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Proof-of-Concept Study

Simple and Scalable hiPSC Expansion in Suspension as Spheroids Using the BioThrust ComfyCell Incu Bioreactor

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Abstract

This study presents a proof-of-concept evaluation of BioThrust's ComfyCell Incu bioreactor for three-dimensional (3D) human induced pluripotent stem cell (hiPSC) cultivation as spheroids. The investigation explored a simplified single-use 300 mL bioreactor (SU300) control setup designed to facilitate the transition from conventional 2D culture to 3D hiPSC expansion. Results demonstrated efficient and high-quality cell growth, yielding up to 2.6 x 10^6 viable hiPSCs/mL and a total of 8 x 10^8 cells. Pluripotency assays confirmed the maintenance of hiPSC identity and phenotype. The simplified design of the ComfyCell Incu intentionally limited process parameter monitoring, emphasizing essential operational functions for early-stage process development. Overall, the ComfyCell Incu system enabled robust and reliable 3D hiPSC expansion, representing an economical and practical solution for early development of hiPSC-derived therapies—particularly in allogeneic applications.

Introduction

Human induced pluripotent stem cells (hiPSCs) have emerged as a cornerstone for the development of (allogeneic) regenerative therapies due to their capabilities for unlimited self-renewal and differentiation into diverse cell types [1, 2]. However, to enable the clinical translation and commercialization of hiPSC-derived products require scalable, reproducible, and well-characterized manufacturing processes that ensure the consistent production of these therapeutic cells [3].

Traditional static culture methods provide limited scalability and process control, making them unsuitable for generating the large numbers of cells needed for therapeutic applications (often in the range of billions of cells per patient) [4]. Large-scale cultivation in stirred-tank bioreactors (STRs) has gained attention as a possible solution, but has so far not overcome all the challenges, especially due to hiPSCs' sensitivity to shear stress (τ). This stress may result from bioreactor agitation, as well as bubble rupture, both negatively affecting viability, growth, and quality of the stem cells [5, 6].

BioThrust'sComfyCell bioreactor platform aims to overcome classical STRs' limitations by employing the proprietary Membrane Stirrer (MemStir) impeller. This innovative stirring system uses hollow-fiber membranes to introduce process gases into the bioreactor via diffusion, eliminating direct aeration (sparging) and thus significantly decreasing shear forces acting upon the cells.

Moreover, the gentle hydrodynamics and efficient gas transfer of the ComfyCell system allowed for the implementation of hiPSC processes based on microcarriers, enabling easy transfer and comparison of these protocols in the MemStir-equipped vessel [7, 8].

The MemStir achieves high volumetric mass transfer rates (kLa) especially at low tip speeds. Through the prevention of foaming, the ComfyCell eliminated the need of antifoam reagents from hiPSC expansion and may decrease downstream processing costs [9].

In this study, a simplified incubator-associated cell culture device was used. The system makes use of the standard incubator atmosphere - oxygen, CO_2, humidity, and temperature - to provide optimal conditions for cell culture and expansion, while incorporating a MemStir impeller that is directly controlled by the Incu device.

Objective

The proof-of-concept (POC) study's objective is to demonstrate and thus provide a blueprint for simple hiPSC expansion using the BioThrust ComfyCellIncu, offering process development teams with limited 3D experience robust and economical equipment to expand high-quality stem cells.

Further process optimization with a fully capable bioprocess controller, which expands on the Incu's capabilities, will provide users with the means to achieve higher cell densities and better-quality cells.

Key Findings

1. In the initial benchmark run, the Comfy*Cell Incu* successfully expanded to ~2.6 x 10^6 iPSCs per mL.
2. A single Comfy*Cell* Incu SU300 was able to produce ~8 x 10^8 iPSCs in a single run with a fold expansion of >17.
3. The *ComfyCell* Incu SU300 allowed robust aggregation and expansion of iPSC-spheroids while running entirely in a standard cell culture incubator.
4. hiPSC maintained a high pluripotency phenotype, with >99 % expression of core pluripotency markers.

Materials & Methods

Figure 1: Isometric view of the BioThrust *ComfyCell* Incu SU300 bioreactor vessel standing on the Incu base unit with the motor coupled on top.

