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Cell therapy solutions

Flexibility and modularity in cell therapy manufacturing: incorporating the CTS Rotea and Xenon systems

Closing the cell wash, concentration, buffer exchange, and electroporation steps

Introduction

The autologous cell therapy process is extremely labor-intensive with many open-process steps and a high degree of complexity, regardless of the cell type in use. The industry is actively working to simplify and close processes to optimize manufacturing, reduce costs, and increase speeds of getting treatments to patients. While viral gene delivery has been in use for quite some time, nonviral delivery methods are attractive because of their reduced cytotoxicity, immunogenicity, and mutagenicity compared to viral vectors. Electroporation is particularly interesting as an alternative to viral delivery because of its simplicity of use and ease of large-scale production.

To better serve the cell therapy industry, Thermo Fisher Scientific has created flexible, modular systems that can be easily adopted into existing workflows. Two fit-for-purpose manufacturing systems have been recently introduced, the **Gibco™ CTS™ Rotea™ Counterflow Centrifugation System** and the **Gibco™ CTS™ Xenon™ Electroporation System**. Individually, these two systems can enable a closed manufacturing process for the wash and concentration of cells and for the nonviral delivery of genetic material, respectively. Together, they represent an important advancement in the evolution of cell therapy manufacturing—reducing risk, time, and complexity of manufacturing. Here we show the ability of the CTS Rotea and CTS Xenon systems to work together and discuss key aspects of working toward optimization and standardization of cell therapy manufacturing workflows.

Materials and methods

PBMC isolation

Peripheral blood mononuclear cells (PBMCs) from 3 donor leukopaks were isolated using the CTS Rotea system. After lysing the red blood cells (RBCs) with **Gibco™ ACK Lysing Buffer** according to the CTS Rotea system isolation protocol, the CTS Rotea system was used to easily isolate the cells of interest by removing the lysed RBCs and performing buffer exchange [1].

T cell activation and debeading

To test the impact of activation time on editing efficiency, PBMCs were activated with **Gibco™ CTS™ Dynabeads™ CD3/CD28** (3 beads for each $CD3^+$ cell) and expanded on the G-Rex™ platform (Wilson Wolf) for 2 or 3 days (37°C with 5% CO_2) in **Gibco™ CTS™ OpTmizer™ T Cell Expansion Serum-Free Medium**, supplemented with **Gibco™ Human IL-2 Recombinant Protein** and **Gibco™ CTS™ Immune Cell Serum Replacement**, per product instructions (complete medium). The aim was to achieve approximately 10 x 10^6 $CD3^+$ cells/cm^2 in 700–800 mL of complete medium. At 2 or 3 days post-activation, volume was reduced on the G-Rex platform, and then T cells were debeaded using the **Gibco™ CTS™ DynaMag™ Magnet** and placed in an incubator for 1 hour. After 1 hour, cells were washed and concentrated (buffer exchange) in **Gibco™ CTS™ Xenon™ Genome Editing Buffer** using either the CTS Rotea system or a manual process.

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Buffer exchange on the CTS Rotea system prior to electroporation

For buffer exchange on the CTS Rotea system, the **Gibco™ CTS™ Rotea™ Single-Use Kit** was primed with wash buffer, cells were loaded to form a cell bed, and then cells were washed, concentrated, and harvested using the protocol outlined in Table 1.

