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Efficacy of a liquid nitrogen-free electronic controlled rate freezer for the cryopreservation of umbilical cord blood.

Comparison between liquid nitrogen and liquid nitrogen-free controlled-rate freezing for the cryopreservation of cord blood.

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1. Introduction

1.1 Background

Successful cryopreservation is a crucial step for manufacturing and effective clinical delivery of advanced therapy medicinal products (ATMPs) and is therefore an integral part of a commercially robust business model. Liquid nitrogen (LN_2) controlled-rate freezers (CRFs) are well established and have been widely used to cryopreserve cellular products such as haematopoietic stem cells (1). However, the use of LN_2 in a controlled environment such as good manufacturing practice (GMP) production areas poses certain challenges. For example, the use of LN_2 can be a source of microbial cross contamination and poses certain serious health risk to operators (2-4). Also, LN_2 cannot be used in cleanrooms where most ATMP manufacturing takes place (Figure 1). There is an alternative to LN_2 CRFs: liquid nitrogen-free, controlled-rate freezers (LN_2-free CRFs), such as the VIA Freeze™ range from Cytiva. Instead, these systems cool samples using conduction cooling, driven by an internal Stirling cryocooler that brings the chamber to cryogenic temperatures with electricity (5). However, there has been little assessment of LN_2-free CRFs using clinical scale materials. In the current study, umbilical cord blood is used as a cellular product starting material to evaluate the efficacy of the VIA Freeze™ Quad system from Cytiva.

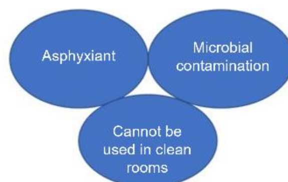

Figure 1: Comparison between LN_2 controlled rate freezing exemplified by the Kryo 560 from Planer PLC, versus LN_2-free controlled rate freezing exemplified by the VIA Freeze™ Quad system from Cytiva.

1.2 Purpose

The purpose of this fieldwork was to evaluate the efficacy of the LN_2-free CRF from Cytiva (VIA Freeze™ Quad system) by comparing it to a classical LN_2 CRF from Planer PLC (Kryo 560) for the cryopreservation of cord blood units (CBUs). Ultimately, the aim was to determine if the VIA Freeze™ Quad system can be used within NHSBT to cryopreserve CBUs and to improve NHSBT's services in the future. To assess the quality of the cryopreserved samples, various essential parameters were analysed such as recovery and viability of CD34+ cells, as well as colony forming units (CFU) and total nucleated cells (TNC).

1.3 **Overview of the study**

Research and development-grade, volume-reduced umbilical cord blood units were used as starting material. Samples were mixed with a cryoprotective solution, split into two equal volumes, and transferred to freezing bags and vials; for each sample, at least one bag and one vial were cryopreserved in the LN_2 CRF and the other ones in the LN_2-free CRF, using set freezing profiles. Pre- and post-thaw analyses were performed to identify any influence of the cryopreservation method. An overview of the sample plan and methods used in the current study are represented in Figure 2; full details are described in the methods section.

