Washing and concentrating T cells at high flow rates on the CTS Rotea Counterflow Centrifugation System

Introduction

The Gibco™ Cell Therapy Systems (CTS™) Rotea™ Counterflow Centrifugation System is designed to support fully closed and scalable automated cell processing to streamline and expedite cell therapy research. The system has a peristaltic pump that delivers flow rates ranging from 5 mL/min to 110 mL/min with the Gibco™ CTS™ Rotea™ Single-Use Kit (Figure 1), and the flow rate can be easily adjusted for specific cell processing applications. At a flow rate of 100 mL/min, an input volume of 1 L can be loaded into the CTS Rotea system in under 10 minutes. Higher flow rates can reduce processing time requirements and make the manufacturing workflow more efficient. The broad range of possible flow rates on the CTS Rotea system allows users to optimize the ratio of flow rate to *g*-force by adjusting centrifugal force.

Before processing cells at high flow rates on the CTS Rotea system, several important parameters must be considered for successful outcomes and consistency. Optimizing the parameters discussed here will improve cell recovery and viability.

Workflow for the CTS Rotea Counterflow Centrifugation System.

T cell isolation and expansion	Wash and concentrate	Analyze
• CTS Dynabeads CD3/CD28 beads • CTS OpTmizer T Cell Expansion SFM • CTS Immune Cell SR • GlutaMAX Supplement • L-glutamine • IL-2 recombinant human protein	• CTS Rotea Counterflow Centrifugation System • Wash with or without CTS DPBS	• Count cells • Attune NxT Flow Cytometer

Prime system → **Load cells** → **Wash cells** → **Harvest**

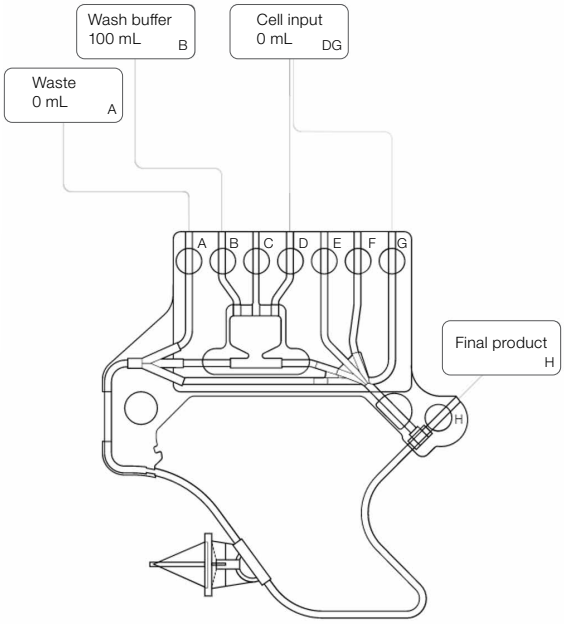

Figure 1. CTS Rotea Single-Use Kit configuration for high-flow T cell washing and concentration.

Materials and methods

T cells were prepared from cryopreserved peripheral blood mononuclear cells (PBMCs). The PBMCs were initially isolated using the CTS Rotea system using a fresh leukopak bag. The PBMCs were thawed, washed, and activated using Gibco™ CTS™ Dynabeads™ CD3/CD28 beads. The cells were then expanded in Gibco™ CTS™ OpTmizer™ T Cell Expansion serum-free medium (SFM) supplemented with Gibco™ CTS™ Immune Cell serum replacement (SR), Gibco™ L-Glutamine, Gibco™ GlutaMAX™ Supplement, and IL-2. Following expansion, the T cells were washed and concentrated on the CTS Rotea system using Gibco™ CTS™ DPBS with or without 2% human serum albumin as a wash buffer. All cells displayed good viability (>85%). A flow rate of 100 mL/min and a centrifugal force of 3,000 x *g* were applied during the loading step in all experiments unless otherwise noted. T cell recovery and viability were evaluated using a ChemoMetec™ NucleoCounter™ NC-200™ automated cell counter before and after processing. The phenotype of the cells before and after washing was analyzed using an Invitrogen™ Attune™ NxT Flow Cytometer. The washed T cells were then cultured to monitor expansion and viability.