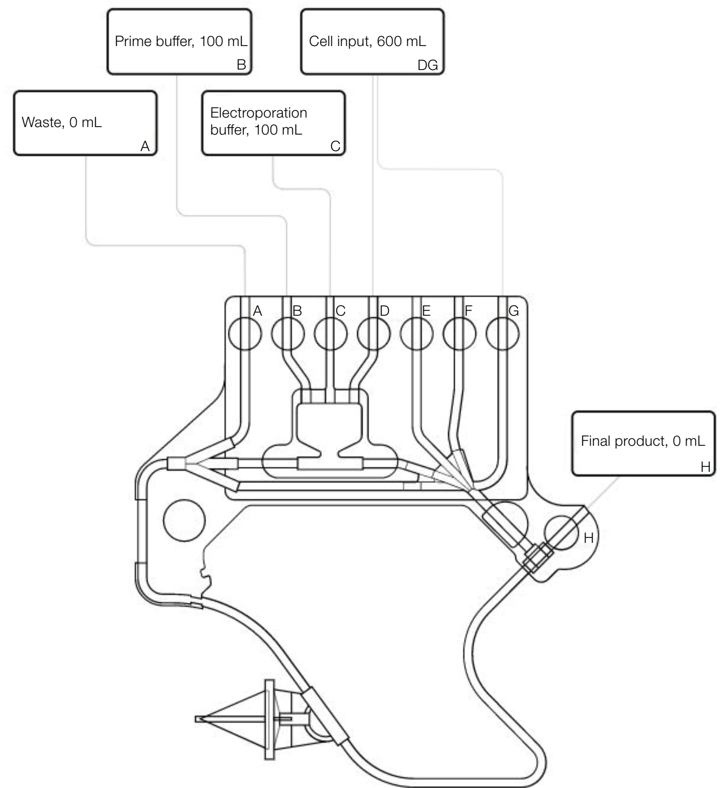

Table 1. Protocol steps for genome editing buffer exchange on the CTS Rotea system.

Step	Description	Flow path	Centrifugal force (x *g*)	Flow rate (mL/min)	Step type	Trigger
1	Pre-prime line A	B to A	0	100	Normal	Input bubble sensor
2	Lubricate rotary coupling	B to A	0	100	Normal	Volume: 15 mL
3	Prime chamber and line A	B to A	10	100	Normal	Volume: 15 mL
4	Add priming volume	B to A	10	100	Normal	Volume: 50 mL
5	Prime bubble trap and line B	A to B	10	100	Normal	Volume: 15 mL
6	Prime line C	A to C	10	100	Normal	Volume: 3 mL
7	Prime line D	A to D	10	100	Normal	Volume: 3 mL
8	Pressure prime	A to EF	10	0	Pressure prime	
9	Prime pause loop	J to K	10	25	Pause	Volume: 3 mL
10	Ramp speeds	J to K	2,000	50	Pause	Time: 20 seconds
11	Form cell bed	D to G	2,000	50	Normal	Volume: 50 mL
12	Load cells to bubble detect	D to A	2,000	35	Normal	Input bubble sensor, pause
13	Concentrate bed for wash	J to K	2,200	20	Pause	Time: 25 seconds
14	Wash cells with CTS Xenon Genome Editing Buffer	C to A	2,200	27	Normal	Volume: 1x wash volume, mL Input bubble sensor, pause
15	Concentrate cells for recovery	J to K	2,800	27	Pause	Time: 5 seconds
16	Harvest cells	C to H	2,800	50	Harvest	Volume: 1x harvest volume, mL
17	Slow down	J to K	100	10	Pause	Time: 10 seconds

Electroporation

Washed and concentrated cells were electroporated in the CTS Xenon Electroporation System with the **Gibco™ CTS™ Xenon™ SingleShot Electroporation Chamber** at a cell density of 5 x 10^7 cells/mL in CTS Xenon Genome Editing Buffer. Cells were electroporated in parallel in the research-use, small-scale **Invitrogen™ Neon™ Transfection System** with an open pipette tip chamber. Respective payload and electroporation conditions are listed in Table 2.

Table 2. Electroporation protocol details.

Experimental details*	
Cells	3 healthy human donors, fresh leukopak product
	Cell concentration during electroporation: 5 x 10^7 cells/mL
Payload	**Invitrogen™ TrueCut™ Cas9 Protein v2**: 120 µg/mL
	Custom sgRNA targeting the T cell receptor (TCR) alpha constant gene (*TRAC*): 30 µg/mL
	Linear donor dsDNA, GFP: 80 µg/mL
Electroporation protocol	Neon system (100 µL): 1,600 V, 10 ms, 3 pulses
	CTS Xenon system (1 mL): 2,300 V, 3 ms, 4 pulses

* For smaller-scale electroporations, the 100 µL Neon tip was used, and the cell number and payload amounts were 1/10 that of the CTS Xenon system.

CAR T cell expansion

After electroporation, a total of 2 x 10^6 cells were plated with complete medium in 6-well plates at 1 x 10^6 cells/mL for expansion. After 3 days of expansion, cells were harvested for phenotypic characterization, viability assessment, and analysis of electroporation efficiency. The remainder of the cells were placed back in culture with complete medium in T-75 flasks at 1 x 10^6 cells/mL for an additional 4 days of expansion, then tested again for viability.

