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Standardized and GMP-Ready, Closed-System Processing of Expanded Adipose-Derived Stem Cells Using the Gibco CTS Rotea System Across 2D and 3D Bioreactor Expansions

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ABSTRACT

The manufacturing of cell-based therapies requires harmonized processing protocols to ensure consistent quality. Expanded adipose-derived mesenchymal stromal cells (ASC) are among the most promising candidates for such therapies due to their regenerative and immunomodulatory properties. However, transitioning these therapies to large-scale production presents challenges related to cell recovery, formulation, and in-process cell counting (IPC).

The Gibco CTS™ Rotea™ multipurpose Counterflow Centrifugation System enables multiple workflow operations—including cell separation, concentration, washing, and buffer exchange—and is designed to operate within a GMP environment. From a cell concentration perspective, the Rotea system can also formulate concentrated cells in different types of media, including fetal bovine serum.

In our laboratory, we developed a protocol for washing and concentrating expanded ASC. However, the initial optimization was performed in DMEM. To implement improvements suitable for industrial manufacturing—such as the use of xeno-free media that does not require media exchange—we sought to validate the performance of this protocol in both 2D and 3D production systems using the RoosterNourish-MSC-XF media. Considering the Rotea as a key improvement in the manufacturing workflow, it is important to recognize that changes introduced by this system also affect IPC requirements. Thus, accurate cell counting at the end of the process, and prior to cryopreservation, becomes essential. Our investigation demonstrated that counting cells formulated in FBS can compromise counting precision, as FBS directly affects cell size and complexity. Therefore, modifying downstream processing protocols must be accompanied by an adapted counting method appropriate for these sample types.

In summary, this work describes the Rotea-based downstream cell concentration method, demonstrates its suitability when using the RoosterBio medium as a growth matrix, and outlines the complete process from cell harvest to formulation. Additionally, we propose an improved IPC strategy for final cell enumeration, integrating a comprehensive downstream workflow with the Rotea system as its central component.

INTRODUCTION

Advanced therapy medicinal products (ATMP) and gene and cell therapies (GCT) are successfully progressing from manual to automated cell manufacturing processes.[1-3] Bioreactor implementations are moving the process to a closed system with automation during the cell expansion workflow.[4] The capacity to work in a controlled environment can reduce costs, improve the volumes, and allow industry to reach production capacities higher than current levels.[5] Moreover, automation also guarantees a more controlled process that

is operator-independent and easier to reproduce later.[6,7]

From this standpoint, developing downstream processes (*e.g.*, wash-volume reduction) that work for most cell types are very important and can be a real challenge.[8] Benchtop centrifugation is the most accepted method for the concentration of cells. High rates of recovery and safety for cell integrity and activity have been demonstrated and reported extensively.[9] The most common problems the GCT industry faces are low cell recovery levels, large dead volumes that cause significant cell loss in tubes, incompatibility between platforms when expansion and centrifugation devices are not produced by the same manufacturer, and devices designed for R&D but aren't really ready for GMP.[10] However, the use of automated centrifugation devices can be an improvement for 2D expansion processes, mitigating contamination risks and reducing processing times.[11,12] Therefore, the objective of the GCT industry should be to develop systems with similar cell recovery percentages to centrifugation while improving overall processing times, and reducing contamination risks[13] and costs.[14]

The Rotea system separates cells using the principle of counterflow centrifugation (CFC), a method for separating cells based on their sedimentation characteristics (size and density). The system suspends cells in a fluidized bed by exerting a constant flow rate force against centrifugal forces. Cells suspended in liquid are gently concentrated without piling up in the cone. They are efficiently washed with acceptable recovery rates at least comparable to that of standard centrifugation[3], and high viability percentages often improved through elutriation.

In our laboratory, we have optimized the Rotea for use with ASC during the harvesting steps of ASC expansion. The concentration and formulation of cells was performed in bags obtained at high concentration (around 50×10^6 cells/mL in FBS) from the Rotea to continue the downstream process in hypothetical formulation devices. The final concentration was 25×10^6 cells/mL in FBS:DMSO (90:10, v/v) prior to freezing. It has been described that FBS can interfere with the accuracy of cell counting. Extracellular vesicles present in FBS can be counted as cells[15] or can modify cell size, impacting the segmentation of cells performed by an automated cell counter. The FBS effect can impact counting protocols because some cells might not be included in the counting.[16] During the Rotea processing setup, it was observed that the cells decreased in size when resuspended in FBS at the end of the process versus cells in complete DMEM (cDMEM). Additionally, the number of cells counted in FBS were lower than when counted in cDMEM.

From a QC standpoint, the accuracy of the counting method is critical.[17] Determining the right number of cells before freezing directly impacts the quality of the product and patient dosing. Thus, having confidence and accuracy in counting methods and protocols is mandatory, from all points of view.

The typical culture method for ASC expansion in clinical and preclinical studies is 2D, with the plastic adherent culture method. But the 3D suspension culture bioreactor process is increasing in use with ASC (and MSC, in general). The industry demands methods that can be controlled, standardized, automated, and capable of scaling production rates to meet future market demands.[9,18]

This paper will cover:

- Process optimization of the Rotea counterflow protocol for ASC in 2D and then 3D expansion in the RoosterBio xeno-free media.
- The ~10 % decrease in cell size after exposure to FBS (or FBS+DMSO), which affected our cell counting accuracy. Both effects were immediately reversed when cells were resuspended again in cDMEM.
- The full process of cell concentration and formulation, including intermediate counting methods that allow the monitoring of harvesting, concentration, and formulation processes to maintain GMP compliance as an in-process control.

MATERIALS AND METHODS

Isolation and Cultivation of Human ASC

After research ethics committee approval, cell isolation was processed according to Costa *et al.*[19] for use as master cell stock. Cells were thawed, centrifuged, resuspended in culture medium, and counted using a NucleoCounter NC-202 (ChemoMetec). Expansion was performed in five-layer CellSTACK culture chambers (2D, Corning) or in 3D using two different systems: the PBS-3 L vertical-wheel bioreactor (PBS Biotech) and a Biostat B Bioreactor Twin CC with 2 L/2 L single-use Univessels (Sartorius). The culture media was a mixture of RoosterBio's RoosterNourish-MSC-XF media and RoosterBasal 2.0-CC (50:1, v/v) plus 30 ng/mL of Gibco gentamicin (Thermo Fisher Scientific). After expansion, cells were frozen at 25×10^6 cells/mL in 1 mL cryovials in a solution of FBS:DMSO (both Thermo Fisher Scientific) 90:10, v/v.

Also, after research ethics committee approval, cell

isolation was processed according to Costa *et al.*[19] and then grown in cDMEM. They were seeded and cultured in flasks, and expanded at 37 °C, 5% CO_2, 90% RH with media exchanges every 3–4 days for use as master cell stock.

