells/ml if leaving the cells five days and 3–4 x 10^5 cells/ml if leaving the cells four days in between passages. However, you should adjust the seeding density to suit your cell line. For recommended culture conditions, see Table I and Table II.

It is recommended that the cells are grown to a confluence of 1–2 x 10^6 cells/ml. If cells are allowed to grow to a higher density, it will have an impact for the next passage, and some cell lines might also be at increased risk of unwanted differentiation. If cultures should appear suboptimal after a few passages, it is recommended to decrease or increase the seeding density. The passage interval may have to be adjusted accordingly.

NOTE: For use of other cell culture vessels or bioreactors, optimization of the protocol will be necessary. As a general rule cells should be seeded at a density of 2–4 x 10^5 cells/ml and grown to a maximum confluence of 2–3.0 x 10^6 cells/ml, unless the bioreactor can handle oxygen control and continuous media change (i.e. perfusion system), in which case higher cell densities can be used.

A. Preparations

Prepare the appropriate volume of Cellartis DEF-CS xeno-free 3D spheroid culture medium for thawing or passaging and warm to 37°C ± 1°C as described in Section VII.A. Warm all other reagents to RT.

B. Passaging

1. Transfer the spheroid suspension to appropriate centrifuge tubes (15 ml or 50 ml).
2. Centrifuge at 50*g* for 2 minutes, optimally in a swing-out rotor. Choose a long deceleration time in order to avoid any disturbance in the spheroid pellet due to “forceful braking”.
3. Aspirate the medium and wash the cell spheroids once with D-PBS (–/–). Repeat the centrifugation (step 2). Aspirate the PBS solution.
4. Add 0.1–0.2 ml Accumax/10^6 cells (estimate 6-fold increase from seeded number of cells) to the cells and incubate for 15–20 minutes. Tap the side of the tube firmly against the bench 3-5 times to detach cells. If necessary, aspirate the cell suspension 1–3 times with a pipette tip in order to completely disperse the spheroids into a single cell suspension.
5. Dilute the cells in Cellartis DEF-CS xeno-free 3D spheroid culture medium for thawing or passaging (1:1 dilution).
6. Centrifuge the cells at 200*g* for 2–3 minutes.
7. Suspend the cells in the appropriate volume of Cellartis DEF-CS xeno-free 3D spheroid culture medium for thawing or passaging.
8. Count the cells in a haemocytometer or in a cell counter (optimized for the cell type).
9. Add the appropriate volume of cell suspension and medium to the cell culture flask to obtain the selected cell concentration (see Table II).

   Note: To minimize the number of spheroids that attach to the walls, pre-wet the conical flask with medium prior to adding the cell suspension.

10. Place the cell culture flask on an orbital shaker at a speed of 60–100 rpm. Place the shaker in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity. If necessary, optimize the speed for the specific shaker used.

XI. Changing Medium for hPS Cells Lines

When the cell density is sparse, you can change the medium every other day; however, it is always important to change medium the day before passage. If the medium turns yellow due to high metabolic activity, increase the medium volume.

Typically, 3–4 ml medium will be consumed per passage and million cells generated, and as a general assumption cells will expand 4–6 times during each passage. For example, per 1 million cells seeded, you will be able to harvest 4–6 million cells and the total volume of medium consumed for passage and medium changes will be in the range of 18–24 ml. For recommended culture conditions, see Table II.

A. Preparation

Prepare the appropriate volume of Cellartis DEF-CS xeno-free 3D spheroid culture medium for maintenance as described in Section VII.B and warm to 37°C ± 1°C. Warm all other reagents to RT.

B. Medium Change

1. Transfer the spheroid suspension to appropriate centrifuge tubes (15 ml or 50 ml).
2. Separate the spheroids from the bulk of the medium either by using option 1 or option 2.
   - *Option 1:* Centrifuge at 50*g* for 2 minutes, optimally in a swing-out rotor. Choose a long deceleration time in order to avoid any disturbance in the spheroid pellet due to "forceful braking".
   - *Option 2:* Leave the tube untouched for approximately 5 minutes to allow the spheroids to sink and become concentrated in the bottom of the centrifuge tube.
3. Carefully aspirate the medium.
4. Suspend the spheroids in prewarmed, Cellartis DEF-CS xeno-free 3D spheroid culture medium for maintenance and transfer back to the cell culture flask.

   Tip: To prevent spheroids from adhering to the plastic surface of the pipette, first aspirate medium up and down one time.

5. Place the cell culture flask on an orbital shaker at a speed of 60–100 rpm. Place the shaker in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity. If necessary, optimize the speed for the specific shaker used.

