Journal of Visualized Experiments

An automated counterflow centrifugal system for small scale downstream process

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60423R1
Full Title:	An automated counterflow centrifugal system for small scale downstream process
Section/Category:	JoVE Bioengineering
Keywords:	Counterflow centrifuge; buffer exchange; cell manufacturing; automated bioprocessing; downstream process; mesenchymal stem cells
Corresponding Author:	Rebecca Lim Monash Institute of Medical Research Clayton, Victoria AUSTRALIA
Corresponding Author's Institution:	Monash Institute of Medical Research
Corresponding Author E-Mail:	rebecca.lim@monash.edu
Order of Authors:	Anqi Li
	Stephen Wilson
	Ian Fitzpatrick
	Mehri Barabadi
	Siow Teng Chan
	Mirja Krause
	Gina Diamanta Kusuma
	David James
	Rebecca Lim
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Melbourne, Victoria, Australia

TITLE:

Automated Counterflow Centrifugal System for Small-Scale Cell Processing

AUTHORS AND AFFILIATIONS:

Anqi Li[1,2], Stephen Wilson[3], Ian Fitzpatrick[3], Mehri Barabadi[1,2], Siow Teng Chan[1], Mirja Krause[1,2], Gina Diamanta Kusuma[1,2], David James[3], Rebecca Lim[1,2,4]

[1]The Ritchie Centre, Hudson Institute of Medical Research, Clayton VIC, Australia
[2]Department of Obstetrics and Gynaecology, Monash University, Clayton VIC, Australia
[3]Scinogy, Melbourne, Australia
[4]Australian Regenerative Medicine Institute, Monash University, Clayton VIC, Australia

Corresponding Author:

Rebecca Lim (Rebecca.Lim@hudson.org.au)

Email Addresses of Co-authors:

Anqi Li (anqi.li@hudson.org.au)
Mehri Barabadi (mehri.barabadi@hudson.org.au)
Siow Teng Chan (siow.chan@hudson.org.au)
Mirja Krause (Mirja.Krause@hudson.org.au)
Stephen Wilson (stephen.wilson@scinogy.com)
Ian Fitzpatrick (ian.fitzpatrick@scinogy.com)
Gina Diamanta Kusuma (gina.kusuma@hudson.org.au)
David James (david.james@scinogy.com)
Rebecca Lim (Rebecca.lim@hudson.org.au)

KEYWORDS:

counterflow centrifuge, buffer exchange, cell manufacturing, automated bioprocessing, downstream process, mesenchymal stem cells

SUMMARY:

Automation is key to upscaling and cost management in cell manufacturing. This manuscript describes the use of a counterflow centrifugal cell processing device for automating the buffer exchange and cell concentration steps for small-scale bioprocessing.

ABSTRACT:

Successful commercialization of gene and cell-based therapies requires manufacturing processes that are cost-effective and scalable. Buffer exchange and product concentration are essential components for most manufacturing processes. However, at the early stages of product development, these steps are often performed manually. Manual dead-end centrifugation for buffer exchange is labor-intensive, costly, and not scalable. A closed automated system can effectively eliminate this laborious step, but implementation can be challenging. Here, we describe a newly developed cell processing device that is suitable for small- to medium-scale cell processing and aims to bridge the gap between manual processing and large-scale automation.

This protocol can be easily applied to various cell types and processes by modifying the flow rate and centrifugation speed. Our protocol demonstrated high cell recovery with shorter processing times in comparison to the manual process. Cells recovered from the automated process also maintained their proliferation rates. The device can be applied as a modular component in a closed manufacturing process to accommodate steps such as buffer exchange, cell formulation, and cryopreservation.

INTRODUCTION:

The landscape of modern medicine has transformed rapidly through recent developments in gene and cell-based therapies (GCT). As one of the fastest growing fields in translational research, the GCT sector also faces unique and unprecedented challenges. In addition to robust clinical outcomes, efficient and cost-effective manufacturing processes are essential for the commercial success of GCT, which is particularly difficult to achieve in small-scale manufacturing[1]. The cost of time, labor, and quality assurances are magnified when each batch of cells only produces a few doses for one patient instead of hundreds or thousands. Unlike allogeneic cell therapies in which the manufacturing processes are more akin to the production of antibodies and recombinant proteins, autologous cell therapies are typically produced as small-scale operations[1]. As a relatively new phenomenon in biopharmaceutical manufacturing[2], options for small-scale cell processing are currently quite limited.

Buffer exchange is essential to cell manufacturing. It is one of the downstream processes where cells are removed from culture media and concentrated for cryopreservation or infusion. Currently, small-scale cell manufacturing often applies processes similar to those in the academic research setting and relies on specialized clean rooms to maintain sterility[3]. Manual downstream processes often use benchtop centrifuges to pellet and resuspend cells for volume reduction and buffer exchange. These open processes are costly (i.e., labor and clean room maintenance) and have limited manufacturing capacity, which are not ideal for commercial production[2,3].

Implementing automation has been proposed as a solution to improve manufacturing efficiency and achieving commercial scale productions[2]. Sterility cannot be achieved in cell-based products through traditional methods used for biologics, such as gamma irradiation or terminal end filtration. Instead, an automated closed system is deployed to reduce risks of contamination and operators relying on clean rooms to maintain sterility[4]. Process automation also addresses the issue of scalability by either having multiple systems running in parallel (scale-out) or increasing the processing capacity of an individual device (scale-up), which in turn minimizes the variability between operators. Furthermore, cost modelling analysis of autologous therapies suggests that automation may reduce the cost of manufacturing[5,6]. However, no cost benefit was found in an autologous stem cell clinical trial where an automated manufacturing platform was used[7], suggesting that the cost benefit of automation may depend on the individual manufacturing process.

There are different strategies in which automation can be introduced into an existing manufacturing process. This can be achieved either by implementing a fully integrated platform or a modular-based processing chain. There are several fully integrated platforms commercially

available for autologous cell manufacturing, such as CliniMACS Prodigy (Miltenyi Biotec), Cocoon (Octane Biotech), and Quantum (Terumo BCT). These integrated platforms, which are often described as "GMP-in-a-box", have low demands on infrastructure and are easy to operate. However, the manufacturing capacity of a fully integrated setup may be restricted by the incubator attached to the system. For example, the culturing capacity of Prodigy is limited to its 400 mL chamber[8] and the Quantum cartridge has a limiting surface area set to 2.1 m^2 (equivalent to 120 T175 flasks)[7], which may not be sufficient for patients requiring higher cell doses[9,10]. Additionally, Prodigy and Quantum have a common attribute that limits their use: the operational unit is occupied by a single batch of cells throughout the cell expansion period, thus limiting the number of batches that can be manufactured by each unit[11]. The modular approach to automation is to create a manufacturing chain with multiple modular units that simulates the commercial manufacturing process[12,13]. This approach, which separates the culture device from the cell washing device, can thereby maximize manufacturing efficiency. An ideal processing device would be one that is adaptable and scalable to manufacturing needs[12].

