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Cryopreservation of Human Embryonic Stem Cell Lines

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Summary

Two different approaches have been adopted for the cryopreservation of human embryonic stem cells (hESCs): vitrification and conventional slow cooling/rapid warming. The vitrification method described here is designed for hESCs that grow as discrete colonies on a feeder cell monolayer, and are subcultured by manual subdivision of the colonies into multicellular clumps. hESCs that are subcultured by enzymatic dissociation can more conveniently be cryopreserved by conventional slow cooling/rapid warming methods. Although both methods are suitable for use in a research context, neither is suitable for cryopreservation of embryonic stem cells destined for clinical diagnostic or therapeutic uses without modification.

Key Words: Human embryonic stem cells; hESCs; vitrification; cryopreservation; controlled-rate cooling.

1. Introduction

In 1998, the successful derivation of a human embryonic stem cell (hESC) line was first reported by Thomson et al. ***(1)***. Since then, the derivation of hESC lines from donated in vitro fertilized embryos has been reported by an increasing number of groups worldwide (*see* **ref. *2*** for review). Two different approaches to cryopreserving hESCs have been adopted: conventional slow cooling/rapid warming and vitrification. The former is based largely on protocols developed for murine embryonic stem cells (mESCs), while the latter is based on vitrification methods developed for embryos. To date, no systematic studies on optimizing cryopreservation protocols for hESCs have been conducted and the few empirical studies that have been undertaken have yielded inconsistent results; not only in terms of "viability" of the cell lines examined, but also in a clear choice of a preferred cryopreservation protocol.

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Methods for the cryopreservation and storage of mESCs are well established ***(3)***. They generally employ a conventional cryoprotectant solution, which consists of 10% (v/v) dimethyl sulfoxide (DMSO) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10–90% (v/v) fetal bovine serum (FBS). Slow cooling in cryovials, at or around 1°C/min, and rapid thawing within a 37°C water bath complete the process. Apart from minor variations, such as preservation on microplates ***(4,5)***, the methods adopted for mESCs are similar to standard protocols routinely used for established cell lines and primary cultures.

Adapting the mouse protocols for hESCs has, in some hands, proved problematic (*see* **ref. *6*** for review). This has been attributed to the highly "cooperative" nature of hESCs (as compared with mESCs), which appear to require intimate physical contact between the cells of the colony (to permit cell–cell signaling) and an optimum clump size of approx 100 cells during serial passage ***(7,8)***. A number of studies of hESCs, comparing vitrification and slow cooling, have reported extremely low viability following the latter ***(9–11)***. In these studies, survival rates of between 6 and 30% (as determined by the ability of colony fragments to reattach, multiply, and retain their undifferentiated state) were reported, with the conclusion that vitrification was the preferred option.

However, a number of stem cell lines have been reported to survive conventional slow cooling/rapid thawing. Whole cell colonies, cryopreserved while still adhering to the culture dish, or embedded in a Matrigel matrix showed an increased level of survival (as measured by Trypan blue dye exclusion or the metabolic assays MTT and Alamar blue) when compared with colony fragments cryopreserved in suspension ***(12)***. Colony fragments, when frozen in suspension, can also show high levels of survival and growth. The HuES cell lines may be cryopreserved in this way, using 10% DMSO and FBS or DMEM ***(13)***. A study using the hESC line H1 also showed that survival rates (as measured by colony number and size) of up to 80% could be achieved using a DMSO-FBS-DMEM cryoprotectant solution. The factors critical for optimizing colony (and cell) survival were a cooling rate of between 1 and 1.8°C/min, and seeding samples at –7 to –10°C ***(14)***. A similar study, again using a DMSO-FBS-DMEM cryoprotectant solution, has reported high levels of survival (>80%) with a cooling rate of 0.5°C/min and seeding at –10°C ***(15)***. The use of a controlled-rate cooling device for slow cooling, although desirable from a quality system and regulatory perspective, is not a requirement as passive cooling devices were no less effective than controlled-rate cooling in a number of studies ***(14,16)***. The protocol used by Ware et al. is published in their paper ***(14)***, whereas that for the HuES cell lines can be found in the technical appendix published separately to the paper ***(13)***. However, details of the cooling rate used have been omitted from the supplement.