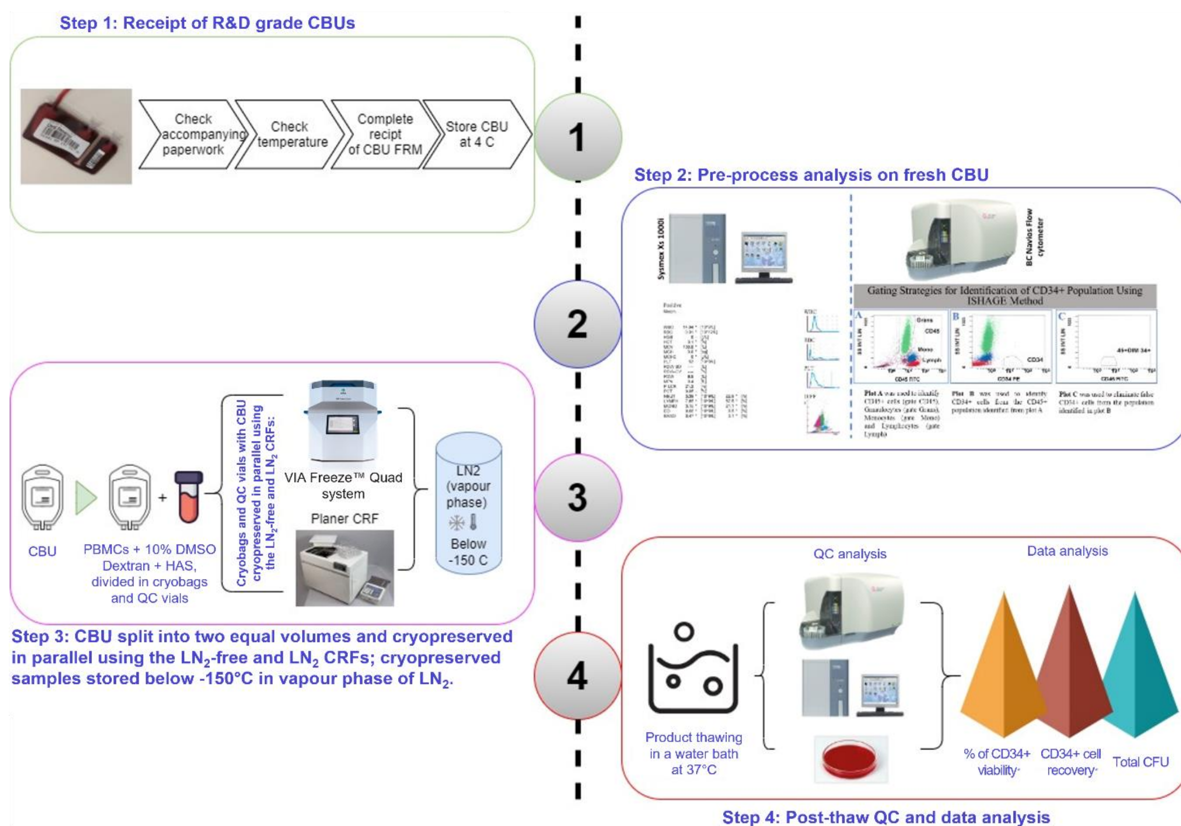

Figure 2: Schematic overview of the sampling plan and methodology used in this study. (1) Cord blood units (CBUs) were stored at 4°C on receipt and (2) pre-process analysis of full blood count, CD34+ count and viability were performed. (3) CBUs were split into two equal volumes and cryopreserved in parallel using a liquid nitrogen-free controlled-rate freezer (LN_2-free CRF) and a liquid nitrogen controlled-rate freezer (LN_2 CRF; using the Kryo 560 system from Planer PLC) in bags as well as quality control (QC) vials. Cryopreserved samples were stored in vapour phase liquid nitrogen below -150 °C for a minimum of two weeks before post-thaw analyses. (4) Thawing was performed by immersing samples in a water bath set at 37°C immediately after removal from storage. The thawed samples were analysed for the recovery of total nucleated cells (TNC) and CD34+ cells, the viability of CD34+ cells and the ability of CD34+ cells to form colonies through the colony-forming units (CFU) assay and ultimately determine any impact of the cryopreservation method.

1.4 Terminology

Term	Comment
CBU	Cord blood unit
7-AAD	7-aminoactinomycin D
CFU, CFU-E, CFU-GM, CFU-GEMM	Colony-forming units, CFU-erythrocyte, CFU-granulocyte/macrophages, CFU-granulocyte/erythrocyte/macrophages/megakaryocyte
CRF	Controlled-rate freezer
DMSO	Dimethyl sulfoxide
HAS	Human albumin solution
HIV	Human immunodeficiency virus
HTLV	Human T-lymphotropic virus
ISHAGE	International Society of Hemotherapy and Graft Engineering
LN_2	Liquid nitrogen
NBL	National bacteriology laboratory
QC	Quality control
RBC	Red blood cell
SEM	Standard error of the mean
TNC	Total nucleated cells
WBC	White blood cell

2. Material and Methods

2.1 Cord blood units (CBUs)

Research and development-grade, volume-reduced, fresh umbilical CBUs (n=6) were obtained from the NHS Blood and Transplant Cord Blood Bank. Informed consent was in place for the cell donations to be used for research and development/service development if not fit for clinical treatment or Haematopoietic Stem Cell (HSC) transplant. All CBUs were microbiology tested at NHS Blood and Transplant Cord Blood Bank and found to be negative for all infectious disease markers (see Appendix 1). This service development project was approved and performed according to the non-clinical issue application number 1282.

2.2 Haematologic cell count

A 500µl sample was transferred from the donation bag to a quality control (QC) tube. Aliquoted samples were analysed using an automated haematology analyser (XS-1000i Sysmex) for determination of TNC, red blood cells (RBC) and white blood cells (WBC) counts.

2.3 Flow cytometry analysis

The phenotype and viability of cell populations were analysed using a Navios flow cytometer (Beckman Coulter). 500µl samples were taken from each CBU bag to determine WBC count using the

haematology analyser. Samples were diluted in 4.5% (wt/v) human albumin solution (HAS; Zenalb 4.5, Bio Products Laboratory) to achieve WBC counts between 1-2×10^7 cells/ml suitable for flow cytometric analysis. To determine the CD34 count and viability, 50µl of sample was stained with 10µl of anti-CD34-PE (Beckman Coulter, #A07776), 10µl of anti-CD45-FITC (Beckman Coulter, #A07782) antibody and 10µl of 7-aminoactinomycin D (7-AAD) dye (Beckman Coulter, #A07704) in duplicate. Stained samples in BD Trucount tubes (BD Biosciences, #340334), were incubated for 15 minutes at room temperature in the dark, thereafter 1ml of lyse solution (Beckman Coulter, #A07799) was added and incubated for a further 5 minutes. The stained cells were gated following guidelines of the International Society of Hemotherapy and Graft Engineering (ISHAGE). CD3 and CD45 positive controls (AQUIOS IMMUNO-TROL, Beckman Coulter), CD34 positive control (CD-Chex Plus, Steck) and negative control (Isotype, Beckman Coulter) were analysed alongside test samples to determine non-specific binding/staining.

