Standardized QC assays and (automated) expansion of pluripotent stem cells

Annett Kurtz, Andrea Bretz, Hannah Johannsen, Tristan Kuchler, Frank Jüngerkes, Annika Last, Andreas Bosio, and Sebastian Knöbel
Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Introduction

Human pluripotent stem cells (hPSCs) hold great promise for disease modeling, drug discovery, and clinical applications. In this regard, working with highly pluripotent, quality-controlled cell stocks during development is crucial to ensure reproducible experimental conditions.

We established a workflow encompassing i) stable expansion of hPSCs using a xeno-free cultivation medium, ii) assessment of pluripotency by quantitative flow cytometry using a defined marker combination, iii) flow cytometric assessment of differentiation potential, based on lineage-specific, complete media, as well as iv) cryopreservation of hPSCs using a chemically defined medium.

Following this workflow, hPSCs could be stably expanded over 20 passages with persistent, high expression of pluripotency markers and almost no expression of differentiation markers. Cultured hPSCs showed the typical morphology and retained a stable karyotype. Quantitative flow cytometry analysis reproducibly confirmed the cells' potential to differentiate into all three germ layers. hPSCs were recovered effectively after cryopreservation. After thawing, a 20-fold hPSC expansion could be achieved, and cells displayed high pluripotency marker expression in p1.

Thus, the workflow assures standardized, robust hPSC expansion and includes characterization and quality control (QC) of the expanded cells as well as efficient cryopreservation. The flow cytometry–based QC strategy was also successfully applied for characterization of hPSCs cultivated automatically in the closed system of the CliniMACS Prodigy®, which is of major relevance for generation of master cell banks (MCB) and working cell banks (WCB) for future clinical cell manufacturing.

Results

1 hPSCs can be stably expanded as single cells or cell clusters using StemMACS™ iPS-Brew XF

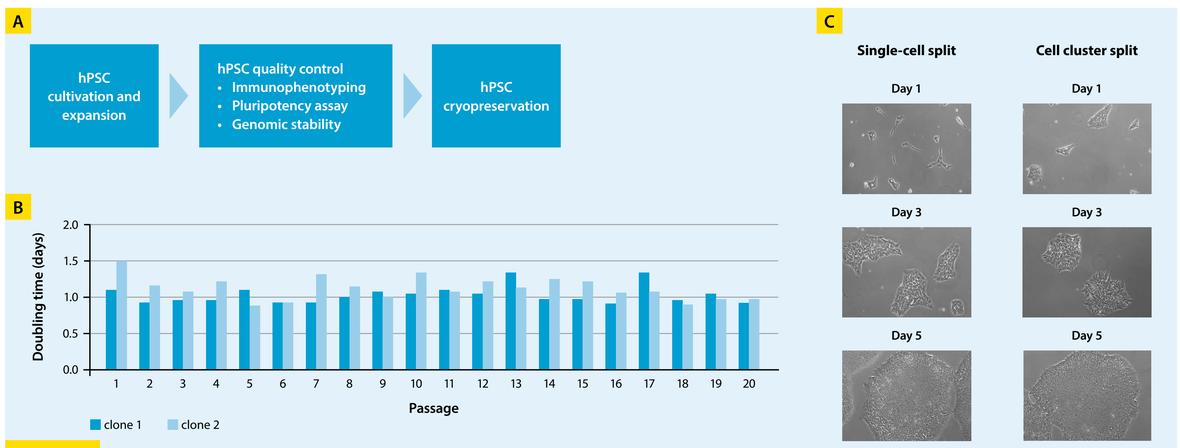

Figure 1

Figure 1A illustrates the workflow combining hPSC expansion, QC of the expanded cells using immunophenotyping, pluripotency assay (differentiation into all three germ layers) and karyotype analysis, as well as cryopreservation. Two hPSC lines were expanded for 20 passages on Matrigel® in StemMACS™ iPS-Brew XF. Cells had a stable doubling time of 24–27 h as assessed for single-cell passaging during long-term cultivation (fig.1B).

Using StemMACS iPS-Brew XF hPSCs could be cultivated as single cells or cell clusters and displayed the typical morphology (fig. 1C, clone 1).

