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DESIGN OF A SIMPLE SLOW COOLING DEVICE FOR CRYOPRESERVATION OF SMALL BIOLOGICAL SAMPLES

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Abstract

BACKGROUND: Slow cooling is a cryopreservation methodology where samples are cooled to its storage temperature at controlled cooling rates. **OBJECTIVE:** Design, construction and evaluation of a simple and low cost device for slow cooling of small biological samples. **MATERIALS AND METHODS:** The device was constructed based on Pye's freezer idea. A Dewar flask filled with liquid nitrogen was used as heat sink and a methanol bath containing the sample was cooled at constant rates using copper bars as heat conductor. **RESULTS:** Sample temperature may be lowered at controlled cooling rate (ranging from 0.4ºC/min to 6.0ºC/min) down to ~-60ºC, where it could be conserved at lower temperatures. An example involving the cryopreservation of Neuro-2A cell line showed a marked influence of cooling rate over post preservation cell viability with optimal values between 2.6 and 4.6°C/min. **CONCLUSION:** The cooling device proved to be a valuable alternative to more expensive systems allowing the assessment of different cooling rates to evaluate the optimal condition for cryopreservation of such samples.

Keywords: slow cooling device, cooling rate, cryopreservation, neural cells

INTRODUCTION

During the preservation at low temperature of biological samples there is often a need for carefully controlled cooling conditions to establish the appropriate protocol to assure viability and functionality of them after cryopreservation (5). Consequently several commercial companies have developed systems for controlled freezing of cells and tissues, most of which are expensive and bench space consuming when dealing with small number of low volume biological samples. On the other hand, there are commercial devices designed to freezing biological samples using an isopropanol bath refrigerated in an -80°C freezer or plastic packs refrigerated with solid carbon dioxide (4, 2). These systems are very useful, however they operates at only one cooling rate of 1°C /min which constitute a most disadvantage to study or compare different cooling protocols. For the present study, and
idea (7), we designed and characterized a simple controlled-rate freezing device for the cryopreservation of a reduced number of small biological samples (up to 5 samples of

1 mL) (3). The thermal control of the sample was achieved by the cooling power of a copper bar submerged in liquid Nitrogen (LN_2).

A specific application involving the cryopreservation of a neural cell line is presented to illustrate some of the potential uses of the device: **a-** incubate the sample plus the cryoprotector agent (CPA) at 10°C to allow its diffusion into cells; **b-** cooling the samples at different rates up to a final temperature of -60°C; **c-** record the freezing curve and **d-** determine the sample nucleation temperature in order to monitor the entire process.

Neuro-2A is a mouse neuroblastoma cell line extensively used for neurotoxicity screening and studies of neuronal differentiation, axonal growth and signaling pathways (8). In our work we use these cells only for an application example and more elaborated analysis of results are out of the scope of this manuscript.

MATERIALS AND METHODS

Experimental procedures

There were three main parts in the study. The aim of part I was the construction of the slow cooling device. Part II of this work was aimed to determine the sample cooling rates obtained using three different copper bars (ø: 1/4, 3/8, and 5/8 inches). We also have previously characterized the system behavior by investigating: **i-** the effect of heat exchange medium stirring rate (480, 780 and 960 rpm) on the sample cooling rates produced by different copper bars and **ii-** the maximal number of samples to be frozen in each protocol.

The ultimate goal of our design was the construction of a device which may be used to optimize cryopreservation protocols. In consequence, a brief description of such application involving Neuro-2A cell line is presented on part III.

Part I

The slow cooling device consists in: **a-** a 1900 mL Dewar flask (cat. 8621/6099, POPE Scientific, Inc. Saukville, MI, USA) containing the refrigerant, **b-** a cooling chamber containing the heat exchange medium (HEM) and a carrier to support the samples, **c-** a custom built constant rate magnetic stirrer, **d-** a cylindrical cooper bar, in contact with the HEM and the refrigerant, used as heat conductor and **e-** a TES-1384 electronic thermometer (*TES Electrical, Taiwan*) connected to a PC. A photograph of the device is shown in Figure 1. In our experiments, Liquid Nitrogen (LN_2) was used as refrigerant and methanol (400 mL) was used as HEM. The methanol was contained in a plastic beaker (Polymethylpentene, Griffin Low-Form Beaker, Nalgene, USA, cat: 1203-0400) isolated with a Polyethylene foam sheet (2 mm, Isolant SA, Buenos Aires, Argentina). The heat transfer, and ultimately, the cooling rate of the bath, was fixed by selecting the proper copper bar diameter (1/4, 3/8, and 5/8 inches diameter), each bar was bended in inverted U form (Figure 1B). The length of left side bar arm was 160 mm and right side bar arm was 60 mm long. The design of the support platform and the bended Copper bar make possible that the bar right side segment (submerged in the HEM) and the bar left side segment (submerged in the refrigerant) were always the same length for all bars.

