Freezing of T cells using the VIA Freeze system

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Cell-based therapies have proven themselves as effective treatments for some of today's toughest diseases. But as these therapies race towards commercialization, questions have arisen on how to scale their manufacture. Cryopreservation is near the end of an intensive and complex manufacturing process. This step is critical, because some of the greatest technical and financial risks are incurred here. Typical workflows use passive freezers or liquid nitrogen. However, these approaches are plagued by safety risks and variability. GE Healthcare Life Sciences' VIA Freeze system was evaluated against both techniques. *Ex vivo* expanded human T cells preserved with all systems showed comparable recovery, expansion, and phenotype after thawing. These results suggest that VIA Freeze system is a viable alternative to conventional freezing approaches, with considerable advantages.

Fig 1. VIA Freeze Quad (right) system was evaluated against current best practices for freezing human T cells. The VIA Freeze series also includes VIA Freeze Research (left) and VIA Freeze Duo (middle) systems.

Introduction

Autologous T cell therapies are no longer limited to small, single-site trials. National and international demand is growing, bringing with it the demand for rapid scaling of the manufacturing. But there is little margin for error, because any variables in the end product could result in a potentially hazardous change to the therapy's potency.

Cryopreservation is a critical tool for quality control and transport of the cells. Liquid nitrogen (LN_2)-based controlled-rate freezers have long been used to freeze clinical-grade T cells. Cells are frozen in either cryogenic vials or bags, using LN_2 to cool them at -1°C/min to the desired temperature. Cryopreserved cells can then be transported or stored long term. Unfortunately, the use of liquid nitrogen creates a safety risk of oxygen depletion in the room where it is used. Therefore, appropriate infrastructure and safety systems must be installed within a clean room environment. This can be expensive and cumbersome. An alternative approach is to use a traditional -80°C freezer or dry ice locker in conjunction with passive containers. These containers modify the thermal transfer process, thereby slowing down the freezing process to a rate of -1°C/min. The challenges here are reproducibility and traceability The various products on the market also struggle to meet current good manufacturing practice (cGMP) guidelines for validation and reproducibility. Because alternatives are lacking, both LN_2 and passive containers are currently used to freeze a variety of cell types, including stem cells, primary cells, and immortalized cell lines.

The VIA Freeze series is a modern solution to the rise of cell-based therapies (Fig 1). These freezers simplify the cell manufacturing workflow and are designed for use in clean rooms and cGMP compliant facilities. By bypassing the need for liquid nitrogen, VIA Freeze systems have no oxygen depletion or air contamination issues. Their small footprint allows systems to sit on a standard laboratory bench. There, they can be easily loaded, unloaded, and cleaned using the quick-release sample holders. VIA Freeze units support multiple cryostorage methods, such as straws, vials, and bags. These freezers include secure data logging, programmable cooling protocols, remote user access control, and report generation.

For this study, *ex vivo* expanded T cells were frozen using three different approaches. A VIA Freeze workflow, a passive container in -80°C freezer, and an LN_2-based controlled- rate freezer were evaluated. After thawing, cell quality attributes (recovery, viability, and phenotype) were analyzed and compared.

Materials and methods

Expansion of T cells in Cellbag™ bioreactors

Briefly, fresh density gradient-isolated human peripheral blood mononuclear cells (PBMCs) were obtained from three donors (netCAD Blood4Research). PBMCs were cultured in T225 flasks at 1 × 10^6 viable cells/mL in ImmunoCult™-XF (STEMCELL Technologies) supplemented with 1% penicillin-streptomycin (1% P-S, GE Healthcare) and 350 IU/mL of Xuri™ IL-2 (GE Healthcare). T cells were activated with Immunocult Human CD3/CD28/CD2 T Cell Activator (STEMCELL Technologies) at a ratio of 25 µL activator per 1 × 10^6 CD3+ T cells. After 3 d, cultures were counted and maintained at a density of 0.5 × 10^6 cells/mL for an additional 2 d.

After 5 d, cells were transferred to a 2 L Xuri Cellbag bioreactor (GE Healthcare) and expanded on the Xuri Cell Expansion System W25 (GE Healthcare). The system parameters were set at 37°C, a rocking rate of 10 rpm, and a rocking angle of 6 degrees. Cells were maintained at 0.5 × 10^6 cells/mL by adding fresh medium up to 1000 mL total volume. After cell concentrations reached a minimum of 2 × 10^6 cells/mL, perfusion was started. Cells were maintained in culture until day 14.

