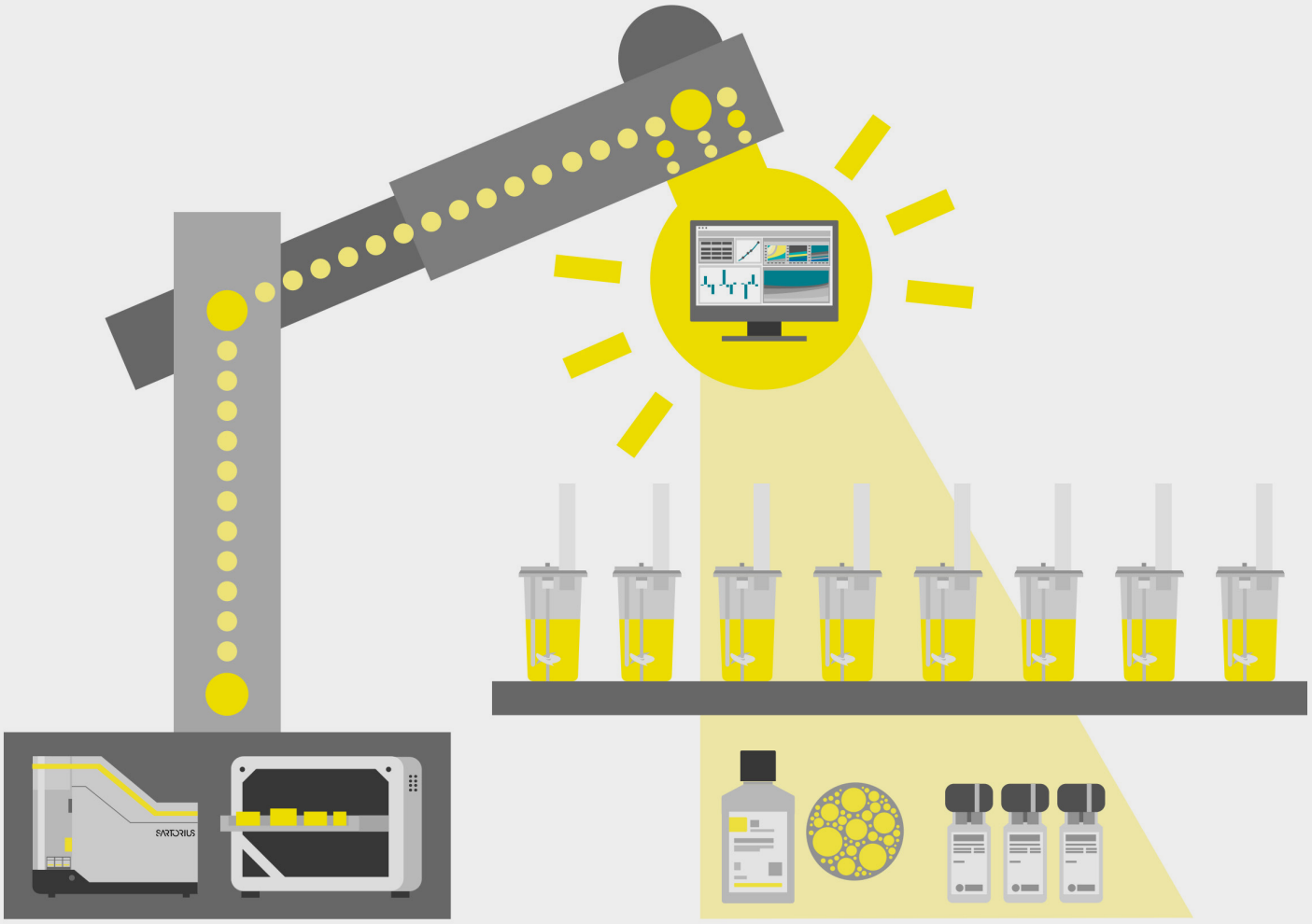


Resource Roadmap to Screening Success

Cell-based Therapy Edition

Simplifying Progress

SARTORIUS

Foreword

With several recent landmark approvals, cell-based therapies have potential to treat a range of diseases. While they've shown success in the clinic, initial therapies still face several challenges, including high production costs and varying response rates to the treatments – leading to a new wave of effort to improve their production.

Compared to traditional biologics, cell-based therapy production comes with unique challenges. In traditional biologics, scientists often use cell culture to isolate a product like a small molecule or protein. In cell-based therapies, the cultured cells are not a means to an end but the product itself. The final drug product quality directly relates to the health of the cells that comprise it, which is linked to the culture conditions in which they were grown.

For this reason, in addition to the usual quality control tests scientists perform on a final product, they also need to screen and validate each new condition – changes in raw materials or process parameters, for example – that enters the production process. However, at the earliest stages of development traditional cell culture approaches rely on methods and technologies (like flasks) that limit design space and exploration. As a result, the limited scope of initial findings using traditional methods can introduce risk to later stages of development, costing significant time and money.

Sartorius Screening Ensemble offers flexibility and speed to help scientists better understand and optimize process parameters and bring the next generation of cellbased therapies to market. The ensemble includes Ambr® 15 high-throughput semi-automated bioreactors guided by MODDE® intuitive DOE software, together with Nutri-T and Nutristem high-performance media, CellGenix reagents, and Solohill® microcarriers to probe the design space and fine-tune cell production.

To characterize the quality of the cell product, the iQue® 3 high-throughput flow cytometer and Incucyte® SX5 livecell imager combine to deliver detailed cell characteristics like biomarker phenotype, tri-lineage differentiation capacity, viability, exhaustion, and killing efficacy. Finally, users can assess their success using SIMCA® multivariate data analysis software. SIMCA® software combines process and cell characterization data to identify the optimal conditions and raw materials that result in the highest quality cell product.

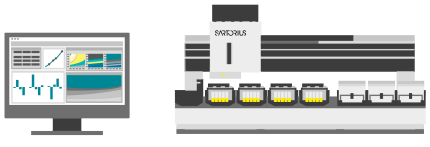
The application notes collected in this eBook demonstrate how you can use the Screening Ensemble to generate process insights. Applying these insights can help you set the stage for process optimization without compromising cell quality.

Sartorius Screening in 5 Steps

Click to be directed to one of the applications notes below

Optimization of T-Cell Expansion Using the Sartorius T-Cell Exploration and Characterization Solution

1. Design Multi-Factor Experiment



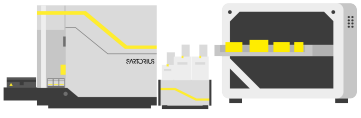
Expansion of specific lymphocyte populations with 4Cell® Nutri-T xeno-free, serum-free medium

Enhancing Cell Growth with Cytokines

2. Validate High Performance Media and Reagents

Phenotypic and Functional Characterization of CAR-T Cells with Advanced Flow Cytometry and Live-Cell Analysis

3. Characterize Cells of Interest



4. Analyze Data for Actionable Insights

Building Screening Capabilities to Accelerate Your Process Development

5. Leverage Screening Platform for Continuous Iteration

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

SARTORIUS

Application Note

October 7, 2021

Keywords or phrases:
CAR-T, immune cell therapy, process development, optimization, DOE

Optimization of T-Cell Expansion Using the Sartorius T-Cell Exploration and Characterization Solution

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Abstract

A single dose of immune cell therapy can include billions of cells. However, the unique complexities of cell-based therapies present major challenges when scaling to meet clinical demand. Process knowledge is paramount to overcome these challenges, yet the process of obtaining it is usually time- and cost-intensive due to extensive open, manual processes, expensive materials and consumables, and limited fit-for-purpose equipment and data-driven insights. Thus, there is a great need for automated ways to efficiently screen and identify critical process parameters (CPPs) and critical quality attributes (CQAs) at early stages of process development. To address this need, the Sartorius T-Cell Exploration and Characterization Solution enables monitoring and control of culture parameters during multi-parallel scale-down expansion and high-throughput analysis of cell product to significantly accelerate process optimization and build process understanding. The experiments described in this application note highlight the use of this Solution to rapidly develop a T-cell expansion protocol that maximizes cell yield and viability. Automated, controlled expansion in the multi-parallel Ambr® 15 Cell Culture system together with the MODDE® Design of Experiments (DOE) software enables thorough screening and iterative optimization of CPPs and sets the stage for seamless, successful scale-up.

Find out more: www.sartorius.com/en/applications/cell-and-gene-therapy/car-t/car-t-pd

Introduction

Gene-modified cell therapy is a rapidly growing industry with hundreds of clinical trials underway. As these trials progress into later phases of development, the need for robust manufacturing processes becomes increasingly important as cell and process complexities grow with scale.[1] Early process optimization sets the stage for successful clinical and commercial manufacturing which plays a foundational role in ensuring cost effective and efficient production of consistent, high-quality CAR-T cell therapies. Optimizing a complex process, however, requires systematic evaluation of many parameters and, as such, can be time-consuming and resource intensive. In fact, optimization protocols that use traditional workflows can take 24 months or longer to complete and cost close to $700,000 (see Discussion for detailed breakdown). While these numbers are prohibitive for many, insufficient optimization, inadequate process understanding, and inefficient processes can translate to higher production costs and lower patient accessibility down the line. Thus, there is a strong need for more cost-effective ways to rapidly screen CPPs and CQAs, set the design space, and build process knowledge during early stages of development.

To ensure seamless future scale-up of immunotherapies, the Sartorius T-Cell Exploration and Characterization Solution is a semi-automated, high-throughput, scale-down model that enables rapid identification and optimization of CPPs and CQAs (Figure 1). This application note describes several successive optimization studies to optimize T-cell fold expansion and viability using the Sartorius T-Cell Exploration and Characterization Solution. As a first step, media screening was performed to identify the optimal media formulation for maximal cell growth and viability. Next, screening of process parameters enabled identification of CPPs and their optimal ranges were determined in subsequent optimization runs. Finally, optimized parameters were transfered to an expansion process in larger volumes to demonstrate the ease of scale-up using the Sartorius solutions for various stages of process development. The automated handling and parallel processing capabilities of the Solution enable such optimization studies to be completed in one fifth of the time it would take when using traditional T-flask based workflows. Taken together with the reduced cost of labor, this provides a strong rationale for using the Sartorius T-Cell Exploration and Characterization Solution to ultimately bring immune cell therapies to patients faster.

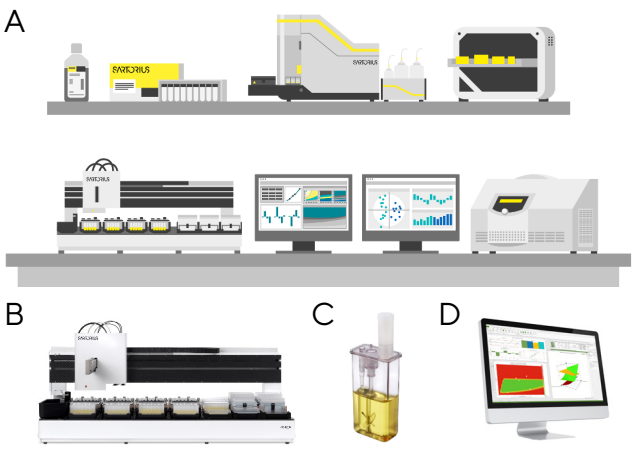

Figure 1. *The Sartorius T-Cell Exploration and Characterization Solution for Early-Stage Process Development.* (A) Schematic of the solution with all components. (B) Ambr® 15 Cell Culture System. (C) Ambr® 15 single-use microbioreactor. (D) MODDE® DOE software.

Materials & Methods

Materials used:

- Ambr® 15 Cell Culture system, 24-way
- Ambr® 15 Cell Culture microbioreactors, no sparge
- Ambr® 250 Modular, 2-way
- Ambr® 250 vessels with dual pitch-blade impellers and baffles
- Ambr® 250 unbaffled vessel with single 'elephant ear' impeller
- MODDE® DOE Software

Optimization of Cell Expansion in Ambr® 15 and MODDE® DOE Software

The Ambr® 15 Cell Culture system with 24 vessel configuration was used for the Media Screening and DOE Optimization experiments together with sparge-less Ambr® 15 single-use microbioreactors (Figure 1B, C). In these bioreactors, gasses flow into the headspace rather than sparge directly into the culture liquid. Each bioreactor has a working volume of 10 - 15 mL with pH and dissolved oxygen (DO) sensors for individual monitoring and control. MODDE® Design of Experiments (DOE) software was used in conjunction with the Ambr® 15 Cell Culture system to accelerate process optimization (Figure 1D). MODDE® DOE enabled the screening of many factors simultaneously to rapidly identify CPPs, a departure from traditional experiments in which one variable at a time is typically evaluated. Data generated by MODDE® was then used to build mathematical relations between factors and responses and de-

velop process knowledge. The MODDE® DOE software provided automatic design recommendations and guided workflows to help analyze the present experiments.

Media Screening

Four commercially available media formulations were screened under stirred-tank conditions, using the Ambr® 15 Cell Culture system (Table 1). Medium 1 and Medium 4 were serum-free formulations, while Medium 2 and Medium 3 were supplemented with 5% human serum (HS). The latter two were evaluated with and without HS in the screening experiments to determine the impact of the added HS. Stirring speed was set to 300 RPM, the DO set point was 60%, and pH was 7.2. A seeding density of 5.0x10^5 cell/mL was used and the working volume was 10 mL. Media were supplemented with 400 IU/mL IL-2. For this and subsequent experiments, precultured T-cells were used and activated with T Cell TransActTM (Miltenyi Biotech). Cells were derived from cryopreserved, healthy human peripheral blood mononuclear cells (PBMCs). All experiments in the Ambr® 15 Cell Culture system were conducted in triplicates. A static control consisted of well plates and T-flasks cultured with the same medium and feeding strategy in a 5% CO incubator.

Parameters	Set Point
Stirring Speed [rpm]	300
DO [%]	60, headspace gassing
pH	7.2
Seeding Density [c/mL]	5 x 10^5 (in 10 mL)
Feed	Batch
IL-2 [U/mL]	400
Cells	Activated T cells from cryopreserved hPBMC

Table 1. *Ambr® 15 Cell Expansion Parameters For Media Screening.*

DOE Optimization Setup for Identifying CPPs

DOE was used to optimize T-cell expansion; six factors were evaluated to determine their impact on cell growth, expressed as fold expansion and viability (Table 2). Three process parameters were held constant: temperature (37 °C), culture volume (10 mL per bioreactor), and use of a batch culture process. As with the media screening step, precultured T-cells obtained from cryopreserved PBMCs were used.

Parameters	1st Optimization
Stirring [rpm]	300; 400
DO [%]	50; 70; 90
pH	7.0; 7.3; 7.5
Medium	M1; M4
Seeding density [c/mL]	5 x 10^5, 1 x 10^6
IL-2 [U/mL]	50; 125; 200

Table 2. *Six Parameters were Evaluated in the DOE for their Impact on T-Cell Expansion.*

To set up the experimental design, the MODDE® software design wizard was used. Input factors were defined and categorized according to whether they were quantitative, qualitative, or whether they would be analyzed on multiple levels. Fold expansion and viability were then designated as "responses," that is, measurements that served as readouts for optimization. Finally, the model and design were selected; given the study objectives, a linear reduced combinatorial design was utilized. Twenty experiments plus two replicates of four conditions resulted in a total of 24 experiments.