2D Cell Expansion and Harvest

Cells were seeded at 600 cells/cm² at 37 °C, 5% CO_2, 90% RH in the xeno-free medium and expanded for 7 days. Then cells were harvested with trypsin/EDTA (Thermo Fisher Scientific), and the reaction was stopped with cDMEM (Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific) in a ratio of 50:50, v/v.

The resulting cell suspension was transferred to a 225 mL tube and centrifuged at 300×*g* for 10 minutes. Following centrifugation, the supernatant was discarded and the cell pellet was resuspended at a final concentration of 25×10^6 cells/mL in FBS containing 10% DMSO (WAK-Chemie Medical).

3D Cell Expansion and Harvest

In the vertical-wheel bioreactor setup, cells were directly seeded post-thaw at a density of 1,750 cells/cm² using 10 g/L Low Concentration Synthemax II Microcarriers (Corning) and the xeno-free medium. On day 0, only 60% of the total working volume was used with a top-off to 2 L performed after 24 hours (h). Culture conditions were maintained at 37 °C, pH 7.2, and 40% dissolved oxygen with a gas flow rate of 0.1 VVM. Continuous agitation was maintained at 15 rpm for 7–9 days.

In the stirred tank bioreactor setup, cells were seeded directly after the thawing step at a density of 3,500 cells/cm² along with 16 g/L of the Synthemax II microcarriers and xeno-free medium. On the first day, 40% of the working volume was used, and a top-off to 2 L was performed after 24 h. The dissolved oxygen level was maintained at 40% with a gassing flow rate of 0.1 VVM while the pH was set to 7.2 and the temperature at 37 °C, for 5–7 days. The agitation strategy started at 90 rpm on the first day and gradually increased by 10 rpm each subsequent day.

For both 3D processes, cells were harvested by allowing the microcarriers to settle to the bottom of the vessels followed by aspiration of the culture medium. The microcarriers were then washed with 1 L of Gibco PBS (Thermo Fisher Scientific), allowed to settle again, and the PBS was aspirated. Subsequently, 1 L of Gibco TrypLE Select 1× (Thermo Fisher Scientific) was added, a non-animal alternative for porcine trypsin. The digestion was carried out at 37 °C using pulses of agitation for 45 min of incubation. The enzymatic reaction was neutralized by adding 500 mL of cDMEM. To separate the cells from the microcarriers, the suspension was passed through a closed-system Harvestainer bag (Thermo Fisher Scientific), which was connected to a 2D Flexsafe 3 L bag (Sartorius) and processed by Rotea.

Rotea Protocol

The basic protocol for Rotea, established by the vendor, is divided into cell loading, elutriation, media exchange/washing, and cell concentration (**Figure 1**), and is divided into the following five principal steps:

(1) Priming. The initial step in which all bubbles and air in the tubes are eliminated. When this step is finished, the system is ready to start.

(2) Bed Formation. Cells are introduced in the cone slowly until a cell bed is established. This stability allows the rest of the cells to be collected without cell loss. Fluid is recirculated.

(3) Cell Loading. Cells are collected in the cone until reaching the cone limit, which is determined by the size of the cells and the flow rate. An increase in flow rate decreases cone capacity, so a balance between cone capacity and flow rate is necessary.

(4) Wash. This is an important step where the culture medium is completely removed in order to prepare cells for the formulation step.

(4) Harvest. Washed cells are collected in the final

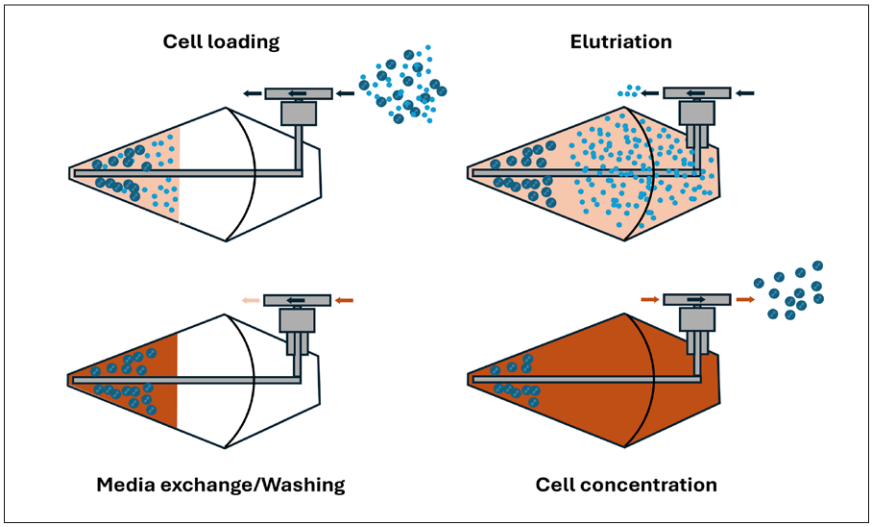


FIGURE 1. Process of centrifugation with Rotea.

TABLE 2. Rotea system details.

Specifications	Description
Centrifugal force	Up to 3000×*g*
Peristaltic pump flow rate	5–160 mL/min
Centrifuge chamber (cone) volume	10 mL
Cell number per chamber load (depending on cell size)	5×10^7–5×10^9 cells
Output cell concentration (depending on cells)	5×10^6–500×10^6 cells/mL
Recommended input volume range	50 mL – 20 L
Minimum output volume	5 mL
Maximum fluid density	1.10 g/mL

storage bag (Miltenyi Biotec) with a capacity of 50 mL.

Aliquots are taken from the bag and counted. Dilution in DMEM is necessary to avoid the negative effects of FBS on cell counting. Finally, cells are frozen in DMSO/FBS solution 1:10 v/v.

Rotea system details are described in **Table 2**. Our Rotea protocol was established for ASC with a total of 28 different experiments (data not shown) during which we defined parameters to be followed for processing our cells (see **Table 3**).

Bed Formation

A fixed cell-bed volume corresponding to 60×10^6 cells was defined as a safety parameter to ensure full cell retention. The "60×10^6 cells/bed/mL" value was obtained by entering an input concentration equivalent to 60×10^6 total cells from which the software automatically calculated the required bed-formation volume. This calculated volume was applied in step 14, as indicated in **Table 4**.

Capacity of the Cone

We established the capacity of the cone at 600×10^6 cells. The cone can harbor more cells—with some donors, we have reached 800×10^6 cells in the cone. Nevertheless, we fixed 600×10^6 cells as a limit to define settings independent of the donor used. This parameter is introduced in step 15 (see "1×processed cells/mL [max 600×10^6]"in **Table 4**). It is calculated from the relationship between the total number of cells and the input concentration. If the resulting volume exceeds the system's capacity, an automatic loop between steps 13 and 24 is executed—triggered by the bubble sensor—until the bag is completely emptied.

