GMP-compliant, stable, and efficient expansion of pluripotent stem cells in a closed cultivation system

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Introduction

Human pluripotent stem cells (hPSCs) hold great promise for clinical use and cell therapy applications. To ensure highest quality and consistency of the resulting cellular products, suitable hPSC lines have to be maintained under standardized cultivation conditions and procedures. We developed a xeno-free iPS-Brew GMP Medium following the recommendations of USP <1043> on ancillary materials, thus enabling expansion of hPSCs for clinical research use. iPSCs were expanded using a pilot lot of this medium. Resulting cells were highly pluripotent, displayed a normal karyotype, and could be easily differentiated into cells of all three germ layers.

To increase the level of process standardization and product consistency we developed a procedure for hPSC cultivation using the integrated cell-processing platform CliniMACS Prodigy®. PSCs could be expanded by a factor of 25 to 60 in a single passage using automated coating, feeding, and harvesting procedures in a closed, single-use tubing set under adherent culture conditions. Subsequently, these cells could be replated and differentiated into neuroectodermal cells within the closed system, illustrating the feasibility of automated cell production for future clinical cell manufacture.

Results

1 iPS-Brew GMP Medium enables stable and efficient hPSC expansion and supports fast culture re-initiation after cryopreservation

In order to qualify the iPS-Brew GMP Medium and analyze the robustness of the culture conditions, two PSC lines (clone 1 and 2) were cultivated on recombinant Laminin-521. We used two different culture techniques: i) cell-cluster passaging with StemMACS™ Passaging Solution XF (A) and ii) single-cell passaging using TrypLE™ (B). Both iPSC clones showed a homogenous morphology over 20 passages regardless of the passaging technique used (A and B, clone1) and had a stable doubling time of 18–24 h (depending on the PSC clone) as assessed for single-cell passaging during long-term cultivation (C). Split ratios of 1:15 and up to 1:20 were suitable for long-term cell-cluster passaging for both clones. Furthermore, cryo-stocks were prepared using StemMACS Cryo-Brew to estimate their recovery after a freeze-thaw cycle. Cells rapidly recovered after thawing (n = 3), almost immediately reaching the standard doubling times (D) and displaying a normal morphology already in p1 after thawing (E).