Counterflow centrifugation (CFC) technology, which dates back to the 1970s, has had a long history in cell processing[14]. It achieves cell concentration and separation by balancing centrifugal force with a counterflow force. Typically, a cell suspension enters from the narrow end of a cell chamber under a constant flow rate while subjected to a centrifugal force (**Figure 1A**). The flow of the fluid is exerted in the opposite direction to the centrifugal force. This is referred to as the counterflow force, which forms a gradient within the cell chamber. The counterflow force then decreases as the cell chamber widens away from the tip of the cone-shaped cell chamber. Cells with higher density and larger diameter have a higher sedimentation rate, and thus they reach force equilibrium towards the tip of the cone-shaped cell chamber. Smaller particles may reach equilibrium towards the base of the chamber or be too small to be retained in the chamber and will be washed away. The CFC technology is mostly known for its application in processing blood apheresis products, such as isolating monocytes for dendritic cell therapies[15,16]. In terms of buffer exchange, the CFC technology has only been applied in large-scale manufacturing[17] and has yet to be used for the smaller scale manufacturing of autologous cell therapies.

To address the need of a suitable device for small-scale cell manufacturing, an automated CFC device (See **Table of Materials**), was recently developed[18]. The automated cell processing device uses counterflow centrifugation technology to remove cell debris and facilitate buffer exchange. The device performs buffer exchange with a single-use kit that can be sterile-connected to a cell transfer bag, which allows the cells to be processed within a sterile, enclosed system. Here, we investigate the use of a counterflow centrifugal device to perform buffer exchange in mammalian cell cultures in automated protocols. In this study, we tested the buffer exchange protocol using Jurkat cells and mesenchymal stromal cells (MSCs) to model nonadherent and adherent cell types, respectively. Jurkat cells are immortalized T cells often used for the study of acute T cell leukemia[19,20]. MSCs are adult stem cells that have been studied in human clinical trials for a wide range of diseases[9].

PROTOCOL:

1. Preparation of reagents and cells for buffer exchange

1.1. Prepare buffers (see **Table of Materials**) in a class 2 laminar flow hood. Using a syringe and needle assembly, remove 50 mL of saline solution from a 500 mL saline bag. Replace this with 50 mL of 20% human serum albumin (HSA) to make 2% HSA in saline, which will serve as the wash buffer.

1.2. Remove the cells from the culture vessels and perform a cell count to determine the starting cell quantity and viability by trypan blue exclusion. Load the cells into a transfer bag.

NOTE: In this protocol Jurkat cells were cultured in RPMI and MSCs were cultured in DMEM:F12. Both media were supplemented with 10% fetal bovine serum (FBS) and 1% Antibiotic-Antimycotic solution. Cells were cultured at 37 °C with 5% CO_2. For MSCs or adherent cells, add a digestion enzyme (e.g., trypsin) into the culture vessels in order to detach the cells, and quench with culture media once the cells are detached. A transfer bag was used in this protocol because the cells were expanded in culture vessels. However, if the cells are cultured in a cell culture bag, it can be directly connected to the single-use kit via sterile tube welding. In this protocol, either 3×10^7 Jurkat cells or 1×10^7 MSCs were used for each run, where cells were suspended in 40 mL of culture media.

1.2.1. Load cells into a transfer bag (see **Table of Materials**) using a syringe and needle assembly if a sterile tube welder is available to connect the transfer bag to the single use processing kit.

1.2.2. Alternatively, use a pair of autoclaved scissors to cut the connecting tube with around 10 cm remainining attached to the transfer bag and add a female Luer connector to the end of the tubing. Use a syringe to aspirate cells and media, then connect the syringe to the Luer connector and load cells to the transfer bag.

1.3. Connect the bag containing the cells, wash buffer bag containing 500 mL of wash buffer prepared in step 1.1, waste bag, and collecting syringe to the single-use kit (**Figure 1B**).

NOTE: The connecting position of each bag depends on the settings in the program. In this protocol, we connected the waste bag at position A, wash buffer at position B, cell bag at positions D and F, and collection syringe at position H (**Figure 1C**).

2. Program for automated buffer exchange protocol

2.1. Open the Graphic User Interface (GUI) of the device and press the "**START**" button on a laptop PC or tablet. Then "**Open**" an existing protocol or press "**New**" to create a new one.

2.2. Use the "**Forward**" and "**Backward**" button to navigate through each step, select the valves to be opened/closed, centrifugal speed, pump speed, pump direction, and action triggers. The settings for each step are summarized in **Table 1**. Then save the protocol.

3. Setting up the machine

3.1. Place the single-use processing kit on the machine and hang the connected bags accordingly. Press the red "**Stop/Reset**" button to reset all valves into the default closed position.

NOTE: The machine will perform a test when the door is closed to ensure the kit has been placed correctly and that all the valves are functioning appropriately.

3.2. Connect the GUI to the processing device and press the "**Connect**" button. Then download the saved program. When the green "**Play**" button lights up, it indicates that the protocol is ready to start.

3.3. Open the manual clamps for the transfer bag tubing.

NOTE: If the operator forgets to open the clamps, the device will stop and the pressure sensor warning will appear. Press the "**Stop**" button to reset the device and open the clamps to start again.

4. Automated buffer exchange

4.1. Press "**Start**" to initiate the buffer exchange program.

NOTE: To manually alter the automated process, press the "**Next**" button to move to the next step before reaching the "**Trigger**" or press "**Pause**" to put the process on hold. This will recirculate the cells through the kit while keeping only valves J and K open (**Figure 1C**).

4.2. At the end of automation step 6 (**Table 1**), the process will pause when air in the now emptied bag triggers the bubble sensor. Press "**Start**" to move the process to the next step if the bag is indeed empty, or press "**Pause**" to resume the processing if the sensor was triggered by a bubble in the line.

5. Collecting and sampling the cells

5.1. When the automated process is finished, close all tubing clamps. Open the door and take the single-use kit out of the device. Disconnect the syringe to collect the cells for subsequent processes. Take a sample of the collected cells for a cell count and viability check (see step 6.2).

6. Process validation

6.1. Perform control experiments using a manual buffer exchange protocol.

6.1.1. Centrifuge cell suspension at 200 x *g* for 5 min. Discard the supernatant and resuspend the cells with 30 mL of cell wash buffer. Centrifuge the cell suspension again at 200 x *g* for 5 min. Discard the supernatant and resuspend the cells with 10 mL of cell wash buffer.

6.2. Perform cell counting via the trypan blue exclusion assay to assess cell viability using an automated cell counter.

6.3. Perform an MTS assay to quantify cell proliferation at 2, 24, and 48 h.

NOTE: The MTS assay was performed on a 96 well tissue culture plate according to the manufacturer's instructions. A 100 µL aliquot of cell culture media (containing 10,000 Jurkat cells or 1,500) per well were plated after the buffer exchange process. At each time point, 20 µL of MTS reagents were added into each well and incubated for 2 h at 37 °C. The absorbance was assessed at 490 nm using a plate reader.

6.4. Perform functional assays to compare the impact of the automated process versus the manual process on the Jurkat cells and MSC function.

6.4.1. Culture Jurkat cells in a 96-well plate at a density of 1 x 10^6 cells/mL stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 µg/mL ionomycin (cell stimulation cocktail) in DMEM:F12 media containing 10% fetal bovine serum (FBS) for 24 h. Incubate the cells at 37 °C with 5% CO_2.

6.4.2. Measure interleukin-2 production from culture supernatant using bead-based ELISA assay (see **Table of Materials**) according to the manufacturer's instructions.

6.4.3. Culture MSCs in a 12 well plate at the density of 10,000 cells/cm^2 in 1 mL of DMEM:F12 media containing 10% FBS supplemented with interferon-γ 50 units/mL for 48 h. Collect supernatant for the kynurenine concentration assay to measure indoleamine 2,3-dioxygenase (IDO) enzyme activity of MSCs as previously described[21].

