A simple improvement of the conventional cryopreservation for human ES and iPS cells

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Abstract

In this study, a simple method for the cryopreservation of human embryonic stem (ES) and induced pluripotent stem (iPS) cells is proposed. It is based on the conventional slow-freezing method with 10% DMSO and modified mainly in a thawing protocol without specific equipment or reagents. Recovery rate of the cells cryopreserved by this method was equally high, which is comparable to that of the cells frozen by the vitrification method. In the case of vitrification method, it requires practiced hand because cells can be terribly damaged upon failure of rapid-warming process and strict maintenance of low temperature is required throughout the cryopreservation. On the contrary, our method is available for novices and cryopreserved cells can maintain cell recovery rate for one week after transfer from -150 °C to -80 °C condition. This simple modified method would gain widespread acceptance.

Introduction

Human embryonic stem (hES) cells1 and human induced pluripotent (hiPS) cells2, 3 have great potential as a possible source of cells for the regenerative medical transplantation of various tissues and also for other research applications, such as developmental biology, toxicology, and drug discovery. Today, a number of hES and hiPS cell lines have been established around the world. To promote these great potential researches using hES and hiPS cell lines, the prerequisite is the foundation of cell banks of well-characterized and safety-tested cells for research which serve as seed stocks for therapeutic applications. Currently, several cell banks are distributing such cell lines all over the world. The International Stem Cell Banking Initiative has developed a consensus on the principles of best practice for the banking, testing, and distribution of hES cells4, 5, 6, 7, 8. During cell banking, although most of the procedures share many of the principles of routine cell culture, a number of significant steps exist in the case of hES and hiPS cells. One of them is cryopreservation9. Both hES and hiPS cells have been well-known to be sensitive to the cryopreservation9, 10, 11. Two cryopreservation methods are commonly used for hES and hiPS cells which are vitrification12, 13, 14, 15 and slow freezing16, 17, 18, 19. At this moment, vitrification method is considered to be preferable for the local storage because of the high survival rate12, 15, 16, 20. However, vitrified

cells can be terribly damaged upon failure of rapid-warming process without practiced hand compared to slow-frozen cells. Furthermore, if the low temperature is not strictly maintained, cell viability decreases rapidly. Therefore, when vitrified cells are transported, it needs liquid nitrogen dry-shippers with careful documentation for the regulatory requirements and transports companies with technical expertise to undertake such shipments, resulting in the high costs involved. As for slow-freezing method with dimethyl sulfoxide (DMSO), although it has been long trusted as a stable cryopreservation for a wide variety of cells including mouse ES cells, it produces low levels of recovery or spontaneously differentiation for hES and hiPS cells.

Until now, several approaches have been investigated for the improvement of cryopreservation for hES and hiPS cells such as the technique based on stabilizing hES colonies adherent to or embedded in a Matrigel matrix17, and the protocol using of Rho-associated kinase (ROCK) inhibitor21, 22. Considering clinical application of hES and hiPS cells, xeno-free defined freezing medium should be required. An effective serum- and xeno-free chemically defined freezing procedure was reported by Hovatta's group23. Another group has proposed in situ cryopreservation using gas permeable culture casettes24. However, there is not any evidence that the cells can stably preserve their phenotypes for more than 10 years, excluding the conventional cryopreservation of the cells with DMSO. Thus, it is suggested that although these new methods would be worth to be challenged, the conventional cryopreservation should be backed up as a precaution.

We here show that a simple modification of the conventional slow freezing cryopreservation with 10% DMSO increased cell viability after thawing. This method requires neither specific equipment nor reagents and only the thawing way is changed, but resulting cells have comparable growth ability to those of vitrified cells. Even when using defined medium, such as our growth factor defined medium hESF925, 26, 27, 28, or Thomson's mTeSR medium29, this method could work well. Because the conventional slow freezing cryopreservation is quite familiar, this simple method would gain widespread acceptance.

Reagents

Critical: All reagents and materials used must be sterile.

Gelatin (0.1%, Millipore, cat. no. ES-006-B)

ES qualified fatal bovine serum (GIBCO, cat. no.10439)

DMEM-F12 (GIBCO, cat. no. 12660-012)

DMEM high glucose (GIBCO, cat. no. 11965-092)

Knockout serum replacement (KSR, Invtirogen, cat. no. 10828-028)

L-Glutamine (200 mM, Invitrogen, cat. no. 25030-081)

β-Mercaptoethanol (55 mM, Invitrogen, cat. no. 21985-023)

CAUTION: β-Mercaptoethanol is toxic. Avoid inhalation, ingestion and skin contact during use.