The initial set of runs was performed using T cells that displayed >90% viability. The cells were expanded for 9–15 days at concentrations ranging from 2.75 x 10^6 cells/mL to 5.2 x 10^6 cells/mL with cell counts of 1.0 x 10^9 to 2.6 x 10^9. Fold expansion and the viability of the T cells were monitored throughout the culturing period and compared to those of manually washed T cells. The cells were washed on days 5, 7, 9, and 11 after bead removal on day 5. Manual washing was performed using a benchtop centrifuge. The T cells were centrifuged in a 50 mL conical tube at 300 x *g* for 5 min, resuspended in complete medium, and cultured for similar amounts of time.

Results

After processing on the CTS Rotea system, the T cells either maintained viability or displayed slightly higher viability.

Initial runs

Cell recovery after processing on the CTS Rotea system was greater than 95% with a slight improvement in cell viability (Figure 2A). Cells from one of the runs were cultured for additional monitoring of expansion and viability. The cultured cells expanded exponentially from 1.0 x 10^6 to 2.0 x 10^6 cells between day 12 and day 14 and from 2.0 x 10^6 to 4.0 x 10^6 cells between day 14 and day 16 (Figure 2B). There was no significant difference in phenotype after the cells were processed on the CTS Rotea System (Figure 2C).

Factors affecting the recovery of T cells after washing and concentration at a high flow rate

Multiple factors affected cell recovery with the high flow rate washing and concentration protocol. These included the initial input concentration, the duration of T cell expansion, the number of cells that could be introduced into the cone per loop, and the washing medium.

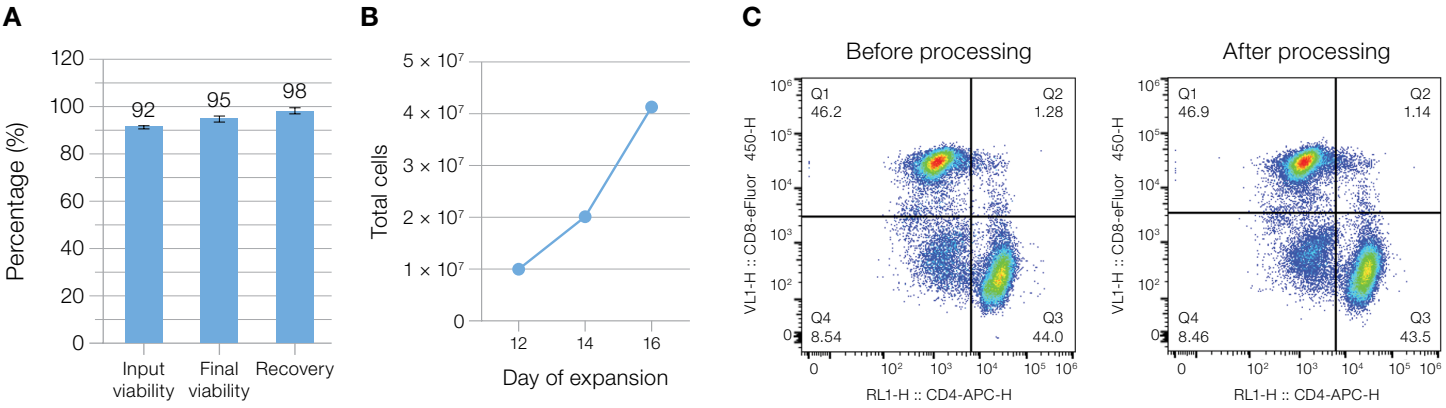

Figure 2. Characterization of cells processed using the CTS Rotea System. (A) Recovery and viability of T cells after washing and concentration at a high flow rate (3,000 x *g*; 100 mL/min; n = 4). **(B)** Expansion of T cells from day 12 to day 16 after washing. **(C)** Flow cytometry results before and after processing the cells on the CTS Rotea system.

Initial input concentration

A successful run on the CTS Rotea system requires the formation of a stable cell bed. Stable cell beds formed more quickly when the initial cell concentration was increased, and more cells were recovered (Figure 3). Three different input concentrations were tested in this study. Recovery was higher in runs with inputs of 2.5 x 10^6 to 5.2 x 10^6 cells/mL than it was in the other two runs. We adjusted the protocol to draw 6.0 x 10^8 to 8.0 x 10^8 cells to establish the cell bed at a flow rate of 60 mL/min at 3,000 x *g* (Table 1). Users can calculate the volume needed to establish a stable cell bed with 6.0 x 10^8 to 8.0 x 10^8 cells based on the initial cell concentration. If the bed is not stable enough, the user can extend the "Establish bed" step until a stable bed forms. Another optimization strategy to minimize cell loss is to increase the CF ratio, which is the ratio of centrifugal force to flow rate, during the "Establish bed" and "Load cells to detect bubbles" steps (Table 1 and "Optimization guide" on page 5).

