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A simple and efficient method of slow freezing for human embryonic stem cells and induced pluripotent stem cells

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Abstract:	Protocols available for the cryopreservation of human embryonic stem (ES) and induced pluripotent stem (iPS) cells are very inefficient and laborious compared to those for the cryopreservation of murine ES/iPS cells or other general cell lines. While the vitrification method may be adequate when working with small numbers of human ES/iPS cells, it requires special skills and is unsuitable when working with large cell numbers. Here, we describe a simple and efficient method for the cryopreservation of hES/hiPS cells that is based on a conventional slow freezing method that uses a combination of Pronase/EDTA for Stem™ and CP-5E™ [final concentrations: 6% hydroxyethyl starch, 5% DMSO, and 5% ethylene glycol in saline]. CP-5E™ is highly effective for the cryopreservation of small cell clumps produced by hES/hiPS colony detachment in the presence of Pronase and EDTA (Pronase/EDTA for Stem™, a formulation containing multiple digestive enzymes from Streptomyces griseus). This novel method would be quite useful for large-scale hES/iPS cell banking for use in clinical applications.

A simple and efficient method of slow freezing for human embryonic stem cells and induced pluripotent stem cells

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Running title: Improved method for slow-freezing of hES/hIPS cells

Summary

Protocols available for the cryopreservation of human embryonic stem (ES) and induced pluripotent stem (iPS) cells are very inefficient and laborious compared to those for the cryopreservation of murine ES/iPS cells or other general cell lines. While the vitrification method may be adequate when working with small numbers of human ES/iPS cells, it requires special skills and is unsuitable when working with large cell numbers. Here, we describe a simple and efficient method for the cryopreservation of hES/hiPS cells that is based on a conventional slow freezing method that uses a combination of Pronase/EDTA for Stem™ and CP-5E™ [final concentrations: 6% hydroxyethyl starch, 5% DMSO, and 5% ethylene glycol in saline]. CP-5E™ is highly effective for the cryopreservation of small cell clumps produced by hES/hiPS colony detachment in the presence of Pronase and EDTA (Pronase/EDTA for Stem™, a formulation containing multiple digestive enzymes from *Streptomyces griseus)*. This novel method would be quite useful for large-scale hES/iPS cell banking for use in clinical applications.

Key words: cryopreservation; human embryonic stem cells; human induced pluripotent stem cells; slow freezing; hydroxyethyl starch; dimethyl sulfoxide; ethylene glycol; Pronase

1. Introduction

Human embryonic stem (hES) cells *(1)* and human induced pluripotent (hiPS) cells *(2)* show great potential for use in numerous biomedical applications, including drug development, disease modeling, and cell therapy *(3)*. Regenerative medicine therapies that use hES/iPS are being explored in Japan and USA for the treatment of retinal diseases such as age-related macular degeneration, Parkinson's disease, spinal cord injuries, etc.*(4)*. Additionally, various iPS cell types have been used to study disease physiology as well as drug targeting. There are, however, several hurdles in the use of hES/iPS cells in cell banking and biomedical applications, including difficulties in the large-scale cryopreservation of hES/iPS cells compared to murine ES/iPS cells or other cells *(5–7)*.

Two methods commonly used for hES/hiPS cell cryopreservation are vitrification *(8–11)* and slow freezing *(12–15)*. While the vitrification method is adequate for hES/hiPS cell cryopreservation, it requires special skills and is not suitable for when working with large amounts of cells. In contrast, slow-freezing methods do not require special skills, as they involve cell resuspension in a cryopreservation medium followed by gradual freezing in a deep freezer or programmable freezer, and also allow the researcher to work with large cell numbers. However, the distinct drawback of their use is that the post-thaw recoveries are low compared to those after vitrification. Until now, several approaches to improve efficiency have focused on the use of various cryoprotectant agents (CPAs) *(16)* and the use of an anti-apoptotic reagent (the

Rho-associated kinase (ROCK) inhibitor, Y-27632) *(17)*.

Recently, we developed a novel slow-freezing method using an animal component-free and protein-free cryopreservation medium, termed CP-5E™ [final concentrations: 6% hydroxyethyl starch (HES), 5% DMSO, and 5% ethylene glycol (EG) in saline] *(18)*. This simple formulation minimizes the risk of exposure to xenogeneic pathogens and eliminates the issues arising from lot-to-lot variations in bovine serum albumin (BSA). The use of CP-5E™ is highly effective in the cryopreservation of small clumps prepared by hES/hiPS colony detachment by the use of Pronase (a mixture of proteases originally isolated from *Streptomyces griseus (19)* cultures*)* in combination with EDTA in the formulation "Pronase/EDTA for Stem™." Using this method, post-thaw recovery frequencies of hES/hiPS cells were above 80% with retention of the typical cellular morphology. Moreover, the cells exhibited a good expansion profile, were positive for pluripotent markers, and could differentiate into the three germ layers.