Freezing of T cells in cryogenic vials and bags

After 14 d of culture, cells were harvested from the Cellbag bioreactor by centrifugation. The cell pellets were resuspended in CryoStor™10 (BioLife Solutions) at 2.5 × 10^6 cells/mL. For each donor, 1 mL of cell suspension was transferred to nine 2 mL cryogenic vials and 20 mL was transferred to six 50 mL cryogenic bags. Three cryogenic vials were loaded into the CoolCell™ Cell Freezing Container (BioCision), which was then placed into a -80°C freezer. Another three cryogenic vials, as well as three cryogenic bags, were loaded into the CryoMed™ Controlled-Rate Freezer (Thermo Fisher Scientific) with a cooling rate of -1°C/min until the temperature reached -80°C. The remaining three cryogenic vials and three cryogenic bags were loaded into the VIA Freeze Quad system with a cooling rate of -1°C/min until the temperature reached -100°C. After freezing, all cells were transferred to LN_2 storage and stored for 1 to 4 wk.

Reactivation of T cells in static culture

Frozen T cells were precisely thawed, washed twice, and cultured in 12-well plates at 1 × 10^6 viable cells/mL in ImmunoCult™-XF supplemented with 1% P-S and 350 IU/mL of Xuri IL-2. After 1 d in static culture, Immunocult Human CD3/CD28/CD2 T Cell Activator was added to the culture at a ratio of 25 µL activator per 1 × 10^6 CD3+ T cells. After 3 d, cells were counted and maintained at 0.5 × 10^6 cells/mL for an additional 4 d.

Live cell recovery and viability

Cell recovery and viability were determined using a NucleoCounter™ NC-200.

Phenotypic analysis

A multicolor flow cytometry assay was used to compare the phenotype of the T cells prethaw and at days 0 and 7 post-thaw. Briefly, 1 × 10^6 cells/mL were stained with CD3-PerCP-Cy™5.5, CD4-V500, CD8-Alexa Fluor™488, CD25-PE, CD57-APC, and CD62L-V450 (all from BD Biosciences). The labeled cells were analyzed on a CytoFLEX™ flow cytometer using CytExpert software, according to the manufacturer's instructions (instrument and software from Beckman Coulter).

Statistics

Results are reported as averages of the technical replicates for each donor, plus or minus their respective standard deviation. 95% confidence intervals were calculated using the CONFIDENCE function in Microsoft® Excel® for the post-thaw fold expansion. The CONFIDENCE function assumes that the sample data are normally distributed with a known standard deviation. The one-way analysis of variance (ANOVA) in Minitab™ 18 was used to determine whether the means of the groups differed for every point of comparison between the freezing methodologies.

Results and discussion

Analysis of T cells frozen in cryogenic vials

The live cell recovery, viability, and expansion potential of T cells frozen in cryogenic vials using CoolCell, CryoMed, and VIA Freeze systems were compared. Day 0 recovery and viability results are presented in Table 1. Comparable viable cell recoveries were obtained across the three systems tested.

Table 1. Recovery of live cells and viability of cells frozen in cryogenic vials using either CoolCell, CryoMed, or VIA Freeze systems on day 0. The values displayed are the means plus-or-minus the standard deviations (three technical replicates for three separate donors). There were no statistically significant differences between group means as determined by one-way ANOVA

	Live cell recovery	Viability
CoolCell	93.0% ± 12.8%	89.0% ± 9.2%
CryoMed	86.7% ± 7.6%	89.3% ± 8.6%
VIA Freeze	91.7% ± 11.7%	88.9% ± 9.0%
p-value	0.444	0.995

A comparison of post-thaw expansion, represented by the achieved cumulative fold-expansion after thawing and reactivation, is presented in Figure 2. Viability remained above 90% for post-thaw expanded cells from all freezing systems (results not shown).

To determine whether the phenotypic profiles of the expanded T cells were similar, the expression patterns of several cell surface markers were compared after 7 d post-thaw expansion. The expression levels of the cell surface markers CD3, CD4, CD8, CD25, CD57, and CD62L on the expanded T cells were equivalent, with most cells showing an activated state based on expression of CD25 (T cells within a single biological replicate showed no statistically different expression levels; results not shown). The proportion of CD4+ and CD8+ T cell populations were also evaluated in the cultures and found to be more donor-dependent than system-dependent (Fig 3). These data indicate that the different freezing or cooling methods have similar effects on T cell subpopulations.