Cell Expansion in Ambr® 250 Modular

Parameters for scaled up cell expansion in the Ambr® 250 Modular were set based on prior optimization studies (Table 3). Baffled and unbaffled Ambr® 250 vessels were both tested in this experiment to optimize cell growth. All variables were held constant except for stirring speed, which varied for baffled and unbaffled bioreactors and was determined based on using the same volumetric power input for both bioreactor types at a lower and higher stirring speed level. A seeding density of 5.0x10^5 cell/mL was used and the working volume was 166 mL. Precultured and activated T-cells were used and cells were derived from cryopreserved, healthy human PBMCs. Static controls consisted of T-flasks kept in a humidified, 5% CO incubator.

Parameters	Ambr® 250 Modular
DO [%]	50, headspace and sparge gassing
IL-2 [U/mL]	200
pH	7.2
Medium	M1
Feed	Batch
Seeding density [c/mL]	5 x 10^5
Vessel type	Baffled | unbaffled
Stirring speed [rpm]	220; 265 | 150; 180

Table 3. *Ambr® 250 Cell Expansion Parameters*

Results

Top media candidate for optimal cell expansion identified via rapid media screening

Media performance was evaluated over the course of nine days in stirred-tank (Ambr® 15 Cell Culture system) and static (flask) conditions (Figure 2). For all the media formulations, fold expansion followed a similar pattern – an exponential growth phase, which then peaked and plateaued, and eventually decreased (Figure 2A, B). This observed pattern likely occurred due to nutrient limitation as the cultures were not fed after Day 2. Cell viability was also measured over culture duration and was consistent with the observed cell growth; as expected, viability dropped when cultures were no longer fed (Figure 2C, D). Some of the media formulations performed better than others. For cells grown in the Ambr® 15 Cell Culture system, Medium 1 and Medium 4 enabled a 20-fold expansion and a high viability for a longer cultivation time (Figure 2A, C). Medium 2, supplemented with HS, also showed comparably high cell growth. However, due to the variability introduced by serum and the consequent preference for serum-free media formulations in manufacturing, Medium 1 and Medium 4 were selected for subsequent experiments. Media performance in cell expansion in the Ambr® 15 Cell Culture bioreactors was also compared to that of static control cultures (Figure 2B, D). In contrast to the high cell growth observed in the Ambr® 15 Cell Culture system, the highest fold expansion in the static culture was approximately 11. While more studies are required to determine the potential significance and underlying cause of the differences observed in cell growth between the Ambr® 15 Cell Culture system and static controls, this result adds to a growing body of evidence that T-cells can thrive in stirred-tank conditions.[2]

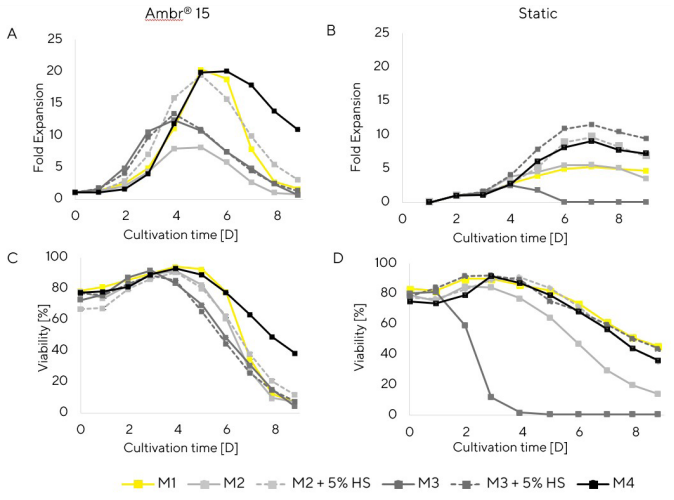

Figure 2. *Rapid Media Screening Identified Optimal Media (M1 and M4) Based on Fold Expansion and Viability.* Four media formulations (M1-4) were rapidly screened for their performance in cell expansion (A,C) using the Ambr® 15 Cell Culture system compared to (B, D) static controls. Media without HS are shown as solid lines, and the two media supplemented with serum are represented by dotted lines. M1, M2 + 5% HS, and M4 showed the highest fold expansion in the Ambr® 15 Cell Culture. M1 and M4 were selected for subsequent experiments. n=3 replicates per experiment.

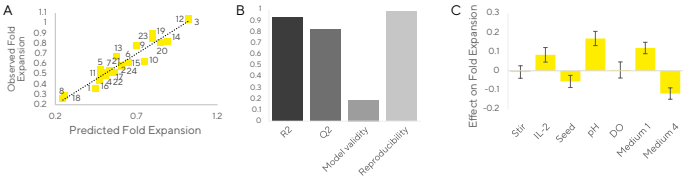

Figure 3. *Prediction of CPPs Based on DOE Optimization Studies Using a MODDE®-Generated Model.* Based on the cell-expansion data obtained in the media screening step, MODDE® DOE software generated a predictive model to identify CPPs. (A) The impact of six input factors on fold expansion was assessed via reduced linear combinatorial design, and observed vs predicted fold expansion was visualized using a replicate plot. (B) Goodness of fit (R^2) and predictive power (Q^2) were both high, as the summary of fit plot showed. (C) Increased IL-2 concentration, higher pH, and Medium 1 had positive impact on fold expansion. Seeding density and Medium 4 had negative impact. Stirring speed and DO had no impact, as shown in the coefficient plot. n = 4 replicates per condition; N = 24 experiments total.

Predictive model identified CPPs based on DOE optimization of protocols with MODDE® data analysis software

To identify CPPs and enable further process optimization, a model was generated using MODDE® DOE software with data from the preceding media screening run. Based on a linear reduced combinatorial design, MODDE® software mapped the observed fold expansion of each of the 24 bioreactors against the predicted values given the six input factors (Table 2, Figure 3). Since stirring speed and DO did not significantly impact fold expansion, they were removed from the model as input factors (Figure 3C, Figure 4). The removal of these two factors improved R^2, Q^2 and the model validity (Figure 4B). A high R^2 indicated a good model fit while the high Q^2 indicated good predictive power of the model. Reproducibility of the model was also high, with low variation of the replicates compared to overall variability. While the calculated model validity was not as high, there was strong linear correlation in the observed versus predicted plot with a narrow replicate variability (data not shown). The coefficient plot displays the impact of each model factor on fold expansion (Figure 4C). IL-2 concentration, pH, and Medium 1 had a positive impact on fold expansion, while seeding density and Medium 4 had a negative impact. These findings were then leveraged as recommendations for iterative optimization runs. For example, based on these predictions, a high pH and concentration of IL-2, a lower seeding density, and Medium 1 were chosen for subsequent runs.

To facilitate interpretation, the data were also visualized in a contour plot (Figure 5). Predicted response values for fold

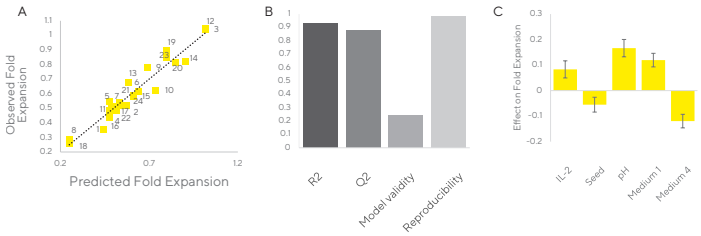

Figure 4. *Predictive Power and Validity of Model Improved by Removing Insignificant Parameters.* Stirring speed and dissolved oxygen were removed as input factors due to their minimal impact on fold expansion. (A) The impact of remaining four input factors on fold expansion was assessed, and observed vs predicted fold expansion was visualized using a replicate plot. (B) Improved Q^2 and increased model validity after removal of factors visualized in the summary of fit plot. (C) The impact of each of the four factors on fold expansion was mapped in the coefficient plot. n = 4 replicates per condition; N = 24 experiments total.

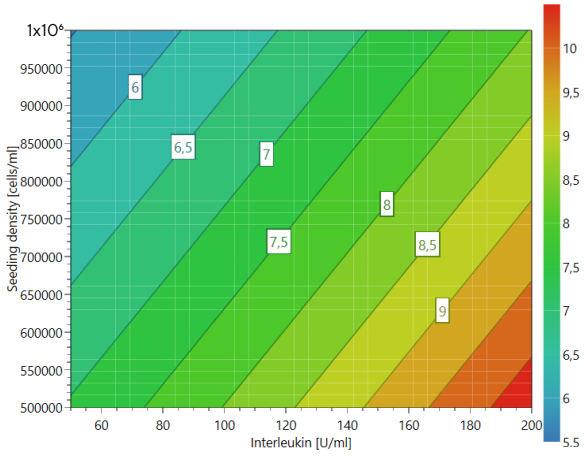

Figure 5. *Optimal Ranges for IL-2 Concentration and Seeding Density Visualized by MODDE®-Generated Contour Plot.* A combination of high IL-2 concentration and low seeding density were predicted to yield the highest fold-expansion. Predicted fold expansion values were displayed as a heat map relative to the level of IL-2 (x-axis) and seeding density (y-axis); all other factors were kept constant.

expansion were plotted relative to the level of IL-2 and seeding density; all other factors were kept constant. Again, the highest predicted values for fold expansion resulted from the combination of high IL-2 concentration and low seeding density.

CPPs fine-tuned with iterative optimization runs

With the knowledge gained in the previous set of experiments, a second set of optimization runs was designed. Now, only the three significant factors identified (DO, pH, and IL-2) were evaluated while the other factors were kept constant: Medium 1, stirring speed of 400 RPM, and seeding density of $5.0x10^5$ cell/mL were used (Table 4). As in the first experiment, the temperature was maintained at 37 °C, and a culture volume of 10 mL was used using batch culture with precultured, activated T-cells.

The same responses of fold expansion and viability were measured. With this setup, the number of replicate experiments

Parameters	2nd Optimization
DO%	50; 70; 90
pH	7.2; 7.4
IL-2 [U/mL]	50; 125; 200

Table 4. *Three Parameters were Evaluated in the DOE for the Second Optimization.*

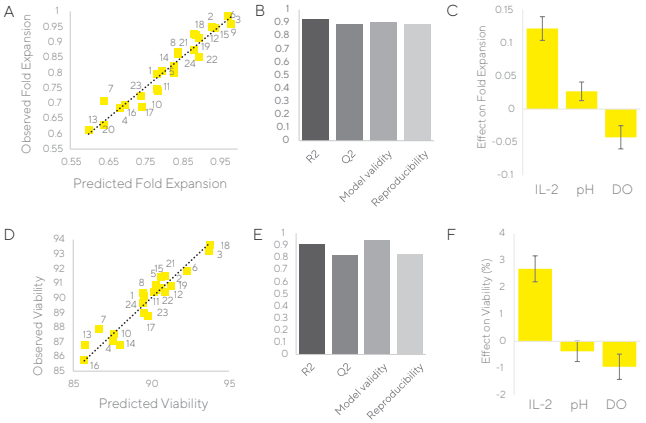

Figure 6. *Second Optimization for Fold Expansion and Viability Identifies IL-2 Concentration as a CPP with Strong Positive Impact.* Based on results from previous DOE experiments, a second optimization run was performed with a reduced number of factors. (A, D) The impact of three input factors on fold expansion (A) and viability (D) was assessed, and observed vs predicted responses were visualized using a replicate plot. (B, E) Goodness of fit (R^2), predictive power (Q^2), and model validity and reproducibility were all high as shown in the summary of fit plots for both fold expansion (B) and validity (E). (C, F) IL-2 concentration had a strong positive impact on fold expansion (C) and viability (F), and dissolved oxygen had a negative impact, as shown in the coefficient plots. n = 6 replicates per condition; N = 24 experiments total.

was increased to six. The analysis of the model showed a strong correlation between observed and predicted values for both responses (Figure 6A). The goodness of fit of the model was confirmed via the summary of fit graph (Figure 6B). The coefficient plots showed that IL-2 concentration had the highest impact on fold expansion and viability (Figure 6C). The model also showed that DO had a negative effect on both responses. Although pH had a significant effect in the first round of optimization when a wider range was tested, there was only a weak effect on fold expansion within the narrower range of pH in the second optimization. This indicates that a robust pH range was identified, since the smaller deviations in pH no longer impacted the cell response.

Sweet Spot analysis with optimal ranges for CPPs enables setting design space for seamless future scale-upp

To identify optimal parameter ranges and set design space to achieve defined fold expansion and viability responses, the MODDE® Sweet Spot analysis was used (Figure 7). The optimal outcome was defined as a minimum fold expansion of 8

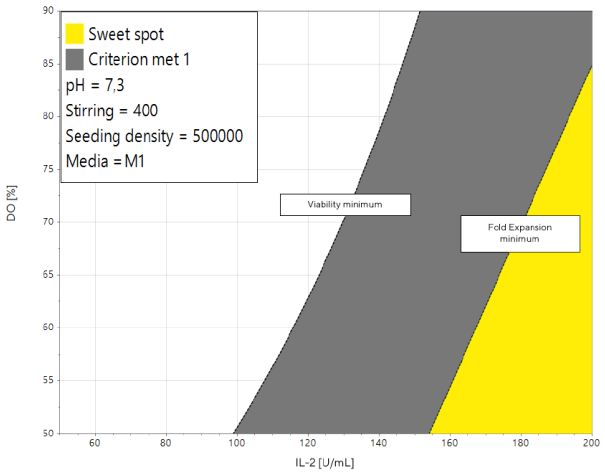

Figure 7. *Design Space with Optimal Process Range Set to Enable Successful Scale-Up.* Optimal process range was identified to achieve a minimum fold expansion of 8 and viability of 90% . IL-2 concentration and DO were the factors studied while pH, stirring speed, seeding density, and medium type were kept constant. The yellow area of the "Sweet Spot" shows the optimal ranges of DO and IL-2 concentration within which both the fold expansion and viability criteria were met; this included IL-2 concentrations above 164 U/mL and DO below 85%.

and a minimum viability of 90%. Since IL-2 concentration and DO had the largest effects on these responses, they were plotted in the Sweet Spot plot while pH, stirring speed, seeding density, and medium type were kept constant. The yellow area of the Sweet Spot plot indicated the optimal ranges of DO and IL-2 concentration within which both the fold expansion and viability criteria were met; this included IL-2 concentrations above 164 U/mL and DO below 85%.

Successful cell expansion in scaled-up volumes enabled by seamless process transfer to the Ambr® 250 Modular

As a final step, to demonstrate proof of concept for the utility of early process optimization to enable seamless scale up, the optimal parameters determined in previous experiments at the 10 mL scale were verified at the 200 mL scale in the Ambr® 250 Modular system. Scaled up cell expansion was tested in both baffled and unbaffled bioreactors to determine the ideal conditions for cell growth. Based on the results from the DOE optimization studies, a high IL-2 concentration and low DO percentage was used (Table 3). Medium, pH, and seeding density values were also set based on the recommendations resulting from the previous experiments, and stirring speed was determined separately for baffled versus unbaffled bioreactor vessels based on volumetric power input. Under these conditions, all samples surpassed the criteria set for successful scale-up, which was based on the design space thresholds of 8-fold expansion and 90% viability (Figure 8A). Cells grown in the Ambr® 250 Modular system in unbaffled vessels showed higher cell growth than those grown in baffled vessels or static controls. Cell viability was comparably high across the four

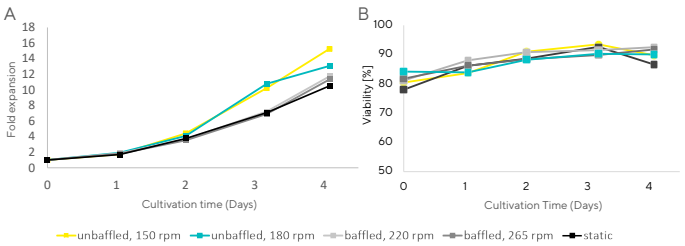

Figure 8. *Seamless Process Transfer of Optimized Parameters Enables Successful Cell Expansion in Scaled-Up Volumes.* Process parameters optimized in previous DOE experiments were transferred to an expansion protocol with scaled-up volumes. (A) Fold expansion of T-cells using the Ambr® 250 Modular expansion process. Unbaffled vessels (yellow and teal) showed greater cell expansion than baffled vessels (light and dark gray), which showed comparable growth on Day 4 to static controls (black) (B) Cell viability was high across all vessels and control samples.

days for all vessels, reaching over 90% by Day 3 (Figure 8B). Taken together, these findings confirm that process optimization performed at the miniaturized scale during early stages of process development can be easily transferred to higher volumes, providing a clear path toward the manufacturing of life-saving immune cell therapies.

