Chapter

Impact of Different Cooling Methods on the Stability of Peripheral Blood Mononuclear Cells (PBMCs)

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Abstract

During cryopreservation of peripheral blood mononuclear cells (PBMCs), there are several recognized cooling methods, which include different cooling rates that might influence the stability of the PBMCs. This chapter will focus on three cooling methods trialled and will describe the different principles they are based on and the outcomes. One cooling method is based on repeatable –1°C/min cooling rate that requires only isopropyl alcohol (method A). The second cooling method is based on the cooling rate of –1° C/min solely (method B). The third cooling method is based on a user-predefined programmable controlled rate of freezing (method C). The first method was discontinued for safety reasons. A small comparative study was performed using 12 cell preparation tubes (CPT) using methods B and C. Cell Viability was measured based on the difference between pre-thaw and post-thaw viability percentages that were obtained from the flow cytometry. From our data, we conclude that although there were no significant differences in the outcomes of the comparative study of cooling methods, the use of either method B or C are the most suitable for long-term storage that will preserve the quality of the sample suitable for future research and clinical applications.

Keywords: cooling, PBMC's, stability, viability, pre-thaw, post-thaw

1. Introduction

PBMCs are white blood cells with round nuclei that includes lymphocytes (i.e. T cells, B cells, and NK cells), monocytes, and dendritic cells [1]. These cells are important biospecimens as researchers use them to recognize circulating disease biomarkers. While fresh viable cells are most often being used, the use of frozen viable PBMC's should be equally considered as they allow the screening of purified monocyte and lymphocyte populations. Denity gradient centrifugation has been utilized for isolating PBMC's because it is not expensive and needs very little specialised

Figure 1.
Whole blood is layered over the density gradient medium. The centrifuged blood is showing the cells in different layers based on different densities. PBMCs settle between the density gradient medium and the plasma, where they can be aspirated [4].

equipment to implement in any lab [1], following cryopreservation, in addition to, functional studies, immunophenotyping, obtain lymphoblastoid cell lines (LCL) by Epstein Barr virus (EBV) transformation, and purification of CD34+ cells [2].

Using cell separation techniques, PBMCs can be isolated directly from whole blood. They are present in peripheral blood, and they have a crucial function of acting as the body's front line to defend against disease [3]. PBMC isolation is based mostly on the method of density gradient medium, and centrifugation [4] as shown in **Figure 1**.

Density gradient centrifugation has been utilized for isolating PBMCs because it is not expensive and needs very little specialized equipment, to implement to any lab [1, 5]. The aim of this chapter is to investigate the effectiveness of different approaches used in freezing rates to ensure that PBMCs are maintained within the optimal viability and functionality, and to preserve the cells with higher viability and biological activity, before and after the thawing processes.

2. Literature review of different cooling rates on PBMCs stability

Our research in this field highlighted that there is a lack of literature about the effects of different cooling rates during sample storage. Some studies provided data that shows the crucial steps of sample storage and handling in maintaining the viability of PBMCs, the recovery of PBMC and T-cell functionality [6]. As such, a review of the available studies will be discussed.

2.1 Quality of frozen PBMCs

Researchers have identified that cryopreservation affects the viability, recovery, and gene expression pattern of PBMCs, when compared to freshly isolated PBMCs [7, 8]. In addition, multiple factors impact the quality of PBMCs including preanalytical, analytical and post-analytical processes. Pre-analytical steps such as the time of sample collection, environmental conditions and calibration of equipment [9]. Analytical steps included the sample processing, type and time of exposure of the cryoprotectant media, viable cells manual mixing conditions, sterile environment and freezing conditions are all critically important for good sample cryopreservation [6]. Post-analytical factors included transportation of viable cells is of paramount consideration as they affect biological specimen viability and functionality [2]. Also, temperature fluctuations happen during retrieval or shipping of stored samples [8]. Nevertheless, an essential step is that the collected PBMCs are conserved in a natural state that renders them from being altered functionally. PBMCs need to be grown in cultures to show viability and to react to immune stimulation to show

phenotypic capability [10] Best practices relating to the maintenance of PBMCs viability is obtained if they are stored below –132°C, the glass transition temperature of water (GTTW) [11]. At this temperature or below, the biological activities of cells are stopped [8]. Additional research suggested that quality control measures in cell repository should be adopted or based on their study findings. The separation of blood, and the storage using a controlled-rate freezer should be within six hours from collection. Environmental safety controls such as a temperature monitoring alarm system should be configured in the liquid nitrogen storage tanks. The study findings recommended to use liquid nitrogen vapor for maintaining a high cell viability through the storage for long-term purposes. However, using gasket threaded vials can also be used if storage is in the liquid nitrogen phase [12].