REPRESENTATIVE RESULTS:

In this protocol, we used Jurkat cells and MSCs as representative examples to demonstrate the automated buffer exchange process. During the process, Jurkat cells and MSCs shared the same processing steps with differences in centrifugal force and pump speed that control the flow rate (**Table 1**). **Figure 2** shows representative images captured by the camera of how the fluidized cell bed may appear during the buffer exchange process. Typically, the fluidized cell bed will resemble the image in **Figure 2A**, where cells accumulate in the middle and towards the front of the cone with a small space at the tip of the chamber, where cells do not accumulate and the opening of the cell loading inlet is visible. The fluidized cell bed may be compressed (**Figure 2B**) when introducing new buffer that is at a different viscosity or density. In this protocol, the pump speed was lowered from 30–35 mL/min to 20 mL/min at the start of the washing step. Once the chamber was filled with the new buffer, the pump speed was returned to normal to prevent pelleting cells at the tip of the chamber. A high flow rate (**Figure 2C**) may be applied to select for live cells because dead cells are smaller and lighter and can be forced out of the chamber by increasing the flow rate.

The automated process of buffer exchange was achieved by first concentrating the cells, then introducing the wash buffer, which was around 10x of the volume of the fluidized cell bed. The cells were then formulated to the desired volume. These three processing steps were designed to follow the same principles as a manual buffer exchange. Typically, a two-cycle centrifugation (200 x *g*, 5 min) is used to perform manual buffer exchange, in which cells are pelleted to concentrate, resuspended to wash, then centrifuged again and resuspended to the final volume. The processing time of the automated processes was shorter compared to the manual ones (**Figure 3**). The recovery rate between manual and automated processes were similar for both Jurkat cells and MSCs, and cell viability was not affected by the process (**Figure 4**). The cell quality was verified by cell proliferation (MTS assay) and cytokine/enzyme production. The recovered cells showed similar proliferation rates between the manual and the automated processes (**Figure 5A**). The level of interleukin-2 production from Jurkat cells and IDO activity of MSCs were also comparable between the two groups (**Figure 5B** and **5C**).

FIGURE AND TABLE LEGENDS:

Table 1: Automated buffer exchange GUI setup for Jurkat cells and MSCs.

Figure 1: Counterflow centrifugal cell processing system. (**A**) A schematic diagram illustrating the principle of counterflow centrifugation. The counterflow force is present in a gradient within the cell chamber. While centrifuging (grey arrow), cells with larger diameters receive a higher sedimentation force, in which the cells reach force equilibrium towards the narrow end of the chamber, forming a fluidized cell bed. Cell debris and small particles that are too small to remain in the chamber are washed away. (**B**) The counterflow centrifugal processing system consists of the processing device and the single-use processing kit. (**C**) The single-use kit configuration for the buffer exchange protocol.

Figure 2: Fluidized cell bed in the cell chamber. Representative images of a fluidized cell bed under (**A**) medium, (**B**) low, and (**C**) high flow rate. The dotted line indicates the area of the fluidized cell bed within the chamber.

Figure 3: Comparison of cell processing time of Jurkat cells and MSCs with manual and automated processing (n = 3–4 in each group, data are presented as mean ± SD).

Figure 4: Comparison of cell viability and live cell recovery of Jurkat cells and MSCs with manual and automated processing. Cell viability (**A**) and live cell recovery (**B**) were measured by trypan blue exclusion assay using an automated cell counter. Live cell recovery was reduced in the absence of serum or when only 3 x 10^6 or 1 x 10^6 cells were processed. (n = 4–9 in each group, data are presented as mean ± SD).

Figure 5: Cell proliferation and cell function from manual and automated processing. (**A**) MTS assay of Jurkat cells and MSCs were performed at 2, 24, and 48 h after buffer exchange. The quality of recovered cells was quantified by Interleukin-2 production from Jurkat cells (**B**), and IDO activity in MSCs (**C**). (n = 4–8 in each group, data are presented as mean ± SD).

Figure 6: Fluidized cell bed stability at various flow rate. Two processes were performed for each cell type. In one process, either 3 x 10^7 Jurkat cells or 1 x 10^7 MSCs. In the second process, 10x the number of cells were used. In both processes, 10 mL of cell elutriate was collected from port A at various flow rates using the centrifuging speed indicated in this protocol (1,600 x *g* for Jurkat cells and 1,500 x *g* for MSCs). The number of cells in the elutriate was determined and presented as percentage of the total amount of cells loaded in the chamber. (n = 3 for each group, data are presented as mean ± SD).

DISCUSSION:

The automated buffer exchange protocol described is simple and user friendly. Nevertheless, there are a few key steps in this protocol that are critical and require particular attention. In our experience, when processing larger cells such as MSCs (average diameter 10–15 µm) each run should include at least 1 x 10^7 cells to achieve optimal cell recovery (**Figure 4B**). Processing smaller cells, such as Jurkat cells (average ~10 µm diameter), requires around 3 x 10^7 cells to achieve a stable fluidized cell bed (**Figure 4B**). When the cell number is too low, cells are more likely to be removed from the fluidized bed, which will affect the final cell recovery. Additionally, we compared cell loss rates at different pump speeds when a constant centrifugal speed is applied. Here, we observed that the cell loss increased with the flow rate (**Figure 6**), which is due to the increasing instability of the fluidized bed at higher flow rates. However, the percentage of cell loss was lower when 10x the number of cells were loaded into the chamber, which suggests that a higher pump speed can be applied when processing a larger number of cells to allow faster processing. In step 5 of the process, 100 mL of culture media was recirculated from the chamber back to the cell transfer bag to allow the fluidized bed to form and stabilize prior to volume reduction and buffer exchange. If the cell concentration is low (e.g., below 0.2 x 10^6/mL), the recirculation step may need to be extended to allow enough cells to form the fluidized cell bed. Likewise, if the cell concentration is high, the recirculation step can be shortened.

The presence of serum in the wash buffer is also critical for cell recovery. Previous studies have shown that hydrodynamic stress can cause necrotic cell death in the absence of buffering proteins[22,23]. The flow dynamic of the counterflow centrifugal system is quite complex. In this protocol, cell recovery rates dropped to around 70% when the process was performed in the absence of serum (**Figure 4B**). Although the exact mechanism is not clear, the presence of serum in culture media or albumin in wash buffers was able to mitigate the potential mechanical damage to the cells. For applications requiring serum-free buffers, hydroxyethyl starch and dextran 40 are examples of non-protein additives that are often used in cell manufacturing to protect against hydrodynamic stress[24,25].

Counterflow centrifugation technology has been used in large-scale biopharmaceutical manufacturing and blood product processing[17]. The applications of CFC technology in small-scale cell manufacturing (i.e., 0.1–10 L) are often limited to isolating mononuclear cells from apheresis products, such as the Elutra (Terumo BCT)[26], in which additional manual intervention is required to perform wash and concentrate steps. Other small-scale cell processing platforms include membrane filtration-based buffer exchange systems such as the LOVO (Fresenius Kabi)[27] and vertical centrifugation separation such as the Sepax (GE Healthcare)[28]. Although it is difficult to

perform a direct comparison between devices, one of the advantages of this counterflow centrifugal device is its capacity to deliver very small output volumes (minimum 5 mL), which is the smallest among currently available cell processing platforms. The small output volume is suitable for applications such as formulating cells for local injections[29] or infusions for neonates[30]. The camera embedded in the device is also a unique feature that provides visualization of the fluidized cell bed during the process development stage.

