Published in final edited form as:

Cryobiology. 2008 April ; 56(2): 120–130. doi:10.1016/j.cryobiol.2007.12.002.

An Improved Cryopreservation Method for a Mouse Embryonic Stem Cell Line

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Introduction

The use of mouse embryonic stem (ES) cells in transgenic mouse production has contributed to a virtual explosion in the number of existing transgenic mouse models that are vital for human biomedical research [1;2]. Coordinated projects to systematically knock out all mouse genes, such as the Knockout Mouse Project (KOMP) [3], Canada's North American Conditional Mouse Mutagenesis Program (NorCOMM, <http://norcomm.phenogenomics.ca/index.htm>), the European Conditional Mouse Mutagenesis Program (EUCOMM, <http://www.eucomm.org>) [4], and Bay Genomics' use of *N*-ethyl-*N*-nitrosurea (ENU) (<http://baygenomics.ucsf.edu/>) [5], will create thousands of mutant ES cell lines as a step towards producing mutant mice that serve as important models of human biology and disease [6]. The C57BL/6 mouse lineage is central to these projects, due to its ease of genetic manipulation, wide accessibility to researchers, and the existence of ES cell lines of this genotype. Storage and maintenance of valuable genotypes as live animal lines would be wholly impractical [7]. On the other hand, banking lines as ES cells is cost-effective and restoration of the ES cells into live, reproductively viable mice is routine in many laboratories across the world. Efficiency of this restoration is greatly improved when freezing and thawing methods produce healthy, rapidly dividing, germ line competent cells.

To our knowledge, there are no published reports quantifying post-thaw recovery of cryopreserved C57BL/6 ES cells. Post-thaw recovery of viable mouse ES cells varies dramatically from one cell line to the next, ranging anywhere from 10–90% (personal communication, Deanna Nielsen, Stem Cell Technologies technical support, 2004; personal communication, Xin Yu, University of California-Davis, 2004). Based on our experience, fewer than 50% of all cells in most mouse ES cell lines survive cryopreservation, which calls into question the efficacy of current methods. Cryopreservation protocols optimized to individual cell lines, if necessary, would allow for the full exploitation of ES cells from all strains, reducing the need for backcrossing in the production of mutant mice as individual backgrounds would be accessible as viable ES cell lines. Optimized cryopreservation protocols maximize post-thaw recovery of intact, pluripotent cells, reducing the time it takes to expand cultures post-thaw and increasing the number of aliquots one can cryopreserve from a single plate.

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The cryobiological approach currently used to preserve ES cells is an equilibrium cooling approach. Equilibrium cooling relies on the formation of extracellular ice which leads to progressive cell dehydration, effectively concentrating the intracellular solution to a vitrifiable state in which, upon further cooling, the cytoplasm becomes a glass [8]. The term, “vitrification” is most often used to describe cooling protocols which bring about an extreme elevation in viscosity of an extracellular solution, *i.e.* the formation of a glass in the absence of ice crystallization [9]. Typically, this requires rapid cooling protocols in the presence of high concentrations of cryoprotective agents (CPAs). However, the use of equilibrium cooling to render a cell to a more vitrifiable state through controlled dehydration, an idea elucidated by Pegg and Diaper in 1990 [8], has only recently gained consistent and systematic support [10; 11;12]. Major damage during a cryobiological protocol using the equilibrium cooling approach is theorized to be due to three major factors: osmotic damage due to water influx and efflux during the addition and removal of CPAs (*e.g.* Me₂SO [13]), mechanical damage due to intracellular ice crystal formation, and solute effects, generally described as chemical damage that occurs due to an increase in the concentration of intracellular ions that occurs during freezing [14]. The degree of damage caused by these factors varies with CPA and cooling and warming profiles.

A direct means to derive optimal cryopreservation protocols for a given cell type is via an exploration of the cell’s fundamental cryobiological parameters and how they relate to the major physical events that occur during the freezing process [15]. Fundamental cryobiology, as it is applied to equilibrium cooling, seeks to define basic cell membrane permeability coefficients such as hydraulic conductivity (L_p), solute permeability (in our case, the permeability of cryoprotectants, P_{CPA}) and their temperature dependence (defined by an Arrhenius equation with activation energy E_a) in order to predict optimal cooling and warming rates for that cell type. To avoid damage during the addition and removal of CPA, osmotic tolerance limits, nominal limits to which a cell can shrink or swell in response to osmotic stress without significant loss of function, are also defined. Each of these parameters can vary greatly according to cell type, species, and even individual (*e.g.* canine erythrocytes *vs.* spermatozoa of mice *vs.* chimpanzee spermatozoa [16;17;18]). Correspondingly, optimal cooling and warming rates can also vary greatly between these groups (*e.g.* human cord blood stem cells *vs.* erythrocytes [19;20]).