2.4 Cryopreservation using LN_2 and LN_2-free controlled-rate freezers

The processed cord blood was transferred to the Cryodoc system (closed processing system) and placed on cool packs (4°C). The syringe of cryoprotectant consisting of 55% DMSO (v/v) and dextran 5% (w/v) (Cryo-sure-DEX40, WAK-Chemie) in HAS was attached to the Cryodoc system (OriGen Biomedical) using a sterile connecting device (Terumo) and cryoprotectant was injected slowly (~1ml/minute) into the cord blood to achieve a 10% DMSO (v/v) concentration. Samples were equally divided and transferred into pre-labelled 50ml CryoMACS® bags (Miltenyi Biotec, #200-074-400) and 2 ml cryovials (Nunc). Bags were fitted inside appropriate metallic cassettes (Thermo Fisher Scientific, #4000610) and subsequently loaded alongside cryovials into the LN_2-CRF (Kryo 560, Planer PLC) and LN_2-free CRF (VIA Freeze™ Quad system, Cytiva with 1000 mL bag SBS adapter, #ASY_30066, and 2x small vial holder SBS adapters, #ASY_30073) for cryopreservation, according to the freezing profiles shown in Table 1. After completion, the cryopreserved samples were transferred to the vapour phase of liquid nitrogen in a 24/7 monitored cryotank (MVE series 1000, Chart Industries) and stored until subjected to post-thaw analyses.

Table 1: Details of the freezing profiles used on the liquid nitrogen controlled rate freezer (LN_2 CRF) versus the LN_2-free CRF (VIA Freeze™ Quad system) for the cryopreservation of cord blood unit samples.

Step	LN_2 CRF freezing profile			LN_2-free CRF freezing profile		
	Cooling rate (°C/min)	Temperature (°C)	Hold time (min)	Cooling rate (°C/min)	Temperature (°C)	Hold time (min)
1		+4	Until samples loaded		+4	Until samples loaded
2	-2	-5		-2	-5	
3	-1	-40		-1	-40	
4	-5	-160		-2	-100	
5			Until samples removed			Until samples removed

2.5 Sterility testing

Sterility testing of CBU samples was performed using Bac T/ALERT system under aerobic and anaerobic conditions. In brief, 2 ml of pre-processed CBU sample was inoculated into the Bac T/ALERT bottles and sent to National Bacteriology Laboratory (NBL) at NHSBT-Manchester for incubation and reading. All CBU samples were tested for mandatory microbiology markers including – but not limited to – human immunodeficiency virus (HIV) HIV-I/II, Cytomegalovirus, Hepatitis C virus, human T-lymphotropic virus (HTLV) HTLV-I/&II, Hepatitis B virus, Syphilis and Epstein-Barr virus, as shown in Appendix 1.

2.6 Thawing

CBU samples (bags and vials) were stored in the vapour phase of liquid nitrogen for a minimum of two weeks before performing post-thaw analyses. Samples were removed from storage and immediately fully submerged (for bags) or half dipped (vials) in a 37ºC temperature-controlled water bath (Grant, Thermo Fisher Scientific), where they were gently agitated. Samples were removed from the water bath once no more visible ice was present and post thaw analyses were performed immediately thereafter.

2.7 Colony-forming unit assays

The colony forming ability of cryopreserved CBU samples (vials and bags) was assessed by CFU assays. Thawed samples were transferred to sterile sample tubes. HAS was added dropwise to cells to perform a 1:2 dilution prior to CD34+ flow analysis to calculate the plating concentration of CD34+ cells. The CFU assay was set up according to NHSBT's standard operating procedure. In brief, a cell suspension adjusted to the desired concentration of 2.5×10^4 cells/ml of CD45/white cell was mixed with MethoCult™ gel (Stemcell Technology), dispensed in gridded Petri culture dishes and incubated for 14 days at 37ºC in a humidified incubator set at 5% CO_2. Petri dishes were removed on the day of counting; CFUs from granulocytes/macrophages (CFU-GM), granulocytes/ erythrocytes/ macrophages/ megakaryocytes (CFU-GEMM) and erythrocytes (CFU-E) were counted under the microscope. Furthermore, the CFU counts were analysed against the number viable CD34+ cells plated to calculate percentages of CD34+ cells that are able to grow into colonies.

2.8 Statistical analysis

Data collected from the full blood count, flow cytometry analyses and CFU assays were analysed using GraphPad prism (version 9). Values shown are means ± standard error of the mean (SEM) from six different donations cryopreserved independently (n=6). After checking that distributions were normally distributed (Shapiro-Wilk test), analyses of variance (ANOVAs) with Tuckey's post-hoc pairwise comparisons of means were performed to compare pre-freeze and post-thaw data of samples cryopreserved in either CRF, in bags and in vials; p-values lower than 0.05 were considered significant.

3. Results

3.1 Total nucleated cells

Full blood count was performed on receipt of CBUs (i.e., pre-freeze), as well as post-thaw on samples cryopreserved in bags and in vials to determine TNC (Figure 3). TNC counts were not significantly different between any of the experimental conditions (p-values > 0.186). In particular, samples cryopreserved in the LN_2-free CRF showed mean post-thaw TNC counts (± SEM) of 63.7 ± 4.3 $\times 10^7$ in bags and 66.7 ± 2.7 $\times 10^7$ in vials, while those cryopreserved in the LN_2 CRF reached 63.6 ± 3.8 $\times 10^7$ and 64.4 ± 4.2 $\times 10^7$, respectively, versus pre-freeze values of 75.2 ± 3.0 × 10^7. The full dataset can be found in Appendix 2, Tables S1 and S2.

