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Review

Advances in automated cell washing and concentration


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ABSTRACT

The successful commercialization of cell therapies requires thorough planning and consideration of product quality, cost and scale of the manufacturing process. The implementation of automation can be central to a robust and reproducible manufacturing process at industrialized scales. There have been a number of wash-and-concentrate devices developed for cell manufacturing. These technologies have arisen from transfusion medicine, hematopoietic stem cell and biologics manufacturing where operating mechanisms are distinct from manual centrifugation. This review describes the historical origin and fundamental technologies underlying each currently available wash-and-concentrate device as well as their relative advantages and disadvantages in cell therapy applications. Understanding the specific attributes and limitations of these technologies is essential to optimizing cell therapy manufacturing.

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Introduction

Regenerative medicine and advanced therapy medicinal products (ATMPs) include gene therapy, cell therapy and tissue-engineering products. As an increasing number of ATMPs enter the market, it is clear that ATMP manufacturing continues to face significant challenges. These include the high cost of materials and specialized equipment, which directly contributes to the high cost of products, which may then struggle to achieve adequate reimbursement for market viability. Complex supply chain logistics, a workforce with limited skills and requirements for Good Manufacturing Practice (GMP) production and quality assurance capacity contribute to the challenges as well [1,2]. One feature that distinguishes ATMPs from other therapeutic products is that many ATMPs continue to be manufactured in academic settings throughout much of the product development phase [3]. It is estimated that 73.2% of phase 1/2 trials are initiated by non-commercial sponsors focusing on addressing unmet medical needs [4]. ATMP manufacturing for these early-phase trials is often performed at smaller scales, with manual procedures, paper-based documentation and little consideration for automation and scalable manufacturing.

Another commonly reported bottleneck in ATMP manufacturing is the shortage of trained specialized staff [5]. A UK-based contract development and manufacturing organization reported a more than 100% increase in demand for bioprocessing roles in 2019 [6]. Additionally, manufacturing facilities have been in high demand, with the purchase of these facilities taking up the majority of 2019 merger and acquisition activities in the cell and gene therapy sector [7,8]. The Center for Breakthrough Medicines, a US-based contract development and manufacturing organization, also announced plans to invest \$1.1 billion in new cell and gene therapy manufacturing facilities early in 2020 [9]. Consequently, it has been predicted that global manufacturing capacity for ATMPs will stagnate without automation [10]. Developers are increasingly aware that a conscious decision must be made as to when and how to transfer a research-based manufacturing process to a market-ready one.

The myriad challenges in ATMP development, coupled with alluring clinical possibilities, have made the implementation of automation increasingly important for those who seek to translate their discoveries into approved and sustainable therapies. There have been a number of upstream and downstream processing devices developed for GMP manufacturing of ATMPs. Instruments such as CliniMACS Prodigy (Miltenyi Biotec, Miltenyi Biotec, Bergisch Gladbach, Germany), Quantum (Terumo BCT, Lakewood, CO, US) and Cocoon (Lonza, Basel, Switzerland) address cell culture and wash-and-concentrate needs

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with a single device. These systems are appealing, as they can reduce the number of handling steps and sterile boundary connections. Furthermore, technical support is consolidated into a single device. Although this may be a simple way of addressing the need for automation, one must also consider that this is at the cost of flexibility and risks complicating the validation process.

Alternatively, automation can be implemented using a modular approach, as opposed to an integrated approach in which multiple processes are integrated into a single device. In the modular approach, several devices, each performing a main function, form a processing chain. The processing material is transferred between devices through sterile welding or a transfer module when an isolator system is in place [11]. The modular approach allows for customized workflows and choice of platforms that the users determine to be most suitable for the product. However, building a process chain requires in-depth knowledge of the enabling technologies as well as the product. One of the most widely used types of modular device is the wash-and-concentrate processing platform.

Volume reduction and buffer exchange are common steps that are essential across all ATMP manufacturing. In a research lab setting, these steps are typically achieved using a benchtop centrifuge followed by repeated manual pipetting to achieve a cell suspension. The same manual process can be performed in a biosafety cabinet in a GMP cell manufacturing facility. However, there is a high risk of contamination because of the opening and closing of vessel lids, particularly when multiple culture vessels are required [12]. The risk of contamination can be greatly reduced by using an automated enclosed processing device for these steps. Additionally, enclosed processing potentially allows more efficient use of higher-rated cleanrooms [13,14]. For the purposes of this review, the authors define an automated wash-and-concentrate process as one that uses a single device for volume reduction, buffer exchange and delivery of the final product. Similarly, a semi-automated process refers to one in which a portion of the aforementioned steps are performed by the operator (e.g., pulling a syringe to recover part of its contents). A manual process refers to one in which the operator is a critical driving force in the process, such as using a pipette for cell resuspension.

Considerations in Device Selection

There are several important considerations when deciding on a wash-and-concentrate device. These are discussed in the following section and summarized in Table 1.

Table 1
Key considerations when selecting wash-and-concentrate devices.

Topic	Key considerations	
Product type	Autologous CAR-T therapy	Expanded MSC therapy
Process requirements	Minimum operating volume	Maximum operating volume
	Percentage viable	Maximum processing rate
	cell recovery	Processing time
	Minimum output	Maximum concentration factor
Integration	Requirement of cell selection (e.g., RBC removal)	
	Physical integration of hardware (footprint, connections), software integration	
Process development	Staff skills and availability	
	Ease of use	
	Cost (consumables and reagents)	
	Regulatory/quality compliance	
	Scalability	
	Time	

Processing requirements

The specific requirements of any given manufacturing process will play into the decision-making process when selecting a wash-and-concentrate device. The wash-and-concentrate step can be applied at the start of a manufacturing process for the purposes of cell enrichment. It can also be applied in the middle of the process for media change or at the end of the process to concentrate the cell suspension for product formulation and filling. In addition to considerations during process development steps, devices should address the critical quality attributes of the product, such as cell number yield, viability and cell concentration. Given that processing requirements differ between autologous and allogeneic products, the considerations in device selection are also different.

Autologous products

The minimal processing capacity and percentage of viable cells recovered are particularly important factors for autologous products because poor cell recovery may lead to a failed manufacturing run. The minimum output volume can also be a critical factor when a concentrated cell suspension is required. For example, one method of chimeric antigen receptor T-cell (CAR-T) manufacturing using electroporation requires a cell concentration of $40\text{--}300 \times 10^6$ cells/mL [15–17]. Given that apheresis may yield, at the lower bounds, approximately 600×10^6 CD3+ or fewer cells in 200–500 mL [18], a cell washing device capable of concentrating the collected product to a volume of less than 10 mL may be required to be suitable for electroporation. This is noteworthy since only a handful of wash-and-concentrate devices are able to deliver this (e.g., Sepax, Ekko and Rotea).

Additionally, ATMP manufacturing from apheresis material often requires red blood cell (RBC) removal or cell selection. Several processing devices (e.g., Sepax, Lovo, Elutra and Rotea) are capable of RBC removal in addition to buffer exchange. Generally, one would select a device that is able to perform multiple steps in the manufacturing process instead of having two separate devices, thereby maximizing the capital investment and minimizing the cost of process development.