Optimal cooling rates are defined as those that cool cell suspensions as rapidly as possible without causing a large difference between the intracellular freezing point and the intracellular temperature, and warming rates are classically optimal if they mimic their cooling rate counterparts [21]. Using principles of the Boyle Van’t Hoff relation [22], the Arrhenius relation [15], a two-parameter mass transport model [23], and Mazur’s two factor hypothesis [14], we explored the fundamental cryobiological parameters of a C57BL/6 mouse ES cell line in order to improve existing cryopreservation protocols and define methods by which cryopreservation methods would be routinely assessed in a repository setting. P_{CPA} and L_p in the presence of CPA (L_p^{CPA}), and the temperature dependence of these values were assessed in the presence of four commonly used CPA, namely ethylene glycol (EG), propylene glycol (PG), Me₂SO, and glycerol (GLY). The osmotically inactive fraction of the cell, V_b , was calculated and osmotic tolerance limits were established using membrane integrity as the endpoint. These parameters were in turn used to predict a theoretically optimized cryopreservation protocol that improved the efficiency of cryopreservation for the C57BL/6 mouse ES cell line by greater than twofold. Finally, this protocol will provide an experimental basis to improve cryopreservation methods for other mouse ES cell lines, and will allow optimization accounting for other endpoints such as gene expression profiles associated with differentiation.

Materials and Methods

Embryonic stem cells

The C57BL/6 mouse ES cell line was acquired at passage 11 from Specialty Media Group (Chemicon International, Temecula, California, now part of Millipore, Billerica, Massachusetts). C57BL/6 mouse ES cell cultures were negative for all pathogens (IMPACT I test, Research Animal Diagnostic Laboratory, Columbia, Missouri; www.radil.missouri.edu/info/index.asp).

Cell culture and standard freezing method

ES cells were cultured on primary mouse embryonic fibroblast cells (MEF) (Chemicon International) at 37°C and 5% CO₂. Culture media contained 15% Defined FBS (Hyclone, Logan, UT), 0.1mM 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 (Chemicon International) in high glucose DMEM (Chemicon International). ES cells were passaged and/or collected every 2 days or at confluence.

For the standard slow cooling freezing 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 1mL aliquots in cryovials (Nalgene Nunc International, Rochester, NY). Cryovials were transferred to a commercially available freezing kit (Nalgene), refrigerated at -80°C overnight (a process which cools at a rate of 1°C/minute), and subsequently transferred to liquid nitrogen (LN₂).

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

Separation of ES cells from feeders

For Coulter counter and osmotic tolerance experiments, ES cells were separated from the feeder cells using a differential sedimentation technique previously described by Doetschman[24]. The separation of MEF from mouse ES cells is routine, and there are many variations of the basic method exploiting the difference in the rate at which fibroblast feeder cells and mouse ES cells settle and adhere to culture dishes [25;26;27]. Briefly, trypsinized ES cell cultures containing MEF were centrifuged, resuspended in 10 mL of culture medium, and plated on the original 100mm cell culture dish for 30 minutes at 37°C. Following incubation, culture medium containing mostly ES cells was transferred to a second culture dish for one hour incubation at 37°C in order to remove remaining fibroblast feeders. Following the second incubation, culture medium containing the ES cells was removed, and the ES cells were counted, centrifuged, and resuspended in either DPBS or culture medium for experimentation. In our hands, the Doetschman sedimentation method resulted in the removal of greater than 99% of contaminating feeder cells from the ES cell suspension (data not shown).

Electronic particle counter

A modified electronic particle counter (EPC) (Coulter Counter model ZM, Beckman Coulter, Inc., Fullerton, CA), equipped with a standard 50 µm aperture tube and computer interface [28], and modified to operate without a mercury-filled manometer as per Benson *et al.* [29] was used for all cellular volumetric measurements. Raw volumetric data were exported into Mathematica (Wolfram Research Inc., Champaign, Illinois) computing package for processing and analysis. Volume was calibrated using standard nominal 10 µm polystyrene latex particles (Beckman Coulter, Inc., Fullerton, CA) at 0, 6, 12, and 22°C. The relationship between

conductivity and latex bead volume was assumed to be the same as that between conductivity and ES cell volume.

