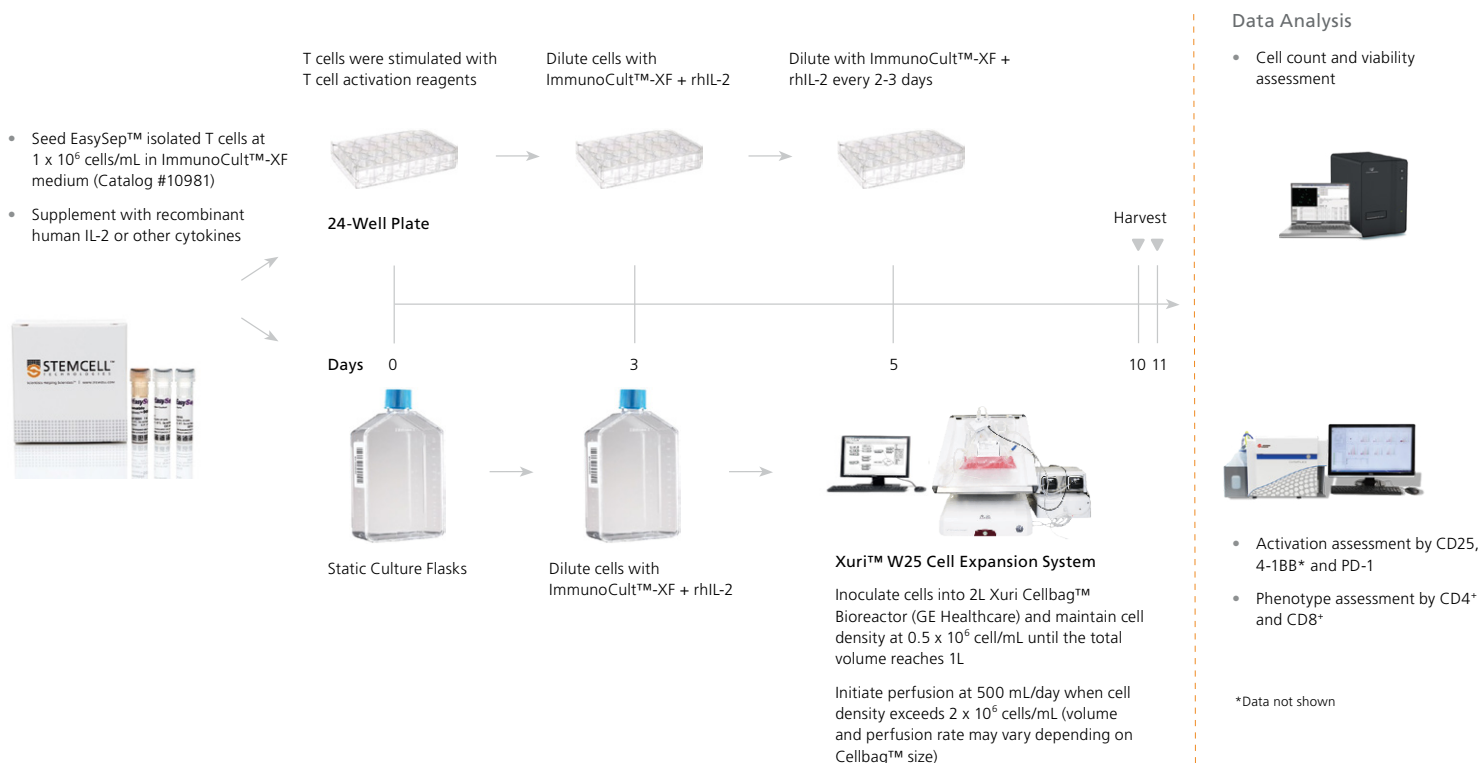


# Optimization of Human T Cell Expansion Protocol: Effects of Early Cell Dilution

Adoptive T cell therapy is a rapidly progressing field, and optimized protocols for the scalable manufacture of T cells are essential to maximize the therapeutic potential of patient-derived T cells. A single administration of adoptive T cell therapy can require billions of expanded T cells. Large-scale production of human T cells for cellular therapy is a complex, multi-step process that presents many opportunities for optimization in order to obtain maximum yield while retaining desired end phenotype and function. In this Technical Bulletin, we highlight the importance of cell density on T cell expansion. Our findings suggest that maintaining T cells at lower cell densities improves T cell growth and viability, especially at early stages of cell expansion. With optimized culture protocols, up to 800-fold expansion of human T cells with > 85% cell