Allogeneic products

Maximum processing capacity is an important factor when selecting a device for the processing of allogeneic products. This is often referred to as the input volume range of the starting material. The input volume range of wash-and-concentrate devices currently available on the market is illustrated in Figure 1. The input volume range of allogeneic products varies from less than 50 mL (e.g., cord blood) [19] to 1000 L or more in expanded cell culture [20–22].

Another critical factor that influences the choice of wash-and-concentrate device is the processing rate and time. Any negative impact on cell quality (e.g., shear stress) can be magnified when cells are subjected to prolonged processing in the downstream device, which typically has no temperature or pH control. Additionally, cells that have been subjected to stressors, such as transfection, transduction, enzyme digestion or cell selection, may become more sensitive to the stress induced by washing and concentrating.

Given that processing rates can vary significantly depending on the composition of culture media and cell density, a first-hand experience with a demo unit may be the only way to aid in the decision-making process. For example, an acoustic-based device (e.g., Ekko) can be more efficient for processing higher-density cell suspensions ($>5 \times 10^5$ cells/mL), where cells can form clusters and fall out of suspension. By contrast, a filtration-based device (e.g., Lovo) is less sensitive to starting cell density but restricted by the minimum output range.

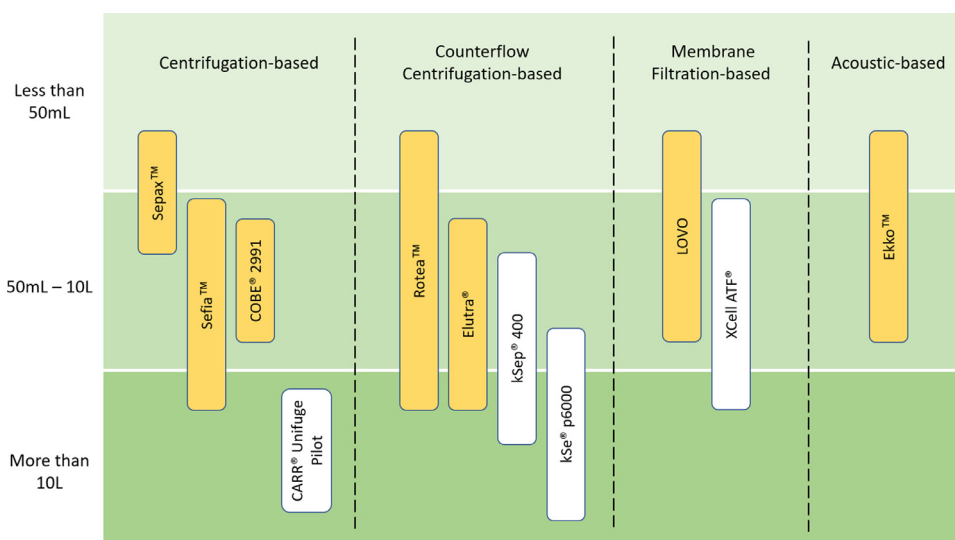


Figure 1. Comparison of cell processing capacity across currently available devices. The devices are grouped by different separation mechanisms. Devices capable of cell selection are in yellow. The size of each block is not to scale but is indicative of the common operating range of each device. (Color version of figure is available online).

Integration

Automated wash-and-concentrate devices typically operate as part of a processing chain; hence, integration of a device into the process chain must be evaluated beforehand. Physical integration refers to how the device fits into the processing chain and connects to other devices. The value of automation is maximized when cells are transferred in a closed manner; hence, it is important to plan and evaluate how the devices connect to each other. Setting up the wash-and-concentrate device can be straightforward if the reagents (e.g., culture media, cryopreservatives or buffers) are already contained within bags. Bags are a format supported by virtually all processing devices. They can be connected through a spike port or sterile welding if the tubing size is compatible. In the absence of this, an extra step of loading the materials into transfer bags before sterile connection to the wash-and-concentrate device is required. If this transfer cannot be achieved through a sterile connection, the value of the automation is significantly discounted, as it is deemed an open process. Ideally, the concentrated product should also be transferred for the downstream process (e.g., fill and formulation) in a closed manner. The footprint and mobility of the device should also be considered since clean room space is often limited. Portability of devices is advantageous, permitting transport between rooms without the need for recalibration. In the event that the device is not portable, it is important to consider the complexity of recalibration.

The importance of software integration was reviewed recently [23]. Replacing manual batch manufacturing and quality control records with centralized electronic batch records allows for operational automation. Additionally, software integration may be extended to multiple devices on a centralized system, which can control all linked devices and provide real-time feedback. This approach is common in the manufacturing of biologics. With ATMPs, process automation is currently restricted to controlling devices from the same manufacturer, as a custom-built system is required to centrally control devices from different manufacturers. The difficulties in developing a custom-built system include substantial capital investment and challenges in extracting encrypted data from different devices. Nevertheless, the need for software integration is unlikely to be critical at the early stages of product development.

Process development efforts

The goal of process development is to develop a robust, reproducible process that can be scaled for clinical production while maintaining product quality and fulfilling regulatory requirements [24]. A robust wash-and-concentrate process is one that has a minimal failure rate regardless of variation in input material. Achieving this involves careful device selection and exhaustively optimized protocols. A reproducible process is one that produces predictable outcomes. In the case of autologous products in which there is substantial intrinsic variation in starting material, a prediction algorithm may also be developed in guiding the manufacturing steps to achieve the desired product profile [25,26]. Process development requires substantial effort to identify and control the critical process parameters of the process, and multivariate analysis is sometimes essential. For example, the critical process parameters for a centrifugation-based process may include flow rate, centrifugal speed and sedimentation time. When optimizing these parameters, one should consider the relationship between process parameters and product quality to ensure that the manufacturing process is suitable for scaled-up production.

The developed process should ideally also consider the cost of goods to ensure the product is profitable by the time it enters the market. The cost of goods typically refers to the manufacturing costs incurred, such as raw materials, labor and quality control testing [27]. Other costs incurred during the development of ATMPs include the cost of clinical trials, regulatory approval and marketing. The capital investment in purchasing a wash-and-concentrate device, single-use processing kits and ongoing maintenance are some obvious costs associated with implementing automation. A direct comparison of the capital investment and the cost of consumables across different wash-and-concentrate devices may assist decision-making in onboarding the technology. However, the pricing may be varied in different regions and enterprise agreements. It is also a limitation of this review that the authors are not able to supply this information. Additional costs, however, may arise through suboptimal operation or if an inappropriate device is selected.

Current Technologies in Automated Wash-and-Concentrate

A recent review compared different wash-and-concentrate technologies for processing blood products [28]. It is worth noting that blood processing begins with fairly predictable starting materials and

involves a relatively standard range of processing volumes. By contrast, cell manufacturing involves a wide range of starting materials and processing goals. Therefore, choosing suitable devices for cell manufacturing is much more complex and requires a firm understanding of the distinct mechanisms of these technologies. It is important to mention that although some of these devices may also be applicable for the production of viral vectors, this is beyond the scope of the current review.

The following section details engineering designs and processing mechanisms in wash-and-concentrate devices that are currently available on the market.

