Perfusion culture of T lymphocytes in the WAVE Bioreactor System 2/10 (software version 2.61)

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This application note presents a robust process for manufacturing up to 6 × 10^{10} human T cells using the WAVE Bioreactor System 2/10 with perfusion. The expanded T cells remain biologically functional and can be re-activated to produce high amounts of cytokines. The cost savings on consumables and labor by using the WAVE Bioreactor system, compared to static bags, are significant due to the handling of one single disposable Cellbag™ bioreactor and the automated exchange of media. Handling only one bag and the high cell densities achieved also reduce the risk of cross-contamination and the time and effort needed to concentrate and harvest cells at the end of the culture.

Introduction

In vivo, T lymphocytes are activated and induced to proliferate upon binding of the T cell receptor to antigen presenting cells. In response to activation, T cells undergo physical, biologic, and phenotypic changes including increased cell size, secretion of cytokines, and up-regulation of CD25 surface expression. This immune response can be mimicked *ex vivo* by the binding of T cells to co-immobilized anti-CD28 and anti-CD3 monoclonal antibodies (1). T cells can be cultured in static bags or flasks but cultures above 2 × 10^9 cells require more than one bag, increasing the amount of consumables and labor. Thus, to meet the need for reproducible expansion of functional T lymphocytes a rapid, scalable, and robust production process is becoming increasingly important. In addition, there is a strong wish to reduce the cost for disposables, media, and labor.

The WAVE Bioreactor System 2/10 (Figs 1 and 2) can be used for cultivation of human T cells and cell densities of more than 1 × 10^7 cells/mL at very high viabilities are readily achieved (2-4). Because Cellbag bioreactors are disposable, presterilized, and completely contained, they are ideally suited for applications where the prevention of cross-contamination is critical.

Fig 1. WAVE Bioreactor System 2/10 with optional Perfusion Controller.

Additionally, the volume in a Cellbag bioreactor can be increased by a factor of 10, reducing inoculum requirements and the need for transfers. For example, a culture in Cellbag-10L can be started with as little as 0,5 L of volume and fresh media can be added to match growth to bring the final batch volume to 5 L. Using the perfusion capabilities of the WAVE Bioreactor system, very high cell densities can be generated while maintaining a closed system. It should be noted that even though the genetic and phenotypic characteristics of the expanded T cells vary between different protocols, the principles for T cell activation and expansion are alike, regardless if for example CD8 positive (CD8+), CD4+, tumor infiltrating, or genetically modified T cells are selected. This suggests that the expansion protocol for enriched peripheral blood T cells described herein can be performed with other T cell subpopulations and/or activation protocols as well. This application focuses on expansion of T cells using the WAVE Bioreactor System 2/10 with a perfusion module but does not include the selection, harvesting or washing of the cells.

Materials and methods

Activation of T cells in static culture

Fresh (maximum 48 h old) density gradient isolated and monocyte-depleted, human peripheral blood mononuclear cells (PBMCs; 3H Biomedical) were washed in phosphate-buffered saline (PBS) plus 5% heat-inactivated human serum (C15-020; PAA Laboratories), counted, and characterized by flow cytometry. The total number of CD3+ T cells was calculated and cells were loaded at a concentration of 1×10^6 cells/mL into gas-permeable VueLife™ culture bags (American Fluoroseal) with preheated T cell media consisting of CellGro™ DC (CellGenix) plus 5% human serum, 1% GlutaMAX™ 100× (Invitrogen), 1% penicillin-streptomycin (Invitrogen), 0.8% acetylcystein (200 mg/mL; BioPhausia), and 100 U/mL interleukin-2 (IL-2; Proleukin™; Novartis). Dynabeads™ ClinExVivo™ CD3/CD28 beads (Invitrogen) were co-cultured with the cells at a ratio of 3:1 (beads: T cells) in a 37ºC incubator with 5% CO_2 atmosphere for 2 to 3 days. On day 3, the cells were counted and if the target cell number was reached the cells were transferred to the Cellbag bioreactor (approximately 1×10^8 for Cellbag-2L or 2×10^8 cells for Cellbag-10L). If the cell number was lower, more media was added to the VueLife bag to maintain 0.5×10^6 cells per mL and cultured for an additional 1 to 2 days.