2.2 Different cooling rates vs. cell viability

PBMCs are being monitored through cell yield, viability, and cell population percentage using fluorescence flow cytometry. Research has concluded that it is important to disclose the temperature and time of processing when data from clinical trial of PBMCs is being published [13].

A study was done to show the impact of multiple temperature fluctuations on cell quality, PBMCs were stored under suboptimal storage condition from 10 different donors. The multiple temperature shifts were compared to optimal storage conditions without temperature shifts. Automated trypan blue dye exclusion and IFN-c ELISpot were used to measure cell viability, recovery, and functionality after cryopreservation in the standardized xeno-free cryomedium IBMT I and cell storage under 3 different conditions. The results were shown to minimize PBMC viability, PBMC recovery and T-cell functionality as measured by IFN-c ELISpot. Hence, temperature fluctuation has been shown to directly affect cell integrity, and the importance of carefully choosing optimal sample storage conditions [6, 14].

2.3 Effects of slow cooling and super cooling on cell viability

Since cooling rate is a major determinant of cell viability following cryopreservation, cryopreserved cells tend to die if it has been linked to intracellular ice formation (IIF) [15]. Research has shown that it has proved beneficial to avoid variable degrees of supercooling in multiple samples by deliberately inducing freezing (nucleation) at a point when the samples have cooled a few degrees below their equilibrium freezing point [16]. Research suggests that cell volume has a pivotal role in the occurrence of IIF than extracellular nucleation temperature or intracellular supercooling. Results indicated that larger cells were more likely to have IIF than smaller cells, and that smaller cells can withstand the supercooling effect before forming intracellular ice [15]. Other research has shown that in post-intracellular freezing, the plasma membrane lost its ability to act as a barrier for extracellular ice, which was similar to the damage caused by osmotic stresses [17].

Optimal slow cooling conditions resulting in retained cell viability are defined by the cooling rate that permits some cell dehydration without the formation of significant amounts of intracellular ice. Tolerances for cell shrinkage and intracellular ice formation vary between cell and tissue types. Ice formation in slowly cooled systems usually begins in the extracellular solution surrounding the biological material. Because ice is pure water, as ice formation occurs, the concentration of solute outside the cells increases and the cells begin to lose water by osmosis resulting in cell

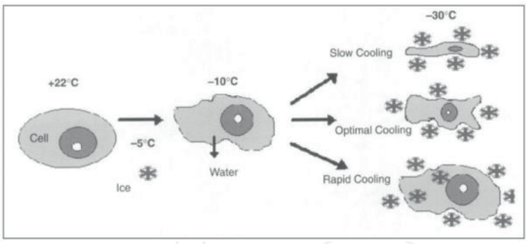

Figure 2.
Different cooling rates showing physical events occurring in cells as they start cooling.

shrinkage. Cooling samples to their freezing point and beyond does not automatically result in freezing the samples at the equilibrium freezing point. Invariably, samples tend to under cool—often referred to as supercooling— to a varying degree that depends on the cooling rate, sample size, presence of nucleating agents, which are foreign particles in a solution that catalyze the formation of an ice nucleus, initiating the freezing process [16]. As samples start the cooling process and as it achieves approximately −5°C, the cells including the surrounding remain unfrozen. Ice starts to form as the temperature drops below −5 down to −15°C. At this stage, the cells and the external medium remain liquefied still, as the plasma membrane blocks the build of ice crystals into the cytoplasm. While the supercooled water in the cells has a greater chemical potential than the water in the partially frozen exterior solution; therefor, water flows out of the cells osmotically and freezes in the external medium. **Figure 2** shows the subsequent events occurring in the cells physically, depending on the cooling rates suggested [18].

Some studies were focused on the observation of the induced IIF which was described earlier in this chapter, which was a result of the water flux across the cells membrane during the freeze–thaw cycle [18]. According to a study model, researchers proposed that IIF may be induced by the plasma membrane through the effects of external ice on the plasma membrane, also known as surface-catalyzed nucleation (SCN), or by the intracellular particles, also known as volume-catalyzed nucleation (VCN). These different effects depend on the freezing conditions used. Also, they suggested that the effects of variables could be minimised if cells were cooled at rapid rates to avoid water flux during the process of freezing [19].