Flows

We established the flow for bed formation at 35 mL/min and found the optimal balance of 80 mL/min between maximum flow and number of cells in the cone to be obtained during the cell-loading step.

Minimal Cell Concentration

We established that the minimum cell concentration that could be processed in the Rotea was 0.12×10^6 cells. With this concentration, cells should be centrifuged or other concentration methods should be used.

Harvest Concentration

We observed the feasibility of concentrating cells to between 45 and 65×10^6 cells/mL. This allowed the following downstream freezing steps to 25×10^6 cells/mL per vial. The harvest volume is the parameter entered into the software. This value is derived by dividing the total number of cells obtained by the target final concentration (see "harvest volume [mL]" in **Table 3**).

TABLE 3. Input parameters set with the Rotea software.

Input Parameter	Description
60×10^6 cells/bed/mL	60×10^6/mL input concentration
Processed cells/mL (max 600×10^6)	600×10^6/mL input concentration
Harvest volume/mL	500×10^6/mL input cells

TABLE 4. Protocol steps used with the Rotea system.

Step	Description	Flow Path	Speed	Flow Rate	Step Type	Triggers
1	Pre-prime A and B	B → A	0×*g*	100 mL/min	Normal	Input bubble sensor, on
2	Lubricate rotary coupling	B → A	0×*g*	100 mL/min	Normal	Input bubble sensor, on Volume: 15 mL
3	Fill CFC chamber and prime A	B → A	0×*g*	100 mL/min	Normal	Input bubble sensor, on Volume: 15 mL
4	Add priming volume	B → A	10×*g*	100 mL/min	Normal	Input bubble sensor, on Volume: 30 mL
5	Fill bubble trap and prime B	A → B	10×*g*	100 mL/min	Normal	Input bubble sensor, off Volume: 20 mL
6	Prime C	A → C	10×*g*	100 mL/min	Normal	Input bubble sensor, off Volume: 5 mL
7	Prime D	A → D	10×*g*	100 mL/min	Normal	Input bubble sensor, off Volume: 5 mL
8	Prime G	B → G	10×*g*	25 mL/min	Normal	Input bubble sensor, on Volume: 7 mL
9	Pressure prime	B → EF	10×*g*	0 mL/min	Pressure Prime	Input bubble sensor, off
10	Prime precirculation	J → K	10×*g*	25 mL/min	Pause	Volume: 3 mL
11	Ramp flow	J → K	10×*g*	100 mL/min	Pause	Time: 31 sec
12	Ramp G	J → K	3,000×*g*	100 mL/min	Pause	Time: 31 sec
13	Ramp to build bed	J → K	3,000×*g*	100 mL/min	Pause	Time: 15 sec
14	Build bed 60M cells/mL	D → G	3,000×*g*	35 mL/min	Normal	Input bubble sensor, off Volume: 1×10^6 cells/bed/mL
15	Load cells to waste at 80 mL/min	D → A	3,000×*g*	80 mL/min	Normal	Input bubble sensor, on Volume: 1× processed cells/mL (max 600×10^6)
16	Pause and decrease flow rate	J → K	3,000×*g*	30mL/min	Pause	Time: 20 sec
17	2 mL pre-wash	B → A	3,000×*g*	30 mL/min	Normal	Input Bubble Sensor, on Volume: 2 mL
18	Pause	J → K	3,000×*g*	30 mL/min	Pause	Time: 20 sec
19	Wash	B → A	3,000×*g*	25 mL/min	Normal	Input bubble sensor, on Volume: 30 mL
20	2 mL pre-wash	C → A	3,000×*g*	25 mL/min	Normal	Input bubble sensor, on Volume: 2 mL
21	Pause	J → K	3,000×*g*	25 mL/min	Pause	Time: 20 sec
22	Wash	C → A	3,000×*g*	25 mL/min	Normal	Input bubble sensor, on Volume: 40 mL
23	Ramp to concentrate	J → K	3,000×*g*	15 mL/min	Pause	Time: 20 sec
24	Harvest	C → H	3,000×*g*	80 mL/min	Harvest	Volume: 1× harvest volume/mL
25	Slow down to stop	J → K	100×*g*	10 mL/min	Pause	Time: 10 sec

Analytical Assays

Viability

Cell count and percent viability were determined with the NucleoCounter following the manufacturer's instructions and using a specially designed protocol for counting ASC.

Adhesion

The percentage of viable cells after 24 h, with respect to total cells seeded, was performed following the protocol described by Valero *et al.*[20] This equation was used:

$$Adhesion\,(\%) = \frac{Number\ of\ viable\ cells\ harvested\ after\ 24\ hours}{Number\ of\ total\ cells\ seeded} \times 100$$

Surface Marker Characterization

After thawing, the cells were resuspended in cell culture medium at 1 to 2.5×10^6 cells/mL, counted and washed with PBS. After washing, cells were stained with paired anti-CD29/CD90 and anti-CD73/CD105 antibodies (Miltenyi Biotec) as well as Zombie violet (BioLegend) as a viability marker. Stained cells were analyzed with a MACSQuant Analyzer 10 Flow Cytometer with the MACSQuantify Software (Miltenyi Biotec).

IDO Activity

Indoleamine 2,3-dioxygenase (IDO) is an intracellular cytosolic enzyme that regulates the degradation of tryptophan in kynurenine. Regulation of tryptophan metabolism is related to the immunomodulatory properties of ASC. For this reason, IDO activity was determined by measuring the kynurenine concentrations in conditioned supernatants. We followed the protocol shown in Valero *et al.*[20]

Lymphoproliferation Assay (LPA)

For this assay, PBMC from healthy human donors were obtained from STEMCELL Technologies. After thawing, PBMC were resuspended and cultured in Roswell Park Memorial Institute 1640 media (RPMI, Thermo Fisher Scientific) supplemented with 10% FBS, L-glutamine (Thermo Fisher Scientific), and penicillin/streptomycin (Lonza).

This LPA method was conducted to evaluate the ability of ASC to inhibit T cell proliferation when co-cultured with activated PBMC, based on DelaRosa *et al.*[21] and Mancheño-Corvo *et al.*[22] The main outcome measured is the ASC's inhibition capacity, which was determined by calculating an inhibition ratio:

$$Inhibition\ Capacity\,(\%) = 1 - \frac{PBMC\ live\ cells\ in\ the\ co\text{-}culture}{PBMC\ live\ cells\ in\ the\ stimulated\ control}$$

The ASC inhibition capacity was considered effective if the inhibition percentage was 40% or higher. In addition, as a secondary outcome, the proliferation of activated PBMC was assessed at 96 h by calculating the fold change between the stimulated and unstimulated conditions.