To ensure maximal thermal contact between the HEM and the specimen, 1.0 mL cryovials (NUNC™, Cat. No.366656) containing 1.0 mL suspension of cells, were positioned on a custom-built carrier (Figure 2), submerged into the methanol and cooled under constant stirring. Sample and methanol bath temperatures were measured every 10 seconds, the data collected, and a temperature *vs.* time graph was generated. Sample cooling rate was estimated using the sample itself as reference. This was done by recording the temperature inside one cryovial containing the sample with a type K thermocouple. The cooling rate was calculated by linear regression of the linear part (between 0ºC and -10ºC) of the cooling curve previous to spontaneous crystallization (see Figure 3). The phase transition time (related to the latent heat of

crystallization) may also be determined measuring the time spent between the temperature at the release of the heat of crystallization (t_1) and the time at which the measured temperature returned to the temperature at which freezing started (t_2) (shown in the insert on Figure 3).

Thermocouple Calibration

The type K thermocouples were calibrated by comparison with a reference thermometer (N-168 glass thermometer, Ever-Ready Thermometer Co, Inc. New Jersey, USA) in a thermally stabilized bath (Mod. 9501, Digital Refrigerated Circulating Bath, Polyscience , ILL, USA) at six fixed-point temperatures (20, 10, 5, 0, -5, -10ºC). In the liquid-filled bath (50 % ethylene glycol/H_2O), the thermocouples and the reference thermometer were immersed with a separation of about 1 cm without contact between them. To develop the comparison calibration, the following measurement sequence was followed: at the fixed temperature, we recorded: S1, X1, X2.... Xn temperatures in the TES-1384 electronic thermometer, then the bath temperature was changed and after 15 minutes of stabilization the second sequence of measurements was registered: S2, X1, X2.... Xn. Where S1 and S2 are the temperatures registered by the reference thermometers and X1, X2.... Xn are the temperatures from thermocouples to be calibrated. This sequence was repeated to give six temperature measurements on each thermocouple. After that, a linear regression of the determined temperature in function of the reference temperature was made and a correction factor determined for each thermocouple.

Part II: Characterization of the system behavior

Different configurations and setups of the proposed design were evaluated for the characterization of the device. An efficient heat transfer between samples and HEM was pursued not only to assure a wider range of cooling rates attainable but mainly to minimize the effect which the liberation of the latent heat during freezing of water may produce on the HEM temperature. Number of samples and stirring rates were evaluated together with cooling rates using different copper bars (or combinations) on two set of experiments:

