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Scalable MSC Suspension-Based Process Adaptation and Optimization in Ambr® 15 Cell Culture Microbioreactors Using DOE

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Abstract

Mesenchymal stem cells (MSCs) are able to self-renew and differentiate into a wide variety of tissues. These multipotent cells are being leveraged against a wide range of diseases, including autoimmune diseases, graft-versus-host disease, and acute myocardial infarction. In order to generate sufficient quantities of cells for clinical applications, MSCs must be transitioned from static cultures to scalable suspension cultures grown in bioreactors. This transfer has traditionally represented a bottleneck during process development. The Sartorius MSC Exploration and Characterization Solution enables rapid MSC process development thus accelerating time-to-market. The experiments presented here demonstrate the critical role of Ambr® 15 Cell Culture system and MODDE® Design of Experiments (DOE) software, as part of the Sartorius MSC Exploration and Characterization Solution, in the development of process knowledge. The automated, controlled, multi-parallel experimental setup and DOE analysis results in rapid identification and optimization of critical process parameters (CPPs).

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Introduction

Since their first clinical application in the 1990s (Lazarus, 1995). mesenchymal stem cells (MSCs) have become a compelling therapeutic modality due to their unique cell biology and wide potential for clinical application, ranging from autoimmune diseases, graft-versus-host disease or acute myocardial infarction to tissue engineering (Pittenger M.D. 2019). Depending on the clinical setting, 1-2 × 10^6 cells/kg are typically used, often with multiple doses being administered over the course of treatment (Lin, 2011).

The clinical need for large quantities of MSCs is hampered by the low number of MSCs found in adult tissue (only 0.001–0.01% in bone marrow (Pittenger M. F. 1999). Therefore, extensive ex vivo expansion is required to reach clinically relevant doses. Various platforms have been evaluated for MSC expansion. Static expansion systems, such as T-flasks or cell factories, are traditionally used for MSC culture. However, these platforms are limited with regards to scalability, process monitoring and control and generally involve many labor-intensive, open-handling steps. To overcome these hurdles and enable transition to a suspension process, microcarrier (MC)-based expansion in stirred bioreactors was investigated to address the need for a robust, automated, scalable and cost-efficient expansion system.

To enable transition to suspension culture, the Sartorius MSC Exploration and Characterization Solution offers a miniaturized, high throughput way to identify and correlate critical process parameters (CPPs) and critical quality attributes (CQAs). By establishing process control via the Ambr® 15 Cell Culture system, the Solution offers automation and high throughput experimentation for the identification of successful culture conditions and reagents, including media and MC.

Selection of suitable MC and medium is an essential step for culture of MSCs in bioreactors. To enable robust, GMP-compliant manufacturing identification of the optimal MC-medium combination early in process development is critical in order to accelerate time-to-market and lower costs, as changes during later stages of clinical development will likely be considered a significant amendment to the manufacturing process (see Chapter 5.23 of the EU GMP Guidelines Vol. 4), requiring extensive comparability testing.

This case study highlights a systematic, cost-efficient approach to screen suitable combinations of MC and media as a first step in the development of a scalable, suspension-based MSC process. We also performed a quality analysis of the expanded MSCs to confirm their MSC phenotype, differentiation potential and cell viability. To accomplish this, we used the Sartorius MSC Exploration and Characterization Solution, including the Ambr® 15 Cell Culture system, DOE Software MODDE®, SoloHill® MC, and the iQue® Screener PLUS advanced high throughput flow cytometer.