Discussion

This study demonstrated the successful optimization of T-cell expansion with the Sartorius Exploration and Characterization Solution. Ambr 15® was effectively used in combination with MODDE® DOE software for rapid and systematic evaluation of CPPs. IL-2 was identified as a CPP with increasing concentrations having a positive effect on cell growth and viability. DO was also identified as a CPP, although with increasing concentrations having negative impact on cell response. pH was identified as a critical factor during screening of a wide pH range; the impact, however, declined when the pH range was narrowed, indicating that a robust pH range had been identified. Design space was thoroughly surveyed using DOE at the miniaturized scale, then verified in scaled up volumes using the Ambr® 250 Modular. Taken together, the studies demonstrate the power of the Sartorius Exploration and Characterization Solution to rapidly optimize processes and build process knowledge to facilitate subsequent scale-up.

While the studies described here successfully demonstrate an approach to tuning CPPs to optimize for cell expansion and viability upstream, the integration of the complete Sartorius T-Cell Exploration and Characterization Solution enables targeted optimization based on T-cell phenotypic and functional profile. For example, iQue® advanced flow cytometer and Incucyte® live-cell analysis combined with

SIMCA® MVDA software enable assessment of CQAs relevant to therapeutic outcomes, such as subtype ratio. Optimizing for these CQAs in addition to cell growth and viability early on in process development ensures consistent quality and effectiveness of the immune cell therapy product. Integration of T-cell phenotypic and functional data via these characterization tools offered in the complete Solution supports even deeper process understanding through correlation of CPPs and CQAs.

Using traditional workflows, an exhaustive process optimization protocol including media screening and CPP tuning could take two years and cost up to $700,000. These calculations are based on the assumptions that in early process development, flasks, fluorescence-activated cell sorting (FACS), cytotoxic T Cell assays (CTL), and standard growth media including serum are used. A similar Cost of Goods Sold (COGS) analysis using the complete Sartorius T-Cell Exploration and Characterization Solution, including the Ambr® 15 Cell Culture system (48-way), iQue®, Incucyte®, and 4Cell® Nutri-T, reveals that the same protocol would only take approximately one year and cost less than $350,000, a 50% reduction in both time and cost. This provides a strong rationale for using the Solution to accelerate process optimization at early stages of process development.

Finally, the Sartorius T-Cell Exploration and Characterization Solution is one in a suite of scalable solutions that span across research and development, early-, mid-, and late-stage process development, and manufacturing. The proof of concept studies presented here demonstrate the compatibility of the Ambr® 15 Cell Culture system with larger stirred-tank bioreactors such as the Ambr® 250 Modular. The latter is part of the Sartorius solution for mid-stage process development, the Sartorius T-Cell Optimization and Characterization Solution (Figure 9). Despite the conventional belief that T-cell and CAR-T cell expansion may be preferable under low-stress conditions and therefore static cultures, higher cell growth was observed under stirred-tank conditions in the Ambr® 15 Cell Culture platform as well as the Ambr® 250 Modular system.[2,3] Taken together, these process optimization data, including the seamless process transfer to larger bioreactors, provide evidence for the scalability and efficiency of the Sartorius Solutions for immune cell therapy production.

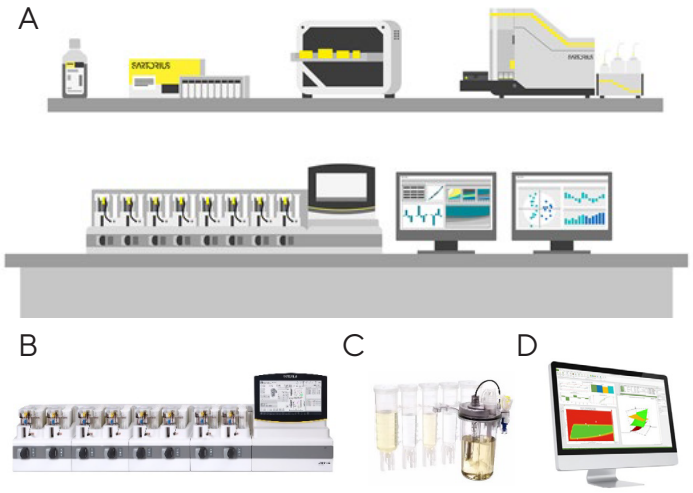

Figure 9. *The Sartorius T-Cell Optimization and Characterization Solution for Mid-Stage Process Development.* (A) Schematic of the solution with all components. (B) Ambr® 250 Modular (B) equipped with eight single-use unbaffled bioreactors. (C) MODDE® DOE software.

Conclusion

Immune cell therapy is a revolutionary and effective approach to treating cancer, yet high costs, labor, and scale-up bottlenecks prevent it from reaching patients. Building process knowledge and performing complete process optimization from the earliest stages of development will help overcome these bottlenecks. However, building process knowledge can be prohibitively costly and time intensive. The studies presented in this application note provide compelling data to recommend the Sartorius T-Cell Exploration and Characterization Solution as a scalable, cost-effective, and efficient approach to early-stage process optimization. Use of the Solution ensures thorough screening of process parameters and establishes a roadmap for future scaling and production of immune cell therapies.

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[3] Costariol E, Rotondi MC, Amini A, Hewitt CJ, Nienow AW, Heathman TRJ, Rafiq QA. Demonstrating the Manufacture of Human CAR-T Cells in an Automated Stirred-Tank Bioreactor. Biotechnol J. 2020 Sep;15(9):e2000177. doi: 10.1002/biot.202000177. Epub 2020 Jul 12. PMID: 32592336.

Ordering Information

Part Number	Part Number
Ambr® 15 Cell Culture system, 24 way configuration	001-8B20
Ambr® 15 Cell Culture system, 48 way configuration	001-8B22
Ambr 15 Cell Culture microbioreactors, no sparge, 20-40°C	001-8A64
Ambr® 250 Cell Culture system, 2 way configuration	001-8A64
Ambr® 250 Cell Culture system, 8 way configuration	001-8A67
Mammalian Vessel with dual pitch-blade impellers and baffles	001-2A23
Mammalian low-shear vessel, w/o baffles, single ‘elephant ear’ impeller	001-2A33
MODDE® DOE Software	UT-210069-M
4Cell® Nutri-T	05-11F2001-1K

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Sartorius Stedim Biotech GmbH
August-Spindler-Strasse 11
37079 Goettingen
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Toll-Free +1 800 368 7178

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SARTORIUS

Application Note

November 20, 2020

Keywords or phrases:
Stem cell memory T-cell, naïve T-cell, CD4/CD8 ratio, consistent T-cell media, serum-free T-cell media

Expansion of specific lymphocyte populations with 4Cell® Nutri-T xeno-free, serum-free medium

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2. Biological Industries Israel Beit-Haemek, Kibbutz Beit-Haemek, 25115, Israel
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Abstract

The success of autologous CAR-T therapy requires robust expansion of specific subpopulations of immune cells derived from the patient. Successful expansion relies on the use of media designed for this specific purpose, preferably without serum which introduces variability which in turn leads to a lack of reproducibility and increases risk to the patient. In this application note, we describe the benefits of a xeno- and serum-free medium for selective expansion of cell subpopulations without the addition of serum. This medium enables robust manufacturing processes by facilitating process control while delivering exceptional performance in terms of fold expansion.

Find out more: www.sartorius.com/car-t

Introduction

Adoptive cell therapy based on chimeric antigen receptor (CAR) T therapy is a remarkable advancement in the fight against cancer. Landmark approvals of Kymriah and Yescarta in 2017 set the stage for rapid growth of this modality, and just a few short years later, hundreds of clinical trials are evaluating adoptive cellular therapies for cancer.[1]

Production of CAR-modified T-cells begins with isolation of peripheral blood mononuclear cells (PBMCs) from the patient, followed by *ex vivo* genetic modification and expansion of cells with the preferred phenotypes for infusion back into the patient. Use of media containing serum of human origin is problematic for cell expansion for several reasons. Serum introduces variability into the process and, as such, it can lead to a lack of reproducibility thus limiting the accumulation of process knowledge. As a result, development of a consistent, robust manufacturing protocol that can be applied across a large patient population becomes challenging. The use of serum also increases the risk of exposing patients to pathogens. At the same time, refrigerated storage of reserved serum lots increases facility costs, which further drives up cost of goods (COGs). Finally, the price and availability of serum can complicate operations as both can vary due to supply limitations.

In contrast, the use of chemically defined, xeno-free and serum-free medium formulations for cell expansion overcomes these challenges. Serum-free media enables robust manufacturing processes by facilitating process control while delivering exceptional performance in terms of fold expansion.

In this study, we demonstrate the robust performance of the xeno- and serum-free 4Cell® Nutri-T medium for the expansion of lymphocytes in comparison to serum-containing and other serum-free medium formulations. 4Cell® Nutri-T, which was developed using patient cells and has been optimized for the cultivation of CAR-T cells, tumor infiltrating T lymphocytes (TILs) and peripheral blood mononuclear cells (PBMCs), delivered superior results for fold expansion and cell viability compared to other medium formulations.

Evaluation of Fold Expansion and Cell Viability

Fig. 1 shows the differences in fold expansion and cell viability of PBMCs from 3 healthy donors cultured in either 4Cell® Nutri-T, a competitor medium A with 5% human AB serum (HS) or in competitor medium B (serum-free). A total of 2×10^6 PBMCs from healthy donors were seeded in 24-well plates (2 mL media/well). After an overnight rest, cells were activated via CD3/CD28 using TransAct™ (Miltenyi) 1:100 and reseeded at 1×10^6 cells/mL in the respective media supplemented with 600 IU/mL IL-2. 24 hrs after activation, cells were transduced with a CD19-CAR lentiviral vector. After transduction, cells were further expanded by splitting and reseeding cells at a density of 0.2×10^6 cells/mL every 2-3 days; fold expansion and cell viability were measured on day 11.

This study demonstrated that 4Cell® Nutri-T medium was superior for expansion of healthy donor transduced T-cells.

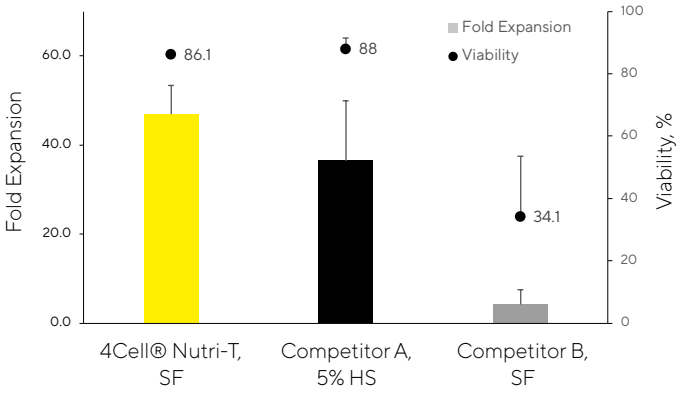

Fig. 1: Fold expansion and cell viability of healthy donor transduced CAR-T cells grown in different media formulations. SF, serum-free. HS, supplemented with human serum. SC, serum-containing.

Fold expansion and the efficiency of transduction were also measured for PBMCs isolated from the blood of a patient with lymphoma and grown in six serum-containing or serum-free media (Fig. 2). Media were supplemented with 50 ng/mL of anti-CD3 OKT3 and 300 IU/mL IL-2. At day 2 post seeding, $2\text{-}3 \times 10^6$ cells were transduced with a CD19-CAR lentiviral vector in 6-well plates pre-coated with RetroNectin(R) (Takara Bio). Post transduction, the cells were collected and reseeded. At day 4, 4 mL of fresh medium and IL-2 were added; at day 6, 50% of the medium was replaced with fresh medium and IL-2. Transduction efficiency was evaluated at day 9 and fold expansion was measured at day 10.

The 4Cell® Nutri-T medium provided the best combination of both fold expansion and transduction efficiency.

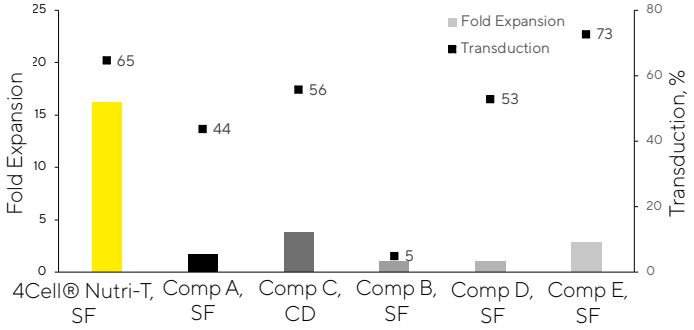

Fig. 2: Fold expansion and transduction efficiency of patient-derived T-cells transduced with CD19-CAR and grown in different medium formulations. SF, serum-free. CD, chemically defined.

Evaluation of Cell Phenotype

The state of differentiation of anti-tumor T-cells, often referred to as their "stemness", can impact the efficacy of immunotherapy.[2] The ratio of CD4+ and CD8+ cells, along with the presence of memory markers, is typically assessed for selection of optimal T-cell subpopulations for more effective cellular immunotherapy.[3] The medium in which T-cells are cultured and expanded can impact the ratio and phenotype of cells and as such, it is important to understand the influence of different medium formulations.[4]

A study conducted at the Ella Lemelbaum Institute for Immuno-Oncology at the Sheba Medical Center in Israel compared 4Cell® Nutri-T medium and a medium supplemented with 5% human serum for the culture and expansion of patient-derived CAR-T cells. Fluorescence activated cell sorting (FACS) analysis was used to assess the presence and ratios of T-cell subsets.[5]

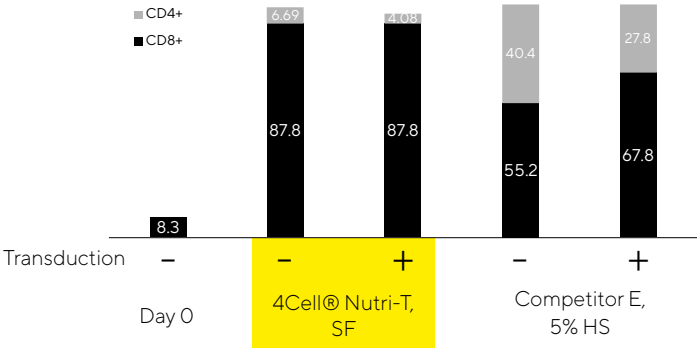

Fig. 3: Percentage of CD8+ and CD4+ cells at day 10 of culture in serum-free versus serum-containing media formulations. SF, serum-free. HS, supplemented with human serum.

