# Clinical-grade culture medium for expansion and large-scale 2D and 3D suspension culture of human pluripotent stem cells

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## Abstract

Here we present the development of a defined, feeder-free medium, without human- or animal-derived components. Human pluripotent stem cells (hPSCs) that are cultured in this medium for an extended period of time express expected stem cell markers, remain diploid, and can differentiate into cell types from the three germ layers. Using this complete, clinical-grade culture medium, eight different hPSC lines that were expanded as a 2D monolayer (2D culture) maintain high expression of pluripotent stem cell markers and lack any expression of differentiation markers over the 12–20 passages tested. In addition, no karyotype abnormalities were reported for any of the tested cell lines. In order to generate clinically relevant quantities of hPSCs—$10^9$ and beyond—it is essential to develop efficient, yet robust 3D suspension cultures maintaining the same stability as 2D monolayer cultures. Previous reports in the literature of suspension cultures have typically described a reduced growth rate compared to monolayer cultures with a final cell concentration of 1–2 million cells per milliliter. We demonstrate that our culture system supports large-scale, 3D, non-adherent expansion of hPSCs in suspension culture in a perfusion bioreactor. Furthermore, by optimizing perfusion rates and dissolved oxygen levels, we were able to expand hPSCs by 800-fold within 3 passages over 11 days to a final concentration of 5 million cells per milliliter using our 3D suspension culture system. In summary, our clinical-grade culture system allows for efficient, robust, and scalable production of hPSCs, thus facilitating the use of hPSCs for research and large-scale 3D suspension clinical applications.

## Materials

Cellartis® DEF-CS™ 500 Xeno-Free Culture Medium (Cat. No.Y30040)

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

Cellartis DEF-CS 500 Xeno-Free GMP Grade Basal Medium (Prototype) (Cat. No.Y30071)

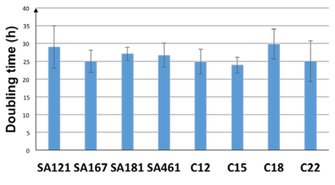
- Human- and animal-free formulation and a defined, feeder-free system
- Robust maintenance medium for manufacturing of research grade or clinical-grade hPSCs in 2D monolayer or 3D suspension cultures
- Single-cell passaging promotes high-yield expansion
- Eliminates the need for cell selection, as there is virtually no background differentiation

iMatrix-511 (Cat. No.T303)

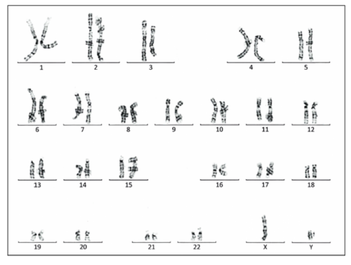
- Chemically defined stem cell culture substrate for animal-free, xeno-free, feeder-free hPSC culture
- Provides greater adhesion of hPSCs than Matrigel or full-length laminin

## Stable proliferation rates and long-term preservation of genomic stability

A




B



C

| hiPSC | Karyotype | hESC | Karyotype |
|---|---|---|---|
| C12 Passage 11 | 46XY, Normal | SA121 | 46XY, Normal |
| C15 | 46XX, Normal | SA167 | 46XX, Normal |
| C18 | 46XY, Normal | SA181 | 46XY, Normal |
| C22 | 46XY, Normal | SA461 | 46XY, Normal |
| C4, passage 21 | 46XY, Normal | | |

**Figure 1. Average doubling time and karyotyping of hPSC lines passaged in Cellartis DEF-CS 500 Xeno-Free Culture Medium.** Four hESC lines (SA121, SA167, SA181, SA461) and four hiPSC lines (ChiPSC12, ChiPSC15, ChiPSC18, ChiPSC22) were grown as a 2D monolayer up to passage 10. **Panel A.** Proliferation rates are displayed as the population doubling time in hours. Cells were passaged every 3–4 days; at most passages, cells demonstrated a population doubling time of 25–28 hours. **Panel B.** Karyotyping results from hESC line SA121 expanded in the xeno-free culture medium for 30 passages. **Panel C.** Summary of karyotyping results from five hiPSC lines and four hESC lines.