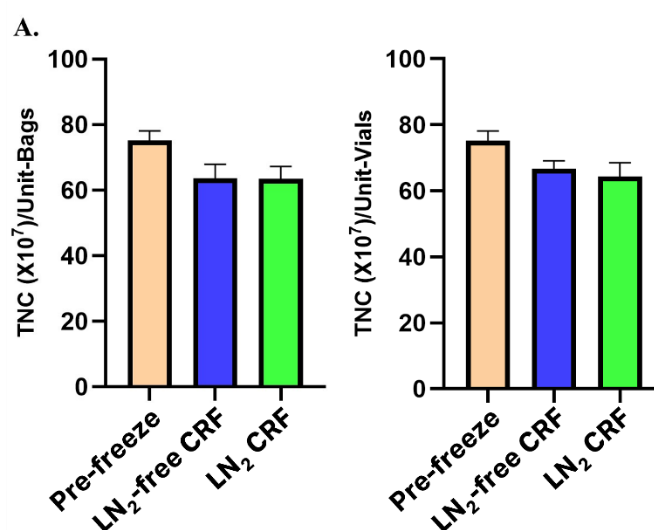

Figure 3: Total nucleated cell (TNC) counts in samples pre-freeze and post-thaw following cryopreservation in bags (A) and in vials (B) using the liquid nitrogen-free controlled rate freezer (LN_2-free CRF) and the LN_2 CRF (n=6; means ± SEM). There was no significant difference between experimental conditions (p-values > 0.186).

3.2 CD34+ cell recovery and viability

CD34+ cell counts and viability were evaluated by flow cytometry. Single platform ISHAGE methodology gating strategy was followed for identification and enumeration of CD34+ population of cells in samples as shown in Figure 4.

No significant difference between any of the experimental conditions was observed on CD34+ cell recovery (p-values ≥ 0.761), although a decline could be observed between pre-freeze and post-thaw values. There was an average ± SEM of 2.4 ± 0.4 $\times 10^6$ CD34+ cells/µl pre-freeze, versus recovered post-thaw values of 1.8 ± 0.3 $\times 10^6$ CD34+ cells/µl from samples cryopreserved in bags in both the LN_2-free CRF and LN_2 CRF, and 1.9 ± 0.4 and 1.8 ± 0.4 $\times 10^6$ cells/µl in vials, respectively (Figure 5A). The full dataset can be found in Appendix 2, Tables S3 and S4.

Similarly, there were no significant differences in CD34+ cell viability between pre-freeze and post-thaw values when samples were cryopreserved in bags, regardless of the CRFs used, with 97.5 ± 0.4%

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CD34+ mean ± SEM cell viability pre-freeze, and 93.8 ± 1.7% and 92.7 ± 1.2% in bags post-thaw following cryopreservation in the LN_2-free CRF and LN_2 CRF, respectively (p-values ≥ 0.493; Figure 6A). Samples in vials also displayed post-thaw CD34+ cell viability that were not significantly different whether cryopreserved in the LN_2-free CRF or LN_2 CRF with 79.6 ± 2.8% and 74.6 ± 3.1%, respectively (p-value 0.460; Figure 6B). However, these figures were significantly lower than pre-freeze values (p-values <0.0001; Figure 6B), as well as post-thaw values of samples cryopreserved in bags (p-values ≤ 0.001). The full dataset can be found in Appendix 2, Tables S5 and S6.

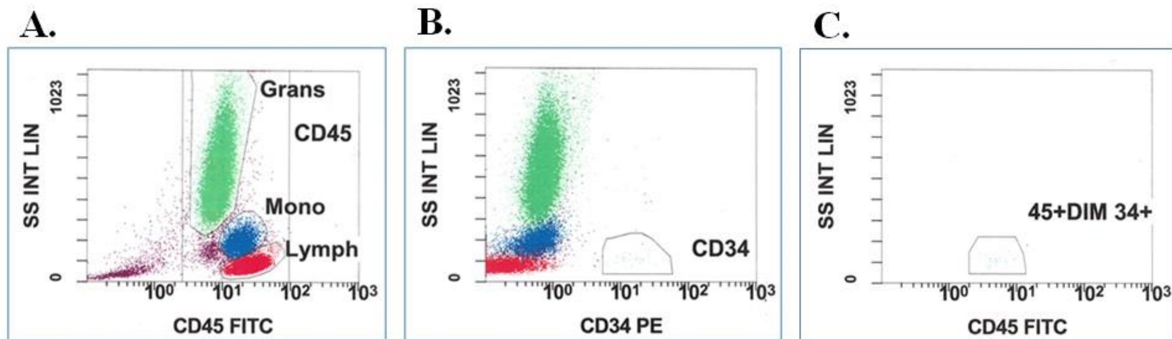

Figure 4: Gating strategies for identification of CD34+ population using ISHAGE method. (A) Plot A was used to identify CD45+ cells (gate CD45), granulocytes (gate Grans), monocytes (gate Mono) and lymphocytes (gate Lymph). (B) Plot B was used to identify CD34+ cells from the CD45+ population identified from plot A. (C) Plot C was used to eliminate false CD34+ cells from the population identified in plot B.