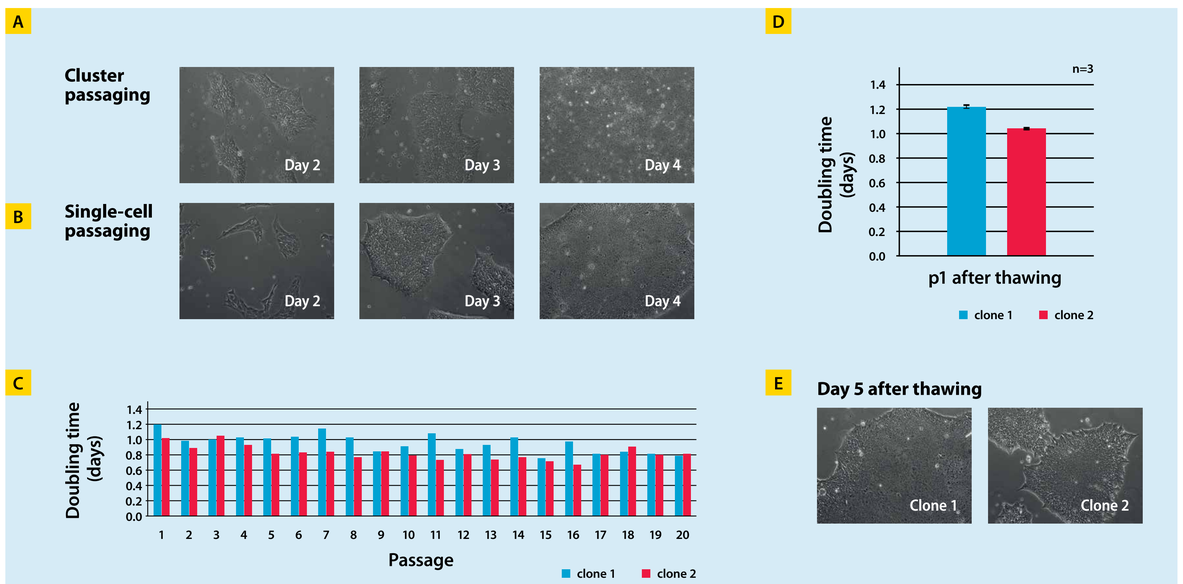


Figure 1

2 Cells cultivated in iPS-Brew GMP Medium stay highly pluripotent and retain a normal karyotype

To determine the quality of the cells cultivated in iPS-Brew GMP Medium, cells were checked for their marker expression profile using multicolor flow cytometry[1]. Both PSC clones showed a high and robust expression of the pluripotency markers TRA-1-60, SSEA-4, SSEA-5, and Oct-4 with >98% positive cells and almost no expression (<14% positive cells) of the differentiation marker SSEA-1 during long-term cultivation regardless of the passaging technique used (A, clone 1, and B). The genomic stability of the cells was analyzed by karyotyping.

No abnormalities could be identified after 20 passages (C, clone 1; p20 of cell-cluster passaging). Moreover, the differentiation potential of each clone, cultivated for at least 10 passages in iPS-Brew GMP Medium, was examined. Standard *in vitro* differentiation assays were used to differentiate the cells into: i) CXCR-4$^+$FoxA2$^+$Sox17$^+$ definitive endoderm cells (endoderm), ii) Sox2$^+$Pax6$^+$PSA-NCAM$^+$ neuroectoderm cells (ectoderm) and iii) CD144$^+$ endothelial precursors and CD140b$^+$ vascular smooth muscle cells (mesoderm) (D).

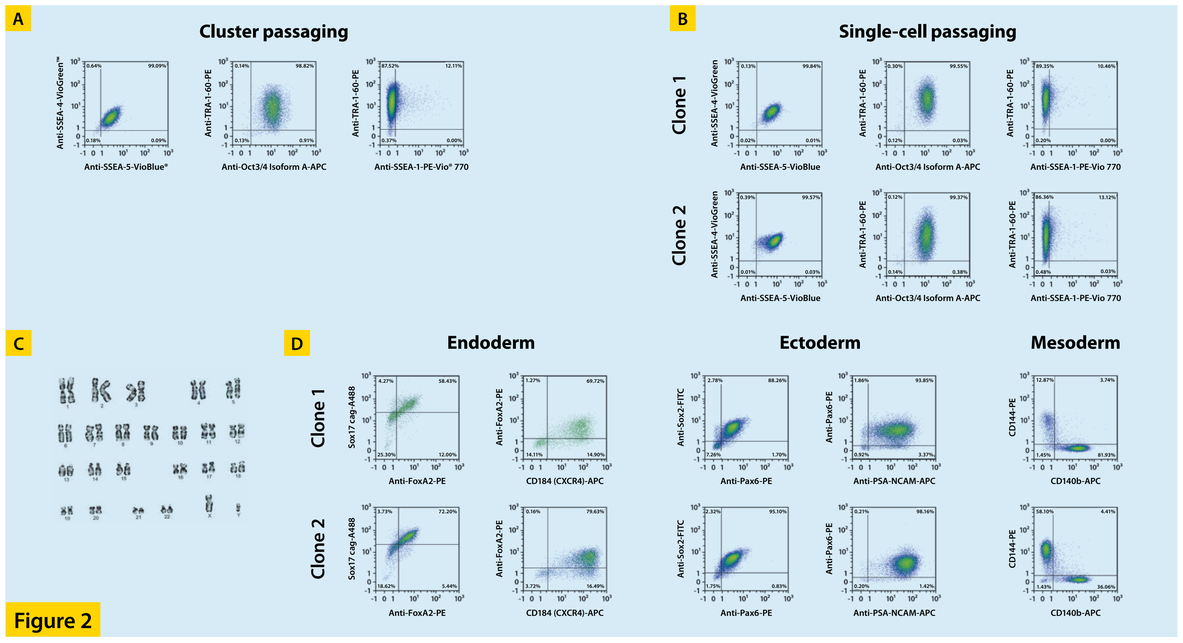
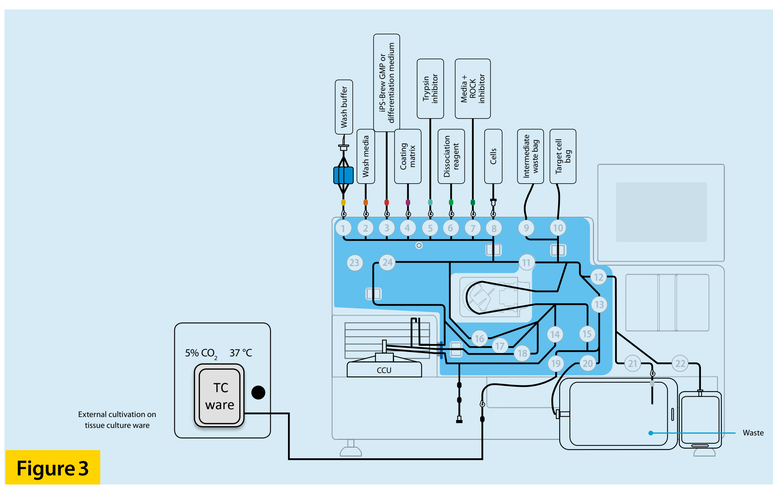