Analysis and characterization to examine electroporation efficiency

At 3 days post-electroporation, T cells were stained with **Invitrogen™ eBioscience™ TCR alpha/beta mAb** (PE conjugated), **Invitrogen™ CD4 mAb** (PE-Cyanine7 conjugated), **Invitrogen™ CD8 mAb** (APC-Cyanine7 conjugated), and **Invitrogen™ SYTOX™ Red Dead Cell Stain** as a cell viability dye. Additionally, T cells were stained with **Invitrogen™ eBioscience™ CD69 mAb** (APC conjugated) and **Invitrogen™ eBioscience™ CD25 mAb** (PE conjugated) for early and late activation markers, respectively. The TCR knockout efficiency was determined by the percentage of TCR-negative cells, and the knock-in efficiency was determined by the percentage of GFP-positive cells. Flow cytometry was performed with the **Invitrogen™ Attune™ NxT Flow Cytometer** with the gating strategy shown in Figure 1.

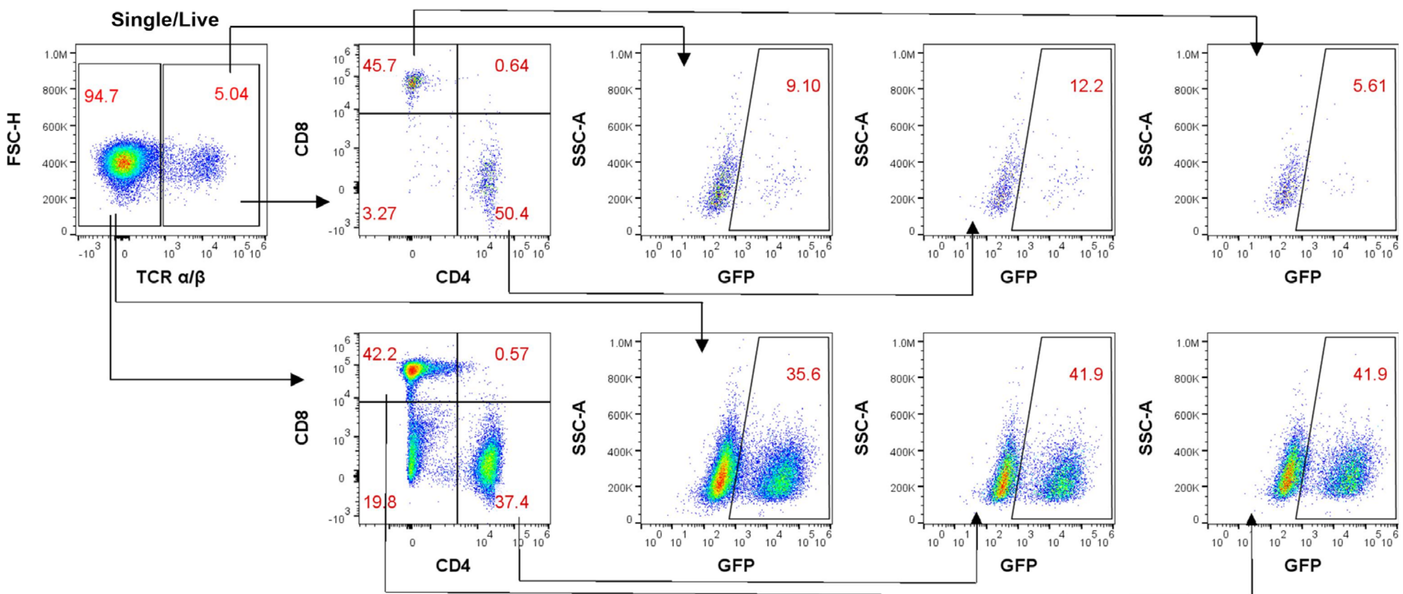

Figure 1. Gating strategy for the Attune NxT Flow Cytometer. The numbers shown in red are percentages of cell populations.

Results

Phenotypic characterization of CD4/CD8 ratios and CD69/CD25 activation markers

Phenotypic analysis showed expected ratios of CD4/CD8 and no significant difference between 2- and 3-day activation (Figure 2), suggesting either a 2- or 3-day activation will result in sufficiently activated CD4/CD8 T cells. We observed no significant difference in these markers between the Neon and CTS Xenon systems for electroporation, manual and CTS Rotea system processes for buffer exchange, or between donors (data not shown).