2.4 Method A, B and C

Different cooling methods are commercially available for PBMCs cryopreservation. Method A samples are placed in isopropanol chambers and into −80°C freezers, or into the vapor of liquid nitrogen at a temperature that varies between −135°C and − 190°C. This method is very simple and low in cost, but it does not provide any evidence for traceability or to verify the cooling rate. Therefore, this method would be avoided in clinical settings where a higher assurance of cell recovery and traceability of the freezing process is needed [20]. The freezing of samples using isopropanol filled devices (method A) requires long equilibration times and can introduce variability based on vial position, so the performance is dependent on vial position and continuous isopropanol replenishment. Method A was eventually excluded from the comparison study due to health and safety concerns relating to the use of the Isopropanol. Using programmable freezers (method C) can keep highly reproducible freeze rates, but are also costly, hard to maintain, susceptible to malfunction, and requires large spaces and energy [21]. Method B which is based mainly

on an alcohol-free freezing at the rate of −1°C/minute combined with a − 80°C freezer [22]. This method ensures high thermal control and reproducibility while maintaining a small footprint [21]. A critical factor that influences the survival of cells during cryopreservation is the choice of an optimal cooling rate [20]. Several studies have been done to assess the cell viability for PBMCs, and it was shown to be consistently above 95% before freezing. An assay was blotted to show viability after freezing using either controlled-rate programmable freezer (method C), or the cell-freezing container (method B). In both methods, cells were frozen and stored at −80°C, then further stored at −150°C for 5 days. PBMCs were analyzed via flow cytometer using propidium iodide as a post-thaw viability. The viability rates were shown to be insignificant in the difference between both methods [21]. Nevertheless, the use of method C is thought to minimize two cell damage effects. The first effect, called solution effect which is extensive cell dehydration. The second effect, called mechanical damage which is intracellular ice crystallization. This is further explained as the continuous adjustments of the temperature reduces based on the temperature of the cells, therefore, compensating for fusion heat and reducing of supercooling effects [23]. This temperature compensation is provided by a programmed decrease in chamber temperature that both initiates nucleation and subsequently compensates for the release of the latent heat of fusion. The major variables involved are the rate of chamber temperature decrease, hold temperature and duration and the rate of temperature increase [16].

2.5 Thawing processes

As PBMCs survive cooling to ultra-low temperature, it is still challenging during the thawing processes at which it can exert effects on survival comparable with those of cooling [18]. So, determining how they survive both cooling and subsequent return to physiological condition is the consideration. An important question would be whether they freeze intracellularly or not, which occurs when cooling is too rapid as explained earlier (**Figure 2**). One study has developed equations that describe the kinetics of water loss and predict the likelihood of intracellular freezing as a function of cooling rate. Although it is necessary to avoid intracellular freezing to accomplish survival, but it is not sufficient. Slow freezing can introduce injury to the cell [24], as described earlier in Section 2.3. A study was developed to identify the risk in the addition of ice-chilled washing media to thawed cells at the same temperature, which was shown to be a high-risk practice that yielded significantly lower viability and functionality of recovered PBMC. This study also compared the previously mentioned outcome to the use of warm cryovials in temperatures of 37°C while adding a warm washing medium. The thawed PBMC in cryovials were kept up to 30minutes at 37°C in the presence of DMSO, and surprisingly showed that exposure to DMSO was a low-risk practice during the thawing process [25].

2.6 Factors that impact PBMCs stability

A major reason to use a freezing equipment or protocol rather than simply placing samples in cold environments is that the temperature compensation provided during controlled rate preservation for release of the latent heat results in improved post-cryopreservation cell viability [16]. In addition, research has focused on improving the interaction between cooling rates and the permeability of the plasma membrane to water and cryoprotectants [26]. The addressed interaction plays a

major role in PBMC stability. So, as the biological metabolism in cells dramatically diminishes at low temperatures, research has shown that cells were unable to endure the low temperatures. However, it is in fact the lethality of an intermediate zone of temperature (−15 to −60°C) that cells must traverse twice—once during cooling and once during warming [18]. Research studies recommend the induction of on-site training that facilitate a standardized method for cell counting, freezing, and thawing in order to maintain an environment with reduced variation in cell recovery. Nevertheless, external quality control programs can also enable the optimization of viability and cell recoveries with higher yields and viability to maximize the value of PBMC to be collected and stored for research studies [12].

3. Materials and methods

3.1 Study sample preparation

A total of 12 blood samples were collected and prepared from healthy adults. These samples were collected and processed according to Qatar Biobank (QBB) procedures. CPT closed sample collection kits with tubes containing additives of sodium citrate were used to collect whole blood [27]. A total of 24 viable cells in 1ml aliquot tubes were divided; every aliquot from the same parent CPT tube was placed in method B and method C, simultaneously. To obtain accurate measurements, viable cells were stored for a minimum of 24 hours in liquid nitrogen vapour.