Specifications

- Laboratory scale working volume: 150 mL - 300 mL
- Culture conditions: standard cell culture incubator
- Bioreactors and expansion vessels:
  - T150 flasks for seed train
  - 1 x *ComfyCell* Incu Single-use Bioreactor (300 mL) "Incu SU300"
- Cell line: UKKi011-A (Cologne University Hospital, Germany)
- Enzyme for dissociation: StemPro Accutase (Gibco, USA)
- Direct inoculation of ~1.5 x 10^5 cells/mL, Viability > 94.0 %
- Process time: 5 days
- Feeding strategy: Batch media change (1vvd)
- Flow cytometry analysis: PSC Analysis Cocktail Kit (Miltenyi Biotec, Germany)

Abbreviations

MemStir = Membrane Stirrer
STR = Stirred-tank Bioreactor
hiPSC = human induced Pluripotent Stem Cell
vvm = Volume of air per working volume per minute
VCD = Viable cell density
Vb = Cell viability
vvd = Vessel volume per day
SU300 = Single-use 300 mL Bioreactor

Parameter/ Reagents	ComfyCell *Incu SU300*
Agitation rate (NS1u)	70 rpm (clockwise)
Atmospheric conditions	~21 % O_2 and 5% CO_2
Temperature	37 °C
pH	7.4
Gassing outlet	MemStir
Aeration volume flow	0.1 vvm
Medium	Chemically defined Medium
Working volume	300 mL

Methods

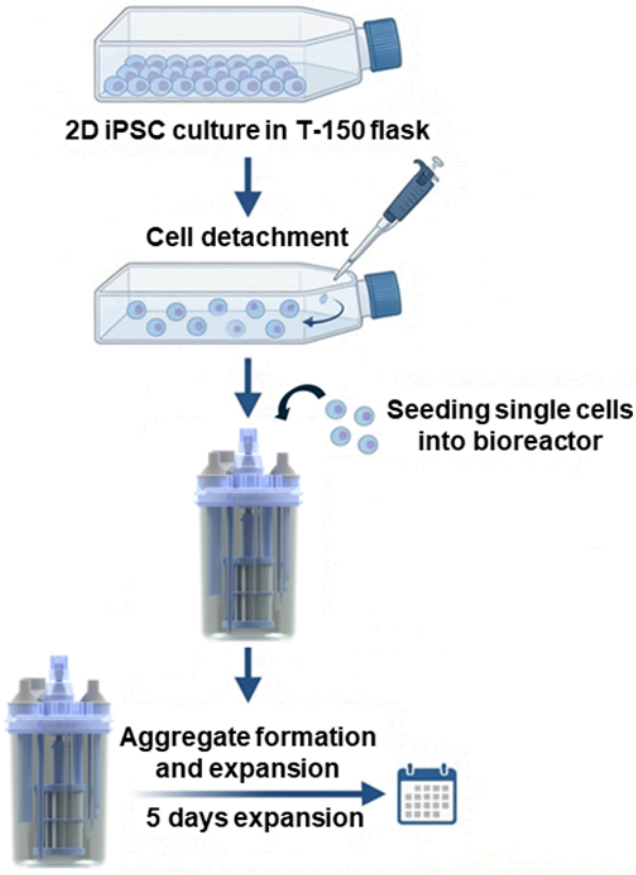

Figure 2: Schematic view of the entire iPSC-spheroid culture process. From the 2D expansion phase to 3D aggregate formation and 5-day expansion using the *ComfyCell Incu SU300* bioreactor.

Results

After equilibration of the medium to 37 °C and the incubator's atmospheric conditions, cells were inoculated at approximately 150,000 cells/mL and cultivated under dynamic conditions at 70 rpm.

Cell concentration and viability were monitored throughout the cultivation to determine the specific growth rate, and microscopic images were acquired to evaluate aggregate size distribution.

