Automated PBMC isolation and T cell wash and concentration by the CTS Rotea system

Introduction

Successful processing and manufacturing in cell and gene therapy workflows is essential to the efficacy of the product. Autologous cell and gene therapy workflows involve isolating cells from an individual, engineering the cells, expanding and concentrating them, and infusing them back into the patient (Figure 1). Certain steps in these workflows could benefit from optimized automation to decrease hands-on time and the cost of the cell manufacturing process.

The Gibco™ Cell Therapy Systems™ (CTS™) Rotea™ Counterflow Centrifugation System is a closed cell-processing system developed for small-batch cell therapy manufacturing (Figure 2).

Key features include:

- **Low output volumes**—as little as 5 mL
- **Process flexibility**—user-programmable software enables creation and optimization of protocols
- **High cell recovery and viability**—fluidized bed supports low-shear processing, enabling greater than 95% cell recovery without decreasing cell viability
- **Gibco™ CTS™ Rotea™ Single-Use Kits**—flexibility to control outputs and inputs based on user discretion, offering scalability from research to clinical manufacturing
- **GMP compatibility**—compliance with industry standards, backed by regulatory documentation and support

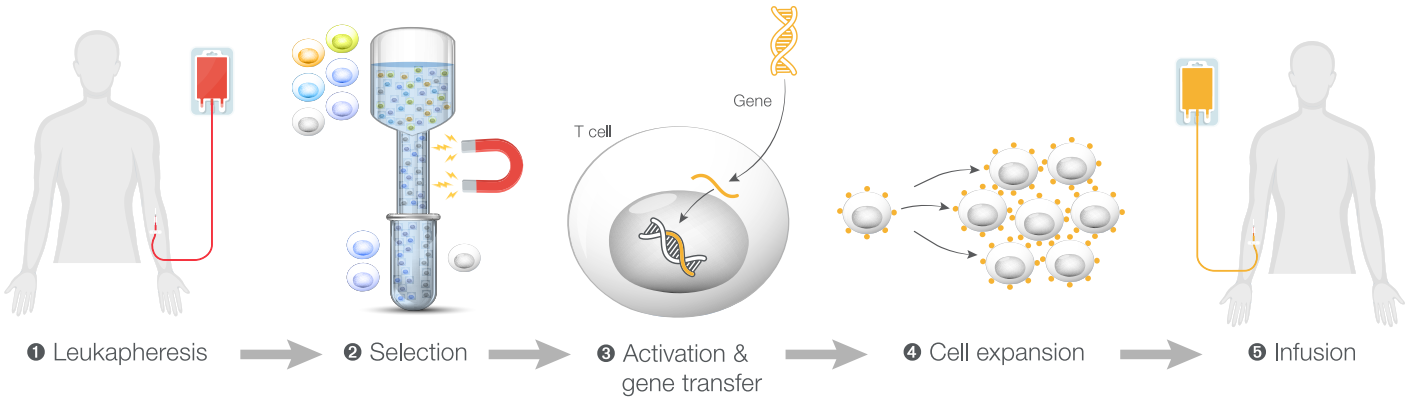

Figure 1. Simplified cell therapy workflow.

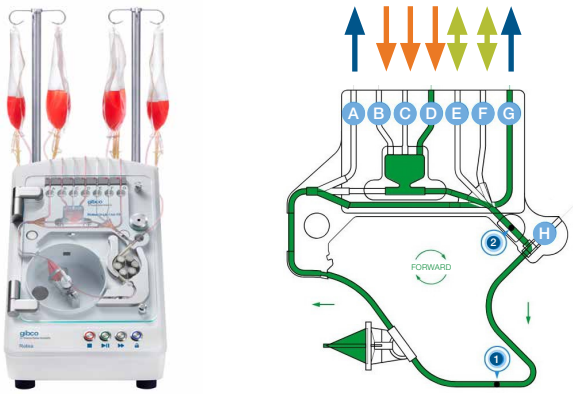

Figure 2. The CTS Rotea Counterflow Centrifugation System.

