Published in final edited form as:

Cryobiology. 2014 April ; 68(2): 176–184. doi:10.1016/j.cryobiol.2013.12.003.

Rationally optimized cryopreservation of multiple mouse embryonic stem cell lines: II—Mathematical prediction and experimental validation of optimal cryopreservation protocols*

Corinna M. Kashuba^{a,*}, James D. Benson^b, and John K. Critser^c

Corinna M. Kashuba: ckashuba@niu.edu

^aDepartment of Biological Sciences, Northern Illinois University, DeKalb, IL 60115, USA

^bDepartment of Mathematical Sciences, Northern Illinois University, DeKalb, IL 60115, USA

^cUniversity of Missouri Comparative Medicine Center, 4011 Discovery Drive, Columbia, MO 65201, USA

Abstract

In Part I, we documented differences in cryopreservation success measured by membrane integrity in four mouse embryonic stem cell (mESC) lines from different genetic backgrounds (BALB/c, CBA, FVB, and 129R1), and we demonstrated a potential biophysical basis for these differences through a comparative study characterizing the membrane permeability characteristics and osmotic tolerance limits of each cell line. Here we use these values to predict optimal cryoprotectants, cooling rates, warming rates, and plunge temperatures. We subsequently verified these predictions experimentally for their effects on post-thaw recovery. From this study, we determined that a cryopreservation protocol utilizing 1 M propylene glycol, a cooling rate of 1 °C/minute, and plunging into liquid nitrogen at –41 °C, combined with subsequent warming in a 22 °C water bath with agitation, significantly improved post-thaw recovery for three of the four mESC lines, and did not diminish post-thaw recovery for our single exception. It is proposed that this protocol can be successfully applied to most mESC lines beyond those included within this study once the effect of propylene glycol on mESC gene expression, growth characteristics, and germ-line transmission has been determined. Mouse ESC lines with poor survival using current standard cryopreservation protocols or our proposed protocol can be optimized on a case-by-case basis using the method we have outlined over two papers. For our single exception, the CBA cell line, a cooling rate of 5 °C/minute in the presence of 1.0 M dimethyl sulfoxide or 1.0 M propylene glycol, combined with plunge temperature of –80 °C was optimal.

Keywords

ES cells; Embryonic stem cells; Embryonic stem; Cryopreservation; Fundamental cryobiology; Membrane permeability; Osmotic tolerance limits; Osmotically inactive cell volume; Optimization; Biophysics

☆Statement of funding: This work was supported, in part, by grants from the National Institutes of Health, National Center for Research Resources (U42RR14821, T32-RR07004, and R41RR025927) and the Cryobiology Research Institute.

© 2014 Elsevier Inc. All rights reserved.

*Corresponding author. Fax: +1 (815) 753 0461.

Introduction

Mouse embryonic stem cells (mESCs) have been developed from numerous genetic backgrounds and have contributed to a virtual explosion in the number of existing genetically engineered mouse (GEM) models available for human biomedical research [4,5]. Thousands of additional mutant mESC lines will be created through coordinated efforts to systematically knock out all mouse genes via projects such as the Knockout Mouse Project (KOMP) [1], the North American Conditional Mouse Mutagenesis Program (NorCOMM, <http://norcomm.phenogenomics.ca>), and the European Conditional Mouse Mutagenesis Program (EUComm, <http://www.eucomm.org>). Storage of valuable genetic material in the form of mESCs is cost-effective and requires minimal space; however, reported variation of 10-90% post-thaw recovery (PTR), depending on the cell line [8,15,16,23,31], raises concerns regarding both the efficacy and reliability of its approach.

Fundamental biophysical cryobiological parameters describe osmotically driven transport across cell membranes and are key to hypothesis-driven predictions of successful outcomes from cryopreservation protocols [10]. They can be used to calculate osmotically driven volume excursions during the addition and removal of cryoprotectants (CPAs) and during the cooling and thawing process [11,12,18,20]. Using these parameters, optimal cooling rates and plunge temperatures can be predicted with the aim of cooling cell suspensions as rapidly as possible without causing a large difference between the intracellular freezing point and the intracellular temperature; warming rates tend to be optimal when they mimic their corresponding cooling rates [19].

We previously described a method to systematically analyze the fundamental cryobiological parameters of a C57BL/6 mESC line [16]. In that study we conducted theoretical simulations utilizing Mazur's two-factor hypothesis [21] to predict optimal cooling rates and plunge temperatures (target temperatures for the rapid plunging of cells into liquid nitrogen for the intent of vitrifying the remaining water that are calculated based on vitrification-favoring concentrations of CPA) for this cell line, and performed experimental validations of our predictions. In Part I of the present study [15], we used the same systematic approach and analysis to characterize the fundamental cryobiological parameters of four additional mESC lines, (BALB/c, CBA, FVB, and 129R1) in order to test the hypothesis that mESCs from different genetic backgrounds would have significantly different biophysical parameters, potentially explaining variation in cryopreservation success among mESC lines. Here in Part II we use the data from Part I to predict and test optimal protocols among the lines.

One very unique aspect of this study is the overall perspective that it gives to “classical” optimization of cryopreservation protocols. Namely, we predict and test multiple hypotheses for multiple cell lines simultaneously, allowing analysis of questions such as seeding, plunge temperatures, superzero vs. subzero permeability, and more.

Materials & methods

Embryonic stem cells

The following mESC lines were acquired at passage 11–12: BALB/c (Thromb-X Group, Chemicon International, Temecula, CA, now part of Millipore, Billerica, MA), CBA (Thromb-X Group), FVB/N (Thromb-X Group), and 129R1 (A. Nagy, Mount Sinai Hospital, Toronto, Canada). Mouse ESC cultures were negative for all pathogens (IMPACT I test, Research Animal Diagnostic Laboratory, Columbia, Missouri; www.radil.missouri.edu).

Cell culture and standard cryopreservation method

Mouse ESCs were cultured on primary mouse embryonic fibroblast cells (PMEF) (Millipore, Billerica, MA) at 37 °C and 5% CO₂. Culture media for the 129R1 mESC line and BALB/c cell line contained 15% Defined FBS (Hyclone, Logan, UT), 0.1 mM non-essential amino acids (GIBCO/Invitrogen, Carlsbad, CA), 1.0 mM sodium pyruvate (GIBCO), 100 µM beta-mercaptoethanol (Sigma Aldrich, St. Louis, MO), 50 IU/mL penicillin (GIBCO), 50 µg/mL streptomycin (GIBCO), and 1000 U ESGRO/mL (Millipore) in high glucose DMEM (Millipore). CBA and FVB mESCs were cultured in RESGRO culture medium (Millipore). Embryonic stem cells were passaged and/or collected every 2 days or at approximately 80% confluence.