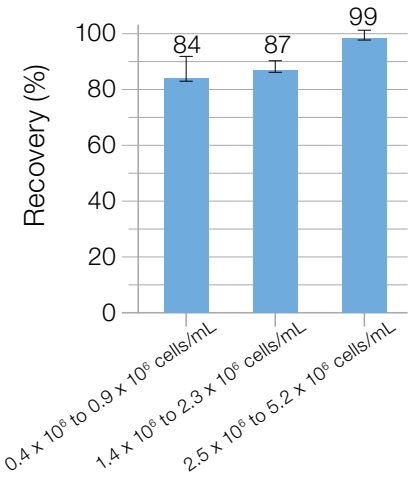

Figure 3. Effect of input concentration on cell recovery.

Duration of cell expansion

T cell recovery and viability were also affected by the duration of the culturing period prior to washing and concentration on the CTS Rotea system. Differences in recovery and viability depended on the number of days the T cells were cultured (Figure 4). Recoveries exceeded 95% when cells were cultured for 9–15 days, while the average recovery was 84% in runs with an expansion period of 5 days.

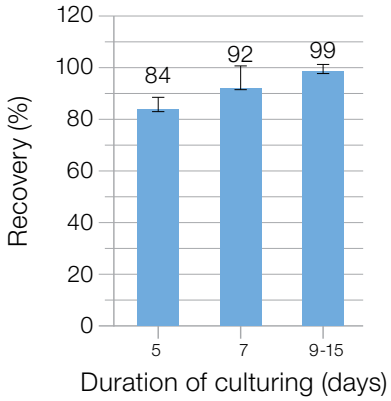

Figure 4. Effect of the duration of culturing and expansion on cell recovery.

Table 1. Conditions for washing and concentrating T cells at high flow rates on the CTS Rotea system.

Step	Description	Flow path	Centrifugal force (x *g*)	Flow rate (mL/min)	Step type	Triggers
1	Pre-prime	B to A	0	100	Normal	Input bubble sensor
2	Lubricate rotary coupling	B to A	0	100	Normal	15 mL
3	Prime chamber and line A	B to A	10	100	Normal	15 mL
4	Add priming volume	B to A	10	100	Normal	50 mL
5	Fill bubble trap and prime B	A to B	10	100	Normal	15 mL
6	Prime D	A to D	10	100	Normal	3 mL
7	Pressure prime	A to E, F	10	0	Pressure prime	–
8	Prime pause loop	J to K	10	25	Pause	3 mL
9	Ramp speed	J to K	3,000	60	Pause	20 sec
10	Establish bed	D to G	3,000	60	Normal	Bed establishment volume
11	Load cells to detect bubbles	D to A	3,000	100	Normal	Input bubble sensor
12	Concentrate bed to wash	J to K	2,400	20	Pause	60 sec
13	Wash cells with buffer	B to A	2,400	25	Normal	Wash volume
14	Concentrate cells for recovery	J to K	2,800	25	Pause	8 sec
15	Harvest	B to H	2,800	100	Harvest	Harvest volume
16	Ramp to stop	K to J	100	10	Pause	10 sec

Chamber capacity

With the standard T cell washing and concentration protocol for the CTS Rotea system, 5 x 10^9 activated T cells can be processed at 2,000 x *g* at a flow rate of 25 mL/min and a CF ratio of 80. The CF ratio for the high flow rate washing and concentration protocol at 3,000 x *g* with a flow rate of 100 mL/min is much lower (30). Higher flow rates expedited the cell bed formation, although fewer cells fit in the chamber. Recovery was 64% after a run with 3.0 x 10^9 activated T cells in the chamber and only 49% after a run with 4.0 x 10^9 cells in the chamber (Figure 5). Cell recovery was improved to >95% after reducing the input to 2.0 x 10^9 to 2.5 x 10^9 cells. Based on these data, the optimal input for washing and concentration at 3,000 x *g* and a flow rate of 100 mL/min is 2.5 x 10^9 cells.