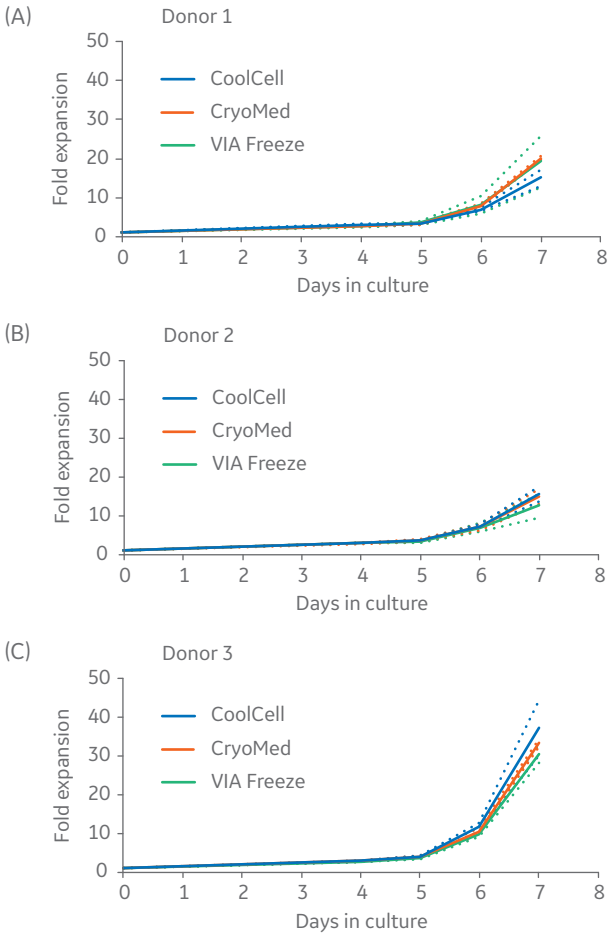

Fig 2. Growth of cells cultured after freezing in cryogenic vials using CoolCell, CryoMed, or VIA Freeze systems for biological replicates (A), (B), and (C). Dotted lines represent the 95% confidence interval of the mean (n = 3 technical replicates for each donor). There were no statistically significant differences between group means as determined by one-way ANOVA.

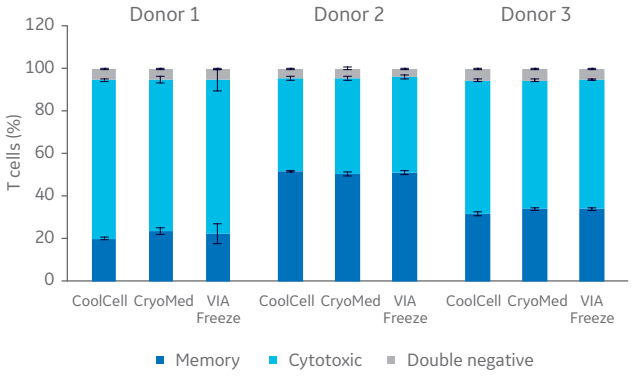

Fig 3. Frequency of CD4+ memory and CD8+ cytotoxic T cells at the end of the culture period for CoolCell, CryoMed, and VIA Freeze systems. Percentages of these populations are shown within the combined lymphocyte/CD3+ populations. Error bars represent the standard deviation of the mean for each biological replicate (n = 3 technical replicates for each donor). There were no statistically significant differences between group means as determined by one-way ANOVA.

Analysis of T cells frozen in cryogenic bags

The VIA Freeze system has the flexibility to freeze cryobags or cryovials. Samples of *ex vivo* expanded T cells frozen in bags were assessed for the same post-thaw attributes as those used in the cryovial study. No difference was observed in the number of viable cells recovered from cells frozen in CryoMed and VIA Freeze (Table 2).

Table 2. Recovery of live cells and viability of cells frozen in cryogenic bags using either CryoMed or VIA Freeze systems on day 0. The values displayed are the means plus-or-minus the standard deviations (three technical replicates for three separate donors). There were no statistically significant differences between group means as determined by one-way ANOVA

	Live cell recovery	Viability
CryoMed	84.9% ± 6.2%	81.8% ± 6.5%
VIA Freeze	80.6% ± 6.5%	81.4% ± 6.6%
p-value	0.171	0.890

To determine whether comparable numbers of expanded cells could be achieved, the cumulative fold expansion after thawing is presented in Figure 4. The average fold expansion growth of peripheral blood T cells was 9-fold for both CryoMed and VIA Freeze systems (average of biological replicates A, B, and C; Fig 4). Viabilities exceeded 90% (results not shown).