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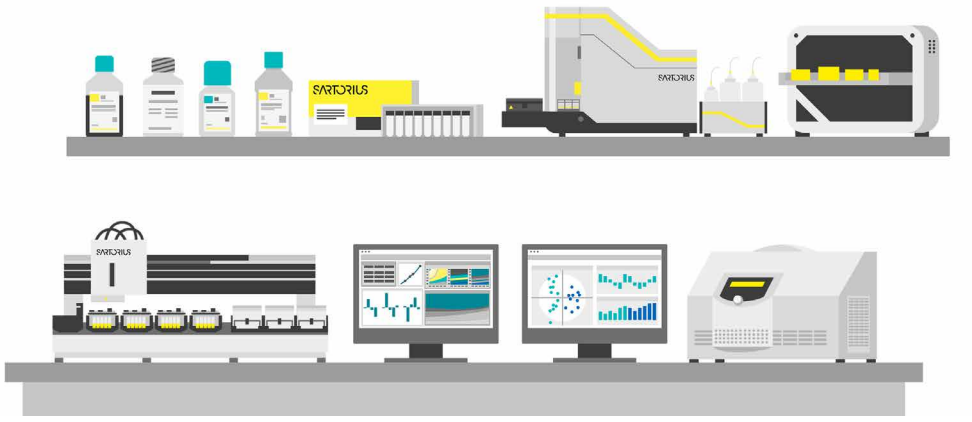


From left to right:
1 Sartorius MSC Exploration and Characterization Solution
2 Ambr® 15 Cell Culture
3 iQue® 3 (the latest from the iQue Screener PLUS line of instruments)
4 MODDE® DOE

2

3

4

Materials

Materials Used:

1. Ambr® 15 Cell Culture system, 24-way
2. 15 mL spargeless microbioreactor vessels
3. MC (SoloHill® Plastic (25 cm^2/mL), Plastic Plus (25 cm^2/mL), Star- plus (25 cm^2/mL), Hillex® II (25 cm^2/mL), MC-1 (25 cm^2/mL), MC-2 (25 cm^2/mL))
4. MSC media (Medium-1, Medium-2, Medium-3, Medium-4, Medium-5, Medium-6)
5. iQue® Screener PLUS

Methods

Static MSC Culture

Cryopreserved human bone marrow MSC (Lonza) were thawed and seeded in T-flasks at a density of 2500 c/cm^2 in a humidified 5% CO_2 incubator at 37°C using six different media (Medium-1 to Medium-6). A half medium exchange was performed every 3-4 days. Upon reaching 80% confluency, cells were harvested by trypsinization, counted, re-seeded in T-flasks and expanded for two additional passages. These cells were used as the seed train for the bioreactor experiments.

MC-Based MSC Culture in the Ambr® 15 Cell Culture System

MC were prepared according to the supplier information. MC were added to the bioreactors at a concentration of 25 cm^2/mL and were incubated overnight at 37°C, pH 7.2 and 40% DO. MSCs from static pre-cultures were used for inoculation at 1200 c/cm^2 in 10 mL media. Using an intermitted stirring regime of 300 rpm for 2 min, followed by no stirring for 30 min, cells were allowed to attach to the MC for 6h. Afterwards, constant stirring at 400 rpm was used and cultures were expanded for 7 days, with daily sampling to assess cell count and viability. 8 mL of media exchange were performed on day 3 and day 6. From day 4 on, we used modified 1 mL tips with wider bore opening for sampling due to the increase in MC-cell aggregate size for homogenous volume.

Cell Counting and Viability

Cell counting and viability was determined using a Neubauer counting chamber and the Trypan Blue exclusion method. For analysis of cells cultured on T-flasks, cells were detached enzymatically by incubation at 37°C for 3 min using 0.1% TrypLE (Sigma). For analysis of cells cultured on MC, MSCs were detached from MC using 0.1% TrypLE at a stirring speed of 700-800 rpm for 6 min. Cells were then stained with trypan blue and counted immediately.

Attachment Efficiency

To analyze the attachment of MSC on MC fluorescent cell staining using calcein (live cell staining) and propidium iodide (dead cell staining) was performed on day 3, 5, and 7 after inoculation. For this, representative samples were drawn from the Ambr® 15 microbioreactors. Samples were stained with 50 µg/mL propidium iodide and 10 mM calcein. To calculate the number of cells attached, stirring was stopped and MC allowed to settle before sampling the cell-containing supernatant media for counting. Cell counting was performed using a Neubauer counting chamber. To calculate the cells attached on MC, the obtained cell count is reduced from the initial seeding density.