Established Minimal Acceptance Criteria for Past Tests

Minimal acceptance criteria considered as passing the above analytical assays are included in **Table 5**.

Statistical Analysis

As the data were not normally distributed, non-parametric paired tests were used to analyze the results. The Wilcoxon matched-pairs, signed-rank test was used to identify significant differences between two groups (final batch vials from the same lot processed by the Rotea and classical centrifugation) while the Friedman non-parametric statistical test was used to identify significant differences among more than two groups (final batch vials from the same lot processed by the Rotea and classical centrifugation). The Kruskal-Wallis test was used to evaluate the comparability between data obtained during the evaluation of cell stability over

TABLE 5. Minimal acceptance criteria.

Parameters	Viability (%)	Adhesion (%)	Cell Surface Markers		IDO Activity		LPA (%)
			CD29+/CD90+ (%)	CD73+/CD105+ (%)	Kynurenine concentration after 24 h (µM)	Kynurenine concentration after 48 h (µM)	
Minimal Acceptance Criteria	70	40	90	90	3	19	40

the time in TrypLE. A p-value of $p<0.05$ was considered statistically significant. All statistical analyses were performed with GraphPad Prism software version 10. The Dunn's multiple comparisons test was performed between each, independent of each three groups in counting assays.

RESULTS

Closed System Processing of 2D-Expanded ASC Material

While developing the protocol in DMEM, we focused on optimizing the washing and concentration steps, after 2D expansion (in the xeno-free media), with the expectation that it would also work for 3D materials.

Figure 2 shows the percentage of recovered cells was more than 80% (83.25±11.4%), which was above the 75% fixed minimum set by the Rotea technicians. Also, elutriation with cDMEM was the same as with the xeno-free media, and viability moved from 93.8±1.7% before the Rotea process to 97.5±2.1% after switching to the Rotea process, increasing 3.7% in viability. Lastly, the average cell concentration was 43.32×10^6 cells/mL, demonstrating the feasibility of using the Rotea to wash and concentrate the cells to a sufficiently high concentration needed to achieve the final formulation at 25×10^6 cells/mL in FBS+DMSO.

Closed System Processing of 3D-Expanded ASC Material

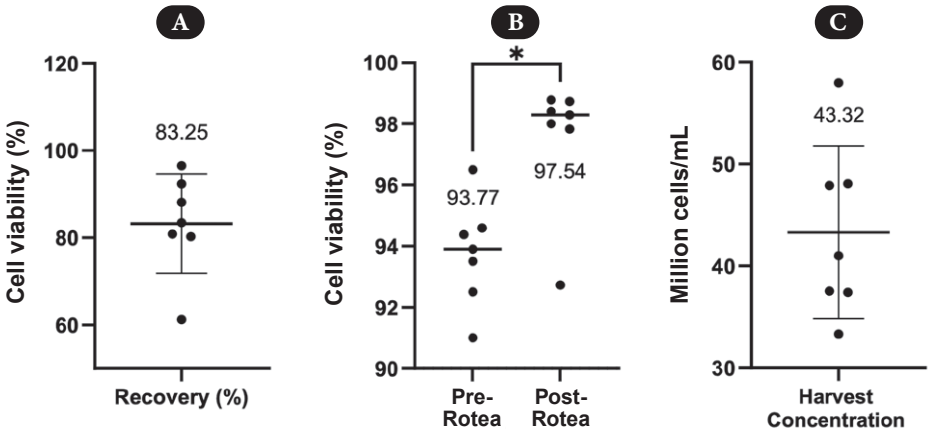
Due to the success of the Rotea process developed for the 2D-expanded cells, we moved forward with the 3D material processing.

As is shown in **Figure 3**, the recovery obtained from cells expanded in both 3D bioreactors was over 75% (78.25±9.7%) of the cells. There was an increase in percentage of viability at the end of the process amounting to 1.6% (elutriation), but this increment was not statistically significant. The average concentration of cells obtained at harvest and after the process was higher than 40×10^6 cells/mL. This concentration is optimal for subsequent downstream freezing processes.

Characterization of Cells Processed by Rotea

Cells expanded in 2D and 3D, and then processed by Rotea, were characterized using a representative number of samples (n=3/6). Viability, adhesion, cell

FIGURE 2. Results obtained after processing the cells expanded in xeno-free media in 2D with the Rotea.

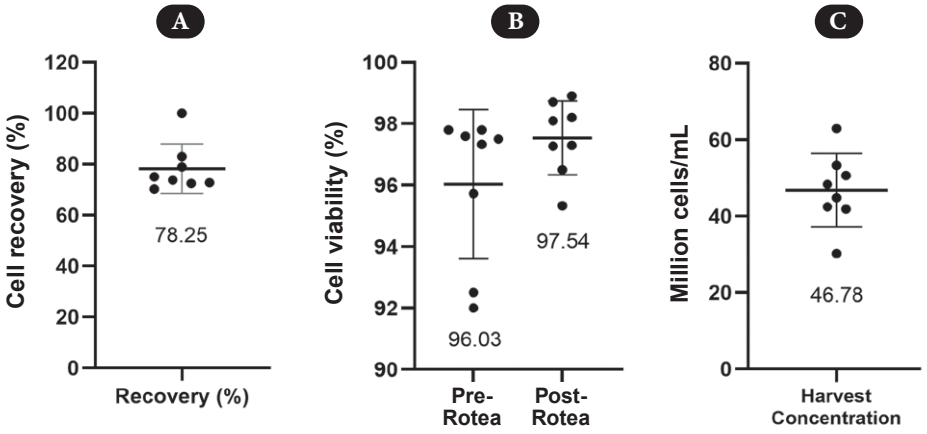


(A) Average percentage of recovery obtained with Rotea after cell harvesting and concentration.

(B) Average percentage of viability before and after Rotea processing. Variation in this percentage showed percentage of elutriation. This variability was statistically significant (Wilcoxon paired test, $p<0.05$, $^*p=0.0031$).

(C) Average of concentration viable cells obtained after processing cells with the Rotea (n=7 technical replicates).

FIGURE 3. Results obtained after processing the cells expanded in xeno-free media in 3D with the Rotea.



(A) Average percentage of recovery obtained with Rotea after cell harvesting and concentration.

(B) Average percentage of viability before and after Rotea processing. Variation in this percentage showed percentage of elutriation. This variability was not statistically significant (Wilcoxon paired test, $p<0.05$).

(C) Average of concentration viable cells obtained after processing cells with the Rotea ($n=8$ technical replicates).

surface markers, IDO activity, and capacity to inhibit the PBMC proliferation were tested. Centrifuged samples were used as control (**Table 6**).