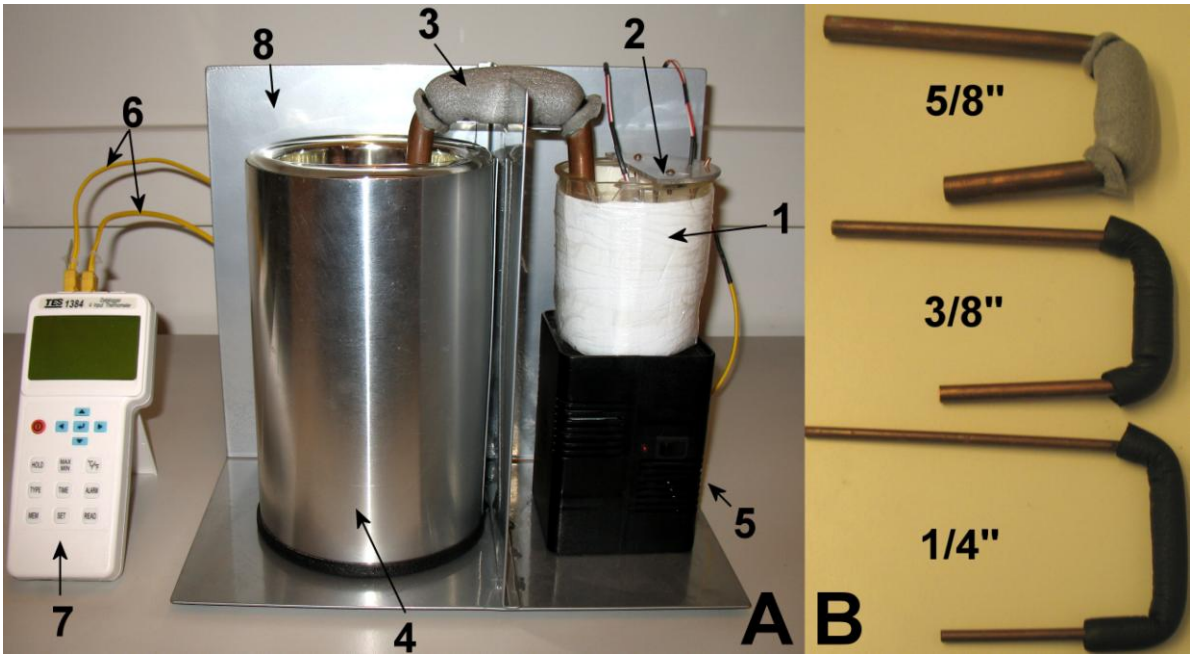

Figure 1. **A**- Slow cooling device: 1-cooling chamber, 2-sample carrier, 3-copper bar, 4-Dewar flask, 5-constant magnetic stirrer, 6-Thermocouples, 7-TES-1384 electronic thermometer and 8-support platform. **B**-Various diameter copper bars used as heat conductors.

I) We studied the effect of HEM stirring rate on the cooling rate produced by different copper bars on five cryovials positioned in the carrier. To do this, we tested three copper bars (ø: 1/4, 3/8, and 5/8 inches) and three stirring rates of the HEM (480, 780 and 960 rpm) in 5 cryovials containing 1.0 mL of inactivated foetal bovine serum (FBS) supplemented with 10% (w/v) of DMSO positioned in the carrier.

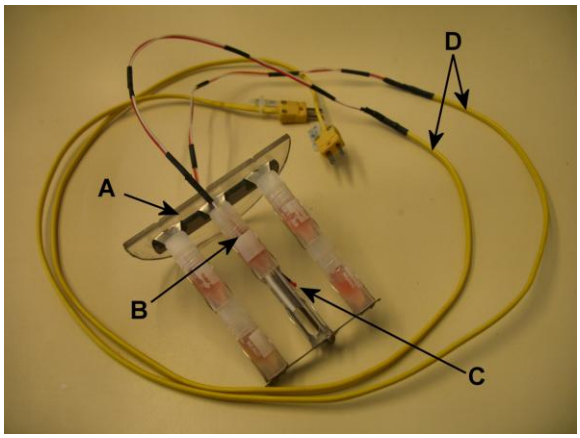

Figure 2. Custom built sample carrier: A- sample carrier, B- cryovial containing a type K thermocouple, C- HEM monitoring type K thermocouple and D- thermocouple connectors.

II) To evaluate the effect of sample number on HEM cooling rate we recorded the HEM temperature time course evolution produced by one to five samples (1 mL) positioned in the carrier.

Part III: Specific application

Finally, a specific application involving optimization of a neuronal cells line cryopreservation protocol is included to illustrate the potential uses of the proposed device. Neuro-2a cells were cultured on 75 cm^2 culture flask at a concentration of 6.8 x 10^5 viable cells/15mL in MEM medium (61100 Gibco, Life Technollogies) plus 10% (w/v) of FBS. After reaching 80% confluence, cells were detached by treating the cell layer with 0.25% (w/v) Trypsin - 0.53 mM EDTA solution for 5 min at 37ºC. Trypsin was inactivated by adding complete medium. Cell number and viability was evaluated by Trypan Blue test. Briefly, 10 µL of cell suspension was diluted 1:50 in 0.4% (w/v) Trypan Blue solution. Living (unstained) and dead (blue stained) cells were counted in a Neubauer chamber and the percentage of viable cells was calculated. Cell suspension was centrifuged 5 min. at 170 g. Pellet was resuspended with inactive FBS supplemented with 10% (w/v) of DMSO at a final cell concentration of 2 x 10^6 viable cells/mL. Cell suspension was aliquoted in four cryovials. One of which contained a type K thermocouple to allow the recording of the sample temperature during the cooling process.