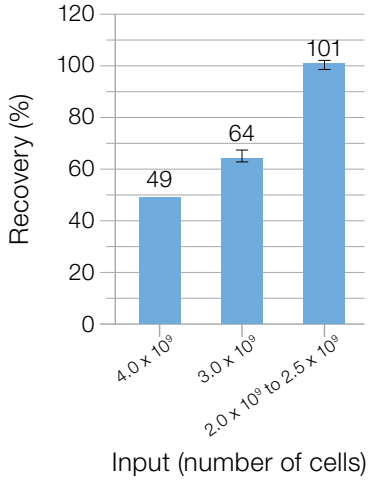

Figure 5. Effect of cell input per loop on recovery.

Washing medium

Differences between the densities of the suspension medium and the medium with which the cells were washed and harvested also affected T cell recovery. This phenomenon can occur with any washing and concentration or buffer exchange protocol. If the density of the medium changes, increasing the CF ratio during the wash step and incorporating wash-pause loops could be beneficial. The number of cells in the chamber is another factor to consider if cells are washed in a medium with a different density than that of the suspension medium. Two runs were performed with 2.0 x 10^9 T cells suspended in CTS OpTmizer T Cell Expansion SFM, and no difference in recovery was observed after the cells were washed either with the culture medium or DPBS (Figure 6A). However, when we processed 1.0 x 10^9 T cells (n = 1), the difference between the densities of the T cell culture medium and DPBS resulted in significantly lower cell recovery (Figure 6B).

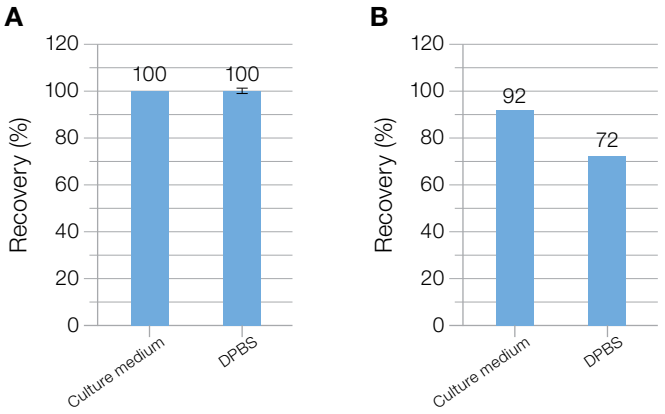

Figure 6. The effect of differences in densities of wash buffers on recovery. T cell recovery was measured for **(A)** an input of 2.0 x 10^9 T cells or **(B)** an input of 1.0 x 10^9 T cells.

Comparison of the high flow rate washing and concentration protocol and manual processing

The T cell expansion rates after washing and concentration with the high flow rate protocol or manual protocol are compared in Figure 7. PBMCs were thawed on day 0 and activated with a 3:1 mixture of CTS Dynabeads CD3/CD28 beads to T cells. Bead removal was performed on day 5 using an Invitrogen™ DynaMag™-50 Magnet. After the beads were removed, the cells were cultured in CTS OpTmizer T Cell Expansion SFM and washed on days 5, 7, 9, and 11. The washed cells were seeded at an optimal density and cultured between washes for a total of 15 days. The fold expansion of the cells washed using the high flow rate protocol was comparable to or better than that of the manually washed cells on all days except day 11 (n = 1).

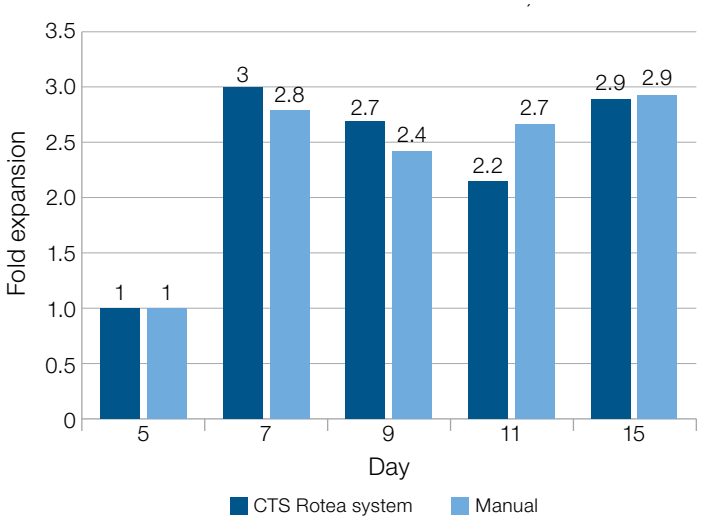

Figure 7. Fold expansion of cells washed on the CTS Rotea system and cells that were washed manually.