Benchtop centrifuge

Conventional centrifugation methods for washing and concentrating cells are open processes. Alternatively, samples may be centrifuged in cell transfer bags to achieve closed processing. This manual approach for closed processing is simple and cost-effective and commonplace in bone marrow transplantation with regard to performing volume reduction of apheresis units prior to cryopreservation [29,30]. In this case, the cell transfer bag is sterile welded to another transfer bag, where the connecting tube is manually clamped during centrifugation. Following centrifugation, the transfer bag containing the cells is placed on a plasma extractor, which gently presses the excess buffer or plasma into a new bag after the manual clamp is released.

A similar volume reduction method is applied in cord blood banking [31], where two-step centrifugation removes part of the RBCs and plasma, leaving the buffy coat for banking. Debulking of the RBCs reduces the volume of dimethyl sulfoxide (DMSO) required for cryopreservation and subsequently minimizes the side effects associated with the cytotoxicity of DMSO and hemolysis. A number of semi-automated devices have been developed to standardize volume reduction and RBC debulking steps following the same operating principles as the manual procedure [32,33]. One such example is the AutoXpress platform system (ThermoGenesis Corp., Rancho Cordova, CA, US), which is a cord blood/stem cell processing system (Figure 2). It is composed of a device and a single-use processing kit. The processing kit contains multiple small pockets that store the separated plasma, mononucleated cells and RBCs (Figure 2A). The cord blood sample is first transferred to the processing kit, which is then fitted

into the device (Figure 2B). The device is then loaded into a centrifuge bucket and centrifuged to separate the sample into plasma, buffy coat and RBC layers. The device directs RBCs into their respective collection bags automatically by changing the position of the stopcock handles (Figure 2C). The buffy coat is then directed to the sampling and cryopreservation bags. Cryoprotectant is added manually afterward. This technology can also be used for cultured cells (X-WASH system; ThermoGenesis Corp.).

Centrifugation-based technology

Centrifugation-based technology is one of the most widely used separation methods. Centrifugation-based devices have different shapes and configurations. The ones that have been applied in cell therapies are mostly the tubular bowl type [34]. Although the mechanism for cell separation is similar among tubular bowl devices, they rely on different mechanisms to deliver the cell concentrate.

Delivered by the flexible diaphragm: COBE 2991

The COBE 2991 (Terumo BCT) was developed by a team of IBM engineers in 1972 [35]. The work was initiated by George T. Judson, an engineer whose 17-year-old son was diagnosed with leukemia and admitted to the National Cancer Institute in Bethesda, Maryland. When Judson visited the blood bank at the National Cancer Institute, he saw how bags of leukapheresis products were being centrifuged to separate the white blood cells. This inspired the development of two unique devices: the IBM blood cell separator and the IBM blood cell processor. The medical unit of IBM was later purchased by Cobe Laboratories in 1984 [36], and these devices are now marketed as the COBE Spectra leukapheresis system and the COBE 2991 cell processor.

The design and development of the COBE 2991 cell processor was led by Jones [35] in 1965. The initial plan was around the use of a donut-shaped blood processing bag lining a concentric centrifuge bowl. The processing bag had four tubes originating from the periphery of the bag to deliver wash solutions and one tube extending from the middle of the bag for supernatant removal. By pumping water into the centrifuge bowl during centrifugation, the water would fill the cavity and push the processing bag to expel the supernatant. The idea of immersing samples in water was unappealing given the risk of contamination and the challenge of sourcing suitable parts for the

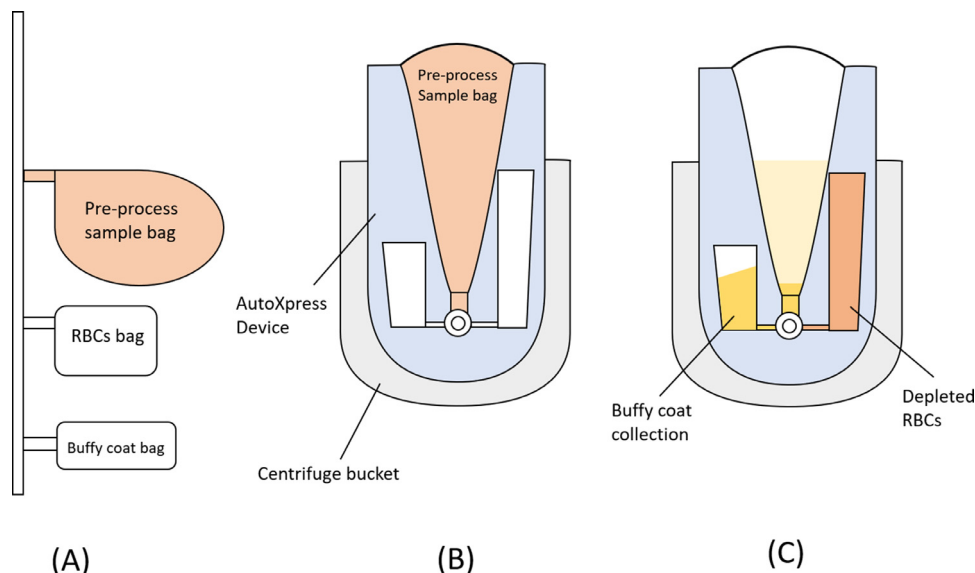


Figure 2. Automated cord blood processing. (A) The cord blood sample is first transferred to the designated bag of the single-use processing kit. (B) The processing kit is then fitted into the AutoXpress device and centrifuged for cell separation using a benchtop centrifuge. (C) The automated system detects the separated fractions with the optical sensor and directs them into different compartments of the processing kit. (Color version of figure is available online).

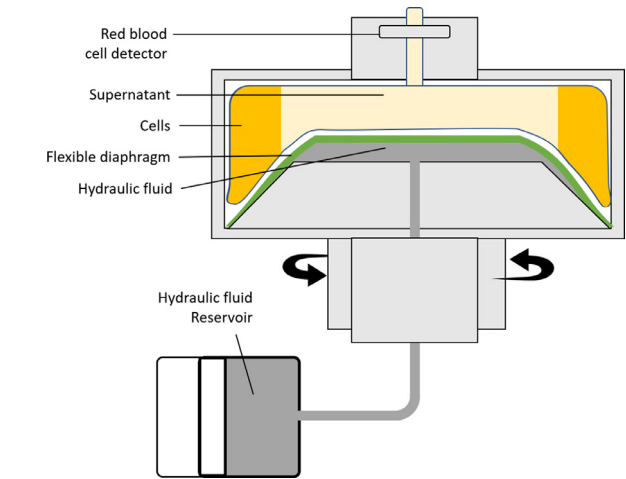


Figure 3. The COBE 2991 cell processor. The final design of the cell processor has a flexible membrane separating the hydraulic fluid (green) from the processing bag. Cells are separated toward the periphery of the processing bag from the supernatant in the center. Figure adapted from Jones [35]. (Color version of figure is available online).

inlets. The water immersion problem was later resolved by inserting a flexible membrane into the centrifuge bowl so that the water was not in direct contact with the processing bag. The fluid path problem was then resolved by having the wash buffer and supernatant enter and leave the processing bag through the same seal port—a key design element of the device.

The final design is illustrated in Figure 3, which demonstrates where blood or leukapheresis products enter through a central port into a donut-shaped processing bag. Upon centrifugation, cells and media separate vertically, and cells migrate toward the periphery of the processing bag. The hydraulic liquid then inflates the flexible membrane inside the centrifuging bowl, pressing against the cell processing bag, thus expelling the supernatant from the center of the processing bag.