One of the limitations of the CFC technology is that it is sensitive to the size and density of the cells. When setting up a buffer exchange protocol for a different cell type, the protocol presented here should serve as a guideline for users to optimize according to their needs, keeping in mind the size of their cells of interest and the density of their cell suspension. Although the current protocol is usable for processing up to 5 L of media, the processing time will be substantially extended (i.e., 1–2 h). It is important to note that the impact of extended processing time on cell quality has not been examined in this current study.

Future studies will evaluate the impact of processing speed and processing time on cell function to determine the maximum processing capacity of the device. Furthermore, future studies may explore other applications such as the removal of dimethyl sulfoxide (DMSO) from cryopreserved products and selective removal of dead cells.

ACKNOWLEDGMENTS:

This work is supported by the Victorian Government's Operational Infrastructure Support Program, and the Victorian Government Technology Voucher provided by the Department of Economic Development, Jobs, Transport and Resources. RL is the recipient of a National Health and Medical Research Council Career Development Fellowship. AL is the recipient of an Australian Postgraduate Award.

DISCLOSURES:

SW, IF, and DJ are COO, CTO, and CEO of Scinogy Pty. Ltd. The access to the CFC device was also provided by Scinogy.

REFERENCES:

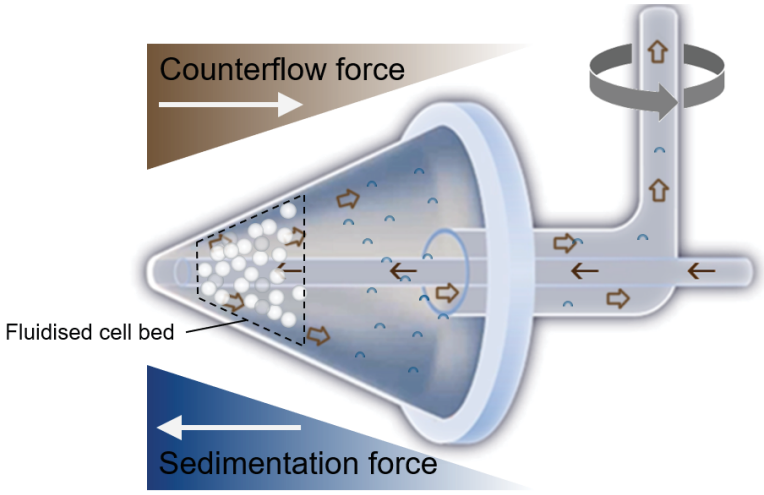
1. Lopes, A. G., Sinclair, A., Frohlich, B. Cost Analysis of Cell Therapy Manufacture: Autologous Cell Therapies, Part 1. *BioProcess International.* **16** (3), (2018).
2. Hampson, B., Ceccarelli, J. Factories of the future: Can Patient-Specific Cell Therapies Get There from Here? *BioProcess International.* **14** (4), (2016).
3. Preti, R., Daus, A., Hampson, B., Sumen, C. Mapping success for commercial cell therapy manufacturing. *BioProcess International.* **13** (9), 33–38 (2015).
4. Heathman, T. R. et al. The translation of cell-based therapies: clinical landscape and manufacturing challenges. *Regenerative Medicine.* **10** (1), 49–64 (2015).
5. Lipsitz, Y. Y. et al. A roadmap for cost-of-goods planning to guide economic production of cell therapy products. *Cytotherapy.* **19** (12), 1383–1391 (2017).
6. Lopes, A. G., Sinclair, A., Frohlich, B. Cost Analysis of Cell Therapy Manufacture: Autologous Cell Therapies, Part 2. *BioProcess International.* **16** (4), 12–19 (2018).

7. Hanley, P. J. et al. Efficient manufacturing of therapeutic mesenchymal stromal cells with the use of the Quantum Cell Expansion System. *Cytotherapy.* **16** (8), 1048–1058 (2014).
8. Leong, W., Nakervis, B., Beltzer, J. Automation: what will the cell therapy laboratory of the future look like? *Cell Gene Therapy Insights.* **4** (9), 679–694 (2018).
9. Galipeau, J., Sensebe, L. Mesenchymal Stromal Cells: Clinical Challenges and Therapeutic Opportunities. *Cell Stem Cell.* **22** (6), 824–833 (2018).
10. Salmikangas, P., Kinsella, N., Chamberlain, P. Chimeric Antigen Receptor T-Cells (CAR T-Cells) for Cancer Immunotherapy - Moving Target for Industry? *Pharmaceutical Research.* **35** (8), 152 (2018).
11. James, D. How short-term gain can lead to long-term pain. *Cell Gene Therapy Insights.* **3** (4), 271–284 (2017).
12. Rafiq, Q. A., Thomas, R. J. The evolving role of automation in process development, manufacture of cell, gene-based therapies. *Cell Gene Therapy Insights.* **2** (4), 473–479 (2016).
13. Rafiq, Q. A. Emerging Automated Approaches for Cell and Gene Therapy Manufacture. *Cell Gene Therapy Insights.* **4** (9), 911–914 (2018).
14. Contreras, T. J., Jemionek, J. F., French, J. E., Shields, L. J. Human Granulocyte Isolation by Continuous Flow Centrifugation Leukapheresis and Counterflow Centrifugation Elutriation (CFCL/CCE). *Transfusion.* **19** (6), 695–703 (1979).
15. Berger, T. G. et al. Efficient elutriation of monocytes within a closed system (Elutra™) for clinical-scale generation of dendritic cells. *Journal of Immunological Methods.* **298** (1), 61–72 (2005).
16. Chen, Y., Hoecker, P., Zeng, J., Dettke, M. Combination of Cobe AutoPBSC and Gambro Elutra as a platform for monocyte enrichment in dendritic cell (DC) therapy: Clinical study. *Journal of Clinical Apheresis.* **23** (5), 157–162 (2008).
17. Whitford, W. G. in *Continuous Processing in Pharmaceutical Manufacturing* (ed. G. Subramanian) (2014).
18. Scinogy. *SMALL BATCH CELL SEPARATION, WASH & CONCENTRATION*, <https://www.scinogy.com/projects> (2019).
19. Yu, D. et al. Targeting Jurkat T Lymphocyte Leukemia Cells by an Engineered Interferon-Alpha Hybrid Molecule. *Cellular Physiology and Biochemistry.* **42** (2), 519–529 (2017).
20. Moharram, S. A., Shah, K., Kazi, J. U. T cell Acute Lymphoblastic Leukemia Cells Display Activation of Different Survival Pathways. *Journal of Cancer.* **8** (19), 4124 (2017).
21. Ling, W. et al. Mesenchymal stem cells use IDO to regulate immunity in tumor microenvironment. *Cancer Research.* **74** (5), 1576–1587 (2014).
22. Tanzeglock, T., Soos, M., Stephanopoulos, G., Morbidelli, M. Induction of mammalian cell death by simple shear and extensional flows. *Biotechnology and Bioengineering.* **104** (2), 360–370 (2009).
23. Aguado, B. A., Mulyasasmita, W., Su, J., Lampe, K. J., Heilshorn, S. C. Improving viability of stem cells during syringe needle flow through the design of hydrogel cell carriers. *Tissue engineering. Part A.* **18** (7–8), 806–815 (2012).
24. Zhu, F. et al. Hydroxyethyl starch as a substitute for dextran 40 for thawing peripheral blood progenitor cell products. *Cytotherapy.* **17** (12), 1813–1819 (2015).
25. Schwandt, S., Korschgen, L., Peters, S., Kogler, G. Cord blood collection and processing with hydroxyethyl starch or non-hydroxyethyl starch. *Cytotherapy.* **18** (5), 642–652 (2016).