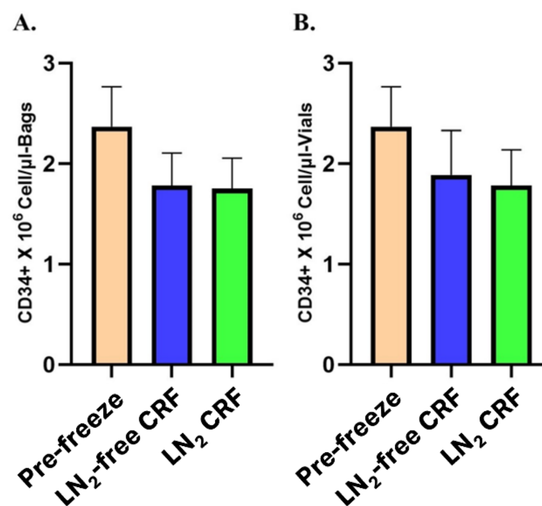

Figure 5: CD34+ cell counts in samples pre-freeze and post-thaw following cryopreservation in bags (A) and in vials (B) using the liquid nitrogen-free controlled rate freezer (LN_2-free CRF) and the LN_2 CRF (n=6; means ± SEM). There was no significant difference between experimental conditions (p-values > 0.761).

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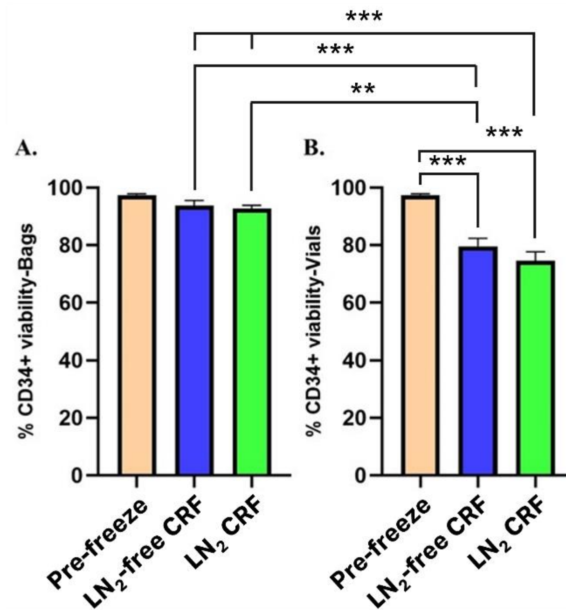

Figure 6: CD34+ cell viability in samples pre-freeze and post-thaw following cryopreservation in bags (A) and in vials (B) using the liquid nitrogen-free controlled rate freezer (LN_2-free CRF) and the LN_2 CRF (n=6; means ± SEM). There was no significant difference between both CRFs, nor between samples cryopreserved in bags and pre-freeze values (p-values > 0.493), however post-thaw CD34+ cell viability of samples cryopreserved in vials was significantly lower compared to pre-freeze values and post-thaw values of samples cryopreserved in bags. A star denotes where pairwise comparisons were significantly different (* 0.01 < p-values ≤ 0.05; ** 0.001 < p-values ≤ 0.01; *** p-values ≤ 0.001).

3.3 Colony-forming unit (CFU) assays

CFU assays were performed on samples post-thaw to assess the functionality of CD34+ cells in cryopreserved CBUs. CFU-GM, CFU-GEMM and CFU-E were identified, counted and summed up to calculate total colony forming units per 10^5 nucleated cells. Means ± SEM of 115.9 ± 21.8 and 92.9 ± 14.9 CFUs were enumerated in bags cryopreserved in the LN_2-free CRF and the LN_2 CRF, respectively (Figure 7A). In vials, total CFUs amounted to 78.5 ± 15.9 and 40.4 ± 42.8 in samples cryopreserved in the LN_2-free CRF and the LN_2 CRF, respectively (Figure 7B). Among these values, the ANOVA revealed that the lower number of colonies formed from samples cryopreserved in vials in the LN_2 CRF as compared to samples cryopreserved in bags in the LN_2-free CRF was significant (p-value = 0.032). Differences between other pairs of comparisons did not appear to be significant (p-values ≥ 0.188). The full dataset can be found in Appendix 2, Tables S7 and S8.

Next, percentages of CD34+ cells able to grow into colonies were investigated: 43.5 ± 4.3% and 36.8 ± 2.8% of CD34+ cells growing into colonies from bag samples cryopreserved in the LN_2-free CRF and the LN_2 CRF, respectively (Figure 8A), and 34.2 ± 4.5% and 18.3 ± 3.9% in vials, respectively (Figure 8B). The latter experimental condition (vials cryopreserved in the LN_2 CRF) appeared to be significantly different from all 3 others, with p-values ≤ 0.016, while differences between other pairs of comparisons did not appear to be significant (p-values ≥ 0.363). The full dataset can be found in Appendix 2, Tables S9 and S10.