Viability

After thawing, all cells showed a percentage of viability over 90%, demonstrating that the cells were in good condition.

Adhesion

Both 2D and 3D cells processed in the Rotea, after freezing and thawing, showed more than 100% cell adhesion. This was in the range of the centrifuged cells control.

Identity

All markers analyzed were over 90% of positive for the 2D and 3D cells, which was higher than the acceptance criteria limits established for identity.

TABLE 6. Minimal acceptance criteria.

Parameters	Viability at thaw (%)	Adhesion (%)	Cell Surface Markers		IDO Activity		LPA (%)
			$CD29^+/CD90^+$ (%)	$CD73^+/CD105^+$ (%)	Kynurenine concentration after 24 h (µM)	Kynurenine concentration after 48 h (µM)	
Centrifuged Cells	95.6 ± 1.1	139.3 ± 3.3	99.92 ± 0.03	98.24 ± 0.30	10.57 ± 1.00	39.5 ± 2.1	63.6 ± 1.8
2D Rotea-Processed Cells	98.8 ± 0.1	129.7 ± 7.9	100 ± 0	94.2 ± 1.8	9.1 ± 0.9	37.4 ± 1.5	68.4 ± 5.4
3D Rotea-Processed Cells	92.6 ± 6.1	97.2 ± 15.1	100 ± 0.1	99.1 ± 1.0	12.8 ± 4.8	47.5 ± 4.3	46.3 ± 4.9

IDO Activity

Kynurenine expression in cells processed with the Rotea and expanded in bioreactors was similar to control, and no statistically significant differences were observed with respect to centrifuged cells.

LPA Study

A LPA was performed to evaluate the capacity of ASC to inhibit stimulated PBMC. Results are shown in **Table 6** as "percentage of inhibition." No differences in the capacity to inhibit proliferation of PBMC were observed between centrifuged cells and cells processed with the Rotea in both 2D and 3D cells.

All cells processed with Rotea passed the characterization test, and results obtained were above the minimum established acceptance criteria.

Cell Stability in Bioreactor Process

TrypLE + cDMEM

One of the principal objectives of this work was to develop a Rotea counterflow washing and concentration system for implementation in a closed system for ASC manufacturing based on bioreactor expansions. Therefore, the total processing time could be long, depending on bioreactor capacity, and a study regarding the capacity of cells to maintain their characteristics during the process of washing and concentration had to be developed. We tested the time that cells could be maintained in the microcarrier detachment media once reaction was stopped. Thus, cells from bioreactors resuspended in TrypLE (following the protocol described earlier) were filtered, an aliquot was taken, and the sample was counted. We repeated the counting every 30 min for a total time of 4 h. During this time, there was no variation in the number of cells counted, percentage of viability, or cell size observed, and cells maintained stable with viability close to 100% (**Figure 4**).

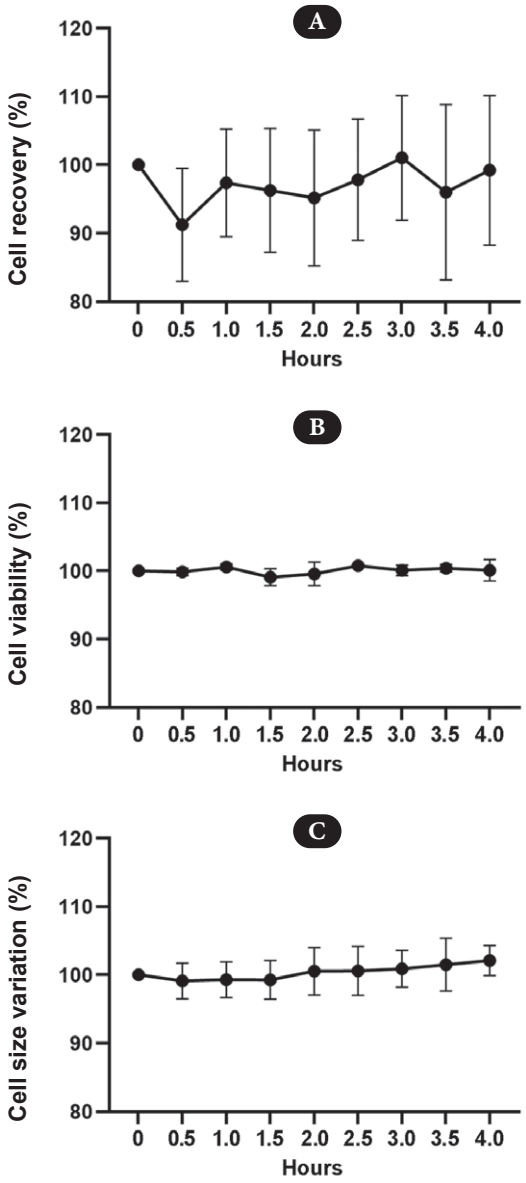
Counting Method After Rotea Centrifugation

After Rotea washing and concentration, the cells were highly concentrated in bags with the FBS matrix. The objective was to move each bag to a formulation device and then dilute to 25×10^6 cells/mL in FBS+DMSO for freezing. The NucleoCounter protocol for ASC was developed by Takeda in collaboration with Chemometec for a cDMEM matrix. So, verification was needed to compare counting in FBS, FBS+DMSO, and DMEM matrices.

Decreasing Size of Cells in FBS

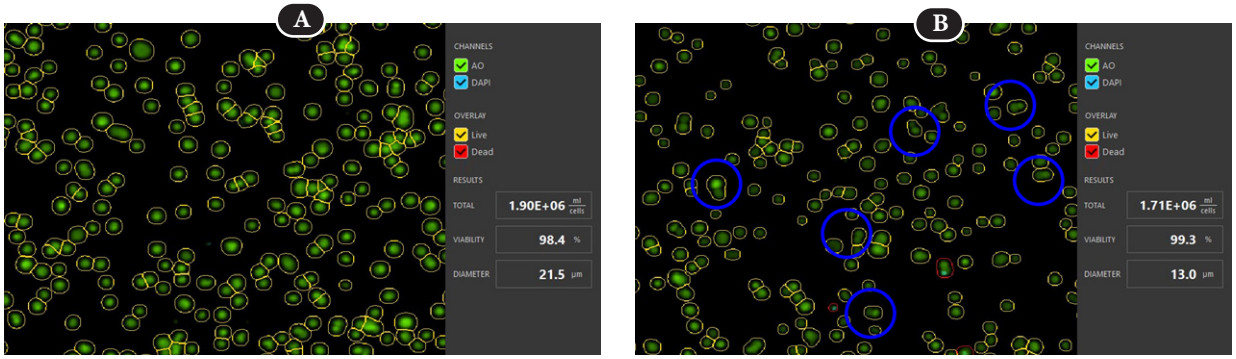
During harvest in the Rotea process, we observed that cell size decreased, as compared to cells in cDMEM. This size decrease occurred after Rotea processing when

FIGURE 4. Stability of cells processed in TrypLE–cDMEM medium using the Rotea system.



