# 5 Slow Cooling Cryopreservation Optimized to Human Pluripotent Stem Cells

Takamichi Miyazaki and Hirofumi Suemori

**Abstract**

Human pluripotent stem cells (hPSCs) have the potential for unlimited expansion and differentiation into cells that form all three germ layers. Cryopreservation is one of the key processes for successful applications of hPSCs, because it allows semi-permanent preservation of cells and their easy transportation. Most animal cell lines, including mouse embryonic stem cells, are standardly cryopreserved by slow cooling; however, hPSCs have been difficult to preserve and their cell viability has been extremely low whenever cryopreservation has been attempted.

Here, we investigate the reasons for failure of slow cooling in hPSC cryopreservation. Cryopreservation involves a series of steps and is not a straightforward process. Cells may die due to various reasons during cryopreservation. Indeed, hPSCs preserved by traditional methods often suffer necrosis during the freeze-thawing stages, and the colony state of hPSCs prior to cryopreservation is a major factor contributing to cell death.

It has now become possible to cryopreserve hPSCs using conventional cryopreservation methods without any specific equipment. This review summarizes the advances in this area and discusses the optimization of slow cooling cryopreservation for hPSC storage.

**Keywords**

Human pluripotent stem cell • Human induced pluripotent stem cells • Human embryonic stem cell • Cryopreservation • Slow-cooling • Vitrification

T. Miyazaki
Institute for Integrated Cell-Material Sciences, Kyoto University, Kyoto, Japan
e-mail: tmiyazaki@frontier.kyoto-u.ac.jp

H. Suemori (✉)
Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan
e-mail: hsuemori@frontier.kyoto-u.ac.jp



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## Abbreviation

| | |
|---|---|
| hPSC | Human pluripotent stem cell |
| hESC | Human embryonic stem cell |
| hiPSC | Human induced pluripotent stem cell |
| DMSO | Dimethyl sulfoxide |
| ROCK | Rho-associated coiled-coil forming kinase |
| MEF | Mouse embryonic fibroblast |
| EG | Ethylene glycol |
| KSR | Knockout serum replacement |
| FCS | Fetal calf serum |

## 5.1 Introduction

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) [1] and human induced pluripotent stem cells (hiPSCs) [2], have an infinite proliferative potential and capacity for differentiation into all cells of the three germ layers. Their unique properties make them promising sources of cells for transplantation therapy, drug discovery and investigations of human congenital abnormalities. For successful applications of hPSCs in regenerative medicine, it is necessary to prepare large numbers of hPSCs with known genetic backgrounds [3] and establish stable cell storage systems (cell banking). To this end, cryopreservation is a key tool that enables long-term preservation of cell stocks. Additionally, cryopreservation is important for easy transportation of cells, enabling the use of hPSCs as desired, and minimizing the loss of their specific cellular potentials. Methodologies for effective hPSC cryopreservation have been actively pursued, in parallel with investigations to improve hPSC yield. However, the progress of hPSC cryopreservation has been ambiguous, and it has also been unclear which factors affect hPSC survival in the cryopreservation process.

Here, we review the cellular characteristics of hPSC cells and the history of hPSC cryopreservation, together with the difficulties that have been encountered with hPSC cryopreservation to date. Finally, we describe an optimized method for hPSC cryopreservation.