26. Stroncek, D. F. et al. Counter-flow elutriation of clinical peripheral blood mononuclear cell concentrates for the production of dendritic and T cell therapies. *Journal of Translational Medicine.* **12**, 241 (2014).
27. Mfarrej, B. et al. Pre-clinical assessment of the Lovo device for dimethyl sulfoxide removal and cell concentration in thawed hematopoietic progenitor cell grafts. *Cytotherapy.* **19** (12), 1501–1508 (2017).
28. Abonnenc, M., Pesse, B., Tissot, J. D., Barelli, S., Lion, N. Automatic washing of thawed haematopoietic progenitor cell grafts: a preclinical evaluation. *Vox Sanguinis.* **112** (4), 367–378 (2017).
29. Panes, J. et al. Expanded allogeneic adipose-derived mesenchymal stem cells (Cx601) for complex perianal fistulas in Crohn's disease: a phase 3 randomised, double-blind controlled trial. *Lancet.* **388** (10051), 1281–1290 (2016).
30. Lim, R. et al. First-In-Human Administration of Allogeneic Amnion Cells in Premature Infants With Bronchopulmonary Dysplasia: A Safety Study. *Stem Cells Translational Medicine.* **7** (9), 628–635 (2018).

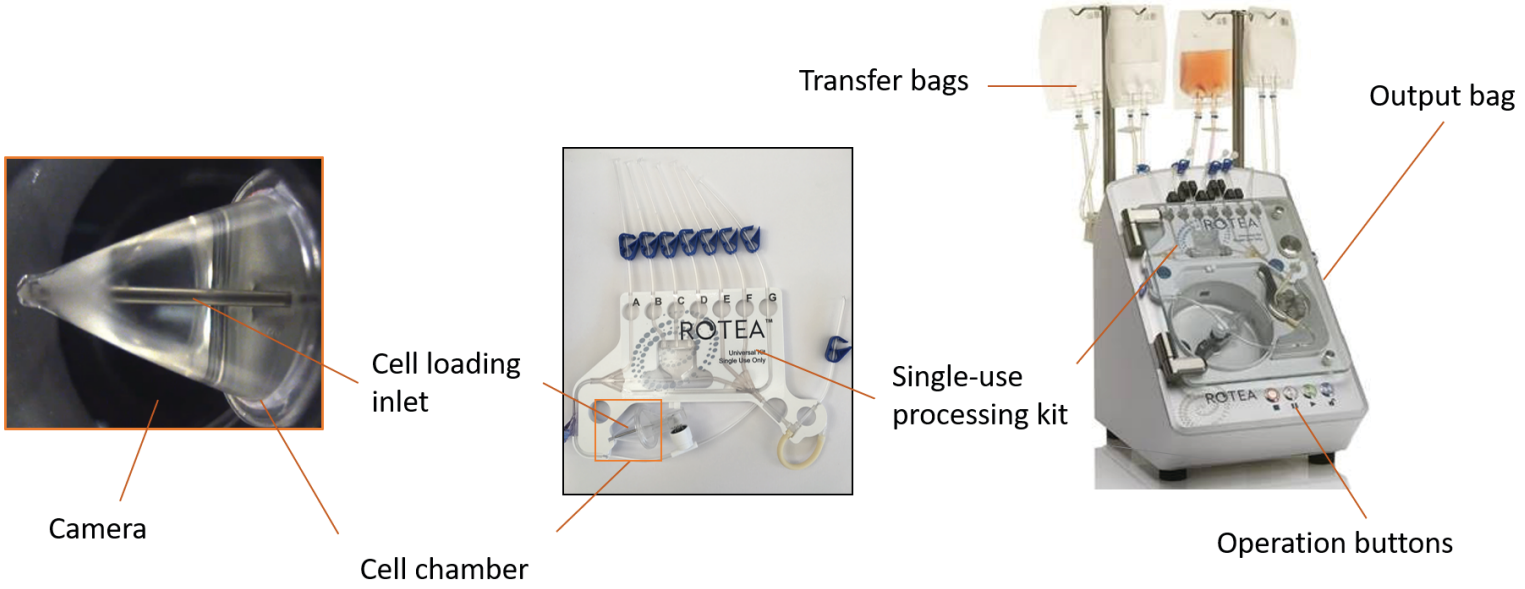
Figure 1

A



Cells
Debris
Direction of fluid entering the chamber
Direction of fluid exiting the chamber