For the standard slow cooling method, cells were resuspended in freezing medium (1.3 M (10%) Me₂SO (Sigma Aldrich), 50% defined fetal bovine serum (Hyclone), and 40% culture medium) in 1 mL aliquots in cryovials (Nalgene Nunc International, Rochester, NY). Cryovials were transferred to a commercially available freezing kit (Nalgene), stored at –80 °C overnight (a process which cools at a rate of 1 °C/minute), and subsequently transferred to liquid nitrogen (LN₂).

Embryonic stem cells were used within 10 passages from the original, and were of normal karyotype at the highest passage. Cell counts were performed using a hemacytometer and Trypan blue stain (Sigma Aldrich) for membrane integrity for all standard cultures.

Separation of mESCs from feeders by differential sedimentation

For all experiments, feeder cells were separated from mESCs by differential sedimentation, a technique previously described by Doetschman [9]. There are many variations of this method [14,25,32], which exploits the difference in the rate at which fibroblast feeder cells and mESCs settle and adhere to culture dishes. Briefly, trypsinized mESC cultures containing PMEF were centrifuged, resuspended in 10 mL of culture medium, and plated on the original 100 mm cell culture dish. After 30 min at 37 °C, the cell suspension was transferred to a second culture dish for one-hour incubation at 37 °C. Following the second incubation, the cell suspension was removed, and these collected mESCs were counted, centrifuged, and resuspended in either DPBS or culture medium for experimentation. Our application of the Doetschman sedimentation method resulted in greater than 99% removal of contaminating feeder cells from the ES cell suspension (data not shown).

Theoretical simulations

We used the method previously described by Kashuba Benson et al. [16] to conduct theoretical simulations in order to determine optimal CPA addition and removal protocols of a 1.0 M solution of dimethyl sulfoxide (Me₂SO), 1,2-propanediol (PG), or ethylene glycol (EG) for BALB/c, CBA, FVB, and 129R1 mESCs using biophysical parameters determined in Part I of this study [15,35]. As previously described, a CPA equilibration (addition or removal) protocol was defined to be optimal if it minimized the number of addition and dilution steps while maintaining mESCs within the defined range of osmotic tolerance. Briefly, we used the temperature dependent two parameter model:

$$\frac{dW}{dt} = -L_p ART \left(M_s + M_n - \frac{S+N}{\rho W} \right), \frac{dS}{dt} = P_s A \left(M_s - \frac{S}{\rho W} \right) \quad (1)$$

where the parameters are defined in Table 1, and the temperature dependence of L_p and P_s follow the Arrhenius relationship

$$P(T) = P_0 \exp \left[\frac{E_a}{R} \left(\frac{1}{T_0} - \frac{1}{T} \right) \right],$$

where P can be either L_p or P_s , and P_0 is the parameter value at room temperature. System (1) is coupled with initial conditions that depend on the protocol being modeled. We determine $M_s(T)$ and $M_n(T)$ from either published or synthetic phase diagrams for Me₂SO and PG, respectively [29,36], and note that A is the volume independent spherical surface area at V_{iso} .

For CPA equilibration (addition and removal) protocols, the overall goal was to minimize cell volume excursion and cryoprotectant exposure time at ambient temperatures. This was accomplished using a numerical optimization scheme in conjunction with the exact solution of Eq. (1) (c.f. [6]), and follows the ideas outlined in [7]. Model conditions included osmotic tolerance limits from Part I of this study, initial intracellular concentration, and temperature. The program automatically optimized addition and removal steps and provided the appropriate concentration of the diluent.

Theoretical optimization of cooling protocols was performed, based on Mazur's two-factor hypothesis [21], in conjunction with measured or predicted ternary phase diagrams [26,29], as previously described for the C57BL/6 cell line [16]. According to Mazur's two factor hypothesis, sub-optimal cooling rates cause damage due to unnecessarily prolonged exposure to the high concentrations of solutes that occur at low temperatures, and super-optimal cooling rates cause damage due to insufficient cellular dehydration that leads to deleterious intracellular ice formation. The optimal cooling rate is that which minimizes cooling exposure time while maintaining at most two degrees of supercooling [21]. In effect, cells should be cooled as quickly as possible without causing the intracellular concentration to be such that the freezing point is more than two degrees above the cellular environment.

We utilize a two-step freezing protocol, where cells are cooled at a controlled rate to a “plunge temperature”, at which point they are immersed in liquid nitrogen, the goal being to minimize intracellular ice formation and promote vitrification [16]. Computer simulations based on the above model estimated the temperature at which the combination of cooling rate and initial CPA concentration would result in an intracellular CPA concentration of 40% by weight, and this was deemed the optimal plunge temperature. Computer simulations of freezing for all cell lines and CPAs were performed iteratively until optimal cooling rates were determined, as previously described [16].

As with the C57BL/6 mESC line [16], the two-parameter mathematical model described above was used to simulate mass transport during warming. The subsequent volume excursion upon equilibration was compared with predicted osmotic tolerance limits [13]. If warming is rapid, the case may occur that cells are hypertonic compared to their surrounding liquid media at room temperature, causing a water influx and potential concomitant osmotic damage [13]. Warming rates up to 1×10^4 °C/minute were simulated to verify that damage due to volume changes would not occur, and the optimal warming rate was considered to be that which was fast enough to prevent devitrification, yet not “too fast” such that after warming, the influx of water would cause the cell to exceed osmotic tolerance limits. A typical method for warming is to place a vial in room temperature or 37 °C water and it remains only to verify that cell volume excursions are within the osmotic tolerance limits[16].

Empirical validation of predicted optima

Empirical validation of predicted freezing rates was conducted with 1.0 M PG and 1.0 M Me₂SO. Each mESC line was cultured in standard conditions to 80% confluence, separated from fibroblast feeders, and resuspended in freezing medium at a concentration of 1×10^6 cells/mL Propylene glycol freezing medium consisted of 1.0 M PG (Sigma), suspended in 129R1/BALB/c culture medium and 50% v/v defined fetal bovine serum (FBS) (Hyclone). Dimethyl sulfoxide freezing medium consisted of 1.0 M Me₂SO (Sigma), suspended in 129R1/BALB/c culture medium and 50% v/v FBS. Cells were cooled in sealed 250 µL cryostraws (IMV International, Maple Grove, MN) in a Planer Kryo 10 Series III programmable freezer (TS Scientific).