3.2 PBMC isolation and cryopreservation

The procedure of PBMC separation was carried out in the laminar flow cabinet, which was turned on for at least 10 minutes before the work was started. The surface was disinfected using sodium hypochlorite followed by 70% ethanol and then type 1 water. CPT tubes were processed following a standard protocol [11] After centrifugation, the tubes appeared to have layers as shown previously in **Figure 1**. PBMCs were transferred using sterile tips into 15 ml sterile prelabelled intermediate tubes. This intermediate tube was connected to a parent tube by a Laboratory Information Management System tool for our labs. 100μl of PBMC were sub aliquoted from the intermediate tube into 5 ml prelabelled plain tubes that were also connected to the parent tube. The plain tube was processed in the cell counter to check the number of WBCs before running the samples in the flow cytometer (Section 3.4). In the 15 ml sterile intermediate tube, sterile phosphate-buffered saline (PBS) was added (in laminar flow cabinet) till 15 ml as first wash cycle. The cells were mixed gently by inverting the intermediate tube 5 times, then it was centrifuged for 15 min at 300 RCF at room temperature. The supernatant was disposed in an empty sterile waste bottle. The cell pellet was resuspended by gently vortexing or tapping tube with index finger. Sterile PBS solution was added until the 10 ml mark as second wash cycle. The tube was mixed by inverting 5 times, then it was centrifuged for 10 min at 300 RCF at room temperature. The supernatant was disposed in an empty sterile waste bottle. 1 ml of 10% DMSO was added to the tube and gently pipetted to mix with the cell suspension. 2 aliquots were created from each one parent tube in the corresponding sterile 1 ml tube. Using our laboratory information management system the parent tube was barcoded and scanned to be linked to 2 aliquots each. The aliquots were placed on a cooling shell to allow the cryoprotectant to enter the cells, and to prevent

heat generation that can damage the cells. Keeping the specific timeline to allow stabilization, which is between 20 to 30 minutes to prevent the toxic effects of DMSO on the cells. 12 out of 24 created aliquots were transferred to method C, where they were gradually cooled in a user pre-defined temperature in the controlled rate freezer that is 1°C per 1 minute until −30°C, after that cooling rate is increased up to 5°C per 1 minute until −100°C is achieved, to ensure that the freezing process runs gradually to keep the cells, membranes, and cellular organelles safe and intact. Eventually, these aliquots were then stored in liquid nitrogen vapour. The remaining 12 created aliquots were transferred to method B, which uses a fridge temperature pre-cooled cool cell box, in which samples were then transferred to be cryopreserved within 4–24 hours of cool cell use time in the -80C freezer. This has been verified with an internally validated method in parallel with method C, in any contingency situation with the goal to cryopreserve high-quality quality PBMC samples.

3.3 Thawing the PBMCs

The cryopreserved PBMCs were retrieved from vapor phase of liquid nitrogen storage and placed directly in -80C portable freezer until samples were thawed. A standard thawing procedure for PBMCs was followed [28]. After thawing, cells were resuspended in PBS buffer as a preparation step for flow cytometer cell viability analysis explained in the next section. A standard thawing procedure is equally as or perhaps more important for obtaining maximum viability and recoveries of cryopreserved PBMC. The thawing procedure should also become part of the validation exercise to ensure reproducible sample preparation and cryopreservation.

3.4 Assessing the cell viability using flow cytometry

Prior to processing samples in the flow cytometer, a cleanse panel was run followed by a fluorescent microspheres suspension check. This step is mandatory as a routine quality control check prior to daily instrument operation. The PBMCs were extracted from the processing of CPT tubes that was previously explained in both sections 3.3 and 3.4. The cells were washed with 400 ul of PBS and centrifuged for 5 minutes at 500xg at 4°C. The supernatant was then discarded, and dyes were added to the cell suspension each prepared as follows, 10 μL of Annexin V-FITC, 20μL of 7-AAD viability dye and 10 μL CD45-APC750. The samples were mixed gently and kept for incubation in the fridge in the dark for 15 minutes. After incubation, 400 ul of binding buffer was added to each sample. Finally, the results were checked for the acceptable viability percentages for each sample as shown in **Figure 3**.

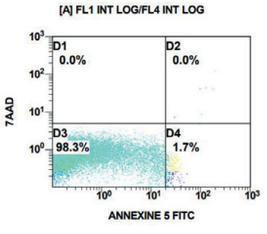

Figure 3.
Dot plot diagram from PBMCs showing cell population. D3. Double negative (Annexin V and 7-AAD negative) healthy cells. D4. Annexin V positive, 7-AAD negative apoptotic cell. D2. Annexin V & 7-AAD double positive necrotic cell.

3.5 Statistical analysis

The temperature for method B was recorded at 10-second intervals over a 4-hour period, which was repeated twice. Also, the temperature for method C was recorded over 2-hour period. Thermocouple probes were calibrated and set up with a temperature data logger for method B to record the temperature every 10 seconds using temperature record data-logger software, while instrument-specific temperature record software was used to generate temperature curves for method C, in addition to, temperature record data-logger. Additionally, Student's t-test was generated based on the comparison of standard deviation and mean values of both method B and method C.