Determination of osmotic tolerance limits

Osmotic tolerance limits were defined by the maintenance of plasma membrane integrity, as indicated by propidium iodide exclusion in 80% of the ES cell population following exposure to anisotonic conditions using sodium chloride as an impermeable solute,. Solutions of varying osmolality were prepared using Dulbecco's Phosphate Buffered Saline (DPBS) that was adjusted to the appropriate osmolality by the addition of either double distilled water or sodium chloride (Sigma Aldrich). The solutions were adjusted to pH 7.1 using NaOH or HCl as necessary. The osmolality of each solution was verified using a vapor pressure osmometer (Wescor, Logan, UT). On three separate days, confluent C57BL/6 mouse ES cells were trypsinized and separated from fibroblast feeders. Equal numbers of cells were then exposed to solutions of 37, 75, 150, 600, 1200, 2400 and 4800 mOsm (n=3 for each solution) for 10 minutes at room temperature. Conditions were abruptly returned to isosmotic by the abrupt addition of appropriate volumes of hyperosmotic solution in the case of hypoosmotic conditions, and hypoosmotic solution in the case of hyperosmotic conditions. Cells were then centrifuged for five minutes at $200 \times g$ and resuspended in isosmotic solution. Cells exposed to anisotonic conditions were compared to controls in which the same quantities of cells were exposed to isosmotic conditions (285 mOsm) following the same protocol. Plasma membrane integrity was assessed by flow cytometry analysis (FACScan, Becton Dickinson, San Jose, CA) of propidium iodide exclusion.

Measurement of cell osmotic response

ES cell volumetric response to variable osmotic stresses was measured at 22°C using an EPC, as previously described [28;30;31;32;33]. Mean cell volume response was measured in real time following abrupt exposure to 206, 285, 600, 900, 1350, and 2880 mOsm solutions prepared from 10X PBS (Sigma) and Milli-Q water and adjusted to pH 7.1 with hydrochloric acid. The osmolality of the solutions was verified using a vapor pressure osmometer (Wescor). Data were averaged over 100 ms intervals prior to analysis. Three replicates were performed for each experimental condition and a representative plot of the output can be seen in Figure 1, Panel A. Equilibrated cell volumes were normalized to their respective isotonic values, and plotted against the reciprocal of normalized osmolality in accordance with the Boyle Van't Hoff relationship [22]. Linear regression was calculated using Mathematica to fit the Boyle Van't Hoff equation to the data. This equation is defined by

$$V = V_{w,iso} M_{iso} / M + V_b,$$

where V is cell volume at osmolality M , $V_{w,iso}$ is isotonic cell water volume, M_{iso} is isotonic osmolality, and V_b is the osmotically inactive cell volume. V_b was determined by performing a linear regression of volume as a function of the reciprocal of osmolality and extrapolating to infinite osmolality (i.e. $1/M = 0$).

Determination of permeability parameters

Volume changes over time were measured by an EPC following abrupt addition of cells to 1.0 M CPA in 1X PBS. Volumetric changes in the presence of 1.0 M Me₂SO, 1.0 M EG, and 1.0 M PG were measured at 0, 6, 12, 22, and 34°C, and a representative plot of the experimental output can be seen in Figure 1, Panel B. Measurements of cells in the presence of 1.0 M GLY were determined at 22 °C only. Three replicates were performed for each treatment on 3 different days.

Data were fit to the following two-parameter mass transport model [34] to determine membrane permeability coefficients for cryoprotective agents (P_{CPA}) and hydraulic conductivity in the presence of cryoprotectants (L_p^{CPA}) at all temperatures:

$$\frac{dV_w}{dt} = -L_p A R T \left(M_s^e + M_n^e - \frac{n_s^i + n_n^i}{V_w} \right),$$

$$\frac{dn_s^i}{dt} = P_s A \left(M_s^e - \frac{n_s^i}{V_w} \right).$$

Here superscripts e and i indicate extra- and intracellular quantities respectively, subscripts s and n indicate permeating and non-permeating quantities, respectively, and A is the volume independent spherical surface area at V_{iso} . Finally, we assume the relationship $n^i = V_w M^i$ where V_w is the intracellular water volume.

The Arrhenius relationship^{c.f.} [15] was used to determine the activation energies, E_a , for the parameters L_p^{CPA} and P_{CPA} by plotting the permeability value (L_p^{CPA} or P_{CPA}) at any absolute temperature T as $R \ln(P(T))$ versus $1/T$:

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

where P_0 is the value at some reference temperature T_0 , R is the gas constant and E_a is the activation energy for the process, expressed in kcal/mol and determined by the slope of the linear regression.