A $2 \times 2 \times 2 \times 2$ factorial design was used to compare protocols using combinations of PG vs. Me₂SO, predicted optimal vs. suboptimal cooling rates, seeding (which is defined as the induced nucleation of intracellular ice crystals, theorized to increase the uniformity of sample cooling [28,33], and was achieved by application of a LN₂-cooled forceps to the freezing vessel until visible ice crystals form) vs. no seeding, and predicted optimal plunge temperatures vs. the standard -80 °C. Please refer to Table 1 for cell line- and CPA-specific predicted optimal cooling rates and plunge temperatures. Standard conditions were considered 1 M Me₂SO, 1 °C/minute, and a plunge temperature of -80 °C.

Non-optimal rates were defined as the predicted optimal rate for the opposite CPA when the predicted optimal cooling rate was 1 °C/minute for at least one CPA. In the case of the FVB cell line, where neither Me₂SO nor PG had predicted optimal cooling rates of 1 °C/minute,

predicted optima of 3.8 °C/minute (Me₂SO) and 3.3 °C/minute (PG) were compared to a non-optimal rate of 1 °C/minute (*i.e.* the current standard cooling rate).

As previously described for the C57BL/6 mESC line [16], theoretical simulations predicted that warming rates between 10 and 1×10^4 °C/minute would have negligible effects on cell survival. Cryostraws were thawed in a room temperature water bath (22 ± 1 °C), resulting in a measured warming rate of approximately 700 °C/minute (data not shown). Embryonic stem cells were thawed, diluted drop-wise by 5 volumes of culture media, centrifuged, resuspended in $1 \times$ PBS, stained with propidium iodide, and passed through a FACScan flow cytometer (BD Biosciences, San Jose, CA) for analysis of percent post-thaw recovery (PTR).

Statistical analysis

Data were analyzed using standard analysis of variance (ANOVA) performed using the SAS General Linear Models program (SAS Institute, Inc., Cary, NC) with a value of $p < 0.05$ used for significance. In situations where a plot of the residuals of a data set was not normally distributed, data were normalized using either natural logarithm or square root transformation. Post-thaw recoveries were compared to standard cryopreservation conditions for each mESC line using Tukey's test. All values are given as mean $\pm$ SEM, unless stated otherwise.

Results

Theoretical simulations

Predicted optimal cooling rates and plunge temperatures for the four mESC lines included in this study are listed in Table 1. To limit the scope of this factorial study, we chose only to use PG and Me₂SO for CPA optimal testing.

Seeding

Initial studies comparing the effects of seeding *vs.* not seeding revealed no significant difference (data not shown); therefore, these comparisons were not included in additional studies.

Empirical validation of predicted optima

It is important to note that predicted optimal plunge temperatures are only optimal for the associated predicted optimal cooling rates, however in order to define them from the standard plunge temperature of -80 °C, we refer to them as “predicted optimal” plunge temperatures in the presentation and discussion of our results.

Overview of the principle experimental factors affecting all four mESC lines

Compared to the standard protocol, there was a significant beneficial effect on PTR of choosing PG. The use of 1 M PG resulted in higher PTR than 1 M DMSO ($p < 0.05$). With the exception of the CBA mESC line, the predicted optimal cooling rates significantly improved PTR ($p < 0.05$) with the use of either cryoprotectant. All results are summarized in Fig. 1.

There was a significant negative effect on PTR of using the predicted plunge temperatures rather than the standard plunge temperature of -80°C . There was also a significant negative effect for the interaction of cooling rate and plunge temperature for the mESC lines CBA and 129R1, where the predicted optimal plunge temperature with the predicted optimal cooling rate resulted in a significantly lower PTR than with sub-optimal cooling rate and the standard plunge temperature of -80°C ($p < 0.05$).

Finally, there was a significant three-way interaction among CPA, cooling rate, and plunge temperature ($p < 0.05$) for all mESC lines, the results of which will be presented per individual mESC line.

Empirical validation of predicted optima for the BALB/c mESC line

The standard cryopreservation method yielded a PTR of $24.0 \pm 3.9\%$ with the BALB/c mESC line.

Three experimental protocols utilizing predicted optimal cooling rates for both Me_2SO and PG resulted in significantly improved PTR by 1.8 to 2-fold; these protocols are listed with their associated PTR in Table 2. The predicted optimal plunge temperature was utilized in one of these protocols; the remaining two protocols that improved PTR utilized standard plunge temperatures.

Predicted optimal cooling rates did not significantly decrease PTR for any cell line except for the BALB/c mESC line, in which one experimental protocol involving 1 M Me_2SO and combining the predicted optimal cooling rate and predicted optimal plunge temperature significantly decreased PTR to $9.8 \pm 3.0\%$ ($p < 0.05$).

Empirical validation of predicted optima for the CBA mESC line

The standard cryopreservation method yielded a PTR of $12.2 \pm 0.7\%$ for the CBA mESC line.

The predicted optimal cooling rate for 1 M Me_2SO combined with standard plunge temperature significantly improved PTR of CBA mESC by 1.7-fold. The use of the predicted optimal plunge temperature with the predicted optimal cooling rate for 1 M Me_2SO resulted in a PTR that was not significantly different from that of the standard method, but was significantly lower than in the same conditions using the standard plunge temperature ($p < 0.05$).

The suboptimal cooling rate for 1 M PG combined with either the standard plunge temperature or the predicted optimal plunge temperature significantly improved PTR 1.9 and 1.7-fold, respectively ($p < 0.05$). Predicted optimal cooling rates for 1 M PG did not significantly improve PTR. Please refer to Table 3 for a summary of protocols that improved PTR of the CBA mESC line.

Empirical validation of predicted optima for the FVB mESC line

The standard cryopreservation method yielded a PTR of $3.6 \pm 0.5\%$ for the FVB mESC line.

Predicted optimal cooling rates for both Me₂SO and PG resulted in significantly improved PTR using both predicted optimal and standard plunge temperatures. The highest PTR ($p < 0.05$) was obtained using Me₂SO, and the associated predicted optimal cooling rate, and predicted optimal plunge temperature.

The suboptimal cooling rate for 1 M PG, in combination with the predicted optimal plunge temperature resulted in a 1.9-fold improvement in PTR as compared to the standard method; however, the improvement in PTR using this method was significantly lower than the 4.4 to 7-fold improvement gained using the predicted optimal cooling rates. Please refer to Table 4 for a summary of protocols that improved PTR of the FVB mESC line.