viability was achieved over 10 - 14 days in static culture. This culture strategy can also promote overall cell growth using the Xuri™ Cell Expansion System W25 (GE Healthcare). The protocol described here can be easily implemented in a T cell expansion workflow to ensure optimal viability and total fold expansion.

## Methods



**Figure 1.** T Cell Isolation, Activation, and Expansion Workflow

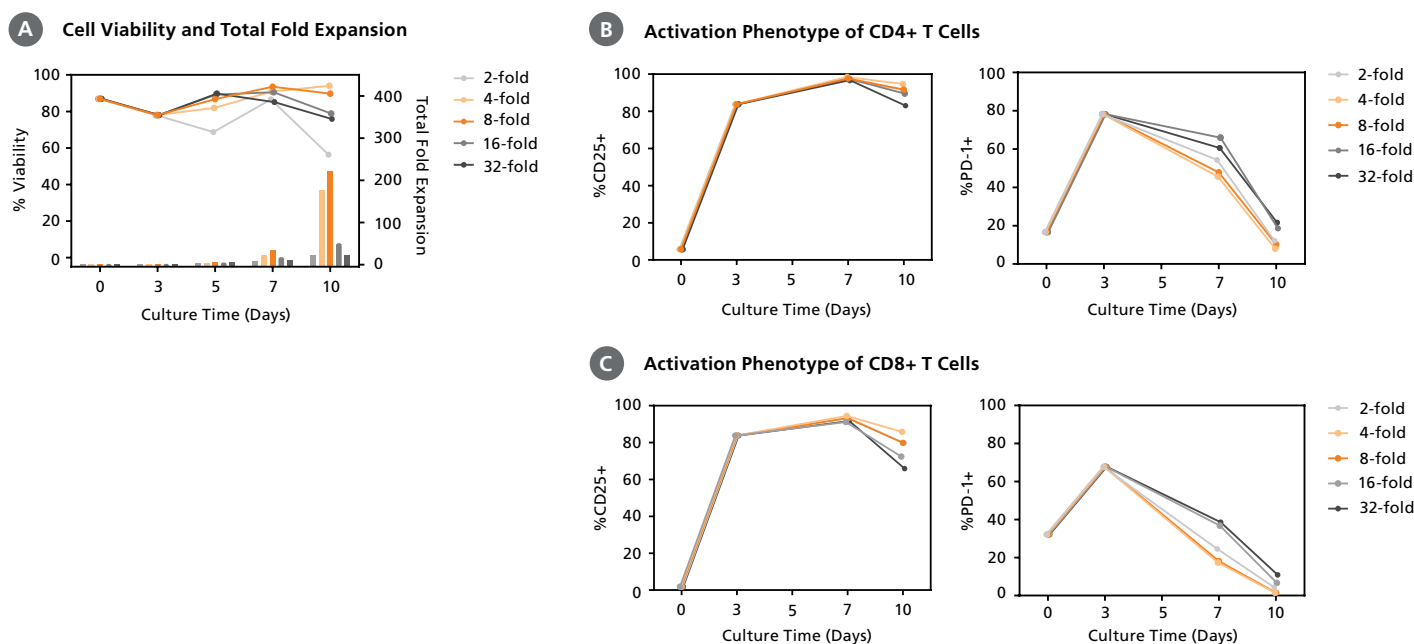
Purified T cells were seeded at  $1 \times 10^6$  cells/mL in ImmunoCult™-XF T Cell Expansion Medium (Catalog #10981) supplemented with 10 ng/mL Human Recombinant IL-2 (rhIL-2). ImmunoCult™ Human CD3/CD28/CD2 or CD3/CD28 T Cell Activators (Catalog #10970 and 10971, respectively) were added at 25  $\mu$ L/mL on day 0. On days 3, 5, 7, and/or 10, cells were passaged according to Figure 1. Cell count and viability assessment were conducted using Nucleocounter® NC-250™ (ChemoMetec), and flow cytometry was performed using CytoFLEX™ S (Beckman Coulter).