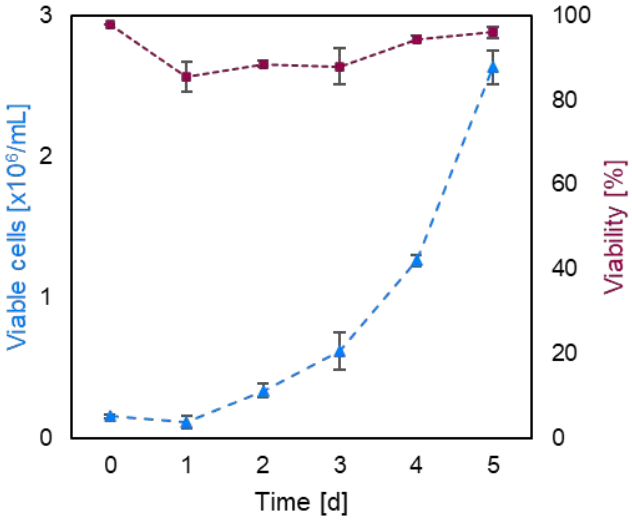

Figure 3: Viable cell density (blue) and viability (purple) kinetics during the 5-day iPSC culture process. Error bars represent standard deviation (SD).

Cellular growth and morphology

During the first 24 hours of iPSC culture in the ComfyCell Incu bioreactor system, a decrease in viable cell density was observed, with cell numbers reaching approximately 1.12 × 10^5 cells/mL, corresponding to ~74 % of the initial inoculum, while viability decreased from ~98 % at inoculation to ~86 % at 24 hours. This early reduction likely reflects transient adaptation effects during the initial aggregation and settling phase, which is commonly accompanied by modest viability loss in dynamic suspension cultures transitioning from 2D to 3D conditions (Figure 3).

From day 2 onward, the culture showed a clear and consistent increase in cell density, with viability stabilizing and improving. At 48 hours, the mean viable cell density reached 3.36 × 10^5 cells/mL, representing a 2.2-fold expansion relative to the starting cell number, and viability recovered to ~89 %. Continued growth was observed over the subsequent days, with cell densities of 6.18 × 10^5 cells/mL at day 3 (4.05-fold expansion, ~88 % viability), 1.26 × 10^6 cells/mL at day 4 (8.26-fold expansion, ~95 % viability), and a maximum of 2.64 × 10^6 cells/mL at day 5 (17.28-fold expansion, ~96 % viability).

Regarding the viability of the formed iPSC aggregates, we observed only a transient decrease in the first 24 hours, followed by consistently high viabilities (≥88 %) from day 2 onward.

Overall, these results demonstrate robust iPSC expansion in the ComfyCell Incu bioreactor system over a 5-day culture period, achieving high cell densities and sustained proliferation while maintaining high cell viability after the initial 24-hour adaptation phase.

These results indicate that while daily batch exchanges support robust iPSC expansion up to mid-culture, higher cell densities might require more continuous nutrient delivery. Implementing a perfusion feeding strategy could therefore extend the active growth phase and enable further increases in final cell yield while maintaining optimal viability.

Aggregate Morphology

During the 5-day iPSC aggregation and expansion process, aggregate morphology was systematically analyzed to assess growth dynamics and culture homogeneity (Figure 4-8).

As shown in Figure 9, a consistent and progressive increase in average aggregate size was observed over time, indicating robust and well-controlled aggregate growth under dynamic culture conditions. Average aggregate diameters increased from ~84 µm at day 1 to ~99 µm at day 2, followed by a further increase to ~142 µm at day 3. Continued aggregate growth was observed at later time points, reaching ~198 µm at day 4 and ~209 µm at day 5. Importantly, aggregate sizes remained highly homogeneous during the early phase of cultivation, particularly up to day 4, reflecting well-regulated aggregation kinetics and stable expansion conditions.

This controlled growth behavior suggests efficient mass transfer and minimal aggregate fusion during the initial expansion phase. Beyond day 4, aggregates continued to increase in size while maintaining a relatively narrow size distribution, demonstrating the robustness and reproducibility of the culture system. Overall, these results support the suitability of this dynamic suspension platform for scalable and reproducible iPSC aggregation and expansion.