Non-essential amino acid solution (0.1 mM, Invitrogen, cat. no. 11140-035)

Basic fibroblast growth factor (bFGF, sigma-Aldrich, cat. no. F0291)

CRITICAL: The activity of bFGF is variable across companies

Dispase (Roche, cat. no. 04942078001)

2.5% trypsin (Invitrogen, cat. no. 15090-046)

Collagenase IV (Invitrogen, cat. no. 17104-019)

Dimethyl sulfoxide (DMSO, Sigma-Aldrich, cat. no. D2650)

CAUTION: Keep this reagent away from sources of ignition. Take precautions to prevent the buildup of electrostatic charge. Personal protection: protective gloves and safety glasses.

CRITICAL: High purification of DMSO should be used. Store in an airtight container after opening.

Feeder cells, Mitomycin C treated primary mouse embryonic fibroblasts (PMEF) (PMEF-CF; DS Pharma Biomedical Co.,Ltd, cat. no. BBASF1214) and (PMEF-H; Chemicon, cat. no. PMEF-H)

CRITICAL: PMEF-CF and PMEF-H are used for hES and hiPS cells respectively.

WA09 hES cells (WiCell, Madison, WI, USA) and JCRB1331 hiPS cells (JCRB1331, Osaka, Japan)

Reagent setup

β-Mercaptoethanol For a 1,000× stock (100 mM), dissolve 70 µl in 10 ml PBS (the final concentration is 0.1 mM). Store at 4 °C in 36 months from date of manufacture.

CAUTION: β-Mercaptoethanol is toxic. Avoid inhalation, ingestion and skin contact during use. Personal protection: gloves, safety glasses and good ventilation.

bFGF Dissolve 25 µg bFGF in 1-ml sterile Tris-base (5 mM). Store at -20 °C in 250–500 µl aliquots in three months. The final concentration should be 100 ng ml− 1.

CRITICAL: Avoid repeated freeze-thaw cycles. Thaw and add bFGF to the medium just prior to the use.

KSR Thaw at 4 °C overnight. Store aliquots at -20 °C up to12 months. CRITICAL: We recommend that KSR is ideally used in a week after thawing.

L-Glutamine Prepare 1:100 dilution, store aliquots at -20 °C in three months.

Non-essential amino acid solution Prepare 1:100 dilution, store aliquots at -20 °C in 12 months from date of manufacture.

Human pluripotent cell medium For WA09 hES cells, add 200 ml KSR, 5 ml L-Glutamine, 1.85 ml β-Mercaptoethanol and 8 ml non-essential amino acid solution into 800 ml DMEM-F12 medium. For JCRB1331 hiPS cells, add 200 ml KSR, 10 ml L-Glutamine, 1.85 ml β-Mercaptoethanol and 8 ml non-essential amino acid solution into 800 ml DMEM-F12 medium. Store at 4 °C and should be used in one week.

Dispase Add 20 mg dispase to 10 ml DMEM medium (high glucose). This gives a final concentration of 2 mg/ml. Sterilize through a 0.22□µm filter. It is possible to dispense enzymes in small aliquots and store at -20 °C for 6 months. Defrost dispase should be ideally used in three days after thawing. Avoid freezing and thawing.

Collagenase IV Dissolve 10 mg in 10 ml distilled water (the final concentration is 1 mg/ml). Sterilize through a 0.22 µm filter. It is possible to dispense enzymes in small aliquots and store at -20 °C for 6 months. Defrost collagenenase should be ideally used in three days after thawing.

CaCl2 Dissolve 0.11 mg in 10 ml distilled water (the final concentration is 0.1 M). Sterilize through a 0.22 µm filter. It is possible to dispense enzymes in small aliquots and store at 4 °C.

Collagenase-Trypsin-KSR solution (CTK) Add 5 ml of 2.5% trypsin, 5 ml of 1 mg/ml collagenase IV, 0.5 ml of 0.1 M CaCl2, and 10 ml of KSR into 30 ml of PBS. It is possible to dispense enzymes in small aliquots and store at -20 °C for 3 months. CTK should be ideally thawed when used. Avoid freezing and thawing.

Slow freezing medium Mix DMSO and KOSR in the ratio 1:9.

CRITICAL: The flow freezing medium should be prepared freshly and stored on ice before use.

