Takara Bio Europe AB

Cellartis® DEF-CS™ Xeno-Free GMP Grade Basal Medium (Prototype) User Manual

Cat. Nos. Y30071
(061616)

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I. Introduction

Cellartis DEF-CS 500 Xeno-Free GMP Grade Basal Medium (Prototype) is a chemically defined basal media that is free from human- and animal-derived components and is used for the efficient expansion of undifferentiated human induced pluripotent stem (iPS) cells. Cellartis DEF-CS 500 Xeno-Free GMP Grade Basal Medium (Prototype) is manufactured as a quality-assured product, according to guidelines for Good Manufacturing Practice (GMP) for Investigational Products.

The procedures described in the manual relate to non-colony type monolayer culture and have been optimised for use with Cellartis human iPS cell lines. If you wish to use Cellartis DEF-CS 500 Xeno-Free GMP Grade Basal Medium (Prototype) for other human induced pluripotent stem cells, please be aware that procedures and protocols may have to be adjusted.

Cellartis DEF-CS 500 Xeno-Free GMP Grade Basal Medium (Prototype) can also be used for dynamic suspension culture of human induced pluripotent stem cells as 3D spheroids. A separate protocol for 3D spheroid suspension culture is available upon request.

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 Xeno-Free GMP Grade Basal Medium (Prototype) (Cat. No. Y30071)

III. Additional Materials Required

The following materials are required but not supplied:

- Culture substrate: Corning Synthemax II-SC Substrate (Corning, Cat. No. 3535)
- bFGF (Recombinant Human FGF basic GMP; Peprotech, Cat. No. 233-GMP)
- Y-27632, MF (Wako Pure Chemical Industries, Ltd. Cat. No. 257-00613)
- Versene Solution (Thermo Fisher, Cat. No. 15040)
- Albix (Recombinant human albumin solution) 10 % (w/v) (Novozymes, Cat. No. 205-005)
- PBS Dulbecco's with Ca^{2+} & Mg^{2+} (D-PBS +/+)
- PBS Dulbecco's w/o Ca^{2+} & Mg^{2+} (D-PBS –/–)
- Sterile water
- Cell culture vessels, tissue culture treated polystyrene surface
- General cell culture equipment used in cell culture laboratory

Compatible research-grade components:

- bFGF (Recombinant Human FGF basic GMP; Peprotech, 100-18B)
- Y-27632 from (Sigma Aldrich, Cat. No. Y0503)
- Culture substrate: iMatrix-511 (Takara Clontech, Cat. No. T303)

IV. General Considerations

A. Storage and Handling

Cellartis DEF-CS Xeno-Free GMP Grade Basal Medium (Prototype) (Cat. No. Y30071) should be stored at 4°C and expires according to the label.

V. Culture of Human iPS Cells in Cellartis DEF-CS 500 Xeno-Free GMP Grade Medium

A schematic picture of the thawing, maintenance and cryopreservation of hiPS cell lines in Cellartis DEF-CS 500 Xeno-Free GMP Grade Medium is shown in Figure 1. The cell expansion capability for 500 ml of Cellartis DEF-CS 500 Xeno-Free GMP Grade Basal Medium (Prototype) is: 20x T25 or 8x T75 or 4x T150 flasks or equivalent.