Nevertheless, the majority of groups deriving stem cell lines have adopted vitrification as the method of choice despite the practical difficulties associated with this methodology. This choice is based largely on the comparative studies previously referred to ***(9–11)***, all of which indicated that vitrification led to increased post-thaw survival of colony fragments when compared with conventional slow cooling. Survival rates of more than 75% were reported in all three studies.

The vitrification protocols reported in all three studies are very similar, based as they are on one previously developed for the cryopreservation of bovine ova and embryos ***(17)*** and applied to hESCs with some modification by Reubinoff et al. ***(9)***. The essence of this protocol has since been described in a manual on hESC culture produced by ESI International ***(18)*** and available via their website. The protocol requires stepwise exposure of colony fragments to two vitrification solutions of increasing cryoprotectant concentration. The common components of which are DMSO and ethylene glycol (EG). The composition of the vehicle solution varies, with differences in sucrose concentration and the presence or absence of FBS, human serum albumin, and buffer (HEPES).

Extremely rapid cooling rates are required to achieve vitrification using this two-component system. This is accomplished by direct immersion into liquid nitrogen (LN_2) of open-pulled straws (OPS) containing small droplets (typically 1–20 μL) of vitrification solution within which were held the colony fragments. Warming, too, has to be sufficiently rapid to avoid devitrification and ice crystallization. This is accomplished by direct immersion of the vitrified samples into a prewarmed, sucrose-based thawing solution followed by stepwise elution of the cryoprotectants using sucrose as an osmotic buffer.

The requirement for rapid cooling and warming has precluded the use of conventional cryovials in the vitrification protocols so far developed for hESCs. The preferred option has been for OPS, though a closed-straw alternative has been shown to provide equally high survival ***(10)***. An alternative method, using intact adherent colonies and a specially designed culture dish and cryovial, has been proposed ***(19)*** but no trials using this method have so far been published.

The vitrification methods so far developed, although suitable for research applications where only small stocks of cells are required, nevertheless impose a number of constraints on large-scale cell banking and storage of embryonic stem cells for use in regenerative medicine or pharmacological and cytotoxicity studies. These applications will require large banks of cells that will have been produced under regulatory frameworks requiring a sterile product prepared in media that is free, as far as possible, from xeno-derived components. Some attempt has been made to address these problems. Substituting closed

straws for open straws, human alternatives such as human serum albumin for animal components such as FBS, and storage in the vapor phase above LN_2 rather than in liquid phase have all been shown to have little or no detrimental effect on colony survival or the level of cell differentiation ***(10)***. However, many of the regulatory requirements, particularly those relating to contamination, and their impact on the cryopreservation and storage of hESCs, have yet to be addressed.

Although the method detailed in this chapter has been shown to be effective for a range of hESC lines under laboratory conditions, it is extremely labor intensive, requiring time-consuming manual dissection and manipulation of the colonies through a series of solutions under a low-magnification stereomicroscope. Transfer of the colony fragments into and out of the various vitrification solutions is time critical, as is the process of transfer to and from liquid nitrogen. Moreover, the use of straws (sealed or otherwise) is incompatible with efficient, routine preparation of large banks of cells. All these procedures require a high level of acquired skills and do not lend themselves to the preparation of the bulk quantities of hESCs required for therapeutic and diagnostic applications.

2. Materials

Appropriate facilities for following good cell culture practice are required (*see* **Note 1**).

2.1. Equipment

1. Laminar flow hood.
2. Cell culture incubator.
3. Binocular stereo-zoom microscope with transmitted light base and tilting substage mirror or darkfield illumination system (*see* **Note 2**).
4. 37°C warm plate or heated microscope stage (optional) (*see* **Note 3**).
5. Appropriate protective clothing and safety equipment for handling LN_2.
6. Small (500 mL or 1.0 L) wide-neck vacuum flask/Dewar.
7. LN_2 vessel for precooling cryovials.
8. LN_2.
9. Pipettors and sterile tips for 1- to 1000-µL volume range.
10. Dual-function countdown timer.
11. Colony cutting tool (*see* **Note 4**).
12. 4- or 5-well cell culture plate or small culture dish (*see* **Note 5**).
13. 6-cm bacteriological Petri dishes (*see* **Note 6**).
14. 5-mL screw-cap cryovials.
15. Aluminium cryovial storage canes (*see* **Note 7**).
16. 21-gage × 1 in. needles.
17. Sterile OPS (*see* **Note 8**).
18. Long (20 cm) and short (12 cm) forceps.
19. Test tube rack to hold cryovials and solutions.