Empirical validation of predicted optima for the 129R1 mESC line

The standard cryopreservation method yielded a PTR of $28.4 \pm 1.3\%$ ($p < 0.05$) for the 129R1 mESC line.

The standard cooling rate of 1 °C/minute was also the predicted optimal cooling rate for Me₂SO, and the use of the predicted optimal plunge temperature with this cooling rate significantly improved PTR by 1.5-fold over the standard method.

Predicted optimal cooling rates for PG did not significantly improve PTR for the 129R1 mESC line, however the suboptimal cooling rates for PG combined with the predicted optimal and standard plunge temperatures significantly improved PTR by 1.4 and 2.1-fold, respectively. Please refer to Table 5 for a summary of protocols that improved PTR of the 129R1 mESC line.

Discussion

The purpose of this study was to test the hypothesis that by applying fundamental cryobiological principles to a given CPA, predicted optimal cooling rates and plunge temperatures would generate mESC cryopreservation protocols that yield improved PTR. Percent post-thaw recovery of mESCs has been demonstrated to vary considerably between cell lines [15,16,23]. Improved PTR, especially via universally applicable cryopreservation protocols, would facilitate banking, maintenance, and inter-laboratory exchange of mESC lines.

Theoretical simulations

Predicted optimal cooling rates ranged from 1.3 to 6.5 °C/minute for 1 M Me₂SO, 1.0 to 3.0 °C/minute for PG, and 1.1 to 1.9 °C/ minute for EG. The relatively narrow range of predicted optimal cooling rates for EG, combined with the uniformly predicted optimal plunge temperature of -41 °C, suggests that a single optimal cryopreservation protocol could potentially be applied to all mESC lines using this CPA. However, to confine our experiments to a manageable level, and to be able to compare our results with those obtained for the C57BL/6 mESC line, we chose two CPAs for experimental validation. PG was selected for its more stable glass-forming properties compared to EG and Me₂SO [2,3] and due to overall P_g being significantly higher for PG than for Me₂SO at room temperature across the four mESC lines studied in Part I of this study, which would enable the most

rapid addition and removal of CPA at room temperature with minimal damage to the cell membrane [13,16]. Dimethyl sulfoxide was chosen for comparative purposes as it is the CPA used in standard cryopreservation protocols.

Empirical validation of predicted optima

The empirical validation experiment was a $2 \times 2 \times 2 \times 2$ factorial design, with 2 levels each of: CPA, cooling rate, plunge temperature, and seeding, and all but seeding had significant main effects on PTR for all lines (with the exception of cooling rate in the CBA line). Specifically, with regards to CPA, protocols utilizing 1 M PG consistently yielded higher overall PTR than those with 1 M Me₂SO. One explanation might be that PG is a more stable glass former than Me₂SO or EG [2,3], thus potentially lethal devitrification during warming is less likely in the presence of PG as opposed to Me₂SO. With regards to cooling rates in the CBA mESC line, only the predicted optimal cooling rate for the Me₂SO protocol yielded improved PTR, however PTR did not decrease with the predicted optimal cooling rate for either CPA. On the other hand, as expected, predicted optimal cooling rates for the remaining three mESC lines yielded higher PTR overall than non-optimal cooling rates. As demonstrated in Table 1, the predicted optimal cooling rate was different for each CPA. At the same time, the predicted optimal plunge temperature was different for each predicted optimal cooling rate.

The main effect of plunge temperatures across mESC lines was contrary to our expectations: the plunge temperature of -80°C yielded overall better PTR than our predicted optimal plunge temperatures. However, plunge temperatures are only “optimal” for their associated, paired, predicted optimal cooling rates, and low temperature plunges would be more appropriate than higher plunge temperatures for cooling rates that were higher than predicted optimal cooling rates. Combined with potential underestimations of E_a due to extrapolations of suprazero E_a measurements to subzero temperatures which would lead to calculations of optimal plunge temperatures that were too high, the standard -80°C plunge temperature may have been advantageous. The significance of the main effect of plunge temperature, however, emphasizes the importance of pairing cooling rates and plunge temperature and the negative effects that occur when plunge temperature is inappropriate. Thus, for example, while cooling rate was not a significant main effect for the CBA strain, the interaction between cooling rate and plunge temperature was significant.

Interestingly, there was no main effect of seeding. We hypothesized that samples subjected to seeding would have improved PTR because they would be subjected to reduced thermal gradients upon ice nucleation. In fact, even with slow freezing protocols, supercooling tens of degrees is possible [24]. The theory that this should be deleterious was not supported by our current experimentation. In practice this is a valuable result because it is very difficult to seed samples in commercially available passive freezing kits.

The factorial design of the empirical validation experiment created a situation where some non-predicted-optimal cooling rates resulted in higher PTR, and some predicted-optimal cooling rates resulted in lower PTR. These situations are difficult to interpret. For example, the experimental plunge temperature was determined by the “optimal” cooling rate. When paired with the non-optimal cooling rate, this plunge temperature would no longer be

optimal according to our modeling. With cooling rates higher than the predicted optimal, the plunge temperature would likely be too high as too little intracellular dehydration should occur which is associated with damaging intracellular ice formation. Contrastingly, with lower than optimal cooling rates, the plunge temperature would likely be too low as intracellular dehydration would be allowed to occur for a lengthier period, and cells would endure potentially damaging solute effects [21]. Ideally one would compare results with “optimal” plunge temperatures for each cooling rate, but this would double again the size of the already large experimental design. A fundamental question in cryobiology is whether intracellular ice formation and solute effects are equally damaging to cells [27]. Depending on which effect is more critical for mESC survival during cryopreservation and warming, bias could be introduced into the experiment in which rates that were “too high” might fare worse than rates that were “too low”. With lower-than-optimal rates, the cell would have the chance to dehydrate before reaching the plunge temperature, and intracellular ice formation would be less likely to occur; if the cell is sensitive to solute effects, PTR might still be suboptimal, but if the cell is less sensitive to solute effects, PTR might actually improve when compared to a predicted optimal rate that is too high.

