

# Closed, automated wash and concentration of expanded human natural killer (NK) cells

Improve the efficiency of NK cell processing with the CTS Rotea Counterflow Centrifugation System and CTS NK-Xpander Medium

## Introduction

One of the key challenges faced by the cell and gene therapy industry is poor efficiency in cell processing. A key improvement being made is the shift toward closed and automated systems, which can help reduce the risk of contamination and error, as well as provide the ability to process multiple products in parallel in less controlled spaces.

The Gibco™ CTS™ Rotea™ Counterflow Centrifugation System is a closed cell processing system developed specifically for small-batch cell therapy manufacturing. It provides output volumes as low as 5 mL with high cell recovery and viability, and is controlled via user-programmable software to enable creation of protocols for many different processes. The CTS Rotea system has been successfully used in various steps of the CAR T cell processing workflow, including isolation of peripheral blood mononuclear cells (PBMCs), cell washing, and concentration of engineered CAR T cells.

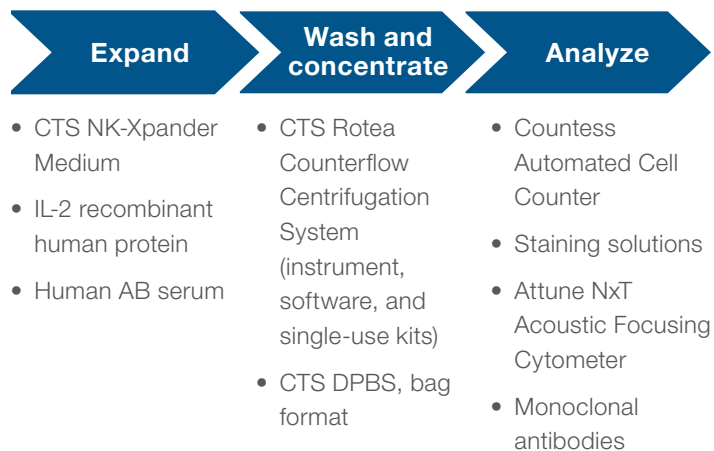
Here we demonstrate use of the CTS Rotea Counterflow Centrifugation System for automated washing and concentration of human NK cells expanded in Gibco™ CTS™ NK-Xpander™ Medium (Figure 1).

## Materials and methods

Enriched NK cells were expanded in CTS NK-Xpander Medium, harvested on day 17, washed, and concentrated using the CTS Rotea Counterflow Centrifugation System. Subsequent to this, phenotypic and functional characterization was performed.



Figure 1. CTS Rotea Counterflow Centrifugation System and CTS NK-Xpander Medium.



## Feeder-free NK cell expansion and activation

Enriched CD56<sup>+</sup> NK cells from PBMCs were cultured per the CTS NK-Xpander Medium **protocol** and scaled up to a final volume of 1.5 L. Briefly, NK cells were plated at  $1.25 \times 10^5$  cells/mL at 200  $\mu$ L per well in Thermo Scientific<sup>™</sup> Nunc<sup>™</sup> non-treated 96-well plates and cultured for 17 days in CTS NK-Xpander Medium (Cat. No. A5019001) containing 500 U/mL recombinant human IL-2 (Cat. No. PHC0023) and 5% human AB serum (Fisher Scientific Cat. No. BP2525100). The cells were fed every 2–3 days beginning on day 5 to maintain an optimal cell density of  $4\text{--}5 \times 10^5$  cells/mL. As cells grew, they were transferred from initial 48-well plates and split into multiple 6-well plates, then T-75 flasks, and finally to multiple T-175 non-tissue culture treated flasks to a final volume of 1.5 L. Cells were stained with trypan blue and counted using the Invitrogen<sup>™</sup> Countess<sup>™</sup> II FL Automated Cell Counter.

