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Application Note

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Scalable MSC Suspension-Based Process Optimization using the Sartorius MSC Optimization and Characterization Solution

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Abstract

Mesenchymal stem/stromal cells (MSCs) are multipotent adult stem cells that are present in many types of tissues, including umbilical cord, bone marrow, and adipose tissue. These cells can differentiate into a number of cell types and are among the most frequently used for regenerative medicine. The ability to manufacture large quantities of MSCs is required for therapeutic applications, and the development of robust and scalable production processes is essential to advance the field. This application note describes the development of a process through rapid identification of critical process parameters (CPPs) and their optimal parameter ranges to yield target ranges of critical quality attributes (CQAs). Such a process can be used for effective scale up to clinically relevant volumes and as a foundation for manufacturing control strategy.

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Introduction

MSCs are the foundational technology for multiple cell-based regenerative therapeutics. These cells have been investigated in more than 1000 clinical trials across dozens of indications and are also used in tissue and organ engineering. MSCs have been shown to be safe and well-tolerated with more than 10 approved therapies globally.

Most commercial indications will require a target production lot size of about 100 billion cells. Thus, manufacturing technologies that can support intensified cell culture and that can be scaled up to meet commercial demand for cGMP manufacturing are essential.[i] Once the target lot size reaches 20 billion cells, production is labor intensive and complex in conventional cell stacks. In addition, static cultivation systems are not amenable to automation due to inefficient mass transfer, presence of nutrient gradients, and inadequate process monitoring, all of which impede scale-up.

Furthermore, process insight is critical to the development of a robust manufacturing protocol for scaling up the production of MSCs. A deep understanding of CPPs and their impact on CQAs enables reliable scale-up and ultimately the establishment of a control strategy for consistent, reproducible manufacturing of a high-quality cell product.

The Sartorius MSC Optimization and Characterization Solution is a scalable, semi-automated solution that enables the development of process insight through the correlation of CPPs and CQAs (Figure 1A). The solution accelerates process development timelines by offering effective tools to establish the optimal process parameters for stirred-tank bioreactor culture and correlate their effect on MSC CQAs to optimize a robust, scalable, well-characterized MSC expansion protocol.

This application note describes a series of key process optimization studies in stirred-tank bioreactor culture using the Ambr® 250 Modular system, MODDE® design of experiments (DOE) software, SoloHill® microcarriers and SIMCA® multivariate data analysis (MVDA) software (Figures 1B-E). Using a systematic DOE approach at the 250 mL scale, process parameters were evaluated in relationship to culture performance and CQAs to identify optimal process parameter range.