4. Results and discussion

4.1 Comparison between method B and method C -cooling rate temperatures

Following manufacturer specification, method B had a cooling rate of 1°C per 1 minute. This fact was verified using probes as mentioned in Section 3.5, and the overall average rate was 0.98°C with slight differences in cooling rate from −15.5°C to −30°C, and from −30°C to −50°C, as shown in **Table 1** and temperature curve is shown in **Figure 4**. Cooling performance of method C was measured by instrument-specific temperature record software configured with the instrument as shown in **Table 2** and **Figure 5**, to detect and record all temperatures and curves, in addition to temperature record data-logger as shown in **Table 3** and **Figure 6**.

Method B, temperature ranges	Cooling rate (°C)
Average rate per 1 min for 4 h	0.98
From 15.5°C to - 30°C	1.33
From −30°C to −50°C	0.71

Table 1.
Method B temperature recorded over 4-hour interval.

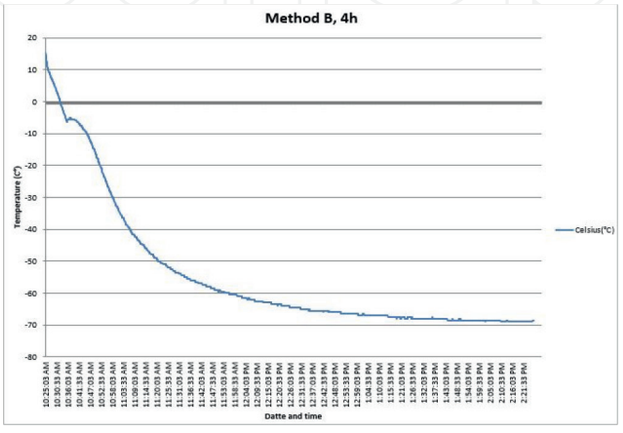

Figure 4.
Method B temperature curve for 4-hour interval.

No.	Temperature (°C)	Duration (min)
1	4	8
2	0	1
30	5	
4	–100	100

Table 2.
Method C freezing cycle program in instrument-specific temperature record.

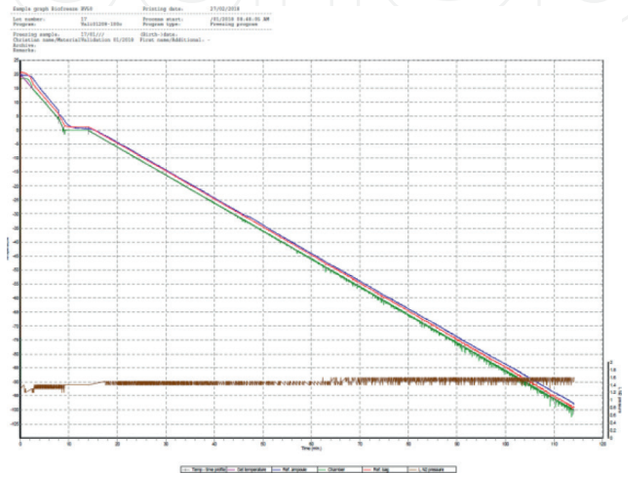

Figure 5.
Curve of method C freezing cycle program in instrument-specific temperature record.

Method C, temperature ranges	Cooling rate (°C)
Average rate per 1 min for 2 h	0.93
From 15.5 °C to – 30°C	1.08
from – 30°C to – 100°C	0.79

Table 3.
Method C temperature recorded over 2-hour interval.

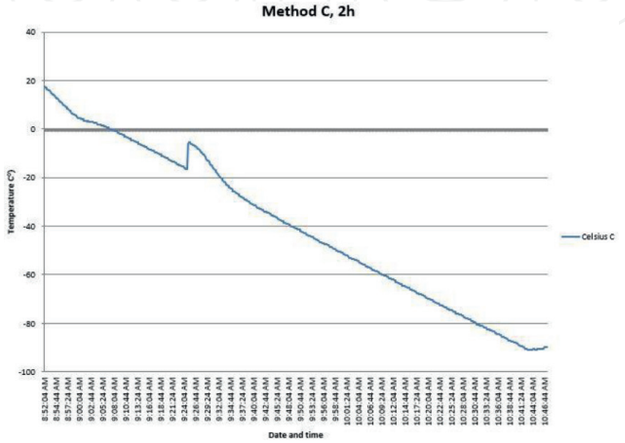

Figure 6.
Method C temperature curve for 2-hour interval, using temperature record data-logger.

As shown in **Figure 4**, latent heat was generated during freezing, an exothermic process, or heat release, known as the latent heat of fusion or crystallization, during ice formation must be conducted away from the material being frozen.

Differences in temperature rate between method B and method C were not statistically significant as shown in **Table 4** below.