Preparing perfusion tubing

Each culture requires two perfusion tubing assemblies (Fig 2). One is connected to the feed line of the Cellbag bioreactor and the other to the harvest line. The tubing assemblies were made as follows: Male luer-lock (MLL) fittings were installed on both ends of two 50 to 70 cm long, 3.2/6.4 mm (ID/OD) platinum-cured silicone tubings (EW-95802-05; Cole-Parmer) and secured in place using zip-ties. One of the autoclaved assemblies was coupled to two PVC extension tubings (with female luer-lock [FLL] and MLL; Sarstedt or Baxa) via a three-way connector, (BD connecta; BD Medical), and connected to the media and VueLife bags. The other end of the silicone tubing was directly connected to the FLL connector of the feed line on the Cellbag bioreactor. Two PVC tubings were connected to each side of the other silicone tubing; one connects with the FLL port of the harvest line and the other connects to a waste bag (Fig 2).

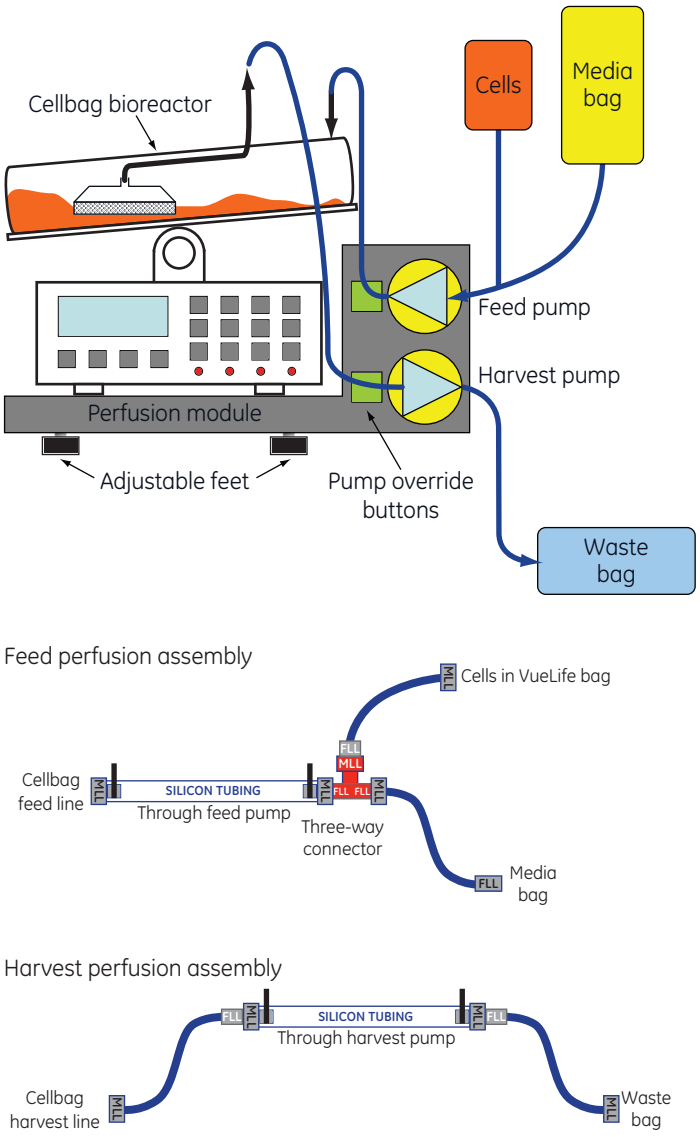

Fig 2. WAVE Bioreactor System 2/10 with Perfusion Controller (top) and perfusion tubing assemblies (bottom).

Preparing the WAVE Bioreactor System 2/10

A WAVE Bioreactor System 2/10 (software version 2.61) with Perfusion Controller (PERFCONT2E) was set up according to the user manual. Cellbag-2L and Cellbag-10L with perfusion filters were used for 1 and 5 L perfusion cultures, respectively. All connections between the Cellbag bioreactors, perfusion tubing assemblies, and waste bags were performed inside a tissue culture hood or by sterile welding (SCBII; Terumo Medical Corporation). A 5 L bag (Hyclone™ HyQtainer; Thermo Scientific), with T cell media supplemented with 200 U/mL of IL-2 and 0,02% Pluronic™ (Invitrogen), was connected to one of the extension tubings of the feed perfusion assembly and hung above the System 2/10. The harvest perfusion assembly was connected to a 20 L waste bag (M*BAG-20L) and the silicone tubings were put in place in the pump heads. The Cellbag bioreactor, with all the tubing fittings and filter heater in place, was filled with 5% CO_2 using the external CO_2 Controller (CO2MIX20) and the weight of the empty Cellbag bioreactor was tared. Media was transferred to the Cellbag bioreactor, using the perfusion pump, and the heating and rocking was started. The temperature (37°C) and pH were allowed to equilibrate for 2 h using a rocking speed of 10 rocks/min (rpm) and an angle of 6°, first ensuring that there was good contact between the temperature probe on the System 2/10 and the Cellbag bioreactor.

