Published in final edited form as:

Cytotherapy. 2010 April ; 12(2): 161–169. doi:10.3109/14653240903377037.

Development of a Reliable, Low-cost, Controlled Cooling Rate Instrument for the Cryopreservation of Hematopoietic Stem Cells

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Abstract

An optimal cooling rate is one of the critical factors influencing the survival of cells during cryopreservation. In this paper we describe a novel device, named the box-in-box, which was developed for optimal cryopreservation of human hematopoietic stem cells (HSC). This work presents the design of the device, a mathematical formulation describing the expected temperature histories of samples during the freezing process, along with actual experimental results of thermal profile tests. In experiments, when the box-in-box device was transferred from room temperature to a -80°C freezer, a cooling rate of $-1\sim-3.5^{\circ}\text{C}/\text{min}$, which has been widely used for the cryopreservation of HSC, was achieved. In order to further evaluate this device, HSC cryopreservation was compared between the box-in-box device and a commercially available controlled rate freezer (CryoMed). The experimental data, including total cell population and CD34+ hematopoietic progenitor cell recovery rates, viability, and cell culture colony assays, showed that box-in-box worked as well as CryoMed instrument. There was no significant difference in either survival rate or the culture/colony outcome between the two devices. In conclusion, the box-in-box device can work as a cheap, durable, reliable and maintenance-free instrument for the cryopreservation of HSC. This concept of a box-in-box may also be adapted to other cooling rates to support cryopreservation in a wide variety of tissues and cells.

Keywords

cryopreservation of hematopoietic stem cells; box-in-box; controlled cooling rate freezer; colony assay

1 Introduction

Stem cell therapy has been used successfully to treat a wide variety of disorders of the hematopoietic system, including immune deficiency, sickle cell disease, bone marrow failure syndromes, and cancer [1–5]. Unfortunately, under standard room temperature or even refrigerated storage conditions, the hematopoietic stem cells (HSC) used in these types of therapies can only survive for a couple of days after extraction from the patient or an allogeneic donor. Thus, with this short shelf life, HSC must either be used immediately after

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procurement, or must be frozen for later usage until the patient has been prepared to receive the transplant. To store cells for these longer periods of time, the biological “clock” of the cells must be stopped⁶. This is accomplished by cooling and then maintaining the cells at extremely low temperatures. This freezing process is called cryopreservation, which typically includes addition of cryoprotective agents (CPA), cooling the cells plus CPA-containing media under optimal conditions until the solution freezes, long-term storage in ultra-cold temperatures, then warming and thawing the cells, sometimes with removal of the CPA media, when the patient is ready for infusion⁷⁻¹⁰.

Currently, there are several methods used to freeze cells. One protocol, termed dump freezing, calls for cell samples to be placed in Styrofoam boxes or in alcohol chambers (Mr. Frosty, Nalgene®) and then placed into -80°C freezers¹¹, or the vapor phase of liquid nitrogen¹². This process has the advantage of being very simple and low cost, however it is limited by the fact that this method produces no recorded documentation to verify the cooling rate, and there is little standardization of the boxes used. Since Styrofoam boxes can vary greatly in size, geometry, density and structure, there is no reason to believe that the cooling rate is consistent or optimal when using this method. Thus, this technique is generally avoided in the clinical setting where a higher assurance of good cell recovery and documentation of the freezing process is required. To achieve this, most transplant programs have adopted the use of controlled rate freezing devices¹³. These instruments can provide a specific cooling rate, can be programmed to execute complex cooling procedures, and can record the actual temperature profiles of the specimens in order to document any failure during the process. However, this highly adaptable cryopreservation tool does so at the price of extreme cost and equipment complexity. Controlled rate freezers require that a minimum of two thermocouples or resistance temperature detectors (RTD) be carefully located in the freezing chamber and on the samples that are to be cooled. If these sensors are not connected appropriately, the control system will no longer receive accurate data and will respond with incorrect inputs of liquid nitrogen and subsequent cooling. Another issue with the controlled rate freezers is that the valves and solenoids are exposed to extreme temperature conditions during the freezing process. When the control valve first opens, it usually starts at room temperature but is rapidly cooled to the temperature of liquid nitrogen, -196°C . This will introduce extremely high thermal strains, and repeated applications may result in fatigue cracks in the components, eventually causing the valves or solenoids to fail. Furthermore, the controlled rate freezer system is very costly to purchase, and considerable amounts of expensive liquid nitrogen are consumed during the cooling process. Thus, there is a need for a consistent, high quality and low cost cooling system that can provide a documented record of the temperature profile of the sample during the freezing procedure. Currently there is no such system that fulfills all of these requirements.