## 5.2 Slow Cooling in hPSC Cryopreservation

In general, cryopreservation is a process of storing cells, tissues, and organs to maintain their properties and viability [4]. Biological material is cooled to freezing temperatures, typically to lower than −140 °C. For cell survival following freezing and thawing, it is necessary to avoid ice crystal formation at icing temperature, which would otherwise cause cell death due to stress from excess intracellular water volume. “Slow Cooling” is one of the cryopreservation methods used to avoid ice crystal formation. The cooling rate is maintained at approximately −1 °C/min (ranging from −1 to −10 °C) in freezing medium that typically contains 10 % (ranging from 2 to 20 %) dimethyl sulfoxide (DMSO) as a cryoprotectant. The cryoprotectant enters the cytoplasm, and dehydration and condensation of the cytoplasm are promoted as the freezing medium cools, inhibiting the generation of ice crystals [4]. This slow cooling technique is widely applied to different cell types, including mouse embryonic stem cells [5]. However, it has been difficult to apply slow cooling cryopreservation to hPSCs. Standard slow cooling resulted in less than 10 % survivability of hPSCs after cryopreservation (as measured 24 h after cryopreservation) [6]. The use of a modified cryoprotectant that contained DMSO, ethylene glycol (EG) and fetal calf serum (FCS) increased hPSC viability to 30 % (as measured by counting cell colonies after 10 days) [7]; while use of propylene glycol or glycerol cryoprotectant solutions resulted in 20–60 % of cells recovering [8]. Most of the improvements to the slow cooling method have focused on modifying the cryoprotectant. The rationale for cryoprotectant modification approaches may come from the impression that hPSCs are fragile and intolerant to the freeze-thawing process; however, the actual reason for the failure of cryopreservation had not been clarified. To better appreciate the

underlying reason for the resistance of hPSCs to cryopreservation using the slow cooling method; it is important to understand the specific cellular characteristics of hPSCs.

## 5.3 Cellular Characteristics of hPSCs

The difficulty with cryopreserving hPSCs might arise from the colony formation pattern that is characteristic of hPSCs. When hPSCs proliferate, they form tightly-packed colonies that are difficult to dissociate without damaging the cells. Cell-to-cell contact and the signals derived from these interactions, such those arising from E-cadherins, are vital for hPSC survival [9]. Indeed, inhibition of cell-to-cell interactions due to E-cadherin antibody leads to death of dissociated hPSCs [10]. To avoid this damage, hPSCs have been cultured in the presence of mild dissociation treatments such as Accutase or Collagenase IV, avoiding the complete single-cell state. Until the discovery of Rho-associated coiled-coil kinase (ROCK) inhibitors' effects on single dissociated hPSC survival [11], sustaining small clusters of cells was essential for hPSC culturing. This approach was applied to the cell detachment process during cryopreservation. Therefore, to cryopreserve hPSCs without single cell dissociation, researchers focused on another cryopreservation manner, vitrification.

## 5.4 Vitrification for hPSC Cryopreservation

Dramatic improvements in cell survival have been achieved by applying vitrification to hPSC cryopreservation. Vitrification involves flash cooling cells directly in liquid nitrogen. In contrast to slow cooling, vitrification avoids ice crystal formation in both the intracellular and extracellular environments. It is achievable when solutes are sufficiently concentrated, or when they are cooled rapidly, thereby increasing the viscosity [12]. Therefore, vitrification requires a system of efficient thermal conduction and is routinely administered in a narrow vessel such as an open pulled straw [13, 14]. Although most standard cell lines are generally cryopreserved by slow cooling, certain cell types and tissues, including oocytes, fertilized eggs and the embryos of several mammalian species and humans, are often preserved using vitrification [15–18]. Moreover, hESCs have also been successfully cryopreserved using vitrification [13]. In early hESC studies cells were stored in an open pulled straw and the maximum cryopreservation volume was extremely limited. However, an improved vitrification approach was soon developed, that instead used a cryotube and a small volume of freezing medium [19]. In comparison with the low cell survival obtained with slow cooling, vitrification sustained relatively higher cell survival rates after freeze-thawing [14, 20].

However, some problems remain with using vitrification for clinical or industrial hPSC applications. The improved vitrification method still restricts the number of cells that may be treated at any one time, and it is fewer than the number of cells that can be processed using slow cooling. Moreover, the freezing medium that is used for vitrification is significantly more harmful to hPSCs than that used in slow cooling. The small volume of freezing medium easily leads to an increase in temperature when transferring cryotubes at room temperature; consequently, rigorous control of the sample temperature is constantly necessary, and skilled handling is required for reliable vitrification results. Additionally, depending on the container used, there is a risk of contamination with infectious agents via direct contact with the liquid nitrogen. Thus, vitrification is unsuitable for the bulk storage of hPSCs, especially when they are intended for future clinical purposes.