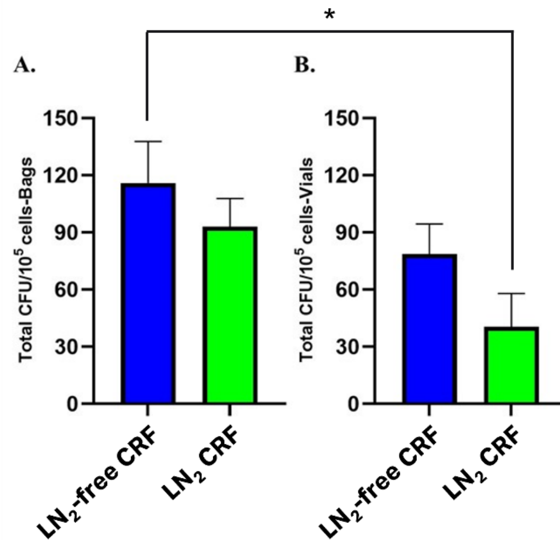

Figure 7: Total colony forming units (CFU) post-thaw in samples cryopreserved in bags (A) and in vials (B) using the liquid nitrogen-free controlled rate freezer (LN$_2$-free CRF) and the LN$_2$ CRF (n=6; means ± SEM). A star denotes where pairwise comparisons were significantly different (* 0.01 < p-values ≤ 0.05; ** 0.001 < p-values ≤ 0.01; *** p-values ≤ 0.001).

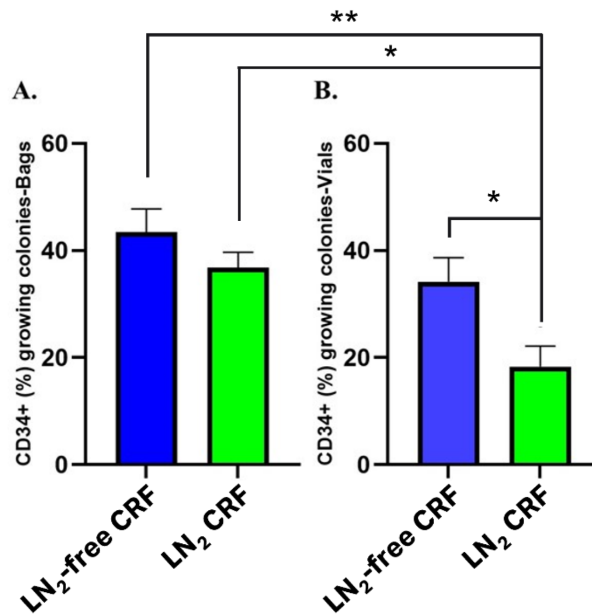

Figure 8: Percentage of CD34+ cells growing into colonies (%) post-thaw in samples cryopreserved in bags (A) and in vials (B) using the liquid nitrogen-free controlled rate freezer (LN$_2$-free CRF) and the LN$_2$ CRF (n=6; means ± SEM). A star denotes where pairwise comparisons were significantly different (* 0.01 < p-values ≤ 0.05; ** 0.001 < p-values ≤ 0.01; *** p-values ≤ 0.001).

4. Discussion and Conclusion

Cryopreservation of cell therapy products eases the constraints on the supply chain and management of medicine administration that unfrozen products impose on manufacturers, couriers, and treatment centres. Due to the pandemic, haematopoietic transplant networks worldwide have recently recommended cryopreserving all stem cell products either autologous or allogenic (7-10). Therefore, effective cryopreservation is a critical step in autologous and allogeneic transplantation.

In this study, the efficacy of Cytiva's liquid nitrogen-free controlled rate freezer, the VIA Freeze™ Quad system (LN_2-free CRF) was tested for the cryopreservation of umbilical cord blood. This LN_2-free CRF is unique as it uses an electrically powered Stirling engine cryocooler instead of LN_2 to reach cryogenic temperatures. Therefore, it has a practical advantage over standard LN_2 CRFs with its ease of implementation within the GMP environment.

In current standard practice, CBU needs to achieve a list of required specifications for cryopreservation and clinical use such as TNC ($\geq 5\times10^8$), viability of CD34+ cells (in pre-and post-thaw samples ≥ 85% and ≥ 70%, respectively), and evidence of potency by CFU or other validated potency assays (11). TNC count in CBU samples is one of the main clinically relevant selection criteria as it correlates with time and probability of engraftment and overall survival (12-15). The haematopoietic potential of a CBU is mostly denoted by the cells expressing the CD34+ antigen (11). It has been widely reported that the engraftment of CD34+ cells is directly related to post-transplantation survival and graft-versus-host disease (16-17). In addition to TNC count, CD34+ cell count is considered a criterion for the clinical use of CBU (15,18). The CFU assay is also a widely used *in-vitro* assay to assess the potency of CBU, as studies have demonstrated a link between pre- or post-thaw CFU and engraftment success (19-21).

All samples met the pre-freeze and post-thaw release criteria for TNC counts, CD34+ cells and potency via CFU counts for clinical use (as per EBMT) (11). They also met the NHSBT release criteria of at least 10% of viable CD34+ cells growing into colonies (validated NHSBT internal quality control assessment method). This suggests that cryopreservation of samples in the new LN_2-free CRF is equivalent to the LN_2 CRF, and that samples cryopreserved in vials provide representative information about those cryopreserved in bags, making them suitable for quality control purposes.