As shown in Fig. 3, culture of CAR-T cells in 4Cell® Nutri-T medium led to a higher percentage of CD8+ cells as compared to the medium supplemented with human serum for both untransduced and transduced cells.

	CD45RA	CCR7
T_N+T_{SCM}	+	+
T_{CM}	-	+
T_{EM}	-	-
T_{EFF}	+	-

Fig. 4: T-cell subpopulation markers. T_N, naïve T-cell. T_{SCM}, stem cell memory T-cell. T_{CM}, central memory T-cell. T_{EM}, effector memory T-cell. T_{EFF}, effector T-cell.

A similar study determined the percentages of four T-cell subpopulations (combined naïve and stem cell memory, central memory, effector memory, and effector (Fig. 4)) following 10 days of culture in the serum-free and serum-containing media (Fig. 5). While both media supported a high percentage of central memory cells (T_{CM}), the percentage of naïve and stem cell memory cells ($T_N + T_{SCM}$) was significantly higher in the untransduced and transduced cultures grown in 4Cell® Nutri-T medium.

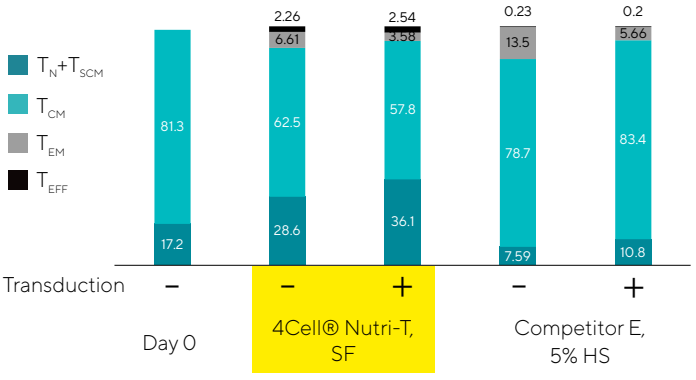

Fig. 5: Percentage of T -cell subsets cultured in 4Cell® Nutri-T serum-free medium or a serum-containing medium. SF, serum-free. HS, supplemented with human serum.

Fig. 6 shows the percentages of T-cell subpopulations achieved following a 10-day static expansion in the serum-free and serum-containing media. While both the serum-free and serum-containing media supported a robust percentage of T_{CM} in the small- and large-scale systems, the combined percentage of T_N and T_{SCM} cells was higher when the CAR-T cells were grown in the 4Cell® Nutri-T xeno- and serum-free medium.

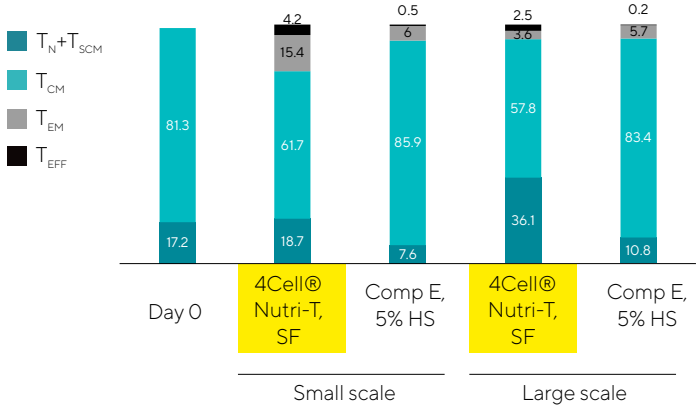

Fig. 6: Percentage of T-cell subsets cultured in 4Cell® Nutri-T xeno- and serum-free medium or a serum-containing medium in large- and small-volume static expansion. SF, serum-free. HS, supplemented with human serum.

Discussion

Adoptive cell therapies such as CAR-T have shown remarkable results for cancer patients who have exhausted available treatment options. A growing population of patients will benefit from this novel modality as the industry further elucidates the underlying biology and optimizes manufacturing processes, including the culture and expansion of CAR-T cells and other types of genetically modified immune cells.

The efficacy of CAR-T therapy relies on a robust, consistent and preferential expansion of a subpopulation of T-cells that retain a degree of stemness. Incorporation of a serum-free medium into culture and expansion processes offers several benefits for this process:

- Delivers improved fold expansion of cells and supports robust cell viability and transduction efficiency
- Eliminates the risk of introducing pathogens present in human serum
- Improves reproducibility of protocols by minimizing variability
- Enables robust manufacturing processes and process control
- Reduces COGs through greater efficiency of cell expansion and by eliminating operational costs associated with serum
- Eliminates reliance on unpredictable serum supply chains with volatile pricing

In this application note, we highlight the performance of the 4Cell® Nutri-T xeno- and serum-free medium for the expansion of T-cells isolated from PBMCs and of CAR T-cells with desirable phenotypes. In all cases, the 4Cell® Nutri-T medium out-performed both serum-containing media and other serum-free formulations.

Acknowledgements

The authors would like to thank the Ella Lemelbaum Institute for Immuno-Oncology at the Sheba Medical Center for a supportive collaboration.

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Ordering information

Description	Part Number
4Cell® Nutri-T Medium, 1L	05-11F2001-1K

Sales and Service Contacts

For further contacts, visit www.sartorius.com/car-t

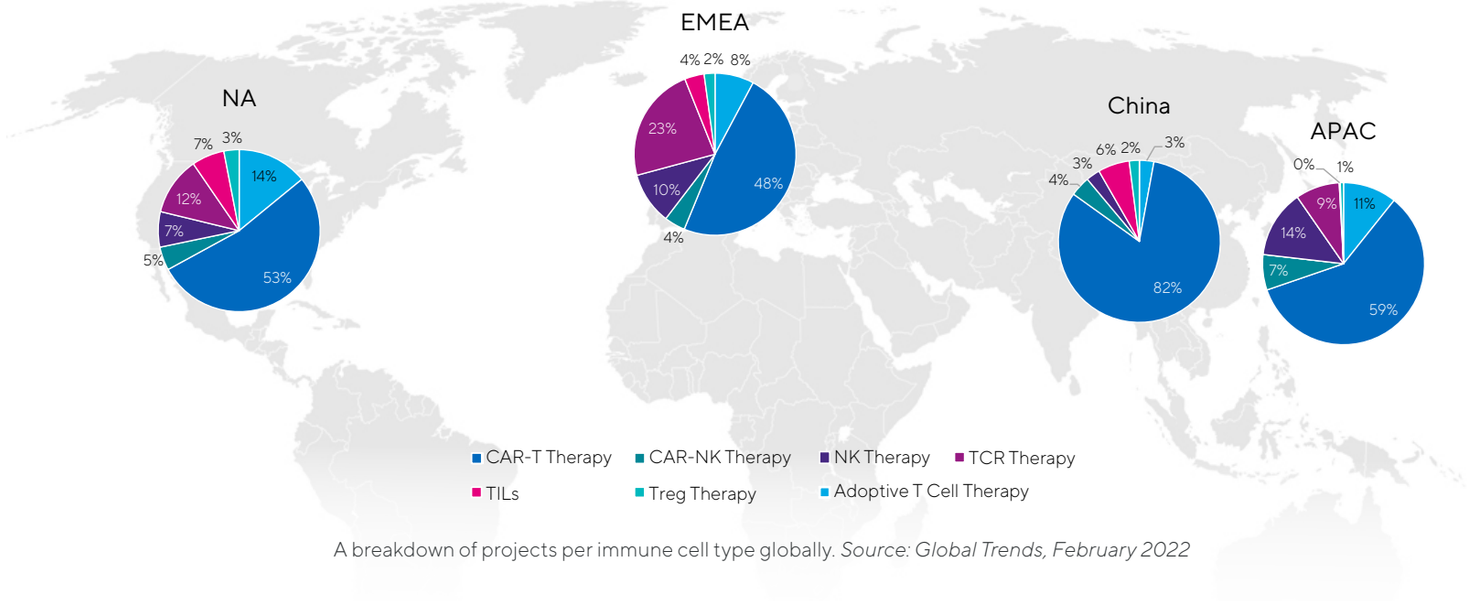
Germany
Sartorius Stedim Biotech GmbH
August-Spindler-Strasse 11
37079 Goettingen
Phone +49 551 308 0

USA
Sartorius Stedim North America Inc.
565 Johnson Avenue
Bohemia, NY 11716
Toll-Free +1 800 368 7178

DIR: 2475299-000-02
Status: 02 | 2020

Enhancing Cell Growth With Cytokines

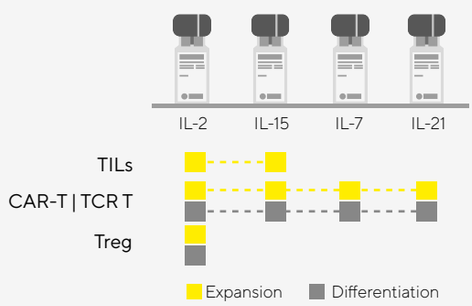
With nearly 4,800 immune cell therapy products in development today, scientists around the world depend on cytokines to support the culture of a variety of immune cell types.



A breakdown of projects per immune cell type globally. *Source: Global Trends, February 2022*

Cytokine Combinations to Tailor Your Immune Cell Culture

Tune cytokines to help promote successful expansion and differentiation of a variety of immune cells.



Combining CellGenix® cytokines makes T cell production more efficient and robust while reducing handling steps.

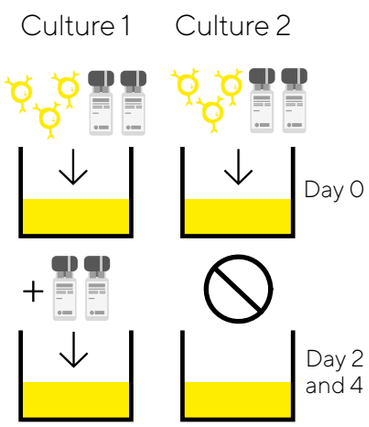
Data Points:

1. CellGenix® IL-7, CellGenix® IL-15, along with anti-CD3/anti-CD28-coupled magnetics beads were added to pre-warmed media containing 5×10⁶ purified CD3+ T cells/well.

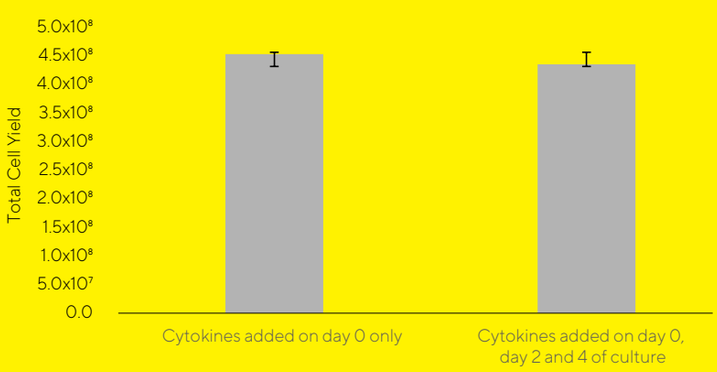
2. The media cultured over an 11-day expansion, with one culture receiving CellGenix® IL-7 and CellGenix® IL-15 on days 2 and 4.

Results:

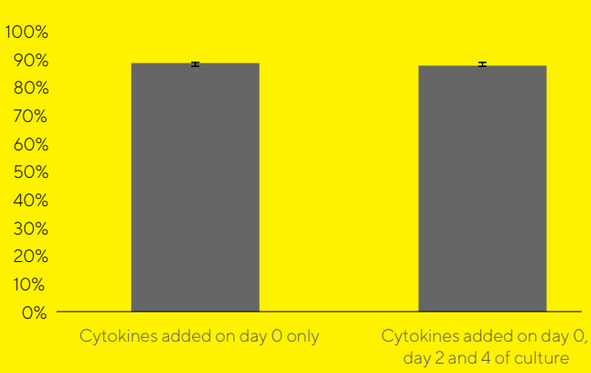
At day 11 of culture, no significant differences in expansion and viability were observed between the two conditions. This demonstrates that **CellGenix® IL-7 and IL-15 support T cell culture over 11 days — meaning you could reduce open handling steps and save up to 15% of aliquot volume.**



Day 11 Cell Yield



Day 11 Cell Viability



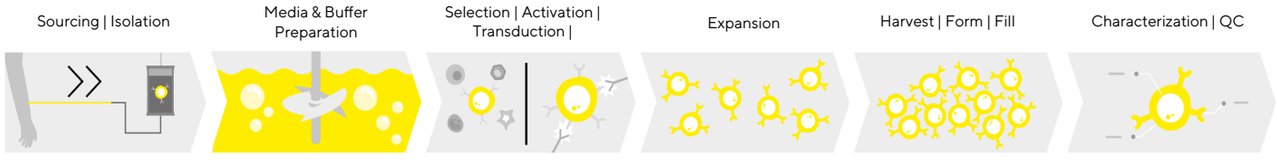
Want to learn more about how our reagents can support your T cell production? Visit us online.

Building Screening Capabilities to Accelerate Your Process Development

Acquire — Find the optimal growth conditions by comprehensively screening media, reagents, and clones.

Analyze — Generate actionable process data by comprehensively and rapidly screening media and components as well as identifying CPPs.

Apply — Build the foundation to acquire process knowledge for the development of clinically translatable protocols.



Process Parameters

Ambr® 15 Cell Culture

iQue®3

Incucyte® SX5

4Cell® Nutri-T NutriStem®

CellGenix® Growth Factors

SoloHill® Mircocarriers

Bioreactors

Consistent, serum-free and xeno-free media, high-performance preclinical reagents, and ready-to-use microcarriers promote target cell growth and phenotype

Bio Analysis

Media & Reagents

Consistent, serum-free and xeno-free media, high-performance preclinical reagents, and ready-to-use microcarriers promote target cell growth and phenotype

iQue®3

Incucyte® SX5

Baseline Characterization

Bio Analysis

Automated, real-time, high-throughput live-cell imaging and advanced flow cytometry platforms for phenotypic and functional characterization of cells

Data Analytics

Design of Experiments (DOE) software to set and verify your design space, and multivariate data analysis software for process parameter and critical quality attribute correlation

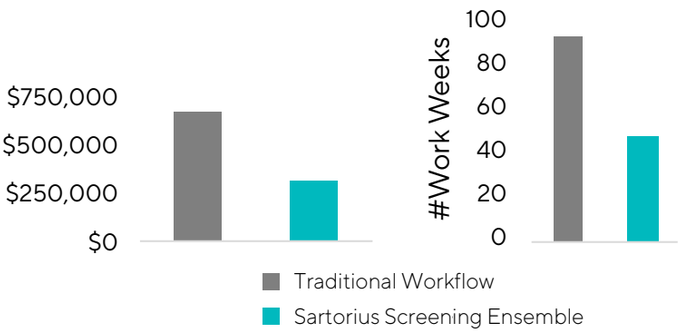
MODDE® | SIMCA®

Characterization of Target CQAs

Reducing COGs and time to screen CPPs and CQAs with Sartorius Screening Ensemble

Screen process parameters and quality attributes

- 7 media to screen
- 4 IL-2 concentrations
- 5 serum concentrations
- 7-10 donors (for validation)
- 3 pH values
- 3 DO values
- 2 seeding densities
- 2 rpm values



Cost reduced by $364k

Time to completion accelerated by 47 weeks

SARTORIUS

White Paper

September 2021

Keywords or phrases:
Chimeric Antigen Receptor (CAR), CAR-T, Immune Cell Killing, Cancer, T Cell Therapy, Immunotherapy

Phenotypic and Functional Characterization of CAR-T Cells with Advanced Flow Cytometry and Live-Cell Analysis

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Sartorius UK Ltd., Hertfordshire, United Kingdom

Introduction

The successful use of immunotherapies to help combat cancer has expanded rapidly in the last few years, with many therapies now approved for clinical use. The precision of the immune system allows a more targeted approach to killing cancer cells, while sparing healthy cells, when compared to traditional chemotherapeutic strategies. One key area of advance has been in the use of gene-modified cell therapies with the introduction of chimeric antigen receptor (CAR) T cells leading the field. The CAR construct is designed to interact with a specific surface epitope or antigen present on the tumor cell, which once in close proximity enables the T cell to kill the tumor cell. Where specific antigens can be identified on the tumor cells, CAR-T cells display targeted effects and, as they are sourced from the patient (known as autologous therapy), there is a lack of rejection. CD19 targeted CAR-T cell therapies, for example Kymriah® (Novarits) or Yescarta® (Kite/Gilead)[1], have shown clinical success against liquid tumors common in lymphoblastic leukemia and non-Hodgkin's lymphoma. Despite this progress, obstacles remain, for instance, the high cost and technical difficulties of phenotyping, profiling and purifying immune cells.[2,3] Also, while some patients have been highly responsive to treatment, others were refractive, and uncovering the mechanistic basis for these differing outcomes is an active area of research. In more recent years, research has progressed to explore the introduction of CAR constructs into alternative immune cells, for example CAR-NK or CAR-macrophages[4-6], and to investigate gene modified cells that target solid tumors.