In light of the limitations of applying vitrification for hPSC cryopreservation, it would be ideal if the slow cooling method could be modified to achieve hPSC cryopreservation. To adapt slow cooling to hPSCs, it is first necessary to recognize what factors underlie the difficulty of applying this technique. A review of previous studies shows that slow cooling of hPSCs has been trou-

blesome, as shown in Table 5.1. To clarify the influence of cryopreservation on hPSC survival, a detailed revision of how cell viability is estimated during cryopreservation is warranted.

## 5.5 The Cryopreservation Process

We often assume that cryopreservation is a simple technique. However, it involves several processing stages, including cell detachment, freezing, storage, thawing, and also 'reseeding'. Significantly, a proportion of cells die as a result of different factors at each processing stage. When the cells are detached there may be a high risk of necrotic cell death due to physical damage from handling. During freezing and thawing, cells may also be at high risk of necrotic cell death due to physical damage; for example, due to the cytotoxicity of cryoprotectant [21], osmotic injury due to excursion of cryoprotectant [22], intracellular ice formation in the cooling process [23], and recrystallization of intracellular ice during warming [24]. On the other hand, at reseeding following freeze-thawing, cells may be at risk of apoptotic cell death due to accumulated damage as a result of freeze-thawing [4] or insufficient adhesion. Together, these possibilities suggest that cell survival during cryopreservation should be individually estimated in a stepwise manner to allow the development of an optimal cryopreservation approach.

## 5.6 Estimation of hPSC Survival

Before a detailed discussion of cell survival efficiency, we should first review the method by which survival is measured. Development of an efficient methodology is most easily achievable when studies can be compared using a common parameter. However, as shown in Table 5.1, the method for estimating hPSC survival efficiency during cryopreservation is not standardized. In addition, previous studies have had trouble estimating the true survival efficiency. One of the issues in the estimation of hPSC viability is selection of the target cells to be counted. The hPSCs are often counted as colonies grown for several days; however, this is quite inaccurate because it easily leads to an overestimation because of the divisions of the original colonies during the freezing and thawing processes. Therefore, even when hPSCs are seeded as colonies, we should estimate the total number of cells by counting the number of single hPSCs after complete dissociation. The other issue is the timing of the counting and the target cells used for comparison. As also shown in Table 5.1, the time at which cell viability is counted is widely variable between different studies. There is a broad time range between 24 h and 1 week that is used for cell counting, while cell survival rates are estimated by the comparison with the number of living cells prior to freezing. Of course, hPSCs proliferate after seeding; therefore, it is inaccurate to calculate cell survival rates by comparing cell numbers a few days after seeding with those prior to freezing. Additionally, counting cells several days after seeding can mask the reason for reduced proliferation, which may be caused by cell adhesion failure or reduced growth. Thus, cell counting should be performed in a stepwise manner, minimizing the time period after the initial cell count to be used for comparison. Accurate cell estimates will allow the development of reliable improvements in cell survival efficiency using this technology.

## 5.7 Optimization of hPSC Cryopreservation

Based on the above background, Miyazaki et al. used a stepwise approach to estimate the detailed survival rates of hPSCs at each stage of cryopreservation [25]. Firstly, they estimated survival up until immediately after freeze-thawing using slow cooling. Dispase treatment was used to detach intact hPSCs colonies, after which survival of individual cells was calculated (42.3–59.4 % for each hPSC line). After freeze-thawing, hPSCs from these colonies had low survival rates (15.5–32.5 %). This data shows that most of the colony hPSCs had already died before reseeding.