There were two instances where a predicted optimal plunge temperature was paired with a higher cooling rate and post-thaw recovery was actually improved as compared to PTR from standard conditions. With the FVB cell line, this situation (1 M PG, a suboptimal cooling rate of 5 °C/minute, and plunge temperature of –41 °C) resulted in PTR that was 1.9-fold greater (at $6.7 \pm 1.2\%$) than PTR under standard conditions ($3.6 \pm 0.5\%$) (Table 4). However, these results were well below the 4.4- to 7-fold improvement in recovery using the predicted optimal cooling rates with or without predicted optimal plunge temperatures. A more interesting example with the CBA cell line using 1 M PG, a five-fold higher-than-optimal cooling rate of 5 °C/minute, and plunge temperature of –41 °C yielded a 1.7-fold improvement in PTR which was not significantly different from improvements in PTR achieved with the predicted optimal cooling rates for 1 M DMSO or with 1 M PG, the same suboptimal 5 °C/minute cooling rate, and a plunge temperature of –80 °C (Table 3), suggesting that despite the increased cooling rate, cellular dehydration was adequate enough to minimize intracellular ice formation.

There were also cases in which predicted optimal plunge temperatures combined with optimal cooling rates resulted in lower PTR than when optimal cooling rates were paired with –80 °C plunge temperature. For the BALB/c mESC line, predicted optimal cooling rates yielded higher PTR than non-optimal cooling rates (Table 2). However, in the presence of 1 M Me₂SO, the predicted optimal plunge temperature combined with the predicted optimal cooling rate resulted in decreased PTR as compared to both PTR under the same conditions with a plunge temperature of –80 °C, and PTR using the standard method. For the CBA mESC line, the predicted optimal cooling rate for 1 M Me₂SO resulted in significantly improved PTR when paired with a plunge temperature of –80 °C; however, when paired with the predicted optimal plunge temperature, the conditions resulted in a PTR that was significantly lower than with a –80 °C plunge temperature and not significantly different from PTR under the standard protocol. Both examples suggest that there was inadequate cellular dehydration using the predicted optimal plunge temperature of –32 °C,

which resulted in damaging intracellular ice formation during either the subsequent cooling or warming phase.

For both the CBA and 129R1 mESC lines, there was a significant interaction of cooling rate and plunge temperature, and both results were unexpected. For the CBA mESC line, the combination of predicted optimal cooling rate and predicted optimal plunge temperature significantly lowered PTR. For the 129R1 mESC line, suboptimal cooling rates combined with the standard plunge temperature significantly improved PTR. These effects originated from the greatly successful outcome of PTR using suboptimal cooling rates for PG.

With the CBA and 129R1 mESC lines, PTR using predicted optimal cooling rates for 1 M PG did not improve PTR, but suboptimal cooling rates for the same CPA did, in fact, increase PTR. In the case of the CBA mESC line, non-optimal cooling rates were five-fold higher than predicted optimal rates, suggesting that our predicted optimal rates were too low. In the case of the 129R1 mESC line, non-optimal cooling rates were three-fold lower than predicted optimal rates, suggesting that our predicted optimal rates were too high.

The three situations listed above can perhaps be explained by constraints of current fundamental cryobiological models, including potential error in extrapolating suprazero E_a to subzero temperatures, questionable model appropriateness at high solute concentrations, and warming rate interactions. Here and in other studies, the assumption is made that suprazero measurements of permeability parameters, including E_a , can be extrapolated to sub-zero temperatures, but this may not be accurate. Hydrogen bonding interactions between water and lipid molecules, and the lipid dynamics required to allow water passage across a cell membrane generally result in high E_a^{LP} [33]. Temperature dependence of the activation energy for L_p may arise from heterogeneity in water transport properties, and temperature-dependent changes in the fluidity of the lipid membrane [34], and thus it is beneficial to measure E_a over as broad a temperature range as possible. However, it is technically difficult to conduct subzero measurements of cryobiological parameters due to the distortion and obscuring of cells by extracellular ice [17]. A recent report by Kleinhans and Mazur [17] utilized an interrupted, rapid cooling method to compare sub-zero E_a to values obtained above 0 °C. Their best-fit value obtained below 0 °C was determined to be 50% higher than the suprazero value, indicating that the suprazero to subzero assumption may not be correct [17]. Inaccurate reflections of subzero permeability parameters by suprazero extrapolations may have led to error in the calculation of predicted optimal cooling rates and plunge temperatures that in turn resulted in little or no improvement of PTR in certain circumstances. While our measurements were conducted over a very broad temperatures ranging from 0 °C to 37 °C, subzero measurements were not conducted. It may be beneficial to allow cells to cool for an additional number of degrees beyond the predicted optimal plunge temperature in order to compensate for inaccuracies of suprazero measurements of E_a .

Additional error may occur when one is applying assumptions made under physiological conditions, *i.e.* relatively dilute solutions, to make predictions for how cells will behave under non-physiologic, nondilute conditions that cells undergo during cryopreservation. Elmoazzen et al. [10] recently described a new nondilute solute transport model that does

not make the previous dilute solution or near-equilibrium assumptions and suggested that previous models may have an unexpected and undesirable concentration dependence of permeability, implying that applications of measurements in one setting may not be appropriate in very different settings. This potential inaccuracy introduced into our model by assumed concentration independence may have contributed to unexpected results encountered in our experiments.

Finally, our results may have been affected by warming rate interactions. The effects of a particular warming rate are strongly dependent on the cooling rate. If cooling rates are high, there is insufficient cellular dehydration and a higher probability of intracellular ice formation. In this case, rapid warming may favor high survival as it tends to inhibit devitrification and the recrystallization of intracellular ice, because small crystal formation is favored by high cooling rates, small crystals have high surface energies which encourages their growth, and slow warming would provide more time for crystal growth upon warming [22]. Warming rate has more unpredictable effects on slowly cooled cells (*i.e.* cells that are cooled slowly enough to allow sufficient dehydration, thus avoiding intracellular ice formation); in this circumstance, warming rate can have no effect (as with human myeloid stem cells frozen slowly in Me₂SO [36]), slow warming may be more damaging than rapid warming (for example, eight-cell mouse embryos cooled slowly in glycerol [30]), or rapid warming may be more damaging than slow [22] (e.g. as with eight-cell mouse embryos cooled in Me₂SO [30]). Our warming rate of approximately 700 °C/minute was predicted by computer simulations to be within a range that would have negligible volumetric effects on cell survival given the CPA addition and cooling protocols. However, this rate may well have influenced cell survival based on whether calculated optimal cooling rates were true, as well as based on how effectively our predicted optimal plunge temperatures provided conditions favoring vitrification at plunge temperatures. As it is difficult to predict warming rate interactions for mESCs, or to know if there is variation between cell lines, it would be beneficial for future studies to include a study on warming rate interactions in multiple cell lines for all CPAs.