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The CTS Rotea system can be integrated into multiple steps in a cell therapy workflow, including cell isolation, washing, and concentration of many cell types. Here we demonstrate the efficacy of the CTS Rotea system for isolating peripheral blood mononuclear cells (PBMCs) from leukapheresis products, and washing and concentrating T cells—two common processes that are central in many cell therapy workflows.

Materials and methods

PBMCs were isolated from healthy donors using the CTS Rotea system, and the recovery, viability, and phenotypes were assessed and compared with those of PBMCs that were manually isolated using a density gradient medium. T cells were expanded in culture before being washed and concentrated using the CTS Rotea system and were similarly assessed and characterized post-processing.

All CTS Rotea system protocols were written using the Gibco™ CTS™ Rotea™ Protocol Builder desktop application.

PBMC isolation

Fresh Leukopak™ bags were purchased from AllCells or HemaCare. After diluting contents of a bag with 3 parts of isolation buffer (HBSS, 0.002 M EDTA, and 0.025% HSA) to obtain a solution of 1/4 the original concentration, PBMCs were isolated using the CTS Rotea system. The processing time for one quarter of a Leukopak bag is approximately 25 min. A summary of the PBMC isolation workflow is diagrammed below. Table 2 shows the full PBMC isolation protocol.

1. Prime the system—the specific sequence of steps shown in Table 1 is necessary to replace all the air in the system with fluid; dilute contents of Leukopak bag.
2. Load cells to form a stable fluidized bed.
3. Introduce lysis buffer to deplete red blood cells.
4. Wash cell bed with medium to stop lysis.
5. Recover cells to intermediate bag for downstream processing.

Table 1. Sequence of priming steps.

Step	Description	Flow path	Centrifugal force (x *g*)	Pump (mL/min)	Step type	Trigger
1	Pre-prime	B to A	0	100	Normal	Input bubble sensor (start of flow)
2	Lubricate rotary coupling	B to A	0	100	Normal	15 mL
3	Fill chamber and prime A	B to A	10	100	Normal	40 mL
4	Fill bubble trap and prime B	A to B	10	100	Normal	15 mL
5	Prime remaining lines	A to user defined	10	50	Normal	5 mL
6	Pressure prime	B to E/F	10	0	Pressure prime	
7	Prime pause loop	J to K	10	25	Pause	Volume: 3 mL
8	Ramp to next step	J to K	User-defined	User-defined	Pause	User-defined

Leukocyte recovery and viability were analyzed using the Invitrogen™ Countess™ II Automated Cell Counter (Cat. No. **AMQAX1000**). Flow cytometry using Invitrogen™ monoclonal antibodies was also performed to assess the relative percentages of constituent cell types: CD45 (Cat. No. **11-9459-42**), CD3 (Cat. No. **48-0038-42**), CD14 (Cat. No. **17-0149-42**), CD19 (Cat. No. **12-0199-42**) and CD56 (Cat. No. **46-0567-42**), corresponding to leukocytes, T cells, monocytes, B cells, and natural killer (NK) cells, respectively.

T cell expansion

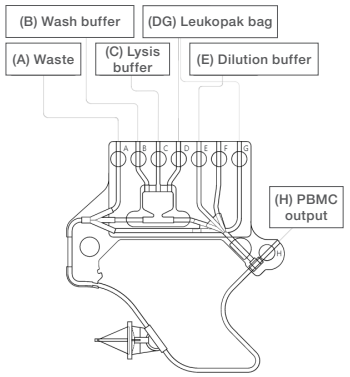
T cells from the Leukopak PBMC isolation using the CTS Rotea system or density gradient medium were expanded using Gibco™ CTS™ Dynabeads™ CD3/CD28 (Cat. No. **40203D**) and subsequently analyzed by flow cytometry after staining with Invitrogen monoclonal antibodies, including CD279 (PD-1)-PE Antibody (Cat. No. **12-2799-42**) and LAG-3 (CD223)-FITC (Cat. No. **11-2239-42**, eBioscience™ antibody), and for CD4 (Cat. No. **17-0049-42**), CD8 (Cat. No.**48-0088-42**), CCR7 (Cat. No. **12-1979-42**), and CD62L (Cat. No. **11-0629-42**) to evaluate T cell subtypes. The expansion lasted 10 days, and cells were counted at 0, 5, 7, and 10 days using the Countess II cell counter.