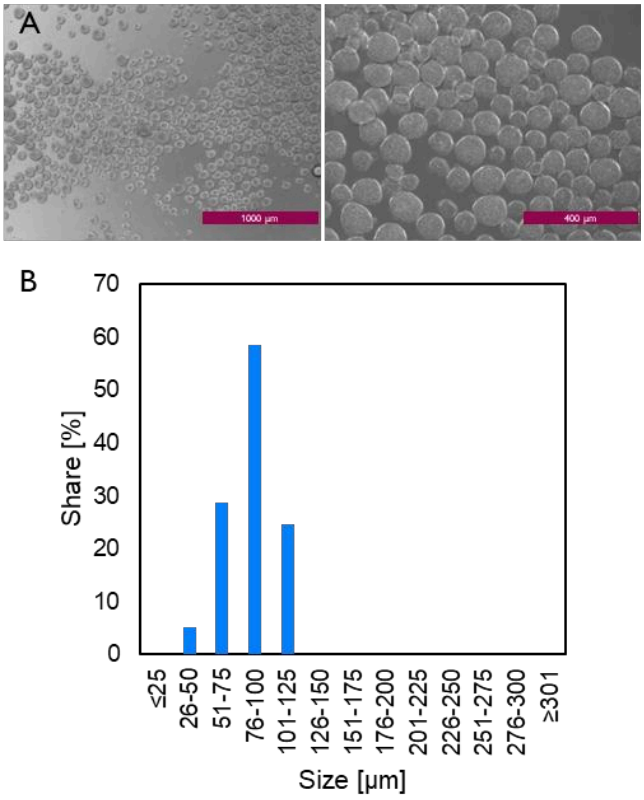

Figure 4: Microscopic images of aggregates morphology at 4 X (left) and 10 X (right) at day 1 (A). Aggregate size distribution at day 1 of iPSC expansion (B).

Results

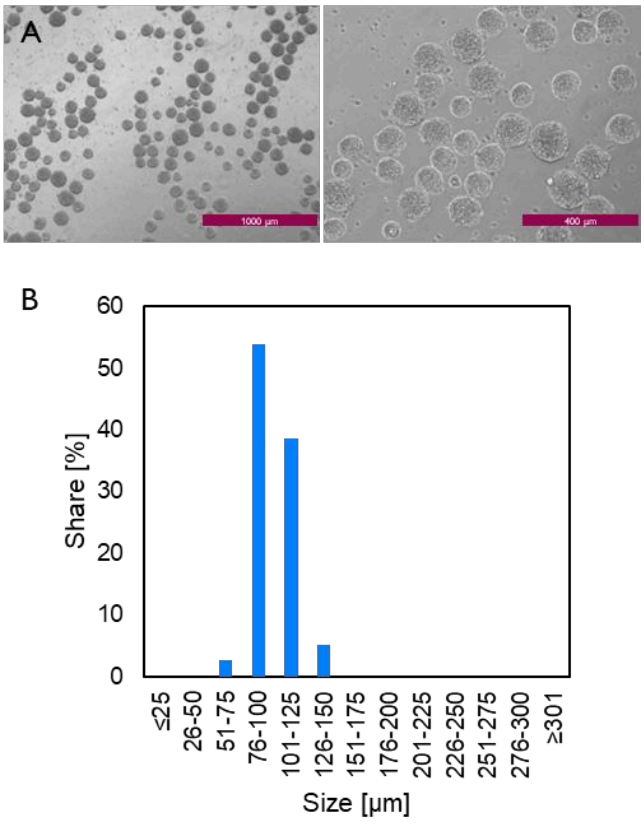

Figure 5: Microscopic images of aggregates morphology at 4 X (left) and 10 x (right) at day 2 (A). Aggregate size distribution at day 2 of iPSC expansion (B).