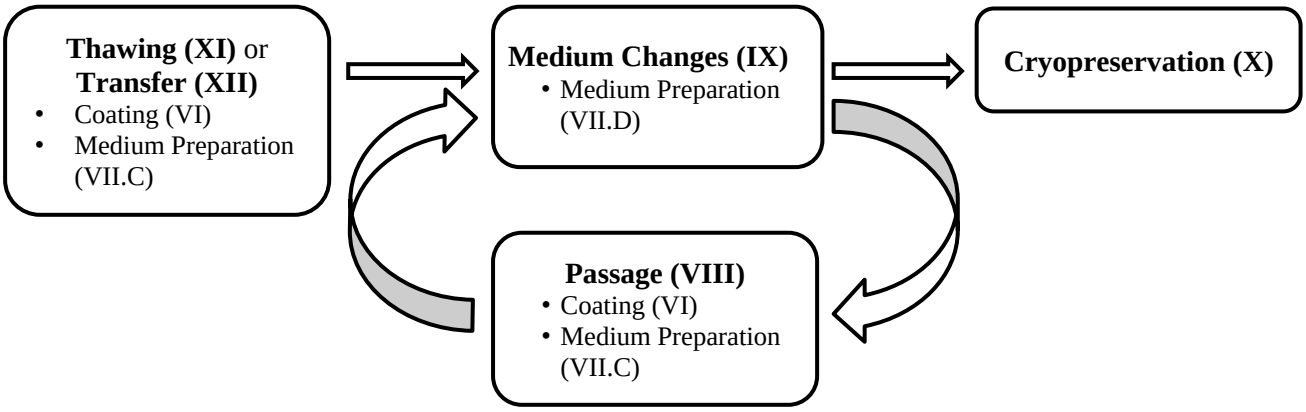

Figure 1. Schematic presentation of the Cellartis DEF-CS Xeno-Free GMP Grade Medium work flow. Corresponding sections of this user manual are referenced in brackets.

Human iPS cell lines that are maintained in Cellartis DEF-CS 500 Xeno-Free GMP Grade Medium should be passaged every 3–4 days with daily medium changes. When the cell density is sparse, you can change the medium every other day, however it is always important to change medium the day after passage or thawing, and the day before passage or freezing. It is recommended that the cells are grown to a maximum confluence of 1.5–3.0 x 10^5 cells/cm^2. A suggestion for a weekly schedule is depicted in Table I.

Table I. Weekly Schedule

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Passage	Medium Change	Medium Change	Passage	Medium Change	-	Medium Change

NOTE: Always work under aseptic conditions.

VI. Coating of Cell Culture Vessels

Coat the appropriate cell culture vessels with Synthemax II-SC Substrate according to manufacturers' instructions. The recommended concentrations may need to be optimized for certain cell lines.

VII. Cellartis DEF-CS Xeno-Free GMP Grade Medium Preparation

A. Preparation of bFGF Stock Solution

1. Decontaminate the external surfaces of all reagents with an appropriate disinfectant and place into the biological safety cabinet.
2. Prepare bFGF stock solution by dissolving the bFGF in 0.1% Albix in D-PBS to a final concentration of 0.1 mg/ml.
3. Aliquot the stock solution and store at –20°C. Aliquots can be stored at –20°C for 12 months after the date of preparation. Thawed vials may be stored at 4˚C for up to one week. Do not subject the aliquots to more than a single thaw and refreeze cycle.

B. Preparation of Y-27632 stock solution

1. Decontaminate the external surfaces of all reagents with an appropriate disinfectant and place into the biological safety cabinet.
2. Prepare the Y-27632 stock solution by diluting Y-27632 in sterile water to a final concentration of 5 mM.
3. Aliquot the stock solution and store at –20°C. Aliquots can be stored at –20°C for 12 months after the date of preparation. Thawed vials may be stored at 4˚C for up to one week. Do not subject the aliquots to more than a single thaw and refreeze cycle.

C. Medium for Thawing or Passage of Human iPS Cells

1. Decontaminate the external surfaces of reagents and the medium bottle with an appropriate disinfectant and place into the biological safety cabinet.
2. Prepare the appropriate volume of supplemented Cellartis DEF-CS Xeno-Free GMP Grade Medium by adding bFGF (dilute stock solution 1:1,000 to a final concentration of 100 ng/ml) and Y-27632 (dilute stock solution 1:1,000 to a final concentration of 5 µM) to Cellartis DEF-CS Xeno-Free GMP-Grade Basal Medium (Prototype).
3. Medium should be freshly prepared on the day of use. Discard any leftover warm medium.

D. Medium for Maintenance of Human iPS Cells

1. Decontaminate the external surfaces of all reagents and the medium bottle with an appropriate disinfectant and place into the biological safety cabinet.
2. Prepare the appropriate volume of supplemented Cellartis DEF-CS Xeno-Free GMP Grade Medium by adding bFGF (dilute stock solution 1:1,000 to a final concentration of 100 ng/ml) to Cellartis DEF-CS Xeno-Free GMP Grade Basal Medium (Prototype). Do not add Y-27632 to maintenance medium.
3. Medium should be freshly prepared on the day of use. Discard any leftover warm medium.