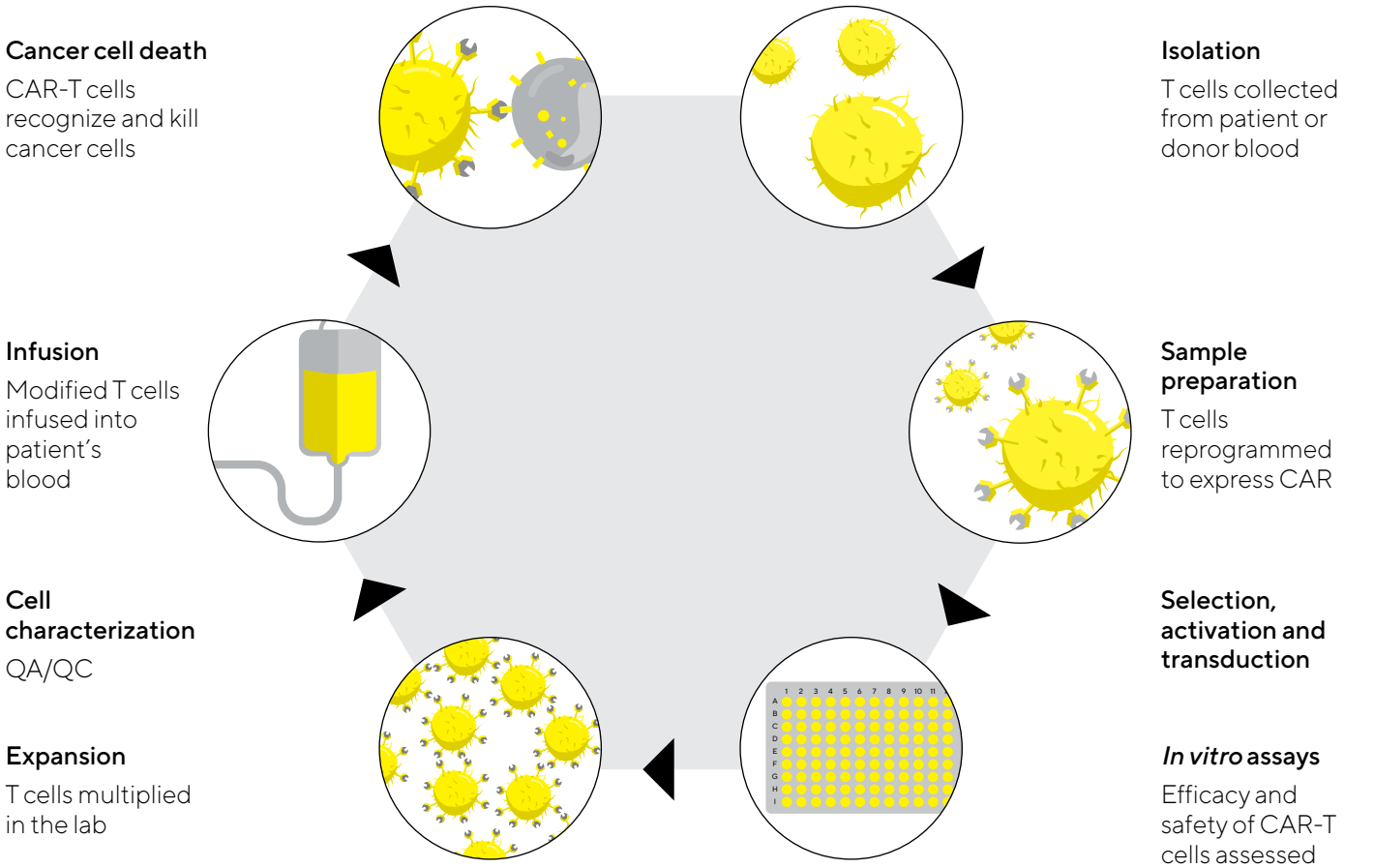
This article will touch on the process of manufacturing and expanding cell therapy products with a focus on CAR-T cells. Key *in vitro* assays used to phenotype and assess function of these modified cells will be introduced with three case studies illustrating the utility of the iQue® Advanced High Throughput Flow Cytometry Platform and the Incucyte® Live-Cell Analysis System (Sartorius).

Find out more: www.sartorius.com

Manufacture and Expansion of CAR-T

In recent years, there has been a large focus on improving the efficiency and quality of CAR-T cells to support their continued clinical use. As shown in Figure 1, there are multiple stages involved in the development and expansion of autologous CAR-T material. At all stages, the resulting product needs to be assessed for quality and functionality, while reducing time from initial donation to re-introduction to the patient.

Figure 1
Development Cycle of CAR-T Material

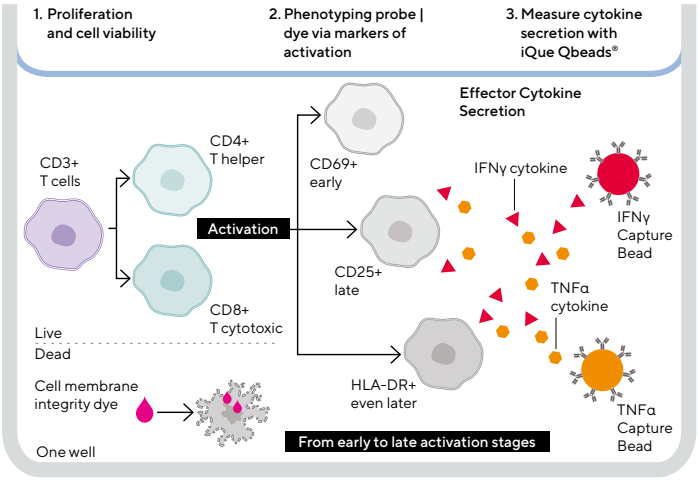


During early development, the CAR construct is optimized to ensure its specificity and engagement with the target of interest. Constructs typically include a recognizable marker, for example, protein L or GFP, which serves as an easy identifier of transduced cells. This marker is used following T cell reprogramming to assess transduction efficiency and can be used for CAR-T enrichment during downstream processing. Once CAR-T cells have been transduced, *in vitro* assays are used to profile the cells and to assess their functional reactivity to the target of interest. For example, flow cytometry can be used to assess the phenotype of cells in combination with functional readouts following a tumor killing assay.[7] These assays need to deliver reproducible and biologically relevant results. An example of the type of flow cytometry readouts that can be easily captured using a high throughput instrument like the iQue® platform are shown below (Figure 2). The panels shown also highlight the capacity for simultaneous quantification of secreted cytokine levels within the same sample.

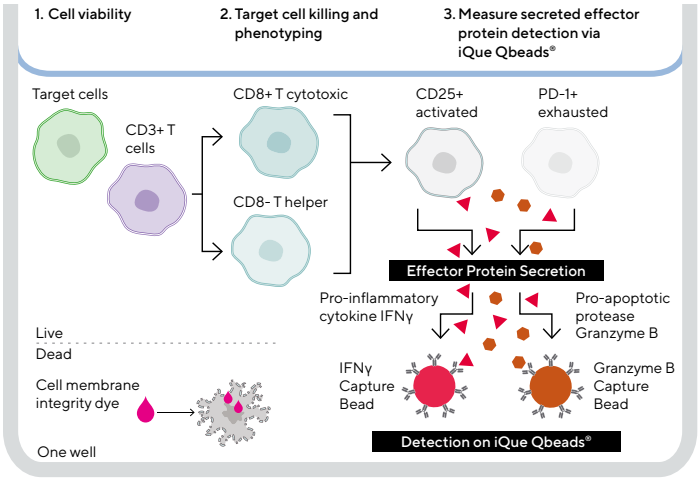
Figure 2

Overview of Phenotype and Function Kits to Characterize T Cells, Compatible With the iQue® Advanced Flow Cytometry Platform

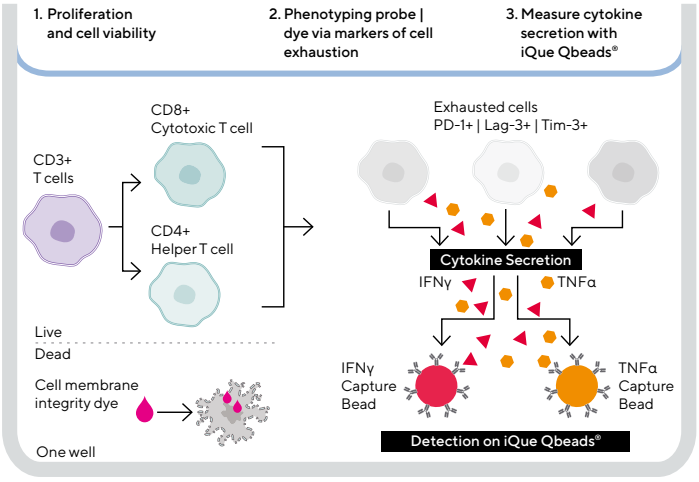
iQue® Human T Cell Activation Kit



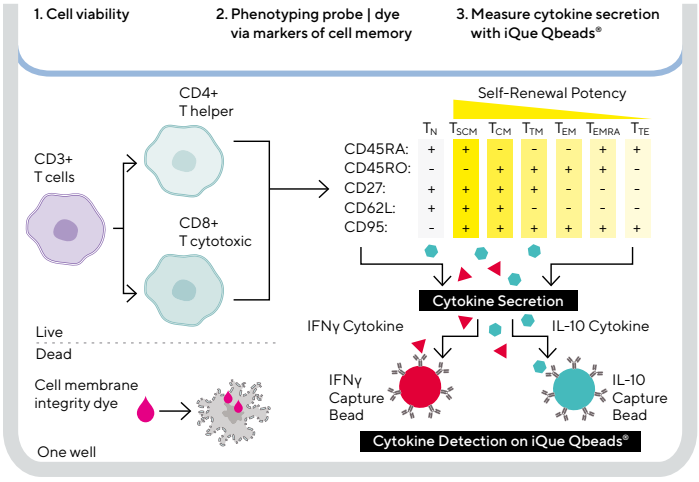
iQue® Human T Cell Killing Kit



iQue® Human T Cell Exhaustion Kit



iQue® Human T Cell Memory Kit



Once development and assessment of the CAR construct is complete, the cells are expanded to provide the quantities needed for re-introduction into the patient. During this phase, phenotype and function of the cells is quantified to ensure required cell profiles are maintained. Expansion is driven by the activation of the cells, often through non-specific mechanisms, for example, by the addition of anti-CD3 and anti-CD28. This can lead to rapid expansion in culture vessels, so nutrient depletion and cell densities need to be closely monitored. To accommodate larger scale CAR-T production (1–10 liters), cells may need to be cultured in stirred tank or wave bag bioreactors.[8,9] When producing clinical grade material, many additional control processes must be introduced to ensure integrity and quality of the samples. For example, cells must be thoroughly characterized, and heightened safety measures must be implemented and carefully documented. These processes are often time-sensitive, so it is important that rapid solutions are available. Real-time screening and analysis techniques enable continuous phenotypic and functional analysis over time to ensure a high-quality product is maintained.

The phenotype of the final CAR-T cell product is of great importance because it strongly links to their clinical potency. Much of the interest in this field has been focused on the influence of exhaustion and memory phenotypes on CAR-T function.[10] For a prolonged anti-tumor response, it is critical that populations of functioning CAR-T cells are maintained once re-introduced into the patient. This relies on preservation of the cells' self-renewal potency coupled with a lack of exhaustion, meaning their ability to kill tumor

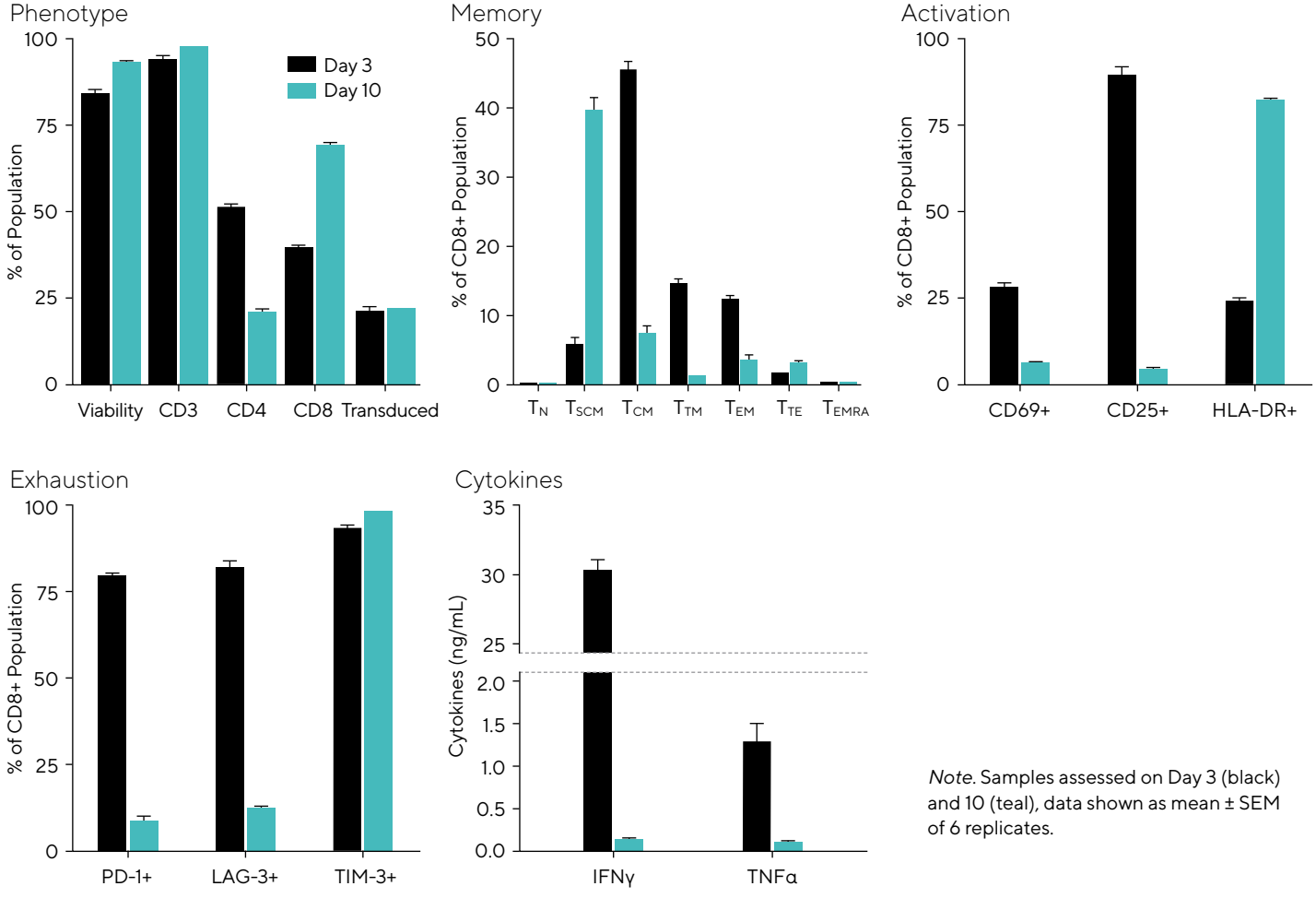
cells is sustained. Some features can be built into the CAR construct to improve longevity of signal[2,3], but expansion protocols can also influence the balance of phenotypes. There are key phenotypes that can be tracked *in vitro* to determine that these parameters have been maintained. Large populations of memory T cells such as stem central memory (T_{SCM}) or central memory cells (T_{CM}) are desirable as they have high self-renewal capabilities. Terminally differentiated cells, such as terminal effector cells (T_{TE}), are undesirable because they have lost their ability to self-renew. Markers such as PD-1, LAG-3 and TIM-3 are important indicators of exhaustion. Expression of these markers will often fluctuate during the expansion phase due to the stimulation added to drive activation and expansion of the T cells.[11] More recently, interest has also been directed towards determining the optimal ratio of CD4 and CD8 cells in a CAR-T product.[12,13]