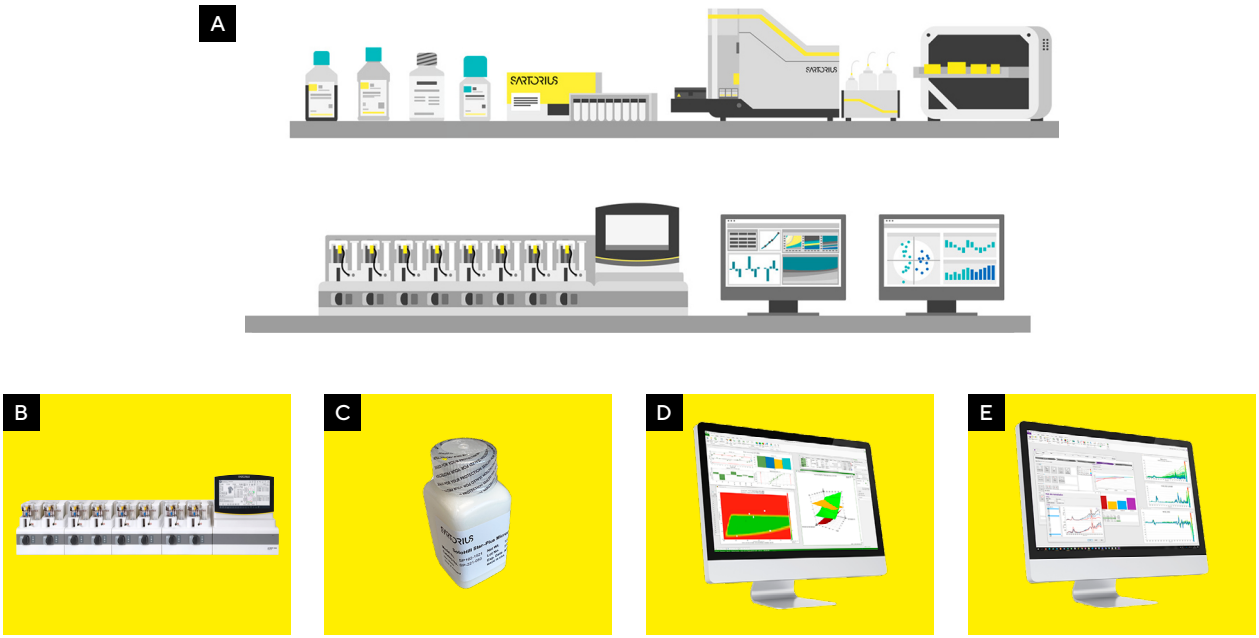

Figure 1: (A) Sartorius MSC Optimization and Characterization Solution, Including (B) Ambr® 250 Modular System, (C) Solohill® Microcarriers, (D) Modde® DOE Software, and (E) Simca® MVDA Software.

Materials

Key materials and equipment used:

- Ambr® 250 Modular System with 8 bioreactor stations (4 modules)
- Ambr® 250 Modular bioreactor vessels, unbaffled, single impeller
- SoloHill® Microcarriers
- MODDE® DOE Software
- SIMCA® MVDA Software
- RoosterBio media and RoosterVial™ human bone marrow-derived and umbilical cord-derived MSCs

Methods

Microcarrier-based MSC culture in the Ambr® 250 Modular

SoloHill® Microcarriers were prepared according to the supplier information. MSC seed train was generated in static 2D cultures (T flask or CellSTACK®). The Ambr® 250 Modular system was setup one day prior to inoculation to allow for medium conditioning (see Table 1 for the overview of Ambr® 250 Modular parameters). For inoculation, different seeding densities were evaluated (Table 2). To allow for cell attachment, an intermittent stirring strategy was employed for the first 6 hours, consisting of 8 cycles of 45 minutes static and 2 minutes stirring at 100 rpm.

Aseptic sampling of Ambr® 250 Modular vessels for cell counting was performed in a biological safety cabinet (BSC) using a serological pipette. Cells were harvested enzymatically by removing 200 mL spent medium from each bioreactor in a BSC. Cells were washed with 200 mL DPBS per bioreactor. For cell detachment, 200 mL TrypLE™ Select (Thermo Fisher Scientific) was added and bioreactors were agitated at the final stir speed (500 rpm, unless otherwise noted) at 37°C for 30 minutes.

Table 1: Overview of Ambr® 250 Modular culture parameters.

Culture Parameter	Setting
Temperature	37°C
pH	Controlled if pH < 7 by headspace gassing (unless otherwise noted)
Agitation	100 rpm for 24 hr, then increase by 50 rpm every 12 hr up to 500 rpm (unless otherwise noted), downward
DO	100% | 50% | 20%
Gassing	Headspace 20 mL/min (Mix of Air, CO_2, O_2 or N_2 *(to regulate DO at 20% & 50%, N_2 had to be used)*)
Feed	Day 3

Design of Experiments (DOE) and Data Analytics

The DOE Software MODDE® was used to design and analyze the experiments to optimize the key process parameters displayed in Table 2. The responses to optimize were viable cell density (VCD) or fold expansion and viability. Based on the obtained results, the software builds a statistical model which can then be used for further analytics and predictions.