Figure 2

3 Workflow for GMP-compliant hPSC expansion and differentiation within the closed cultivation system CliniMACS Prodigy®

The CliniMACS Prodigy® provides the possibility of setting up complex protocols for cell manufacturing in a closed system (fig.3). To increase the level of process standardization and product consistency, a prototype program for hPSC cultivation and differentiation was developed using the TS 730 tubing set, which provides eight connections for media, buffer, and coating solutions as well as the possibility to pre-warm media during transfer from an external 4 °C storage compartment into the cultivation and centrifugation unit (CCU). At day –1 media and solutions or external tissue culture ware are connected sterilely before installing the tubing set on the instrument.

Bags with cell suspensions or cultivation media are connected via sterile welding just before use at day 0. Typically, 1×10^6 hPSCs are inoculated into the CCU and cultivated over the next days, with automated daily media exchange, until cells are automatically harvested on day 5 yielding 25–60×10^6 hPSCs depending on the cell line used. The harvested hPSCs can subsequently be used for further differentiation purposes, e.g., using sterilely connected external large-area tissue culture (TC) ware (Corning® CellSTACK®) kept in an adjacent incubator.



Day –1: Device setup
- Install tubing set
- Connect all media and solutions
- Coat centrifugation and cultivation unit (CCU)

Day 0: Culture start
- Inoculate culture in CCU

Day >1: Culture maintenance
- Automated media exchange (flexibly programmable schedule)
- Automated harvest and split

Day 5: Harvest of hPSC culture
- Automated harvest of hPSC culture
- Connect differentiation media
- Inoculate hPSCs into coated external TC ware for further neuroectodermal differentiation

Days 5–12: Culture differentiation
- Automated media exchange

Day 12: Harvest of differentiated culture
- Automated harvest of differentiated cells