For **(A–C)**, measurements were taken every 30 min. Values are expressed as percentage change relative to baseline (0 h). Mean and standard deviation (SD) were represented. No statistically significant differences were observed over time (Kruskal–Wallis test, $n = 3$ independent assays, each measured in triplicate).

FIGURE 5. Imagery obtained during automated cell counting.



(A) ASC in cDMEM matrix. **(B)** ASC in FBS matrix.

Cell sizes were significantly lower when counted in the FBS matrix media.

cells were formulated in FBS and counted. Moreover, we observed that the number of cells counted in FBS was lower than the number of cells counted in cDMEM (aliquots from same mother solution, centrifuged and resuspended in cDMEM or FBS in parallel).

As shown in **Figure 5**, this reduction in the number of cells counted was due to the fact that the algorithm designed to count our cells in the NucleoCounter did not correctly segment groups of nearby cells, treating these groups as a single cell (**Figure 5B**, blue circles). So, we needed to determine if these reductions in cell number were due to Rotea processing, FBS resuspension, or a combination of both factors.

Factors Affecting Cell Size Decrease and Loss of NucleoCounter Accuracy in Rotea Processing

In order to determine if FBS itself, or the combination of FBS/Rotea process, was the root cause of cell size reduction and loss of NucleoCounter accuracy, the following experiment was designed. Cells were thawed, diluted in cDMEM, and divided into two separate tubes. The tubes were centrifuged and resuspended in cDMEM (tube 1) and FBS (tube 2). Then, cells from both tubes were counted (**Figure 6A**). Following this, tubes 1 and 2 were centrifuged, resuspended in cDMEM, and counted once again (**Figure 6B**). Total and viable cells, viability percentage, and cell size were taken into account (see experimental design in **Table 7**).

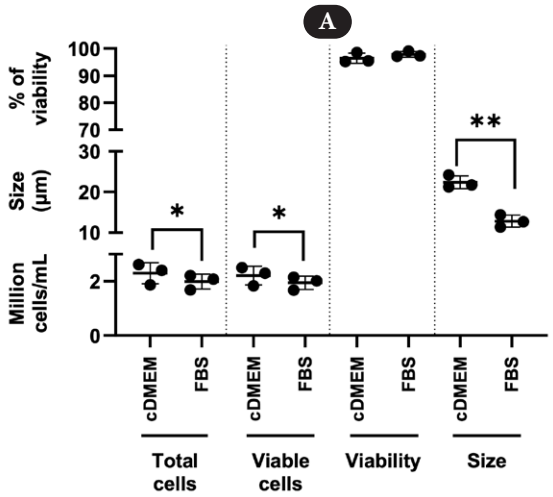
As shown in **Figure 6A**, when cells were resuspended in FBS, the number of cells counted was significantly lower (around 13% lower for total cells and 12% lower for viable cells). Viability was almost the same with both matrices, but the size of the cells resuspended in FBS was 40% lower than the size observed in cells resuspended in cDMEM. Nevertheless, when the cells were centrifuged and resuspended in cDMEM, this decrease in size and in the number of cells counted was reversed, and data from tubes 1 and 2 were practically the same. Observing these data, it was apparent that the matrix had a major impact on the counting method.

Exploring Methods of Cell Dilution

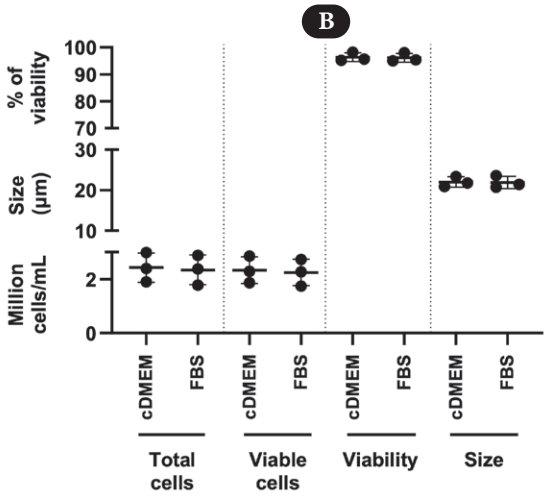
Based on the results shown in **Figures 6A and B**, which showed that cells in FBS cannot be counted in the NucleoCounter with the desired accuracy, we evaluated dilution methods to ensure accurate, consistent counts. Specifically, we aimed to confirm that the NucleoCounter maintained its standard accuracy—without matrix-dependent variability—when cells were either concentrated with the Rotea in FBS or formulated post-Rotea for cryopreservation in FBS+DMSO.

A vial of frozen cells was thawed, diluted, and separated into three different tubes, with the same number of cells per tube. Then, tubes were centrifuged and resuspended at 25×10^6 cells/mL in DMEM (control), FBS, and FBS+DMSO. The selected cell concentration represented the worst-case scenario based on the target formulation concentration for frozen cells. We assumed that cells from the Rotea would be more concentrated, meaning that higher dilutions would be required, and the matrix would be less likely to affect the cell count. This concentration was also the lowest we expected to count at any point during the downstream cell production process. Aliquots from the three tubes were

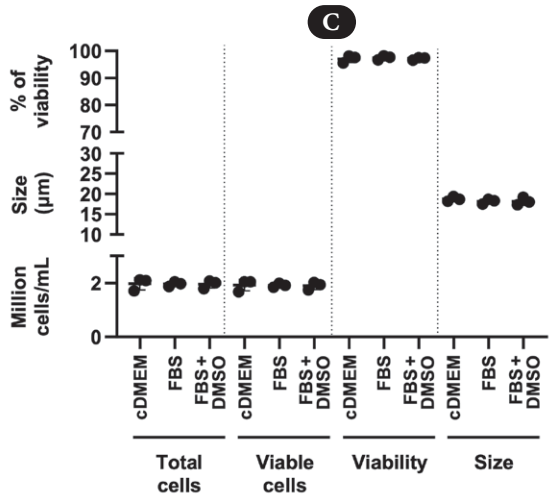
FIGURE 6. Results of counting with different matrices.



(A) Comparisons between counting cells in cDMEM and in FBS. Both samples were taken from same original cell dilution to assure same initial cell number (mean ± SD). (Paired *t*-test, $*p<0.05$, $**p<0.01$.)



(B) Comparisons between cells from cDMEM after centrifugation and resuspended in cDMEM, and cells from FBS and resuspended in cDMEM to determine if effects were reversible. (Paired *t*-test, $p<0.05$.)