To investigate the effects of early cell dilution on T cell expansion, T cells were activated with ImmunoCult™ Human T Cell Activator in ImmunoCult™-XF T Cell Expansion Medium supplemented with rhIL-2 at a cell density of  $1 \times 10^6$  cells/mL. On day 3, T cell culture volume was increased by 2-, 4-, 8-, 16-, or 32-fold by the addition of fresh ImmunoCult™-XF T Cell Expansion Medium supplemented with rhIL-2. On days 5 and 7, T cell culture volume was increased by 4-fold. Where indicated, T cells were transferred into a 2L Xuri™ Cellbag™ Bioreactors, and maintained in the Xuri™ Cell Expansion System W25 on day 5. T cells were harvested for analysis on day 10 or 11 as indicated.

## Results

### Effect of Early Cell Dilution on Human T Cell Expansion

To investigate the effect of cell density on human T cell expansion, we activated T cells on day 0 using ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator or ImmunoCult™ Human CD3/28 T Cell Activator. On day 3, we increased the total culture volume by 2-, 4-, 8-, 16- or 32-fold, and determined the effects on total expansion. The 4- and 8-fold culture volume increases resulted in the highest total fold expansion and cell viability without any significant effects on the activation phenotype of expanded cells (Figure 2).



**Figure 2. Total Cell Expansion is Affected by the Culturing Protocol Three Days Following Initial T Cell Activation**

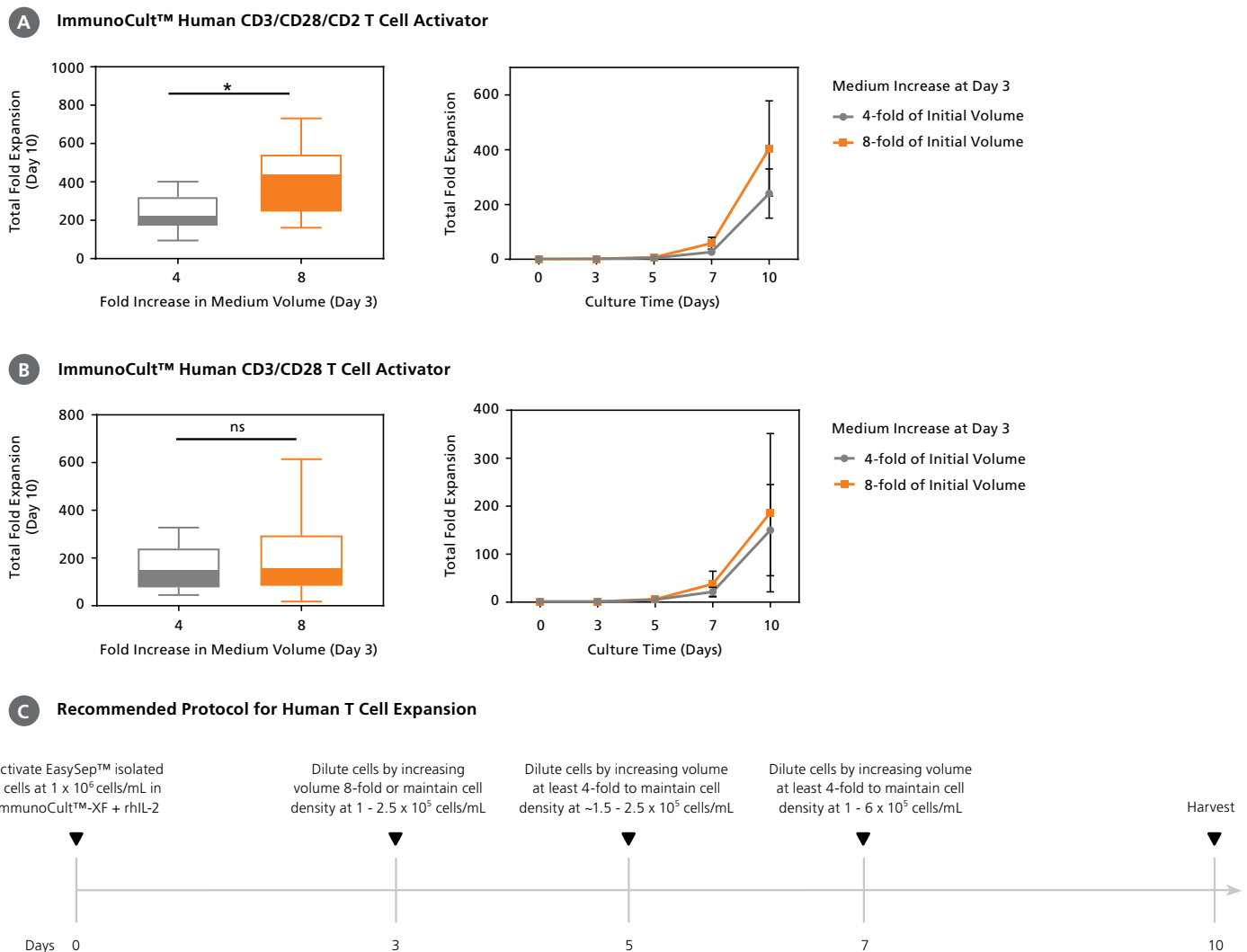
(A) Optimal viable T cell expansion at day 10 was observed with 4- and 8-fold culture volume increases on day 3 of culture. Total fold expansions of 176 and 222 were observed in 4- and 8-fold culture volume increases, respectively. (B) CD4+ and (C) CD8+ T cell subsets had subtle differences in activation marker expression (CD25 and PD-1) that did not correlate with fold expansion or the subculture protocol on day 3 (data shown for one representative donor).