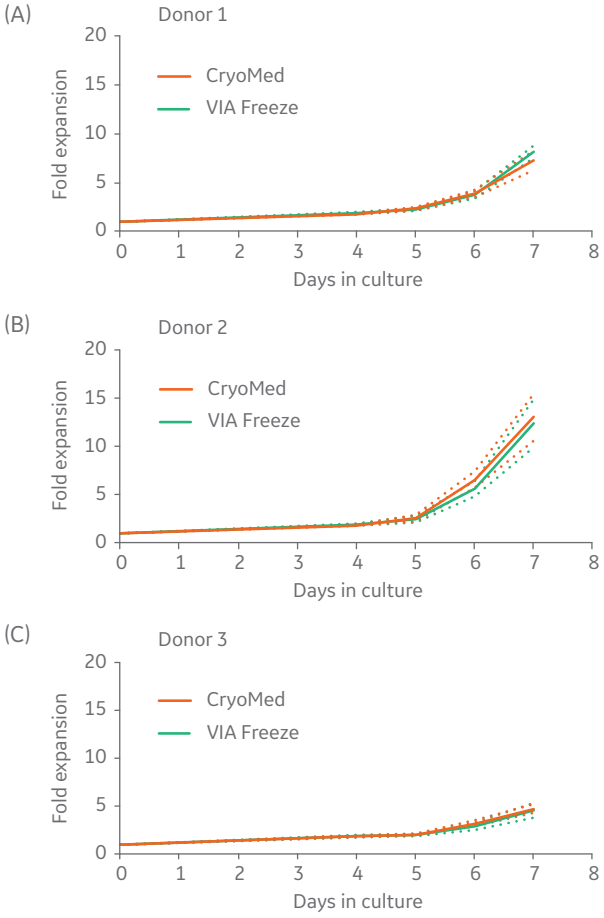

Fig 4. Growth of cells cultured after being frozen in cryogenic bags using either CryoMed or VIA Freeze systems for biological replicates (A), (B), and (C). Dotted lines represent the 95% confidence interval of the mean (n = 3 technical replicates for each donor). There were no statistically significant differences between group means as determined by one-way ANOVA.

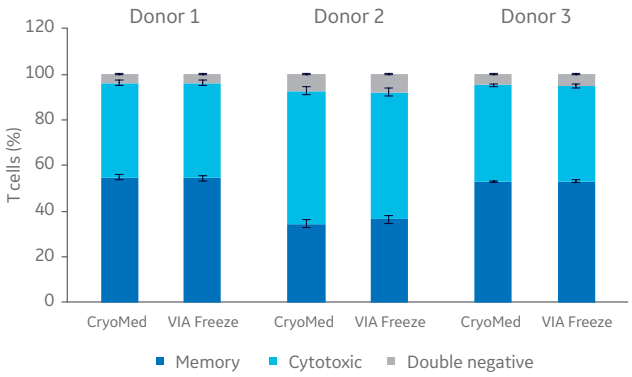

Fig 5. Frequency of CD4+ memory and CD8+ cytotoxic T cells at the end of the culture period for CoolCell, CryoMed, and VIA Freeze systems. Percentages of these populations are shown within the combined lymphocyte/CD3+ populations. Error bars represent the standard deviation of the mean for each biological replicate (n = 3 technical replicates for each donor).

To determine whether the phenotypic profiles of the expanded T cells were similar, the expression patterns of several cell surface markers were compared after 7 d expansion. The expression of the cell surface markers CD3, CD4, CD8, CD25, CD57, and CD62L on the expanded T cells were equivalent, with most cells showing an activated state (T cells within a single biological replicate showed no statistically different expression levels; results not shown). The proportion of CD4+ and CD8+ T cell populations were also evaluated in the cultures and found to be equivalent between freezing methods, although donor variability was noted (Fig 5). There were no statistically significant differences between group means as determined by one-way ANOVA. These data indicate that the different freezing methods have equivalent effects on T cell subpopulations.

Conclusion

Post-thaw evaluation of cryogenic bags showed comparable T cell recovery, viability, and phenotype for CryoMed and VIA Freeze systems. Results for cryogenic vials were comparable across CoolCell, CryoMed, and VIA Freeze systems. The observed differences could be attributed to the different T cell donors, not the technology. This study shows that, in addition to the VIA Freeze system's ease of use, it can deliver results on par with liquid nitrogen-based freezers and passive freezing containers. The VIA Freeze system provides uniform cooling, scalability, and digital logging for compliance with cGMP guidelines.

Acknowledgements

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Ordering information

Product	Product code
HyClone Penicillin-Streptomycin 100X Solution	SV30010
Xuri IL-2	29062789
VIA Freeze Quad	VFQ_30010
Xuri Cell Expansion System Cellbag Perfusion, pH and DO, 2 L	29105498
Xuri Cell Expansion System W25	29064568

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GE Healthcare Bio-Sciences AB, Björkgatan 30, 751 84 Uppsala, Sweden
GE Healthcare Bio-Sciences Corp., 100 Results Way, Marlborough, MA 01752, USA
GE Healthcare Europe GmbH, Munzinger Strasse 5, D-79111 Freiburg, Germany
GE Healthcare Japan Corp., Sanken Bldg., 3-25-1, Hyakunincho Shinjuku-ku, Tokyo 169-0073, Japan
GE Healthcare UK Ltd., Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK
For local office contact information, visit gelifesciences.com/contact.

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