Table 2: Key process parameters optimized using DOE.
*displayed as the final stirring speed obtained on Day 4 or 5 (200 - 500 rpm) in the results figures

Factor	Abbreviation	Level
Microcarrier density	Mic	2.5 cm^2/L | 5 cm^2/L | 10 cm^2/L
Cell density	Cel	11,500 cells/mL | 23,000 cells/mL | 46,000 cells/mL
Agitation speed	Spe	After 24 hr: stepwise increase every 12 hr by 12 rpm | 25 rpm | 37 rpm*
Culture duration (day of harvest)	Har	4 days | 5 days
pH control	pHC	Uncontrolled (5% CO_2) | controlled (if pH < 7: no CO_2 gassing)
DO concentration	DO	100% | 50% | 20%
Feed	Fee	24 hr | 48 hr | 72 hr post inoculation
Switch of stir direction	Swi	Switch every 0 min | 2.5 min | 5 min

Cell Characterization Assays

Flow Cytometry

The MSC identity was confirmed by flow cytometry analysis performed on cryopreserved cells harvested from bioreactors to determine expression of MSC surface markers (positive markers: CD73, CD90, CD105, CD166 and negative makers: CD14, CD34, CD45). As a reference, cell surface marker expression of static 2D control cells was evaluated.

Multilineage Differentiation Assays

Multilineage differentiation of MSCs into adipocytes, osteoblasts and chondroblasts were evaluated qualitatively on cryopreserved cells harvested from bioreactors. For adipogenic differentiation, cells were cultured in StemPro® Adipogenesis Differentiation Kit medium (Gibco) for 7-14 days, then fixed and stained with Oil Red O Solution (MilliporeSigma). For osteogenic differentiation, cells were cultured in StemPro® Osteogenesis Differentiation Kit medium (Gibco) for 14 days, then fixed and stained with 2% Alizarin Red Stain (Lifeline Cell Technology). For chondrogenic differentiation, cell pellets were generated in ultra-low attachment plates and cultured in StemPro® Chondrogenesis Differentiation Kit medium (Gibco) for 21 days, then fixed, sectioned, and stained with Alcian Blue. All differentiated samples were imaged following staining and compared with respective undifferentiated samples as well as 2D control cells.

IDO Assay

Cryopreserved cells harvested from the bioreactors were plated at 60,000 cells/cm², cultured for 20-28 hours, then washed and switched to medium containing 2% FBS and 10 ng/mL interferon- γ (IFN-γ) for 24 hours. Exposing MSCs to the proinflammatory cytokine IFN-γ induces indoleamine 2,3-dioxygenase (IDO) activity and is central to the immunosuppressive function of MSCs.[ii] The cell supernatant was collected, and the kynurenine concentration was measured using a spectrophotometric assay. Kynurenine concentration was normalized to the number of cells and days of incubation to obtain the amount of IDO secreted. The kynurenine level was compared to the levels from the 2D control cells.

Angiogenic Cytokine Secretion

Cryopreserved cells harvested from the bioreactors were plated at 60,000 cells/cm², cultured for 20-28 hours, then washed and switched to a medium supplemented with 2% FBS. After 24-hour incubation, the supernatant was collected and assayed for FGF, HGF, IL-8, TIMP-1, TIMP-2 and VEGF concentration using a MultiPlex ELISA (Quansys Biosciences). Cytokine secretion rates were obtained by normalizing the cytokine concentration to the number of cells and days of incubation. The cytokine secretion rate of the bioreactor cells was compared to the 2D control cells.

Results

Determination of optimal expansion parameters using DOE

Understanding of CPPs and their impact on process outcome is a foundational step in process development. Furthermore, identification of the optimal process design space is needed to define process ranges that meet quality specifications. These considerations can also support Quality by Design (QbD) submissions.

In this study, the understanding of optimal expansion process parameters for suspension-based MSC culture on microcarriers was rapidly gained by using the Ambr® 250 Modular in combination with the DOE software MODDE® as part of the Sartorius MSC Optimization and Characterization Solution. For this, eight different factors were evaluated for their impact on fold expansion, viable cell density (VCD) and cell viability (Table 2). A total of 50 runs were performed in the Ambr® 250 Modular, including several replicate experiments.