## Comparison of T Cell Activators

The 8-fold volume increase resulted in significantly higher cell expansion compared to the 4-fold volume increase for cells activated using ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator (Figure 3A). On the contrary, the 8-fold volume increase did not cause a statistically significant increase in expansion compared to 4-fold volume increase for cells activated using ImmunoCult™ Human CD3/CD28 T Cell Activator (Figure 3B).

## Recommended Human T Cell Expansion Protocol

The findings above have led us to revise our human T cell expansion protocol with optimized cell dilutions (Figure 3C). It is optimal to dilute cells by increasing volume 8-fold or maintaining cell density at  $1 - 2.5 \times 10^5$  cells/mL at day 3 post-activation. We further dilute the expanding cells by increasing culture volume 4-fold at days 5 and 7 post-activation.



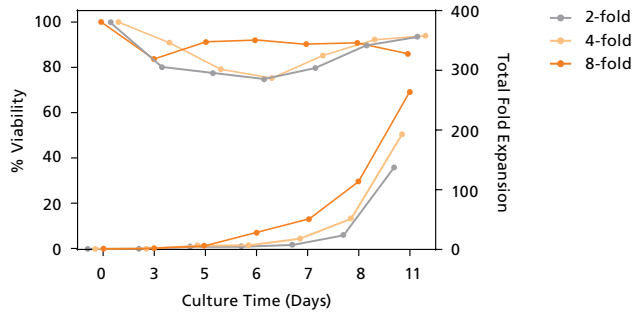
**Figure 3.** Comparison of ImmunoCult™ Human T Cell Activators with Optimal Culture Protocols