The data below (Figure 3) shows an example phenotype profile of CAR-T cells during a 10-day expansion process with anti-CD3/anti-CD28 activation beads as a static culture in flasks (cells supplied by Dr. Qasim Rafiq's lab at University College London). Samples were analyzed on Day 3 and 10 post CAR transduction using the iQue® Human T Cell Kits. The data quantifies the general T cell population for CD3, 4 and 8 alongside viability and transduction efficiency across the sample days. For this example, the Day 10 memory phenotypes display a higher proportion of the desired T_{SCM} and T_{CM} cells with negligible populations of the more differentiated phenotypes of effector memory (T_{EM}), T_{TE} and effector memory cells re-expressing CD45RA (T_{EMRA}). The activation profile shows early activation markers, CD69 and CD25, are more highly expressed on Day 3 and reduce by Day 10, while HLA-DR, a later marker for activation, increases from Day 3 to 10. PD-1 and LAG-3 display a similar trend to the early activation markers, in that they are initially high but reduce by Day 10. Interestingly, TIM-3 expression remains high throughout. Both IF and T F concentrations are high in the Day 3 sample but dramatically drop by Day 10.

This type of profiling data can help support optimization of expansion processes and the complete understanding of the phenotype ratios present in the final product.

Figure 3

CAR-T Phenotyping Using iQue® T Cell Characterization Kits During an Anti-CD3/Anti-CD28 Driven Expansion of Transduced T Cells



Note. Samples assessed on Day 3 (black) and 10 (teal), data shown as mean ± SEM of 6 replicates.

Quantification of the Functional Activity of CAR-T Cells

The following case studies will be used to exemplify the power of the combined use of the iQue® Advanced Flow Cytometry Platform and the Incucyte® Live-Cell Analysis System for the functional profiling of CAR-T cells. All data shown has been generated using commercially obtained CAR-T cells (Creative BioLabs) which were supplied as frozen cultures of transduced T cells alongside control mock transduced T cells from a matched donor. The CAR-Ts have been transduced with a second-generation CAR construct, specific for either CD19 or HER2, with reported transduction efficiencies of around 50% for these samples.

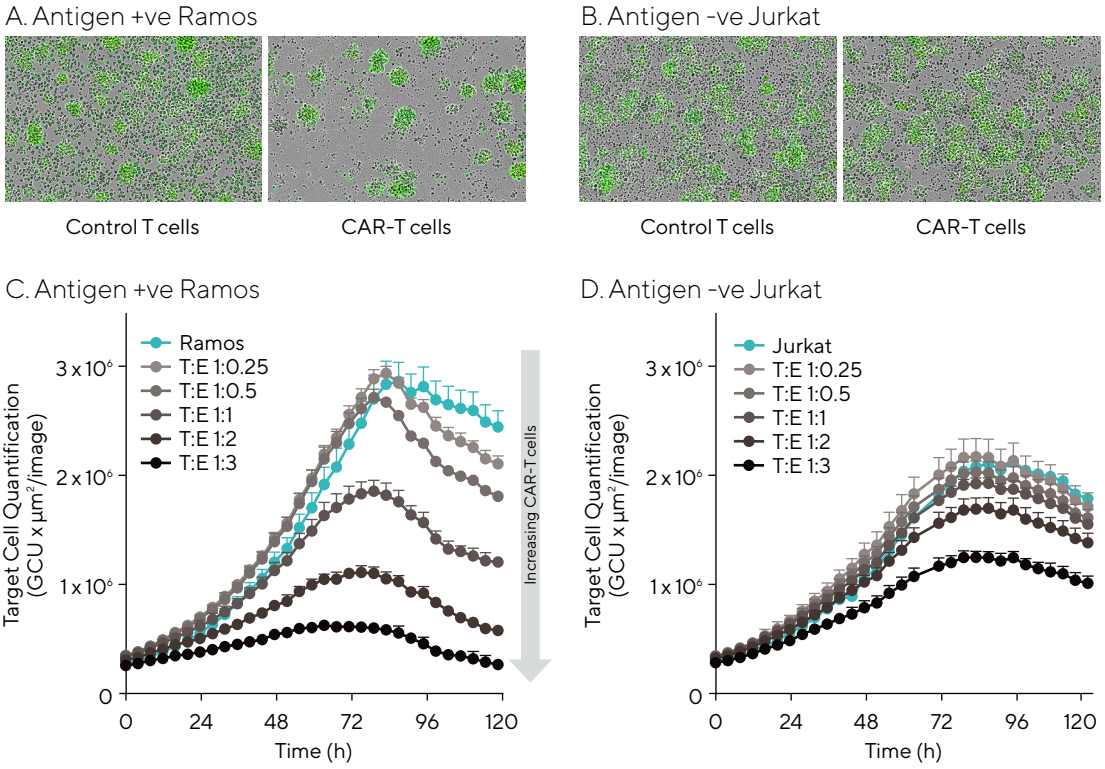
Case Study 1: Specific Killing Profile of CD19-Targeted CAR-T Cells

CAR-T cells are designed to selectively target and kill tumor cells through interaction with a specific surface antigen, while limiting off-target side effects.

To demonstrate this specificity *in vitro*, anti-CD19 CAR transduced T cells or donor matched mock transduced T cells were used in an Incucyte® immune cell killing assay. CD19 antigen positive Ramos or CD19 antigen negative Jurkat cells were seeded in combination with T cells at various target to effector ratios (T:E). The target cells were transduced to express a nuclear restricted green fluorescent protein (Incucyte® Nuclight Green Lentivirus) to aid quantification. Images of the co-culture were collected over the next four days and quantified for area of green fluorescence in each well. Images showed a clear reduction in the antigen positive Ramos cells when they were co-cultured with anti-CD19 CAR-T cells (Figure 4A), which was not seen with antigen negative target cells or with mock transduced T cells (Figure 4A and B). Quantification of images demonstrates a clear CAR-T cell density related decrease in Ramos target cells over time (Figure 4C). Maximal effect was measured using a T:E ratio of 1:3, representing a 73.2 ± 0.7% reduction in target cell numbers at 72 hours. There was some death of antigen negative Jurkat cells at the higher CAR-T ratios (Figure 4C), representative of 36.5 ± 2.6% of Jurkat cells at 72 hours. This effect highlights the possibility of off-target events either by non-transduced T cells in the culture or due to the high number of effector cells in the well. No killing was induced by mock transduced control T cells, unless they had been non-specifically activated with CD3/CD28 Dynabeads® (ThermoFisher) (data not shown).

Figure 4

CD19-Targeted CAR-T Cell Killing of Antigen Positive Target Cells



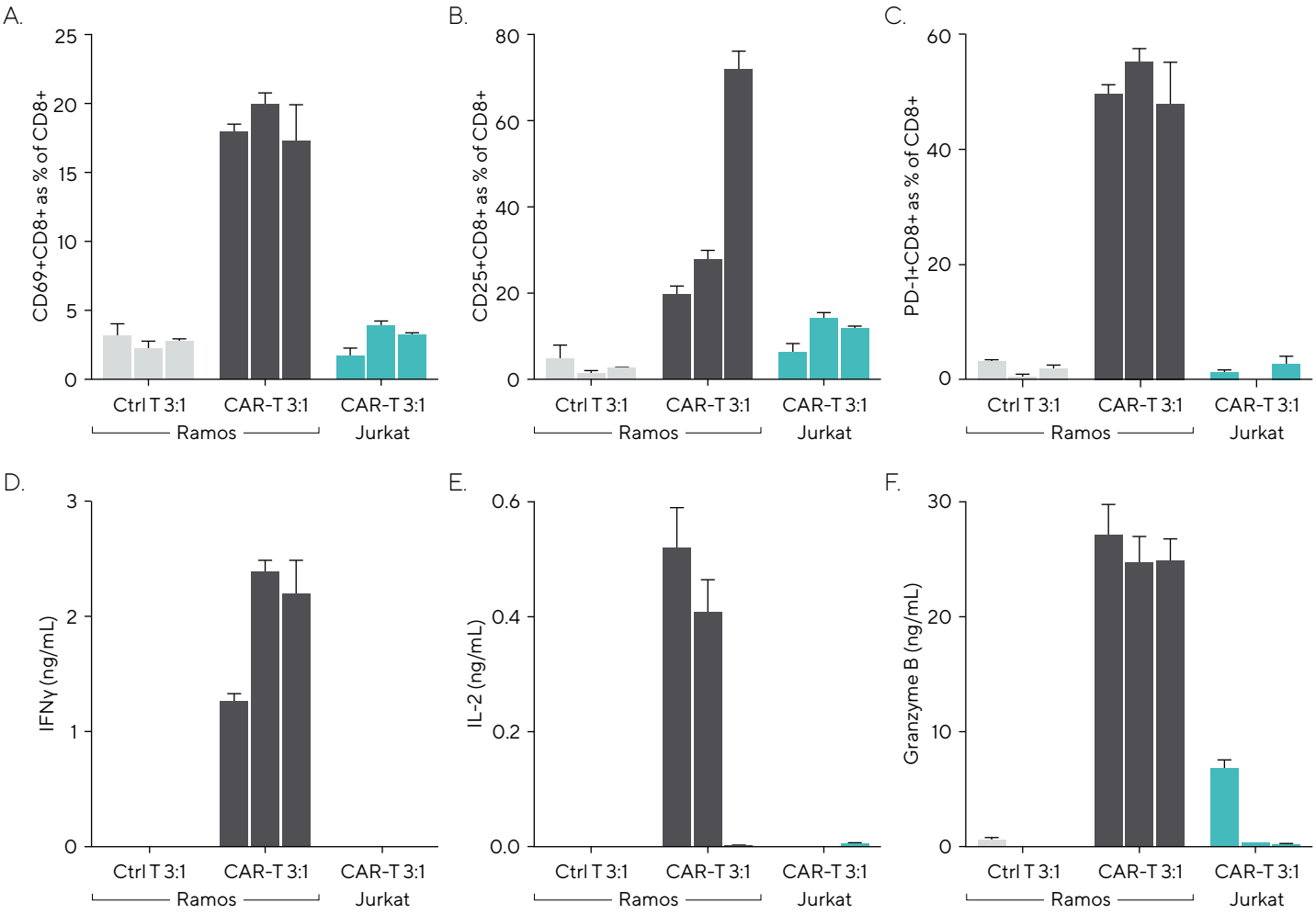
Note. A co-culture of Incucyte® Nuclight Green labeled Ramos or Jurkat cells with either anti-CD19 CAR-T or control T cells was set up at various T:E ratios in a 96-well plate. Cultures were imaged in the Incucyte® every 4 hours over 4 days and quantified for green fluorescent area. Images taken at 72 hours (A) show a clear reduction in green area of Ramos cells in combination with CAR-T cells (1:2 T:E). Time course graphs (B and C) demonstrate increased killing of antigen positive Ramos cells compared to antigen negative Jurkat cells. Data shown as mean ± SEM of 3 wells.

After Incucyte® images had been collected, on Day 2, 4, and 7, samples were subsequently analyzed on the iQue® platform to assess phenotype and function using the iQue® Human T Cell Activation and iQue® Human T Cell Killing Kits, as well as quantification of IL-2 via the iQue® Human T Cell Companion Kit. The kits enable quantification of T cell surface markers and secreted proteins indicative of T cell activation and tumor cell killing. Results show that, when combined with antigen positive Ramos cells, there was a rapid upregulation of T cell activation markers CD69, CD25, and PD-1 (Figure 5A-C, respectively) on the CD8+ cells. This upregulation demonstrated some time dependence, with the highest levels observed on Day 7, but there was little difference between CAR-T cell densities. Expression of all 3 activation markers was low in co-cultures with antigen negative Jurkat cells or in the presence of mock transduced T cells (< 7%). In the presence of Ramos cells, concentrations of secreted cytokines IF and IL-2 indicators of activation increased at early time points, but then dropped by Day 7, indicating a transient response. Release of Granzyme B, an indicator of cell killing, increased in co-cultures containing CAR-T cells, but only in the presence of antigen positive Ramos cells. For all secreted proteins, there was a general increase in levels with increasing CAR-T density.

Overall, this complete quantification demonstrates a clear antigen specific activation of anti-CD19 CAR-T cells as measured by both surface markers and secreted proteins in combination with the functional readout of killing antigen expressing tumor cells.

Figure 5

Antigen Specific Activation of Anti-CD19 CAR-T



Note. Samples were quantified on Day 2, 4, and 7 for surface marker expression and secreted protein using either iQue® Human T Cell Activation Kit or iQue® Human T Cell Mediated Killing Kit with iQue® Human T Cell Companion Kit (for IL-2). Graphs (A-C) show expression levels in CD8+ T cells of CD69, CD25 or PD-1, and graphs (D-F) show levels of IFNγ, IL-2 or Granzyme B. Grey bars represent Ramos with mock transduced T cells, black bars are CD19 CAR-T with Ramos cells, and teal bars are CAR-Ts in combination with Jurkat cells. The 3 bars represent Day 2, 4, and 7, all data shown as mean ± SEM of 3 wells.