B



C

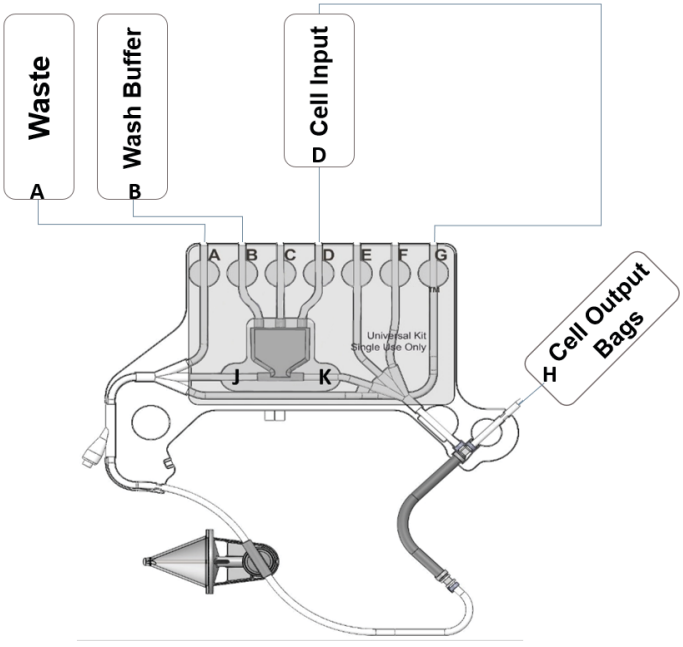


Figure 2

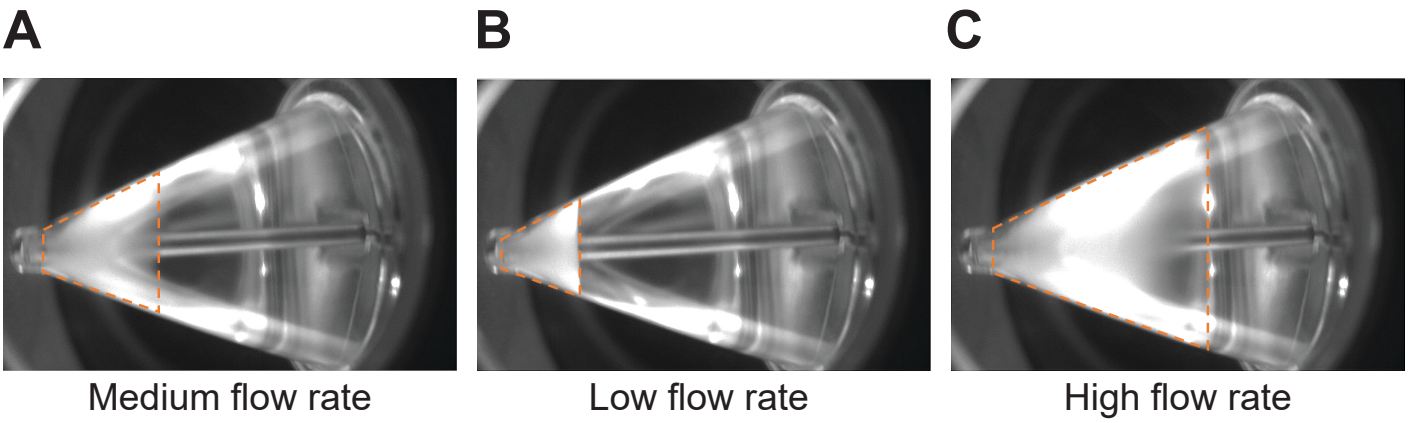


Figure 3

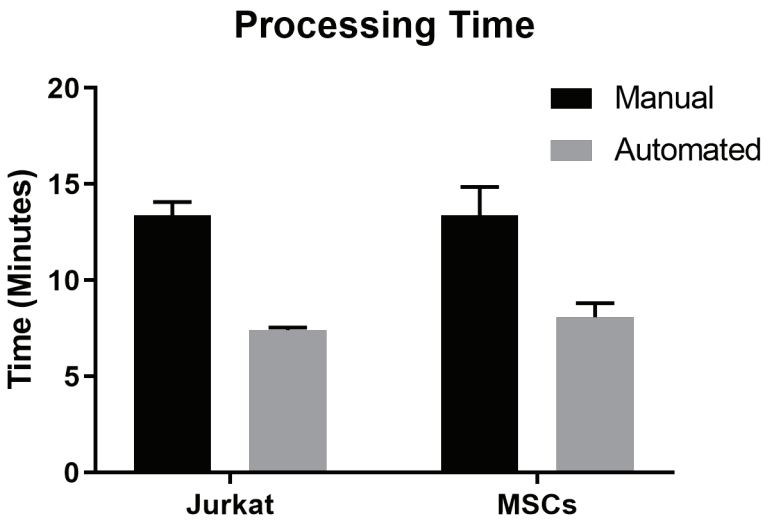


Figure 4

A

Cell type	Jurkat		MSC	
Processing method	Manual (n=4)	Automated (n=8)	Manual (n=6)	Automated (n=9)
Cell viability (before)	88.3 ±3.2%	85.6 ±5.5%	88.9 ±6.8%	93.3 ±4.2%
Cell viability (after)	86.9 ±5.8%	88.9 ±5.1%	94.3 ±3.1%	95.9 ±1.5%

B

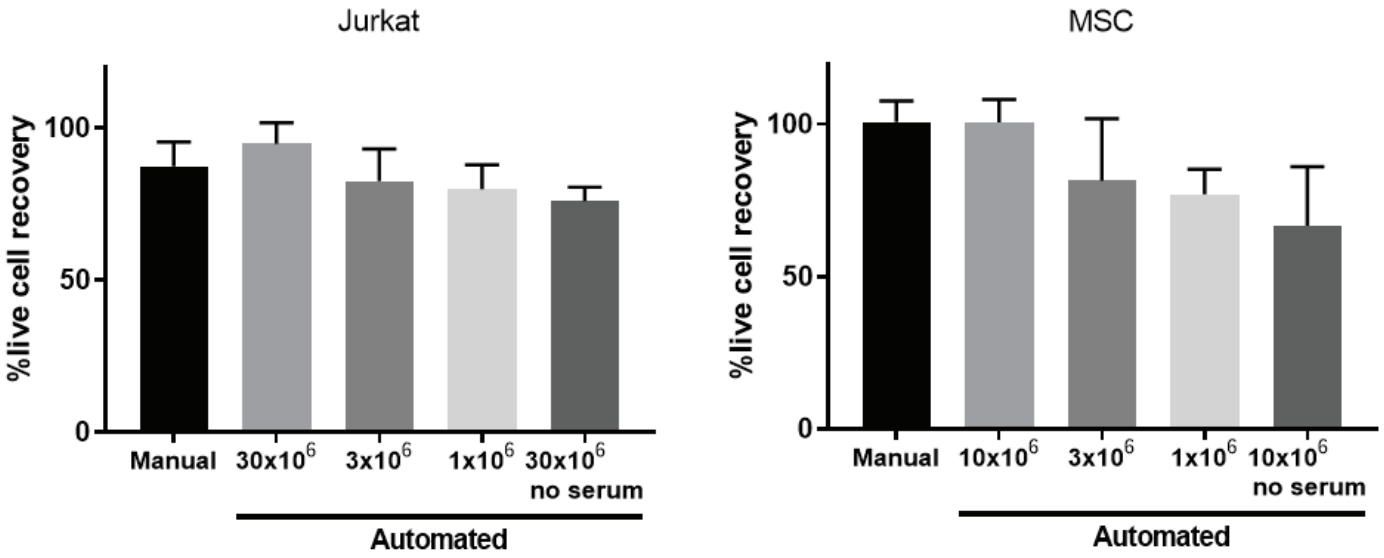


Figure 5

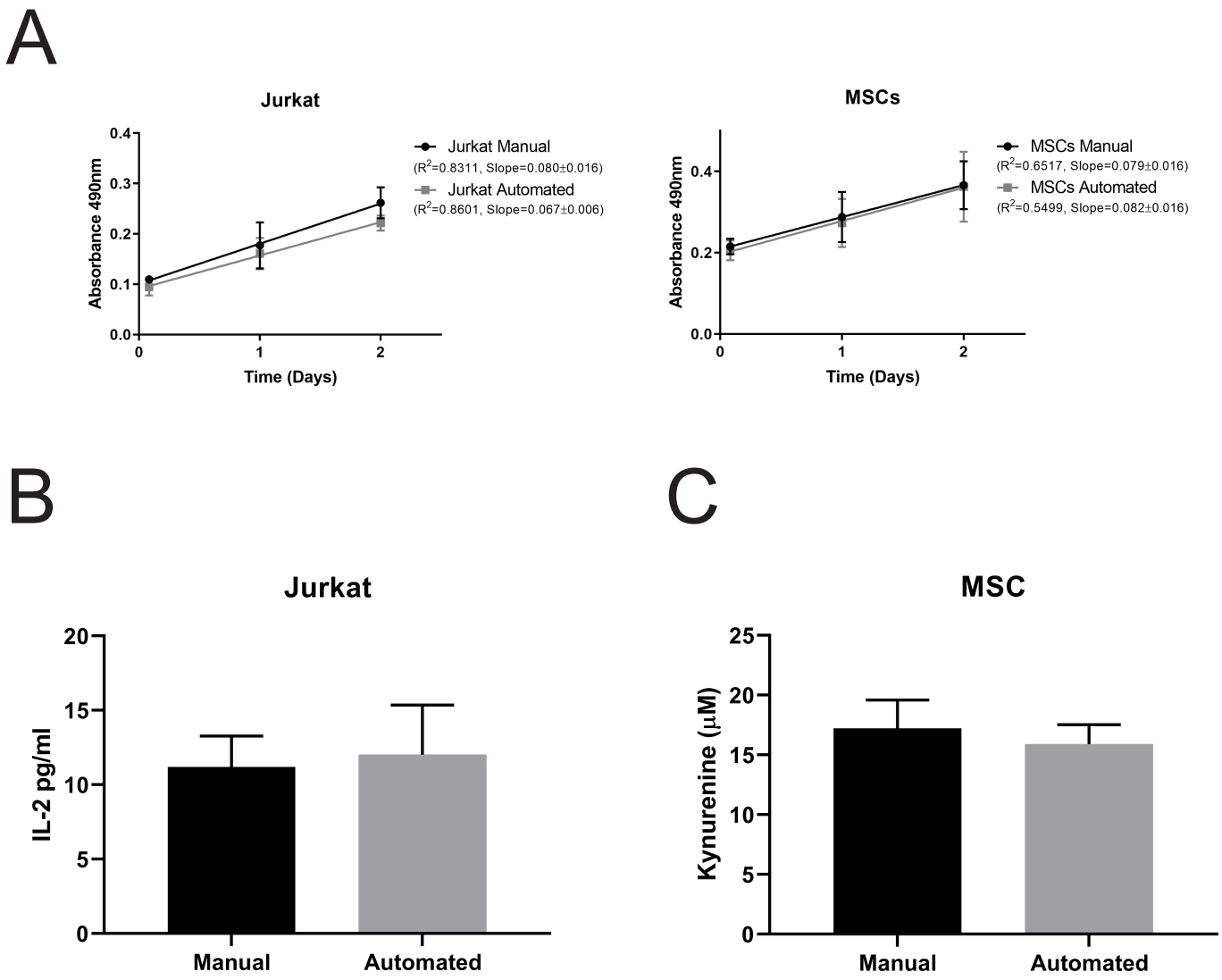
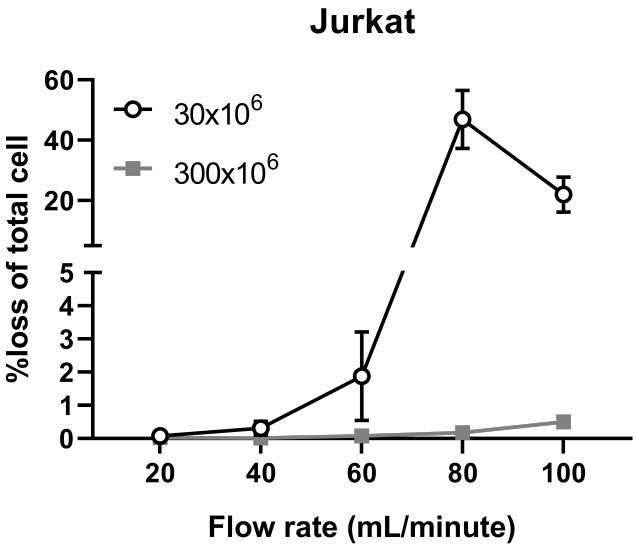