Flow Cytometry Acquisition and Analysis

Cells were detached from MC and stained using the following antibodies and their respecitve isotype controls: anti-CD14, anti-CD19, anti-CD 34, anti-CD 45, anti-CD73, CD 90, anti-CD105, anti-HLA DR.

For compensation BDTM CompBeads Anti-Mouse Ig, κ and Negative Control beads were used according to the manufacturer's instructions.

Data was obtained using the iQue® Screener PLUS flow cytometer and data analysis was performed using the Forecyt® software. A minimum of 15,000 cell events were recorded for each sample.

Adipogenic and Osteogenic Differentiation of MSCs

Cells grown in the Ambr® 15 Cell Culture system were harvested enzymatically and seeded at 1000c/cm^2 in 24 well plates. Cells were kept overnight in standard culture medium and were then exposed to adipogenic (Gibco, cat no: A10070-01) or osteogenic (Gibco cat no: A10072-01) differentiation medium. As negative controls, cells were kept in standard culture medium. Medium was exchanged every 3-4 days. After 21 days, cells were fixed in 4% formaldehyde. To analyze adipogenic differentiation, staining with 0.3% Red Oil O (cat no: 1320-06-5) was performed. For osteogenic analysis, cells were stained with 2% Alizarin Red S (cat no: A5533). After washing, samples were analyzed microscopically.

MODDE®

The DOE software MODDE® was used to design and analyze the experiments and data generated out of the Ambr® 15 Cell Culture system.

Two factors were defined;
1. MC
2. Media (med)

As response criteria; attachment efficiency and cell count were chosen. A full factorial experimental design was used.

Results

As a first step in developing a MC-based MSC expansion process, we screened six commercially available MC and six MSC media for their performance in an MSC suspension culture. Criteria for selection of MC were based on choosing solid, non-macroporous MC consisting of animal-origin free components. For culture media, we focused on serum- and xeno-free media. These media were used according to the manufacturers' recommendations and therefore, additional supplements and/or surface coating were added if required. For comparison purposes, we also included alpha-MEM medium supplemented with 10% FCS (Medium-2) as a widely used, standard R&D MSC culture medium.

For the MC-based MSC suspension culture in the Ambr® 15 bioreactors, we used pre-cultured cells derived from static cultures using the respective medium. Bioreactor cultures were performed as outlined above. As read-outs for the experimental design (see below) we defined the responses "attachment efficiency" (after 24h) and "cell count on day 7".

For an unbiased design of the bioreactor experiments, we used the DOE software MODDE®. We chose to screen all combinations of the six media and MC (36 experiments). As we did not have previous data on our experimental error, we also included replicates of 12 combinations, resulting in a total of 48 experiments. The experimental design and corresponding worksheet were generated using the MODDE design wizard. The 48 experiments were performed using the Ambr® 15 Cell Culture system. Afterwards, the resulting data was analyzed using MODDE®.