T cells were stimulated on day 0 using ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator or ImmunoCult™ Human CD3/CD28 T Cell Activator in ImmunoCult™-XF T Cell Expansion Medium supplemented with rhIL-2 in static culture. Total culture volumes were increased by 4- or 8-fold using fresh medium on day 3. Total culture volumes were further increased by 4-fold on days 5 and 7 until day 10 of culture. Cumulative fold expansion during culture and total fold expansion at day 10 were analyzed relative to the initial cell seeding number. (A) With ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator, increasing the culture volume by 8-fold on day 3 resulted in high cell growth and an overall  $405 \pm 174$  total fold expansion (mean  $\pm$  SD,  $n = 14$ ). Increasing the culture volume by 4-fold on day 3 resulted in a total fold expansion of  $240 \pm 90$ . ( $*p < 0.0001$ , paired t-test). (B) With ImmunoCult™ Human CD3/CD28 T Cell Activator, increasing the culture volume by 8-fold or 4-fold resulted in total fold expansions of  $150 \pm 95$  and  $186 \pm 165$ , respectively (mean  $\pm$  SD,  $n = 14$ ) (ns indicates no significant difference, paired t-test). (C) The recommended human T cell expansion protocol with ImmunoCult™ reagents.

## Human T Cell Expansion Using the Xuri™ Cell Expansion System

Obtaining billions of T cells for cell therapy requires large volumes of cells to be expanded in a controlled environment. The optimized T cell expansion protocol above can be adapted for use with the Xuri™ Cell Expansion System W25 for T cell expansion in a closed system for cell therapy manufacturing.

T cells were initially seeded in cell culture flasks as outlined in Figure 1. On day 3 of culture, fresh medium was added to increase the total culture volume by 2-, 4-, or 8-fold. Cells were then transferred into a 2 L Xuri™ Cellbag™ Bioreactors on day 5. We found that the 8-fold culture volume increase on day 3 resulted in the highest total fold expansion (Figure 4).



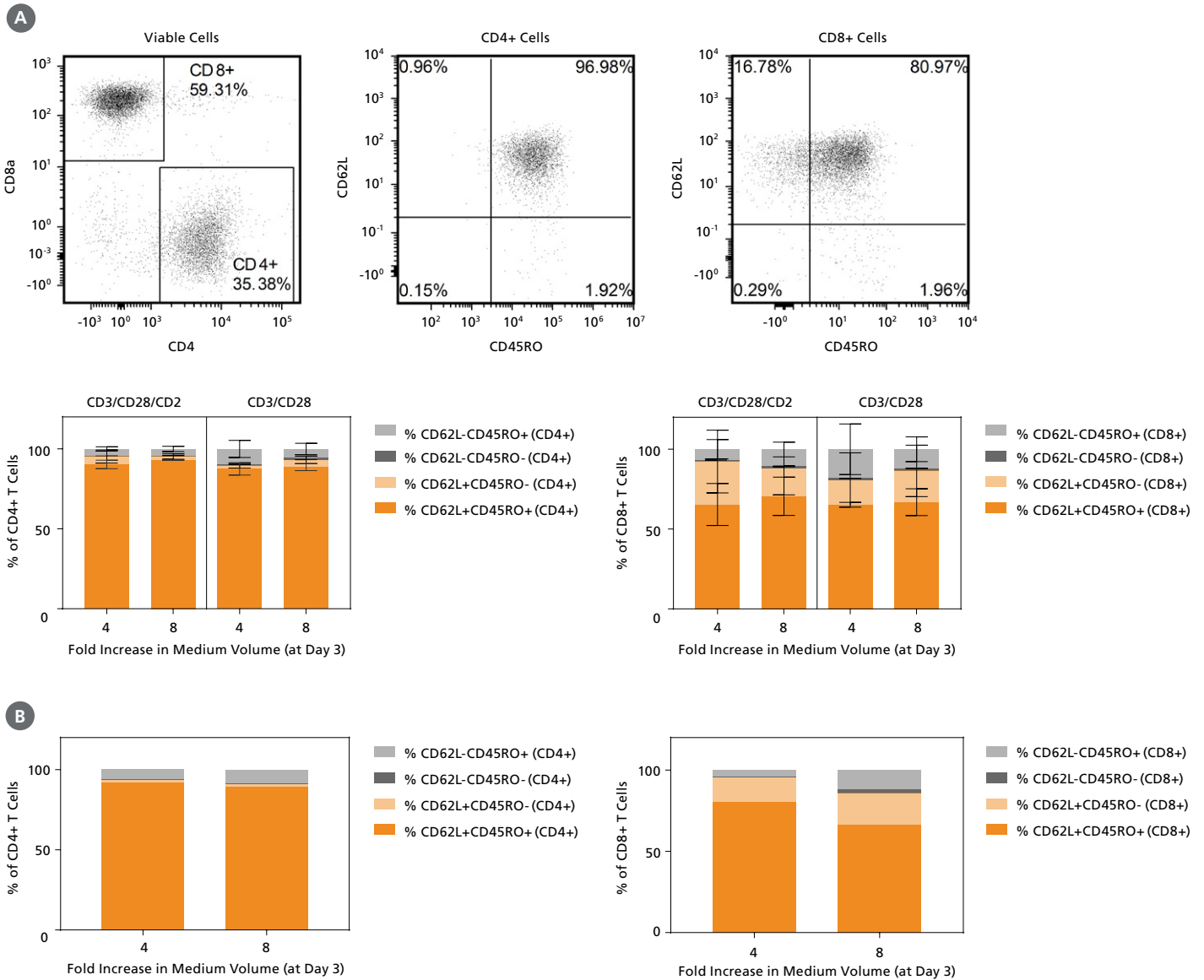
**Figure 4. T Cell Expansion in Xuri™ Cell Expansion System W25 Also Benefits from Early Cell Dilution for Optimal T Cell Expansion**