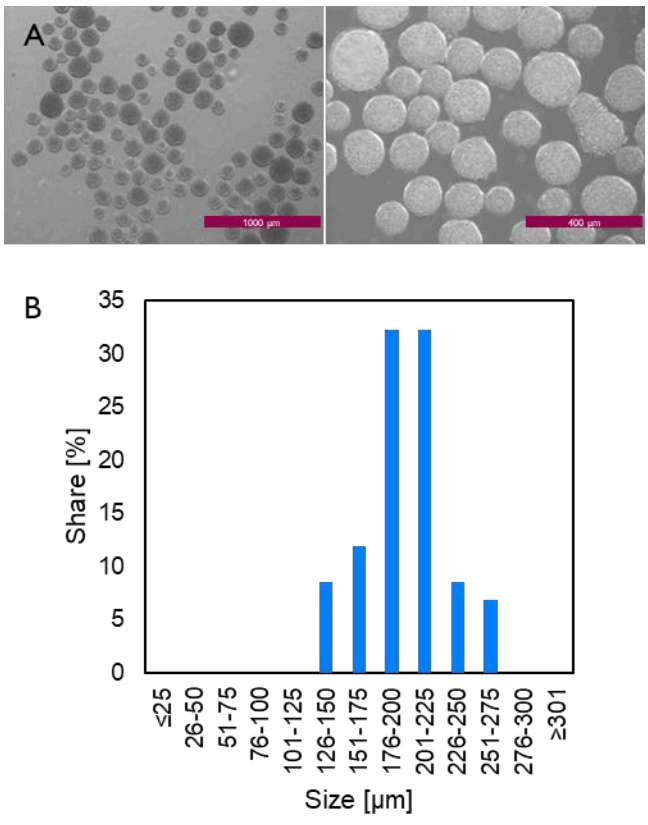

Figure 6: Microscopic images of aggregates morphology at 4 X (left) and 10 x (right) at day 3 (A). Aggregate size distribution at day 3 of iPSC expansion (B).

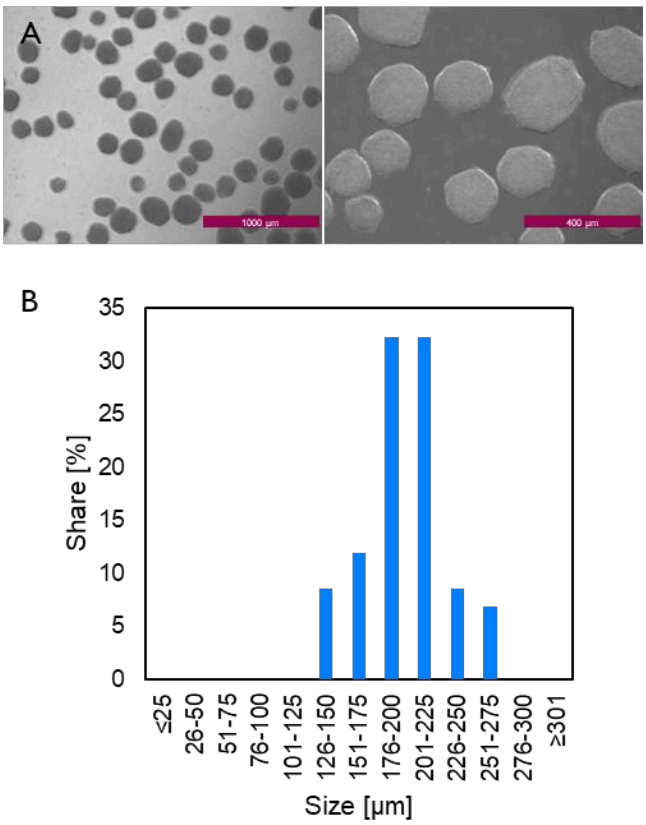

Figure 7: Microscopic images of aggregates morphology at 4 X (left) and 10 x (right) at day 4 (A). Aggregate size distribution at day 4 of iPSC expansion (B).