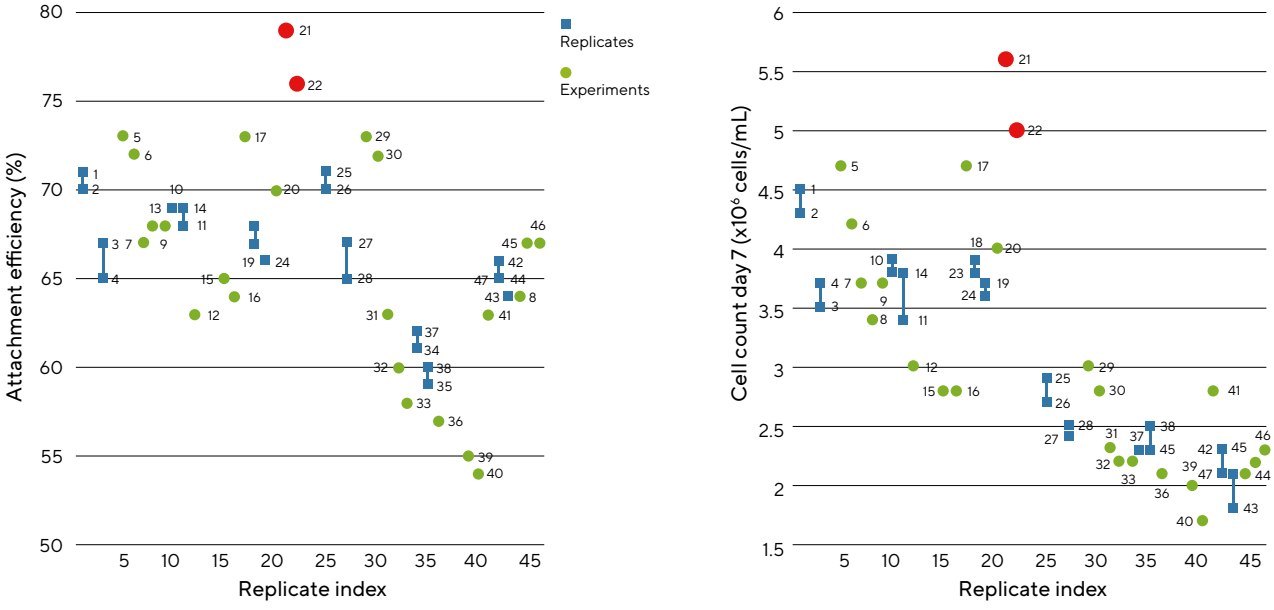
Data Analysis Using MODDE® Software

The DOE data analysis wizard in MODDE® software provides a guided step-by-step approach to verifying, visualizing, and analyzing the experimental data. We describe in the following two sections a tool for raw data evaluation (replicate plots) and a tool for interpretation of the data (coefficient plot).

1. Replicate Plot

Replicate plots display the measured response values for all experimental runs. They allow to visualize the variation in the responses for all experiments in a quick raw data evaluation and allow for easy graphical discrimination of single and replicate experiments. In our case study, 12 experiments were replicated (Figure 1, blue). For the two responses chosen in our case study – attachment efficiency and cell count – the variation in all replicates is smaller than the variation across all experiments, showing that replicate error is small, and a good model is likely to be obtained. Notably, two experiments (N21 and N22) showed the highest values for both responses with attachment efficiencies of 79% and 76% and cell counts of 5.6 x 10^6 cells/mL and 5.0 x 10^6 cells/mL, respectively. These experiments were performed using the same medium (Medium-3) and SoloHill® Plastic (N21) or Plastic Plus (N22) MC.