2.2. Cells

hESC colonies of 1–3-mm diameter, growing on a feeder cell layer in a single or multiwell cell culture dish.

2.3. Solutions

1. Growth medium (GM): use appropriate GM recommended for your particular hESC line.
2. Base medium (BM) for vitrification and thawing: 89% (v/v) 25 m*M* HEPES-buffered DMEM (Invitrogen, cat. no. 42430-025, Paisley, Scotland, UK), 10% (v/v) FBS, and 1% (v/v) nonessential amino acids (Invitrogen, cat. no. 111430-035, 10 m*M* solution). Store for up to 1 wk at 4°C.
3. Base medium with 1 *M* sucrose (BMS): for a 10-mL final volume, add 3.42 g sucrose to 6 mL 25 m*M* HEPES-buffered DMEM and warm to 37°C to dissolve. Add 2 mL FBS, plus enough DMEM to bring the final volume to 10 mL. Sterilize by passing through a 0.2-µm syringe filter. Store for up to 1 wk at 4°C.
4. Vitrification solution 1 (V1): 80% (v/v) BM, 10% (v/v) DMSO, 10% (v/v) EG. Filter-sterilize as above can be aliquotted and stored at –80°C.
5. Vitrification solution 2 (V2): 50% (v/v) BMS, 10% (v/v) BM, 20% (v/v) DMSO, 20% (v/v) EG (*see* **Note 9**). Filter-sterilize as above can be aliquotted and stored at –80°C.
6. Thawing solution (T1): 80% (v/v) BM and 20% (v/v) BMS. This solution is only required for the alternative thawing method (*see* **Note 10**).
7. Thawing solution with 0.1 *M* sucrose (T2): 50% (v/v) T1 and 50% (v/v) BM. This solution is only required for the alternative thawing method (*see* **Note 10**).

3. Methods

All culture manipulations should be carried out following good cell culture practice/aseptic technique in a laminar flow hood if possible (*see* **Note 1**).

3.1. Vitrification

1. Prepare all solutions in advance. Fill the wide-neck vacuum flask/Dewar with LN_2 using the appropriate safety equipment.
2. Set out equipment in the work area (laminar flow hood) including the flask/Dewar containing LN_2.
3. Prepare a well or dish containing 1 mL of fresh GM to hold the colony fragments after dissection.
4. Prepare a vitrification plate. This can be the base or lid of a 6-cm Petri dish (*see* **Note 6**). Pipet one 200-µL drop of V1 and two 100-µL drops of V2 spacing the drops well apart on the plate and avoiding air bubbles.
5. Program one timer to count down 1 min, and the other to count down 25 s.
6. Place the culture dish containing hESC colonies on the microscope stage and cut one or more colonies into appropriate-sized fragments (*see* **Notes 11–13**).
7. Using a pipettor set on 5–10 µL, prewet the tip with GM from the culture dish and transfer the colony fragments to the holding well containing fresh GM (*see* **Notes 14** and **15**). Return the culture dish to the incubator.