**Table 5.1** Summary of cryopreservation protocols for human pluripotent stem cells

| Freezing medium | Cell lines/type of culture | ROCK inhibitor | Cellular state | Cryo-method | Recovery rate | Estimation | Reference |
|---|---|---|---|---|---|---|---|
| Slow cooling: 90 % FCS, 10 % DMSO, DMEM | hESC(HES-1,HES-2)/MEF feeder | No | Colony | Slow cooling, | Slow cooling: 16 % | Slow cooling: colonies after 2 days, compared with pre-freezing colonies | [13] |
| Vitrification: 20 % DMSO, 20 % EG, 0.5 mol/L sucrose, DMEM | | | | Vitrification | Vitrification: 100 % | Vitrification: measuring area of colonies | |
| 10 % DMSO, 50 % FBS, 35 mM trehalose, culture medium | hESC(H1,H9)/MEF feeder Or Matrigel | No | Colony | Slow cooling | <25 % | Increase ratio of cells, compared with DMSO alone | [27] |
| 90 % KSR, 10 % DMSO, 0.2 mol/L trehalose | hESC(original)/MEF feeder | No | Colony | Slow cooling | <48 % | Colonies at 7 days after seeding, compared with preseeding colonies | [20] |
| 5 % DMSO, 10 % EG, culture medium | hESC(SNUhES-3)/STO feeder | No | Colony | Slow cooling | <30 % | Colonies at 10 days after seeding | [7] |
| 10 % DMSO, 25 % FBS, culture medium | hESC(H1)/MEF feeder | No | Colony | Slow cooling | <79 % | Area of colonies, relative to non-freezing control | [28] |
| 10 % DMSO, 90 % culture medium | hESC(H9,BG01V/hOG), hiPS | Yes | Single | Slow cooling | <8-folds | Increase ratio compared with cells without ROCK inhibitor at 4 days after seeding | [29] |
| 10 % DMSO, 90 % KSR | hESC(H9) | Yes | Single | Slow cooling | After freezing: <80 % | After thawing: cells by flow cytometry analysis | [30] |
| | | | | | After seeding: <65 % | After reseeding: cells at 24 h after seeding | |

(continued)

**Table 5.1** (continued)

| Freezing medium | Cell lines/type of culture | ROCK inhibitor | Cellular state | Cryo-method | Recovery rate | Estimation | Reference |
|---|---|---|---|---|---|---|---|
| 10 % DMSO, 90 % culture medium | hESC(HS207,HS401)/human foreskin fibroblast feeder | Yes | Single | Slow cooling | <56 % | Cells after thawing by flow cytometry analysis, compared with prefreezing cells | [31] |
| 10 % DMSO, 90 % FCS | hESC(Royan H5,H6), hiPSC(Royan hiPSC1,4)/Matrigel | Yes | Single | Slow cooling | After thawing: <90 % | After thawing: cells, compared with prefreezing cells | [32] |
| | | | | | After seeding: <4–32 % | After seeding: colony formation, compared with initially seeded cells | |
| 10 % DMSO, 90 % KSR | hESC(H9,CHA-hES3)/MEF feeder | NO | Colony | Slow cooling | <51 % | Colonies at 7 days after seeding (relative to non-freezing control) | [33] |
| 10 % EG, 90 % culture medium | hESC(H9), hiPSC/MEF feeder or Matrigel | Yes | Single | Slow cooling | <60 % | Cells at 2 days after seeding | [8] |
| 10 % DMSO, 90 % culture medium | hESC(H9)/MEF feeder | Yes | Colony | Slow cooling | <10 % | Cells at 24 h after seeding, compared with postthawing alive cells | [6] |

As described earlier, slow cooling requires sufficient penetration of cryoprotectant and adequate dehydration inside the cells. Therefore, it is thought that hPSCs under a colony state are not tolerate to the process of slow cooling. To overcome this problem, Miyazaki et al. tried changing the state of hPSCs prior to freeze-thawing and estimated hPSC survival immediately after thawing. The hPSCs, dissociated into single cells by treatment with EDTA and TrypLE Select, had much high survival rates before freeze-thawing (68.1–77.7 %). More importantly, hPSCs remained viable even after thawing (59.7–66.1 %, corresponding with 80–90 % survival). This data shows that physical damage during freeze-thawing is minimized when hPSCs are cryopreserved in a single cell state, and suggests that colony morphology enhances direct damage that lead to necrotic cell death at the first step of cryopreservation. Together, these results suggest that the majority of hPSCs that are killed are a result of the process of traditional slow cooling itself, and are already dead before the reseeding stage.