Theoretical simulations

Theoretical simulations were performed to determine optimal CPA addition and removal protocols of a 1.0 M solution of Me₂SO, PG, or EG for C57BL/6 mouse ES cells. A protocol was defined to be optimal if it minimized the number of addition and dilution steps while maintaining ES cells within the defined range of osmotic tolerance. The overall goal was to minimize cell volume excursion and cryoprotectant exposure time at ambient temperatures. A computer model was used for these procedures. In given experimental conditions, which included osmotic tolerance limits, initial intracellular concentration, and temperature, the program automatically optimized addition and removal steps, and also provided the appropriate diluent concentration.

Theoretical optimization of cryopreservation protocols based on Mazur's two factor hypothesis was also performed. Briefly, Mazur's two factor hypothesis [14] states that 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, leading to deleterious intracellular ice formation. Mazur suggests that the optimal cooling rate is that which minimizes cooling exposure time while maintaining at most two degrees of supercooling [14]. In other words, he suggests that cells are 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. This optimization can be achieved by pairing solute-solvent flux models with the appropriate ternary phase diagram (NaCl-CPA-Water) to determine to what degree supercooling would occur.

In a two-step freezing protocol, cells are cooled at a controlled rate to a temperature, termed the "plunge temperature", at which point they are plunged into liquid nitrogen. The purpose of this procedure is to minimize intracellular ice formation and promote vitrification. Optimal plunge temperatures were calculated using computer simulations based on the above model that 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. Computer simulations of freezing for all cell lines and CPAs were performed iteratively until optimal

cooling rates were determined, i.e. simulations were run at increasing cooling rates until more than two degrees of supercooling occurred before the intracellular concentration of CPA reached 40%. For an illustrative curve of determination of optimal cooling rates, please refer to Figure 2.

Warming was simulated using the two parameter mathematical model [34] described above, and the subsequent volume excursion upon equilibration was compared to the predicted osmotic tolerance limits. Warming rates up to 1×10^4 °C/min were simulated to verify that damage due to volume changes would not occur. An optimal warming rate is that which is fast enough to prevent devitrification, yet not too fast that after warming, the influx of water causes the cell to exceed osmotic tolerance limits (please see [12] for a discussion on the avoidance of devitrification using rapid warming rates, and [35] for a discussion of the damaging effects of volume change from overly rapid warming rates). Since the fastest practical method for warming is placing a straw in room temperature or 37°C water (the difference in cooling rates is reasonably negligible) it remains only to verify that cell volume excursions are within the osmotic tolerance limits. For an illustrative curve of optimal warming rates, please refer to Figure 2.

Empirical validation of predicted optima

Empirical validation of predicted freezing rates was conducted with 1.0 M PG and 1.0 M Me₂SO. C57BL/6 mouse ES cells, cultured in standard conditions, were 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 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 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). Comparisons were made with standard conditions (Me₂SO, 1°C/minute, -80°C plunge temperature) and predicted optimal conditions that included cooling rate (4°C/minute, Me₂SO; 1°C/minute, PG) and two plunge temperatures of predicted optimal (-32.5 ± 0.5 °C, Me₂SO; -40.5 ± 0.5 °C, PG) vs. low (-80° C). Initial studies comparing the effects of seeding (the induced nucleation of intracellular ice crystals by applying a LN₂-cooled forceps to the freezing vessel until visible ice crystals form, theorized to increase the uniformity of sample cooling [36;37]) vs. not seeding revealed no significant difference ($p < .05$) (data not shown); therefore these comparisons were not included in additional studies. Finally, ES cell recovery using cryostraws in conditions mimicking standard ES cell freezing conditions were compared to ES cell recovery in standard conditions using cryovials (Nalge Nunc International, Rochester, NY) that are commonly used to freeze and distribute ES cells.

Theoretical simulations predicted that warming rates between 10 and 1×10^4 °C/minute would have negligible effects on cell survival. Therefore, cryo-straws 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). Immediately post-thaw, ES cells were diluted, drop-wise, by 5 volumes of culture media, centrifuged, resuspended in 1XPBS, 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

A *t*-test was used to compare percent PTR in standard conditions using cryo-straws and cryovials. A Dunnett's test was used in the analysis of experimental validation studies of predicted optimal cooling rates and plunge temperatures as compared to standard conditions [38]. For all other comparisons, standard analysis of variance (ANOVA) was performed with

the SAS General Linear Models program (SAS Institute, Inc., Cary, NC). An alpha value of $p < .05$ was used for all tests. All values are stated as mean $\pm$ SEM, unless stated otherwise.

Results

Osmotically inactive cell volume

C57BL/6 mouse ES cells behaved as ideal linear osmometers over a range of 200 mOsm to 2800 mOsm. The calibrated measurements with the Coulter counter gave a mean isosmotic volume of $695 \pm 3.4 \mu\text{m}^3$. Extrapolation of the regression line to infinite osmolality gave an osmotically inactive cell volume of $49.7 \pm 1.3\%$ of isosmotic cell volume with an r^2 value of 0.945. These values are depicted in Figure 3.