Cryovials were allocated in the sample carrier and immersed on a previously cooled methanol bath at 10ºC during 10 min to ensure the proper diffusion of CPA. Four different cooling rate protocols were performed until sample temperature reached -50ºC. Finally cryovials were placed in LN_2 for storage for at least 2 days.

For rewarming, cryovials were thawed by gentle agitation in a 37ºC water bath. Dilution of the CPA was performed by adding 9 mL of complete medium and 5 min. centrifugation at 170 g. Supernatant was discarded and cells were resuspended by adding 3 mL of complete medium. Post rewarming cell number and viability was evaluated by Trypan Blue test in a Neubauer chamber.

Significant differences between groups were determined by one-way ANOVA, with a Tukey-Kramer multiple comparisons post test.

RESULTS

Thermocouple Calibration

All the utilized thermocouples showed a linear response in the range of temperatures used for calibration yielding a linear regression of the form:

$$T_{ref} = T_c \times \boldsymbol{a} + \boldsymbol{b}$$

Where T_{ref}: glass thermometer temperature; T_c: thermocouple temperature measurement; $\boldsymbol{a}$ and $\boldsymbol{b}$ where linear regression coefficients. This regression was used to correct all the temperature values. In all cases $\boldsymbol{a}$ values

were always between 0.9 and 1.1 and ***b*** values were never higher than ± 0.5ºC

cells) the temperature of the bath was decreased at a fixed cooling rate, using a

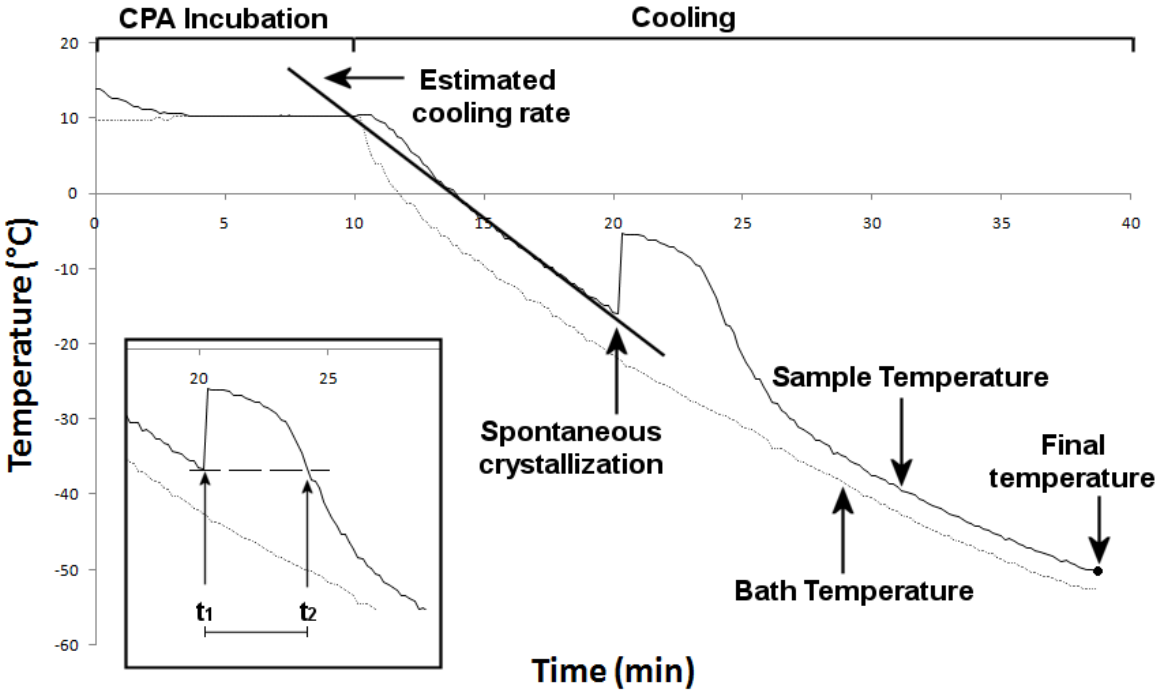

Figure 3. A typical temperature profile for a given sample treated as described in text. The first 10 minutes represent the incubation at 10°C. Thick line represents the estimated cooling rate. The insert shows the phase transition time (heat of crystallization) (t_2-t_1)