T cell washing and concentration

T cell recovery and viability were analyzed before and after processing on the CTS Rotea system. Viability and recovery were determined using an average of three Countess II cell counter readings. To ensure that relative percentages of constituent immune cell types were not altered due to processing on the CTS Rotea system, flow cytometry after staining with Invitrogen monoclonal antibodies for CD4 (Cat. No. **17-0049-42**) and CD8 (Cat. No. **48-0088-42**) was performed.

Table 2. PBMC isolation protocol.

Step	Description	Open valves	Centrifug. force (x *g*)	Pump (mL/min)	Step type	Trigger(s)
1	Pre-prime	E to A	0	100	Normal	Bubble sensor input (start of flow)
2	Lubricate rotary coupling	E to A	0	100	Normal	15 mL
3	Fill chamber and prime A	E to A	10	100	Normal	40 mL
4	Fill bubble trap and prime B	A to B	10	100	Normal	15 mL
5	Prime C	A to C	10	50	Normal	5 mL
6	Prime D	A to D	10	50	Normal	5 mL
7	Pressure prime	A to F	10	0	Pressure prime	50 mL
8	**Prime pause loop**	**J to K**	10	**25**	**Pause**	**3 mL**
9	Dilute Leukopak bag contents	E to G	10	80	Normal	User-defined volume for 1:3 dilution
10	Ramp speed	J to K	2,100	25	Pause	10 sec
11	Establish cell bed	D to G	2,100	12	Normal	50 mL
12	Load cells	D to A	2,000	18	Normal	Bubble sensor input (end of flow)
13	Recirculate	J to K	2,000	18	Pause	10 sec
14	Wash with medium	B to A	2,000	16	Normal	30 mL
15	Lyse red blood cells	C to A	2,000	14	Normal	20 mL
16	Continue to lyse red blood cells	C to A	2,000	16	Normal	50 mL
17	Recirculate to complete lysis	J to K	2,000	16	Pause	30 sec
18	Stop lysis and wash with medium	B to A	2,000	16	Normal	50 mL
19	Recirculate	J to K	2,100	16	Pause	15 sec
20	Harvest cells	B to H	2,100	25	Harvest	35 mL
21	Ramp to stop	J to K	500	25	Pause	5 sec



The T cell washing and concentration protocol takes approximately 15 min on the CTS Rotea system, based on an input volume of 0.25 L. The workflow is summarized below, and the protocol steps are listed in Table 3.

Prime system → Load cells in chamber → Wash cells → Recover T cells

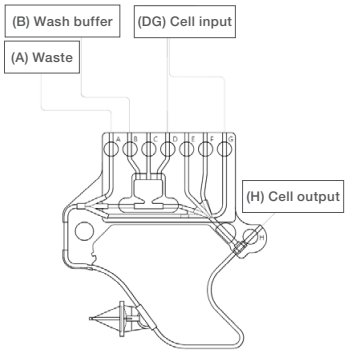
Results

PBMC isolation

Across three separate runs, both viability and recovery of PBMCs isolated from a Leukopak bag using red blood cell lysis buffer with the CTS Rotea system were consistently high (Figure 3A). In Figure 3B, the relative proportions of cell types in the Leukopak bag and in the output from a CTS Rotea PBMC isolation protocol are shown. Red blood cells are greatly reduced while T cells are significantly increased.