In this paper, we describe a novel, yet simple, low cost and reliable device, the box-in-box system, which was developed to achieve all of these requirements. For some types of cells it is only required to achieve a single constant cooling rate. The optimal cooling conditions for HSC cryopreservation are well documented ($-1\sim-2.5^{\circ}\text{C}/\text{min}$)¹⁴⁻¹⁶, and it is possible to use thermal inertia to achieve this desired cooling rate. By applying a lumped capacitance analysis, a specific device was built which provides optimum cooling over a broad range of temperatures. The advantage of this method is that it eliminates the need for expensive controlled rate freezing systems, liquid nitrogen consumption and failure prone components. At the same time the box-in-box approach represents a significant improvement over the dump freezing method by providing a consistent, high quality freezing process along with documented temperature recordings of the sample during the cryopreservation procedure. The overall efficiency of this box-in-box device was assessed by comparison with a commercially available controlled rate freezer (CryoMed TM 1010, Cryogenic Tech., FL) for HSC cryopreservation and function recovery after thawing.

2 Materials and Methods

2.1 Design of Box-in-Box system: Lumped Capacitance Model

The goal of developing a model of the box-in-box system was to be able to define an insulation thickness required to achieve the desired cooling rate. In order to simplify the modeling, this system was assumed to behave as only a one-dimensional conduction problem since the whole box is flat (the dimensions of 2 of the axes are much larger than the third one), as shown in Figure 1-A. The third axis with direction of the thickness of the canisters, which is referred to as the primary axis, is set to a thickness that can provide a desired rate of heat transfer. The other two axes are then insulated so that heat transfer along those axes is negligible. Symmetric design can further simplify the model of the box-in-box system. In this model the system has 3 parts: an aluminum enclosure with a thickness L_c for rigidity, a polyethylene insulation layer with thickness L_i and, by symmetry, half of the cell bag with thickness L_b as shown in Figure 1-B. The boundary at the centerline of the bag is assumed to be insulated and by solving the energy equation a temperature profile can be found analytically

$$-Q_{out} = \frac{dE}{dt} \quad (1)$$

where Q_{out} is the heat transfer rate through the surface (W), E is the thermal energy stored within the bag (J), and t is time (s). The total thermal resistance of the insulation and aluminum shell (R_{eq}) is modeled as a resistor in a planar thermal circuit with the same surface area A (m^2)¹⁷.

$$R_{eq} = L_s / K_s A + L_i / K_i A = T_{ib} - T_s / Q_{out} \quad (2)$$

where k_s and k_i are thermal conductivities (W/mK) of the aluminum and insulation material, respectively. Assuming constant density ρ (kg/m^3), specific heat capacity C (kJ/kgK) and volume V (or $L_b A$, m^3) of the bag, the rate of stored energy change in the lumped system may be expressed as:

$$\frac{dE}{dt} = \rho c V \frac{dT_{ib}}{dt} \quad (3)$$

θ can be defined as given in equation (4)

$$\theta = \frac{T_{ib} - T_s}{T_{ib,t=0} - T_s} \quad (4)$$

Substitute equations 2 and 3 and θ into equation 1 and solve it with the initial condition of $\theta_{t=0} = 1$ to yield the following solution:

$$\theta(t) = \exp\left(-\frac{t}{\rho c R_{eq} L_b A}\right) \quad (5)$$

The cooling rate can then be found by differentiating equation 5 as follows:

$$\frac{dT_{ib}}{dt} = -(T_{ib,t=0} - T_s) \cdot \frac{1}{\rho c R_{eq} L_b A} \cdot \exp\left(-\frac{t}{\rho c R_{eq} L_b A}\right) \quad (6)$$