4.2 Adjusting the cooling rate for better stability of PBMCs

A critical variable factor that is the main scope of this chapter is the effect on the survival rate of cells by the cooling rate. During slow cooling the cells are exposed to DMSO, which is harmful for cell viability, and the concentration of external liquid is increasing, leading to dehydration as a consequence of efflux of water from cells due to change in osmotic status. Therefore, cells can shrink and become deformed. Using fast cooling rate, the dynamic characteristic of the cell membrane must be considered. The intracellular water cannot pass through membrane fast enough and freezes inside the cell. This process is lethal for the cell. During freezing, the following phases are appearing in the cell media: liquid phase cooling 1°C/min, supercooling (undercooling) is happening below the freezing point of media (DMSO freezing point is −5°C), this phase is followed by thermal increase as its exothermal reaction. Phase change-liquid to solid phase change followed immediately after supercooling. Solid phase I freezing is same 1C°/min. End solid phase I freezing is usually between −25°C to −50°C. Protocol of freezing can be shortened in the duration of the reaching the solid phase freezing, and cooling rate can be increased 5°C/min to the end of solid phase II freezing, which is between −80 and − 90°C. This provides adequate temperature security by preventing sample warming above End solid phase I. As the optimal cooling rate is essential for cell viability during cryopreservation, and specifically for that purpose, 4 control rate protocols were developed and compared in terms of temperature stability and viability. First program was following the control rate according to method B protocol with 1°C per 1 minute. Second program was designed to have cooling rate 1°C per 1 minute until −30°C, after that, cooling rate was increased up to 5°C per 1 minute until −100°C is achieved, as shown in **Figure 6** and **Table 5**.

A third programme was created to prevent the impact on cells by latent heat generation, which is clearly shown in **Figure 7** below.

		Method B	**Method C**
1 °C/1 min	Mean	0.98	0.93
	St dev	0.47	1.83
	N	200	200
	Sp2	198	14.07
	t calc	0.0036	
	t value from t table, CI 99%	2.62	
	ItI < It tabI	0.003 < 2.62	

Table 4.
Comparison between method B and method C, program of 1°C/1 min.

No.	Temperature (°C)	Duration (min)
1	4	8
2	0	1
3	0	5
4	−30	6
5	−100	14

Table 5.
Method C freezing cycle program in instrument-specific temperature record, with addition to the program.

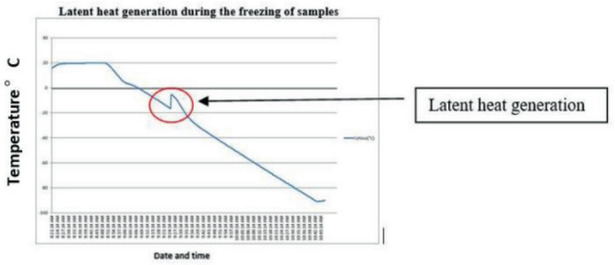

Figure 7.
Latent heat generation during the freezing of samples.

To improve the viability of the cells, an additional plot between −12°C and − 30°C was added (4°C/min), as shown in **Table 6** below.

The shape of the peak for latent heat generation in program 4 as shown in **Figures 8** and **9** was not as sharp as at the first program. Both of the programs were reaching the point of freezing media −5°C. And cooling rate was increasing for 4°C per minute to prevent the supercooling. To keep the cooling rate during crystallization as close to 1–2°C/min, a much greater difference between gas and sample had to be maintained as illustrated in **Table 7**. Heat of fusion was transferred through the wall of ampoule and heat capacity of the sample. After the intracellular phase transition was done (at −30°C), cooling rate can be increased to 5°C per minute.

The next improvement step was done at the temperature level of −30°C to achieve equal temperatures between the chamber and reference ampule (equal to sample) before increasing the cooling rate, as shown in **Table 8** below.

No.	Temperature (°C)	Duration (min)
1	0	5
2	−12	12
3	−20	2
4	−30	10
5	−100	14

Table 6.
Method C freezing cycle program in instrument-specific temperature record, with addition to the program.

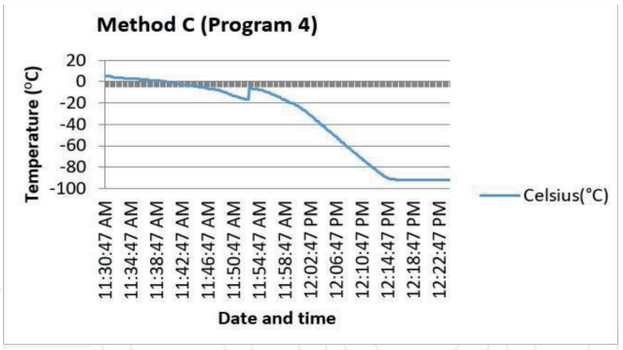

Figure 8.
Method C temperature curve for program 4, using temperature record data-logger.