The MODDE® software was used to analyze experimental data. The effect of the different factors on VCD was evaluated using coefficient plots (Figure 2). In the original plot, all factors are displayed, including factors that had small values but large confidence intervals, indicating that these factors were non-significant for the model (Figure 2A). The Auto Fit function was used to automatically optimize the model by removing non-significant factors (Figure 2B). The optimized model showed that stirring speed had a negative impact on VCD, therefore a lower stirring speed should be chosen. Dissolved oxygen (DO), on the other hand, had a positive impact, indicating the process should be operated at higher DO concentrations (Figure 2). The model further indicates that a longer culture duration also had a positive impact on VCD suggesting cells should be harvested on Day 5 rather than Day 4 in this process.

To make predictions on the optimal operational range of the process, MODDE® provides tools such as the Sweet Spot Plot (Figure 3). Because DO concentration and stirring speed had the highest influence on VCD, these two factors were used as variables in the following predictions on the optimal process ranges, while the other factors were kept constant. The Sweet Spot Plot highlights the ranges (in green) for DO and stirring speed which yielded pre-defined process outcomes of VCD > 0.3×10^6 cells/mL, fold expansion > 10 and viability > 95%, thereby indicating the optimal ranges for these process parameters (Figure 3). The optimal space for these two process parameters was found to be in the area of higher DO concentration and lower stirring speed, though a wide range of process parameters met VCD specifications (Figure 3, green area).

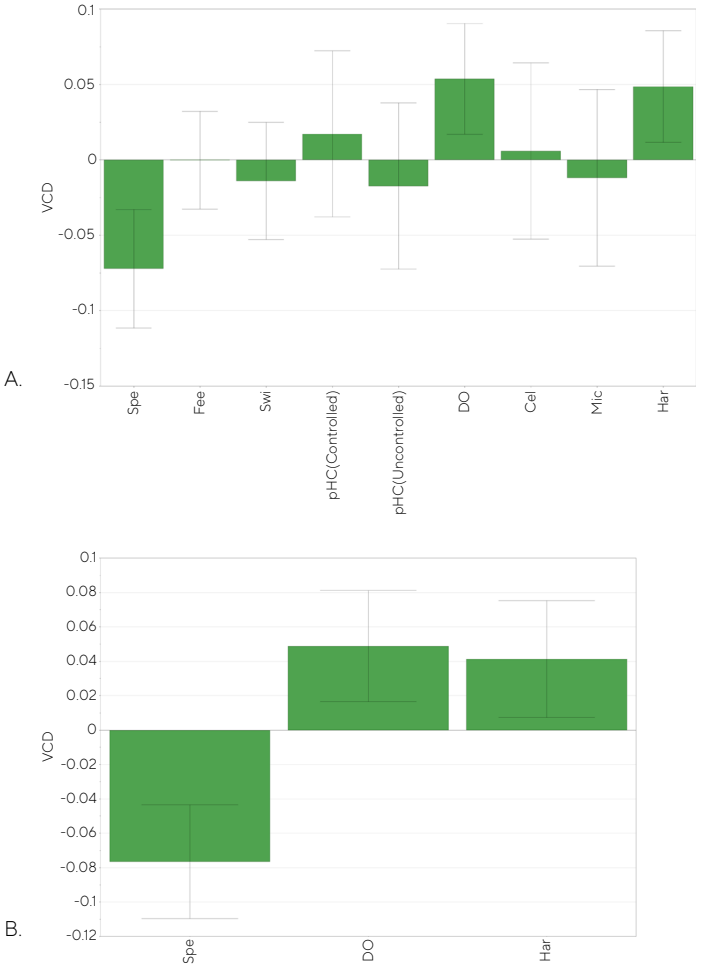

Figure 2: Stirring Speed, DO, and Culture Duration Are Factors With Statistically Significant Impact on VCD, Displayed via Coefficient Plots. *(A) Original plot with all factors. (B) Using the Auto Fit function of MODDE® the plot was optimized by removing factors that are not statistically significant.*