2.2 Parameters Selection

To develop the box-in-box system, the first parameter chosen was the box material. 14 gauge (1.63mm) aluminum alloy 2024-T6 ($k_s = 177 \text{ W/mK}$ at 300K)¹⁷ was chosen because of its high level of structural strength, relatively low price, and with this thickness it would be easy to machine. Polyethylene foam was chosen as the insulating material because it is tough, deformable, easily machined, low cost, can survive well at low temperatures, and has a low thermal conductivity. Commercially available foams have thermal conductivities ranging from 1.00 W/mK to 0.07 W/mK, which at the low end is near the conductivity of stationary air (0.026 W/mK at 298K)¹⁷.

From the lumped capacitance model¹⁷, the thickness of the insulation layer to achieve a specific cooling rate can be determined. Since most important osmotic and thermodynamic processes that might cause cryoinjury occur between 0°C and -33°C¹⁸, the target cooling rate should be maintained within this temperature range. Since the cooling rate and the fraction of cooling completed have the same dependence on time, it is a simple matter to determine an ideal initial cooling rate and maintain the desired cooling velocity until -33°C. Let $b = dT_{ib}/dt$, and denote the initial fraction of cooling as θ_i , the initial cooling rate as b_i , the fraction of cooling and the cooling rate at a specific temperature as θ_c and b_c , respectively. Then an ideal initial cooling rate, which leads to a specific cooling rate at a specific temperature can be obtained as

$$b_i = b_c \cdot \theta_i / \theta_c \quad (7)$$

Using -1°C/min at -33°C, 25°C for an initial temperature, -80°C for the freezer temperature in equation 7 yields

$$b_i = b_c \cdot \theta_i / \theta_c = -1^\circ\text{C/min} \times 1/0.45 = -2.23^\circ\text{C/min} \quad (8)$$

Where $\theta_i = 1$, $\theta_c = -33 - (-80) / 25 - (-80) = 0.45$.

Equation 6 can then be solved using $t = 0$ for the thermal resistance required to provide this cooling rate, which is, in this system, a 36kg/m³ (or 2.2lb/ft³) foam with conductivity of 0.072W/mK, and the ideal thickness of insulation was found to be 7.62mm. The lowest cost cutting tool for the foams had a 6.35mm (1/4 inch) minimum width, so it was chosen to vary the foam density to fine tune the cooling rate after the box-in-box was constructed.

The final design, shown in Figure 2-A&B, has an aluminum enclosure that houses a polyethylene foam insert with three slots for 4"×4" canisters, and a top foam sheet that establishes the insulation layer. The resulting box was contracted (Bauer Cases, Vancouver, WA) with thermocouples installed.

Since the HSC are very precious and the volumes of the samples are generally very small, sometimes they are contained in 1.5ml vials. In order to cryopreserve the samples in this kind of vials, the box-in-box device was also updated as shown in Figure2-C. The details of the design were the same as that for samples in bags, except that there were a few slots to fit the vials. Thermocouples were also installed inside this system.

2.3 Cooling Rate Test

Since HSC may be cryopreserved in plastic vials or bags, the cooling rates of samples inside the vials and bags were tested. Vials (1.5ml, Corning Life Science, Lowell, MA), cell bags (25ml, Pall Life Sciences, East Hills, NY) with aluminum canisters approximately in the size of 4"×4"×½" were used during cryopreservation, shown in Figure 2. 20ml and 1ml water were put in the cell bags and vials respectively. To check the cooling rates in the system, tests were performed with T-type thermocouples (SA-1T, Omega, Stamford, CT) located at center point of the sample inside the vials and bags during the freezing experiments. For each test the box-in-box system was transferred from room temperature to a -80°C freezer and cooled until the temperature inside the samples was lower than -70°C.