8. Place the vitrification plate alongside the holding plate on the microscope stage or nearby.
9. Set the pipettor to 5 µL, prewet a tip with GM and take up six fragments from the holding well (*see* **Note 13**). Focus the microscope on the 200-µL drop of V1 solution on the vitrification plate. Submerge the open end of the pipet tip in the center of the V1 drop and gently expel the colony fragments into the drop avoiding the meniscus.
10. Start the timer (set for 1 min). The fragments will gradually sink to the bottom of the V1 solution.
11. While the timer is counting down, place a new tip on the pipettor and prewet it in the V1 drop. At the end of the 1-min period collect the colony fragments in the pipet tip and transfer them into the center of the first 100-µL drop of V2 solution avoiding the meniscus.
12. Start the other timer (set to 25 s). The fragments will float in the V2 solution.
13. While the timer is counting down, change the pipet tip and prewet it in the first V2 drop. At the end of the 25-s period transfer the fragments to the second 100-µL drop of V2 solution. Proceed immediately with the next step.
14. Change the tip and prewet it in the second drop of V2 solution. Collect the colony fragments in 3–5 µL and pipet this onto a clean area of the vitrification plate to form a small drop. It is important to avoid introducing an air bubble into this drop. Make sure that the tip is prewetted and take a small volume of V2 solution into the tip ahead of the fragments so that they can be deposited onto the plate without depressing the plunger to its full extent, leaving a small residue of liquid in the pipet tip.
15. Set the pipettor aside and pick up a pulled straw (*see* **Note 16**). Holding the straw at a slight angle to the vertical, touch the narrow open end of the straw against the surface of the drop containing the fragments. The liquid and fragments should enter the straw by capillarity (*see* **Note 17**).
16. Vitrify the fragments by holding the straw vertically (narrow end down) and immersing the lower end of the straw immediately in LN_2. Rest the straw against the edge of the Dewar, allowing approx 1 cm of the straw to protrude above the surface of the LN_2. Multiple straws can be lined up around the edge of the Dewar and held in this way until transferred to cryovials. Take care that the vitrified samples always remain submerged in LN_2.
17. Repeat the procedure (**steps 5–16**) with further batches of fragments using the drops of vitrification solutions V1 and V2 on the vitrification plate up to three times. For further repetitions set up a new vitrification plate with fresh drops of vitrification solution (*see* **step 4**).
18. When the required number of straws has been processed, label an appropriate number of 5-mL cryovials and puncture each vial several times with a 21-gage needle. Remove the lids, attach each vial to the bottom of an aluminium cane, and precool in vapor phase or LN_2 (*see* **Note 7**).
19. Remove one precooled cane at a time and transfer it to the Dewar containing the straws, taking care not to disturb them. Use sterile forceps to transfer the appropriate number of straws to the cryovial (*see* **Note 18**).
20. Replace the cap and transfer to cryostorage below –160°C (*see* **Note 19**).

3.2. Thawing

The choice of method for thawing vitrified hESCs will depend on the particular embryonic stem cell line. The method described next is relatively quick and simple (for an alternative method, *see* **Note 10**).

1. Prepare a culture dish or a well containing the appropriate feeder cell layer incubated in advance in GM.
2. Retrieve a cryovial containing vitrified straws from cryostorage. Transport the vial to the work area in a container capable of maintaining the temperature below –160°C (*see* **Note 19**).
3. Fill the wide-neck vacuum flask/Dewar with LN_2 using the appropriate safety equipment.
4. Prepare a holding dish or well containing 1 mL of GM, warmed to 37°C in a cell culture incubator.
5. Remove the cryovial from the transport container, attach it to the bottom of an aluminium cane, remove the cap, and submerge the vial in the small dewar of LN_2.
6. Set a pipettor to 20 µL and attach a tip that will fit snugly into the wide end of a straw.
7. Place the holding dish/well under the microscope and remove the lid.
8. Raise the cryovial to the surface of the LN_2 and remove one straw with sterile forceps.
9. Take the straw between the thumb and middle finger and submerge the narrow end in the well of GM. As soon as the liquid inside the straw melts, place a finger over the upper end of the straw to help force the vitrification solution and colony fragments into the well. Take care not to damage the fragments with the end of the straw as they fall into the well. It is important to minimize any mechanical disturbance or mixing of the medium at this stage.
10. If the contents of the straw do not empty readily into the holding well, insert the tip of the prepared pipettor into the upper (wide) end of the straw, twisting to obtain a seal, and depress the plunger gently. If necessary, rinse out the end of the straw by gently drawing up and expelling some of the GM.
11. Carefully place the holding well in the cell culture incubator and leave to equilibrate for 5 min.
12. Bring the holding well and culture dish containing prepared feeder cells to the microscope. Using a fresh, prewetted tip gently transfer the colony fragments from the holding well to the culture dish, spacing them out evenly on the feeder layer.
13. Allow the fragments to sink onto the surface of the feeder layer before carefully returning the culture dish to the cell culture incubator.
14. Viable colony fragments will generally attach to the feeder layer within 24–72 h after thawing.

4. Notes

1. The use of appropriate facilities, good cell culture practice, and good aseptic technique should prevent contamination and provide viable colony fragments.

However, this cryopreservation method is not intended *per se* to provide cell material for clinical, diagnostic, or therapeutic use. Such material will be required to comply with regulatory requirements, which encompass the derivation, processing, storage, and distribution of the cell line, as well as cryopreservation when destined for such uses.