The COBE 2991 was first used for deglycerolizing RBCs [36]. High glycerol (40%) concentrations allow RBCs to be frozen in temperatures of -80°C . However, glycerol concentration must be reduced to $<2\%$ to prevent hemolysis upon transfusion [33], thus requiring a washing step. The COBE 2991 provides automated RBC washing with approximately 90% RBC recovery [37,38], which addresses unexpectedly high blood volume demands and/or requirements for rare blood types [39]. The application of the COBE 2991 has since expanded to other blood products, such as platelets and apheresis material [40–42].

To date, the use of the COBE 2991 has largely been limited to minimally manipulated cell products, and this may in part be due to limited scalability. Additionally, the minimal processing volume of the COBE 2991 has further limited its use for small-volume products such as cord blood (Table 2). Moreover, the maximum volume of the processing bag is <1 L. Repeated processing runs are thus required to process larger volumes with the COBE 2991, which substantially increases processing time [20]. Repeated washing cycles are also required to reduce residuals due to the carryover volume in the donut-shaped disposable [43].

Delivered by the reverse flow: cell savers

Cell Saver (Haemonetics, Boston, MA, US) was the first commercial blood salvage device introduced in 1974 [44]. Subsequently, “cell saver” became a term that referred to a class of blood salvage devices used in perioperative care. These devices are designed to collect blood, replace plasma with saline and concentrate RBCs for reinfusion. This process is achieved by a bell-shaped centrifuge chamber (bowl) in which a centrifugal force gradient is formed, allowing RBCs to sediment toward the bottom and lateral sides of the chamber (Figure 4A) [45]. Once the RBCs are concentrated and washed, cells are delivered by reversing the pump direction and replacing the chamber with filtered clean air.

Choosing the correct bowl size is critical to the operation of a cell saver, where the bowl size can range from 70 mL to 375 mL. The 225- to 250-mL size bowl is most widely used in surgery, requiring a minimum input volume of 500–740 mL of shed blood to fill a bowl [45]. Partial filling of the bowl may result in insufficient washing, as platelets and leukocytes can accumulate in the chamber, whereas excess volumes may result in cells leaving the chamber because of overflow and, hence, reduced recovery [46,47]. The bowl size also dictates the output volume, which is typically around the same volume as the chamber [48].

The repurposing of Cell Saver 5 (Haemonetics) has been suggested for CAR-T manufacturing to process leukapheresis material and cultured T cells [49–51]. There are several blood salvage devices that are able to achieve cell wash-and-concentrate following a similar principle, including Electa (Sorin Group, Saluggia, Italy), CATS (Freseinius Kabi, Bad Homburg, Germany) and OrthoPAT (Haemonetics) [52]. In an early human study, the blood salvage device Hemalite-2 (Haemonetics), now discontinued, was utilized for the wash-and-concentrate of *ex vivo*-expanded hematopoietic progenitor cells before infusion [53]. Later, the Cell Saver system also supported early T-cell therapy development by allowing the adjustment of both centrifugal speed and pump speed. Another study investigated the use of Cell Saver 5 for the isolation of mononuclear cells from apheresis products, achieving approximately 90% RBC removal and approximately 64% mononuclear cell recovery [54]. One possible challenge to applying the device in ATMP manufacturing is the relatively large output volume, which may not be suitable for some autologous products.

Delivered by the syringe plunger: Sepax and Sefia

In the late 1980s, the feasibility of using umbilical cord blood as an alternative source of hematopoietic stem cells for bone marrow reconstitution was explored [55,56]. Each umbilical cord blood unit (50–200 mL) is reduced to a standard volume of approximately 20 mL [57]. The need to automate and standardize this procedure led to the development of several automated processing devices, such as the AutoXpress platform mentioned earlier and, more recently, Sepax from BioSafe Systems (later marketed by Cytiva, Marlborough, MA, US).

The Sepax 2 is a medical device for cord blood processing, whereas the Sepax C-Pro, which has a similar design, is a cell manufacturing device. Sepax is an enclosed automated processing benchtop device designed for the processing of cord blood, bone marrow and similar starting materials. It utilizes a syringe-like, cylinder-shaped cartridge for cell concentration and washing. In its automated cell separation protocol, the processing chamber first draws up the

Table 2
Summary of centrifugation-based devices.

	COBE 2991	Sepax	Sefia	CARR Unifuge Pilot
Process volume	150–630 mL per cycle [36]	30–220 mL per cycle, up to four cycles [60]	50 mL to >10 L [61]	>1000 L [63]
Minimum output volume	60–80 mL [134]	8 mL [60]	15 mL [61]	N/A

N/A, not applicable.

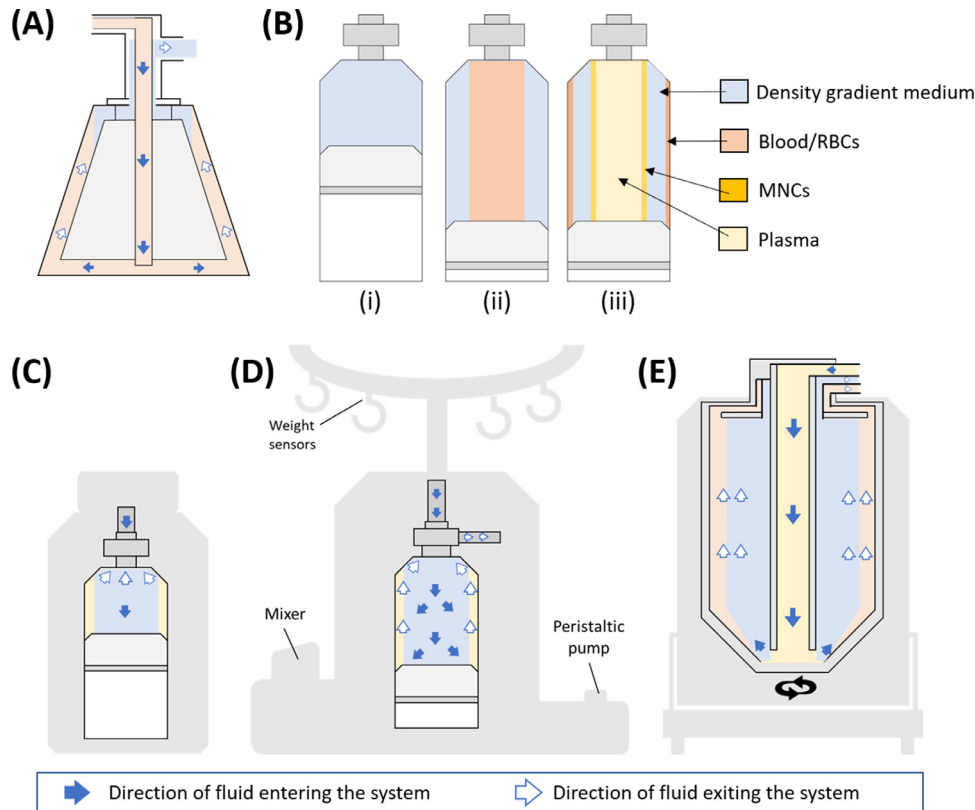


Figure 4. Operation principle of the cylinder-type centrifugation cell separating system. (A) The Latham bowl design allows cells to sediment toward the lower lateral side of the bowl while excess liquid exits through the top opening. (B) The cell processing chamber first draws in a density gradient medium (first image) and then slowly draws in the blood sample while the centrifugation is underway (second image). The RBCs slowly move through the density gradient medium and migrate to the periphery of the chamber (third image), which achieves a vertical separation of RBCs, density gradient buffer, MNCs and plasma. The fluid path of the Sepax (C), Sefia (D) and CARR Unifuge (E) systems, where the blue arrows indicate the direction of fluid entering the chamber and the white arrows indicate the direction of fluid exiting the chamber. The Sefia and CARR Unifuge systems are able to process the medium in a continuous fashion in which the fluid enters and exits through separate channels. Figure adapted from Reeder [45] and Aktas *et al.* [59]. MNCs, mononuclear cells. (Color version of figure is available online).