The effect of heat exchange medium stirring rate on the sample cooling rate

Tested HEM stirring rates showed no effect on samples cooling rate, so an intermediate rate (780 rm.) was selected (data not shown).

The maximal number of samples to be frozen

Trough the monitoring of the HEM temperature time course evolution at different number of samples to be cryopreserved, no differences on the cooling rates were observed. In consequence, the maximal number of samples positioned on the carrier was established in six (including the reference sample).

A typical Cooling curve and parameters

Figure 3 shows a representative temperature profile obtained during the cryopreservation of Neuro-2A cell line. After the incubation period (10 min. at 10 °C - used to facilitate the ACP diffusion into the liquid nitrogen (LN_2) reservoir as a "heat sink" in the configuration described by Pye (7). A cylindrical cooper bar in contact with the methanol bath and the LN_2 was used as heat conductor. The heat transfer, and ultimately, the cooling rate of the bath, was fixed by selecting a proper copper bar diameter.

In the selected example using the 3/8" copper bar, the cooling rate was 1.43°C/min, the spontaneous crystallization occurs at -15.9°C, the melting point was -5.2°C, the phase transition time was 4 min and the final cooling temperature was -50.2°C.

Cooling rates and copper bar diameter

Table 1 shows the cooling rates obtained using different diameters of the heat conductor. Five cooling rates were obtained by running the system using different single and combined copper bars. A volume of 2 L of LN_2 was required for each run.

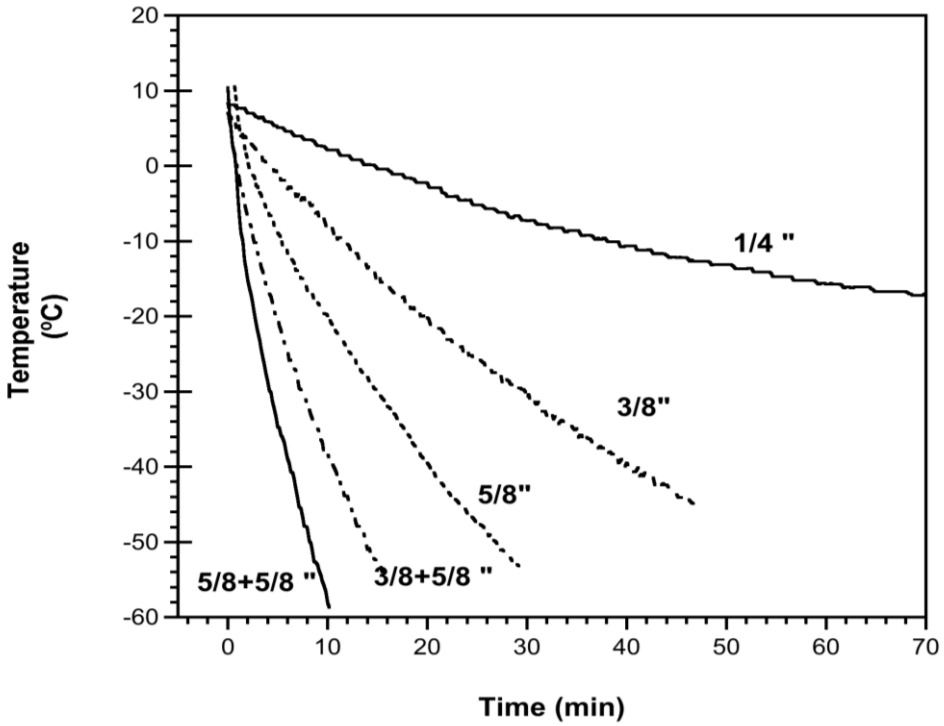

Figure 4. Time course evolution of HEM temperatures during cryopreservation protocols carried out with different copper bars.