2 QC of hPSCs can be standardized using immunophenotyping, pluripotency testing by StemMACS™ Trilineage Differentiaton Kit, and karyotype analysis

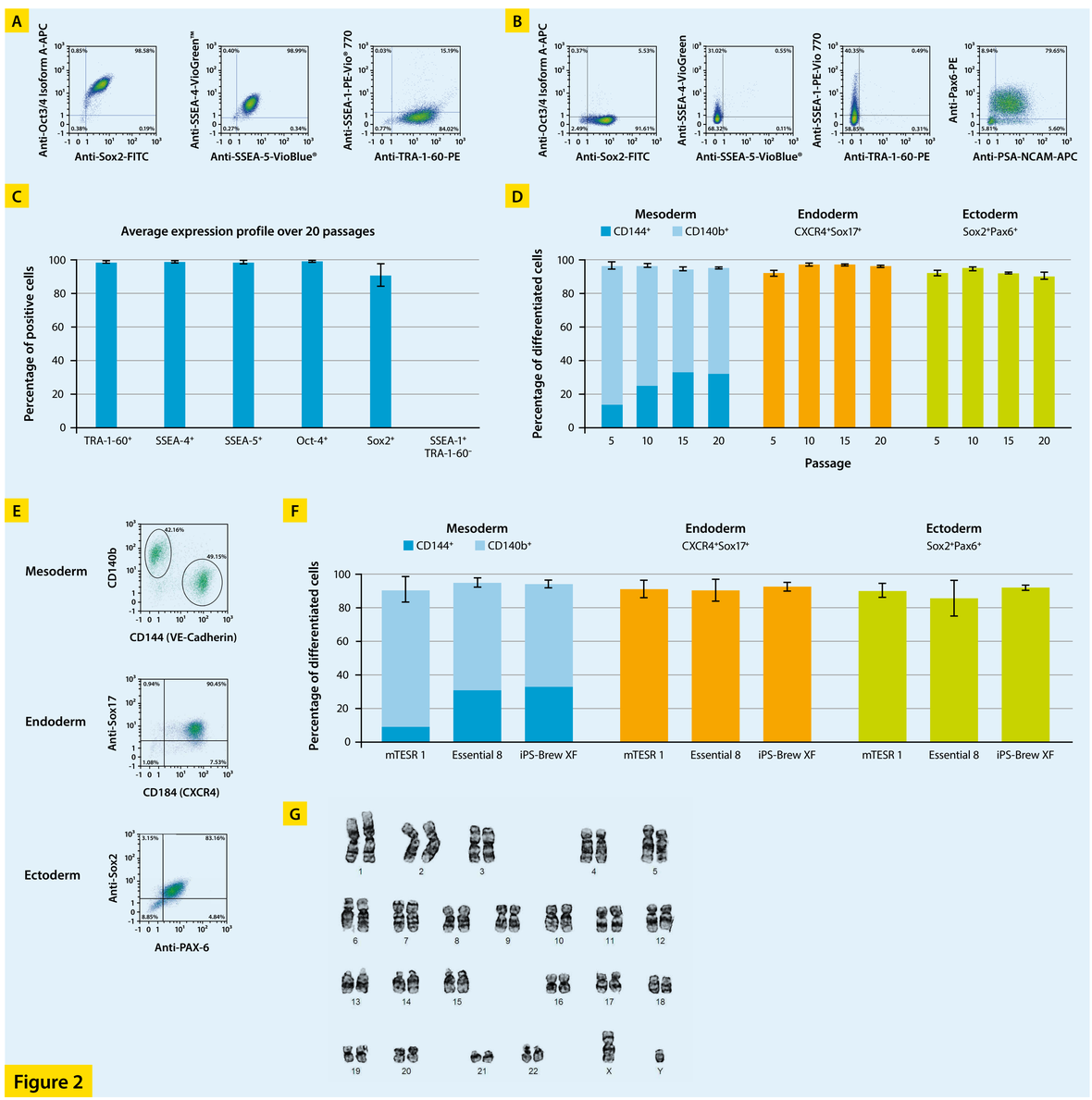

Figure 2

3 hPSCs show high recovery, normal doubling time and high pluripotency marker expression after cryopreservation in StemMACS™ Cryo-Brew