T cell culture in the WAVE Bioreactor System 2/10

PVC extension tubing was connected to the FLL of the VueLife bag and sterile welded to the feed perfusion assembly. The CO_2 Controller was turned off and inlet and outlet air filters clamped before the cells were transferred by gravity flow. The three-way connector was turned to allow fresh media from the media bag to wash out the remaining cells before the VueLife bag was sealed off. The inlet and outlet air filters were opened and rocking started (10 rpm and an angle of 6°). Fresh media was added each day to maintain a cell density of around 0.5 × 10^6 cells/mL until the maximum volume of the Cellbag bioreactor was reached and perfusion started (1 L for Cellbag-2L and 5 L for Cellbag-10L). For the first days of perfusion, 0.35 volumes of media were exchanged per 24 h (perfusion typically started on day 5 to 7). The perfusion rate was then gradually increased to 0.75 and finally 1.0 culture volume to maintain glutamine of around 2 mM and glucose of around 0.5 g/L. The perfusion was performed in a semi-continuous mode and a shot-volume of 50 mL was used throughout the entire culture. Media bags were exchanged by sterile welding to maintain a closed system. The IL-2 concentration was increased to 300 U/mL when perfusion was changed to 0.75 culture volumes, and to 500 U/mL when the perfusion was changed to 1 culture volume. Cells were sampled daily through the needleless sample port: first increasing the rocking speed to 20 rpm for 1 min to ensure collection of a representative sample. Viable cell counts were performed using the standard Trypan blue exclusion method (5). The glucose, lactate, and glutamine levels were measured daily on a BioProfile™ 100+ analyzer (Nova Biomedical) and pH, CO_2, and O_2 levels were measured on an ABL5 blood gas analyzer (Radiometer) according to the manufacturer's instructions.

Phenotypic analysis

The cells were immunophenotyped by flow cytometric analysis at various days of culture: 1 × 10^6 cells were stained with CD3 FITC (345763)/CD45 PerCP-Cy™5.5 (332784)/CD4 PE-Cy7 (348809)/CD8 APC-H7 (641400)/CD28 PE (555729)/CD25 APC (340907), CD3 FITC/CD45 PerCP-Cy5.5/ CD19 APC (345791)/ CD16 PE (332779), or CD45 PerCP-Cy5.5/CD14 PE (345785) and analyzed on a FACSCanto™ flow cytometer using FACSDiva™ software according to the manufacturer's instructions (reagents, instrument, and software from BD Biosciences).

The cell cultures were monitored for cell size and volume on days 0, 3, 5, 7, 9, and 11 using a Multisizer™ 3 COULTER COUNTER™ (Beckman Coulter).

T cell reactivation and cytokine production

On day 11, cells were depleted from Dynabeads using a Dynal MPC magnet (Invitrogen), washed with PBS plus 5% human serum, and re-seeded in 2 mL of fresh culture media with 200 U/mL of IL-2 at 1 × 10^6 cells per mL. The cells were left unstimulated or re-activated with new CD3/CD28 Dynabeads at a ratio of 3:1 (beads:T cells) for 18 to 20 h. The supernatants were collected and the level of cytokine production was determined using the CBA Th1/Th2 cytokine kit (BD Biosciences) and analyzed by flow cytometry according to the manufacturer's instructions. The concentrations of IL-2, IL-4, IL-5, IL-10, INF-γ, and TNF-α were based on the standard curve for each cytokine.