Figure 3

4 Efficient hPSC expansion and neuroectodermal differentiation with the CliniMACS Prodigy®

We assessed the feasibility of an automated iPSC expansion and subsequent neuroectodermal differentiation. First, iPS clone 2 was expanded within the CCU using iPS-Brew GMP Medium. Cells grew comparably to iPSCs cultivated on Laminin-521–coated standard 6-well TC plates in iPS-Brew GMP Medium (A) and displayed typical hPSC morphology during expansion (B). After five days of closed-system expansion an in-process control (IPC) was conducted on a cell sample obtained from a sampling pouch, using multicolor flow cytometry. The cells expressed the pluripotency markers at a high level (>91% positive cells), with almost no expression of the differentiation marker (<4% positive cells) (C). Of the 5.9×10^7 harvested iPSCs, 2.5×10^7 cells were transferred into a sterilely connected single-layer CellSTACK (636 cm^2) and compared to a standard 6-well plate differentiation culture. A larger number of 2.23×10^8 neuroectodermal cells (3.5×10^5 cells/cm^2) could be generated in the closed-system cultivation compared to only 1.32×10^7 neuroectodermal cells (2.3×10^5 cells/cm^2) in the standard 6-well plate (D), demonstrating the successful upscaling for generating differentiated cells. Cells showed the normal neuroectodermal morphology during differentiation in the closed system (E) and expressed the expected markers Sox2, Pax6, and PSA-NCAM comparably to cells differentiated in a 6-well plate (F).

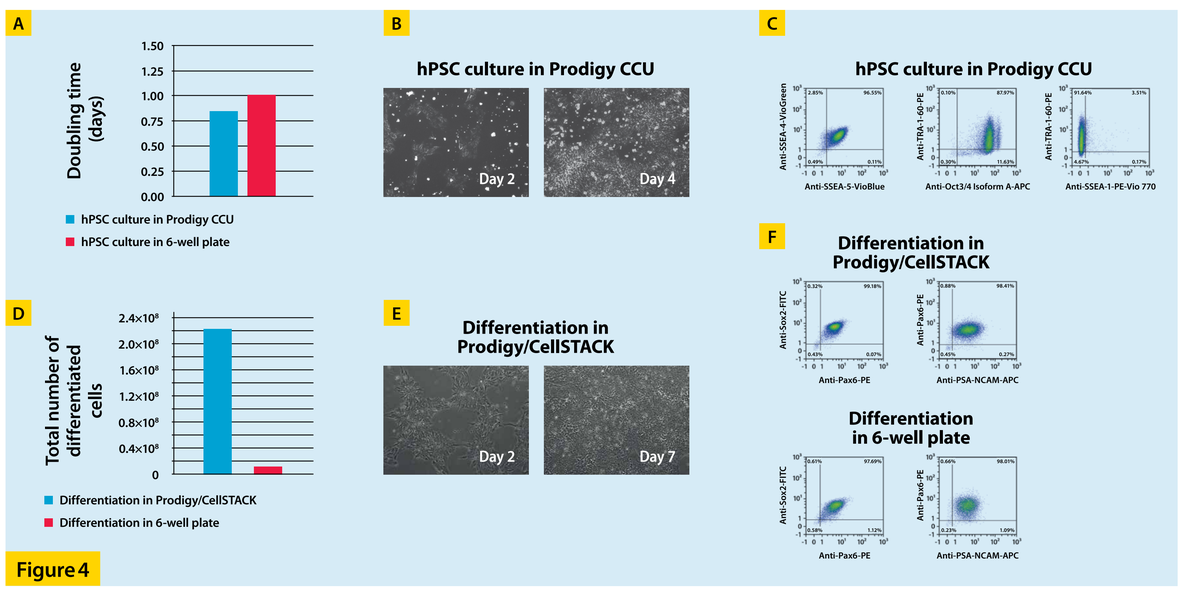


Figure 4

Conclusion

Here we developed a xeno-free cultivation medium for hPSCs, following the recommendations of USP <1043> on ancillary materials, which:

- allows the maintenance and stable expansion of hPSCs on standard cell attachment matrices like recombinant Laminin-521,
- supports rapid culture initiation after cryopreservation,
- keeps hPSCs highly pluripotent for more than 20 passages while retaining a normal karyotype and the capacity to differentiate into cells of all three germ layers.

iPS-Brew GMP Medium can be used with the CliniMACS Prodigy for hPSC cultivation in a closed system offering the perspective of an automated GMP-compliant cell manufacture.

Reference:
1. Miltenyi Biotec (2016) Multicolor flow cytometry analysis of human pluripotent stem cell cultures. http://www.miltenyibiotec.com/~/media/Files/Navigation/Cell%20analysis/resources/AppNote_Flow_cytometry_of_PSC_cultures.ashx)