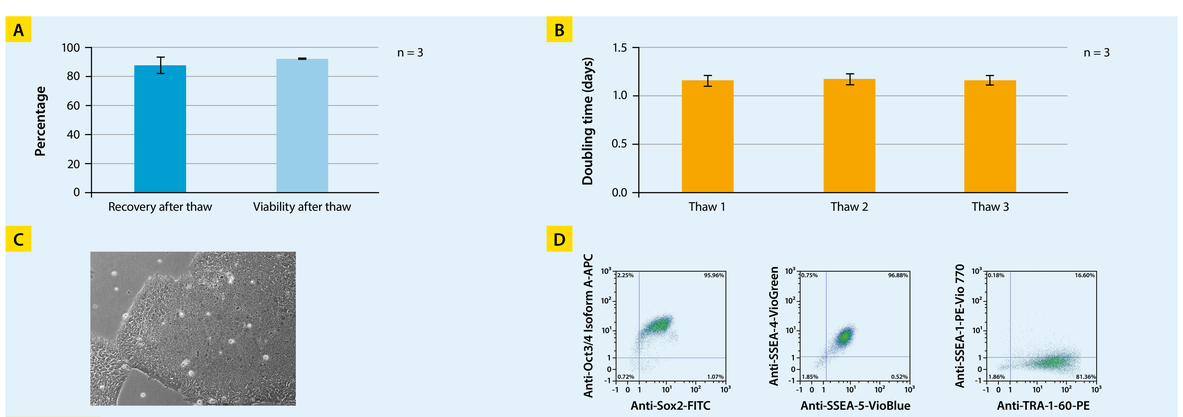

Figure 3

QC of the expanded cells is exemplified for clone 1 in figure 2. Immunophenotyping was performed using a multicolor flow cytometry protocol[1] based on well-known pluripotency markers and a differentiation marker. Analysis in passages 5, 10, 15, and 20 showed persistently high expression of pluripotency markers TRA-1-60, SSEA-4, SSEA-5, Sox2, and Oct-4 (92–99%) and almost no expression of differentiation marker SSEA-1 (0.1% SSEA-1$^+$TRA-1-60$^-$) (fig. 2A, C). During development of the flow cytometry protocol, clone 1 was differentiated into neural lineage and analyzed using this marker panel. All pluripotency markers were down-regulated except for Sox2, which is also a marker for neural precursors, whereas SSEA-1 was up-regulated (fig. 2B). This confirmed the reliability of the chosen markers for hPSC immunophenotyping. Additionally, PSA-NCAM and Pax6 were stained to prove the neural identity.

Differentiation potential was assessed in passages 5, 10, 15, and 20 using the StemMACS Trilineage Differentiation Kit, which supports directed differentiation into all three germ layers based on lineage-specific, complete media. Quantitative flow cytometry analysis[2] confirmed a stable capacity of the cells to differentiate into CD140b$^+$ vascular smooth muscle or CD144$^+$ endothelial cells (mesoderm), CXCR4$^+$Sox17$^+$ definitive endoderm cells (endoderm) and Sox2$^+$PAX-6$^+$ neuroectoderm cells (ectoderm) during long-term cultivation (fig. 2D, E). During development of the differentiation kit, clone 1 was cultivated in either StemMACS iPS-Brew XF, mTeSR™ 1, or Essential 8™ Medium and subsequently differentiated. Regardless of the PSC expansion medium used, the overall differentiation efficiency was the same for each germ layer (fig. 2F). Thus, the potency assay can be used in combination with various PSC cultivation media for QC. At last, genomic stability after long-term cultivation was confirmed by karyotype analysis. No abnormalities were observed (fig. 2G).

hPSCs frozen in the animal component–free, chemically defined StemMACS Cryo-Brew showed a reproducible recovery of 88% and high viability of 93% after thawing (fig. 3A, clone 1, n = 3). Cells rapidly recovered in p1 after thawing (n = 3), almost immediately reached a standard doubling time of 27–28 h (fig. 3B, clone 1, n = 3), and displayed a normal morphology (fig. 3C, clone 1) and high expression level of pluripotency markers (fig. 3D, clone 1).