density gradient buffer (Figure 4B). The density gradient medium typically has a density of approximately 1.077 g/mL, which allows the sedimentation of RBCs (density approximately 1.11 g/mL) while keeping the mononuclear cells (density approximately 1.067–1.077 g/mL) in suspension [58]. Subsequently, the blood sample is drawn into the chamber while the chamber centrifuges from the base. Mononuclear cells and RBCs are separated vertically by differential density and centrifugation. The syringe slowly expels the separated layers, which results in separation of plasma followed by the mononuclear cell fraction. The optical sensor located at the top of the device detects the contents passing through and directs them into pre-specified bags by controlling the stopcock manifold [59].

The Sepax system utilizes single-use kits specific to the clinical application (e.g., cord blood collection, bone marrow mononuclear cell collection), which is user-friendly with minimal programming. The main disadvantage of this system is its limited flexibility. When a syringe-like vacuum force is the only active drive within the system, it limits the flow direction of its contents (Figure 4C). The company also developed a more comprehensive version of the device, the Sefia (Figure 4D), which utilizes the same core technology as the Sepax. The Sefia can handle volumes greater than 10 L through continuous-flow technology (Table 2). The Sepax processing kit shares one opening for fluid entry and exit, and processing is limited to one chamber load (220 mL) at a time [60]. However, the Sefia (CT-800.1 processing kit) allows fluids to exit through a separate opening from fluid entry, which thus allows continuous processing of larger volumes (Figure 4D) [61]. Furthermore, the Sefia possesses extra accessory features, such as sample mixer [62].

Delivered by continuous processing: CARR UniFuge

The CARR UniFuge (Pneumatic Scale Angelus, Stow, OH, US) is a continuous processing system with a >1000-L capacity (Figure 4E) [63]. The operating principle of the CARR UniFuge is similar to that of the Sefia system, relying on a centrifuging cylinder. The cells accumulate around the cylinder wall while the supernatant passes through the center of the cylinder, similar to the mechanism utilized by Cell Saver. The main difference is that the overflowing concentrated cells are collected continuously through a separate channel from the fluid, which thus achieves continuous processing. The CARR UniFuge Pilot is the smallest version in the series, with a cylinder chamber volume of 1.7 L. This minimal input volume may render it unsuitable for early-phase process development. The company has proposed to release a scaled-down version with a smaller chamber size (275 mL) to address the early development need [64].

Counterflow centrifugation-based technology

The initial concept and design of a counterflow centrifugal device dates back to 1932, when Charles Lindbergh designed a device to wash RBCs in suspension [65]. In 1948, Lindahl reported successful cell separation using a counterflow centrifugal system [66]. The technology has since been further developed and marketed by Beckman Instruments (now Beckman Coulter, Pasadena, CA, US) for cell separation [67]. The Beckman design includes a specialized centrifuge (Avanti J-26 series and J6-MI) fitted with an elutriation rotor (JE-5.0) that allows the fitting of tubing and a separation chamber (Figure 5) [68]. However, the buffer flow rate control is managed by a separate

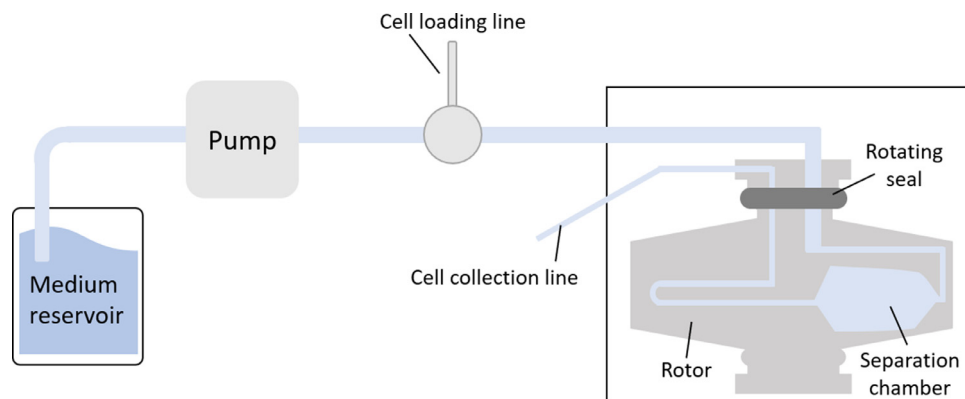


Figure 5. Conceptual design of a counterflow centrifugation chamber. Medium is pumped from the medium reservoir into the separation chamber. Cells are introduced through the cell loading line into the rotating chamber and are then separated in the chamber according to cell size and density. Different fractions of cells are collected from the collecting tube by increasing pump flow rate. Figure adapted from Sanderson and Bird [67]. (Color version of figure is available online).

peristaltic pump. This technology has been applied in cell enrichment and clinical trials of lymphocyte depletion before allogeneic bone marrow transplantation [69–76]. The clinical trials showed that the procedure was safe and had a reduced incidence of acute graft-versus-host disease, but a higher incidence of engraftment failure was also observed, possibly due to the loss of CD34+ cells during elutriation [71,74–76].

In the early 2000s, the Elutra (Terumo BCT), modified from the COBE Spectra apheresis system with a platelet leukoreduction system kit into a cell processing device, was developed [77]. The Elutra has been applied in separating monocytes from apheresis products for dendritic cell-based cancer vaccines [78] as well as lymphocyte enrichment for T-cell therapies [79,80]. A larger-scale counterflow centrifugal device, the Ksep (Sartorius, Goettingen, Germany), was later developed in 2011.

The two applications of counterflow centrifugation—cell separation of subpopulations of cells and cell wash-and-concentrate—are widely applied in ATMP manufacturing. Berger *et al.* [78] reported monocyte enrichment from leukapheresis products using the Elutra. This represents a significant step in the development of immunotherapy products given that monocyte removal has been found to facilitate T-cell activation in CAR-T manufacturing [81,82]. This has been attributed to the release of soluble factors such as prostaglandin E2 by monocytes, which suppresses T-cell activation [83]. Furthermore, monocyte removal enables the use of granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells for CAR-T manufacturing [84].