However, some parameters did appear lower for samples that were cryopreserved in vials as compared to their bags counterparts. This was particularly evident for CD34+ cell viability, regardless of the CRF used (Figure 6), while only vial samples cryopreserved in the LN_2 CRF were characterised by lower percentages of CD34+ cells able to grow into colonies compared to other cryopreservation conditions (Figure 8). Finally, for total CFUs, the experimental condition leading to the poorest outcome was again vials cryopreserved in the LN_2 CRF, which was significantly different from the condition leading to the highest outcome, that is bags cryopreserved in the LN_2-free CRF (Figure 7). This phenomenon of volume-dependant cellular outcome post-thaw has been observed previously in cord blood samples, in QC tubing segments compared to bulk bags. This was attributed to ice nucleation occurring at higher sub-zero temperatures in samples of lower volume (i.e. QC segments vs bags), and therefore to a higher degree of supercooling, which resulted in poorer cell outcome post-thaw (6). Despite some lower post-thaw parameters in vials compared to bags, QC vials still provide vital information in the estimation of the expected performance of cells in the bag. However, as they

may slightly underestimate the quality of the bag sample, TNC should perhaps be considered as the main release criterion in samples whose QC vial reveal a post-thaw CD34+ cell viability that is slightly below the specification.

When comparing the LN_2-free CRF versus the LN_2 CRF more closely, they did not appear to significantly influence the recovery of TNC post-thaw, nor the recovery and viability of CD34+ cells post-thaw – both for samples cryopreserved in bags and in vials. However, when it comes to total CFUs, post-thaw values were on average higher following cryopreservation in the LN_2-free CRF compared to the LN_2 CRF (in bags as well as in vials), although this was not statistically significant (Figure 7). The same trend was observed with the post-thaw percentage of viable CD34+ cells growing into colonies, and the difference was significant in vial samples (Figure 8B).

In conclusion, the data obtained from this study demonstrates that the LN_2-free CRF is a viable option to cryopreserve CBUs. Although the LN_2 CRF can achieve cryogenic temperatures rapidly, it poses the risks of product contamination and health and safety hazards to the operator (2-4). In comparison, the LN_2-free CRF that we tested – the VIA Freeze™ Quad system from Cytiva – reduces these risks. On the other hand, its maximum cooling rate is - 2.00ºC/min, hence taking a longer time (approx. 1 hour 30 minutes) to freeze CBUs compared to the LN_2 CRF (approx. 1 hour). However, the results indicate that the variation in the cooling rates/time between LN_2 CRF and LN_2-free CRF did not impact post-thaw outcome of cryopreserved samples, making the LN_2-free CRF a viable alternative to the LN_2 CRF for the cryopreservation of CBUs.

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6. Appendices

Appendix 1: Example test report for the infectious disease markers performed on a cord blood unit

Fresh Processed R&D report

Cord Blood Unit: [redacted] **Type of Report: R&D**

Maternal IDM Screening	**Date:**
HBsAg	Negative
anti-HBc	Negative/ Low risk
HBV NAT	Negative
anti-HCV	Negative
HCV NAT	Negative
anti-HIV I/II	Negative
HIV NAT	Negative
anti-CMV	Negative
CMV NAT	Not tested
anti-syphilis	Negative
anti-HTLV I/II	Negative
HEV NAT	Negative

Infectious Disease Screening

- Routine mandatory microbiology markers performed by testing laboratory, NHSBT Manchester Department of Health Medicines and Healthcare Products Regulatory Agency (MHRA) license.
- Additional microbiology markers and confirmatory testing performed by National Transfusion Microbiology Reference Lab (NTMRL), NHSBT Colindale; Clinical Pathology Accreditation.

*** Copies of Laboratory Reports available upon request if required.**

Storage Temperature Requirements

This cord blood unit must be stored below -150°C.

Appendix 2: Raw datasets, average and standard deviation (SD) for each cellular parameter assayed and corresponding Tukey's multiple comparison test results

Table S1: Total nucleated cell (TNC) counts pre-freeze and post-thaw: raw data, average, standard deviation (SD) and standard error of the mean (SEM).

TNC ($\times 10^7$)	Pre-freeze	Bags LN_2-free CRF	Bags LN_2 CRF	Vials LN_2-free CRF	Vials LN_2 CRF
CBU 1	70.2	55.3	60.2	67.9	53.4
CBU 2	74.0	58.2	66.8	67.5	73.6
CBU 3	75.9	58.3	68.7	73.0	66.2
CBU 4	79.0	74.3	59.7	63.8	62.0
CBU 5	65.3	56.4	49.6	56.3	53.1
CBU 6	86.6	79.7	76.4	71.5	77.8
Average	75.2	63.7	63.6	66.7	64.4
SD	7.3	10.5	9.2	6.0	10.2
SEM	3.0	4.3	3.8	2.7	4.2

Table S2: Tukey's multiple comparison test on total nucleated cell counts pre-freeze and post-thaw: p-values of pairwise comparisons between experimental conditions

Experimental condition					
Pre-freeze	Pre-freeze				
Bags LN_2-free CRF	0.194	Bags LN_2-free CRF			
Bags LN_2 CRF	0.186	1	Bags LN_2 CRF		
Vials LN_2-free CRF	0.471	0.977	0.972	Vials LN_2-free CRF	
Vials LN_2 CRF	0.242	1	1	0.991	Vials LN_2 CRF

Table S3: CD34+ cell counts pre-freeze and post-thaw: raw data, average, standard deviation (SD) and standard error of the mean (SEM).