Vitrification medium (DAP213) Dissolve 0.59 g of acetamide in 6 ml of Human pluripotent cell medium, subsequently add 1.42 ml of DMSO and 2.2 ml of propylene glycol. Fill up to 10 ml with the medium. Store aliquots at −80 °C for three months.

Fixation buffer (pH9.5) for Alkaline phosphatase staining Refer Table 1 for the recipes.

Equipment

• 15 and 50 ml Conical tube (BD, cat. no. 352096, cat. no. 352070)

• 25 cm2 vent bulb culture flask (Corning, cat. no. 430639)

• 5, 10, and 25 ml Plastic disposable pipette (VWR, cat. no. 89130-896, 89130-898, 89130-900)

• transfer pipette (Scientific, cat. no. SC-233-J).

• 0.22 µm Pore size filter (Millipore, cat. no. SLGP033RS)

• 0.22 µm Vacuum filtration (500 ml, Thermo Scientific, cat. no. 291-4520)

• Disposable syringes, 10 and 20 ml (Terumo, cat. no. ss-10ESz, ss-20ESz)

• Glass Pasteur pipettes, 9 inches. Sterilize by autoclave (Fisher Scientific, cat. no. 13-678-6B)

• Cryovial (Thermo Scientific, cat. no. 5000-0020)

• Centrifuge (KOKUSAN, H-60R)

• Inverted phase-contrast microscope (4, 10, 20 and 40× objectives) (OLYMPUS, CKX41)

• Purifier class II biosafety cabinet (LABCONCO, cat. no. 302310030)

• Micropipette (0.5–10, 10–100 and 100–1,000 µl) (Eppendorf, cat. no. 4910000018, 4910000042, 4910000069)

• Pipette aid (Drummond Scientific Company, PA-400)

• Tissue culture incubator, with humidity and gas control to maintain 37 °C and 95% humidity in an atmosphere of 5% CO2 in air (YAMATO, IP 400)

• Water bath (TAITEC, SDminiN)

• Hemocytometer (Onecell, OC-C-SO2)

• Cryovial storage rack (Thermo Scientific Nalgene, cat. no. 238-024)

• Liquid nitrogen tank (Thermo Scientific, TY509X4)

Procedure

Passaging of hES/iPS cells:

Maintain WA09 and JCRB1331 cells on feeder layers of mitomycin C-treated primary mouse embryonic fibroblasts (PMEF) in gelatin-coated 25 cm2 tissue flasks. Passage colonies every 5-7 days depending on colony growth. Exchange culture medium every day except the day following the passaging.

Freeze stock of hES/iPS Cells:

1. Remove the differentiated area completely by using the Pasteur pipette in advance.
2. Examine all the plates containing hES/iPS Cells by inverted phase-contrast microscopy. Cultures used for cryopreservation should show no signs of microbial contamination and should be subconfluent (the distance between the colonies is < 10-20% of the average colony diameter). Confluent cultures may be less amenable to freezing.
3. Label cryogenic vials with the name of the cell line, passage number, lot number and the date.
4. Remove the culture medium from flasks.
5. Add 1.5 ml dispase (1U/ml) per the T25 flask and incubate for 2-5 min until the edges of the colonies start to curl. CRITICAL: Treatment time of dispase varies and depends on the cell lines and quality of colonies used.6. Gently scrape with scraper and pipetting with 10 ml pipette so as not to be small clumps.

CRITICAL: Cell colonies should not be disrupted in small size.

7. Transfer to a 15 ml centrifuge tube and spin at 20 X g, 2 min, 4 °C. CRITICAL: It is recommended to centrifuge at low g forces (20 x g) to prevent feeder cells collecting at the bottom of the tube together.
8. Carefully aspirate supernatant without disturbing the cell pellet. CRITICAL: Finger tap the tube gently to disperse the pellet.
9. Resuspend the cells in freshly prepared ice-cold freezing medium (10% DMSO/culture

medium). CRITICAL: The freezing medium should be prepared freshly and kept on ice before use.

10. Transfer 200 µl aliquots into each labeled cryogenic vial. CRITICAL: Small volume may result in higher cell survival rates. It is recommended the volume of fluid during cryopreservation is 200 ul rather than 500 ul.
11. Place the vials on ice for a few minute. CRITICAL: Vials are allowed to sit on ice for 15 min. A prolonged storage on ice is likely to cause a further decline of cell viability.
12. Store them in the freezing container (BICELL or Mr.Frosty®) and keep it in a -80 °C deep freezer overnight. CRITICAL: It is recommended to finish suspension within 30 min. Prolonged immersion cause decline in cell viability.
13. Next day transfer the vials to the liquid nitrogen tank (in the vapor phase, -150 °C). CRITICAL: Immersion of vials into the liquid phase of liquid nitrogen may lead to viral or cross contamination.