Viability and recovery

Viability of cells was measured 3 days after electroporation for all conditions tested: 2-day versus 3-day activation protocols, manual versus CTS Rotea system processes for buffer exchange, and Neon versus CTS Xenon systems for electroporation. Results were well above 85% for all conditions tested, suggesting minimal impact on viability (Figure 3).

Cell recovery was also measured after debeading and buffer exchange for both 2- and 3-day activation protocols (62% and 79% average recovery, respectively, results not shown). Cells are typically fragile at various steps of the workflow, including after debeading and electroporation. Some of the optimization techniques used to improve recovery were increasing the CTS Rotea system input cell number, increasing the input cell concentration by volume reduction during debeading, and resting the cells for a short time (~1 hour) after debeading.

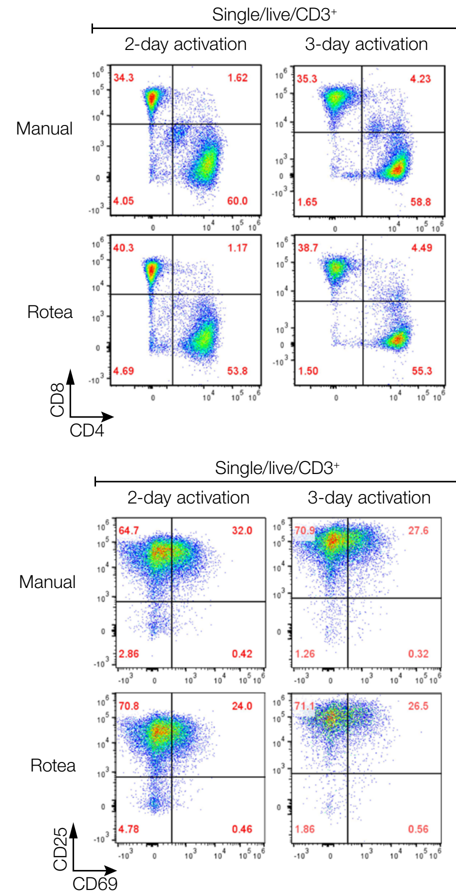

Figure 2. Phenotypic characterization of CD4/CD8 T cell markers and CD25/CD69 activation markers. The numbers shown in red are percentages of cell populations.