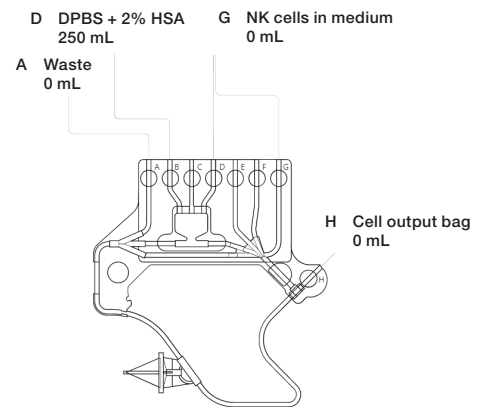
## Closed, automated washing and concentration of expanded NK cells

Following expansion, NK cell washing and concentration were performed using the CTS Rotea Counterflow Centrifugation System. Prior to loading onto the CTS Rotea system, a single-use kit was constructed using bag connections made via standard welding techniques (Figure 2). The CTS Rotea System was primed by replacing air in the system with buffer. The cells were loaded into the chamber to form a fluidized bed. Fresh wash buffer, consisting of Gibco<sup>™</sup> CTS<sup>™</sup> DPBS (without CaCl<sub>2</sub> and MgCl<sub>2</sub>) and 2% human serum albumin (Nova Biologics, Cat. No. 68982-0643-02), was allowed to flow

through the bed to wash the cells. The cells were then concentrated and harvested from the system for further downstream processing.



All CTS Rotea System protocols were written using the Gibco<sup>™</sup> CTS<sup>™</sup> Rotea<sup>™</sup> Protocol Builder desktop application. Table 1 lists the steps of the NK cell washing and concentration protocol, and Figure 2 illustrates the configuration of the Gibco<sup>™</sup> CTS<sup>™</sup> Rotea<sup>™</sup> Single-Use Kit.



**Figure 2.** CTS Rotea Single-Use Kit configuration for NK cell washing and concentration.

**Table 1.** Sequence of NK cell washing and concentration protocol on the Rotea system, including initial priming steps.

| Step                                    | Description                  | Flow path | Speed     | Flow rate  | Step type      | Trigger  |
|---|------------------------------|-----------|-----------|------------|----------------|--|
| <b>Priming sequence</b>                 |                              |           |           |            |                |  |
| 1                                       | Pre-prime                    | B to A    | 0 x g     | 100 mL/min | Normal         | Input bubble sensor  |
| 2                                       | Lubricate rotary coupling    | B to A    | 0 x g     | 100 mL/min | Normal         | Volume: 15 mL  |
| 3                                       | Prime chamber and line A     | B to A    | 10 x g    | 100 mL/min | Normal         | Volume: 15 mL  |
| 4                                       | Add priming volume           | B to A    | 10 x g    | 100 mL/min | Normal         | Volume: 50 mL  |
| 5                                       | Prime bubble trap and line B | A to B    | 10 x g    | 100 mL/min | Normal         | Volume: 15 mL  |
| 6                                       | Prime line D                 | A to D    | 10 x g    | 50 mL/min  | Normal         | Volume: 5 mL   |
| 7                                       | Pressure prime               | A to EF   | 10 x g    | 0 mL/min   | Pressure prime |  |
| 8                                       | Prime pause                  | J to K    | 10 x g    | 25 mL/min  | Pause          | Volume: 3 mL   |
| 9                                       | Ramp speed to initiate bed   | J to K    | 2,200 x g | 50 mL/min  | Pause          | Time: 10 sec   |
| <b>Loading and washing the NK cells</b> |                              |           |           |            |                |  |
| 10                                      | Initiate bed                 | D to G    | 2,200 x g | 50 mL/min  | Normal         | Time: 4 min  |
| 11                                      | Load input material          | D to A    | 2,250 x g | 35 mL/min  | Normal         | Volume: 1 x input aliquot (mL)<br>Input bubble sensor, pause |
| 12                                      | Adjust speed for wash        | J to K    | 2,400 x g | 25 mL/min  | Pause          | Time: 15 sec   |
| 13                                      | Wash                         | B to A    | 2,400 x g | 25 mL/min  | Normal         | Volume: 30 mL  |
| 14                                      | Concentrate bed for harvest  | J to K    | 2,500 x g | 15 mL/min  | Pause          | Time: 10 sec   |
| 15                                      | Harvest                      | B to H    | 2,500 x g | 50 mL/min  | Harvest        | Volume: 1 x harvest volume (mL)                              |
| 16                                      | Ramp to stop                 | K to J    | 500 x g   | 50 mL/min  | Pause          | Time: 5 sec  |

## NK cell phenotypic characterization

Expanded NK cells were gated for live cells using Invitrogen™ LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit. Their CD56, CD3, and CD16 levels were then measured using appropriate antibodies and the Invitrogen™ Attune™ NxT Acoustic Focusing Cytometer.