CRITICAL: It is recommended to abort long term storage in a -80 °C deep freezer. An extended storage at -80 °C causes the decline in cell recovery. Storage in a -80 °C deep freezer within 7 days after transferring from liquid nitrogen tank is permissible.

CRITICAL: It is reported that vitrification protocols may be superior to slow freezing. We showed our slow freezing method was equally successful as vitrification method.

PAUSE POINT: The cells can be stored indefinitely in the vapor phase of liquid nitrogen.

14. Document the details of vials and storage location in the appropriate inventory.

Preparation of feeder seeded culture flasks before thawing of hES/hiPS cells

15. Coat the flasks with 0.1% gelatin (3 ml/25 cm2 flask).
16. Incubate the flasks for 1 hour at 37 °C or overnight at room temperature.
17. Rinse the flasks with PBS.

18. Inoculate mitomycin c-treated primary mouse embryonic fibroblasts (PMEF) at 5-7x103 cells/cm2 in DMEM (high glucose) containing 10% heat inactivated FBS. CRITICAL: Prepare the feeder seeded culture flasks in a few days in advance of the thawing or passaging of hES/hiPS cells, and use them within 5 days.

CRITICAL: Density of feeder cells should be determined by colony morphology of hES/hiPS cells in advance.

Thawing of hES/hiPs cells

19. Prior to using of feeder seeded flasks, replace the medium with the human pluripotent cell medium twice.
20. Pre-warm 10 ml of the human pluripotent cell medium in a 15 ml tube in a 37 °C water bath.
21. Rapidly thaw out frozen cells by adding the pre-warmed medium directly into a vial with the transfer pipette. CRITICAL: In a case of thawing the frozen cells in a 37 °C water bath or in a 37°C incubator, the cells may be seriously damaged.
22. Gently collect the cells in the 15ml tube and wash vial with the pre-warmed medium.
23. Centrifuge the tube at 90x g, 2 min, room temperature.
24. Aspirate the medium and finger tap the tube gently to disperse the pellet.
25. Resuspend the cells in 5-7 ml of pre-warmed culture medium and transfer the suspension into a 25 cm2 flask.
26. Gently move the flask several times along horizontal and vertical directions to distribute the cells evenly.
27. Incubate the flasks in an incubator at 37 °C in a humidified atmosphere of 5% CO2.
28. Culture medium should be exchanged every other day until hES/hiPS cells are ready for passaging. CRITICAL: Do not move the flask on the day following the passaging.

Alkaline phosphatase staining to determine the growth of hiPS/hES cells

29. Remove the culture medium and rinse once with PBS for 1 min at room temperature.

30. Fix the colonies with the fixation buffer for 1 min at room temperature.

31. Wash twice with DDW.

32. Start the color reaction by incubating in the reaction buffer blocking off the light.

33. Stop the reaction after 15 min by washing twice with DDW.

34. Count the stained colonies.

Timing

Steps 1–14, Freeze stock of hES/iPS: 1hour

Steps 15–18, Preparation of feeder seeded culture flasks before thawing of hES/hiPS cells: 5-10 days

Steps 19–29, Thawing of hES/hiPS: 10–15 minutes

Steps 30–35, Alkaline phosphatase staining: 20–30 minutes

Anticipated Results

Simple modified method of conventional slow freezing cryopreservation for hES and hiPS cells described here have equally successful recovery rate and ability to maintain undifferentiated state as those of vitrification method (Fig. 1, Fig. 2). Thawing method by addition of pre-warmed medium directly into the frozen vial also leads to high cell viability after recovery from cryopreservation (Fig. 3). Additionally, shortening keeping time on ice before freezing (Suppl. Fig. 1), use of dispase (Suppl. Fig. 2), shortening cell suspension time after the dissociation process (Suppl. Fig. 3), reducing fluid volume from 500 to 200 ul (Suppl. Fig. 4), implementation of freezing process in vapor phase of liquid nitrogen (-150 °C) within 7 days after slow freezing process in a -80 °C deep freezer (Suppl. Fig. 5) increase viability of the cryopreserved cells by the slow freezing method. Moreover, cryopreserved cells are maintained high recovery rate and undifferentiated state within 7 days if the vials were transferred from a liquid nitrogen gas tank to a -80 deep freezer (Suppl. Fig. 6).