## Cells maintain their pluripotency without differentiation

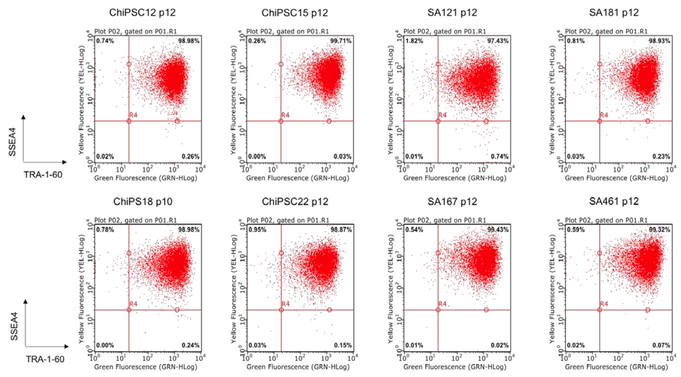



**Figure 2. Pluripotency marker expression in cells maintained in Cellartis DEF-CS 500 Xeno-Free Culture Medium.** Flow cytometry analysis shows more than 98% of cells were positive for cell surface markers TRA-1-60 and SSEA4. Four hiPSC lines (ChiPSC12, ChiPSC15, ChiPSC18, and ChiPSC22) and four hESC lines (SA121, SA167, SA181, and SA461) were analyzed after 12 passages in conventional 2D monolayer cultures.

## Cells maintain their differentiation potential into the three germ layers

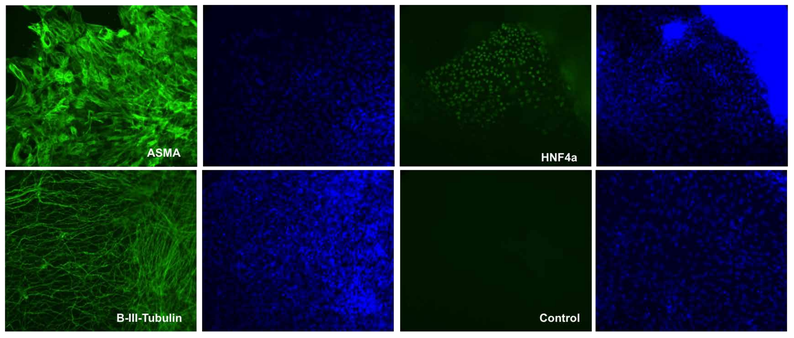



**Figure 3. Spontaneous differentiation into the three germ layers.** The cell lines described in figures 1 and 2 were analyzed for their ability to spontaneously differentiate into the three germ layers after 12 consecutive passages using Cellartis DEF-CS 500 Xeno-Free Culture Medium. Undifferentiated cells were centrifuged in 96-well plates to form aggregates. After one week, the aggregates were transferred to cell culture plates for further spontaneous differentiation. Two weeks later, the differentiated cells were fixed and stained for the presence of ASMA (mesodermal marker), β-III-Tubulin (ectodermal marker), and HNF4a (endodermal marker). The images show representative stainings from cell line ChiPSC22.

## Expansion workflow for 3D suspension culture

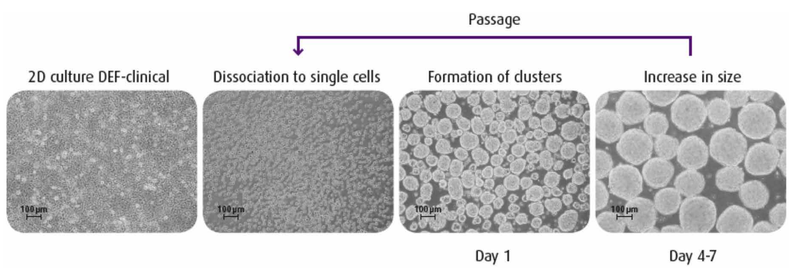



**Figure 4. Optimal spheroid generation from hPSCs using Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium.** To create a workflow for scale-up to produce a large number of cells, 3D suspension culture was optimized for the expansion of aggregates of pluripotent stem cells. Cells grown as 3D suspension cultures in the culture system formed homogeneous, round spheroids and exhibited a robust proliferation rate.