Figure 1



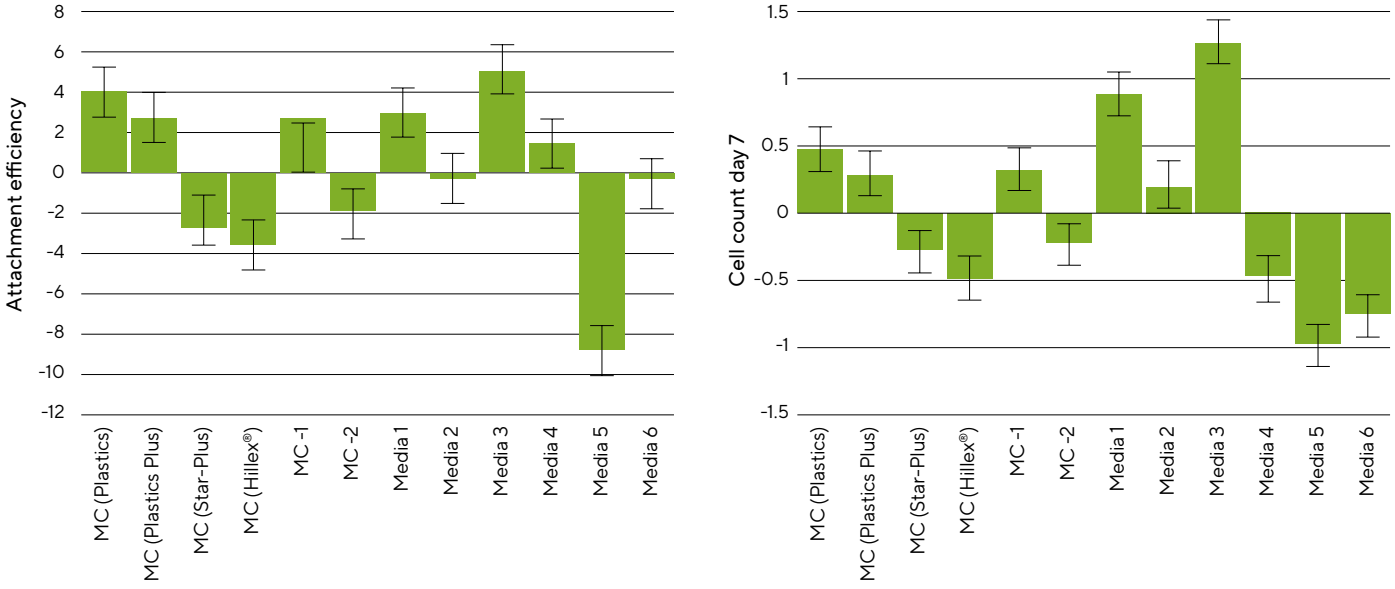
Note. Replicate plots of attachment efficiency (left) and viable cell count on day 7 (right). Experiments are numbered 1 – 48. Replicate experiments are shown in blue color, connected with a bar. Experiments which were not replicated are shown in green. Experiments 21 and 22 are marked in red for highlighting purposes. Experiment 21 – SoloHill® Plastic MC; Experiment 22- SoloHill® Plastic Plus MC; both experiments used Medium-3

2. Coefficient Plot

Coefficient plots provide a graphical illustration of the significance of each model term. Using coefficient plots, we evaluated whether each MC and medium had a significant positive or negative effect on attachment efficiency and cell count combination of MSC culture in the Ambr® 15 Cell Culture system (Figure 2).

SoloHill® Plastic, SoloHill® Plastic Plus and MC-1 MC and media 1, 3 and 4 supported the cell attachment on MC (Figure 2, left). As for attachment efficiency, SoloHill® Plastic, SoloHill® Plastic Plus and MC-1 MC as well as Medium-1 and Medium-3 had a positive effect on cell growth as shown by the cell count on day 7 (Figure 2, right).Medium-4, which had a positive effect on attachment efficiency, did not support cell growth as illustrated by its negative coefficient value for cell count. On the other hand, Medium-2, which had an insignificant effect on attachment efficiency, had a positive effect on cell count.

Figure 2



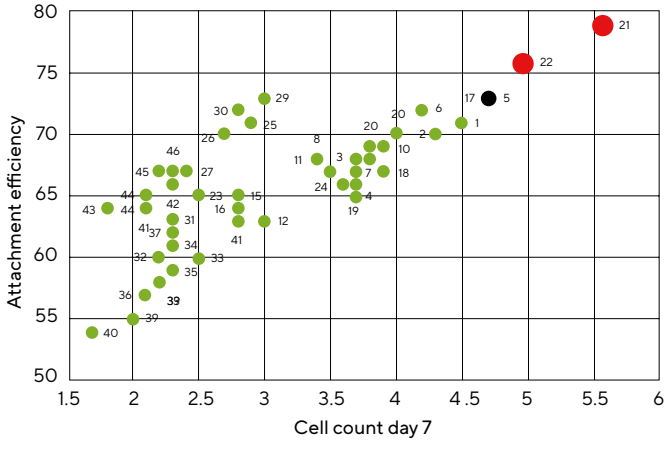
Note. Coefficient Plots for model interpretation. Displayed are the coefficients of each model term (in this case for each factor) and their confidence intervals for the responses attachment efficiency (left) and cell count on day 7 (right).