## NK cell functionality

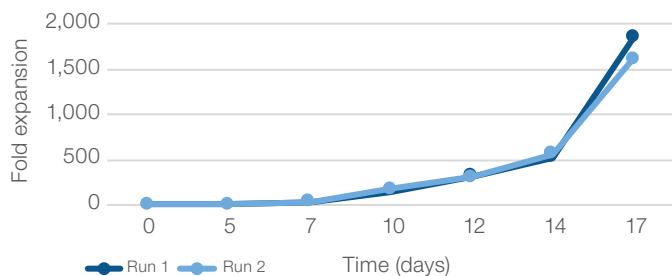
NK effector cells expanded in CTS NK-Xpander Medium were coincubated with K562 target cells labeled with the Invitrogen™ CellTrace™ CFSE Cell Proliferation Kit at NK:K562 cell ratios of 0.625:1, 1.25:1, 2.5:1, and 5:1 for 2 hours. Following incubation, degranulation was assessed based on the expression of CD107a by CD56<sup>+</sup> NK cells, measured on the Attune NxT Acoustic Focusing Cytometer. NK cell cytotoxicity was assessed by measuring K562 cell death on the Attune NxT system by gating for CFSE-labeled K562 cells and measuring the percentage of dead cells using the LIVE/DEAD stain kit.

## Results

NK cells were expanded to  $1.83 \times 10^9$  cells in a final volume of 1.62 L using CTS NK-Xpander Medium. Cells washed and concentrated using the CTS Rotea Counterflow Centrifugation System showed high recovery and viability post wash and maintained their phenotype and functionality.

## Feeder-free NK cell expansion and activation

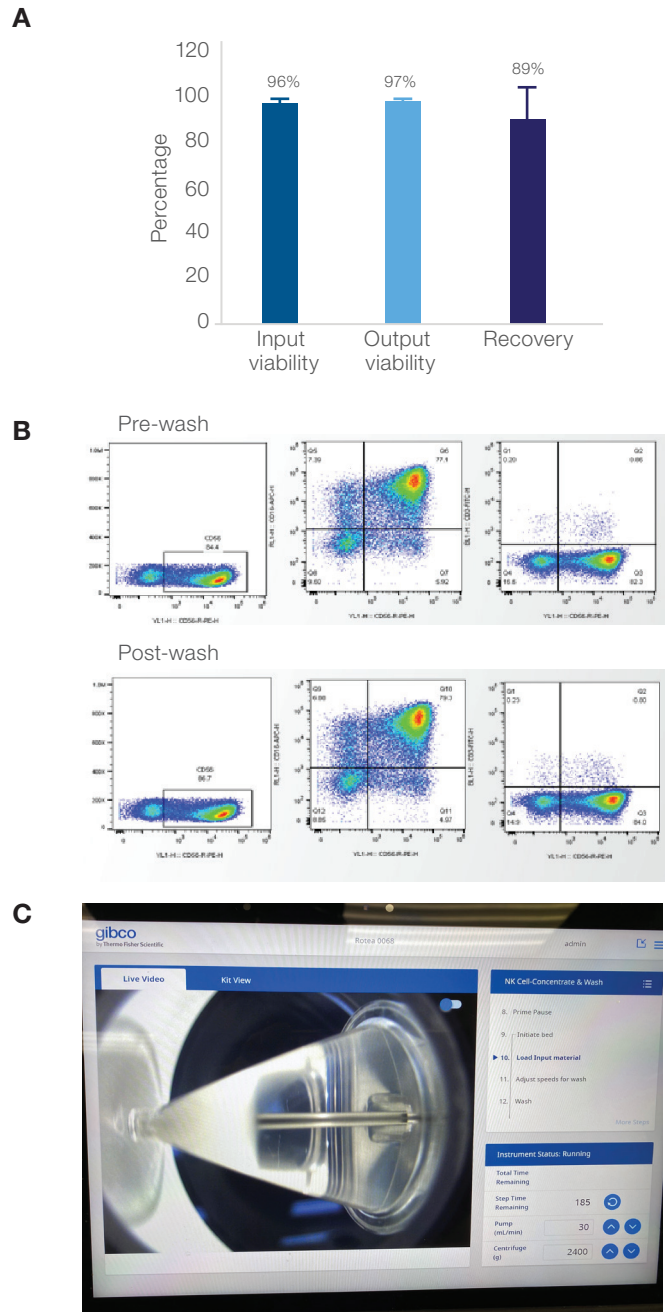
PBMC-derived NK cells cultured in CTS NK-Xpander Medium expanded by 1,700-fold on average after 17 days (Figure 3). The cultures started at  $1.13 \times 10^6$  cells in 9 mL and increased to  $1.83 \times 10^9$  cells in 1.62 L.