## Pluripotency markers are maintained in suspension cultures

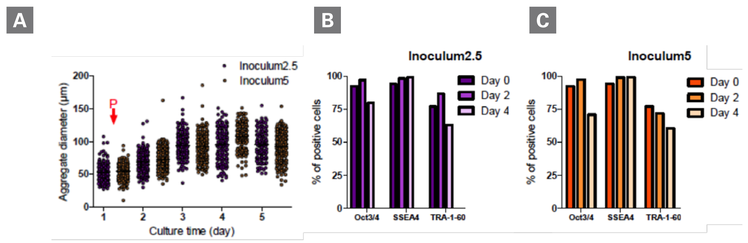



**Figure 5. Scalable spheroid suspension culture in bioreactors with maintained pluripotency.** Analysis of the scalability in suspension culture was performed for the human induced pluripotent stem cell line ChiPSC4. **Panel A.** The bioreactor was inoculated with undifferentiated single cells at 2.5 x $10^5$ cells/ml (inoculum 2.5) or 5.0 x $10^5$ cells/ml (inoculum 5). Perfusion (P) was initiated after 1.5 days. Samples of spheres were collected every 12 hr and the size of the formed spheres was measured over five days. **Panels B, C.** The maintenance of the pluripotency in the spheroid culture system was measured by flow cytometry, detecting the pluripotency markers OCT4, TRA-1-60, and SSEA4.

## Scalable expansion of hiPSCs in bioreactors facilitates preclinical applications

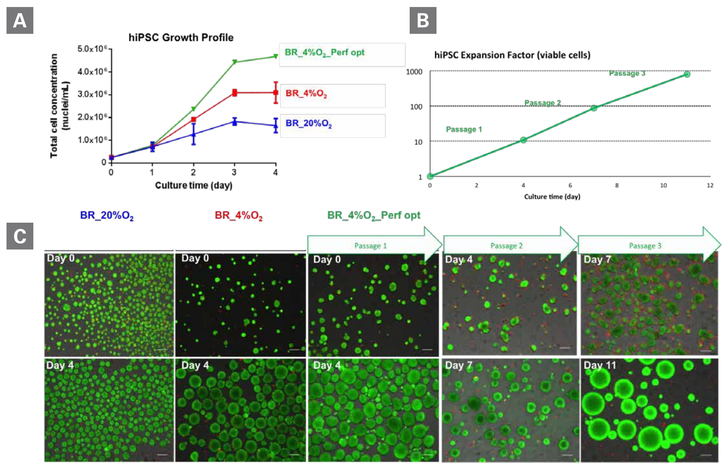



**Figure 6. Successful establishment of a scale-up strategy to expand hiPSCs by a factor of 800 in 11 days. Panel A.** hiPSCs were expanded as 3D aggregates in a stirred-tank bioreactor for comparison of the effects of oxygen and different perfusion rates on growth rate. **Panel B.** Expansion factor of hiPSCs during 11 days of culture, including 3 passages. **Panel C.** Representative images of hiPSC aggregates at day 0 and day 4 for the three bioreactor strategies evaluated, and images during passage for the optimized strategy. Viable cells were labeled in green with fluorescein diacetate and dead cells in red with propidium iodide. Scale bar = 200 µm.

## Conclusions

- Cellartis DEF-CS 500 Xeno-Free Culture Medium is defined and completely free of human- and animal-derived components. (Cellartis DEF-CS 500 Xeno-Free GMP Grade Basal Medium [Prototype] is now available.)
- The system is optimized for feeder-free culturing of hPSCs in 2D monolayer or 3D suspension culture formats. (Cellartis DEF-CS 500 Xeno-Free 3D Spheroid Culture Medium is now available.)
- hPSCs cultured in this system express the expected stem cell markers, remain diploid, and differentiate into cell types from the three germ layers.
- The system allows for robust and scalable hPSC production in a perfusion bioreactor, facilitating the use of hPSCs for research and large-scale clinical applications.