2.4 HSC Preparation

Peripheral blood stem cells (PBSC) were collected by apheresis from mobilized volunteer normal donors at the Fred Hutchinson Cancer Research Center (Seattle, WA). Cell numbers were obtained using a Beckman Coulter Cell Counter (model: ZM, Beckman Coulter, Fullerton, CA). PBSC were stained with CD14 FITC, CD34 PE (Becton Dickinson, Franklin Lakes, NJ) and 7AAD (Sigma, Milwaukee, WI), and then the cellular phenotype and viability were analyzed by flow cytometry (BD FACSCalibur, Franklin Lakes, NJ). CD34+ cells were enriched using the Miltenyi CliniMACs system (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's instructions. After the selection process was finished the purified CD34+ cells were evaluated by flow cytometry and for colony-forming activity, and were used in the cryopreservation experiments as described below.

2.5 Cryopreservation of HSC

The enriched CD34+ cells were concentrated by centrifugation at 1600rpm for 10min to a final concentration of approximately 10×10^6 cells / ml. 2X cryoprotective media, composed of Normosol-R (Hospira, Lake Forest, IL), 25% Human Serum Albumin (Octapharma, Hoboken, NJ) and DMSO (Cryoserv, Edwards Lifesciences, Irvine, CA) with the volume ratio of 4:4:2, was precooled to 4°C, then added into the cell suspension drop-wisely and slowly until the original volume had been doubled. One ml of the cell suspension was placed into multiple 1.5ml freezing vials, and then the vials were divided randomly into two groups: cryopreservation with the box-in-box system or with the CryoMed instrument.

A picture of the box-in-box system for freezing vials is shown in Figure2-C. The vials were put in the slots, and included were 2 vials containing only the freeze solution for the temperature history recording (dummy samples). The box-in-box system was put into the -80°C freezer for about 2 hours until the temperature of the samples inside the vials was lower than -70 °C. During the whole process the temperatures of the samples were recorded with the thermocouples and data acquisition system (USB-TC, Measurement Advantage, Measurement Computing, Middleboro, MA). After this, the system was moved out of the -80°C freezer and the frozen vials containing cell suspensions were transferred into the vapor phase of a liquid nitrogen tank (<-150°C).

For the cryopreservation with CryoMed, the cooling program was set as: -1°C/min from 4°C to -4°C, -25°C/min from -4°C to -40°C, 15°C/min from -40°C to -12°C, -1°C/min from -12°C to -40°C, and then -10°C/min from -10°C to -90°C. After the vials were equilibrated in the CryoMed machine, they were also transferred into the vapor phase of the liquid nitrogen tank.

2.6 The Thawing of HSC and Removal of CPA

When thawing cells, the vials containing the cryopreserved CD34+ cells were removed out from the liquid nitrogen tank and immediately placed into a 37°C water bath, gently shaken

until no ice crystals remained, then transferred into 50 ml Falcon tubes, and serially diluted with an isotonic solution containing Dulbecco's Phosphate Buffered Saline +1% fetal bovine serum (DPBS). In each dilution step, the total volume was doubled, and there was 3-minute interval between the dilution steps to allow equilibration. Once the stepwise dilution was completed, the cells were centrifuged at 1600rpm for 10 min. The supernatant was removed, and the cells were resuspended in 1ml of DPBS and assessed for cell counts, flow cytometry analysis for CD34 and viability, and colony generation activity.