**Figure 3.** Fold expansion of NK cells cultured in CTS NK-Xpander Medium for 17 days.

## Closed, automated washing and concentration of expanded NK cells

Expanded cells were loaded into the CTS Rotea system to form a stabilized bed for subsequent washing in CTS DPBS. Recovery was ~90% with high viability and maintenance of cellular phenotype (Figure 4).



**Figure 4.** NK cell washing and concentration. **(A)** NK cell viability and recovery averaged over four washing and concentration runs. **(B)** Flow cytometry staining for CD56, CD16, and CD3 before and after washing with the CTS Rotea System NK cell wash and concentration protocol. **(C)** CTS Rotea System software image with a chamber of NK cells in a fluidized bed.

## NK cell functionality

Cells washed and concentrated using the CTS Rotea system maintained cytolytic function and were able to degranulate (Figure 5) and kill K562 target cells (Figure 6) in a dose-dependent manner.

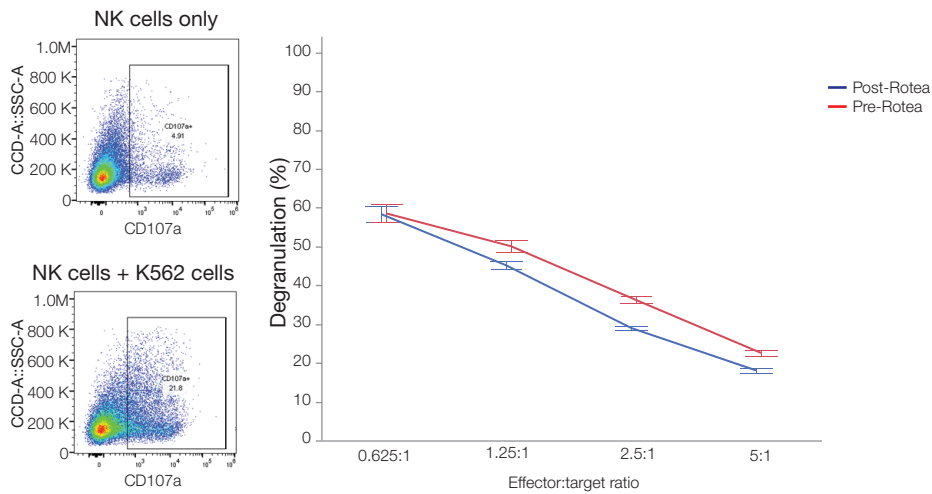


Figure 5. Maintenance of NK cell degranulation capability after washing and concentration.