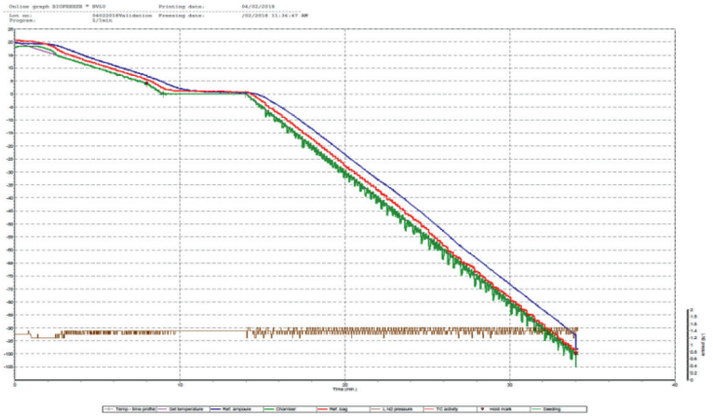

Figure 9.
Curve of method C freezing cycle program in instrument-specific temperature record using program 4.

Program	Temperature	Average difference between chamber and reference probe in ampule	Average difference between reference probe in ampule and set temperature	Parameter criteria
1 °C/1 min	16–0 °C	3.83	1	3
	0 °C to −100°C	1.99	2	
5 °C/1 min	16–0 °C	2.7	1.6	
	0 °C to −100°C	4.9	1.8	
5 °C/1 min [4 °C]	16–0 °C	2.7	1.5	
	0 °C to −100°C	3.4	1.32	

Table 7.
Difference between chamber and reference probe in ampule, and difference between reference probe in ampule and set temperature with regards to different programs.

The instrument-specific temperature recording software has an option to define the ΔT between the set value and the actual value. If ΔT becomes smaller value, the program continues automatically. This is to assure that sample temperature has been stabilized at the chamber temperature before it is cooled down with the defined freezing rates of the freezing program.

No.	Temperature (°C)	Duration (min)		Heat	Hold
1	**0**	**5**	**5**		
2	−12	12	17		
3	−20	2	19		
4	−30	10	29		Stop
5	−100	14	43		

Table 8.
Method C freezing cycle program in instrument-specific temperature record, with addition to the program.

4.3 PBMCs viability using method B and method C (comparative study)

Viability was measured and compared before and after thawing [28]. Lowest result obtained was 95.9% and highest was 98.9% and the difference between each was between 0 and 1% which is in the acceptable range, shown in **Table 9**:

Then, method C protocol with first program that had the same cooling rate as in method B, with 1°C per 1 minute. Viability was measured and compared before and after thawing [28]. Lowest result obtained was 95.6% and the highest as 98.4% and difference between each was between 0 and 1.5% which is within an acceptable range, as shown in **Table 10** below:

Viability was measured based on the difference between pre-thaw and post-thaw viability percentages that were obtained from the flowcytometry. Viability 80% and 75% recoveries are recommended [28], and both methods are within acceptable ranges.

The initial hypothesis was that difference between method B and method C in terms of viability will not be statistically significant, which was proven by Student t test, as shown in **Table 11**. Statistical significance during this comparative study

Sample # in Method B	Pre-thaw Viability %	Post-thaw Viability %	Difference %
TUR0014631	96.7	96	0.7
TUR0014632	98.5	96.7	1.8
TUR0014633	97.3	96.8	0.5
TUR0014634	96.7	97.1	0.4
TUR0014635	97.5	96.5	1
TUR0014636	96.3	96.3	0
TUR0014637	99.9	98.9	1
TUR0014638	97.5	97.8	0.3
TUR0014639	97.2	97.1	0.1
TUR0014640	98.2	98.3	0.1
TUR0014621	97.5	97.4	0.1
TUR0014622	96.4	95.9	0.5

Table 9.
Method B viability overview.

Sample # in Method C (Program 1)	Pre-thaw Viability %	Post thaw Viability %	Difference %
TUR0014631	96.7	96.2	0.5
TUR0014632	98.5	97	1.5
TUR0014633	97.3	96.8	0.5
TUR0014634	96.7	96.7	0
TUR0014635	96.2	97.2	1
TUR0014636	96.3	95.6	0.7
TUR0014637	99.9	98.4	1.5
TUR0014638	97.5	97.8	0.3
TUR0014639	97.2	97.1	0.1
TUR0014640	98.2	98	0.2
TUR0014621	97.5	97.4	0.1
TUR0014622	96.4	96.4	0

Table 10.
Method C viability overview.