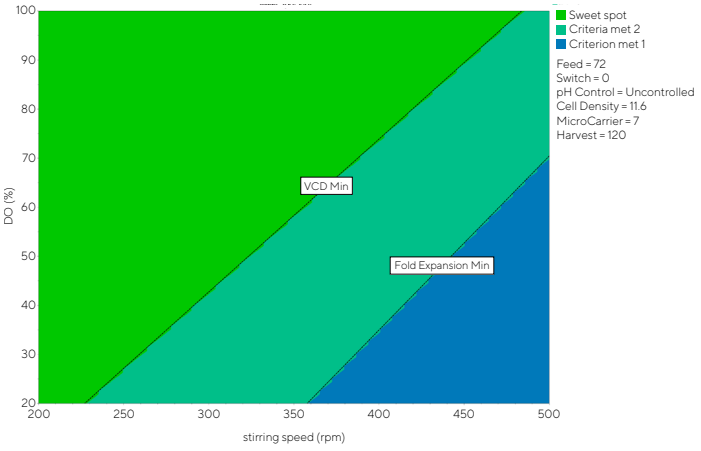

Figure 3: Optimal Process Range Identified to Achieve a Minimum VCD > 0.3 X 10⁶ Cells/mL, Fold Expansion > 10 and Viability > 95% Using Predictive Analysis in Modde® (Displayed via Sweet Spot Plot). The green area ("Sweet Spot") indicates the optimal process parameter space.

Verification of the predicted process parameter ranges with different donors

A well-known source of variability in cell therapy manufacturing is the cell starting material due to the inherent variability of living cells derived from different donors. As this study aimed to develop a robust process applicable to different donors and tissues, the process was verified with MSCs from three different donors. Cells derived from bone marrow (BM, two donors) and umbilical cord (UC, one donor) were used.

While viability is often used as a measure of process outcome, in this process optimization, VCD was used instead. VCD is a key measure of process outcome, and unlike viability (which remains constant despite changing process parameters), it showed variability in the process optimization, making it a more sensitive readout of process outcome.

As illustrated by the growth curves in Figure 4, expansion in the Ambr® 250 Modular system is robust and works with cells from different donors including different tissue sources. Not unexpectedly, donor-to-donor variability was observed. BM Donor 1 showed slower growth than BM Donor 2. A culture- duration of five days yielded the highest abundance for all donor-derived cells, and UC-derived MSCs showed the greatest increase from Day 4 to Day 5.

Using the data analysis software SIMCA®, the predictive power of the process model built was evaluated for the expansion of MSCs from three different donors. Based on this model, the fit of the verification runs to the previous runs was evaluated. Verification runs were a good fit to the model (R^2= 0.8569), indicating that the model successfully predicts process outcome (VCD) for a number of donors (Figure 5).

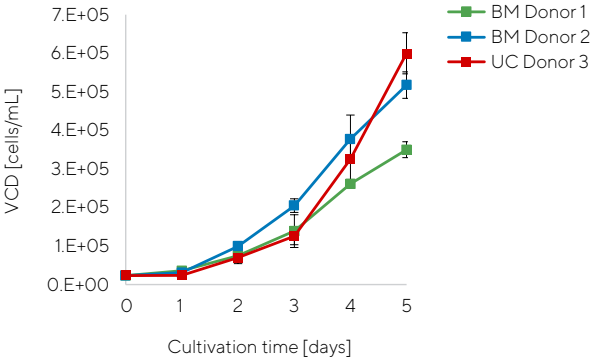

Figure 4: Successful MSC Expansion for 3 Different Donors and 2 Tissue Sources. Growth curves of MSC expansion using the Ambr® 250 Modular expansion process. Displayed are average values +/- standard deviation of n= 2 (BM Donor 1), n=3 (BM Donor 2) and n=4 (UC Donor 3) runs.