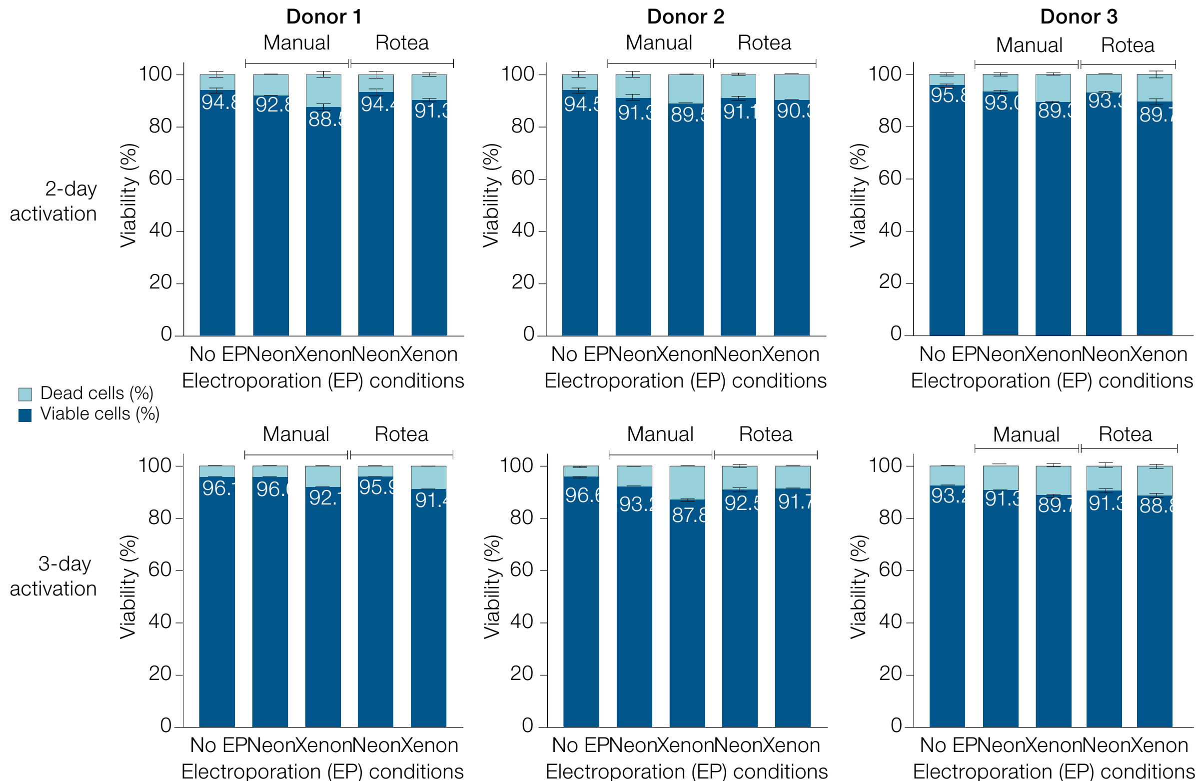

Figure 3. Viability of cells at 3 days post-electroporation.

Electroporation efficiency

Electroporation efficiency (knock-in versus knockout) was assessed 3 days post-electroporation for all conditions tested: 2-day versus 3-day activation protocols, manual versus CTS Rotea system processes for buffer exchange, and Neon versus CTS Xenon systems for electroporation. As expected, there was donor-to-donor variation, with knock-in efficiency of up to 36.8% (Figure 4). When analyzing individual conditions tested, the following results were seen:

- Knock-in efficiency of the CTS Xenon system (13.2–36.8%) was consistently higher than the Neon system for all 3 donors across all conditions (Figure 4). These results were supported by evaluating the total number of edited cells for donor 3 (Figure 5).
- Two-day activation showed higher electroporation efficiency compared to 3 days across all donors and conditions (Figure 4).
- For the 2-day activation protocol, efficiency was similar using manual versus CTS Rotea system processes for buffer exchange (Figure 4). The 3-day activation resulted in lower efficiency for donor 1 and 2 on the CTS Rotea system, but similar efficiency for donor 3.

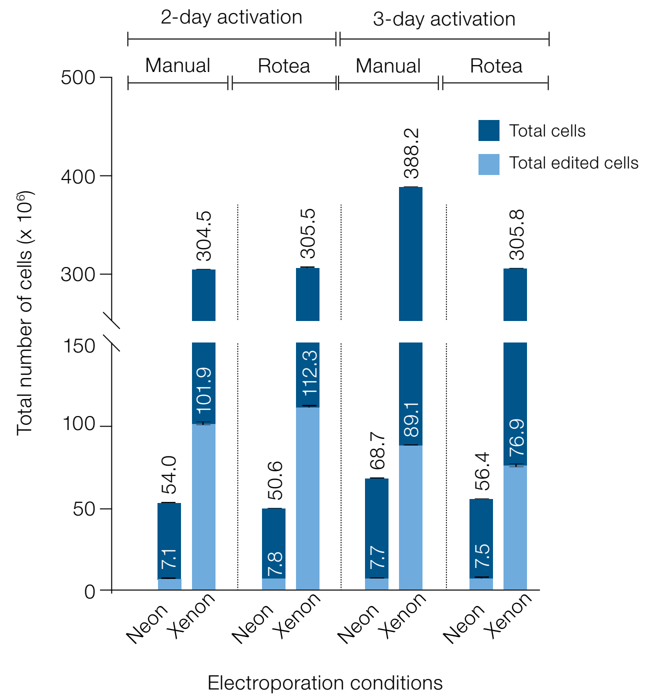

Figure 5. Superior knock-in efficiency of the CTS Xenon system compared to the Neon system for donor 3. Values lower on the bars are edited cells; values above the bars are total cells.