Sample No.	Parent tube sample #	Viability % in Method B	Viability % in Method C
1	TUR0014631	96	96.2
2	TUR0014632	96.7	97
3	TUR0014633	96.8	96.8
4	TUR0014634	97.1	96.7
5	TUR0014635	97.5	97.2
6	TUR0014636	96.3	95.6
7	TUR0014637	98.9	98.4
8	TUR0014638	97.8	97.8
9	TUR0014639	97.1	97.1
10	TUR0014640	98.3	98
11	TUR0014621	97.4	97.4
12	TUR0014622	95.9	96.4
	Mean	97.2	97.1
	St dev	0.86	0.76
	N	12	12
	Sp2	11.2	3.3
	t calc	0.03	
	t value from t table for 34 df,	3.06	
	CI 99%		

Table 11.
Comparison between method B and method C, viability percentage.

was calculated using t test for temperature protocols (cooling rate variation): ItI < It tabI 0.003˂2.62 and post thaw viability detected on flow cytometer ItI < It tabI 0.03 < 3.06. Expected accuracy for post thaw viability interval was ±20% and obtained post thaw viability was 0.55%. Precision-reproducibility estimation during 5 different days was post thaw viability 0.66% when the acceptable interval was ±20%.

Precision estimation was designed through 4 days, 6 samples every day, for a total of 24 results from 12 CPT tubes. Between day variation was expected to be low as samples were in a stable frozen state. The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the reproducibility conditions. Statistical calculations were made in Microsoft Excel -Tools> Data Analysis> ANOVA, one factor test ordering Sw interserial standard deviation, and using Microsoft Excel functions to calculate Six, Sb and Stott. Results obtained are shown in **Table 12** below. Coefficient of variation (%) (Stot*100/Xsr) was shown to be 0.79%. Hence, results of precision met the requested acceptable criteria. Viability percentages and difference in pre and post thaw viability was in acceptable criteria, lowest was 0.3 and highest was 4.1, as shown in **Figures 10** and **11**, respectively. **Table 13** illustrates all the validation study parameters that were taken and calculated.

Average	**Xsr**	**95.23**
St dev of average of groups	Sx	0.53
	Sx2	0.29
	Sw2	0.57
	Sb2	0
Total st dev	Stot	0.76
Source of Variation Within Groups	Sw	0.76
Source of Variation Between Groups	Sb	0
Coefficient of variation (%) (Stot*100/Xsr)	CV[%]	0.79

Table 12.
CV % calculated.

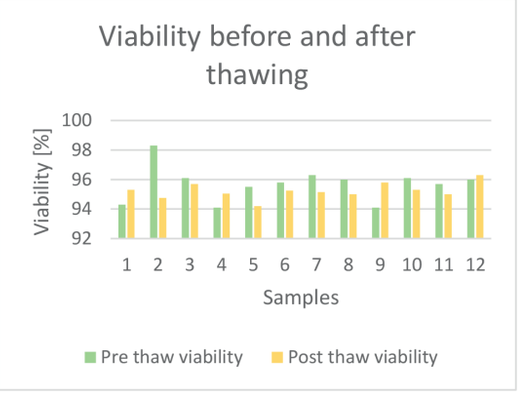

Figure 10.
Pre-thaw and post-thaw viability percentages.

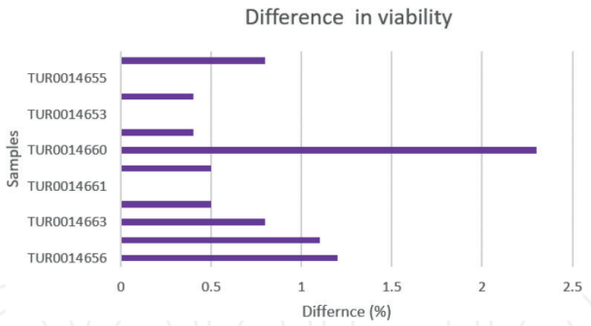

Figure 11.
Pre-thaw and post-thaw viability differences.

Parameter	Required	Obtained	Acceptance	Improvement action
Comparison	T test	Temperature protocols: ItI < It tabI 0.003 < 2.62	pass	
		Viability ItI < It tabI 0.03 < 3.06		
Sensitivity	1 °C/min	1 °C/min	pass	
		5 °C/min (from-30°C)		
		5 °C/min (from −20 °C 4 °C/min)		
Accuracy	Temperature interval ± 3C	Temperature interval < 1.5 °C Post thaw Viability interval 0.55%	pass	
	Post thaw Viability interval ± 20%			
Precision	Temperature interval ± 3C	Temperature interval < 1.5 °C	pass	
	Post thaw Viability interval ± 20%	Post thaw Viability interval 0.66%		

Table 13.
Showing all values obtained through the validation study.

5. Conclusions

The presented study showed that there was no statistically significant difference in cooling methods. However, advantage of Method C is demonstrated in a major decrease in cooling time by reducing the PBMCs processing life cycle, without a need for intermediate storage space while sample traceability is enhanced by using the device software which can be integrated with our LIMS system. The risk of human error, which might occur with Method B, is minimized by reducing operator intervention. Both methods can be used in accordance with laboratory preferences, budget, and guidelines with integrated risk assessments and instrument downtime contingency plans.

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Conflict of interest

The authors declare no conflict of interest.

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