From examining PTR results of interactions between CPA, cooling rate, and plunge temperature, predicted optimal cooling rates for 1 M Me₂SO seemed in general to be more successful than predicted optimal cooling rates for 1 M PG in generating at least moderate improvement in PTR. In all four mESC lines, the predicted optimal cooling rates for Me₂SO resulted in improved PTR as compared to standard protocols. However, improved PTR occurred in only two out of four mESC lines using predicted optimal cooling rates for 1 M PG. When examining PTR using 1 M PG in all mESC lines, including the previously published C57BL/6 mESC line [5], the PTRs of four out of five mESC lines (BALB/c, C57BL/6, FVB, and 129R1) were significantly improved ($p < 0.05$) simply by changing from 1 M Me₂SO to 1 M PG, and for the fifth mESC line, the CBA line, changing from 1 M Me₂SO to 1 M PG did not significantly diminish PTR. A cooling rate of 1 °C/minute was predicted to be optimal in three out of the five mESC lines.

Improvements in PTR were most dramatic for the FVB mESC line, where the predicted optimal cooling rate combined with the predicted optimal plunge temperature for 1 M Me₂SO resulted in a 7-fold improvement in PTR. The very low PTR achieved with the

standard freezing protocol for the FVB mESC line made any significant increase in PTR sound impressive when expressed in fold recovery. In general, the PTR of the CBA and FVB mESC lines, which, out of the four mESC lines, yield the lowest PTR under standard conditions, was raised to more practical levels (at best, $23.3 \pm 3.9\%$ and $25.2 \pm 4.0\%$ vs. $12.2 \pm 0.7\%$ and $3.6 \pm 0.5\%$, respectively), but the highest PTR was approximately less than half that of the highest PTR for the BALB/c and 129R1 mESC lines. For the BALB/c and 129R1 mESC lines, the greatest improvement in PTR was approximately 2-fold, raising PTR from $24.0 \pm 3.9\%$ and $\pm 1.3\%$ to, at best, $48.7 \pm 7.1\%$ and $59.4 \pm 2.6\%$, respectively.

Summary

We predicted optimal cooling rates and plunge temperatures in the presence of 1 M Me₂SO and 1 M PG using fundamental cryobiological parameters, including OTL, V_b , L_p , P_{CPA} , and their associated E_a , for the BALB/c, CBA, FVB, and 129R1 mESC lines. The results of these experiments as well as the previous C57BL/6 mESC study by Kashuba Benson et al. [16] suggest that a protocol utilizing 1 M PG, a cooling rate of 1 °C/minute, and plunge temperature of -41 °C will yield improved PTR relative to standard methods for most mESC lines. This protocol can be easily applied in a laboratory setting (Fig. 2). For the FVB line in particular, the improvement in PTR was considerable under these conditions. From these observations, it seems probable that this optimized protocol can be applied to all mESC lines to generate improved PTR relative to the current standard method. The exception in our studies was with the CBA mESC line, in which the use of these conditions did not significantly diminish PTR and as such these conditions would not be contraindicated (however, to improve PTR for this cell line, we recommend a cooling rate of 5 °C/minute in the presence of either 1 M Me₂SO or 1 M PG, and a predicted optimal plunge temperature of -80 °C).

The CBA line is an example of what this series of papers intended to investigate: we have clearly demonstrated that there are important fundamental physiological differences among mESC lines that may point to biophysical reasons for differential post-thaw survival. These differences indicate that, while the proposed new standard approach outlined above and in Fig. 2 is generally successful, there may be considerable room for improvement. Moreover, there are enough mESC lines in existence such that exceptions to this universal protocol will occur. However, these exceptional lines can be approached on a case-by-case basis using the systemic fundamental cryobiological approach to improving PTR that was originally described for the C57BL/6 mESC line [16], and further developed here. Further, if these first-pass approaches to optimize post-thaw recovery are not satisfactory for a particular strain, there are myriad additional approaches for further protocol refinement that may be warranted, including the examination of CPA induced toxicity and detailed studies of post-thaw phenotype instead of membrane integrity, among others. However, with the rapid growth of novel mESC lines, and the need for facile cryopreservation of these valuable research tools, we believe a default protocol is needed.

Future studies addressing potential sources of error, including the examination of warming rate interactions, subzero vs. suprazero measurements of E_a , and exploration of alternative models appropriate for high solute concentrations could result in further improvements to

mESC protocols. Considering that mESC lines have been characterized and banked while utilizing cryopreservation methods solely utilizing Me₂SO, it would be advisable to study the effects of PG and additional prospective CPAs on mESC gene expression, growth characteristics, and germ-line transmission. Future studies should also include the empirical validation of cooling rates and predicted plunge temperatures for EG in comparison to the standard method, as predicted optima for this CPA seem to be very similar for all mESC lines studied to date.