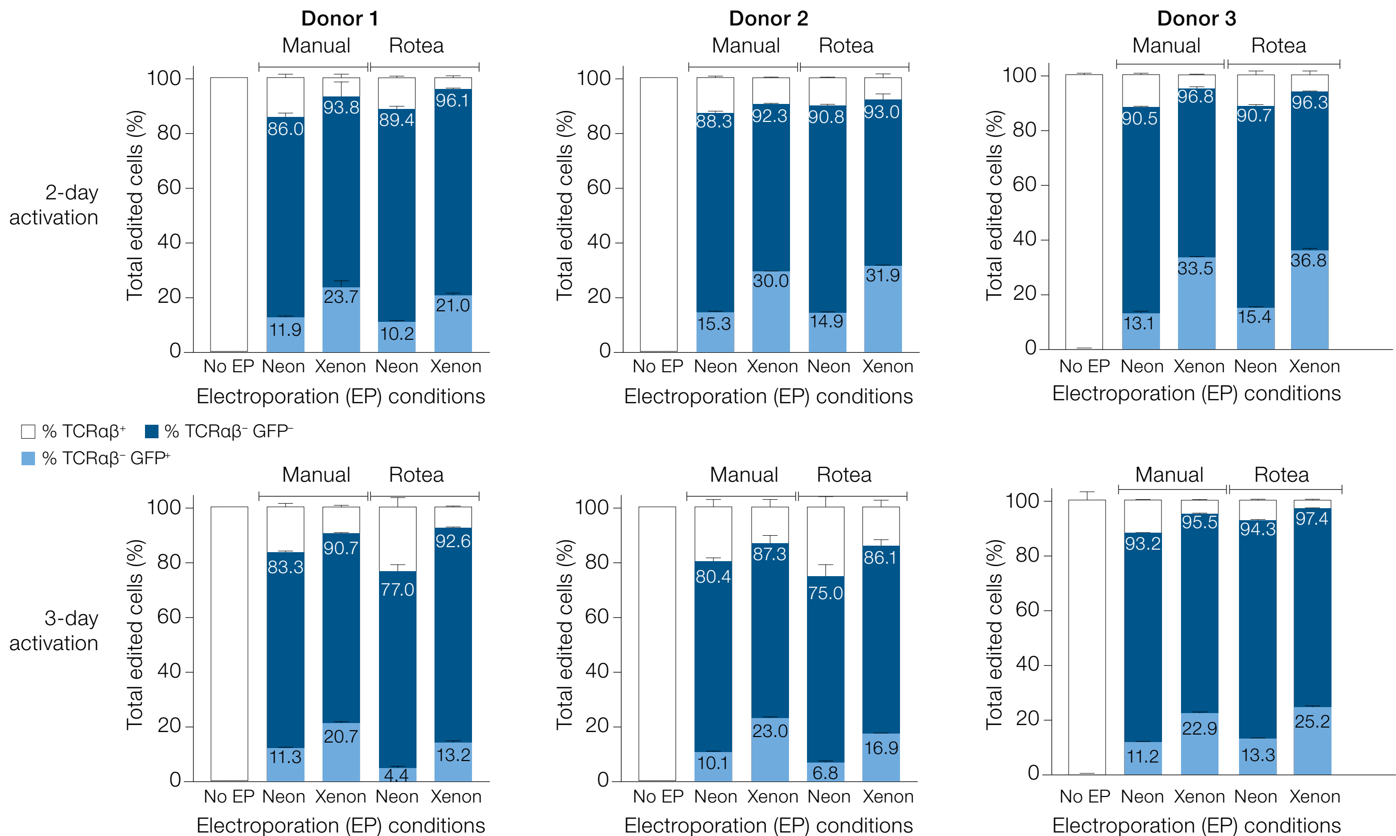

Figure 4. Electroporation efficiency 3 days post-electroporation. Values lower on the bars are for TCRαβ⁻ GFP⁺ cells; values higher on the bars are total edited cells (TCRαβ⁻ GFP⁺ and TCRαβ⁻ GFP⁻).

Cell health and growth

Cells were evaluated for viability and growth after electroporation on the CTS Xenon system. Good viability (>80%) was observed for all conditions compared to no-electroporation controls (Figure 6). Cells from the 2-day activation protocol showed slightly improved growth over those from the 3-day activation protocol, for cells edited using the CTS Xenon system. Edited cell yield for the 2-day activation was 102 x 10^6 (manual processing) and 112 x 10^6 (CTS Rotea system processing). For the 3-day activation protocol, edited cell yield was 89 x 10^6 (manual processing) and 77 x 10^6 (CTS Rotea system processing). Data shown are from donor 3 only (cells from donors 1 and 2 were only expanded to day 3 post-electroporation).

It is important to note that the work completed for this application note included buffer exchange and electroporation steps that were closed. Other steps of the workflow were kept open, including cell culture and expansion. Unfortunately, contamination occurred during the media exchange from day 3 to day 7 of culture, resulting in the exclusion of one data point from analysis (3-day activation, manual wash, CTS Xenon system EP). No contamination was observed when the closed CTS Rotea system was used for media exchange. As many have experienced, contamination is more common in open processes, further emphasizing the importance of our goal to enable the cell therapy industry with closed manufacturing systems.