Results

Various protocols to optimize the expansion of T cells in the WAVE Bioreactor system have been tested and here we present results from two 1 L and one 5 L T cell cultures using a WAVE Bioreactor System 2/10 with software version *2.61*. This software has an improved temperature control, which is particularly important when small media volumes are used in the Cellbag bioreactor. The perfusion control is also markedly improved because each media exchange starts with a harvest of one shot volume, which is immediately followed by a feed-shot. Thus, the volume in the Cellbag bioreactor never fluctuates more than one shot volume (e.g., 50 mL), making the environment for the cells more stable. With software version 2.61, both harvest and feed shots are divided in to several mini-shots, decreasing the risk of small cells being trapped in, or going through the filter. Several studies were performed with software version *2.61* and results showed up to 60% better cell growth after 7 days of culture compared to the prior software version (*2.55*) (results not shown). The two 1 L cultures were cultured in parallel using the same starting material but with different rocking modes: either a constant rocking speed of 10 rpm and angle of 6° throughout the entire culture was used or the rocking speed and angle were incrementally increased from 10 rpm and 6° at the start to 17 rpm and 7° at the end of culture.

Freshly isolated PBMCs were counted and characterized with respect to various cell surface markers, viability, and size/volume, and cultured with CD3/CD28 activation beads in static cultures for two days. This period ensured close contact between beads and cells and allowed the T cells to reach log phase and sufficient cell number before being transferred to the System 2/10 (typically on day 3 to 4). 1.25 × 10^8 and 2.77 × 10^8 MNC were used as starting material for the 1 L cultures and 5 L culture respectively. At the time of activation, the percent of CD3+ T cells was 76% for the two 1 L cultures and 72% for the 5 L culture, of which most were CD25 negative (i.e., inactive) (Table 1). Thus, the calculated number of CD3+ T cells at the start was 9.5 × 10^7 and 1.88 × 10^8 cells, for the 1 L cultures and 5 L culture respectively. After approximately five days of culture, the fraction of CD3+ T cells had increased to above 90% and nearly all of these expressed CD25, demonstrating the high degree of activation.

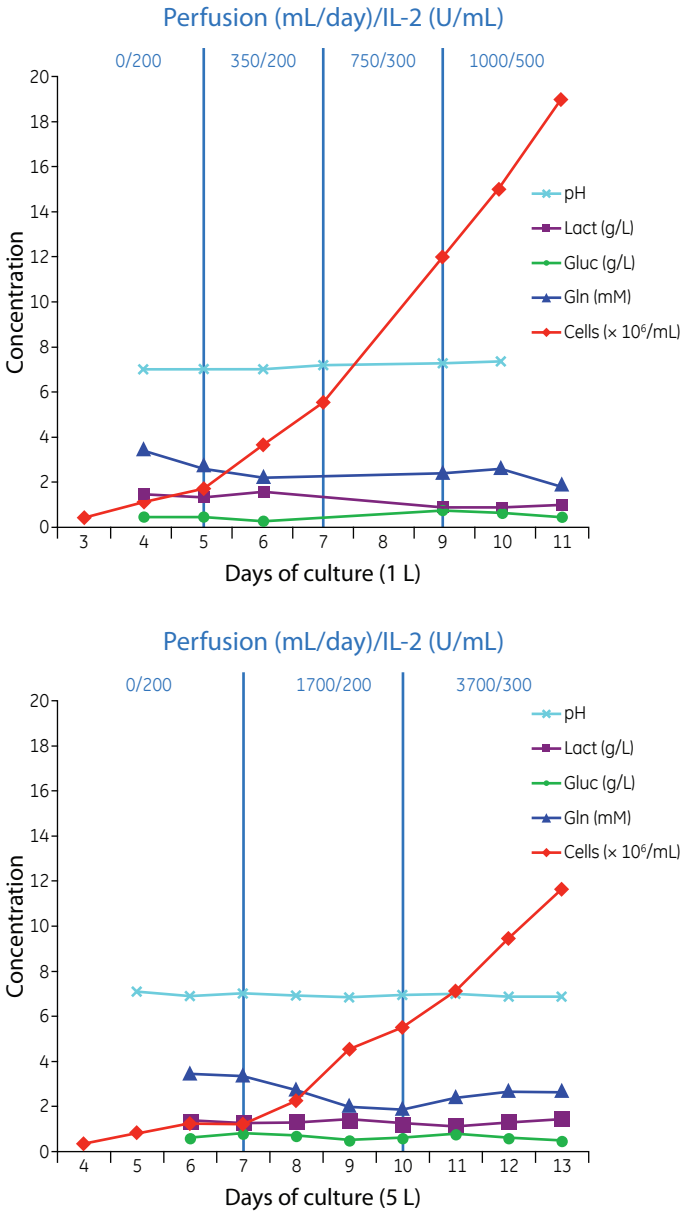

Fig 3. Cell density gradually increases during expansion in the WAVE Bioreactor System 2/10 whereas pH and levels of metabolites and nutrients are kept constant by increasing the perfusion rate. Data from one 1 L culture (with increasing rocking) (top) and the 5 L culture (bottom) are shown.