Table 3. T cell wash and concentrate protocol.

Step	Description	Flow path	Centrifugal force (x *g*)	Pump (mL/min)	Step type	Trigger
1	Pre-prime	B* to A	0	100	Normal	Bubble sensor input (start of flow)
2	Lubricate rotary Coupling	B to A	0	100	Normal	15 mL
3	Fill chamber and prime A	B to A	10	100	Normal	40 mL
4	Fill bubble trap and prime B	B to A	10	100	Normal	15 mL
5	Prime D	A to D	10	50	Normal	5 mL
6	Pressure prime	B to EF	10	0	Pressure prime	
7	Prime pause loop	J to K	10	25	Pause	3 mL
8	Establish cell bed	D to G	2,500	25	Normal	1 min
9	Load cells	D to A	2,500	30	Normal	Bubble sensor input (end of flow)
10	Concentrate bed for wash	J to K	2,600	30	Pause	10 sec
11	Wash	B to A	2,600	30	Normal	30 mL
12	Concentrate bed	J to K	2,700	15	Pause	10 sec
13	Harvest cells	B to H	2,700	50	Harvest	10 mL
14	Ramp to stop	K to J	500	50	Pause	5 sec



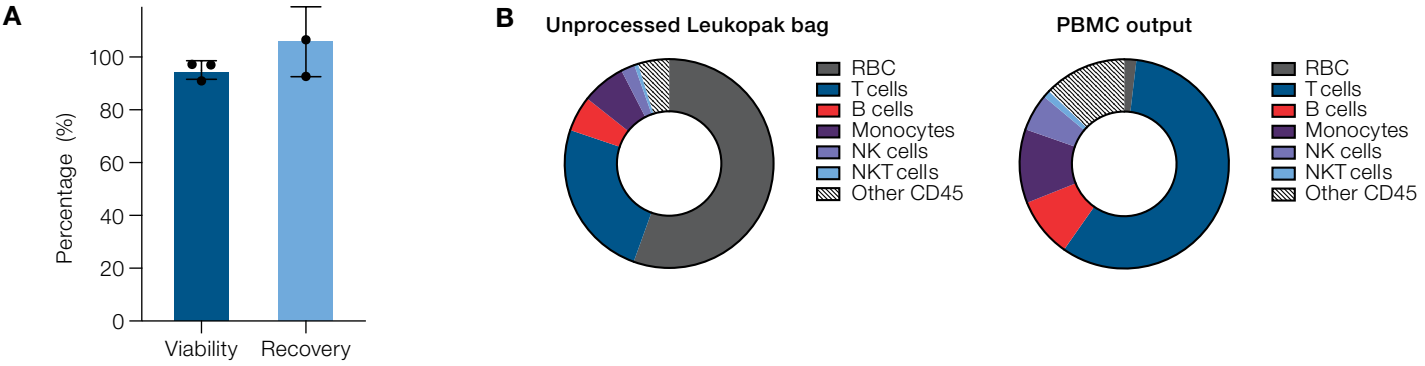


Figure 3. PBMC isolation using the CTS Rotea system. (A) Viability and recovery of PBMCs and **(B)** relative proportions of cell types present in an unprocessed Leukopak bag and after PBMC isolation.

T cell expansion

Expansion rates were nearly identical between T cells isolated using the CTS Rotea system and using a density gradient medium, showing that cells processed using the CTS Rotea system do not behave differently from those processed using manual methods (Figure 4A). The expanded T cells also have similar percentages of helper ($CD4^+$), cytotoxic ($CD8^+$), $CD62L^+CCR7^+$, and exhausted ($PD1^+$, $LAG\text{-}3^+$) T cells, all of which are of vital importance in many cell therapies (Figure 4B).