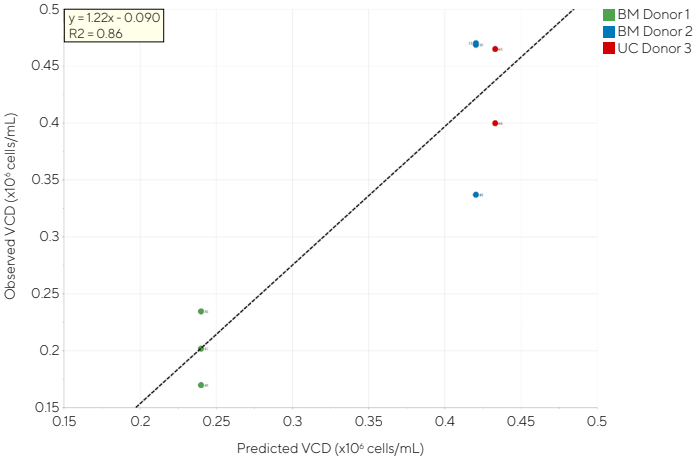

Figure 5: The Developed Process Model Was Used for the Successful Expansion of MSCs From Three Different Donors. Fit in SIMCA® software of the process data from verification runs to the process model using three different donors from bone marrow (BM Donor 1 and BM Donor 2, green and blue, respectively) and umbilical cord (UC Donor 3, red). The fit achieved R^2= 0.8569.

Confirmation of MSC characteristics after bioreactor expansion

To ensure that MSC characteristics were maintained after bioreactor expansion, several quality control assays were performed using MSCs harvested from bioreactor cultures and static, 2D expanded cells as a reference. Assays included the evaluation of minimal MSC criteria (such as surface marker expression and trilineage differentiation potential) as well as functional assays (such as IDO activity and cytokine secretion assays).[iii]

Surface marker expression of the seven analyzed markers was comparable between all three donors (Figure 6A). Furthermore, marker expression was also comparable to the reference 2D control cells. In addition to surface marker expression analysis, trilineage differentiation analysis of MSCs into osteoblasts, chondroblasts and adipocytes was performed. Representative images show that bioreactor-expanded MSCs differentiated into all three lineages, comparable to the reference 2D static culture control cells (Figure 6B, Donor 2).

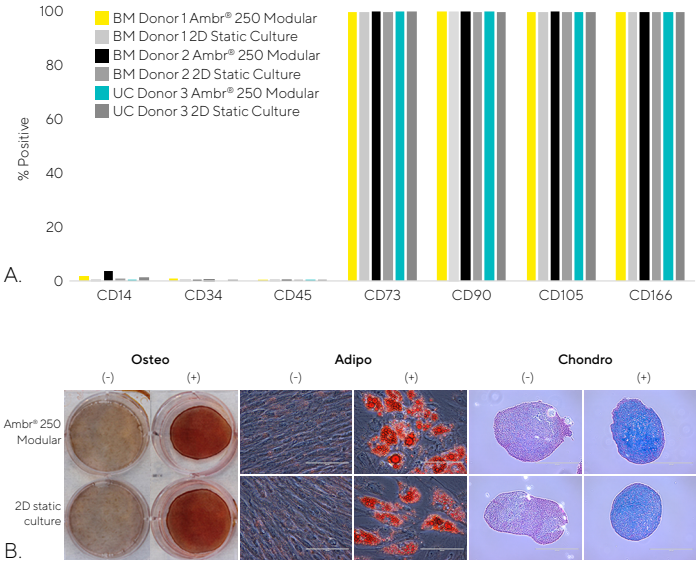

Figure 6: Quality Attribute Assessment of MSCs Expanded in Ambr® 250 Bioreactors. *(A) Phenotypic analysis of MSC surface marker expression of MSCs from three different donors. (B) Trilineage differentiation potential of MSCs expanded in Ambr® 250 bioreactor vessels (top row) and 2D static control cultures (bottom row). Representative results shown for BM Donor 2 cells. Scale bar 100 µm Adipo, 400 µm Chrondro.*