Conclusion

With the CTS Rotea Counterflow Centrifugation System and our high flow rate T cell washing and concentration protocol, we efficiently washed and concentrated T cells while retaining excellent viability and obtaining superior recoveries. The consideration of factors such as the initial T cell concentration, the total number of cells being loaded, the duration of culturing prior to cell processing, and the wash medium is critical to achieving good outcomes. Importantly, the total number of cells that can be loaded must be determined before running this protocol. This is because the relatively low CF ratio limits the number of cells that can be loaded into the Counterflow centrifugation (CFC) chamber. A higher concentration of cells can expedite cell bed formation, which will enable faster loading. A multi-bite process with loops can enable you to process all of your cells in a single run without overloading the chamber.

The duration of expansion and the type of wash medium can significantly affect cell recovery. However, this is not unique to the high flow rate washing and concentration protocol. Each of these factors should be considered for any cell processing method when using the CTS Rotea system. If a flow rate above 100 mL/min is desired, the Thermo Scientific™ CTS™ Rotea™ Hi-Flow Single-Use Kit can accommodate a maximum flow rate of 165 mL/min. The CTS Rotea Counterflow Centrifugation System offers process flexibility for a range of cell processing applications, and the CTS Rotea Hi-Flow Single-Use Kit and standard CTS Rotea Single-Use Kit provide users with the freedom to design runs with a wide range of flow rates, total cell counts, and cell concentrations.

Optimization guide

Step	Description	Factor	Optimization
10	Establish bed	Input concentration	To eliminate variability that can affect the establishment of a stable bed, aim for 6.0×10^8 to 8.0×10^8 cells to establish the cell bed.
		Day of expansion	If the cells are in the early phase of expansion (days 3–7), consider increasing the CF ratio. This can usually be accomplished by decreasing the flow rate from 60 mL/min, as the *g*-force is at maximum.
		Unsure of input concentration	Input 100 mL to establish the bed and manually extend the step until a stable bed forms. Ideally, the surface of the bed will be mostly flat with minimal turbulence. The stainless steel cannula should be just visible at the tip of the CFC chamber.
11	Load cells to detect bubbles	Input concentration	When the input concentration is low, try stepped loading. For example, once a stable bed forms at a flow rate of 60 mL/min, try to load at 70 mL/min for a few minutes. Then increase the flow rate incrementally up to 100 mL/min.
		Cell quantity in chamber	If you are starting with more than 2.5×10^9 cells, consider doing runs in loops of 2.5×10^9 cells.
		Day of expansion	If your cells are in the early phase of expansion (days 3–7), consider increasing the CF ratio. This is usually accomplished by decreasing the flow rate from 100 mL/min, as the *g*-force is at maximum.
13	Wash cells with buffer	Cell quantity	If you have fewer than 2.0×10^9 cells, use a wash buffer with a density that is close to the density of the medium the cells are in. To increase the density of the wash medium, add a protein like human serum albumin. Alternatively, increase the CF ratio of the wash step or add wash-pause loops.
		Media with different densities	Try an optimization technique like increasing the CF ratio or adding wash-pause loops. If this does not work, add an intermediate bag to dilute the cells with the fresh wash buffer before the wash step. 1. After loading the cells, recover them as a concentrate in the intermediate bag. 2. Pump fresh wash buffer into the intermediate bag for pre-dilution. 3. Reestablish the fluidized bed by recirculating and reload the diluted cells from the intermediate bag. 4. Continue washing with the new medium.

Ordering information

Product	Quantity	Cat. No.
T cell culture		
CTS Immune Cell SR	50 mL	A2596101
GlutaMAX Supplement	100 mL	35050061
L-Glutamine (200 mM)	100 mL	25030081
Human IL-2 Recombinant Protein	1 mg	PHC0023
CTS DPBS (1X)	1 L	A1285801
CTS Dynabeads CD3/CD28	10 mL	40203D
Analysis		
Attune NxT Flow Cytometer, blue/red/violet/yellow	1 each	A24858
Cell washing and concentration		
CTS Rotea Counterflow Centrifugation System + 2 year warranty (including PM)	1 each	A50757* A47695**
CTS Rotea Single-Use Kit	10 pk	A49585
Sorvall ST 8 Small Benchtop Centrifuge	1 each	75007200

* North America and Europe.

** Rest of world.

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