Osmotic tolerance

The effects of anisotonic conditions on C57BL/6 mouse ES cell membrane integrity, as represented by propidium iodide (PI) exclusion, are shown in Figure 4. There was a significant main effect of osmolality on ES cell membrane integrity ($p < .05$). Post-anisotonic exposure cell membrane integrity varied by day of experiment ($p < .05$). Overall, an increase in the proportion of cells staining positive for PI, indicating decreased membrane integrity, was observed as conditions departed from isosmotic. As shown in Figure 4, as compared to isosmotic conditions, the membrane integrity of ES cells was decreased significantly in anisotonic conditions of 37.5, 75, 1200, 2400, and 4800 mOsm ($p < .05$). Ninety percent or greater of C57BL/6 mouse ES cells excluded propidium iodide, *i.e.* maintained cell membrane integrity, following exposure to osmolalities [34] between 150 mOsm and 600 mOsm. Extrapolation from the regression line between data points indicated that 80% of the C57BL/6 mouse ES cell population retains membrane integrity between 139 mOsm and 1075 mOsm, or 63% and 153% of isosmotic cell volume, respectively.

Permeability parameters for C57BL/6 mouse ES cells

Changes in ES cell volume in the presence of 1.0 M CPA, measured over time by electronic particle counter (EPC) at 0, 6, 12, 22, and 34°C, were fitted to compute P_{CPA} and L_p^{CPA} . Room temperature values of P_{CPA} , L_p^{CPA} , and their associated activation energies are shown in Table 2. Room temperature L_p^{CPA} values did not differ significantly for Me₂SO, EG, or PG; however L_p^{GLY} was significantly lower than L_p^{CPA} for Me₂SO, EG, and PG ($p < .05$). Room temperature values for P_{PG} were significantly higher than for P_{EG} , P_{Me_2SO} , and P_{GLY} . P_{GLY} values were significantly lower than P_{EG} and P_{Me_2SO} values ($p < .05$). Due to these markedly lower room temperature values for L_p^{GLY} and P_{GLY} , GLY was deemed an unsuitable CPA for equilibrium freezing, and measurements at additional temperatures were not conducted. There was no significant difference in E_a values of Me₂SO, EG, and PG for either L_p^{CPA} or P_{CPA} ($p < .05$).

Theoretical simulations

A two-step freezing protocol was determined to be optimal for the C57BL/6 mouse ES cell line, whereby cells are slowly cooled in a controlled-rate freezer to a temperature ("plunge temperature") at which they would be rapidly transferred to liquid nitrogen (LN₂). Predicted optimal freezing rates and plunge temperatures, which together in theory create conditions favoring intracellular vitrification, are listed in Table 3. The predicted optimal freezing rates were determined to be 4.1°C/minute for Me₂SO, 1.4°C/minute for EG, and 1.2°C/minute for PG, with plunge temperatures of -33°C, -41°C, and -41°C, respectively. PG was chosen as the most effective CPA for empirical validations, and compared against Me₂SO, the CPA of standard protocols.

Empirical validation of predicted optima

Percent PTR in standard conditions did not differ significantly when using cryo-straws ($34.2 \pm 6.0\%$) or cryovials ($31.9 \pm 1.6\%$) ($p < .05$).

In concurrent studies comparing predicted optimal cooling rates and plunging temperatures, there was no significant effect of seeding ($p < .05$) (data not shown). PTR under standard freezing conditions (Me_2SO , cooling rate of $1^\circ\text{C}/\text{minute}$, and plunge temperature of -80°C) was $31.8 \pm 4.5\%$. PTR using Me_2SO at the predicted optimal cooling rate of $4^\circ\text{C}/\text{minute}$ was $39.0 \pm 4.9\%$ using predicted optimal plunge temperature, and $44.8 \pm 6.1\%$ using low plunge temperature. PTR using PG at the predicted optimal cooling rate of $1^\circ\text{C}/\text{minute}$ was $48.4 \pm 5.2\%$ at low plunge temperature. These results did not differ significantly ($p < .05$). PTR using PG at the predicted optimal cooling rate of $1^\circ\text{C}/\text{minute}$ and predicted optimal plunge temperature of -41°C was $63.9 \pm 6.3\%$, a twofold, significant increase as compared to standard freezing conditions ($p < .05$).