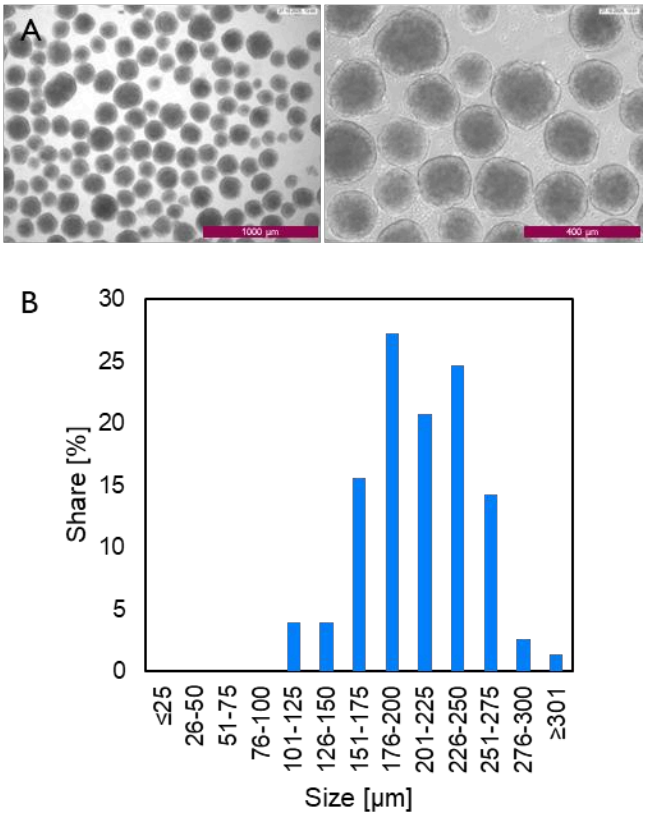

Figure 8: Microscopic images of aggregates morphology at 4 X (left) and 10 x (right) at day 5 (A). Aggregate size distribution at day 5 of iPSC expansion (B).

Results

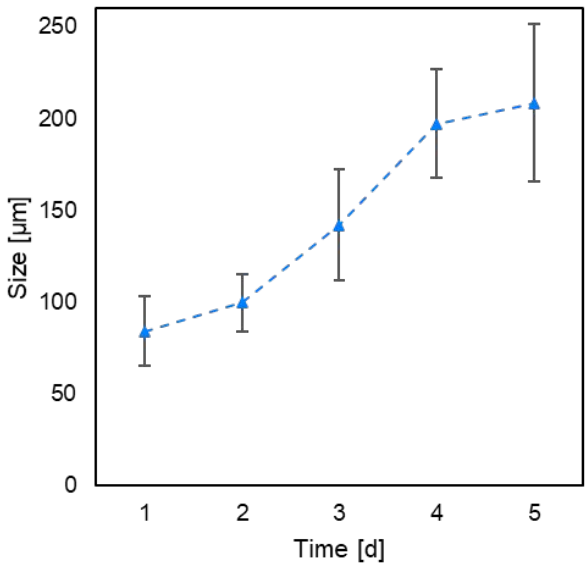

Figure 9: Average aggregate diameter over the 5-day culture period.

Pluripotency assessment

To evaluate whether pluripotency was maintained during the expansion process, harvested iPSCs from the bioreactor were phenotypically characterized by flow cytometry at day 5 of culture. A panel of well-established pluripotency markers (TRA-1-60, Oct3/4, and SSEA-4) and the differentiation-associated marker CD15 was used to assess cell identity and differentiation status.

Representative two-parameter flow cytometry dot plots demonstrate that iPSCs retained a highly pluripotent phenotype following the 5-day expansion period (Figure 10A–B). Co-staining for TRA-1-60 and CD15 (Figure 10A) revealed a cell population dominated by TRA-1-60–positive cells, with only a negligible fraction of CD15-positive events (98.7 %). Overlay of isotype controls and antibody-stained samples confirmed the specificity of the observed marker expression and minimal background signal. Similarly, analysis of Oct3/4 and SSEA-4 expression (Figure 10B) showed that the majority of cells co-expressed both pluripotency markers (98.1 %), indicating preservation of core transcriptional and surface marker profiles associated with undifferentiated iPSCs.

Quantitative analysis of marker expression across the expanded population further confirmed robust maintenance of pluripotency after 5 days of culture (Figure 10C). High expression levels were observed for all pluripotency markers analyzed, with 99.1 % TRA-1-60–positive, 99.8 % SSEA-4–positive, and 99.0 % Oct3/4–positive cells. In contrast, expression of the differentiation marker CD15 remained extremely low (0.2 %).

Together, these results demonstrate that the applied expansion conditions support efficient iPSC proliferation while preserving pluripotent identity and minimizing spontaneous differentiation over a 5-day expansion period.