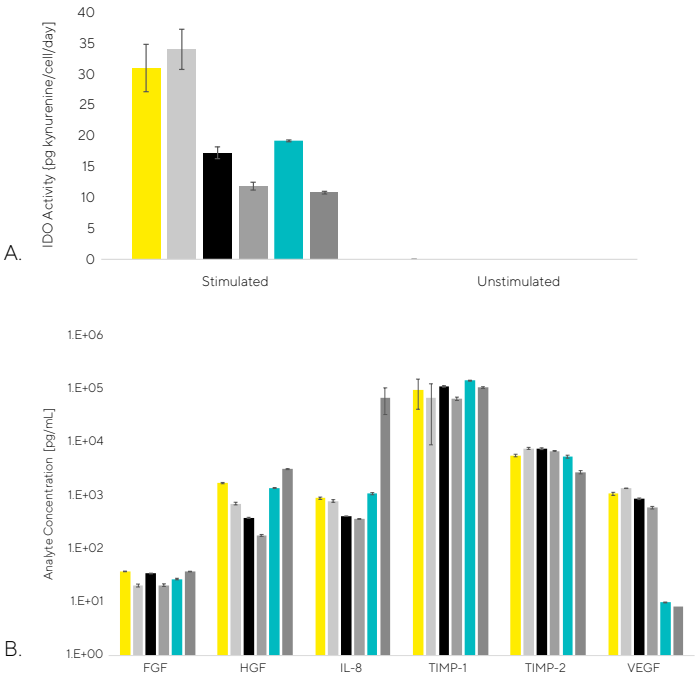

Figure 7: Functional Analysis of MSCs From Three Different Donors Expanded in Ambr® 250 Bioreactors Compared to 2D Static Culture Control. *(A) Immunomodulatory potential induced by IFN-γ stimulation. (B) Angiogenic cytokine secretion.*

Furthermore, functional assays analyzing IDO activity and angiogenic cytokine secretion were performed (Figure 7). This study did not detect an impact of process parameters on functional quality attributes. However, in comparison to 2D static cultures, MSCs expanded in Ambr® 250 bioreactors showed higher IDO activity for two of the donors tested, whereas one donor (BM Donor 1) showed comparable IDO secretion (Figure 7A). This may indicate a donor-dependent, higher immunosuppressive function of bioreactor-expanded cells compared to standard 2D static culture. This finding requires further experimental confirmation.

Angiogenic cytokine secretion was in general comparable between bioreactor- and 2D static culture control cells (Figure 7B). Differential expression of IL-8 was only observed in the UC-derived MSC cultures. This observation requires further experimental evaluation to confirm whether there is an effect of the different culture methods on IL-8 secretion for UC-derived MSCs.

Correlation of CPPs and CQAs for process insights

Undertanding how CPPs can influence CQAs of the final product is a critical aspect in biopharmaceutical manufacturing. In particular, this is also an important aspect for cell-based processes, such as MSC expansion, because the cell-based product is even more complex than traditional biologics, such as large-molecule therapeutics, including proteins and peptides.

In this study, not unexpectedly, no significant influence of process parameters on the analyzed CQAs, such as surface marker expression, was observed. As the MVDA SIMCA® analysis for CD166 indicates, the observed variation in marker expression was within a narrow range (Figure 8). For CD166, expression ranged from 99.9% to 100.0%, a negligible variation in range, especially when considering flow cytometry assay variability. However, it is expected that with further research into critical surface markers or other CQAs as well as an adapted choice of CQAs, the impact of CPPs on MSC characteristics will be measurable. The loss of MSC stemness due to shear stress has previously been described.[iv] Some examples of candidate CQAs may be apoptotic markers (Caspase 3/7 activity, Annexin V affinity, membrane integrity or mitochondrial depolarization) or lineage marker gene expression (alkaline phosphatase, runt-related transcription factor 2 (Runx2) or aggrecan).