VIII. Passage of Human iPS Cells

As a general rule, cells should be seeded at a density of 3–4 x 10^4 cells/cm^2. Use 4 x 10^4 cells/cm^2 if leaving the cells three days and 3 x 10^4 cells/cm^2 if leaving the cells four days in between passages. This can be adjusted to suit the cell line as appropriate.

When passaging the cells, it is highly recommended that the cells are grown to a density of 1.5–3 x 10^5 cells/cm^2; see **Error! Reference source not found. Error! Reference source not found.**for corresponding images of human iPS cells in culture. Please note that the cells will cover the surface of the culture vessel (be confluent) at a cell density of approximately 0.8–1 x 10^5 cells/cm^2. Do not passage the cells for at least another day, to achieve the recommended density at passage. If cells are passaged too soon, it might have a negative impact on the growth rate during the next passage, and some cell lines might also be at increased risk of unwanted differentiation. If cells are allowed to grow to density of > 3 x 10^5 cells/cm^2, it might have a negative impact on the growth rate during the next passage. 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.

A. Preparations

Cell culture vessels should be coated as described above. The appropriate volume of supplemented Cellartis DEF-CS Xeno-Free GMP Grade Medium for Thawing or Passage should be prepared as described in Section VII.C and warmed to 37°C ± 1°C before use. Warm all other reagents to RT before use.

B. Passage

1. Check cells under microscope; photo document as necessary.

2. Aspirate medium from the 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. Tap the side of the cell culture vessel firmly against the bench 3-5 times to detach cells. 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 with Versene to detach cells.
4. Dilute the cells in supplemented Cellartis DEF-CS Xeno-Free GMP Grade Medium (1:1 dilution) and pipette up and down several times to achieve a single cell suspension.
5. Centrifuge the cells at 300*g* for 2–5 minutes.
6. Resuspend the cells in the supplemented Cellartis DEF-CS Xeno-Free GMP Grade Medium.
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 newly coated cell culture vessel to obtain the selected density. The seeding volume of supplemented Cellartis DEF-CS Xeno-Free GMP Grade Medium should be 0.3 ml/cm^2.
9. Tilt the vessel backwards and forwards gently to ensure the cell suspension is dispersed evenly over the surface and place in the incubator.

IX. Medium Change for Human iPS Cells

Medium change is recommended daily (except day of passage). Use 0.3–0.4 ml/cm^2. If the medium turns yellow due to high metabolic activity, increase the volume of medium.

A. Preparation

The appropriate volume of supplemented Cellartis DEF-CS Xeno-Free GMP Grade Medium for Maintenance should be prepared as described in section VII.D and warmed to 37°C ± 1°C before use. Discard any leftover warm medium.

B. Medium Change

1. Check cells under microscope; photo document as necessary.
2. Carefully aspirate the medium and pipette newly warmed medium into the cell culture vessel. Avoid flushing medium directly on the cell layer.
3. Place the cell culture vessel in the incubator.

X. Cryopreservation of Human iPS Cells

Cellartis human iPS cells cultured in Cellartis DEF-CS Xeno-Free Culture System can be cryopreserved by using common slow freezing protocols for cell suspensions using STEM-CELLBANKER® GMP Grade (Takara Clontech, Cat. No. CB045). As a general guide, 2.5–3.5 x 10^6 cells in 1 ml freezing medium should be frozen in a 2 ml cryovial.

XI. Thawing of Human iPS Cells

When thawing human iPS cells in Cellartis DEF-CS Xeno-Free Culture Medium, approximately 1.5–2.0 x 10^5 cells/cm^2 should be seeded in 0.3–0.4 ml medium/cm^2.

A. Preparations

Cell culture vessels should be coated with Synthemax II-SC Substrate according to manufacturers' instructions; the recommended concentrations may need to be optimized for certain cell lines Supplemented Cellartis DEF-CS Xeno-Free GMP Grade Medium for Thawing or Passage should be prepared as described in Section VII.C and warmed to the appropriate temperature; see below for the recommended volumes and temperatures.