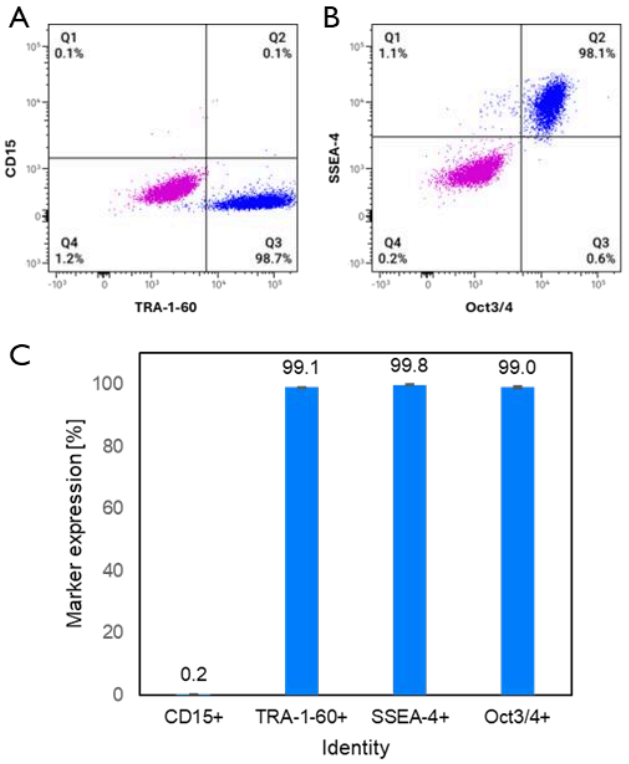

Figure 10: Phenotypic characterization of iPSCs on day 5 of culture. Representative flow cytometry dot plots showing TRA-1-60/CD15 (A) and Oct3/4/SSEA-4 (B) expression. In both panels, overlaid dot plots display isotype controls (purple) and specific antibody staining (blue). Quantitative summary of overall pluripotency marker expression together with CD15 expression as an indicator of differentiation status (C). Error bars represent standard deviation (SD).

Summary & Outlook

This application note demonstrates robust and controlled iPSC aggregation and expansion under dynamic suspension culture conditions over a 5-day cultivation period.

Aggregate morphology analysis revealed a consistent, progressive increase in average aggregate size, from ~84 µm at 24 h to ~209 µm by day 5, with highly homogeneous aggregate populations. This controlled aggregate growth indicates stable culture conditions and efficient mass transfer, which are critical for scalable iPSC manufacturing.

In parallel, cell expansion followed a clear growth trajectory. Starting from an initial inoculation density of 1.53 × 10^5 cells/mL (day 0), cell concentrations slightly decreased to 1.12 × 10^5 cells/mL (day 1), followed by a marked recovery to 3.36 × 10^5 cells/mL (day 2) and accelerated growth to 6.18 × 10^5 cells/mL (day 3). By day 4, cell densities of 1.27 × 10^6 cells/mL were achieved, reaching a maximum of 2.64 × 10^6 cells/mL by day 5. This growth profile highlights sustained expansion rates at early stages, followed by a pronounced increase in expansion rate at later stages of cultivation. Although no decline in expansion rate was observed within the 5-day period, a plateau in growth would be expected beyond this window due to the inherent limitations of batch-media changes at higher cell densities, particularly for iPSC culture.

Summary & Outlook

To overcome these constraints and further increase achievable cell densities while preserving aggregate quality and pluripotency, a transition to a perfusion-based culture strategy is proposed. Continuous medium renewal, combined with targeted supplementation on top of the basal medium, would enable sustained nutrient availability, improved waste removal, and tighter process control [10].

Pluripotency was confirmed by flow cytometry after 5 days of culture using TRA-1-60, Oct3/4, SSEA-4, and the differentiation marker CD15. Quantitative analysis revealed uniformly high pluripotency marker expression (99.1 % TRA-1-60–positive, 99.8 % SSEA-4–positive, 99.0 % Oct3/4–positive) and very low CD15 levels (0.2 %), demonstrating robust maintenance of pluripotent identity and neglectable spontaneous differentiation over the 5-day expansion period.

Overall, these results underscore the robustness of the aggregation and expansion platform while highlighting a clear pathway for further process intensification. Implementation of perfusion culture with optimized supplement feeding represents a promising next step to push cell densities beyond current batch limits while maintaining homogeneous aggregate morphology and high iPSC quality, thereby supporting future scale-up and translational manufacturing efforts.

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