Discussion

Me_2SO has been used for decades to decrease solute effects during cryopreservation of cells [39;40;41]. The Me_2SO slow freezing protocol, involving a cooling rate of $1^\circ\text{C}/\text{minute}$ and plunging into LN_2 at -80°C , has been applied to a variety of cell types with variable results, indicating that modification of this protocol may be beneficial in many circumstances. For example, cryopreservation of human ES cells using this protocol has been found to diminish *Oct-4* expression [42], and studies of cord blood $\text{CD}34^+$ cells have suggested that PG is a more appropriate CPA than Me_2SO [13]. Potential variation in response to any cryopreservation protocol can largely be attributed to wide-ranging differences in fundamental cryobiological parameters specific to individual cell types and species [43;44].

Three short technical reports have been published that relate to mouse embryonic stem cell cryopreservation [45;46;47]. These discuss small-scale, 96-well plate protocols for the freezing of mouse ES cell transgenic clones. To our knowledge, there have been no rigorous, cell-line specific, published studies on the optimization of cryopreservation protocols for mouse ES cells that would be applicable to ES cell banking and culture needs. This study demonstrates that it is possible to dramatically increase post-thaw recovery of mouse ES cells using a fundamental approach to predict optimal equilibrium freezing conditions and CPA. The resulting new protocol greatly increases mouse ES cell cryopreservation efficiency, maximizing the use of frozen aliquots within the laboratory as well as in transport and exchange of ES cells between researchers.

Our fundamentally-derived approach required the determination of optimum CPA, CPA concentration, equilibrium cooling rate, warming rate, and plunging temperatures. The optimum CPA was considered to be PG for two reasons. First, its high P_{CPA} values at room temperature, as compared to Me_2SO and EG, would enable the most rapid addition and removal of CPA at room temperature with minimal damage to the cell membrane [48]. Secondly, PG is a more stable glass former, *i.e.* vitrifies more easily, than either Me_2SO or EG [49;50], therefore devitrification resulting in intracellular ice formation (IIF) during either cooling or warming is less likely in the presence of PG as compared to Me_2SO or EG. For empirical validation of predicted optima, the effectiveness of PG as a CPA was compared against the CPA used in standard freezing protocols, Me_2SO , using cryo-straws vs. vials as vessels. There was no significant difference ($p < .05$) in percent PTR when standard conditions were utilized with either cryo-straws or vials.

Computer simulations of cell response during cooling and warming were conducted by applying mathematical equations describing mass transfer (water, solutes) across cell

membranes [51]. A key assumption of this model is that cells respond as “ideal osmometers,” meaning the equilibrium cell volume is linearly related to the reciprocal of the extracellular osmolality. This is described using the Boyle Van’t Hoff equation and a single parameter, V_b [52;53]. C57BL/6 mouse ES cells behaved as linear osmometers over the range of 200–2800 mOsm, indicating that cell volume is not regulated through an active process in the hypo- or hyperosmotic range studied. V_b was determined to be 49.8% of isosmotic cell volume. This value is comparable to the V_b of human spermatozoa (50% of isosmotic cell volume) [54] and human red blood cells (43% of isosmotic cell volume) [55], and is notably higher than the range of V_b measured for other stem cells, including human umbilical cord blood CD34+ cells (27% and 32% of their respective isosmotic cell volumes) [13;56], and human bone marrow-derived hematopoietic progenitor cells (20.5% of isosmotic cell volume) [57].

Osmotic tolerance limits were established based on the retention of membrane integrity in 80% of the cell population following exposure to anisomotic solutions using NaCl as the impermeable solute. These 80% limits were established for C57BL/6 mouse ES cells to be between approximately 139 and 1075 mOsm. Therefore, 1.0 M CPA, added drop-wise [58], would maintain the cell within its osmotic tolerance limits, and from a practical standpoint, this concentration was comparable to the standard freezing protocol which employs 10% Me₂SO (1.3 M) and 50% FBS in cell culture medium. Cell injury has been found to vary at similar anisomotic exposures depending on whether ionic or nonionic solutes are used to induce anisomolality. For example, hypertonic injury to human spermatozoa has been found to be greater with ionic NaCl solutions than with nonionic sucrose solutions [59], and other studies have shown that in concentrated ionic solutions, cellular membranes can become increasingly permeable to these ions over time [60;61;62]. While this effect is not universal [63], it is possible that the use of NaCl in our osmotic tolerance experiments had an effect independent of osmolality that was not discernible with the present experiments.