3. Summary of Data Analysis Using MODDE®

In conclusion, the results showed that a combination of SoloHill® Plastic or SoloHill® Plastic Plus MC with Medium -3 led to the highest attachment efficiency as well as the highest cell count in MSC suspension cultures. An attachment efficiency of 79% and cell concentration of 5.6×10^6 cells/mL was obtained using SoloHill® Plastic MC and Medium-3. For a graphical presentation of this conclusion, MODDE® provides a scatter plot tool (Figure 3) best combinations highlighted in red).

Furthermore, the scatter plot illustrates that good results could also be obtained using SoloHill® Plastic or SoloHill® Plastic Plus MC in combination with Medium-1 (Figure 3 combinations highlighted in black).

Figure 3



Note. Scatter Plot showing the correlation of cell count and attachment efficiency. This graphical tool allows a graphical illustration of the best MC – medium combinations. Experiments marked in red are the two MC – medium combinations showing the highest attachment efficiency and cell count. Experiment marked in black is the third best combination. Experiment 5: SoloHill® Plastic MC; Experiment 17: SoloHill® Plastic Plus MC; both experiments used Medium-1 | Experiment 21: SoloHill® Plastic MC ; Experiment 22: SoloHill® Plastic Plus MC; both experiments used Medium-3

Quality Analysis of Expanded MSC

To evaluate MSC quality after expansion in MC-based suspension culture, we analyzed cell viability (also monitored during expansion), cell phenotype and differentiation potential of MSCs harvested from bioreactor cultures using the different MC and media combinations described previously.

1. Cell Viability

Cell viability was monitored throughout MSC expansion in the bioreactors. Generally, we observed an increase of viability over culture time in the MC – media combinations that supported cell growth, which may be explained by an initial phase during which the MSCs derived from static cultures had to adapt to the suspension environment. With the best MC- medium combinations of Medium-3 and SoloHill® Plastic or SoloHill® Plastic Plus MC, a viability of 81% and 86% was observed on day 7, respectively.

2. Phenotypic Analysis of MSC from Bioreactor Cultures

To confirm that the basic MSC phenotype of cells is maintained in bioreactor suspension cultures, cell phenotype was assessed after 7 days of culture in the Ambr® 15 Cell Culture system. The results showed that the expanded cells were positive for CD90, CD105, and CD73 and negative for hematopoietic lineage and human leukocyte markers CD14, CD19, CD45, CD34 and HLA-DR (Table 1).

Table 1

Phenotypic Analysis of MSCs Expanded in Ambr® 15 Bioreactors.