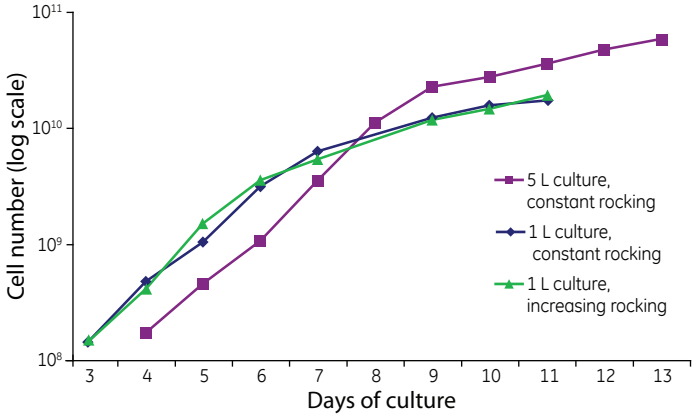

Fig 4. Cumulative growth of cells in the WAVE Bioreactor System 2/10. 145 × 10^6 cells were transferred to the Cellbag-2L on day 3, and 172 x 10^6 cells for Cellbag-10L on day 4. Note that the y-axis shows cell number in log-scale.

Table 1. Phenotypic analysis of fresh untreated cells, activated expanded cells, and re-activated cells from a 1 L culture (constant rocking) and the 5 L culture.

	Percent of CD45+ white blood cells						Percent of CD45+/CD3+ T cells		
	Day of culture	Cells	CD3+ T cells*	CD14+ monocytes	CD19+ B cells	CD16+ NK cells	CD4+ T cells	CD8+ T cells	CD25+ T cells
1 L culture	0	Fresh cells	76	0	6	9	68	24	1
	11	Activated cells	97	–	0	0	74	21	68
	12	Re-activated cells	99	–	0	0	80	19	99
5 L culture	0	Fresh cells	72	3	5	14	43	49	1
	5	Activated cells	93	–	0	0	73	25	99
	14	Activated cells	94	–	1	0	53	40	41

*All CD3+ cells also expressed CD28 (results not shown).
Note: Activated cells are sampled during culture, whereas re-activated cells were sampled on day 11 and stimulated with new CD3/CD28 beads for 18 to 20 h.

The cells were counted daily from day 3 and media was added to maintain a density of approximately 0.5×10^6 cells/mL until the maximum volume of the Cellbag bioreactor (i.e., 1 or 5 L) was reached and the perfusion was started. In addition to cell counts and viability; pH, CO_2, lactate, glucose, and glutamine levels were monitored. By increasing the perfusion rate, the lactate levels were minimized and the pH, glucose, and glutamine levels were kept constant throughout the entire culture period despite a dramatic increase in cell densities (Fig 3). The cumulative cell growth in a Cellbag-2L and Cellbag-10L are presented in Figure 4. The cumulative fold expansion growth of peripheral blood T cells was approximately 200-fold for the 1 L cultures and 300-fold for the 5 L cultures with a final yield of 2×10^{10} and 6×10^{10} viable cells, respectively. The cell densities reached 2×10^7 cells/mL in Cellbag-2L and above 1×10^7 cells/mL in Cellbag-5L (Fig 3) with viabilities above 90% (results not shown). We could not observe any significant difference in cell growth or viability between the two 1 L cultures, suggesting that adjustment of the rocking mode to regulate gas exchange in the Cellbag bioreactor is not crucial for T lymphocytes as long as the cells are kept in suspension (Fig 4).

Peripheral blood T cells showed robust and reproducible activation as determined by cell size (Fig 5). Before CD3/CD28 activation, the average cell volume was approximately 200 fL (200×10^{-15} L). The size increased as a response to activation and peaked at approximately 800 fL on day 5 (Fig 5). At the end of culture the cells had almost returned to resting cells size, as expected.