Temperature profiles and copper bar diameters

Figure 4, shows the HEM temperature profiles obtained using the 1/4, 3/8, 5/8 and the combination of 3/8+5/8 and 5/8 + 5/8 copper bars.

The relationship copper bar diameter and cooling rate

Figure 5 expose the lineal relationship between the copper bar diameter and the cooling rate obtained when using single copper bars. On the other hand, this relationship was not maintained when the combination of two copper bars was used. In addition, it is possible to machining a copper bar to obtain a specific cooling rate in the studied cooling rates interval (0.4 to 3.5°C).

Cell line cryopreservation

Cell viability, before and after cryopreservation, was evaluated at different cooling rates. Post rewarming viable cell yield (number of viable cells after rewarming, normalized to the number of viable cells before cryopreservation) was also evaluated.

Table 2 shows cryopreservation parameters for different cooling rates obtained with the proposed device. In this particular case, optimal cooling rate was established between 2.6ºC/min and 4.9ºC/min. At these cooling rates, cell viabilities were significantly higher when compared with 0.4ºC/min and 6.0ºC/min. Nevertheless, no statistical differences were detected when viable cell yields were evaluated.

Copper bar diameter (inches)	Sample cooling rate (°C/min)	S.D. (°C/min)	n
1/4	0.40	0.05	4
3/8	1.46	0.09	4
5/8	3.44	0.30	4
3/8 + 5/8	4.61	0.67	4
5/8 + 5/8	6.25	0.07	2

Table 1. Estimated cooling rates for different copper bars used as heat transfer units. (*S.D: standard deviation; n: number of replicates*)

These experiments briefly illustrate the possible applications of the suggested device.

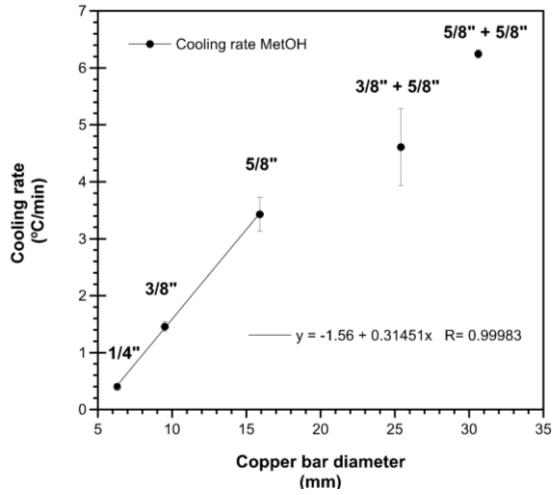

Figure 5. The relationship of sample cooling rate vs. copper bar diameter.

DISCUSSION

The proposed device has a simple design, with many potential applications in cryopreservation of biological samples such as cells suspensions and tissues from various sources. In our laboratory we have recently used this device to cryopreserve rat neural cells (3). Ware et al (9) have applied the Pye´s idea to the controlled-rate freezing of human embryonic stem cells, but they used only one cooling rate running on a basic system. With the cryopreservation device used in this work it became possible to determine optimal parameters, such as the cooling rate, the final cooling temperature, the nucleation temperature and the time spent on the transition phase. The design of the sample carrier was appropriate for the spontaneous nucleation of samples. Furthermore, the device could be useful to set up standard conditions for the controlled seeding of the samples by touching the cryovial with a chilled needle or forceps. By modifying the sample carrier it is also feasible to employ the proposed design with plastic straws widely used for preservation of sperm and other samples.

For traditional cryopreservation, the first step in the preservation protocol of cells or tissues is often the loading of a CPA into the biological sample. The time during which cryoprotectants penetrate within the tissue is known as ‘‘equilibration time’’. The success of a particular protocol is usually influenced by the type and concentration of cryoprotectant, the equilibration time and the temperature range over which the CPA loading ought to be controlled (1). The operation of our device using two coiled copper wires (ø 2.8 mm) bended as described in M&M, could maintain a 10ºC temperature during the time required for CPA loading into neural cells (see Figure 3).