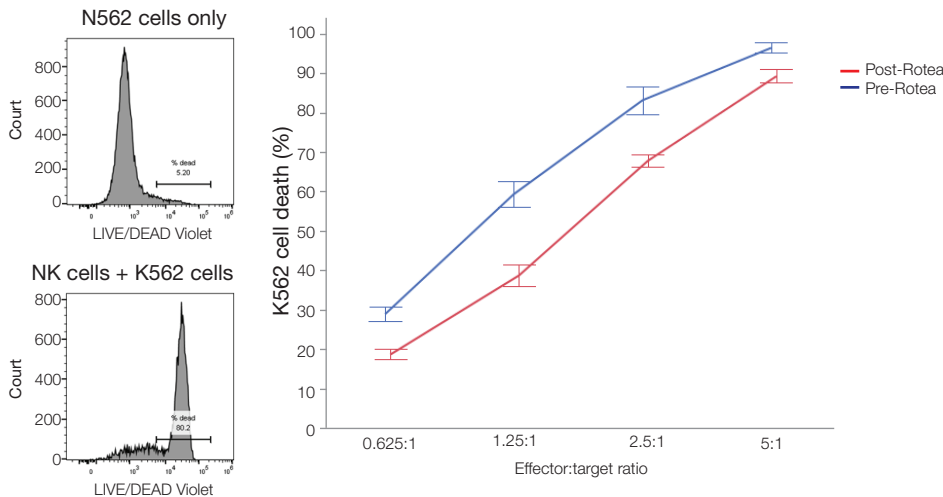


Figure 6. Maintenance of NK cell cytotoxicity after washing and concentration.

## Conclusions

Critical improvements to cell and gene therapy manufacturing can be achieved by reducing risk and hands-on time using regulation-compliant reagents and closed manufacturing systems. We have demonstrated efficient NK cell expansion in a feeder-free culture system as well as high recovery after washing and concentrating the cells using a closed, automated counterflow centrifugation system. Here we have demonstrated efficient expansion of NK cells in a feeder-free culture system using CTS NK-Xpander Medium, and high recovery of cells during wash and concentration using the CTS Rotea Counterflow Centrifugation System.

## Ordering information

| Product   | Quantity      | Cat. No.                     |
|---|---------------|------------------------------|
| <b>Expansion</b>  |               |                              |
| CTS NK-Xpander Medium   | 500 mL bottle | A5019001                     |
|   | 5 L bag       | A5019002                     |
| Human IL-2 Recombinant Protein  | 1 mg          | PHC0023                      |
| Human AB Serum  | 100 mL        | Fisher Scientific, BP2525100 |
| Nunc non-treated 96-well plates   | Case of 160   | 268200                       |
| Nunc non-treated 48-well plates   | Case of 75    | 150787                       |
| <b>Analysis</b>   |               |                              |
| Countess 3 FL Automated Cell Counter  | 1 instrument  | AMQAF2000                    |
| Trypan Blue Solution, 0.4%  | 100 mL        | 15250061                     |
| CellTrace CFSE Cell Proliferation Kit   | 1 kit         | C34570                       |
| eBioscience Flow Cytometry Staining Buffer  | 600 mL        | 004222-26                    |
| Fc Receptor Binding Inhibitor Polyclonal Antibody   | 100 tests     | 14-9161-73                   |
| UltraComp eBeads Compensation Beads   | 100 tests     | 01-2222-42                   |
| ArC Amine Reactive Compensation Bead Kit  | 1 kit         | A10346                       |
| Attune NxT Acoustic Focusing Cytometer  | 1 instrument  | A24858                       |
| CD56 Monoclonal Antibody (CMSSB)  | 100 tests     | 120567-42                    |
| CD3 Monoclonal Antibody (OKT3)  | 100 tests     | 11-0037-42                   |
| CD16 Monoclonal Antibody (CB16)   | 100 tests     | 17-0168-42                   |
| CD107a (LAMP-1) Monoclonal Antibody (eBioH4A3)  | 100 tests     | 25-1079-42                   |
| LIVE/DEAD Fixable Violet Dead Cell Stain Kit, for 405 nm excitation                               | 400 assays    | L34964                       |
| <b>Wash and concentration</b>   |               |                              |
| CTS Rotea Counterflow Centrifugation System with 2-year warranty, including OQ after PM plus IQOQ | 1 instrument  | A50757*                      |
|   |               | A47695**                     |
| CTS Rotea Counterflow Centrifugation System with 2-year warranty, including PM                    | 1 instrument  | A50760*                      |
|   |               | A47679**                     |
| CTS Rotea Single-Use Kit  | 10 pack       | A49585                       |
|   | 5 pack        | A49313                       |
| CTS Rotea Hi-Flow Single-Use Kit  | 10 pack       | A46575                       |
|   | 5 pack        | A49239                       |
| CTS DPBS  | 2 L bag       | A1285602                     |
| CTS Rotea Kit Tube Clamps   | 100 pack      | A49127                       |
| CTS Rotea Kit Sterile Luer Connectors   | 10 pack       | A50110                       |
| CTS Rotea Kit Sterile Sample Ports  | 10 pack       | A50111                       |

\* North America and Europe.

\*\* Rest of the world.

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