Our method may be available for novices, because it is simple and easy method that requires neither specific equipment nor reagents (Fig. 1). Moreover, our method allows for keeping in a -80 °C deep freezer within one week after transferring from a liquid nitrogen tank, which make favorable to transportation as compared to vitrification method. Also, this cryopreservation is available for serum-

free culture such as culture with hESF9 medium and hESF9a2i medium25, 26, 27, 28, 29.

We believe that our method can be employed by stem cell banking and researchers and will most likely lead to the expected outcome.

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Figures

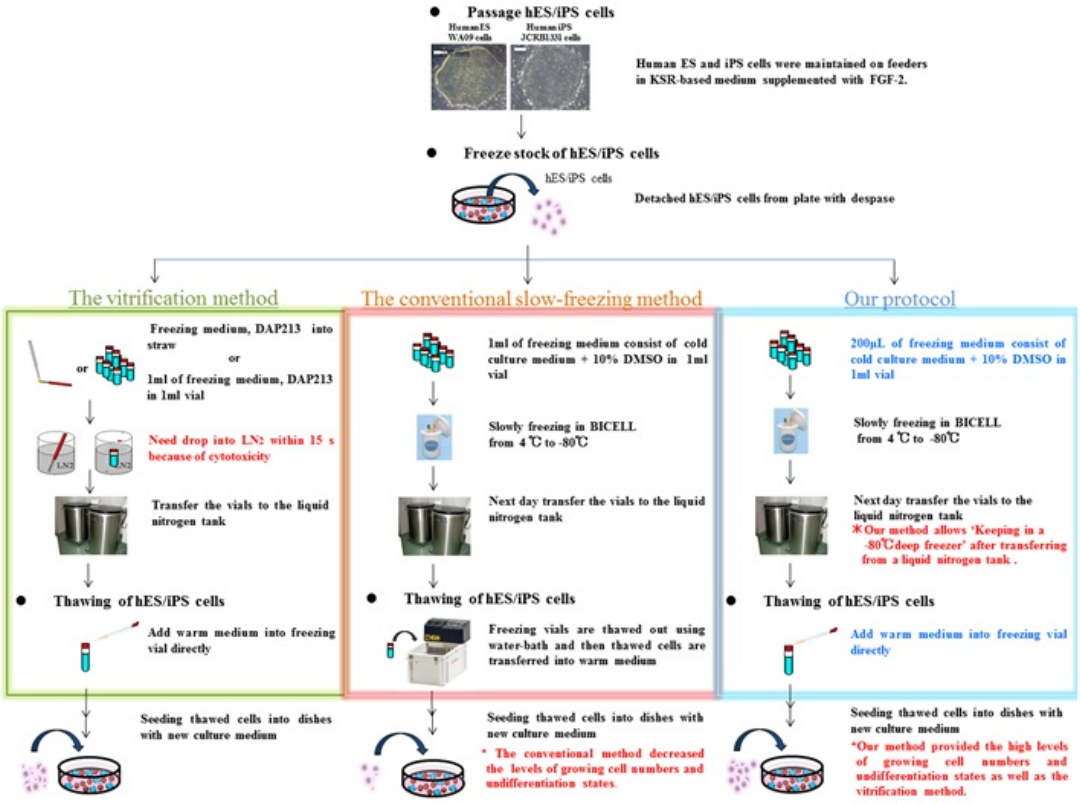

Figure 1

Schematic diagram illustrating the process of our cryopreservation method Our cryopreservation protocol is based on the conventional slow-freezing method with 10% DMSO and modified simply without specific equipment or reagents. Recovery rate of the cells cryopreserved by this method was equally high in comparison with the vitrification method. Even when using defined medium, such as our growth factor defined medium hESF925, 26, 27, 28, or mTeSR medium29, this cryopreservation method could work well.