Figure 6

Figure 6



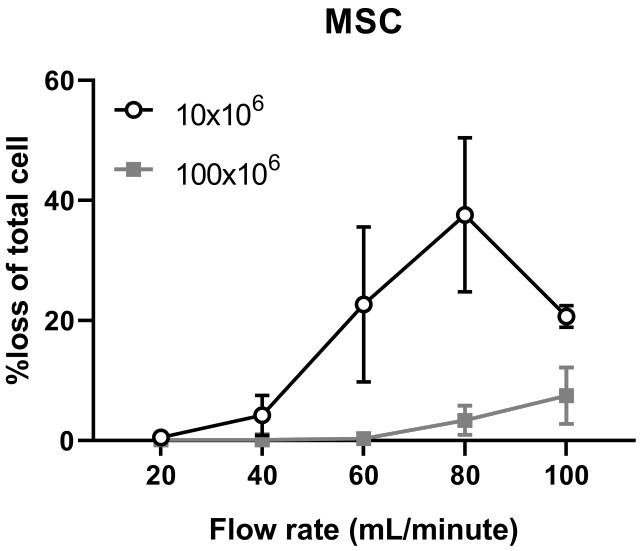


Table 1 Click here to access/download;Table;Table 1.xlsx

Step Number	Description	Open valves	Centrifuge speed (*g*)	Pump speed (ml/min)
1	Prime tubing from B to A	A, B, K	10	50
2	Fill bubble Trap from A to B	A, B, K	100	50 (reverse)
3	Prime tubing A to D	A, D, K	100	50 (reverse)
4	Prime tubing J to K	J, K	100	50
5	Load cells – initial start D to G	D, K, G	1600 (Jurkat)	25 (Jurkat) 30 (MSCs)
6	Load cells with bubble detect [At the detection of bubble/ empty tubing, the program will pause and wait for operator's command, press 'pause' or 'next']	A, D, K	= last step	30 (Jurkat) 35 (MSCs)
7	Empty remaining media on port D tube	A, D, K	= last step	= last step
8	Wash cells 1	A, B, K	= last step	20
9	Ramping up washing speed	A, B, K	= last step	35
10	Wash Cells 2	A, B, K	= last step	= last step
11	Prepare to harvest	J, K	= last step	= last step
12	Recover cells to output and dilute to target volume	B, J, K	= last step	60 (Reverse)

Note: '= last step' is a setting option for centrifuging speed and pump speed in the GU

Triggers
Volume: 45 ml
Volume: 10 ml
Volume: 2 ml
Volume: 3 ml
Volume: 100 ml
Bubble sensor D
Volume: 1.5 ml
Volume: 20 ml
Timer: 5 seconds speed ramping
Volume: 20 ml
Timer: 2 seconds
Volume: 10 ml

I.

Table of Materials

Click here to access/download;Table of Materials;Table of materials (1).xlsx

Name	Company	Catalog Number
20 ml Luer lock syringes	BD	302830
20% Human serum albumin (HSA)	CSL Behring	AUST R 46283
4-(Dimethylamino)benzaldehyde	Sigma-Aldrich	156477-25g
500ml IV saline bag	Fresenius Kabi	K690521
Antibiotic-Antimycotic	Thermo Fisher Scientific	15240112
Automated cell counter (Countess)	Thermo Fisher Scientific	N/A
Cell counting chamber slides	Thermo Fisher Scientific	C10228
Cell stimulation cocktail (500x)	Thermo Fisher Scientific	00-4970-93
Cell transfer bags	Terumo	T1BBT060CBB
CellTiter AQueous One Solution Cell Proliferation Assay (MTS)	Promega	G3582
Centrifuge	Eppendorf	5810R
DMEM: F12 media	Thermo Fisher Scientific	11320082
EnVision plate Reader	Perkin Elmer	N/A
Fetal bovine serum (FBS)	Thermo Fisher Scientific	10099141
Human Interleukin 2 (IL2) Kit	Perkin Elmer	Al221C
Luer (female) fittings	CPC	LF41
PC laptop or PC tablet device	ASUS	N/A
Plate reader (SpectraMax i3)	Molecular Device	N/A
Recombinant Human IFN-γ	PeproTech	300-02
Rotea counterflow centrifuge cell processing device	Scinogy	N/A
Rotea single-use processing kit	Scinogy	N/A
RPMI media	Thermo Fisher Scientific	11875119
Surgical scissors	ProSciTech	420SS
Trichloroacetic acide	Sigma-Aldrich	T6399-250g
Trypan Blue stain	Thermo Fisher Scientific	T10282
Trypsin digestion enzyme (TrypLE Express Enzyme)	Thermo Fisher Scientific	12604013

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	An automated counterflow centrifugal system for small scale downstream process
Author(s):	Anqi Li, Stephen Wilson, Ian Fitzpatrick, Mehri Barabadi, Siow Teng Chan, Mirja Krause, Gina Diamanta Kusuma, David James, Rebecca Lim

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

[X] Standard Access [ ] Open Access

Item 2: Please select one of the following items:

[X] The Author is **NOT** a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Rebecca Lim
Department:	The Ritchie Centre
Institution:	Hudson Institute of Medical Research
Title:	Associate Professor
Signature:	
Date:	14th June 2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

17th September 2019

Dear Editor-in-chief,

On behalf of my co-authors, I am pleased to submit the revision of our manuscript, "**An automated counterflow centrifugal system for small scale downstream process**" for publication in the Journal of Visualized Experiments.