CD34+ ($\times 10^6$/µl) counts	Pre-freeze	Bags LN_2-free CRF	Bags LN_2 CRF	Vials LN_2-free CRF	Vials LN_2 CRF
CBU 1	3.8	2.9	2.8	3.3	3.3
CBU 2	1.0	0.6	0.6	0.01	0.8
CBU 3	2.5	1.8	2.0	1.9	2.1
CBU 4	1.6	1.2	1.2	1.7	1.4
CBU 5	2.5	2.1	2.0	2.5	1.2
CBU 6	2.9	2.1	1.9	2.0	1.9
Average	2.4	1.8	1.8	1.9	1.8
SD	1.0	0.8	0.7	1.1	0.9
SEM	0.4	0.3	0.3	0.4	0.4

Table S4: Tukey's multiple comparison test on CD34+ cell counts pre-freeze and post-thaw: p-values of pairwise comparisons between experimental conditions

Experimental condition					
Pre-freeze	Pre-freeze				
Bags LN_2-free CRF	0.790	Bags LN_2-free CRF			
Bags LN_2 CRF	0.761	1	Bags LN_2 CRF		
Vials LN_2-free CRF	0.886	1	0.999	Vials LN_2-free CRF	
Vials LN_2 CRF	0.792	1	1	1	Vials LN_2 CRF

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Table S5: Percentage of CD34+ cell viability pre-freeze and post-thaw: raw data, average, standard deviation (SD) and standard error of the mean (SEM).

%CD34+ viability	**Pre-freeze**	**Bags LN_2-free CRF**	**Bags LN_2 CRF**	**Vials LN_2-free CRF**	**Vials LN_2 CRF**
CBU 1	97.0	92.2	96.4	87.1	87.2
CBU 2	98.1	91.7	90.1	73.3	67.4
CBU 3	97.1	98.3	88.9	77.3	73.2
CBU 4	96.2	87.2	91.0	76.1	77.6
CBU 5	98.8	96.3	94.7	74.7	66.5
CBU 6	97.7	97.1	94.9	89.1	75.9
Average	97.5	93.8	92.7	79.6	74.6
SD	0.9	4.2	3.1	6.7	7.6
SEM	0.4	1.7	1.2	2.8	3.1

Table S6: Tukey's multiple comparison test on CD34+ cell viability pre-freeze and post-thaw: p-values of pairwise comparisons between experimental conditions

Experimental condition					
Pre-freeze	Pre-freeze				
Bags LN_2-free CRF	0.725	Bags LN_2-free CRF			
Bags LN_2 CRF	0.493	0.995	Bags LN_2 CRF		
Vials LN_2-free CRF	< 0.0001***	0.0005***	0.001**	Vials LN_2-free CRF	
Vials LN_2 CRF	< 0.0001***	< 0.0001***	< 0.0001***	0.460	Vials LN_2 CRF

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Table S7: Total colony forming units (CFU) post-thaw: raw data, average, standard deviation (SD) and standard error of the mean (SEM).

Total CFU/10^5 cells	**Bags LN_2-free CRF**	**Bags LN_2 CRF**	**Vials LN_2-free CRF**	**Vials LN_2 CRF**
CBU 1	172.6	133.4	106.4	117.8
CBU 2	34.6	34.1	40.4	7.9
CBU 3	171.8	117.6	87.9	31.6
CBU 4	83.1	79.1	31.1	12.7
CBU 5	126.3	116.0	133.1	11.6
CBU 6	107.0	77.4	72.1	61.0
Average	115.9	92.9	78.5	40.4
SD	53.3	36.5	39.0	42.8
SEM	21.8	14.9	15.9	17.5

Table S8: Tukey's multiple comparison test on total colony forming units (CFU) post-thaw: p-values of pairwise comparisons between experimental conditions

Experimental condition				
Bags LN_2-free CRF	Bags LN_2-free CRF			
Bags LN_2 CRF	0.796	Bags LN_2 CRF		
Vials LN_2-free CRF	0.460	0.938	Vials LN_2-free CRF	
Vials LN_2 CRF	0.032*	0.188	0.445	Vials LN_2 CRF

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Table S9: Percentage of CD34+ cells growing into colonies post-thaw: raw data, average, standard deviation (SD) and standard error of the mean (SEM).

%CD34+ CFU	Bags LN_2-free CRF	Bags LN_2 CRF	Vials LN_2-free CRF	Vials LN_2 CRF
CBU 1	37.3	28.9	25.3	23.5
CBU 2	35.4	40.7	47.9	20.2
CBU 3	62.2	45.2	44.6	13.7
CBU 4	49.3	42.9	20.5	9.7
CBU 5	34.9	30.7	38.1	9.1
CBU 6	41.8	32.6	28.7	33.7
Average	43.5	36.8	34.2	18.3
SD	10.6	6.9	11.0	9.5
SEM	4.3	2.8	4.5	3.9

Table S10: Tukey's multiple comparison test on percentage of CD34+ cells growing into colonies post-thaw: p-values of the pairwise comparison between experimental conditions

Experimental condition				
Bags LN_2-free CRF	Bags LN_2-free CRF			
Bags LN_2 CRF	0.641	Bags LN_2 CRF		
Vials LN_2-free CRF	0.363	0.962	Vials LN_2-free CRF	
Vials LN_2 CRF	0.001**	0.016*	0.045*	Vials LN_2 CRF

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