Optimisation of current Good Manufacturing Practice compliant controlled rate freezing for human embryonic stem cells

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Introduction

The generation of clinical grade human embryonic stem cell lines (hESCs) to a level of current Good Manufacturing Practice (cGMP) requires a validated, reproducible and safe method of freezing cells for the creation of a Master Cell Bank. In combination with a suitable chemically-defined cryopreservation reagent, Controlled Rate Freezing offers the highest level of compliance with cGMP standards for human cells. The Asymptote EP600 is a compact, cleanroom-compliant controlled rate freezer that is not dependent on liquid nitrogen for operation. Our aim in this study was to determine the efficiency in recovery of hESCs using the EP600 in combination with a suitable defined cryopreservative. In addition, to optimise hESC viabilities by assessing the effect of nucleation and different freeze profiles on the recovery post-thaw.

Materials and Methods

Culture and freezing - RCM-1 hESC line was cultured in a feeder-free system comprising StemPro (Invitrogen) supplemented with 8 ng/ml BFGF, using CELLSart (Invitrogen) as the matrix. Cells were cultured in a 12-well plate (approximately 1x10\textsuperscript{6}cells/well), refreshed with fresh supplemented media 1 hour prior and harvested mechanically using an EZ passage tool (Invitrogen). The hESCs from each of the wells were processed discretely in triplicate or quadruplicate. The cells were centrifuged and either resuspended into ice-cold media and an equivalent volume of x2 freeze reagent (RC x2 Freeze) was added or cells were resuspended according to manufacturers instructions (Synth-a-Freeze, Invitrogen; Profreeze, Lonza and Cryostor CS10, BioLife Solutions). The cells were frozen down either using the programmed profiles indicated on the EP600 controlled rate freezer before being stored in a -150°C freezer, or alternatively placed in a ‘MR Frosty’ isopropanol tub (Nunc) and then placed in a -40°C freezer overnight, before being transferred to a -150°C freezer for at least 24 hours.

Resuscitation and assessment of viability – Cells were resuscitated and 10% of the cells were reserved, trypsinised and subjected to a Trypan Blue (Invitrogen) assessment of immediate viability. The remaining cells were re-seeded into a 12-well plate and cultured for 48 hours in StemPro media supplemented with 8 ng/ml BFGF on CELLSart. Following 48 hours incubation, the cells were subjected to an Alkaline Phosphatase stain (Sigma) to visualise successfully plated and growing ES cell colonies, to determine post-thaw (i.e. longer term) viability. These wells were imaged and a viability assessment was made qualitatively. Each experiment was performed at least in triplicate and a student t-test performed (i.e. indicate statistically significant differences (p<0.05, **p<0.001, ***p<0.0005).

Temperature measurements were made using a Squirrel data logger (Grant Instruments) with the thermocouples situated within the sample vials for each run, and subjected to nucleation, where indicated as above.

Cryostor CS10 yields the highest viability of hESCs post-thaw

Three commercially available xeno-free and defined products - Synth-a-Freeze (Invitrogen), Profreeze (Lonza) and Cryostor CS10 (BioLife Solutions) were evaluated in addition to our own x2 Freeze reagent using passive freezing in a Mr Frosty. Immediate recovery was not significantly different with each reagent with Cryostor CS10 provided the highest viability when assessing post-thaw recovery and growth (figure 1).

![Figure 1: Immediate viability of hESCs does not vary significantly when frozen in various cryopreservatives and assessed with Trypan Blue (A), images taken following an incubation period previous to indicate that Cryostor CS10 provides the highest level of recovery (B).](image1)

Efficient nucleation is essential for high levels of hESC viability

The EP600 was used to freeze hESC in a linear freeze profile, using Cryostor CS10, with an ice nucleation step at -9.0°C where indicated. Efficient nucleation of the vias resulted in an improvement of immediate viability of 26% compared to that of non-nucleated cells (from 39.9 ± 2.0% to 65.9 ± 4.5% - see Table 1) and a consequent increase in hESC colony survival following the 48 hr culture period (figure 2).

![Figure 2: Efficient nucleation of vias results in a significant increase in hESC viability when compared to non-nucleated control samples (A). Images of wells following an extended culture period (B)].(image2)

![Figure 3: Temperature profiles of samples frozen in Cryostor CS10 using the linear and non-linear type 'A' and type 'B' freeze profiles without (A) and with (B) a nucleation step. The temperature profile of a sample frozen using a 'Mr Frosty' is included for comparison (A).](image3)

![Figure 4: Immediate viability (A) and images from an extended culture period following recovery of vias (B) indicating the differences between profile 'A' and profile 'B'. Nucleation significantly improves recovery of cells frozen using profile 'A' but not that of 'B'.](image4)

Improvements to viabilities using non-linear freeze profiles

To investigate further optimisation of the recovery rate, two additional freeze profiles were evaluated in conjunction with nucleation. Using Cryostor CS10 as the cryopreservant, hESCs were frozen down in a non-linear ‘A’ profile (with an initial shallow temperature drop) or in a ‘B’ type profile (with a steeper initial temperature drop) with and without nucleation (figure 3).

![Figure 5: Immediate viability (A) and images from an extended culture period following recovery of vias (B) indicating the differences between profile 'A' and profile 'B'. Nucleation significantly improves recovery of cells frozen using profile 'A' but not that of 'B'.](image5)

Conclusions

Cryostor CS10 provides the highest hESC viability post-thaw in a comparison of commercial defined, xeno-free cryopreservatives.

Efficient nucleation is essential for high recovery of cells when applying controlled rate freezing. Different freeze profiles can provide varying viabilities in combination with nucleation. The Asymptote EP600 represents an efficient solution for the cryopreservation of hESCs within a cleanroom environment.