



BioArchive[®] Freezing Curve for Cord Blood

*Determination of the Specific Freezing Rate in the BioArchive System
to Provide the Highest Viability of Cord Blood MNC
as Measured by CFU-C Assays*

Introduction

Freezing in liquid nitrogen has been used for cryopreservation of cell suspensions from blood and other biological tissues for several decades. Storage of cells at cryogenic temperatures stops or significantly reduces their metabolism and thus preserves them for prolonged periods of time. During freezing, however, formation of large ice crystals tends to occur inside the cells, which cause irreversible damage and must be avoided. Use of intra-cellular cryoprotective agents such as glycerol or dimethyl sulfoxide (DMSO) and extra-cellular cryoprotectants, such as hydroxyethylstarch or dextran, along with careful control of the rate of freezing, helps retard or prevent the formation of ice crystals and thus protects the cells from injury during freezing.

In the BioArchive, the computerized user interface allows definition and selection of a freezing profile by entering the freezing and ending temperatures of the freeze process and entering the cooling rates that are to be maintained during each of the three freezing phases i.e. pre-freeze, freezing, post-freeze.^[1]

Purpose

The aim of this study was to evaluate the effect on viability of cord blood derived MNC using different freezing profiles for the controlled rate freezing in the BioArchive System. The experiments were performed at the New York Blood Center's National Cord Blood Program under the direction of Ludy Dobrila, Ph.D., Associate Director.

Materials and Methods

Processing of Placental/Cord Blood

Placental blood was collected in disposable collection bags containing anticoagulant (25.0 ml CPD). The red blood cells were sedimented using hydroxyethyl starch (final concentration: 1% w/v) followed by gentle centrifugation (50 g at the expected red blood cell-plasma interface for 6 minutes). The leukocyte-rich plasma was then transferred to a processing bag set. The cells were concentrated to a volume of 20 ml by a second centrifugation step (400g, 13 minutes) and removal of the excess supernatant plasma. 5 ml of the cryoprotective solution (55% w/v DMSO / 5% w/v Dextran40), was then slowly added to the concentrated cells, and the mixture transferred to a THERMOGENESIS CORP.- approved two-compartment freezing bag for programmed freezing and storage in the BioArchive System.^{[2], [3]}

The 25 ml units were kept frozen for at least 48 hours. After storage, the units were retrieved from the BioArchive, kept in the vapor phase of liquid nitrogen for 5 min before being immersed in a 37°C water bath for thawing. The units were subsequently diluted with 25 ml of a 2.5% albumin and 5% Dextran40 solution and mixed gently for a few minutes to allow complete equilibration and then further diluted with 50 ml of the same 2.5% albumin and 5% Dextran40 solution and centrifuged at 400 g for 20 min. The excess supernatant was carefully removed and the sedimented cells were suspended in albumin/Dextran40 solution.

Samples for viability testing were taken before freezing and after thawing of the unit.

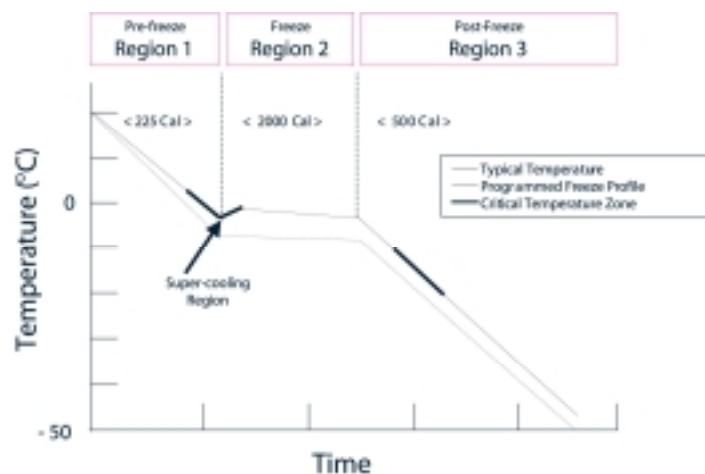
Assessment of Viability by Hematopoietic Colony Formation (CFU-C)

Cord blood was diluted 1:500 in culture tubes containing Iscove's modified Dulbecco's culture medium. The following human recombinant growth factors were added: 50 ng erythropoetin, 25 ng granulocyte/macrophage colony-stimulating factor, 250 ng stem cell factor, 250 ng granulocyte colony-stimulating factor and 25 units of IL3. Triplicate samples were cultured in Petri dishes for 2 weeks at 37°C in humidified controlled atmosphere (5% O₂/ 5% CO₂ / 90 % air). Colonies were identified and enumerated with a phase contrast microscope.^[3]