References

1. Austin CP, Battey JF, Bradley A, Bucan M, Capecchi M, Collins FS, Dove WF, Duyk G, Dymecki S, Eppig JT, Grieder FB, Heintz N, Hicks G, Insel TR, Joyner A, Koller BH, Lloyd KCK, Magnuson T, Moore MW, Nagy A, Pollock JD, Roses AD, Sands AT, Seed B, Skarnes WC, Snoddy J, Soriano P, Stewart DJ, Stewart F, Stillman B, Varmus H, Varticovski L, Verma IM, Vogt TF, von Melchner H, Witkowski J, Woychik RP, Wurst W, Yancopoulos GD, Young SG, Zambrowicz B. The knockout mouse project. *Nat Genet.* 2004; 36(9):921–924. [PubMed: 15340423]
2. Baudot A, Alger L, Boutron P. Glass-forming tendency in the system water-dimethyl sulfoxide. *Cryobiology.* 2000; 40:151–158. [PubMed: 10788314]
3. Baudot A, Odagescu V. Thermal properties of ethylene glycol aqueous solutions. *Cryobiology.* 2004; 48:283–294. [PubMed: 15157777]
4. Bedell MA, Largaespada DA, Jenkins NA, Copeland NG. Mouse models of human disease Part II: recent progress and future directions. *Genes Dev.* 1997; 11:11–43. [PubMed: 9000048]
5. Bedell MA, Jenkins NA, Copeland NG. Mouse models of human disease Part I: techniques and resources for genetic analysis in mice. *Genes Dev.* 1997; 11:1–10. [PubMed: 9000047]
6. Benson JD, Chicone CC, Critser JK. Exact solutions of a two parameter flux model and cryobiological applications. *Cryobiology.* 2005; 50:308–316. [PubMed: 15904913]
7. Benson JD, Kearsley AJ, Higgins AZ. Mathematical optimization of procedures for cryoprotectant equilibration using a toxicity cost function. *Cryobiology.* 2012; 64:144–151. [PubMed: 22248796]
8. Chan SY, Evans MJ. In situ freezing of embryonic stem cells in multiwall plates. *Trends Genet.* 1991; 7:76. [PubMed: 2031286]
9. Doetschman, T. Gene targeting in embryonic stem cells: I. History and methodology. In: Pinkert, CA., editor. *Transgenic Animal Technology: A Laboratory Handbook*. 2nd. Academic Press; San Diego: 2002.
10. Elmoazzen HY, Elliott JA, McGann LE. Osmotic transport across cell membranes in nondilute solutions: a new nondilute solute transport equation. *Biophys J.* 2009; 96:2559–2571. [PubMed: 19348741]
11. Gao DY, Liu J, Liu C, McGann LE, Watson PF, Kleinhans FS, Mazur P, Critser ES, Critser JK. Prevention of osmotic injury to human spermatozoa during addition and removal of glycerol. *Hum Reprod.* 1995; 10:1109–1122. [PubMed: 7657750]
12. Gilmore JA, Liu J, Gao DY, Critser JK. Determination of optimal cryoprotectants and procedures for their addition and removal from human spermatozoa. *Hum Reprod.* 1997; 12:112–118. [PubMed: 9043914]
13. Gilmore JA, Liu J, Peter AT, Critser JK. Determination of plasma membrane characteristics of boar spermatozoa and their relevance to cryopreservation. *Biol Reprod.* 1998; 58:28–36. [PubMed: 9472919]
14. Gordeeva OF, Manuilova ES, Grivennikov IA, Smirnova YA, Krasnikova NY, Zinov'eva RD, Khrushchov NG. Expression of regulatory genes Oct-4, Pax-6, Prox-1, Ptx-2 at the initial stages of differentiation of embryonic stem cells in vitro. *Ontogenez.* 2003; 34:174–182. [PubMed: 12816048]
15. Kashuba CM, Benson JD, Critser JK. Rationally Optimized cryopreservation of multiple mouse embryonic stem cell lines: I-Comparative Fundamental Cryobiology of Multiple Mouse

Embryonic Stem Cell Lines and the Implications for Embryonic Stem Cell Cryopreservation Protocols. Companion Paper.

16. Kashuba Benson CM, Benson JD, Critser JK. An improved cryopreservation method for a mouse embryonic stem cell line. *Cryobiology*. 2008; 56:120–130. [PubMed: 18191827]
17. Kleinhans FW, Mazur P. Determination of the water permeability (L_p) of mouse oocytes at -25°C and its activation energy at subzero temperatures. *Cryobiology*. 2009; 58:215–224. [PubMed: 19161999]
18. Liu J, Woods EJ, Agca Y, Critser ES, Critser JK. Cryobiology of rat embryos II: a theoretical model for the development of interrupted slow freezing procedures. *Biol Reprod*. 2000; 63:1303–1312. [PubMed: 11058533]
19. Mazur P. Freezing of living cells: mechanisms and implications. *Am J Physiol*. 1984; 247:C125–C142. [PubMed: 6383068]
20. Mazur P. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *J Gen Physiol*. 1963; 47:347–369. [PubMed: 14085017]
21. Mazur P, Leibo SP, Chu EH. A two-factor hypothesis of freezing injury. Evidence from Chinese hamster tissue-culture cells. *Exp Cell Res*. 1972; 71:345–355. [PubMed: 5045639]
22. Mazur, P. Principles of cryobiology. In: Fuller, BJ.; Lane, N.; Benson, EE., editors. *Life in the Frozen State*. CRC Press LLC; New York: 2004. p. 3-65.
23. Miszta-Lane H, Gill P, Mirbolooki M, Lakey JRT. Effect of slow freezing versus vitrification on the recovery of mouse embryonic stem cells. *Cell Pres Tech*. 2007; 5:16–24.
24. Muldrew, K.; Acker, JP.; Elliott, JAW.; McGann, LE. Water to ice. In: Benson, E.; Fuller, B., editors. *Life in the Frozen State*. CRC Press; Boca Raton: 2004.
25. Nagy, A., editor. *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, New York: 2003. Preparation of ES Cells for Injection; p. 469
26. Pegg DE. Simple equations for obtaining melting points and eutectic temperatures for the ternary system dimethyl sulfoxide/sodium chloride/ water. *Cryo Letters*. 1986; 7:387–394.
27. Pegg DE, Diaper MP. On the mechanism of injury to slowly frozen erythrocytes. *Biophys J*. 1988; 54:471–488. [PubMed: 3207835]
28. Petersen A, Schneider H, Rau G, Glasmacher B. A new approach for freezing of aqueous solutions under active control of the nucleation temperature. *Cryobiology*. 2006; 53:248–257. [PubMed: 16887112]
29. Prickett RC, Elliott JAW, McGann LE. Application of the osmotic virial equation in cryobiology. *Cryobiology*. 2010; 60:30–42. [PubMed: 19665010]
30. Rall W, Polge C. Effect of warming rate on mouse embryos frozen and thawed in glycerol. *J Reprod Fertil*. 1984; 70:285–292. [PubMed: 6363690]
31. Sambu S, Xu X, Schiffter HA, Cui ZF, Ye H. RDGS-functionalized alginates improve the survival rate of encapsulated embryonic stem cells during cryopreservation. *Cryo Letters*. 2011; 32:389–401. [PubMed: 22020461]
32. Schenke-Layland K, Angelis E, Rhodes KE, Heydarkhan-Hagvall S, Mikkola HK, Maclellan WR. Collagen IV induces trophectoderm differentiation of mouse embryonic stem cells. *Stem Cells*. 2007; 25:1529–1538. [PubMed: 17363553]
33. Songsasen N, Leibo SP. Cryopreservation of mouse spermatozoa I. Effect of seeding on fertilizing ability of cryopreserved spermatozoa. *Cryobiology*. 1997; 35:240–254. [PubMed: 9367612]
34. Verkman AS. Water permeability measurement in living cells and complex tissues. *J Membr Biol*. 2000; 173:73–87. [PubMed: 10630923]
35. Weast, RC.; Astle, MJ., editors. *CRC Handbook of Chemistry and Physics*. CRC Press; Boca Raton, Florida: 1981.
36. Wells JR, Sullivan A, Cline MJ. A technique for the separation and cryopreservation of myeloid stem cells from human bone marrow. *Cryobiology*. 1979; 16:201–210. [PubMed: 477366]

Fig. 1.