2.7 Assessment of the population recovery rates

The numbers of total nucleated cells (TNC), total viable cells, total CD34+ cells were determined for the samples both before and after cryopreservation. From those data, the recovery rates were assessed as follows.

$$\text{recovery rate of TNC} = \frac{\text{TNC after cryopreservation}}{\text{TNC before cryopreservation}} \quad (9)$$

$$\text{recovery rate of CD34+cells} = \frac{\text{total number of CD34+cells after cryopreservation}}{\text{total number of CD34+cells before cryopreservation}} \quad (10)$$

$$\text{recovery rate of viable cells} = \frac{\text{total number of viable cells after cryopreservation}}{\text{total number of viable cells before cryopreservation}} \quad (11)$$

2.8 CD34+ Cell Culture and Colony Assay

The enriched CD34+ cells were cultured in MethoCult GF (StemCell Technologies H4434, Vancouver, BC, Canada) at a concentration of 500cells / 1ml plate in triplicate. The plates were incubated with humidified atmosphere, 5% CO₂ at 37°C for 14 days, then the numbers of BFU-E (BFU-E: burst forming unit erythroid) and CFU-GM (CFU-GM: colony-forming units-granulocyte macrophage) per plate were quantified under microscope. Total colony forming activity in the original 1ml vial after cryopreservation was compared with activity before freezing.

Since we want to get the “total colony generation ability recovery rate”, we need to consider the cell loss post cryopreservation. The seeding density of 500 cells per plate included both viable and dead cells. So the colony generation ability recovery rate was calculated as follows.

$$\text{recovery rate of colony generation} = \frac{\text{colony number after cryopreservation}}{\text{colony number before cryopreservation}} \times \text{recovery rate of TNC} \quad (12)$$

2.9 Statistical Analysis

The results were presented as mean ± SD of independent experiments. The statistical analysis was performed with one-way ANOVA test.

3 Results

3.1 Cooling Rate Measurement

The cooling temperature profiles of the samples in the cell bags (25ml) and vials (1.5ml) are shown in Figure3-A &B. The cooling rates at each temperature point were calculated and shown in Figure3-C&D. Since the data acquisition system was set as one temperature data per second, the cooling rate of each point was calculated as:

$$\text{cooling rate at } N^{\text{th}} \text{ second} = \frac{\text{temperature at } N^{\text{th}} \text{ second} - \text{temperature at } (N - 10)^{\text{th}} \text{ second}}{10} \times 60 (^{\circ}\text{C}/\text{min}) \quad (13)$$

For the samples in the bags, the cooling rate was about $-1^{\circ}\text{C}/\text{min}$, supercooling was observed at $-14.4 \pm 0.8^{\circ}\text{C}$ ($n=7$). For the samples in vials, the cooling rate was in the range of $-1 \sim -3.5^{\circ}\text{C}/\text{min}$, and the supercooling was at $-6.2 \pm 0.2^{\circ}\text{C}$ ($n=6$), but the temperature oscillation and solidification latent heat release at the supercooling point were much weaker than those of samples in bags.

3.2 Population Recovery Rates

Absolute recovery rates, calculated based on total numbers pre and post cryopreservation, are shown in Table-1. For TNC, the population recovery rates were $72.5 \pm 10.9\%$ and $75.4 \pm 13.8\%$ ($n=10$) for the samples cryopreserved with the box-in-box system and CryoMed machine, respectively. For CD34+ cells, the population recovery rates were $73.5 \pm 13.0\%$ and $73.9 \pm 13.9\%$ ($n=10$), and for viable cells, the recovery rates were $71.4 \pm 12.0\%$ and $71.7 \pm 13.2\%$ ($n=10$). There was no significant difference comparing box-in-box with the CryoMed for any of these results ($p=0.6 \sim 0.9$).

3.3 BFU-E and CFU-GM Colony Recovery Rates

Colony generation recovery rates are shown in Table-2. For BFU-E, the recovery rates were $64.5 \pm 14.6\%$ and $62.3 \pm 12.9\%$ ($n=8$) for the samples cryopreserved with box-in-box system and CryoMed respectively. For CFU-GM, recovery rates were $59.4 \pm 22.6\%$ and $53.1 \pm 18.9\%$ ($n=8$). There was no significant difference comparing box-in-box with CryoMed for any of these results ($p=0.6 \sim 0.7$).