Freezing Profiles

Proper freezing of 25 ml volume reduced cord blood^[2] must take into consideration three distinct temperature regions in which the cord blood unit exhibits markedly different thermodynamic properties. In the pre-freeze region (6°C to -3°C), the specific heat of the 25 ml liquid cord blood solution is approximately 1 and the heat that has to be removed is ~225 calories. In the freeze region, the entire latent heat of fusion (~2,000 calories) must be removed as the temperature of the cord blood falls to ~ -12°C and is frozen solid. Finally, in the post-freeze region, the specific heat of the frozen cord blood reduces to 0.5 and the reduction in temperature from -15°C to -50°C requires the removal of only 500 calories of heat.^[4]

Consequently, optimizing a freezing curve for this cellular product requires a system which is able to apply different rates of heat removal for each of the three temperature regions.



The BioArchive Controlled Rate Freezing (CRF) System allows six parameters which define the freezing curve to be varied by the operator: Pre-freeze rate (1°C/min to 10°C/min), start freeze temperature (0°C to -15°C), fan power (10 % to 100%), end freeze temperature (-3°C to -30°C),

post freeze rate (1°C/min to 10°C/min), and end temperature (-10°C to –55°C) which is the temperature at which the controlled-rate freeze profile ends and the frozen cord blood unit is robotically lowered into the -196°C liquid nitrogen. As a result of precise measurements of the initial temperature at which the release of the latent heat of fusion begins, the start freeze was set at –3°C in all experiments. Numerous combinations of the other parameters were evaluated.

In the interest of maximizing the productivity of the CRF capacity of the BioArchive, if two freezing rates within any of the three regions of the freezing profile provided the same high recoveries, the more rapid of the two rates was chosen for the final freezing profile validation.

In order to define the zone in which optimum recoveries will occur, the following freezing profiles were tested:

Pre-Freeze Rate (°C/min)	Start Freeze Temp. (°C)	Fan Power during Freeze Region (%)	End Freeze Temp. (°C)	Post Freeze Rate (°C/min)	End Temp. (°C)
2	-3	10	-10	2	-50
2	-3	100	-15	2	-50
10	-3	100	-15	2	-50
10	-3	100	-15	4	-50
10	-3	10	-15	2	-50
10	-3	50	-20	2	-50
10	-3	100	-10	2	-50
10	-3	100	-12	2	-50
10	-3	100	-15	2	-50
10	-3	100	-12	2	-30
10	-3	100	-12	2	-35
10	-3	100	-12	2	-40

Result

1. Evaluation of Freezing Profile

Each table shows the variations in the freezing profile and the percentage recovery of colony forming units (CFU). The recovery was calculated as the percentage of the number of CFU after thawing compared to the number of CFU before freezing.

Variation of Freezing Rate Prior to Freezing and Fan Power During Freeze (Removal of Latent Heat of Fusion)

Pre-freeze: 2°C/min

Pre-Freeze Rate (°C/min)	Start Freeze Temp. (°C)	Fan Power during Freeze Region (%)	End Freeze Temp. (°C)	Post Freeze Rate (°C/min)	End Temp. (°C)	n	CFU Recovery (%)
2	-3	10	-10	2	-50	1	74
2	-3	100	-15	2	-50	2	91

Pre-freeze: 10°C/minute:

Pre-Freeze Rate (°C/min)	Start Freeze Temp. (°C)	Fan Power During Freeze Region (%)	End Freeze Temp. (°C)	Post Freeze Rate (°C/min)	End Temp. (°C)	n	CFU Recovery (%)
10	-3	10	-10	2	-50	2	73.5
10	-3	50	-15	2	-50	2	70.5
10	-3	100	-15	2	-50	2	97.5

Conclusion: Recoveries were similarly high with low and high pre-freeze rates and were better with high fan power during freeze.

Variation of End Freeze Temperature Using 100% Fan Power During Freeze and a Post Freeze Rate of 2°C/min.