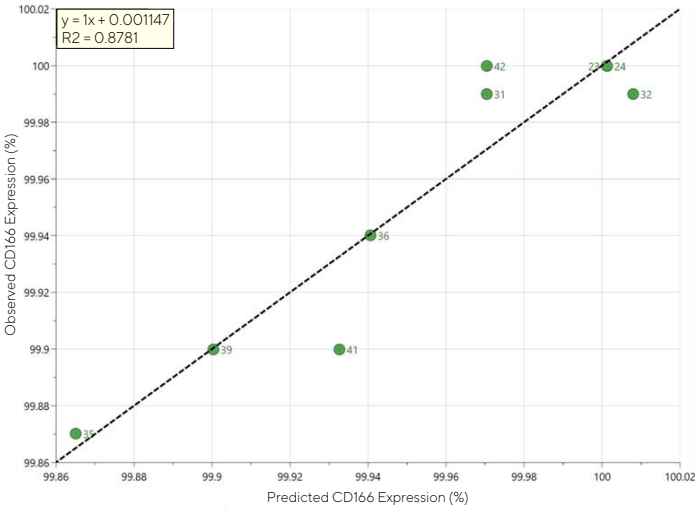

Figure 8: The Impact of Process Parameters on the Expression of the Subset of Surface Marker Was Limited. The range of surface marker CD166 expression variation was narrow (99.86 to 100.02), as expected. Observed values on y-axis, predicted values on x-axis. Analysis was carried out using SIMCA® MVDA software.

Discussion

Identifying CPPs and gaining a thorough understanding of their influence on process outcome is a critical step for establishing a robust and scalable MSC expansion process for therapeutic development. Furthermore, identification of the optimal process design space is needed to define process ranges and to consider the risk of not meeting quality specifications if the process operates outside of defined process ranges.

Studies presented demonstrate that the Sartorius MSC Optimization and Characterization Solution enables a scalable, semi-automated, DOE-guided approach to identifying CPPs and establishing optimal expansion parameter ranges at the 250 mL volume for rapid, cost-effective development of a scalable, robust production process. The Ambr® 250 Modular bioreactor system, in combination with MODDE® DOE software, SIMCA® MVDA software and SoloHill® Microcarriers, enabled rapid identification of the optimal design space to optimize MSC suspension culture performance.

Process optimization studies were accelerated through the use of the multiparallel Ambr® 250 Modular bioreactor system. Advanced data analysis using MODDE® identified DO, stirring speed, and the duration of the culture as CPPs, while other parameters, such as cell and microcarrier density, control of pH, time of feeding or switching the stirring direction, had negligible effect on VCD, fold expansion and viability. The following SIMCA® MVDA analysis of process characterization data yielded a model that successfully predicted VCD process outcome for the expansion of MSCs derived from three different donors and two tissue types. The addition to the model of the MSC phenotyping data, albeit limited, indicated the potential to

further develop this approach to evaluate the impact of CPPs on CQAs. Developing process knowledge, including identification of CPPs and their impact on CQAs, not only enables the formulation of control strategy for manufacturing, but also demonstrates QbD and supports regulatory submissions.

Future studies involving the measurement of more specific MSC phenotypic markers will inform an even more powerful process understanding. This process knowledge will be critical for effective scale-up. As the development progresses to clinically relevant volumes, this model can serve as a foundation for control strategy development.

Conclusion

MSCs represent a promising modality for a wide range of clinical applications in regenerative medicine. MSCs can be isolated from several sources including bone marrow and umbilical cord and differentiate into a variety of cell types with pleiotropic effects. The ability to produce sufficient quantities of MSCs with the requisite CQAs is essential for continued advancement of the field and expansion of the application of these powerful cells in new therapeutic areas. The Sartorius MSC Optimization and Characterization Solution is an effective tool for developing a robust, scalable and well-characterized MSC expansion process.

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