

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.

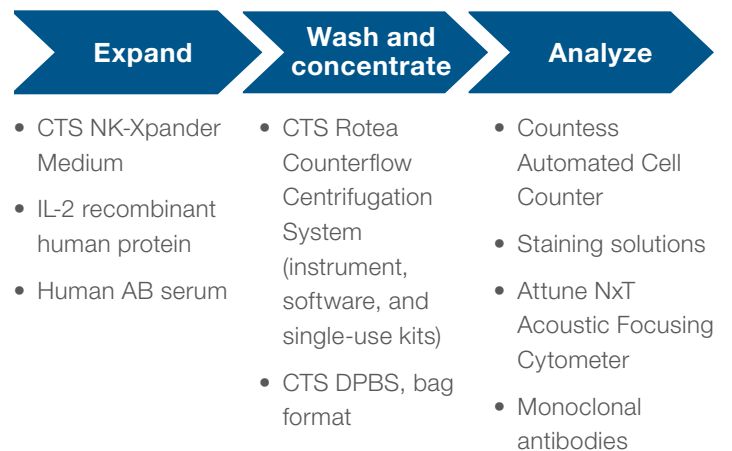
Here we demonstrate use of the CTS Rotea Counterflow Centrifugation System for automated washing and concentration of human NK cells expanded in Gibco™ CTS™ NK-Xpander™ Medium (Figure 1).

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⁺ 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 1.25×10^5 cells/mL at 200 μ L per well in Thermo Scientific™ Nunc™ 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™ Countess™ 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™ CTS™ DPBS (without CaCl₂ and MgCl₂) 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™ CTS™ Rotea™ 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™ CTS™ Rotea™ Single-Use Kit.

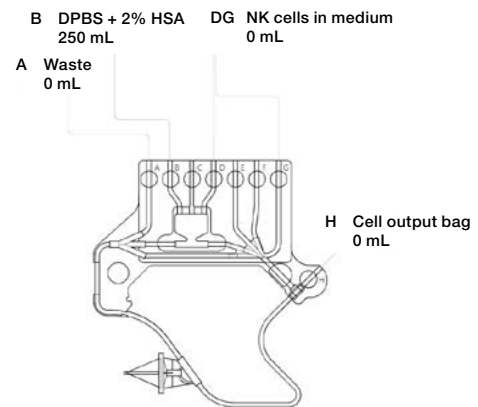


Figure 2. CTS Rotea Single-Use Kit configuration for NK cell washing and concentration.

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
Priming sequence						
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
Loading and washing the NK cells						
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) 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⁺ 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 1.83×10^9 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 1.13×10^6 cells in 9 mL and increased to 1.83×10^9 cells in 1.62 L.

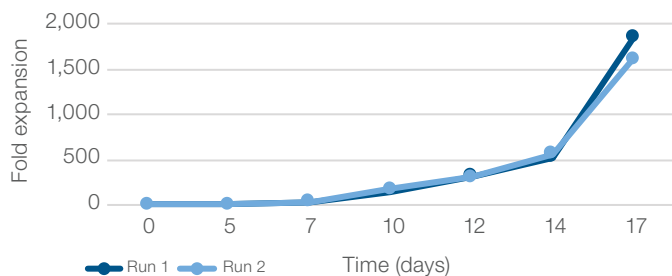


Figure 3. Fold expansion of NK cells cultured in CTS NK-Xpander Medium for 17 days.

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).