Pre-freeze: 2°C/min

Pre-Freeze Rate (°C/min)	Start Freeze Temp. (°C)	Fan Power During Freeze Region (%)	End Freeze Temp. (°C)	Post Freeze Rate (°C/min)	End Temp. (°C)	n	CFU Recovery (%)
2	-3	100	-15	2	-50	2	91

Pre-freeze: 10°C/min

Pre-Freeze Rate (°C/min)	Start Freeze Temp. (°C)	Fan Power During Freeze Region (%)	End Freeze Temp. (°C)	Post Freeze Rate (°C/min)	End Temp. (°C)	n	CFU Recovery (%)
10	-3	100	-15	2	-50	2	92
10	-3	100	-20	2	-50	2	74

Conclusion: An end freeze temperature of -15°C gives acceptable recoveries.

Variation of Post Freeze Rate Using 100% Fan Power and an End Freeze Temperature of -15°C.

Pre-freeze: 2°C/min

Pre-Freeze Rate (°C/min)	Start Freeze Temp. (°C)	Fan Power During Freeze Region (%)	End Freeze Temp. (°C)	Post Freeze Rate (°C/min)	End Temp. (°C)	n	CFU Recovery (%)
2	-3	100	-15	2	-50	2	91

Pre-freeze: 10°C/min

Pre-Freeze Rate (°C/min)	Start Freeze Temp. (°C)	Fan Power During Freeze Region (%)	End Freeze Temp. (°C)	Post Freeze Rate (°C/min)	End Temp. (°C)	n	CFU Recovery (%)
10	-3	100	-15	2	-50	2	92
10	-3	100	-15	4	-50	1	89
10	-3	100	-15	10	-50	1	33

Conclusion: A post-freeze rate of 2°C/min gives acceptable recoveries.

Variation of End Temperature

Essentially the End Temperature is the temperature at which the frozen cord blood unit can be robotically transferred directly to the -196°C liquid nitrogen environment. Temperatures explored were at -30°C, -35°C, -40°C and -50°C.

Pre-Freeze Rate (°C/min)	Start Freeze Temp. (°C)	Fan Power During Freeze Region (%)	End Freeze Temp. (°C)	Post Freeze Rate (°C/min)	End Temp. (°C)	n	CFU Recovery (%)
10	-3	100	-12	2	-30	3	31
10	-3	100	-12	2	-35	3	115
10	-3	100	-12	2	-40	3	108
10	-3	100	-12	2	-50	3	115

Conclusion: Recoveries were acceptable below -35°C as End Temperature.

2. Validation of Recommended Freezing profile

Pre-Freeze Rate (°C/min)	Start Freeze Temp (°C)	Fan Power During Freeze Region (%)	End Freeze Temp. (°C)	Post Freeze Rate (°C/min)	End Temp. (°C)	n	CFU Recovery (%)
10	-3	100	-10	2	-50	5	114
10	-3	100	-12	2	-50	5	107
10	-3	100	-15	2	-50	5	104

Conclusion

The evaluation shows that a pre-freeze rate of 10°C/min and a fan power of 100% during the critical Freeze phase (-3°C to -15°C) will give acceptable recoveries. Lower fan powers may cause loss of viability. In contrast, the importance of a slow post freeze rate was established. A post-freeze rate of 2°C/min proved to be superior to 10°C/min.

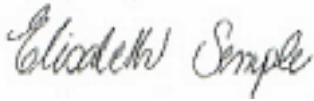
During the validation of the Recommended Freezing Profile, no difference in the number of CFUs was found when comparing samples taken pre-freezing with samples taken post-thawing.

The result shows that a freezing profile with a pre-freeze rate of 10°C/min, fan power during fusion of 100%, post-freeze rate of 2°C/min, an end freeze temperature set between -10 and -15 °C and a final controlled freeze temperature of -50°C will consistently give post-thaw cell viability comparable to pre-freezing values.

Recommended Freezing Profile

Pre-Freeze Rate (°C/min)	Start Freeze Temp (°C)	Fan Power during Freeze Region (%)	End Freeze Temp (°C)	Post Freeze Rate (°C/min)	Final Controlled Freeze Temp (°C)
10	-3	100	-12	2	-50

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^[1] See BioArchive Operator's manual for further information.

^[2] See In service guide-lines 'New York Blood Center Protocol for Reduction of Excess Red Cells and Plasma from Placental Blood Followed by Cryoprotection using the Medsep Processing bag Sets and the THERMOGENESIS CORP. Auto-volume Expressor', THERMOGENESIS CORP.

^[3] Rubinstein et al. Processing and cryopreservation of placental/umbilical cord blood for unrelated bone marrow reconstitution. PNAS 1995;10119-22

^[4] Handbook of Chemistry and Physics, 56th edition, CRC Press, 1996