Summary of all percent post-thaw membrane integrity (top) and fold-increase of post-thaw membrane integrity over “standard cryopreservation protocols” (bottom) as a function of strain and protocol type, grouped by CPA and then protocol type. The standard protocol was defined to be 1 M Me₂SO at 1 °C/minute to –80 °C then plunge to LN₂. Because the fold-improvement over standard protocols was considerably higher for the FVB line compared to the other lines, the top panel of the fold increase portion includes results from only the FVB line, and the bottom panel includes the other lines. Labels SO-SO, SO-PO, PO-SO and PO-PO indicate protocol type where SO and PO indicate Sub- and Predicted-Optimal, respectively, the first term of the pair is the “optimality” of the cooling rate, the second term, the “optimality” of the plunge temperature. Particular protocol parameters are found in Table 1, and specific results for protocol are given in Tables 2 through 5.

Fig. 2.

A proposed new standard protocol for mouse embryonic stem cells (mESCs). Here we use the abbreviation PG for propylene glycol and FBS for fetal bovine serum.

Table 1

Predicted optimal cooling rates for the BALB/c, CBA, FVB, 129R1, and previously published C57BL/6 [16] mouse embryonic stem cell lines for three cryoprotectants.

Cell line	EG	PG	Me ₂ SO
BALB/c	1.1 (–41)	1.0 (–41)	6.5 (–32)
C57BL/6	1.4 (–41)	1.2 (–41)	4.1 (–33)
CBA	1.1 (–41)	1.1 (–41)	5.0 (–32)
FVB	1.9 (–41)	3.3 (–40)	3.8 (–33)
129R1	1.6 (–41)	3.0 (–41)	1.3 (–33)

Predicted optimal plunge temperatures are listed in brackets adjacent to cooling rates. Cooling rates are in °C/minute; plunge temperatures are listed in °C. EG, ethylene glycol; PG, 1,2-propanediol; Me₂SO, dimethyl sulfoxide.

Table 2

Experimental cryopreservation protocols that resulted in improved percent post-thaw recovery (PTR) of BALB/c mouse embryonic stem cells as compared to the standard cryopreservation method.

Protocol	CPA (1 M)	CR (°C/minute)	PT (°C)	PTR (%)	Fold improvement
Standard	Me ₂ SO	1.0 (SO)	-80 (SO)	24.0 ± 3.9 ^a	–
1	Me ₂ SO	6.5 (PO)	-80 (SO)	43.8±4.9 ^b	1.8
2	PG	1.0 (PO)	-80 (SO)	48.7 ± 7.1 ^b	2.0
3	PG	1.0 (PO)	-41 (PO)	42.6 ±4.9 ^b	1.8

CPA, cryoprotectant; CR, cooling rate; PT, plunge temperature; SO, suboptimal value; PO, predicted optimal value. While predicted optimal plunge temperatures are only optimal for predicted optimal cooling rates, they are indicated to be “predicted optimal” in order to easily define them from the standard plunge temperature. Different superscripts indicate significantly different values ($p < 0.05$).

Table 3

Experimental cryopreservation protocols that resulted in improved percent post-thaw recovery (PTR) of CBA mouse embryonic stem cells as compared to the standard cryopreservation method.

Protocol	CPA (1 M)	CR (°C/minute)	PT (°C)	PTR (%)	Fold improvement
Standard	Me ₂ SO	1.0 (SO)	-80 (SO)	12.2 ± 0.7 ^a	-
1	Me ₂ SO	5.0 (PO)	-80 (SO)	20.9 ± 1.6 ^b	1.7
2	PG	5.0 (SO)	-80 (SO)	23.3 ± 3.9 ^b	1.9
3	PG	5.0 (SO)	-41 (PO)	20.3 ± 2.4 ^b	1.7

CPA, cryoprotectant; CR, cooling rate; PT, plunge temperature; SO, suboptimal value; PO, predicted optimal value. While predicted optimal plunge temperatures are only optimal for predicted optimal cooling rates, they are indicated to be “predicted optimal” in order to easily define them from the standard plunge temperature. Different superscripts indicate significantly different values ($p < 0.05$).

Table 4

Experimental cryopreservation protocols that resulted in improved percent post-thaw recovery (PTR) of FVB mouse embryonic stem cells as compared to the standard cryopreservation method.

Protocol	CPA (1 M)	CR (°C/minute)	PT (°C)	PTR (%)	Fold improvement
Standard	Me ₂ SO	1.0 (SO)	-80 (SO)	3.6 ± 0.5 ^a	–
1	Me ₂ SO	5.0 (PO)	-33(PO)	25.2 ± 4.0 ^b	7.0
2	Me ₂ SO	5.0 (PO)	-80 (SO)	20.7 ± 2.9 ^c	5.8
3	PG	1.0 (PO)	-41 (PO)	16.7 ± 3.8 ^c	4.6
4	PG	1.0 (PO)	-80 (SO)	15.8 ± 3.2 ^c	4.4
5	PG	5.0 (SO)	-41 (PO)	6.7 ± 1.2 ^d	1.9

CPA, cryoprotectant; CR, cooling rate; PT, plunge temperature; SO, suboptimal value; PO, predicted optimal value. While predicted optimal plunge temperatures are only optimal for predicted optimal cooling rates, they are indicated to be “predicted optimal” in order to easily define them from the standard plunge temperature. Different superscripts indicate significantly different values ($p < 0.05$).

Table 5

Experimental cryopreservation protocols that resulted in improved percent post-thaw recovery (PTR) of 129R1 mouse embryonic stem cells as compared to the standard cryopreservation method.

Protocol	CPA (1 M)	CR (°C/minute)	PT (°C)	PTR (%)	Fold improvement
Standard	Me ₂ SO	1.0 (PO)	-80 (SO)	28.4 ± 1.3 ^a	-
1	Me ₂ SO	1.0 (PO)	-33 (PO)	43.0 ± 2.3 ^b	1.5
2	PG	1.0 (SO)	-41 (PO)	59.4 ± 2.6 ^b	2.1
3	PG	1.0 (SO)	-80 (SO)	40.8 ± 2.7 ^b	1.4

CPA, cryoprotectant; CR, cooling rate; PT, plunge temperature; SO, suboptimal value; PO, predicted optimal value. While predicted optimal plunge temperatures are only optimal for predicted optimal cooling rates, they are indicated to be “predicted optimal” in order to easily define them from the standard plunge temperature. Different superscripts indicate significantly different values ($p < 0.05$).