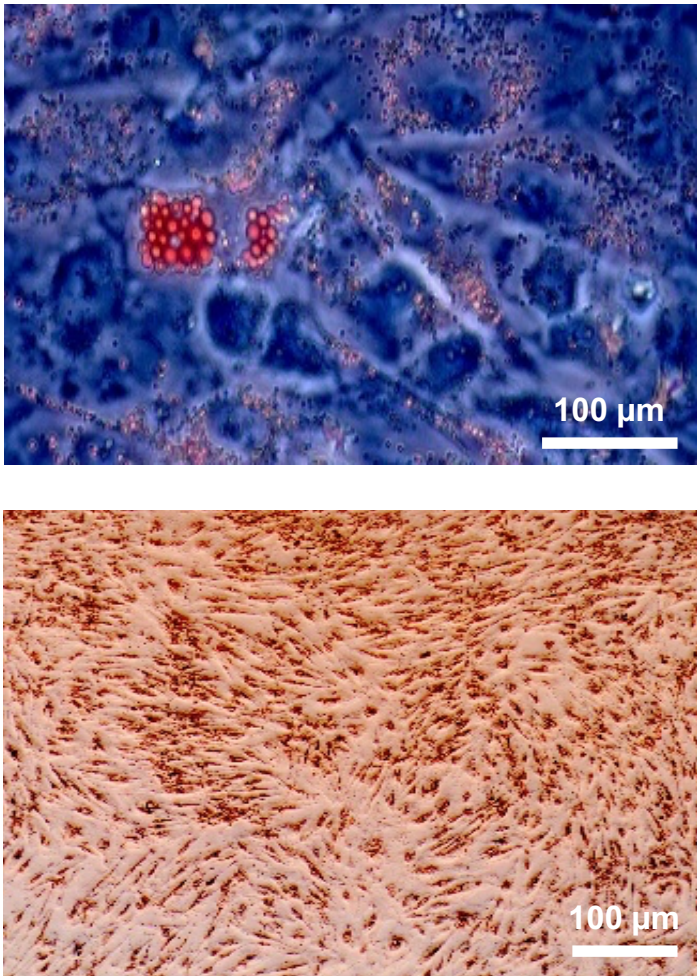
(+) ve markers	(+) ve - %	(-) ve markers	(-) ve - %
CD 73	99	CD 14	4%
CD 105	99	CD 19	4%
		CD 34	4%
		CD 45	4%
		HLA-DR	2%

3. Lineage Differentiation of MSC Analysis

To further evaluate the differentiation potential of MSCs after MC-based suspension culture in bioreactors, we analyzed the adipogenic and osteogenic differentiation potential of expanded cells. As depicted in Figure 4, MSCs maintained their ability to differentiate.

In summary, cells cultured in the Ambr® 15 Cell Culture system achieved good cell viabilities and maintained MSC characteristics.

Figure 4



Note. Adipogenic (top picture) and osteogenic (bottom picture) differentiation of MSCs after MC-based suspension culture in Ambr® 15 bioreactors. Exemplary staining of cells expanded SoloHill® Plastic MC and Medium-1. Presence of lipid vacuoles in adipogenic differentiated cells are confirmed via Oil red O staining and calcium deposits in osteogenic differentiated cells are confirmed via Alizarin red stain. Scale bar 100 µm.

Discussion | Conclusion

Process transfer of MSC expansion from traditional static culture to scalable, suspension culture in bioreactors is a common bottleneck in MSC process development. With this case study, we present Sartorius MSC Exploration and Characterization Solution for miniaturized, high throughput development of MC-based MSC culture in stirred bioreactors.

The Ambr® 15 Cell Culture bioreactor system in combination with MODDE® DOE software allows rapid screening of different MC and media combinations. For screening of these combinations, the systematic DOE approach allows for unbiased experimental design. Furthermore, the DOE software MODDE® provides an easy to-use, user-friendly interface for experimental design as well as statistical data analysis and visualization.

Due to the small scale (10-15 mL) culture volumes and multiparallel experiments run on the Ambr® 15 Cell Culture platform, offline analytical assays for cell characterization and function require low assay volumes and fast data acquisition and analysis. To address this volume and speed demand, we used the iQue® advanced flow cytometry platform, allowing for the fastest sample acquisition in the industry using small sample volumes provided in 96 or 384 well plates. In this case study, we showed how the iQue® platform can be used to assess the MSC phenotype criteria.

The Sartorius MSC Exploration and Characterization solution addresses customer needs by providing tools for rapid, robust MSC process development, thereby decreasing development times and COGs. In particular, the Ambr® 15 Cell Culture platform allows cultures to be run in parallel (up to 48 at a time) and at low working volumes (10-15 mL), all in an automated workstation. Moreover, it provides a stirred bioreactor environment and integrated PAT, such as pH and DO sensors, to investigate and define process control early on in the development process. This facilitates transfer and scale-up in later development stages. Furthermore, the combination of the Ambr® 15 Cell Culture system and DOE allows the identification and optimization of critical process parameters, leading to increased process understanding.

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