(C) Comparisons between samples concentrated at 25M cells/mL in cDMEM, FBS, and FBS+DMSO, and diluted 1:15 in cDMEM. (Dunn's multiple comparisons test, $p<0.05$.)

Cells were counted in the NucleoCounter with protocol specially developed for our ASC. Results represent the mean ± SD. There were no statistically significant differences between compared groups. ($n=3$)

TABLE 7. A summary of the experimental design.

Tube #	Process	Matrix 1 Counting	Process	Matrix 2 Counting
1	Cell thawing and distribution into two different tubes	cDMEM	Centrifugation	cDMEM
2		FBS		cDMEM

diluted 1:15 (v/v) in cDMEM before counting. Then, aliquots were taken from each condition, diluted 1:15 in cDMEM, and counted in the NucleoCounter. Studies were performed in triplicate (see **Figure 6C**).

As shown in **Figure 6A**, the total cell count, viable cell count, viability percentage, and cell size were similar in the three conditions analyzed for each sample. No significant differences were observed among cells resuspended in cDMEM, FBS, or FBS:DMSO (90:10, v/v) and diluted 1:15 in cDMEM, indicating that diluting a sample 1:15 was enough to reverse effects observed when cells were resuspended in FBS.

DISCUSSION

Implementing the Rotea system in our ASC manufacturing has proven to be an efficient and safe approach for cell washing and concentration in closed systems, and is applicable to both 2D and 3D expansions using xeno-free media. The optimized protocol achieved recovery rates above 75%, cell concentrations suitable for downstream formulation ($\geq 40\times10^6$ cells/mL), and improved post-process viability due to elutriation. These results were consistent across multiple donors and expansion platforms, with no negative impacts on cell identity, potency, adhesion, or immunomodulatory capacity, complying with internally established GMP release criteria.

All tests for characterization of cells processed by the Rotea after 2D expansion in xeno-free media were within specified criteria. The Rotea did not have any negative impact on identity, potency, lymphocyte proliferation inhibition capacity, adhesion, or viability of the cells. Taking into account that processing ~8 L of media by Rotea (including serial repetition of all steps) would take around 3 h, we demonstrated that it can be a feasible option for batches of 40 culture chambers. This was determined by the data obtained from experiments evaluating the viability of cells resuspended in cDMEM:TrypLE (2:1, v/v) up to 4 h. Cells continued to show a similar size and viability, which were established as indicators of healthy cells.

In some initial 3D runs, we observed reduced recovery, approximately 65% (data not shown). Analysis of selected samples indicated that ~35% of the missing cells were present in the waste bags, and according to NucleoCounter measurements, these cells were viable. Nevertheless, further characterization using an apoptosis assay (data not shown) revealed that a higher proportion of these cells were in early to mid-stage apoptosis with membrane still intact. These findings may explain the high viability measured in waste-bag samples despite the lower overall recovery, suggesting that Rotea elutriation may remove not only dead cells but also pre-apoptotic cells.

Accordingly, this effect could contribute to consistently high recovery rates and yield an improved, higher-quality cell population, ultimately supporting a more robust cryopreserved final product.

When the 2D expansion protocol for ASC (with xeno-free media) was transferred to a 3D bioreactor-based process, we found that the same protocol could be applied. Across all bioreactor expansion runs initiated and evaluated using the Rotea system, cell recovery and viability were comparable to those obtained in 2D expansions (**Figure 3**).

All samples processed in the Rotea successfully passed the characterization analyses performed, and results obtained were above the limited acceptance criteria established in the manufacturing area for the release. Working with all data obtained in this study, we can conclude that the Rotea process is feasible for 3D in up to 10 L bioreactors that can be processed in less than a total of 4 h.

For freezing the cells or formulating in a high serum concentration, we observed variations between cell counting in cDMEM and cell counting in FBS. For this reason, we designed a dilution protocol for counting highly concentrated cells in cDMEM instead. We found that diluting the cells 1:15 was enough to correct the accuracy issue with automated cell counting. From this, we infer that cell samples at lower concentration, requiring less dilution, could be counted without problems.