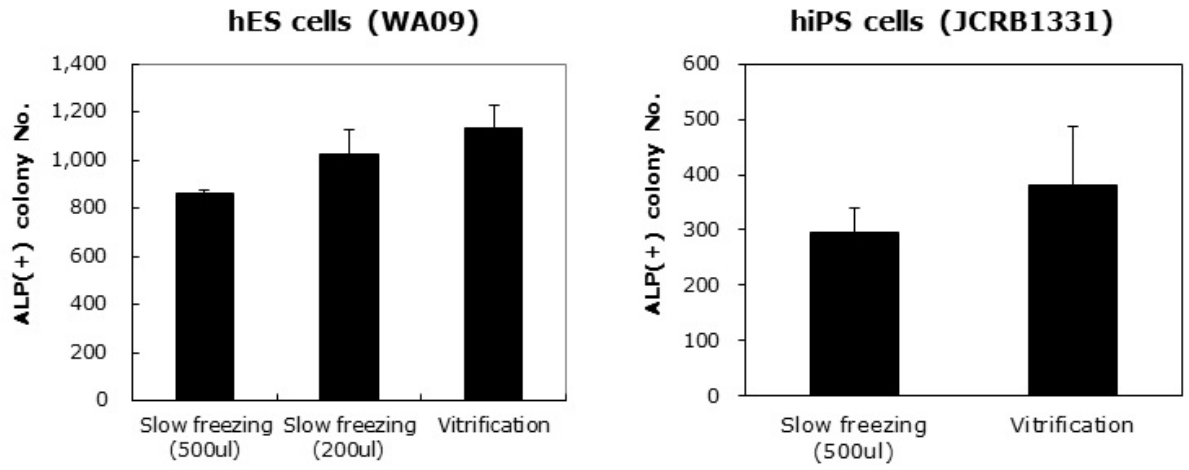

Figure 2

Comparison between slow-freezing and vitrification method The cells were frozen by each cryopreservation method. The colonies with ALP positive were measured at 6 days (H9) or 7 days (JCRB1331) after thawing. Vitrification method was performed according to Fujioka, T. and Suemori, H16. Briefly, cells were resuspended with 200 µl of DAP213 and rapidly placed into cryovials and then transferred to the vapor phase of the liquid nitrogen (within 15 sec). Bars indicate the means ± S.D. (n=3).

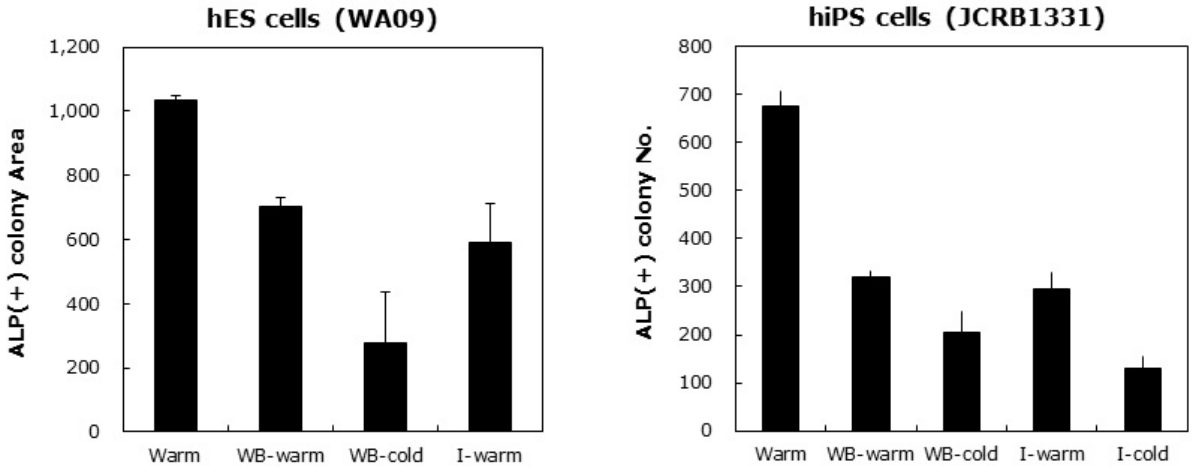

Figure 3

Comparison among several thawing method after cryopreservation The cells were frozen by slow-freezing method and thawed out by each thawing method. Then, the colonies with ALP positive were measured at 6 days (H9) or 7 days (JCRB1331) after thawing. Warm, directly add pre-warmed medium into frozen cells; WB-warm, thaw with water bath and add pre-warm medium into vial; WB-cold, thaw with water bath and add cold medium into vial; I-warm, thaw with incubator and add pre-warm medium into vial. Bars indicate the means ± S.D. (n=3).

component	Working concentration	Volume
Citric acid	4.5 mM	0.864 g
Sodium citrate	2.25 mM	0.581 g
Sodium chloride	3.0 mM	0.174 g
Methanol	65 %	650 ml
formaldehyde	3 %	30 ml

Adjust pH to 9.5. Fill up to 1,000 ml in distilled water

Figure 4

Table 1. Fixation buffer (pH9.5) for Alkaline phosphatase staining

Supplementary Files

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