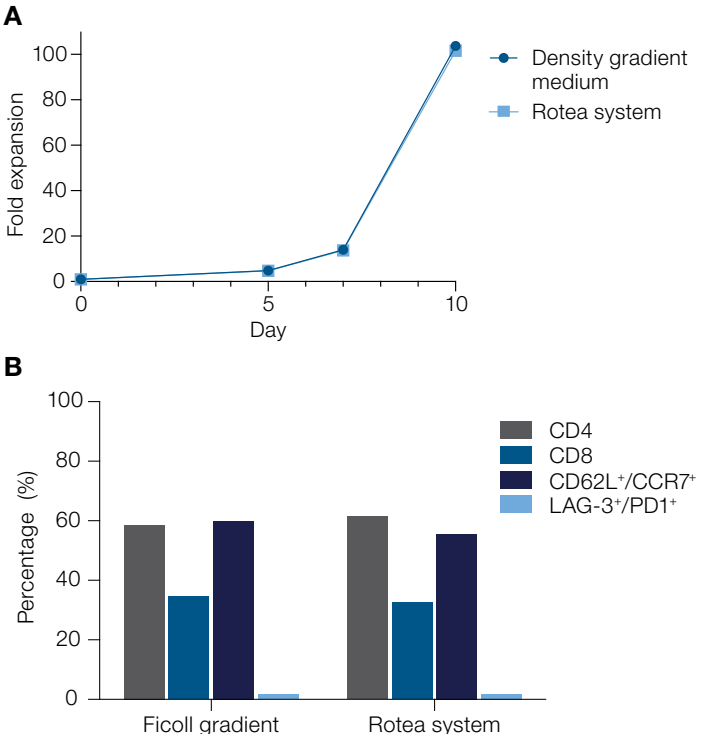


Figure 4. T cell expansion. (A) Expansion of T cells isolated using the CTS Rotea system and a density gradient medium. **(B)** Characterization of the expanded T cells isolated using the CTS Rotea system and a density gradient medium.

T cell washing and concentration

In addition, we tested the output capability of the CTS Rotea system by completing a series of T cell washing and concentration runs to determine viability and recovery of T cells post-processing (Figure 5). One liter of culture medium containing 5 x 10^8 cells was used as input, and the output volume was then changed from 5 mL to 10 mL, and finally, to 20 mL. After processing on the CTS Rotea system, viability and recovery were >90% for all output volumes, including 5 mL.

The CTS Rotea system chamber is capable of capturing up to 5 x 10^9 T cells in a stable fluidized bed while maintaining over 90% viability and recovery, as can be seen in Figure 6. The CTS Rotea system can easily process more than 5 billion cells using the same consumable by looping the protocol multiples times.

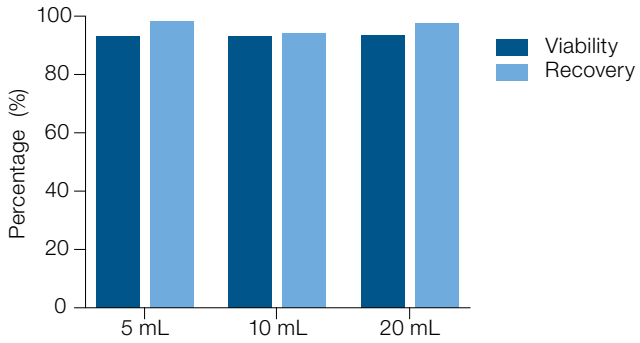


Figure 5. Recovery and viability for T cell washing and concentration with 5 mL, 10 mL, and 20 mL output volumes.

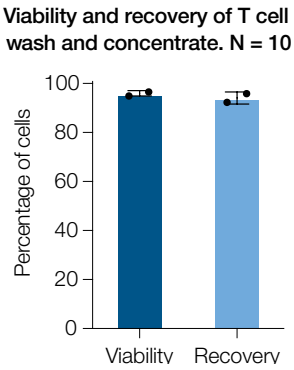


Figure 6. Average recovery and viability for two T cell wash and concentrate runs with an input of 5 x 10^9 cells.