Purified T cells were seeded at  $1 \times 10^6$  cells/mL in cell culture flasks and stimulated with the ImmunoCult™ Human CD3/CD28 T Cell Activator. On day 3 of culture, fresh medium was added to increase the total culture volume by 2-, 4-, and 8-fold. On day 5 of culture, the cells were transferred to 2 L Xuri™ Cellbag™ Bioreactors. Perfusion with fresh medium was initiated on day 8 as outlined in Figure 1. Following 11 days of culture, the highest total fold expansion (263) was observed with the 8-fold culture volume increase on day 3. Viability was similar across the different cell dilutions.

## Phenotype of Expanded T Cells

The T cell phenotype plays a critical role in the efficacy of cellular therapy products in addition to cell number. For example, studies in mouse models suggest that minimally differentiated T cells (naïve T cells, stem cell memory T cells, or central memory T cells) exhibit superior antitumor activity compared to highly differentiated T cell subsets.<sup>1</sup>

The majority of T cells expanded using ImmunoCult™ reagents in static culture alone or in the Xuri™ Cell Expansion System had a central memory phenotype as determined by their co-expression of CD62L and CD45RO (Figure 5).



**Figure 5.** The Majority of Expanded T Cells had a Central Memory T Cell Phenotype (CD62L+CD45RO+) Following Stimulation and Expansion with ImmunoCult™ Reagents

Purified T cells were stimulated with ImmunoCult™ Human T Cell Activators on day 0 and expanded for 10 days in ImmunoCult™-XF T Cell Expansion Medium supplemented with rhIL-2. Summary of the resulting subset composition in CD4+ or CD8+ T cells using a (A) static culture alone (n = 2) with both ImmunoCult™ Human T Cell Activators (the representative FACS plots show T cells stimulated with ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator) or (B) GE Xuri Cell Expansion System W25 (n = 1) with ImmunoCult™ Human CD3/CD28 T Cell Activator. There are subtle differences in phenotype marker expression with various activators and expansion protocols.

## Discussion

The implication of having an optimized method to produce T cells for cell therapy is significant. As described in this Technical Bulletin, we have identified cell density following T cell activation as a key factor in optimizing T cell expansion.




We show that maintaining T cells at lower cell densities during early T cell expansion improves T cell growth and viability. Our study identifies day 3 as a key timepoint for culture volume adjustment when optimizing T cell expansion protocols. At day 3 post-activation, cultured T cells display high levels of cell activation, as indicated by the expression of CD25 and PD-1, but have not significantly expanded (Figure 2). We speculate that lowering cell density at this time point improves T cell expansion by increasing the amount of available nutrients and space per cell after they have received their activation signals. On the other hand, lowering cell density too much may reduce the concentration of autocrine survival factors or cell-cell contact necessary to support optimal fold expansion and cell viability.