B. Thawing Cells

NOTE—FOR YOUR PROTECTION: Wear a protective face mask and protective gloves. Use forceps when handling a frozen vial. Never hold the vial in your hand as the cryo vial may explode due to rapid temperature changes.

1. Transfer 9 ml of supplemented Cellartis DEF-CS Xeno-Free GMP Grade Medium to a sterile centrifuge tube and warm to RT.
2. Using forceps, transfer the vial directly into a container with 37°C ± 1°C water. Thaw the vial by gently pushing it under the surface of the water. Do not submerge the cap of the vial in the water bath as this could contaminate the cells.
3. Allow the vial to thaw until the cell suspension can be poured out of the vial, with frozen parts of cell suspension still left in the vial.
4. Decontaminate the vial in appropriate disinfectant.
5. Pour the contents of the vial into the sterile tube containing 9 ml supplemented Cellartis DEF-CS Xeno-Free GMP Grade Medium at RT.
6. Use 1 ml supplemented Cellartis DEF-CS Xeno-Free GMP Grade Medium, warmed to RT, to rinse the vial. Add to the cell suspension.
7. Centrifuge at 300*g* for 1 minute.
8. After centrifugation, aspirate the supernatant and gently resuspend the pellet in a volume corresponding to 0.3–0.4 ml supplemented Cellartis DEF-CS Xeno-Free GMP Grade Medium/cm^2 (37°C ± 1°C), resulting in approximately 1.5–2.0 x 10^5 cells/cm^2.
9. Pipette the cell suspension into the cell culture unit.
10. Ensure the cells and medium are evenly distributed across the surface of the cell culture unit and place the cell culture unit in the incubator.

XII. Transfer of Human iPS Cells to Cellartis DEF-CS Xeno-Free GMP Grade Medium from Other Culture Media

Human pluripotent stem cells maintained in other culture systems can readily be transferred to Cellartis DEF-CS Xeno-Free GMP Grade Medium. Fresh cultures can be transferred and cryopreserved cultures can be thawed directly into the Cellartis DEF-CS Xeno-Free GMP Grade Medium. The cells might need up to five passages to adjust to the new culture medium.

The normal Cellartis DEF-CS Xeno-Free GMP Grade Medium protocol should be followed although some considerations might need to be taken into account regarding the passage interval, as described directly below (Section XII.A).

A. Passage Interval

When seeding human pluripotent stem cells previously cultured in a different culture system, the cells might initially grow differently than in the former system. Depending on the confluence of the cell monolayer, the suitable interval might be between three to seven days for the first passage. The cells will adapt to the morphology displayed in **Error! Reference source not found.** prior to passage. However, if the cells remain sparse after seven days in culture, a passage is still recommended.