Case Study 2: Exhaustion Profiling of CAR-T Cells Under Antigen Challenge

Repeated exposure to tumor cell antigens can lead to CAR-T cell exhaustion. Examining the phenotypic profile of exhausted CAR-T cells can help to improve our understanding of how this exhaustion affects the longevity of the clinical response. To investigate this *in vitro,* CAR-T cell exhaustion was induced by continuously challenging the anti-CD19 CAR-T cells with the antigen positive Ramos cell line. Every 2–3 days for 10 days, the CAR-T cells were counted and re-stimulated with fresh Ramos cells (1:1 T:E). On Day 11, the stimulated CAR-T cells were counted and seeded into a 96-well plate with Ramos cells (1:1 T:E). A fresh batch of non-exhausted CAR-T cells were plated both in co-culture with Ramos and as a monoculture for comparison. Daily cytokine samples (10 µL) were taken from all wells of the assay plate and, after 72 hours, cells and supernatants were quantified using the iQue® Human T Cell Exhaustion Kit.

Cytokine secretion, both of IF and T F , was low in wells containing the exhausted CAR-T and Ramos cell co-culture (Figure 6A and B). Comparatively, fresh CAR-T cells secreted significantly higher levels of IF and T F , with peak concentrations at 48 hours of 3.5 ± 0.2 ng/mL and 0.6 ± 0.1 ng/mL, respectively. Fresh CAR-T cells in monoculture produced low levels of each cytokine. This distinct loss of cytokine secretion in wells with the repeat antigen challenged T cells is a clear sign of their exhaustion.

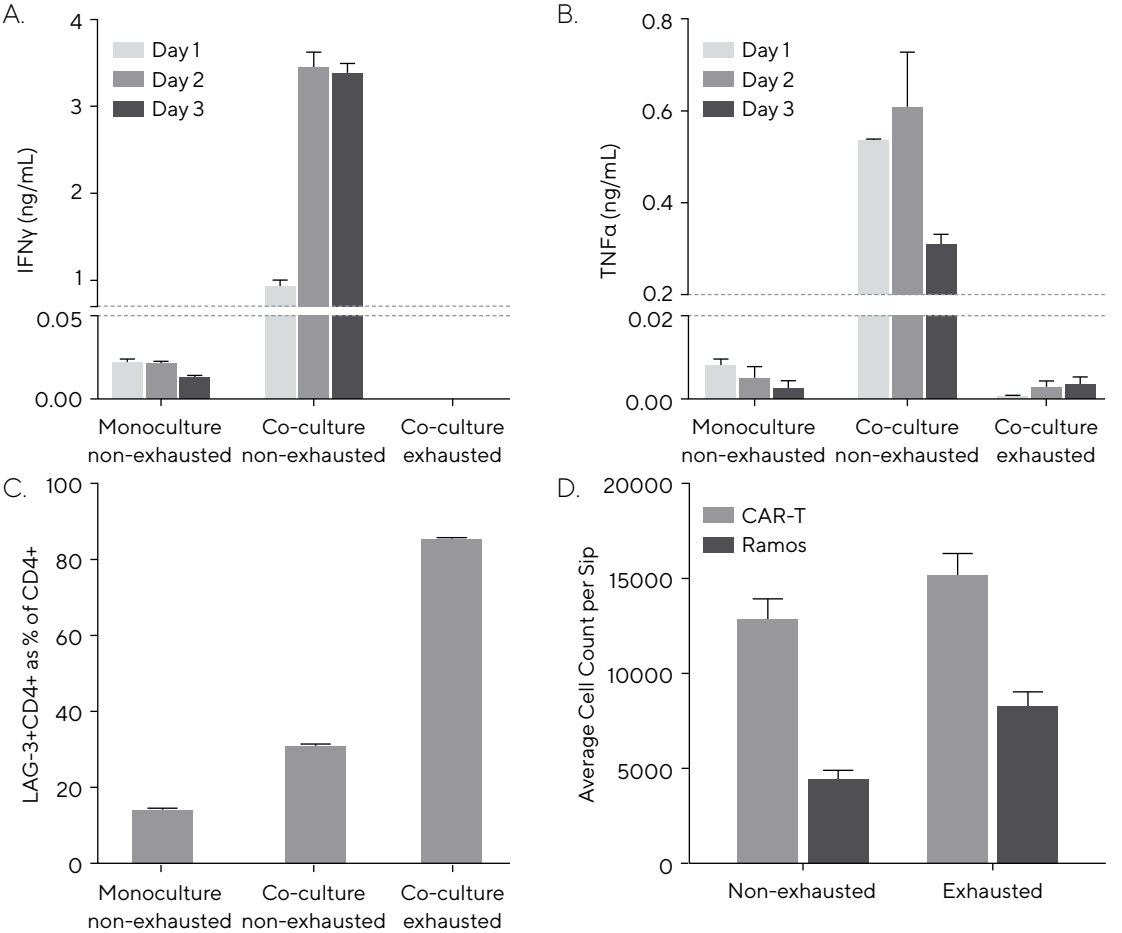
After 72 hours, expression of the LAG-3 exhaustion marker was highly elevated in the challenged CAR-T cells, with 86 ± 0.3% of the CD4+ population positive for this phenotype, whereas the freshly stimulated CD4+ CAR-T cells had just 31 ± 0.6% expression (Figure 6C). The CAR-T cell monoculture had a small population positive for LAG-3 (17 ± 0.8%).

A reduction in the exhausted CAR-T cells' ability to kill the target cells was also observed in the co-culture incubations (Figure 6D). Non-exhausted CAR-T cells were able to reduce Ramos cell numbers more effectively than exhausted CAR-T cells, with iQue® acquired values having an average of 4606 ± 463 cells per sip from wells containing non-exhausted CAR-T cells compared to 8483 ± 688 cells per sip with the exhausted CAR-T cells. The reduced ability of T cells to kill target cells is another hallmark of exhaustion.

Overall, this complete quantification demonstrates a clear antigen-specific driven exhaustion profile in these anti-CD19 CAR-T cells. The data demonstrates the utility of the exhaustion profiling kit in this type of cellular profiling.

Figure 6

Anti-CD19 CAR-T Cells Challenged With the CD19+ Ramos Cell Line Exhibited a Clear Exhaustion Phenotype



Note. Exhausted and non-exhausted anti-CD19 CAR-T cells were seeded separately at 50K/well. Incucyte® Nuclight Green labeled Ramos cells were then added at 50K/well (T:E ratio of 1:1). Non-exhausted CAR-T cells grown as a monoculture were used as controls. Cytokine samples were taken every 24 hours. After 72 hours, all cells and supernatant samples were analyzed using the iQue® Human T Cell Exhaustion Kit. Each data point represents mean ± SEM, n = 4 wells.

Case Study 3: Solid Tumor Killing and "On Target Off Tumor" Profiling with HER2 CAR-T Immune Cell Killing

Post clinical success of anti-CD19 CAR-T therapies for liquid tumors, there has been increased interest in applying similar therapies to solid tumors, for example, in the fight against breast cancer. An obvious target of interest in this area is the HER2 (ERBB2) receptor which has been identified to be over-expressed in many breast cancers. Unfortunately, in early trials, there were serious adverse events in the clinic linked to "on target off tumor" effects and further testing was stopped.[14, 15] It was indicated that the CAR cells had attacked other "off tumor" cells throughout the body that expressed low levels of HER2 epitope and were, therefore, defined as "on target." There is additional evidence in the literature that the affinity of the CAR-T interaction with the HER2 antigen can also contribute to this effect.[16]

To model potential "on target off tumor" effects *in vitro*, a spheroid co-culture with anti-HER2 CAR-T cells was used to mimic the immune killing of a solid tumor. Three cell lines were profiled for their HER2 expression (Figure 7A) showing a spectrum of expression levels relative to IgG isotype control. AU565 display the highest expression of HER2 (median fluorescence intensity (MFI) with IgG background subtracted 1×10^6). MDA-MB-231, often used as triple negative, control line, in our experiments show a very low level of expression relative to IgG (MFI 1.1×10^4) while MDA-MB-468 show minimal expression (MFI 4.2×10^3).

The three cell lines, modified to express a nuclear restricted green fluorescent protein (Incucyte® Nuclight Green Lentivirus), were seeded into ultra-low attachment (ULA) plates and allowed to form single spheroids over 3 days in the presence of Matrigel® (1.25%). Once formed, anti-HER2 CAR-T cells or mock transduced control T cells were added to the wells at various T:E ratios. Spheroids were imaged in the Incucyte® for 7 days and green fluorescence intensity was quantified as a measure of spheroid health.

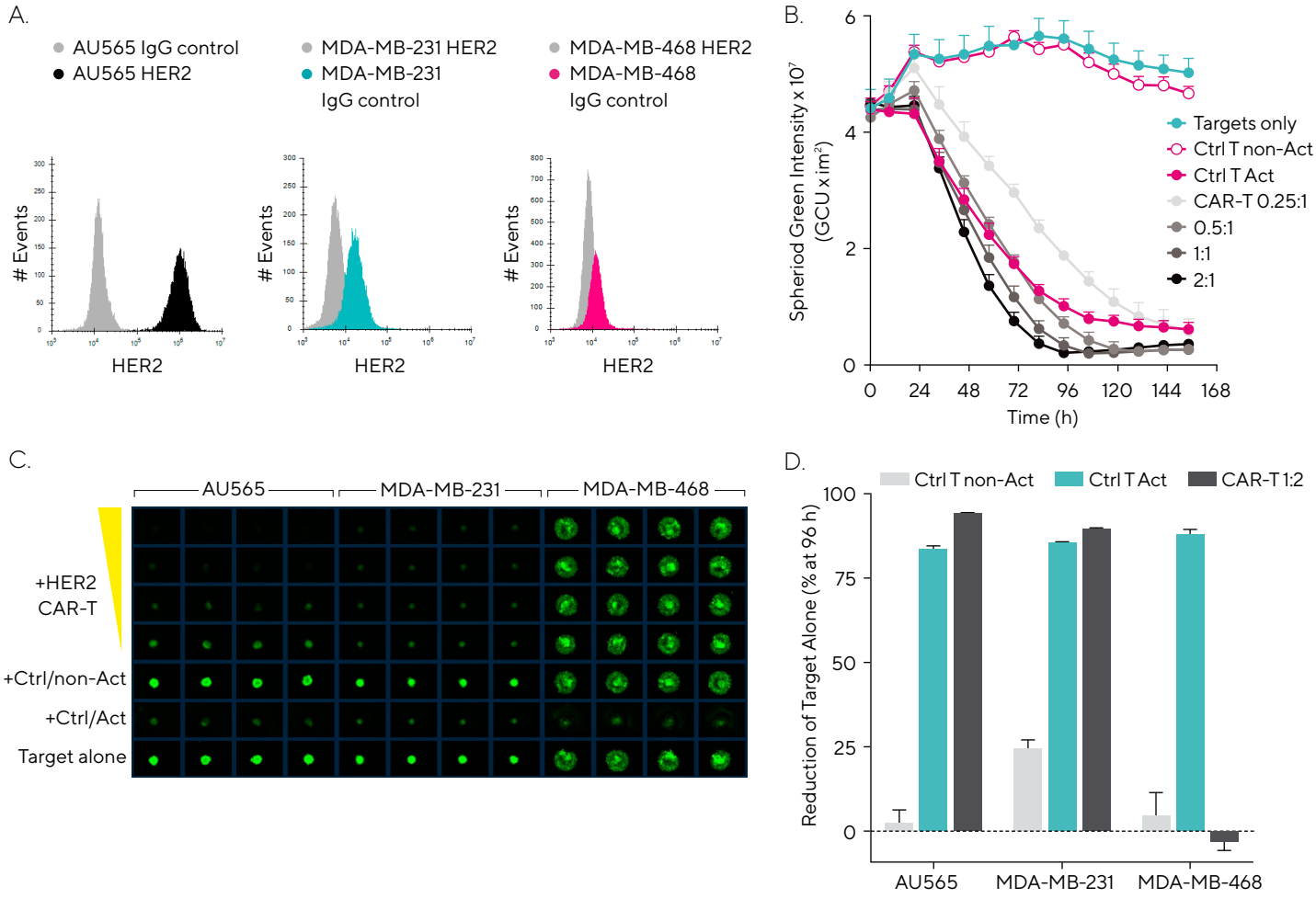
Results with the high expressing AU565 cells demonstrate a clear CAR-T cell driven reduction in green fluorescence (Figure 7B), indicating spheroid death (96% reduction at 96 h compared to target cells alone). No death was measured with non-activated, mock transduced T cells unless in the presence of CD3/CD28 Dynabeads®. When using low level expressing MDA-MB-231 cells, there was also a strong killing effect with the anti-HER2 CAR-T cells, indicating an "on target off tumor" effect (Figure 7C and D). The effect on MDA-MB-231 was seen across two separate CAR-T cell preparations and is in line with previously reported anti-HER2 CAR-T data.[16] The extent of killing measured was similar in strength to that seen with the AU565 cells (91% reduction at 96 h). When using MDA-MB-468 target cells, which have minimal expression of HER2, no CAR-T driven death was measured (Figure 7C and D).

As in the previous example, on Day 2, 4, and 7, samples were analyzed on the iQue® platform. Supernatants were collected for secreted protein analysis before cultures were gently dissociated to remove Matrigel® and break up the spheroids. Samples were assessed for phenotype and function using the iQue® Human T Cell Activation and iQue® Human T Cell Killing kits. The results show an increase in CD69 and CD25 activation markers on the CD8+ population for both AU565 and MDA-MB-231 co-cultures with anti-HER2 CAR-T cells (Figure 8A and B). This effect was absent in the presence of mock transduced T cell. The MDA-MB-468 cells showed no change compared to control T cells for CD69 and low levels for CD25 which decreased by Day 4. Supernatants were assessed for IF and Granzyme B levels using iQue Qbeads® detection as part of the kits. Once again both AU565 and MDA-MB-231 CAR-T co-cultures demonstrated high levels for both proteins while nothing was detected in the MDA-MB-468 co-culture wells.

Both the live-cell analysis and flow data indicate anti-HER2 CAR-T driven killing or activation of T cells in co-cultures with high expressing AU565 and low expressing MDA-MB-231 cells, indicating the potential for "on target off tumor" effects with these cells. The lack of any activity in the presence of MDA-MB-468 cells demonstrates the expected specificity of the anti-HER2 CAR-T cells. Similar data was observed in a 2D monolayer version of the assay (data not shown).