4 Discussion

HSC transplantation has become a very important treatment option for many patients, which in turn has made optimal long-term cryopreservation a critical issue. Controlled rate freezing systems, such as CryoMed devices, are widely used to provide a suitable cooling rate of about $-1^{\circ}\text{C}/\text{min}$. To cut down on complexity and cost, other researchers have used dump freezing methods such as Styrofoam boxes placed into -80°C freezers to cryopreserve their samples. However, as detailed in the introduction, there are significant shortcomings with this approach. In this paper, a novel, simple, and inexpensive device, termed the box-in-box, has been designed and evaluated. This device can provide a controlled and consistent cooling rate for samples in either bags or vials, and can record the temperature of the samples during freezing process.

The device had been optimized for specimens contained in 25ml bags, and the cooling rate tests showed that the system could provide $-1^{\circ}\text{C}/\text{min}$ cooling rate. However, for samples in smaller 1ml cryo-vials, the cooling rate was about $-1 \sim -3.5^{\circ}\text{C}/\text{min}$, most likely a reflection of the smaller heat capacitance of the vials. This higher cooling rate doesn't exactly match the published requirements for optimal cryopreservation of HSC. Nevertheless, the results

for TNC, CD34, viability, and BFU-E and CFU-GM were similar between the box-in-box and the controlled-rate CryoMed system (Tables 1 and 2). This may have been due to the fact that the supercooling and solidification heat release were much weaker than those of samples in bags (Figure 3), which could have been beneficial to the cell preservation results in the box-in-box testing. In the future the device could be further optimized for such smaller volume samples, by modifications such as increasing the thickness of the insulation layer. This finding implies that the cooling rate is related to the heat capacitance of the samples, indicating the importance of standardizing the volume of the samples in future application. Unfortunately, enriched CD34+ cells from volunteer donors are only available in limited quantities, which meant that direct experiments to compare recoveries could not be evaluated in the bags, but only in the cryo-vials.

In conclusion, the concept of thermal inertia was applied to achieve a specific cooling rate. The box-in-box device described in this report can act as an alternative method for the controlled rate freezing of HSC specimens. The materials to manufacture this device are inexpensive to obtain and to machine, there are no delicate parts such as valves or solenoids that could fail, and there is no need for liquid nitrogen during the freezing process. The results from the cooling rate tests have shown that the system can provide very consistent cooling rates of $-1 \sim -3.5$ °C /min, and the use of thermocouples allows the temperature history of the samples to be easily recorded during the freezing process. Furthermore, it is clear that with minor modifications to the existing box-in-box design, other rates of freezing that might be optimal for different kinds of cells and tissues could be developed.

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Figure 1.
The design of box-in-box system

Figure2.
Final design of the box-in-box for bags and vials. A, the bags and vials for HSC cryopreservation. B, box-in-box for samples in bags.C, box-in-box for samples in vials.

Figure3. Temperature profiles and cooling rates of the samples in bags and vials. A, the temperature history of samples in bags. B, the temperature history of samples in vials. C, the cooling rates of samples in bags. D, the cooling rates of samples in vials.

Table 1

Population recovery rates of all cells, CD34+ cells and viable cells

Sample#	NO. of all cells (10 ⁶)	Recovery rate of TNC (%) ^a	NO. of CD34+ (10 ⁶)	Recovery rate of CD34+ (%) ^b	NO. of viable cells (10 ⁶)	Recovery rate of viable cells (%) ^c
1	pre-cryo		4.68		4.75	
	box-in-box	84.0	3.73	79.8	3.77	79.2
	CryoMed	92.8	3.90	83.3	3.93	82.7
2	pre-cryo		6.56		6.70	
	box-in-box	84.1	5.23	79.7	5.26	78.5
	CryoMed	86.7	4.69	71.6	4.72	70.5
3	pre-cryo		4.69		4.80	
	box-in-box	70.4	3.27	69.8	3.33	69.3
	CryoMed	77.0	3.39	72.3	3.45	71.8
4	pre-cryo		1.16		1.39	
	box-in-box	70.0	0.95	81.8	0.98	70.7
	CryoMed	74.0	0.99	85.6	1.03	74.2
5	pre-cryo		5.84		5.91	
	box-in-box	81.5	4.25	72.8	4.32	73.2
	CryoMed	96.7	5.16	88.5	5.24	88.8
6	pre-cryo		3.28		3.48	
	box-in-box	71.1	2.54	77.2	2.58	74.3
	CryoMed	65.1	2.32	70.4	2.36	67.7
7	pre-cryo		4.14		4.34	
	box-in-box	71.5	2.97	71.8	3.02	69.7
	CryoMed	63.0	2.49	60.3	2.54	58.5
8	pre-cryo		2.93		3.11	
	box-in-box	77.8	2.77	94.6	2.85	91.5
	CryoMed	74.6	2.74	93.6	2.81	90.3
9	pre-cryo		3.35		3.52	