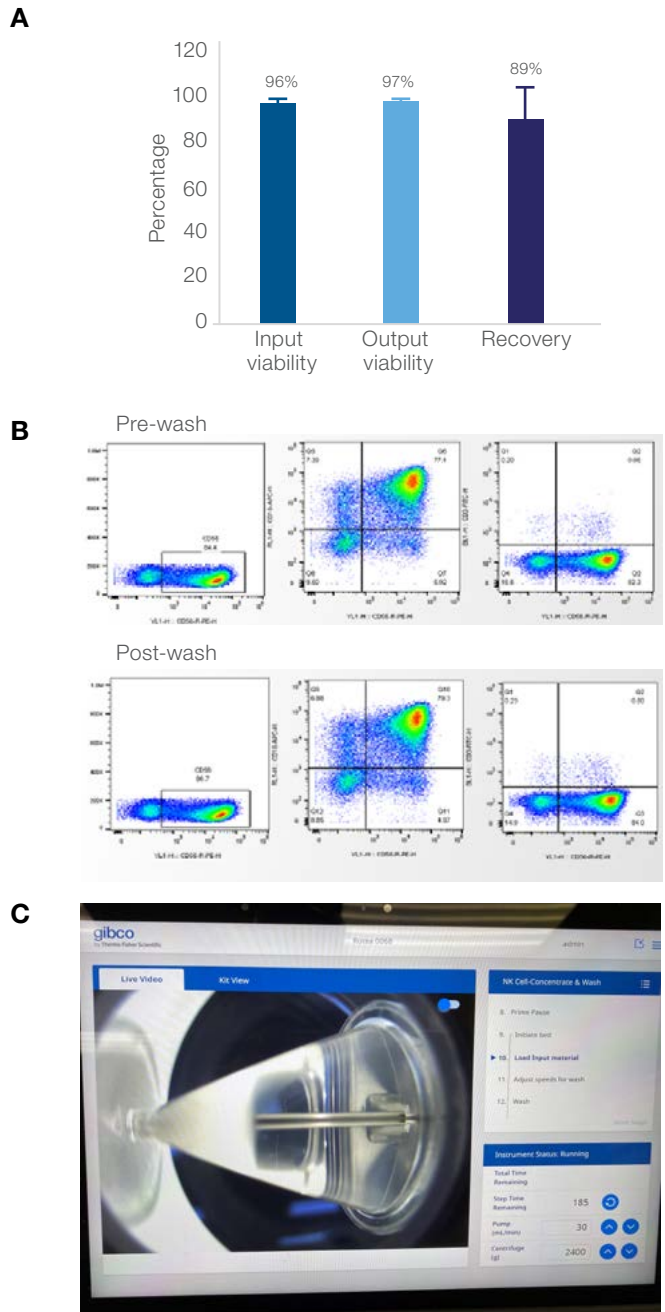


Figure 4. NK cell washing and concentration. (A) NK cell viability and recovery averaged over four washing and concentration runs. (B) Flow cytometry staining for CD56, CD16, and CD3 before and after washing with the CTS Rotea System NK cell wash and concentration protocol. (C) CTS Rotea System software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

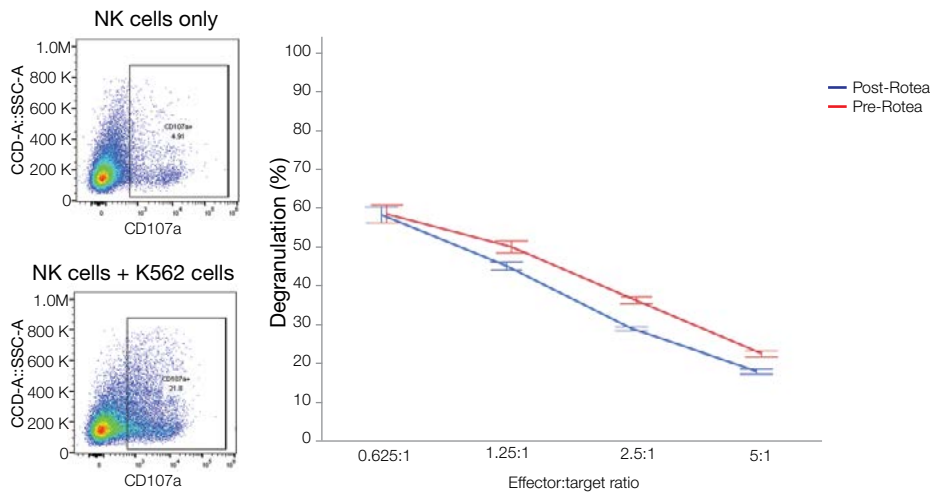


Figure 5. Maintenance of NK cell degranulation capability after washing and concentration.

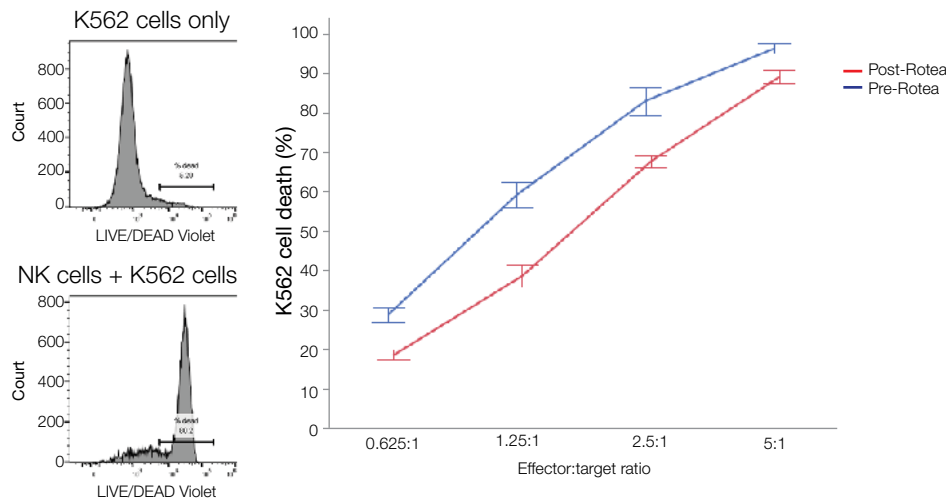


Figure 6. Maintenance of NK cell cytotoxicity after washing and concentration.

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.
Expansion		
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
Analysis		
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
Wash and concentration		
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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