XIII. Images of Human iPS Cells Maintained in Cellartis DEF-CS Xeno-Free GMP Grade Culture Medium

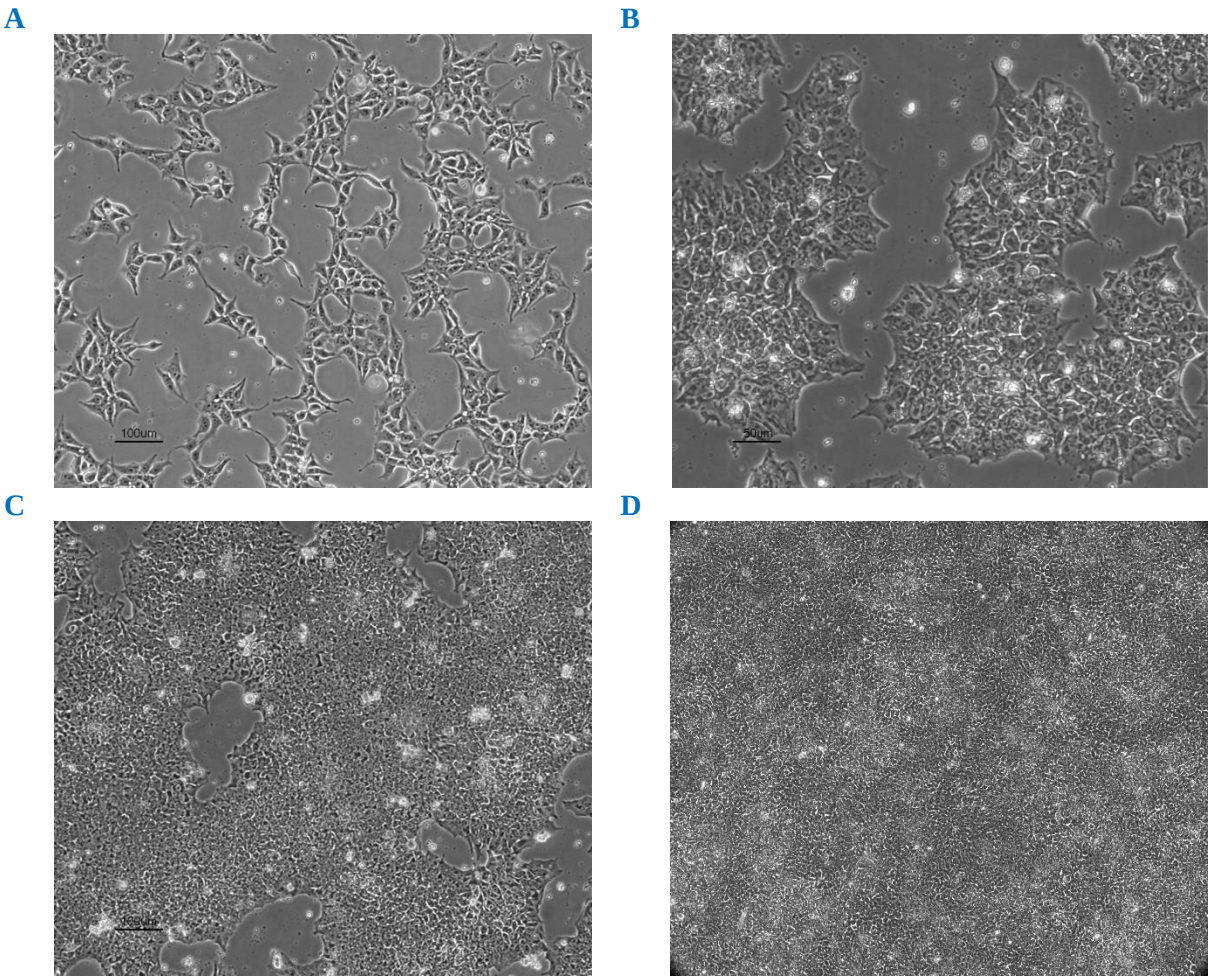

Figure 2. Human iPS cells in Cellartis DEF-CS Xeno-Free GMP Grade Culture Medium using Synthemax. Panel A. Day 1 after passage (seeded at 3 x 10^4 cells/cm^2) (10X). **Panel B.** Day 2 after passage (approximate density 1 x 10^5 cells/cm^2) (20X). **Panel C.** Day 3 after passage (approximate density 3 x 10^5 cells/cm^2) (10X). **Panel D.** Day 4 after passage (approximate density 4 x 10^5 cells/cm^2) (10X).

Appendix A. Troubleshooting Guide

Table II. Troubleshooting Guide

Problem	Possible Explanation	Solution
Cells detach/round up	Synthemax coated surface has dried out.	Add some medium to the surface directly after the coating solution has been removed.
Cells detach prior to passage	Too low concentration of coating solution, or too short period of coating.	Try other concentrations of coating solution. Coat for a longer period.
Cells do not detach at passage	Too small volume of Versene, too short treatment.	Increase volume to 0.2 ml/cm^2. Use warmed solution. Treat the cells longer in incubator (up to 30 minutes).
Cells do not detach even though Versene is used as described	Different cell lines can react differently to Versene.	Flush off the cells with pipette. Though the cells are quite robust during Versene treatment and flushing, one should account for increased cell death and try to adjust the seeding density accordingly.
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	Too small media volumes used between passages. Some cell lines have a higher metabolic activity, though they do not necessarily divide faster.	Increase the media volumes used, especially if the medium has turned yellow at higher densities before medium change.
Transferred cells do not adapt to Cellartis DEF-CS Xeno-Free Culture Medium	The cells are not used to the new environment.	The cells could benefit from a higher seeding density for the first few passages, e.g. 6–8x10^4 cells/cm^2.

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