The fluid dynamics within a counterflow centrifugation chamber are often modeled through two formulae. The sedimentation velocity is described by Stoke's law:

$$SV = \left[\frac{d^2(\rho_p - \rho_m)}{18\eta} \right] \omega^2 r \quad (1)$$

The sedimentation velocity (SV) increases with increasing diameter of the particle (d), density difference between particles and buffer ($\rho_p - \rho_m$), centrifugal angular velocity (ω) and radial position of the particles (r). The counterflow velocity is described as follows:

$$CV = \frac{F}{A} \quad (2)$$

The counterflow velocity (CV) in the direction opposite to the sedimentation velocity is a ratio of flow rate (F) to cross-sectional area (A) [85,86]. Typically, a counterflow centrifugation chamber is cone-shaped, and cells arrive in the narrower end of the cone and travel toward the wider end. A counterflow velocity gradient is created across the chamber and is highest at the narrow end of the chamber and lowest at the widest part of the chamber. Cells within the chamber reach velocity equilibrium when the sedimentation velocity is equal to the counterflow velocity. Larger cells with higher sedimentation velocity will accumulate in the region of the chamber where the highest counterflow velocity is encountered (i.e., at the narrower end of the chamber), whereas smaller cells with lower sedimentation velocity will accumulate at the wider end of the chamber, where counterflow velocity is lowest. Once equilibrium is achieved within the chamber, the application of a higher flow rate will force smaller cells out of velocity equilibrium (i.e., elutriation).

Chamber size

The size of the chamber dictates the maximal and minimal processing capacity. Table 3 summarizes the chamber size of various counterflow centrifugation devices and the minimum cell numbers required for a stable fluidized cell bed. The formation of a fluidized bed requires a critical number of cells within the chamber, where collisions of cells minimize the whirling effect caused by incoming cells. The stability of a fluidized bed increases with cell number [87]. Devices such as the Rotea (developed by Scinogy Pty Ltd, Melbourne, Australia and marketed by Thermo Fisher Scientific, Waltham, MA, US)

Table 3
Comparison of counterflow centrifugal devices.

	Beckman Coulter Elutriator [68]			Elutra chamber [135]	Rotea chamber [87,136]	Ksep system [88]	
	Standard chamber	Large chamber	Sanderson chamber			Ksep 400	Ksep 6000
Chamber volume	4 mL	40 mL	5.5 mL	40 mL	10 mL	100 mL × 4	1000 mL × 6
Minimum number of cells	2×10^7	2×10^8	2×10^6	5×10^9 ^c	5×10^7	N/A	N/A
Maximum centrifuge speed	4700 g ^a	4700 g ^a	4700 g ^a	1000 g ^b	3000 g	1000 g	2000 g

N/A, not applicable.

^a Based on the Beckman Coulter JE-5.0 elutriation rotor.

^b Estimated from 2600 rpm.

^c Recommended operating range in which lower number of cells may still be feasible with optimization [79].

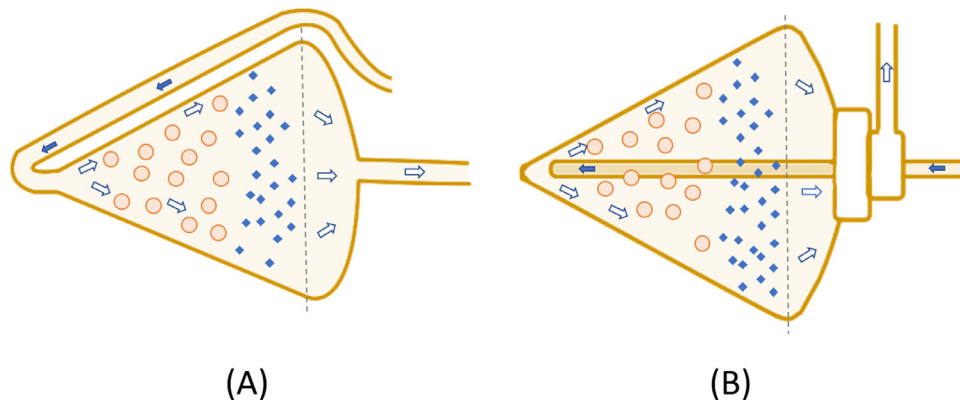


Figure 6. Design of two counterflow centrifugation chambers and inlet designs. Counterflow centrifugation chamber with the cell inlet exterior to the chamber (A) compared with the cell inlet within the chamber (B). The fluid path is demonstrated using blue arrows (entering the chamber) and white arrows (exiting the chamber). The light orange circles indicate cells (or large particles) and the blue squares indicate cell debris (or small particles). (Color version of figure is available online).

are equipped with a camera to permit real-time assessment of the cell bed stability within the chamber, which facilitates process optimization.

Chamber inlet design

The Elutra and Ksep series have similar chamber designs in which the cell inlet is external to the chamber connected from the narrow end (Figure 6A). The Rotea chamber design differs in that the cell inlet is inside the chamber (Figure 6B), which allows a higher centrifugal speed (Table 3) and minimizes dead space. Higher centrifugal speed is also beneficial, as it allows cells to accumulate in the chamber at a higher flow rate and efficiency.

Despite these promising aspects of counterflow centrifugal technology, there is a scarcity of information on the efficiency of this approach in cell washing and concentration. The Ksep 6000 has six processing chambers with a volume of up to 1 L each and is capable of processing a volume of up to 6000 L in a continuous fashion [88,89]. A recent study reported that the Ksep 400 performed the wash-and-concentrate process with 50 L of pluripotent stem cells in under 2 h, achieving cell recovery above 90% with no negative impact on cell viability [90]. At the other end of the spectrum, the Rotea is able to wash and concentrate samples with a volume of less than 50 mL [87]. The study reported the process time is under 15 min, with cell recovery close to 100% without loss of cell viability. These studies exemplify the large operational range of counterflow

centrifugal devices, which is ideal for progressing from early clinical studies to commercial production.

Filtration-based separation

Filtration uses a size-exclusion process to retain larger particles by means of a porous structure (filter) while allowing smaller particles to pass through. The principle of filtration dates back to pre-historic times, when humans used filtration as a method for water purification [91,92]. Today, filtration is applied across a wide range of industries, including food, material and pharmaceutical manufacturing [93,94]. There are three filtration methods commonly used in the pharmaceutical industry: normal flow filtration (NFF), tangential flow filtration (TFF) and spinning membrane filtration [95].

Normal flow filtration

Also known as dead-end filtration, NFF is the most basic form of filtration, where the input material is passed from one side of the filter to the other (Figure 7A) [94]. NFF is a cost-effective method for the removal of cells or cellular debris in biological manufacturing where the filtrate contains the target product. This technique is most efficient when the percentage of solid contents in the sample is low, as high solid content causes membrane clogging (fouling) [96]. For cell therapy applications, NFF is most commonly used in the harvesting of Mesenchymal stromal cells (MSCs) to separate microcarriers from the cells after enzymatic dissociation [97].