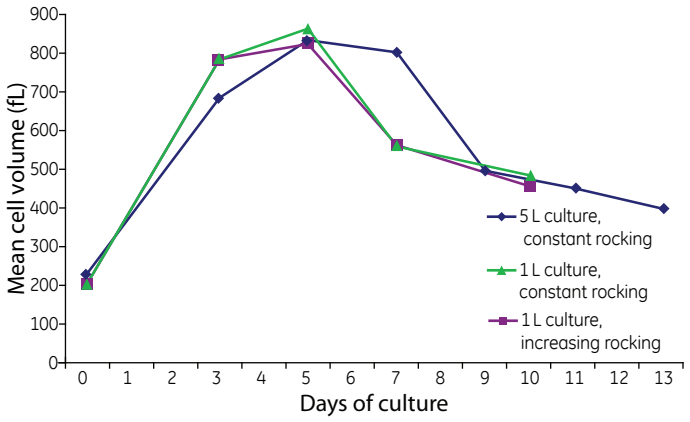

Fig 5. Cell size changes during culture in response to activation. Note that the cells increase in volume as a response to activation.

Re-stimulation of activated T cells is a natural biologic event that takes place during an immune response. To assure that the expanded cells were biologically functional at the end of culture, the cells were re-activated for 18 to 20 h with new CD3/CD28 beads, and the induction of cytokine secretion and CD25 expression was measured. As shown in Table 1, the percentage of cells expressing high levels of CD25 increased from 68% to 99%. Moreover, a dramatic increase in production of IL-2, IL-4, IL-5, IL-10, TNF-α, and INF-γ secretion was observed in response to re-stimulation (Fig 6).

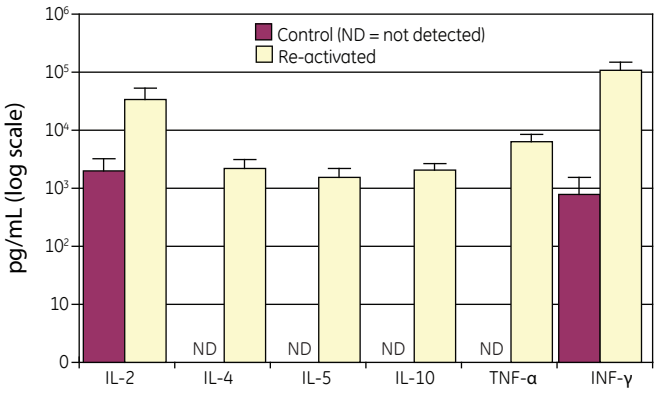

Fig 6. Concentration of soluble cytokines after re-activation. On day 11 of culture, cells were left unstimulated or re-activated with new CD3/CD28 beads for 18 to 20 h and cytokine production was assessed. The results are mean ±SD from three cultures. Note that the concentration of IL-4, IL-5, IL-10, and TNF-α was undetectable in the unstimulated control cells and that the culture media is supplemented with 200 U/mL of IL-2.

Conclusion

This application note presents a robust process for expanding large numbers of biologically active T cells at high concentrations using the WAVE Bioreactor System 2/10 (software version *2.61)* with perfusion. Major advantages of the WAVE Bioreactor system for culture, compared to static culture, are that cells are cultured in one single disposable bag and that perfusion supports high cell densities, reducing the costs of goods and labor. As an example; to produce 6×10^{10} T lymphocytes at a density of 2×10^{6} cells/mL in static bags requires 30 × 1-L bags and at least 30 L of media. Expanding the same amount of cells using the WAVE Bioreactor System 2/10, according to the protocol described herein, requires only two culture bags (one Cellbag-10L and one gas-permeable bag) and 16 L of media. The cost savings on labor by using the WAVE Bioreactor system are considerable due to the handling of one bag instead of 30 and the automated exchange of media, which reduces the hands-on-time during expansion. In addition, handling only one bag with high density cells reduces the risk of cross-contamination and the time and effort needed to concentrate and harvest cells at the end of culture.

References

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Ordering information

Product	Code no.
WAVE Bioreactor System 2/10 (BASE2/10 EH)	28-9377-86
System 2/10 Perfusion Controller (PERFCONT2E CellTech)	28-9884-64
Filter Heater (FLTHTR2)	28-4116-39
Protective White Opaque PVC Lid (LID2/10W, OPAQUE)	28-9376-33
CO_2/Air Aeration Controller (CO2MIX20)	28-9377-95
Cellbag-2L (BioClear™ 10, Perfusion, DO)	28-9376-52
Cellbag-10L (BioClear 10, Perfusion, DO)	28-9376-62
M*BAG-20L	MB0020L10-01

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