Sample#	NO. of all cells (10 ⁶)	Recovery rate of TNC (%) ^a	NO. of CD34+ (10 ⁶)	Recovery rate of CD34+ (%) ^b	NO. of viable Cells (10 ⁶)	Recovery rate of viable cells (%) ^c
10	box-in-box	46.9	1.55	46.3	1.60	45.3
	CryoMed	52.0	1.65	49.3	1.70	48.4
	pre-cryo		3.25		3.42	
	box-in-box	68.0	1.20	61.4	2.13	62.4
	CryoMed	72.0	2.08	64.1	2.18	63.9

TNC after cryopreservation

$$a\text{recovery rate} = \frac{\text{TNC after cryopreservation}}{\text{TNC before cryopreservation}}$$

total number of CD34+cells after cryopreservation

$$b\text{recovery rate} = \frac{\text{total number of CD34+cells after cryopreservation}}{\text{total number of CD34+cells before cryopreservation}}$$

total number of viable cells after cryopreservation

$$c\text{recovery rate} = \frac{\text{total number of viable cells after cryopreservation}}{\text{total number of viable cells before cryopreservation}}$$

Table 2

Recovery rates of colony generation

Sample#	BFU-E/ 500 cells ^a	CFU-GM/500 cells ^a	Recovery rate of TNC (%)	BFU-E recovery rate(%) ^b	CFU-GM recovery rate(%) ^c
1	pre-cryo	58.00			
	box-in-box	42.38	84.1	61.5	89.4
	CryoMed	37.63	86.7	56.3	58.5
2	pre-cryo	41.50			
	box-in-box	43.25	70.4	73.4	68.4
	CryoMed	41.75	77.0	77.5	57.8
3	pre-cryo	21.63			
	box-in-box	31.25	70.0	75.2	34.7
	CryoMed	20.38	74.0	69.7	42.0
4	pre-cryo	47.88			
	box-in-box	40.00	81.5	68.1	54.3
	CryoMed	38.63	96.7	78.0	62.7
5	pre-cryo	22.88			
	box-in-box	26.50	71.1	82.4	37.6
	CryoMed	22.30	65.1	63.5	26.6
6	pre-cryo	24.00			
	box-in-box	15.75	71.5	46.9	41.7
	CryoMed	16.83	63.0	44.2	29.0
7	pre-cryo	11.13			
	box-in-box	9.88	77.8	69.1	55.6
	CryoMed	9.38	74.6	62.9	69.5
8	pre-cryo	41.25			
	box-in-box	34.88	46.9	39.7	93.3
	CryoMed	36.63	52.0	46.2	78.9

^a All the numbers were calculated as the means of the colony numbers of at least 4 plates.

$$\begin{aligned} \text{BFU-E recovery rate} &= \frac{\text{NO. of BFU-E after cryopreservation}}{\text{NO. of BFU-E before cryopreservation}} \times \text{recovery rate of TNC} \\ \text{CFU-GM recovery rate} &= \frac{\text{NO. of CFU-GM after cryopreservation}}{\text{NO. of CFU-GM before cryopreservation}} \times \text{recovery rate of TNC} \end{aligned}$$