Figure 7

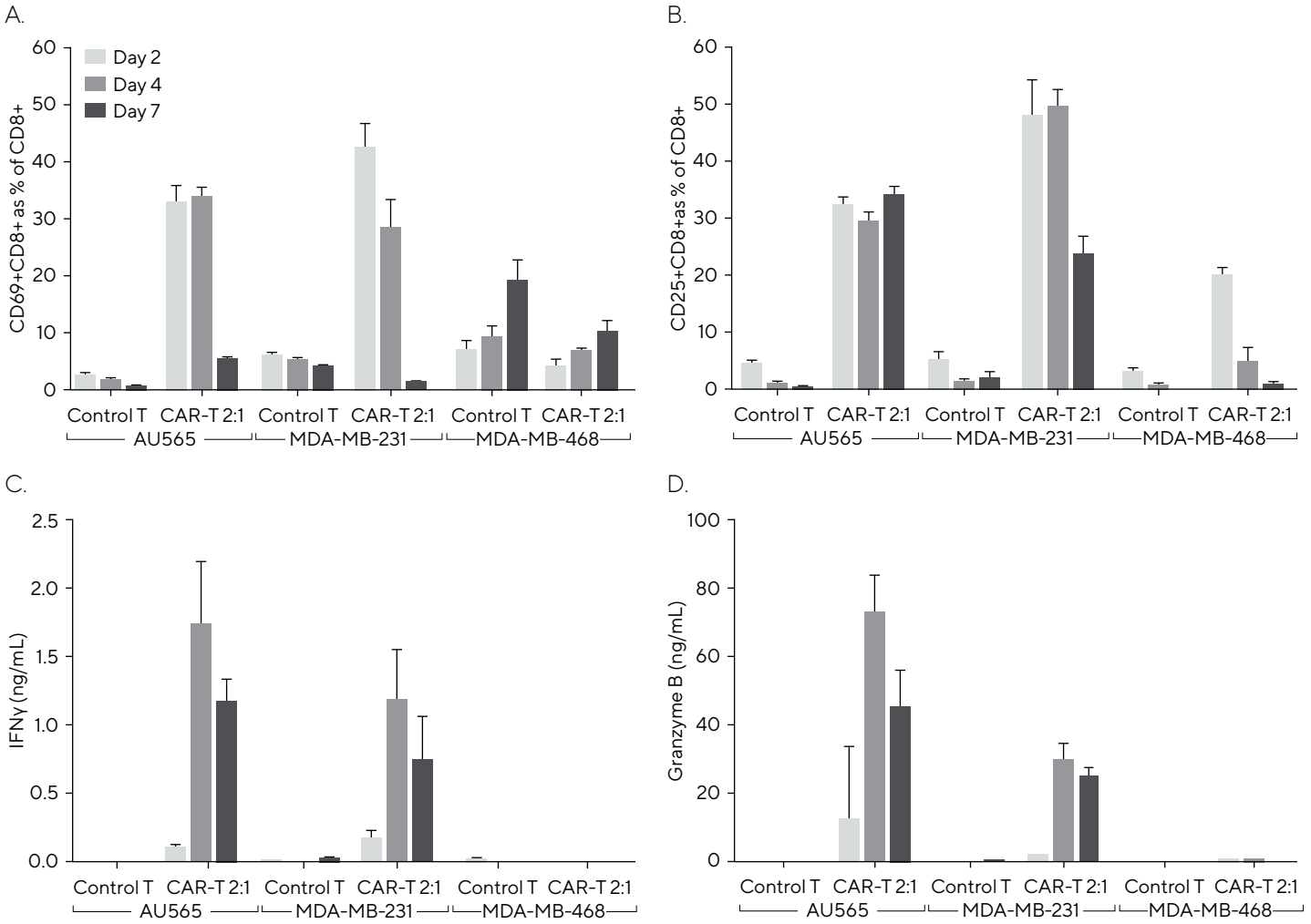
"On Target Off Tumor" Anti-HER2 CAR-T Driven Killing in a Solid Tumor Co-Culture Model



Note. Histograms (A) show AU565 > MDA-MB-231 > MDA-MB-468 for HER2 expression relative to IgG isotype control using iQue®. Time course graph (B) shows the reduction in AU565 spheroid green intensity over 7 days when in co-culture with CAR-T but not mock transduced T cells. The deep well view (C) and bar graph (D) indicate an "on target off tumor" effect when using MDA-MB-231 cells and no effect on MDA-MB-468 cells. All data shown as mean ± SEM of 4 wells.

Figure 8

"On Target Off Tumor" Activation of T Cells in a Solid Tumor Co-Culture Model



Note. Samples were quantified on Day 2, 4, and 7 for surface marker expression and secreted protein using either iQue® Human T Cell Activation Kit or the iQue® Human T Cell Killing Kit. Graphs (A and B) show expression levels in CD8+ T cells of CD69 or CD25, and graphs (C and D) show levels of IFNγ or Granzyme B for each target cell co-culture with either non-activated mock transduced T cell or anti-HER2 CAR-T cells. The 3 bars represent Day 2, 4, and 7, all data shown as mean ± SEM of 4 wells.

Summary and Conclusions

The use of advanced cell therapies is rapidly developing, with increased focus on improving the efficiency of cell production for use in the clinic. Development is focusing on improving construct longevity, selectivity, manufacturing, and delivery to the patient. The potential switch to the use of allogeneic, off-the-shelf products offers a number of potential benefits for the clinic, such as reduced cost of manufacturing, improved long-term storage of cells, and increased consistency of larger batches. As mentioned, development is ongoing in the area of CAR-NK cells which have the benefit of HLA-independent therapies and are attractive for potential allogeneic therapies.

The data examples shared in this whitepaper demonstrate how the use of both live-cell analysis and advanced flow cytometry can add value when developing and characterizing T cell therapies. These techniques have value at multiple stages in the development and expansion of cell products and can be applied to multiple cell types.

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Application Note

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Keywords or phrases:
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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).

Find out more at:
www.sartorius.com/msc

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).

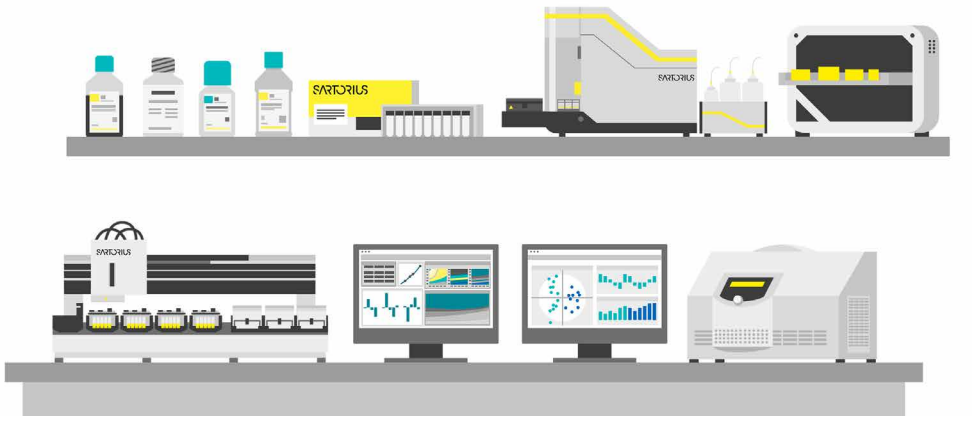
The clinical need for large quantities of MSCs is hampered by the low number of MSCs found in adult tissue (only 0.001–0.01% in bone marrow (Pittenger M. F. 1999). Therefore, extensive ex vivo expansion is required to reach clinically relevant doses. Various platforms have been evaluated for MSC expansion. 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.

1



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 BDT CompBeads Anti- ouse Ig, and Negative Control beads were used according to the manufacturer's instructions.

Data was obtained using the iQue® Screener PL S ow 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®.

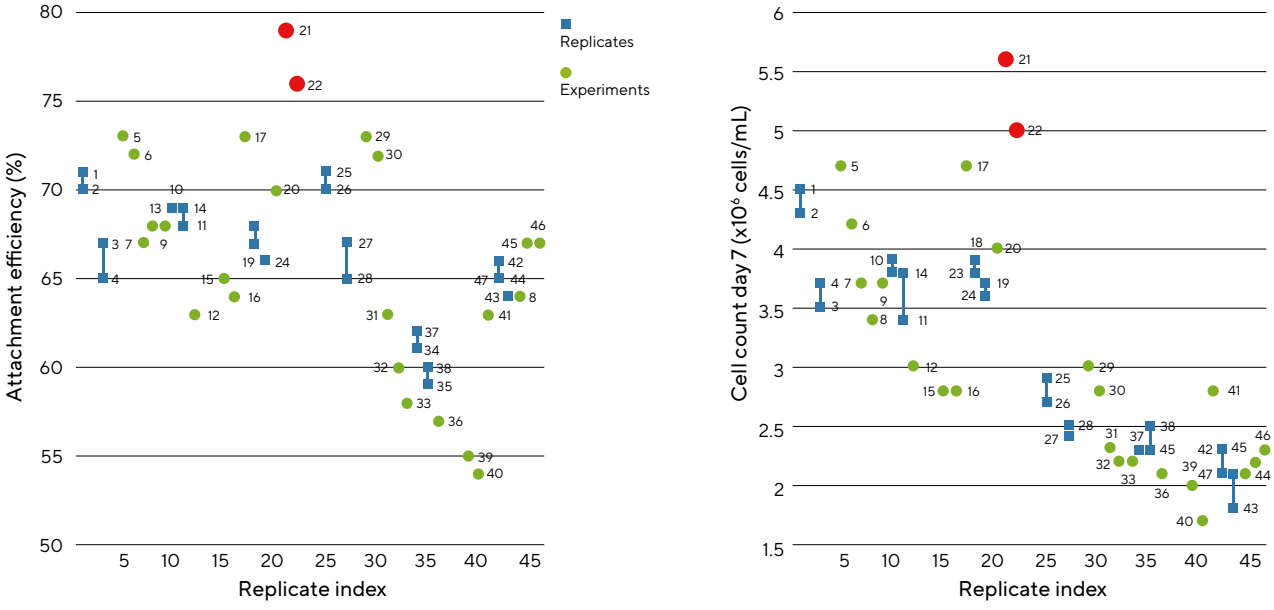
Data Analysis Using MODDE® Software

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



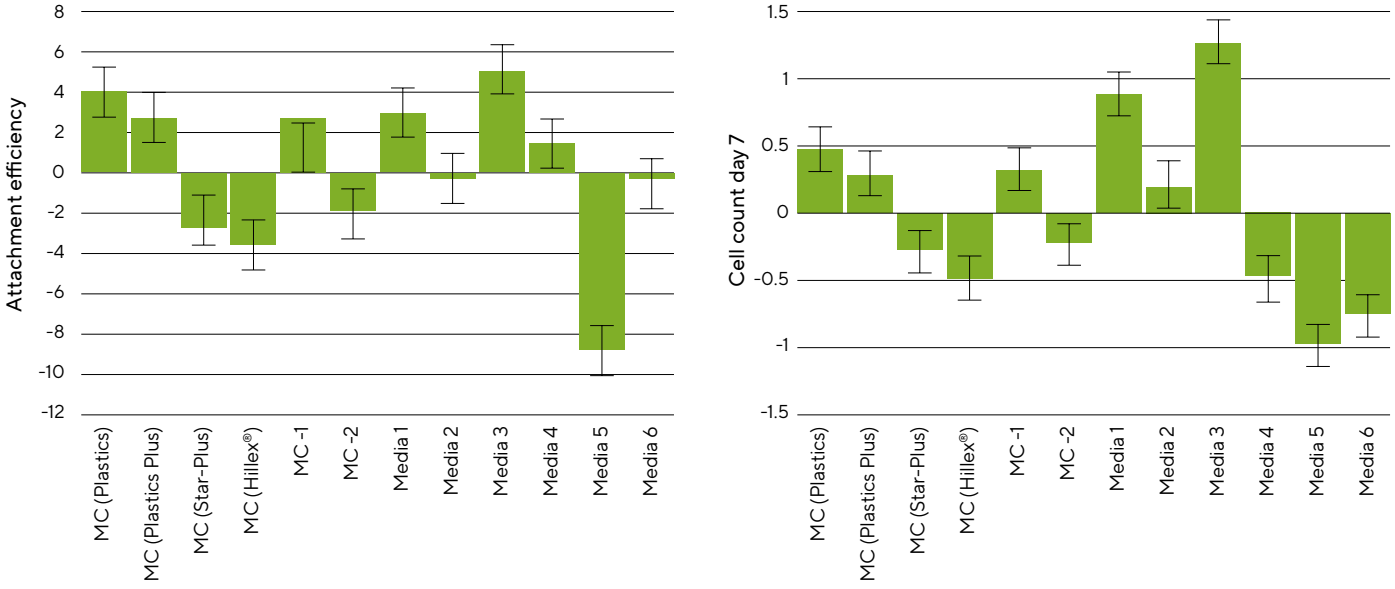
Note. Replicate plots of attachment efficiency (left) and viable cell count on day 7 (right). Experiments are numbered 1 – 48. Replicate experiments are shown in blue color, connected with a bar. Experiments which were not replicated are shown in green. Experiments 21 and 22 are marked in red for highlighting 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



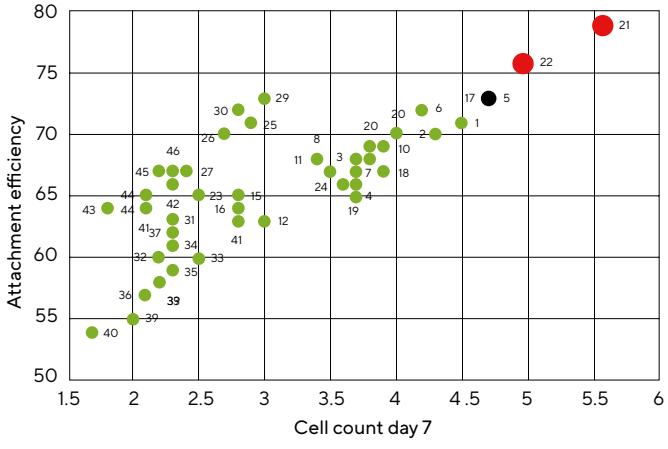
Note. Coefficient Plots for model interpretation. Displayed are the coefficients of each model term (in this case for each factor) and their confidence intervals for the responses attachment efficiency (left) and cell count on day 7 (right).

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.

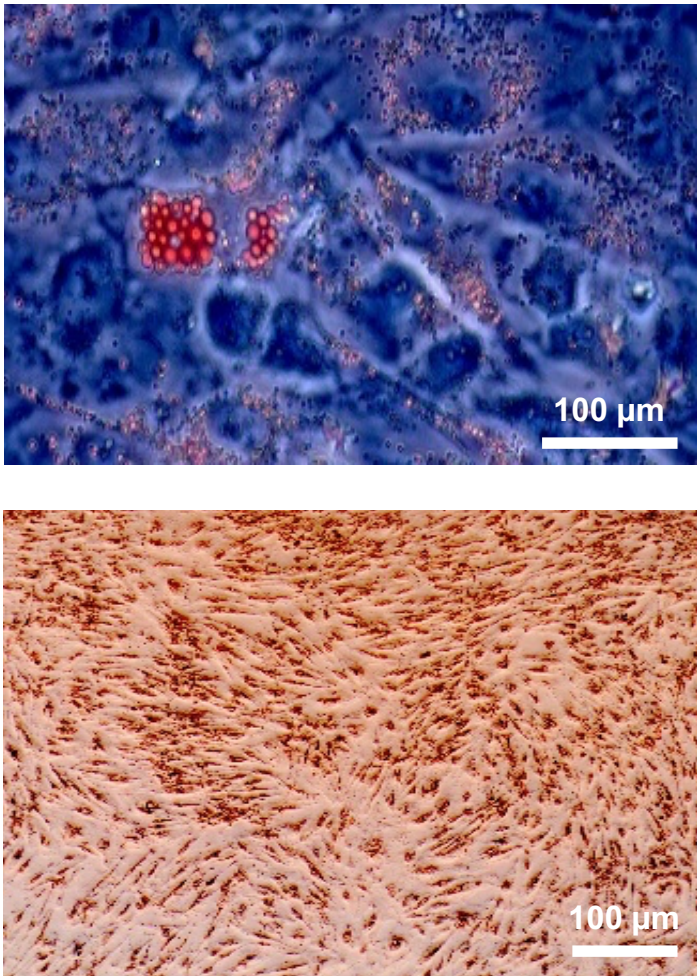
(+) ve markers	(+) ve - %	(-) ve markers	(-) ve - %
CD 73	99	CD 14	4%
CD 105	99	CD 19	4%
		CD 34	4%
		CD 45	4%
		HLA-DR	2%

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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