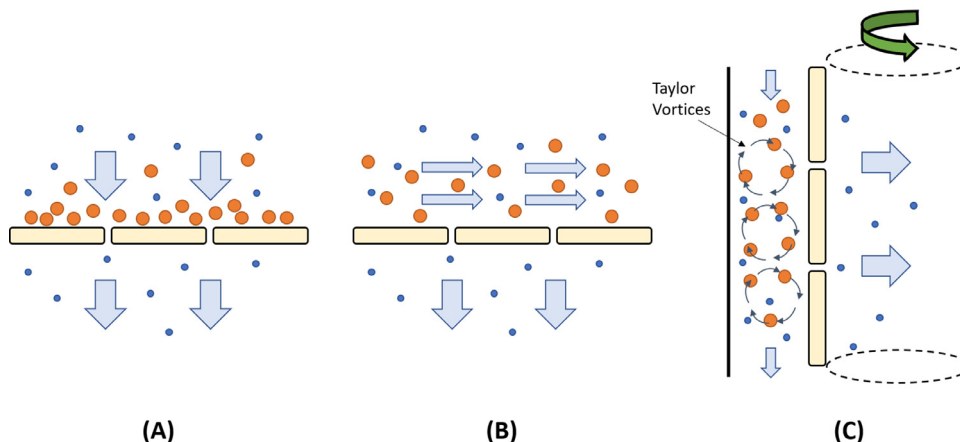


Figure 7. Comparison of different filtration modes in bioprocessing. (A) Normal filtration, (B) tangential flow filtration and (C) spinning membrane filtration in which Taylor vortices are formed from membrane rotation in the annular gap. Blue arrows indicate the direction of fluid, the green arrow indicates the rotating direction of the spinning cartridge, orange circles indicate cells and blue dots indicate water molecules. Figure adapted from Liderfelt and Royce [94]. (Color version of figure is available online).

Table 4
Summary of filtration-based cell concentrate systems.

	Sartoflow slice 200 benchtop crossflow system [137]	XCell ATF system [138]		Lovo [139]
		XCell ATF 2	XCell ATF 10	
Maximal Process volume	500 mL	1 L	1000 L	22 L
Minimum output volume	20 mL	0.1 L	6 L	50 mL

Tangential flow filtration

TFF is a dynamic filtration technology whereby the input material is fed in parallel flow to the filtration membrane, which minimizes membrane fouling (Figure 7B). A study using TFF for MSC harvesting demonstrated cell recovery above 80% when optimized (Sartoflow slice 200 benchtop crossflow system; Sartorius) [97,98]. The Kaneka cell concentration wash system (Kaneka Corporation, Tokyo, Japan) is one of the latest TFF devices developed for cell manufacturing. It has additional features, such as intermediate bags for enzyme digestion and a programmable fluid path, which makes it ideal for adipose tissue-derived MSC processing [99].

One of the issues with the TFF system is that variations in transmembrane pressure can occur within a cassette system, and this may lead to damaging shear forces [100]. The alternating tangential flow (ATF) system is a variation of the TFF that has gained popularity in cell harvesting. Instead of utilizing a unidirectional flow, as is the case with TFF, the ATF system allows fluid to flow back and forth across the hollow fiber membrane, thereby improving efficiency [101]. An example of a commercial ATF system is the XCell ATF (Repligen, Waltham, MA, US), which is able to scale up from 1 L to 2000 L (Table 4).

Spinning membrane filtration

The spinning filtration membrane system comprises a cylinder-shaped membrane within a container. The input material is loaded into the annular gap between the container and the membrane. The fluid dynamic within the gap of two concentric rotating cylinders is described as Couette flow [102]. Notably, toroidal vortices, known as Taylor vortex flow, are generated when the rotation of the inner cylinder reaches a threshold speed [103]. Filtration is then facilitated through the formation of Taylor vortices by membrane rotation in the annular gap (Figure 7C).

The first commercial spinning membrane filtration devices were the Biodruckfilter (Sulzer, Winterthur, Switzerland) [104,105] and Benchmark rotary biofiltration unit (Membrex Inc, US) [106,107], which were developed for the biotech industry. Neither device is scalable, as they comprise a single cylinder and the filtration area is limited to the wall of the cylinder. Subsequent development to scale up spinning membrane filtration systems is achieved by maximizing the filtration area with a multi-disk or multi-shaft setup [103]. Nevertheless, the single cylinder design has remained the mainstay of plasmapheresis and cell therapy manufacturing.

In the 1980s, porous hollow fiber plasma filtration was presented as a simpler and cheaper alternative to the centrifugal system for plasmapheresis [108,109]. However, hollow fiber filtration has a typically low efficiency due to increased osmotic pressure resulting from platelet and RBC accumulation as well as membrane fouling [102]. Spinning membrane-based plasmapheresis was introduced in 1985 and substantially improved the efficiency of plasma collection. This technology was subsequently commercialized by Baxter and developed into an automated cell wash-and-concentrate device—the Cytomate (discontinued in 2010).

The Cytomate was first developed in the early 2000s for DMSO removal from thawed bone marrow transfusion products [110–112]. The Cytomate was reported to achieve approximately 75% cell recovery in comparison to manual processing but required 1 h to process approximately 500 mL of material [110,113]. This technology has since been further developed in the form of the Lovo, a spinning membrane wash-and-concentrate device able to process 500 mL of material in less than 30 min [114,115]. The Lovo is able to perform RBC and immunomagnetic bead removal, buffer exchange and volume reduction. Notably, the minimum output volume of the Lovo is 50 mL, which may not be ideal for applications where input volume is <100 mL (Table 4).

Ultrasonic acoustic wave-based devices

The modern application of ultrasound technology began in 1917 with submarine detection [116]. Ultrasound later found its use in medical imaging, food processing, wastewater treatment and bioprocessing [117,118]. Ultrasound is able to separate particles based on their density since particles with a higher density have a tendency to move toward the pressure node in an ultrasonic field, whereas particles with a lower density move toward the pressure antinode (Figure 8A) [119–121]. The force that drives particles toward the pressure node is the primary acoustic force, which causes particles to accumulate and form bands across the acoustic field. Particles with a larger radius have a high primary acoustic force at a given particle density, which then allows further separation of the particles. The secondary acoustic force is applied within the particle bands, driving particles toward or away from each other depending on the angles between the center line of two particles and the direction of the waves. The particles are attracted to each other when the angles are between 54° and 90° (Figure 8B) [120], forming clusters, which eventually sediment out of suspension (Figure 8C).

A number of acoustic-based cell separation devices have been developed since the 1990s [122]. A transducer is used to generate a macroscale ultrasonic wave on one side, which is reflected on the opposite side to generate an acoustic wave field. When the acoustic force reaches critical strength, cells or particles are trapped in the standing wave, forming packed clusters that then sediment. Acoustic-based cell separation is most efficient when the starting material has a relatively high cell density or involves large cells because the formation of cell clusters is key to sedimentation.

Currently, there are a few commercially available acoustic devices that target different processing steps (Table 5): BioSep (Applikon Biotechnology, Schiedam, Netherlands), Cadence acoustic separator (Pall Life Sciences, Port Washington, NY, US) and Ekko (FloDesign Sonics, Wilbraham, MA, US). The BioSep is designed to be part of a perfusion culture system for suspending cells and performing media exchange. As a result, the processing rates are relatively low in these systems [123]. The Cadence acoustic separator was developed for cell harvest and media clarification and is able to process at a maximum of 3.6 L/h (Table 3). One disadvantage of this system is that it operates with a minimum input cell density of 2×10^7 cells/mL, which is much higher than the typical culture density at the end of expansion in cell manufacturing [124]. The Ekko is a newly developed cell processing device that overcomes some of the issues with traditional acoustic devices. The Ekko system has a unique chamber design in which cells enter through the fluid inlet on the two lateral sides and move upward. Cells are then subjected to a multi-dimensional acoustic standing wave generated by a proprietary piezoelectric setup. The chamber design maximizes cell clustering through the combined gravity, fluid drag and primary and secondary acoustic force, which allows the “trapping” of low-density cells, such as lymphocytes [125,126]. The same device is also being developed for cell selection and media exchange for perfusion culture protocols [127].