One variable that needs to be taken into account when researchers optimize their cell expansion protocol is genetic modification. Producing T cells for therapy often requires genetic modification of the T cells, which can be achieved using viral vectors<sup>2</sup> or gene-editing nucleases.<sup>3</sup> How these methods may affect optimal culture dilutions remain to be investigated.

The protocol described here provides a basic framework for testing the effects of other variables that can affect T cell expansion, including starting cell populations (T cell subsets), cytokines, and growth factors. The optimal culture volume increases will vary depending on these variables, so we recommend that researchers find the culture volume increases that work best with their specific protocols. Ultimately, having a well-validated, fully optimized protocol can contribute to solving some of the major challenges of T cell therapy, including lack of standardization.

## T Cell Isolation, Activation, and Expansion Reagents

Optimize your human T cell therapy research and manufacturing with the following isolation, activation, and expansion reagents.

	PRODUCT	CATALOG #	DESCRIPTION
Isolation	 <p>EasySep™ Release Human CD3 Positive Selection Kit</p>	17751	<ul style="list-style-type: none"> <li>Isolate up to 99% pure CD3<sup>+</sup> cells free of magnetic particles in under 30 minutes</li> <li>Obtain highly viable, functional cells without the need for columns</li> </ul>
Activation	 <p>ImmunoCult™ Human CD3/CD28 T Cell Activator</p> <p>ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator</p>	10971 10970	<ul style="list-style-type: none"> <li>Soluble reagents for robust activation and expansion without magnetic beads</li> <li>Not exclusively licensed for use in T cell immunotherapy manufacturing</li> </ul>
Expansion	 <p>ImmunoCult™-XF T Cell Expansion Medium</p>	10981	<ul style="list-style-type: none"> <li>Rapidly expand T cells without the use of serum</li> <li>Consistent expansion functional human T cells</li> </ul>

These products are designed for cell therapy research applications following the recommendations of USP <1043> on Ancillary Materials.

Contact us to qualify these reagents under an approved investigational new drug (IND) or clinical trial application (CTA).

Learn more at [www.stemcell.com/t-cell-therapy](http://www.stemcell.com/t-cell-therapy)

## References

1. Gattinoni L et al. (2011) A human memory T cell subset with stem cell-like properties. *Nat Med* 17(10): 1290–7.
2. Li G et al. (2017) Gammaretroviral production and T cell transduction to genetically retarget primary T cells against cancer. *Methods Mol Biol* 1514: 111–8.
3. Osborn MJ et al. (2016) Evaluation of TCR gene editing achieved by TALENs, CRISPR/Cas9, and megaTAL nucleases. *Mol Ther* 24(3): 570–81.

Copyright © 2018 by STEMCELL Technologies Inc. All rights reserved including graphics and images. STEMCELL Technologies & Design, STEMCELL Shield Design, Scientists Helping Scientists, EasySep, and ImmunoCult are trademarks of STEMCELL Technologies Canada Inc. Cellbag and Xuri are trademarks of General Electric Company or one of its subsidiaries. CytoFlex is a trademark of Beckman Coulter Inc. NucleoCounter is a registered trademark, and NC-250 is a trademark of ChemoMetec A/S. All other trademarks are the property of their respective holders. While STEMCELL has made all reasonable efforts to ensure that the information provided by STEMCELL and its suppliers is correct, it makes no warranties or representations as to the accuracy or completeness of such information.

STEMCELL TECHNOLOGIES INC.'S QUALITY MANAGEMENT SYSTEM IS CERTIFIED TO ISO 13485. PRODUCTS ARE FOR RESEARCH USE ONLY AND NOT INTENDED FOR HUMAN OR ANIMAL DIAGNOSTIC OR THERAPEUTIC USES UNLESS OTHERWISE STATED.