XII. Cryopreserving hPS Cell Lines

Any hPS cells cultured using this workflow can be cryopreserved by using common slow freezing protocols for cell suspensions using STEM-CELLBANKER (Zenoaq Resource Co. Ltd., Cat. No. ZR636). As a general guide, freeze 2.5–3.5 x 10^6 cells in 1 ml freezing medium in a 2-ml cryovial. Freeze the cells as a single cell suspension. Follow steps X.B 1–9 to achieve a single cell suspension.

NOTE: Cryopreservation of cells in spheroid formation is not recommended.

XIII. Images of hPS Cells Maintained in Cellartis DEF-CS Xeno-Free 3D Spheroid Culture Medium

A B C

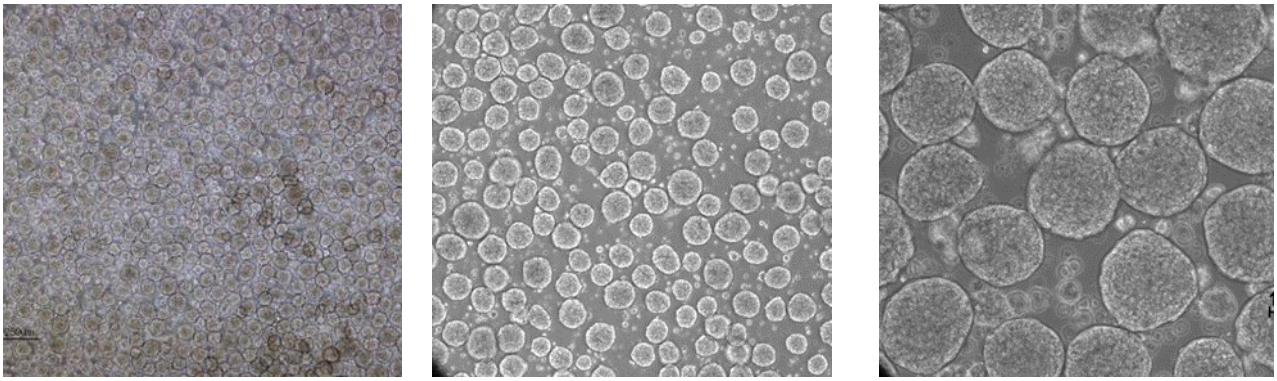

Figure 2. hPS cells in Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium w/o antibiotics. Panel A. Day 1 after passage (4X). Panel B. Day 1 after passage (10X). Panel C. Day 1 after passage (20X).

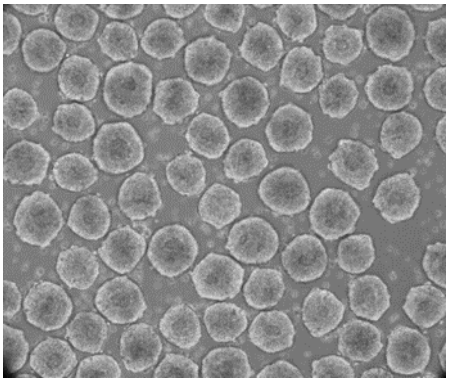

Figure 3. hPS cells in Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium w/o antibiotics (prior to passage, 10X).

Appendix A. Troubleshooting Guide

Table III. Troubleshooting Guide

Problem	Possible Explanation	Solution
The cell density at passage varies considerably	Over-compensated cell seeding at previous passages.	Try to keep passage intervals and seeding densities as consistent as possible, i.e. try to not compensate a slow growth for the next passage, or vice versa.
The cells seem to differentiate	Higher concentration of Additive 2 needed.	Increase the additive solution 1.5–2 times compared to the recommended concentration.
The cells seem to differentiate	Too small media volumes used between passages. (Some cell lines have higher metabolic activities, although they do not necessarily divide faster.)	Increase the media volumes used, especially if the medium has turned yellow at higher densities before medium change.
The cells seem to differentiate	Initial cell concentration was too high	Decrease the cell concentration. A good starting concentration is $2x10^5$ cells per ml medium, in case the cell line tends to differentiate.
Transferred cells do not adapt to Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium w/o antibiotics	The cells are not used to the new environment.	The cells could benefit from a few passages in Cellartis DEF-CS 500 Xeno-Free Culture Medium prior to transfer to suspension conditions.
Transferred cells do not adapt to Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium w/o antibiotics	The cells are not used to the new environment.	The cells could benefit from a longer initial passage interval. Use a passage interval of 7 days for the first few passages in suspension culture.
The spheres bind to the plastic medium/air interphase	The media volume is not optimal.	It normally helps to increase the media volume 30–50%.
The spheres form big clusters, either directly or after media change	To high initial cell concentration, or non-optimal media volume.	The media volume needs to be optimized in conjunction with used shaker platform and cell culture device.

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