Regarding other technical requirements, the high specific heat of liquids renders them appropriate as HEM. Alcohols are widely used in liquid cooling baths for temperatures below -70ºC, but cannot be used much below because they become very viscous and eventually freezes (6). In our work we have chosen methanol as HEM (specific heat: 2.47 J/g ºC, freezing point: -97ºC). Other alcohols such as ethanol (specific heat: 2.29 J/g ºC, freezing point: -114ºC) or isopropanol (specific heat: 2.68 J/g ºC, freezing point: −89°C) could also be used as HEM. Nevertheless these alcohols

Cooling Rate (ºC/min)	Initial Cell Viab. (%)	Post rewarming Cell Viab. (%)	Post rewarming Viab. Cell Yield (%)
0.4 ± 0.1[a]	90.0 ± 0.6	52.1 ± 5.7***	41.8 ± 6.3
2.6 ± 0.5	86.2 ± 8.2	90.3 ± 1.3	70.4 ± 19.4
4.6 ± 0.5	85.7 ± 6.2	88.3 ± 4.3	67.6 ± 19.4
6.0 ± 0.5	91.5 ± 1.0	76.9 ± 4.2*	61.6 ± 19.9

Table 2. Cell line cryopreservation results at different cooling rates. [a] Final temperature -25ºC. *** $p<0.001$ when compared with 2.6ºC/min, 4.6ºC/min and 6.0 C/min. * $p<0.05$ when compared with 2.6ºC/min and 4.6ºC/min.

are not identical and the system must be tested and calibrated (for attainable cooling rates) for each potential HEM before being used routinely in the lab. Also, required security measures must be taken according to the selected media. Particularly when using methanol, the device should be operated on a well-ventilated space or inside a fume hood.

Liquid nitrogen (LN_2) was selected as refrigerant because is easily available and it has a boiling point of -196 ºC at 1 atm of barometric pressure, which is suitable as an efficient cold sink. Each freezing protocol consumes approximately 2 L of LN_2 which render economical the operation of the device.

During the freezing procedure, the samples release the latent heat of fusion due to ice formation, which may increase the cooling chamber temperature depending on the mass and dimensions of the samples. This fact could change the cooling rate altering the cryopreservation protocol. In our device it was possible to monitor the temperature of the HEM during the ice crystallization in order to evaluate the effect of the latent heath of fusion on the HEM temperature and the cooling rate. We are aware of the fact that, under spontaneous nucleation, the samples do not crystallize simultaneously and that heat of fusion of each sample is not necessarily released at the same time. To validate the assumption of constant cooling rate under these circumstances we decided to record the temperature of the HEM during the entire process.

However, there are some small disadvantages to consider. Special care must be taken in order to maintain LN_2 level in the Dewar flask. This is particularly important when using high diameter copper bars or combinations. A decrease of the LN_2 level ultimately results on a modification of the cooling rate of the methanol bath and the sample. As proposed by Pye, insulation of the cooling bar may be extended to below the liquid nitrogen level reducing the effect of level modifications on cooling rate. Nevertheless, this option reduces the range of cooling rates attainable with the reported configuration. In the present work, multiple rates have been tested so flexibility on the cooling rate was a requirement, and constant refilling of the Dewar flask was adopted as a solution. Further studies, to be performed at single cooling rates may, however, use the Pye's solution of extended insulation of the copper bar.

The inclusion of a custom builds magnetic stirrer in the design was based on the need of a controlled and quantified stirring rate and may be replaced by commercial equipment without affecting the performance of the cooling device.

Another point to be taken under consideration is the final temperatures limitation when using small diameter bars. Especially in the case of the 1/4" bar, only temperatures as low as ~-25ºC could be achieved. Around these temperatures the heat exchange between methanol bath and LN_2 equals the heat exchange between methanol bath and surrounding air resulting on a cooling rate of virtually 0ºC/min.

Although the design has proven to be robust and reliable, pertinent testing and calibrations are required before the system may be used regularly for sample processing. Also, further mechanical modifications of copper bars may improve heat exchange between the HEM and the LN_2 adding extra flexibility on the attainable cooling rates.

In summary, the proposed device meets the requirements of a simple controlled cooling system to be used with small number of relative low volume biological samples.

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