After establishing the importance of the cellular state prior to freeze-thawing, appropriate colony dissociation methods for slow cooling were mostly addressed. The next consideration is avoiding the apoptotic cell death of single hPSCs when they are reseeded. To solve this problem, there are two approaches that may be used to improve the survival of single hPSCs. The first approach is the adjustment of the cell density at reseeding. Adequate cell density is required to allow cell-cell interactions, which promotes hPSC survival [26]. By optimizing the seeding density of freeze-thawed hPSCs, hPSCs showed robust adhesion and increased survival (40–60 %, compared with numbers of seeded alive cells) at 12 h after seeding [25].

The second approach is the use of ROCK inhibitor at cell reseeding, which effectively increased the survival of single hPSCs (55 % on average in comparison with the number of seeded viable cells). Importantly, higher cell density (for example, $3 \times 10^5$ cells/cm$^2$) did not require the use of ROCK inhibitors for hPSC survival, but cells at lower densities (for example, $0.5 \times 10^5$ cells/cm$^2$) benefited from its use. To achieve a density of $0.5 \times 10^5$ cells/cm$^2$, a slightly higher seeding density is required when the cells are subcultured. Therefore, cryopreserved single-hPSCs should be seeded at higher cell densities without the use of ROCK inhibitor, but it should be used for lower seeding densities. The described experiments were carried out using Matrigel-coated culture dishes. However, some culture substrates, such as specific laminin isoform and its fragment, have been shown to enhance the survival of single hPSCs [26]. When these substrates were used to coat culture vessels instead, cryopreserved single hPSCs were efficiently recovered at much lower cell densities (60 % at 12 h, compared against the number of cells seeded). Therefore, following optimization of the culture conditions, hPSCs that have been cryopreserved and thawed can easily and efficiently recover to subculture.

## 5.8 Conclusion

We have summarized the main difficulties encountered with using the slow cooling method for hPSC cryopreservation. During freeze-thawing, hPSCs that have been dissociated from the culture media and frozen as intact colonies are mostly killed, presumably having suffered necrotic cell death due to insufficient penetration of the cryoprotectant into the colony state. Following thawing, hPSCs survival has also been low, due to cells being seeded at an inadequate density, which is thought to lead to apoptotic cell death in response to earlier damage from the freeze-thawing process. By solving those problems; that is, dissociating hPSCs into a single cell state and optimizing the reseeding density after freeze-thawing, slow cooling becomes an effective method for hPSC cryopreservation. Importantly, because this is a routine cell culture technique, specific equipment is unnecessary.

The use of slow cooling for cryopreservation of hPSCs will be further optimized in the future to suit a wider range of clinical material. In doing so, it is highly recommended that the cellular state of target hPSCs be taken into consideration. If this is done, there should be no significant trou-

ble in the cryopreservation of undifferentiated hPSCs. Where differentiated cells that are derived from hPSCs are to be cryopreserved, the dissociation manner of target cells should be considered. This is because most differentiated cells form robust tissue structures, and these are more sensitive to disruption than those of cells in an undifferentiated state. As long as insufficient penetration of cryoprotectant into tissues remains a problem, it will be necessary to dissociate target cells into a single cell state. Although slow cooling is highly recommended for hPSC cryopreservation, vitrification is recommended instead for certain types of differentiated cells. We conclude that in any situation requiring hPSC cryopreservation it is important to optimize each step of the procedure and consider the cellular state of the hPSCs to be freeze-thawed. This approach is non-specific and may also be applied to the cryopreservation of pluripotent stem cells from other animal species.

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