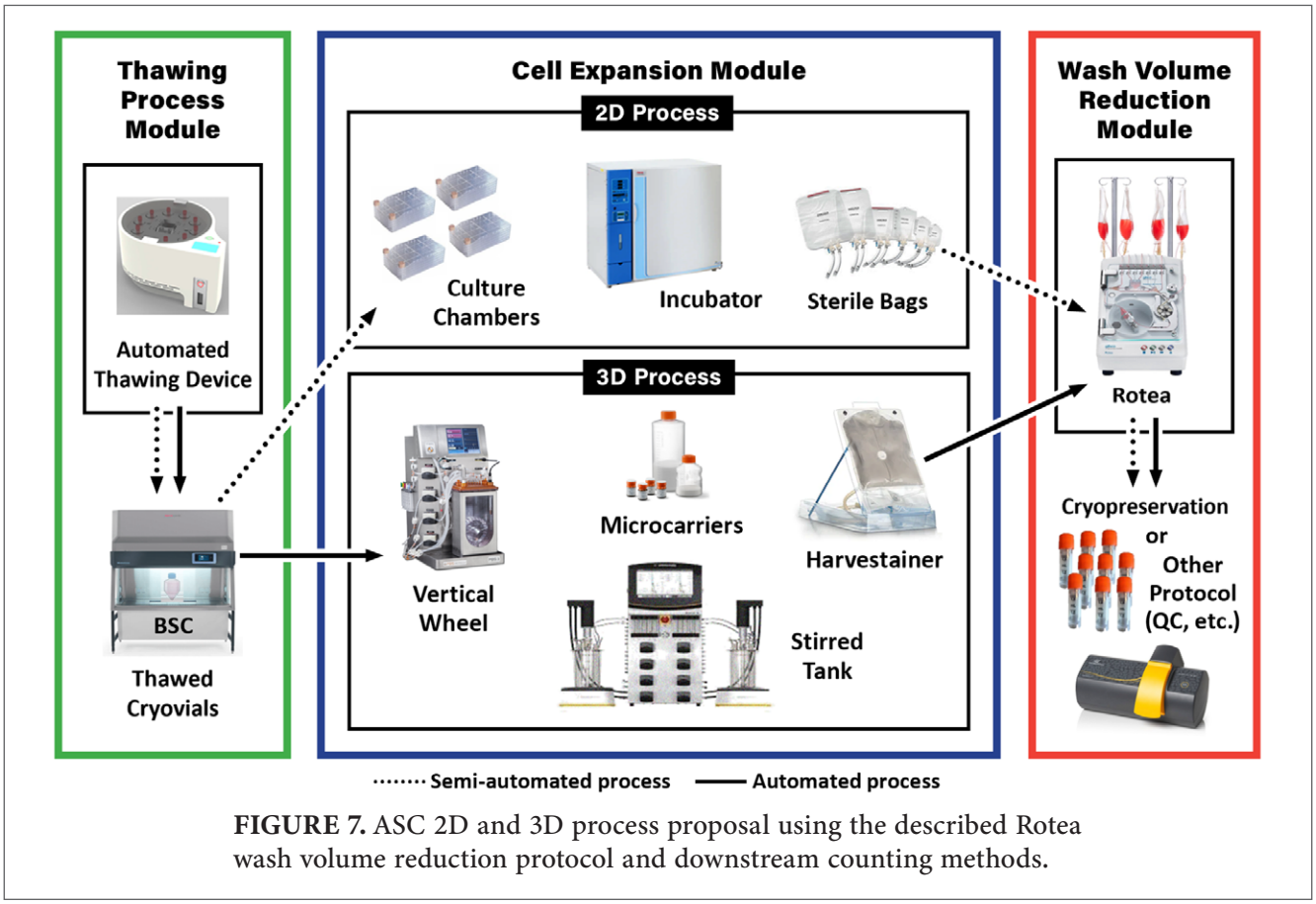


FIGURE 7. ASC 2D and 3D process proposal using the described Rotea wash volume reduction protocol and downstream counting methods.

CONCLUSION

For ASC manufacturing processes in 2D (culture chambers using cDMEM and xeno-free media) and 3D (bioreactors with microcarriers using xeno-free media), the Rotea system represents a suitable option for cell batch processing. It can accommodate total washing and volume-reduction workflows lasting up to 4 h. Rotea incorporates software that can be aligned with manufacturing regulatory requirements, is compliant with 21 CFR 11, and is straightforward for operators to use. The hardware and single-use kits are suitable for operation in up to Grade B cleanrooms. The overall manufacturing workflow was clearly defined, which ensured efficient execution.

Finally, after significant research and experience in cellular therapy product development and production, we propose the following suggestion for our 2D and 3D process in **Figure 7**, which shows thawing (green square)[20], expansion (blue square)[19], and as focused on in this article, the downstream processes of ASC (red square). To review the processes used in our study:

- Cells are thawed in an automatic thawing device.[20]
- After counting, cells are seeded in the 5-layer culture chambers (2D) or in a bioreactor with microcarriers (3D vertical wheel or stirred tank[19]).
- After expansion with xeno-free media, cells are transferred from culture chambers to a bag for working in a closed system, or from bioreactors directly to a Harvestainer for filtration.
- After filtration, cells are concentrated and washed with the Rotea until reaching around 40 to 55×10^6 cells/mL in FBS. Allow a later dilution to reach 25×10^6 cells/mL in FBS:DMSO (90:10, v/v) freezing solution.
- QC with the NucleoCounter at the end of the process, diluting samples in DMEM at least 1–15 times.
- While not described here, automation can continue with processes for formulation and fill-and-finish[23] before cell freezing.

To conclude, the CTS Rotea represents a robust solution for harmonizing downstream processes in ASC manufacturing, supporting scalability, process consistency, and regulatory compliance. Its integration into 3D bioreactor-based workflows and 2D expansions facilitates the transition from manual to automated or semi-automated systems, ultimately enabling faster, safer, and more reproducible production of ATMP.

ADDENDUM — Authors' Perspective

Automated solutions for ATMP manufacturing should be considered early in process development, with a clear view of the intended commercial-scale process. It is also important to recognize that upstream and downstream operations in automated settings often differ substantially from their manual counterparts. Therefore, the choice of an automation platform must be guided by a thorough understanding of the process's critical requirements and a balanced assessment of each technology's advantages and limitations.

In 2D culture workflows, integrating the Rotea system can shorten processing time for large cell banks and reduce the number of operators needed to manage multiple, five-layer culture chamber stacks (CS5). Before the adoption of a manufacturing setting, the workflow should be evaluated in the target environment. One practical option is to harvest trypsinized cells directly into a single-use bag connected to the CS5 via weldable or aseptic connectors. This supports the transition to a closed process in which cells can be concentrated and washed with the Rotea system before formulation or cryopreservation. Overall, a CS5-to-bag approach reduces manual handling and physical workload, and helps optimize staffing by freeing resources for other critical steps.

Using this approach, Valero *et al.* describe (in this article) how the Rotea cone capacity limitation can be addressed with a continuous, recirculating (loop) process, as detailed in the Materials and Methods section. This strategy enables processing of larger cell batches and may reduce costs.

Kit customization and pre-process filtration are critical to enable rapid, aseptic tube welding or connections. In cell therapy manufacturing, adapting materials and workflows is inherent to the field and should be planned from the outset. The Rotea system is versatile enough to be integrated across different manufacturing workflows, whether 2D or 3D, making it a suitable solution, regardless of the cleanroom classification required.

From a 2D standpoint, replacing standard centrifugation with Rotea may streamline operations, reduce total processing time, and lower contamination risks. This would shift CS5 culture processing from a manual workflow to a semi-automated one because, from cell harvest onward, the process can be performed in a closed system. A robust validation and implementation plan is especially important if the therapy is approaching commercialization or late-stage clinical development. Once cells are in an enclosed system, moving the operation to a lower-classified environment (*e.g.*, from Grade B to a lower grade) may further improve efficiency by reducing capital and operating expenditures.

For 3D (bioreactor) implementation, a clearly defined upstream and downstream workflow is essential to integrate all devices within the bioreactor ecosystem and meet GMP release criteria. In addition, Rotea may reduce contamination risks by minimizing operator-driven cell handling.

In this setting, the cell detachment and harvest step from the microcarriers represents a critical interface with downstream processing. The manufacturer specified the cell concentration limit for Rotea operation as 0.5×10^6 cells/mL. However, based on our experience, lower concentrations can also be processed with acceptable recovery, although efficiency may vary, depending on the process parameters. When operating below this threshold—commonly encountered in microcarrier-based systems—it is essential to minimize the detachment volume prior to loading onto the Rotea. Developing detachment strategies that enable efficient harvest in smaller volumes is particularly critical at this interface with downstream automation, ensuring compatibility with subsequent automated operations.

While operating below the manufacturer's defined concentration limit appears to be feasible, excessively large volumes may prolong overall processing times and could negatively impact cell quality. Therefore, this should be carefully evaluated during process development.

In general, the early alignment of upstream culture conditions, detachment strategies, and downstream automation constraints are key to realizing the full benefits of semi-automated ATMP manufacturing. A well-designed, integrated workflow that minimizes manual handling, reduces processing volumes, and supports closed system operation can improve robustness, scalability, and cost efficiency, thereby facilitating the translation of advanced cell therapies toward commercial manufacturing.

Author Disclosure Statement

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