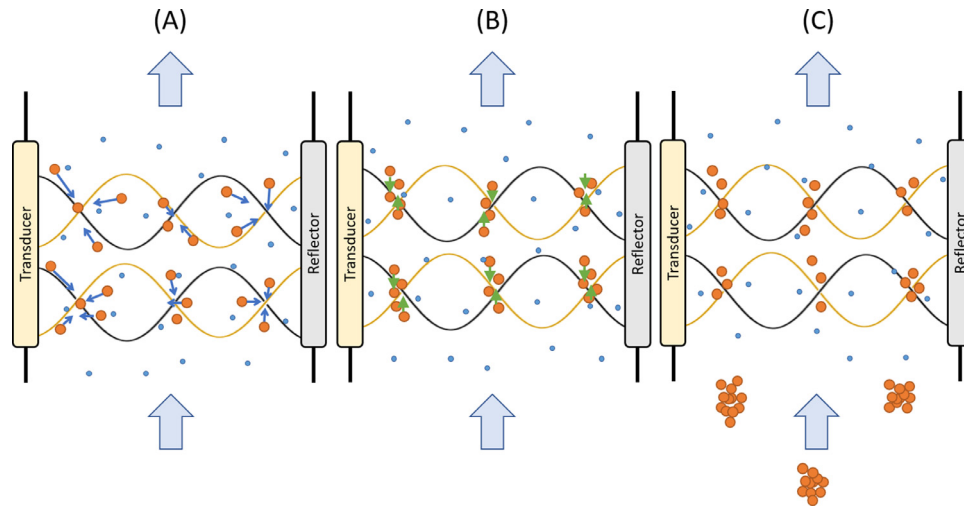


Figure 8. Schematic diagram of cell concentration under the action of ultrasonic acoustic waves. (A) Cells moving toward the acoustic pressure node when medium passes through the acoustic field. (B) Cells are further clustered under the secondary acoustic force when they are in the vicinity of each other. (C) Clustered cells result in accelerated sedimentation and settle out into a collection port, whereas cell debris follows the movement of the fluid and separates from the cell concentrate. Orange circles indicate cells and cell clusters and blue dots indicate small particles (such as cell debris). Green and blue arrows indicate direction of particle movement and light blue arrows indicate fluid movement. Figure adapted from Lenshof *et al.* [121]. (Color version of figure is available online).

Table 5
Summary of processing capacity of acoustic devices.

	BioSep acoustic cell retention system [123]		Cadence acoustic separator [141]	Ekko cell processing system [140]
	1 L	1000 L		
Batch volume, L	0.1–1	100–1000	3–25	0.150–5
Minimum cell concentration per mL	2×10^5	2×10^5	2×10^7	5×10^5
Maximum cell concentration per mL	N/A	N/A	5×10^7	4×10^7
Processing rate, L/h	0.041	41.2	3.6	1–1.3

N/A, not applicable.

Challenges and Future Directions

Despite the variety of wash-and-concentrate devices available to users seeking to automate this step of their manufacturing process, there is still a significant gap between manufacturing needs and the technologies that are available. Based on the specifications of some recently approved products [128–131], most wash-and-concentrate devices can deliver the required range of cell concentration and volumes. Nevertheless, pediatric indications may require doses as low as 1.2×10^6 cells [128], which falls well below the operational range of currently available wash-and-concentrate devices.

The minimal output volume of a wash-and-concentrate device is associated with the dead spaces within the system (i.e., tubing, water trap), which are set to meet the needs of the maximum processing volumes. Future developments for pediatric applications may arise from simple closed processing using dead-end centrifugation in a semi-automated fashion. Additionally, current wash-and-concentrate devices generally target products that are delivered intravenously; hence, the final products are usually dispensed into bags. However, this may not be optimal for engineered/scaffold products or hydrogel-like formulations, where process requirements are usually product-specific. The bag format is also not ideal for therapies that require local injections [132,133]. The diversity in the ATMP field makes it impossible to have a “one-size-fits-all” automated solution. Furthermore, it is unrealistic to expect to have automated devices developed for these applications until there is sufficient market demand. Therefore, a modified processing kit could be a possible solution in the meantime.

It is very likely that both autologous and allogeneic therapies will continue to occupy the market in the foreseeable future. Therefore, wash-and-concentrate devices will be expected to serve the manufacturing needs of both classes of ATMPs, and flexible programming of these devices will allow them to cater to diverse processing needs.

Conclusions

The implementation of automated solutions should be considered in the early stages of process development, with a vision of how the ATMP will be produced at commercial scales. It is also important to appreciate that upstream and downstream processes are often achieved by automated solutions using an approach that is completely different from the manual process. Hence, selecting suitable automated solutions should be based on intimate knowledge of the critical requirements in the manufacturing process as well as the advantages and disadvantages of each technology.

Generally speaking, the newer generations of wash-and-concentrate devices are more desirable because of their smaller footprints, ease of use and software support. It is important to keep in mind that process development requires a substantial effort. Devices such as Sepax and Sefia are centrifugation-based systems, which have processing parameters similar to benchtop centrifuge systems. As a result, process development is more intuitive, which is ideal for teams that are less experienced. The Lovo system may favor pediatric applications, where DMSO removal is required and cell numbers are sometimes low. The Ekko system is best for processing materials with high

acoustic contrast (e.g., microcarriers) and high cell density (e.g., apheresis units). The Rotea system has great flexibility in automation programming, which appeals to those who wish to customize processes for their needs (e.g., organoid processing or concentrating cells to $>300 \times 10^6$ cells/mL).

Given that an array of wash-and-concentrate devices have been developed, future developments in this space may come in the form of more flexible and customized processing kits rather than the development of novel devices. One of the challenges in a modular setup or the use of a wash-and-concentrate device is the connection between different devices. The processing kit is a mechanism to connect two devices and can effectively address the issue through design modifications. Future developments are also expected to encourage increasing collaborations between therapy developers and industry partners to bring forth innovative solutions in cell manufacturing.

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Declaration of Competing Interest

BL is a consultant for Novartis and Terumo Medical Corporation; on the scientific advisory board for Avesta, Patheon/Thermo Fisher Scientific Viral Vector Services, Immuneel Therapeutics Private Limited, IN8bio, Ori Biotech and Vycellix; and co-founder of and equity holder in Tmunity Therapeutics. DJ is chief executive officer of Scinogy Pty Ltd.

Author Contributions

Conception and design of the study: AL, DJ and RL. Acquisition of data: AL. Analysis and interpretation of data: AL, NS and BL. Drafting or revising the manuscript: AL, GK, DD, NS, DW, BL, DJ and RL. All authors have approved the final article.

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