We thank the editors and reviewers for their insights and the opportunity to respond to their comments. We appreciate the time taken to collectively improve this manuscript. We have addressed each concern as detailed in our response below.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

This manuscript has been subjected to more proof reading and all grammatical and syntax errors have been corrected.

• **Protocol Numbering:** All steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.
One-line space has now been added between each protocol step.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
Filming protocol has been highlighted, including the sub-steps and excluding notes

2) Some of your shorter protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
A few short protocol steps has been combined.

3) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
The highlighted steps follow the operational order of the protocol.

4) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
The total length of the highlight is around 1.5 pages.

5) Notes cannot be filmed and should be excluded from highlighting.
No notes were highlighted.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
The modifications and troubleshooting are discussed in 2 paragraphs, lines 260-276. Limitations of the technique are listed in line 302-306. Significance with respect to existing methods is discussed in lines 288-300. Future applications in lines 308-311. Critical steps within the protocol is lines 278-286.

• **Figure/Table Legends:** Define error bars in all figures.
All error bars are now defined.

• **References:** Please spell out journal names.
The spelling of journal names has been checked.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Rotea™
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

The commercial name of the product has been removed. The Rotea is now referred to as the 'CFC device'.

2) Please remove the name ROTEA from fig 1
The Rotea name in figure 1B has been removed.

• Please define all abbreviations at first use.
All abbreviations have been checked and defined.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
The authors described the use of a counterflow centrifugal cell processing device for automating the buffer exchange and cell concentration steps for small-scale bioprocessing. The protocol is useful

and could be used for small-scale processes in a closed manner, compatible with GMP guidelines. My only concern is about the COG. Since the system is single-use, the COG will not be lower than the traditional method used (centrifugation), especially in a small-scale scenario. Please try to focus in the fact that the system is closed and automated, not of being cost-effective.

The benefit of automation in the introduction has been revised in Line 78-89, which includes improved sterility and scalability and minimisation of operator variation. Cost-effectiveness is discussed but is not the focus of the manuscript, as suggested by Reviewer 1.

Minor Concerns:
-Page 3, line 80: "A recent cost analysis report identified labour as the main cost in GCT, accounting for almost 50% of the cost of goods (CoG)"

Is this for autologous or allogeneic product? Which process are you referring? Please provide more details for this important information.

The cost analysis study was in reference to an autologous product and refers to the entire manufacturing process. Details have been added to the manuscript (lines 85-86).

-Page 3, line 90: The authors state that automated platforms commercially available for cell manufacturing include Cocoon and CliniMACS Prodigy that is not suitable for adherent cell cultures. However, the Quantum system (Terumo BCT) provides protocols for adherent or suspension cells in a fully automated system. Please revise.

We thank Reviewer 1 for pointing this out and we have now revised our manuscript to include the Quantum system (lines 95-103).

Cell viability after the process is the most important information. The results is presented in the table 2. How the authors measured the cell viability? Flow cytometry? Please add this information.

Cell viability was measured by trypan blue exclusion and an automated cell counter. This information has been added to the figure legend, lines 441-442.

As already mentioned, the authors describe that implementing automation, the COG can be reduced. However I am not sure if in this case, the costs will be truly reduced especially due to the fact that the method proposed is intended for small-scale. Could authors estimate the costs of the traditional method (centrifugation, etc.) and Rotea including labour, time, consumables, reagents? Just to justify and extol the work.

Reviewer 1 raises a valid point and in staying true to our claims, the emphasis of our protocol has been refocussed to closed and automated process.

Reviewer #2:
Manuscript Summary:
It appears sufficient work has been done to optimize the process. It may be useful to provide data demonstrating the observations noted in the discussion, such as minimum cell numbers, flow rates,

pump speeds, and presence of serum. For example, a graph showing recovery across multiple centrifuge and pump speed configurations.

We agree with Reviewer 2 that adding observations noted in the discussion would be useful. To this end, we have added cell recovery data from runs with a suboptimal number of cells and in the absence of serum (Figure 4b). Additionally, we have also added a new figure demonstrating the percentage cell loss across different flow rate (Figure 6).

Major Concerns:
The addition of at least one functional characterization assay to complement the viability and recovery data per cell type would be suggested.

We agree with Reviewer 2 that adding functional characterisation assays would complement our viability and recovery data. Accordingly, we have added the measurement of cytokine/enzyme production from each cell type as an additional assay to complement the protocol. The data have been provided in Figure 5.

More focus on both the advantages and limitations of the Rotea could be outlined in the discussion, in addition to suggested use cases.

We agree with Reviewer 2 that this would benefit the quality of our paper and have this included the advantages of Rotea in lines 299-304 and 306-309.

Lines 86-95: The highlighting of fully-automated platforms appears here, but the capabilities of these two options doesn't match with the function of the Rotea. Instead, the mention of more similar CFC processing technology was listed in lines 254-268 in the discussion. Comparison of these offerings is more pertinent to match the Rotea's functionality as demonstrated in this manuscript. Pros and cons of the device could be placed either completely in the introduction or discussion for comparison to other platforms.

The fully automated platforms discussed in the introduction part (line 90-101) was intended to inform the reader of the different ways of implementing automation, in particular, highlighting the need of the modular-based approach. However, we understand the concerns of Reviewer 2, and as such the wording in this paragraph has been updated to clarify our intention. Additionally, the significance of the protocol is discussed in the discussion in comparison to current existing technology (lines 292-298), in accordance to requirements of the journal.

Line 100-107: A figure or schematic illustrating the centrifugal force, opposite fluid flow, flow gradient, and separation of live cells and debris would be helpful here. This could also be supported by the current Figure 1, where an additional device schematic could show these concepts.

We have now included a schematic illustration is added in Figure 1 to describe all the forces.

Minor Concerns:

Line 90: Further elaborate on the types of operating protocol limitations here.

We have elaborated on the operating protocol limitations in lines 98-103 to describe how a fully integrated system may be restricted by its the culturing capacity.

Line 92: It would be useful here to identify examples of "typical adherent mammalian cell cultures" that are being used/investigated as cell therapies.

In our response to Reviewer 1, we have added Quantum (Terumo BCT) system to this paragraph and as such we have removed the statement in line 92.

Line 107-111: This use case of CFC seems to diverge from the focus of the paper. Specific cell population isolation isn't investigated for the protocol.

We have removed the statement around use of the CFC device for cell population isolation.

Line 132: It is unclear from the protocol only if the cultured cells were grown in flasks or culture bags. This would be important to identify at which step the open versus closed system processes take place for this protocol.

Thank you for pointing this out. Our protocol used cells cultured in flasks, and we have now clarified this in protocol step 1.2. line 142-149

Figure 4: Further elaboration in the figure caption would be recommended to accurately label what constitutes as the "cell bed" as described in the manuscript.

We have now updated this (now Figure 2) to include a dotted line to outline the area that is occupied by the 'cell bed'.

We thank the editor and reviewers again for taking time to review this re-submission and look forward to hearing from you in due course.

Kind regards,

A/Prof Rebecca Lim
Research Group Head
The Ritchie Centre
Hudson Institute of Medical Research
27-31 Wright St, Clayton VIC 3168
Email: Rebecca.Lim@hudson.org.au
Tel: +613 8572 2794