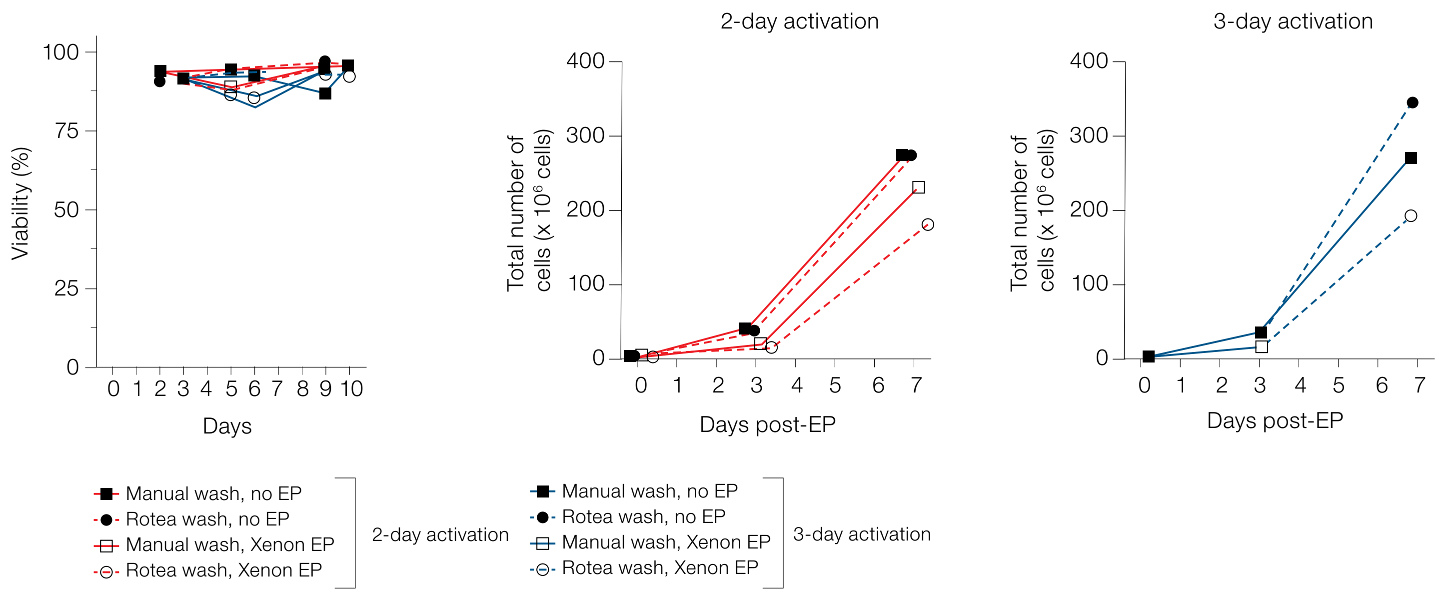

Figure 6. Viability and expansion of cells up to 7 days post-electroporation.

Discussion

Optimization of cell and gene therapy workflows can be quite complex because of the large number of process steps and variables included. Here we tested various conditions for the buffer exchange and electroporation steps across 3 different donors. Comparisons included the closed and semiautomated CTS Rotea system versus an open and manual process for buffer exchange, the CTS Xenon system versus the Neon system for electroporation, and the length of time for activation of T cells.

As expected, we observed some donor-to-donor variability, especially for the more sensitive workflow steps such as electroporation. Given the number of variables that can impact each process step, it is recommended that each step be optimized individually prior to linking them together.

Overall, the 2-day activation protocol resulted in higher knock-in efficiencies. When looking at the 2-day protocol more closely, specifically at the impact of closed versus manual operations on electroporation efficiency, the CTS Xenon system (which can support closed-system electroporation) outperforms the Neon system (open-system electroporation), and the CTS Rotea system (closed cell processing) outperforms manual buffer exchange. In addition, these two modular systems can be connected to each other by sterile welding to create a fully closed wash, concentration, buffer exchange, and electroporation protocol.

In conclusion, the CTS Rotea and CTS Xenon systems can provide superior performance and help reduce contamination in the cell therapy manufacturing workflow, thus making these two instruments powerful, modular tools in the quest toward creating a closed cell therapy manufacturing process.

Reference

1. Thermo Fisher Scientific. Application note: Automated PBMC isolation and T cell wash and concentration by the CTS Rotea system.

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