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The CTS Rotea system shows consistency in performing T cell wash and concentrate protocols that maintain high viability of cells with high recovery. Figure 7A shows that across 10 runs of T cell wash and concentrate, both viability and recovery are consistently over 90%. The variability between runs is also low with standard deviations of 1.1% for viability and 3.4% for recovery. Flow cytometry was performed on T cells before and after being washed and concentrated using the CTS Rotea system. Populations of CD4 (helper) and CD8 (cytotoxic) T cells were consistent between the two sample groups, exemplifying the fact that the relative proportions of cell subpopulations are unaffected by CTS Rotea system processing (Figure 7B).

Processing of samples by the Rotea system can be viewed in real time in the "Live Video" view (Figure 7C). This allows for active monitoring of all steps within a protocol, including the fluidized cell bed formation and stabilization.

Conclusion

The CTS Rotea Counterflow Centrifugation System is capable of isolating, washing, and concentrating various cell types with no phenotypic change and no loss in recovery or viability. PBMCs isolated from a leukapheresis product had recovery and viability comparable to PBMCs manually isolated using a density gradient medium, with no change in phenotype or cell type composition. T cells isolated from PBMCs were successfully activated with Dynabeads magnetic beads, expanded, and characterized after processing with the CTS Rotea system. Expanded primary T cells that were washed and concentrated using the CTS Rotea system showed high recovery, cell concentration, and viability with no change in phenotype across various output volumes. The flexibility and efficiency of the system and user-programmable software allow it to be incorporated into multiple steps of various cell therapy workflows.

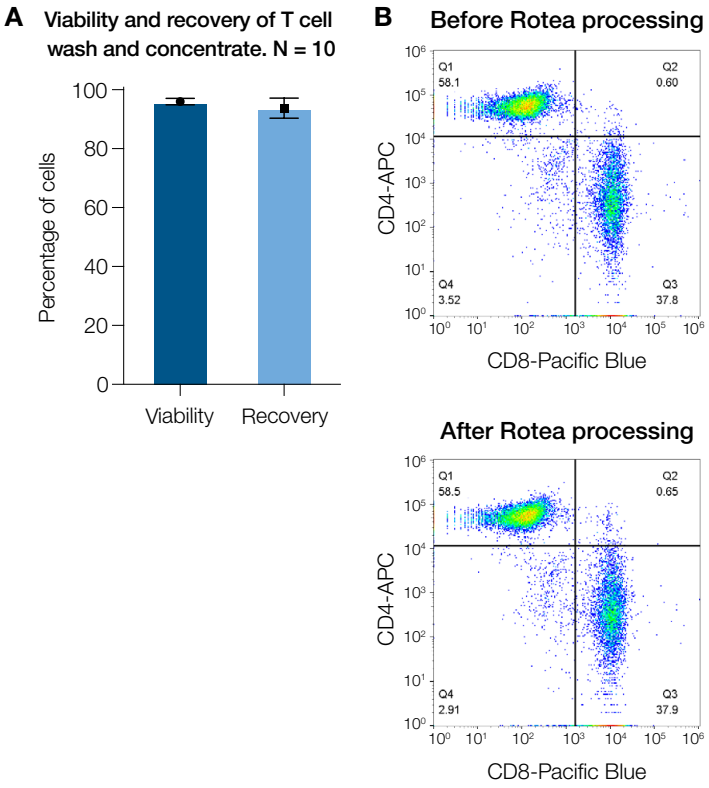

Figure 7. T cell washing and concentration. (A) T cell recovery and viability averaged over ten T cell washing and concentration runs. **(B)** Flow cytometry after staining of CD4 and CD8, before and after the CTS Rotea system washing and concentration protocol. **(C)** CTS Rotea system operating software with a chamber of T cells in a fluidized bed.

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