2. It is important that the microscope has an objective lens with a wide enough field of view to include the whole hESC colony and its environs, with sufficient working distance to allow the use of the pipettor and cutting knife.
3. Using the method described here, we have not found it necessary to warm the holding well or processing plate during vitrification or thawing. However, it may be advantageous to use a warming plate during the alternative (longer) thawing method (*see* **Note 10**) to obviate moving the thawing plate repeatedly to and from the incubator during the washing steps.
4. A number of options are available as cutting tools, including pulled glass Pasteur pipets, plastic pipet tips, sterile manufactured stem cell knives, or glass holding-pipets.
5. Multiwell cell culture plates (e.g., Medical Technology Ventriebs-GMbH Altdorf, Germany, cat. no. 19021/0005) or in vitro fertilized/organ culture dishes (e.g., Corning/Costar, Corning, NY, cat. no. 3260MTG) are required for holding the colony fragments after dissection. Shallow wells with sloping sides are preferable for ease of pipetting under the microscope.
6. Dishes that have not been coated or treated for cell attachment are preferred so that solutions can be pipetted onto the surface in discrete drops that will not spread over the plastic.
7. For easier handling of 5-mL cryovials, use canes designed to hold 2-mL vials so that the 5-mL vial will stand out at an angle from the cane affording better access when adding or removing the cap or contents.
8. OPSs are available from LEC Instruments (Victoria, Australia; www.lecinstruments.com).
9. The vitrification solution V2 used in this protocol contains 0.5 *M* sucrose. Other protocols use different sucrose concentrations, e.g., 0.33 *M*, *see* protocols at www.escellinternational.com.
10. An alternative thawing method utilizing a stepwise elution protocol involves thawing the vitrified fragments and incubating for 1 min in thawing solution (T1), followed by sequential 5-min incubations in T2, and two aliquots of BM before being plated onto the appropriate feeder cell layer. Details can be found in the ESI Methodology Manual Embryonic Stem Cell Culture, July 2005, available at www.escellinternational.com.
11. Only choose undifferentiated colonies for vitrification, and preferably those that appear relatively thick. Colonies with a flatter, thinner morphology (typically those growing on sparser areas of the feeder layer) tend to be more difficult to cut cleanly.
12. The fragments should be the largest size that can be taken up into the pipet tip (in 3–5 μL of liquid). The fragments will shrink significantly as they are passed through the vitrification solutions.

13. Cut fragments in multiples of six in order to vitrify six fragments per straw. Until practiced, it is recommended to process just six fragments at a time. With experience it is possible to process enough fragments for several straws in one operation, the limiting factor being the number of fragments that can be taken up in 5 µL of liquid. Increasing this volume is not advisable, as this may lead to excessive dilution of the vitrification solutions.
14. When transferring colony fragments from one solution to another, or whenever you use a new pipet tip, it is important to prewet the tip by drawing up and expelling some of the appropriate solution before picking up the fragments. This will help to prevent the fragments from sticking to the tip and avoid air bubbles.
15. Always minimize the pipetting of fragments. Pipet slowly, using the smallest volume pipettor (5- or 10-µL size), and set it to the minimum volume necessary to transfer the fragments. With practice it is possible to pipet 12 or more fragments in 3 µL.
16. As there may be some variability in the length of the OPSs within a packet, check that your straws will fit inside your 5-mL cryovials before picking up the processed fragments.
17. Taking up the fragments into the straw can take several seconds, and may be helped by resting the tip of the straw on the plate and slowly tilting it into a vertical orientation. Because of the short incubation time in V2, the fragments do not become fully equilibrated and will tend to float toward the top of the column of liquid as it enters the straw. If all of the liquid from the small drop is not taken up, leaving the fragments close to the open end of the straw, it is often possible to move them further into the straw by dipping the end of the straw into one of the larger drops of V2.
18. In terms of safe storage and stock inventory, it is advisable to place only one or two straws per cryovial. This enables the minimum number of straws to be removed from cryostorage when cells are required for culture, and obviates the need for relabeling or replacing vials when part of their contents have been removed.
19. Vitrified cells must always be maintained below –160°C to avoid devitrification. Straws must not be stored or transported on dry ice.

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