Computer simulations using P_{CPA} , L_p^{CPA} , and V_b predicted an optimal cooling rate of 1.2°C/minute for 1.0 M PG and 4.1°C/minute for 1.0 M Me₂SO, indicating that the standard cooling rate of 1°C/minute is inadequate for Me₂SO when applied to this cell line. Use of the predicted optimal cooling rate of 1°C/minute and a plunge temperature of –41°C for 1.0 M PG significantly improved percent PTR of viable C57BL/6 mouse ES cells. The PTR we achieved under these conditions was $63.9 \pm 6.3\%$, a significant twofold improvement over standard freezing conditions ($p < .05$). Interestingly, but not unexpectedly, percent PTR did not increase significantly when the optimal cooling rate was continued below the predicted optimal plunge temperature to a lower plunge temperature (a standard condition) of –80°C ($p < .05$). Continued dehydration beyond –41°C would further minimize intracellular ice formation by favoring vitrification; however, this dehydration would lead to damaging solute effects with deleterious effects on post-thaw recovery. This effect of dehydration beyond the predicted optimal plunge temperature may also be applied to cells frozen in 1.0 M Me₂SO, frozen at the predicted optimal cooling rate, but plunged into LN₂ at –80°C.

The predicted optimal cooling rate for Me₂SO failed to improve percent PTR even when paired with the optimal plunge temperature. For Me₂SO, there may be a broad range of protocols that would produce similar PTR if a predicted optimal lies in this range [19]. Reduced PTR may also be due to the damaging effects of IIF during either or both cooling and warming. Total solute concentration (>40% volume), the glass-forming capacity of the CPA, and the interaction between cooling and warming rates influence the likelihood of IIF and whether or not a vitrified solution will become crystallized [64;65]. It is possible that the criterion of 40% CPA by weight is an inadequate concentration for vitrification in the presence of Me₂SO; however, investigation of this concept is beyond the scope of this study. Another potential source of cell injury could be that the devitrification of extra- or intracellular glass that formed during the plunge into LN₂ occurs during warming if rates are too slow. Previous studies of

two-step, interrupted slow freezing of eight-cell mouse embryos have demonstrated the warming rate dependence of survival [66;67]. While cell dehydration occurred during the initial slow freezing step, the cells still contained freezable water at -40°C . Due to the high viscosity of the intracellular environment in the presence of CPA, the solution vitrified to form a metastable glass. However, with slow warming, the glass devitrified with subsequent recrystallization, damaging the embryos. It is possible that the warming rate of approximately $700^{\circ}\text{C}/\text{minute}$, although predicted by computer simulation to be within a range that would have negative effects on cell survival, was actually inadequate for the prevention of ice recrystallization during the warming process. As stated previously, Me_2SO tends to have less stable glass-forming properties than PG [49], which would increase the likelihood of this effect.

The improved cryopreservation protocol for the C57BL/6 mouse ES cell line utilizes 1.0 M PG, a cooling rate of $1^{\circ}\text{C}/\text{minute}$, warming rate of approximately $700^{\circ}\text{C}/\text{minute}$ and a plunge temperature of -41°C (Figure 5). Our hypothesis-based, fundamental cryobiological approach resulted in a twofold increase in percent post-thaw recovery of membrane-intact ES cells as compared to the standard freezing protocol, as measured by propidium iodide exclusion. While increased post-thaw recovery in terms of membrane integrity is an important step in facilitating banking, transport, and post-thaw reconstitution of ES cell cultures, future studies must additionally determine the effect of this new protocol on C57BL/6 ES cell characteristics such as colony morphology, ES cell marker gene expression, and germ-line transmissibility. The methods to improve cryopreservation protocols outlined in this study will speed analysis of future mouse ES cell lines, which is especially important for current mouse ES cell lines that have poor post-thaw recovery, and for future, perhaps rare mouse ES cell lines involved in projects such as the “Knock-Out Mouse Project”. Finally, defining methods to improve cryopreservation in mouse protocols gives clues as to how to improve cryopreservation of human ES cell lines.

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Figure 1.

Representative plots of calibrated cell volume versus time data from the Coulter counter. (A) Representative plot of calibrated cell volume versus time data from the Coulter counter and (inset) a histogram of the equilibrated volume distribution. Cells equilibrated in isosmotic media were abruptly injected into anisosmotic media, the resulting equilibrated volumes were recorded, noise was filtered out digitally, and mean volumes were recorded. (B) Representative plot of calibrated cell volume versus time data from the Coulter counter and a representative two parameter model fit. Cells equilibrated in isosmotic media were abruptly injected into media containing 1M CPA in isotonic buffer. The resulting “shrink-swell” volume versus time data were fit to a two parameter solute solvent flux model.

Figure 2.

Plot of theoretical cell volume versus temperature during cooling (panel A), warming (panel B), and versus time at room temperature (panel C) for cells in Me_2SO (solid line) and in PG (dashed line). In panel A, cooling rates were $4^\circ\text{C}/\text{min}$ and $1^\circ\text{C}/\text{min}$ for Me_2SO and PG, respectively, and plunge temperatures were -32.5°C and -40.5°C for Me_2SO and PG, respectively.