4 Quality control of hPSCs cultivated under standardized, automated, closed-system conditions using the CliniMACS Prodigy®

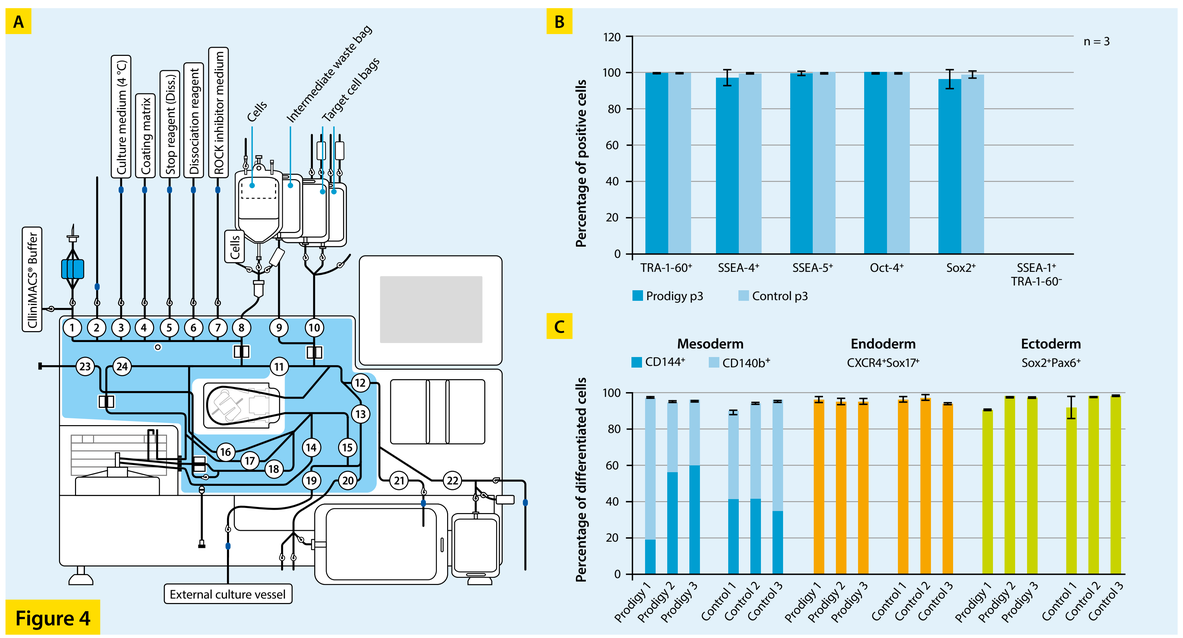

Figure 4

Clone 1 was expanded automatically in the closed system of the CliniMACS Prodigy, on Laminin-521 in iPS-Brew GMP Medium until p3 (fig. 4A, n = 3). Afterwards, the flow cytometry–based QC was applied. Immunophenotyping revealed that these expanded cells expressed pluripotency markers comparably to the manually expanded control cells (96–99%, control: 98–99%), with almost no expression of the differentiation marker (<0.1% SSEA-1$^+$TRA-1-60$^-$, control: <0.1% SSEA-1$^+$TRA-1-60$^-$) (fig. 4B). The differentiation potential of the automatically expanded cells was also comparable to the manually expanded control (fig. 4C, n = 3). Additionally, karyotype analyses of cells expanded in the CliniMACS Prodigy were without pathological findings (data not shown).

Conclusion and outlook

We established a workflow which allows:

- efficient and stable expansion of hPSCs,
- standardized characterization and QC of the expanded cells by immunophenotyping as well as assessment of differentiation potential and genomic stability,
- efficient hPSC cryopreservation including rapid recovery of highly pluripotent cells.

QC of hPSCs can also be used to monitor cell cultures maintained under standardized, automated, closed-system conditions, for example, during generation of master cell banks (MCB) and working cell banks (WCB).

References

1. Miltenyi Biotec (2016) Multicolor flow cytometry analysis of human pluripotent stem cell cultures.
2. Miltenyi Biotec (2017) StemMACS™ Trilineage Differentiation Kit – Protocol for flow analysis.

Both articles are available as PDF at **www.miltenyibiotec.com**