Figure 3.

Boyle Van't Hoff relationship for C57BL/6 mouse ES cell line. ES cell volumetric response to variable osmotic stresses was measured at 22°C using an electronic particle counter. Cell volume response was measured in real time following abrupt exposure to 206, 290, 600, 900, 1300, and 2800 mOsm solutions, $n=6$. Error bars represent SEM. V , equilibrated cell volume; V_{iso} , isosmotic cell volume; iso , isosmotic; osm , osmolarity of treatment. V_b is expressed as mean percent of isosmotic cell volume $\pm$ SEM.

Figure 4. Mean ($\pm$ SEM) osmotic tolerance limits of C57BL/6 mouse ES cells as determined by plasma membrane integrity. Equal numbers of ES cells were exposed to solutions of 38, 75, 150, 600, 1200, 2400 and 4800 mOsm ($n=6$ for each solution) for 10 minutes at room temperature, and compared with controls in which the same quantities of cells were exposed to isosmotic solution (285 mOsm) in the same manner. Plasma membrane integrity was assessed by flow cytometry analysis of propidium iodide exclusion. The shaded area of the graph represents the osmotic range in which 80% of cells maintain cell membrane integrity. Data points with similar subscripts are not significantly different as compared to isosmotic solution ($p<.05$).

Figure 5.
Improved freezing protocol for C57BL/6 mouse ES cells. FBS, fetal bovine serum.

Table 1

Definition of major symbols and terms.

Symbol or abbreviation	Description	Units	Value
e, i	Superscripts: e, external; i, internal	None	None
s, n, w	Subscripts: s, solute; n, nonpermeating; w, water	None	None
L^{CPA}	Hydraulic conductivity	$\mu\text{m min}^{-1} \text{atm}^{-1}$	Parameter
P	Solute permeability	$\mu\text{m min}^{-1}$	Parameter
P_{CPA}	Temperature	K	295
T	Cell surface area	μm^2	Parameter
A	Cell volume	μm^3	Variable
M_n^e	Osmotically inactive cell volume	μm^3	Parameter
V_b	Intracellular water volume at time t	μm^3	Variable
$V_w(t)$	Moles of internal permeating solute at time t	Moles	Variable
$n_s^i(t)$	Time	Seconds	Variable
T	Osmolality of initial (isotonic) intracellular salts	$\text{Osm kg}^{-1} \text{H}_2\text{O}$	0.290
M_{iso}	Osmolality of extracellular salts	$\text{Osm kg}^{-1} \text{H}_2\text{O}$	0.290
M_n^e	Osmoles of intracellular salts	Osm	Variable
n_n^i	Molality	$\text{mol kg}^{-1} \text{H}_2\text{O}$	Variable
M	Partial molar volume of solute	L mole^{-1}	Variable
$\bar{V}$	The universal gas constant	$\text{kcal mole}^{-1} \text{K}^{-1}$	1.987×10^{-3}
R			

Table 2

Room temperature solute permeabilities and hydraulic conductivities, and their associated activation energies for C57BL/6 mouse ES cells with respect to 4 cryoprotective agents (CPA). Due to the relatively low room temperature values for hydraulic conductivity and solute permeability, glycerol was deemed an unsuitable CPA for equilibrium freezing, and measurements at additional temperatures were not conducted. All CPA were at 1.0 M concentration. Hydraulic conductivity and solute permeability values are expressed as mean $\pm$ SEM. Superscripts indicate no significant difference between values.

	Hydraulic conductivity $\mu\text{m}/\text{min}/\text{atm}$	Solute permeability $\mu\text{m}/\text{min}$
Dimethyl sulfoxide	0.41 ± 0.03^a	4.59 ± 0.41^c
Activation energy	14.29^b	15.47^d
r^2	0.9847	0.9119
Ethylene glycol	0.48 ± 0.06^a	4.17 ± 0.24^c
Activation energy	15.35^b	13.19^d
r^2	0.9680	0.8293
Propylene glycol	0.42 ± 0.03^a	6.58 ± 0.38
Activation energy	14.12^b	14.08^d
r^2	0.9829	0.8943
Glycerol	0.14 ± 0.01	1.05 ± 0.07
Activation energy	-	-
r^2	-	-

Table 3

Predicted optimal freezing rates for the C57BL/6 mouse ES cell line.

1.0 M CPA	Rate (°C/min)	Plunge Temperature (°C)
Dimethyl sulfoxide	4.1	-33
Ethylene glycol	1.4	-41
Propylene glycol	1.2	-41