CP-5E™ contains both high- and low-molecular weight CPAs. HES is a plant-derived high molecular weight CPA that cannot enter cells and remains in the extracellular space to participate in cell dehydration and minimize intracellular ice crystal formation, thus helping in membrane stabilization *(20)*. This CPA has been used as a plasma volume expander and drug stabilizer, suggesting its biological safety. In contrast, DMSO and EG are low molecular weight CPAs (78.13 Da and 62.07 Da, respectively), and can penetrate the cellular membrane and prevent the formation of ice crystals during cooling or warming.

Our method has the following advantages:

1) Cell detachment with Pronase/EDTA for Stem™ is rapid and can be accomplished in less than 5 min.

2) The reagents are not complex in formulation and are relatively inexpensive.

3) The freeze-thaw method used is simple and does not require intensive training.

4) There is no need for a programmable freezer.

5) Rapid thawing in a water bath is simple and does not require any special post-thaw recovery solutions.

Because the conventional slow freezing method is quite familiar, this easy and robust cryopreservation method can be used widely for basic research and in clinical applications as well.

2 Materials

All reagents and materials used must be sterile.

2.1 Reagents

1. Dulbecco's modified Eagle's medium (DMEM)/F12 + GlutaMax™ (Life Technologies, cat no. 10565-018)
2. KnockOut™ serum replacement (KSR: Life Technologies, cat no 10828-028)
3. MEM non-essential amino acids (100×) (Life Technologies, cat no. 1140-050,)
4. 2-Mercaptoethanol (Wako cat no. 139-06861, Japan)
5. Penicillin Streptomycine, Liquid (100×) (Life Technologies, cat no. 15140-122)
6. Basic fibroblast growth factor (bFGF) (Wako, cat no. 068-04544, Japan)
7. DMEM, high glucose, pyruvate (Life Technologies, cat no. 11995-065)
8. Fetal Bovine Serum (Tissue Culture Biologicals, cat no.101, USA)
9. PBS (Phosphate Buffered Salts) tablets (TaKaRa, cat no. T900)
10. Mitomycin C (Kyowa Hakko Kirin, Japan)
11. 0.05% Trypsin/EDTA (Life Technologies, cat no. 25300-062)
12. Gelatin from porcine skin Type A (Sigma-Aldrich, cat no G1890-100G)
13. 2.5% Trypsin (10×), no phenol red (Life Technologies, cat no. 15090-046)
14. Collagenase, Type IV (Life Technologies, cat no. 17104-019)
15. $CaCl_2$ (Wako cat no. 039-00475, Japan)

16. Y-27632 (WAKO, cat no. 253-00513, Japan)

17. VECTOR Alkaline Phosphatase Substrate Kit IV (Vector laboratories, cat no. SK-5400)

18. Pronase/EDTA for Stem™ (Kyokuto Pharmaceutical Industrial, Japan)

    0.075 mg/mL Pronase and 0.2 mM EDTA in D-PBS (-) (*see* **Note 1**)

19. Freezing medium CP-5E™ (Kyokuto Pharmaceutical Industrial, Japan)

    6% (w/v) Hydroxyethylstarch, 5% (v/v) dimethyl sulfoxide, and 5% (v/v) ethylene glycol in physiological saline

20. Human ESC line, KhES-1 (Riken BRC, Japan)

21. Human iPS cell line, 201B7 (Riken BRC, Japan)

22. Mouse fibroblast SNL76/7 feeder cell line [an STO cell line that expresses both G418 resistance and leukemia inhibitory factor, European Collection of Cell Culture (ECACC), UK]

2.2. Reagent setup

1. Human ES/iPS medium

   DMEM/F12 containing 20% KSR, 1% non-essential amino acids, 0.2 mM 2-mercaptoethanol, 1× penicillin/streptomycin, and 2 ng/mL bFGF: To prepare 500 mL of this medium, mix 100 mL KSR, 5 mL GlutaMax, 5 mL of the 100× non-essential amino acid solution, 0.5 mL of 0.1 M 2-mercaptoethanol, and 5 mL of 100× penicillin/streptomycin, and

then make up to 500 mL with DMEM/F12. Add 0.5 mL of 5 μg/mL bFGF before use. Store at 4°C for up to a week.

2. SNL medium

DMEM (high glucose, pyruvate, 2 mM L-glutamine) containing 10% Fetal Bovine Serum, 1× MEM-NEAA, and 1× penicillin/streptomycin: To prepare 500 mL of this medium, mix 50 mL of FBS, 5 mL of 10× MEM-NEAA and 5 mL of 100× penicillin/streptomycin. Store at 4°C for up to a week.

3. Gelatin-coated culture dishes

To prepare a 0.1% gelatin solution, dissolve 0.5 g gelatin powder in 500 mL of distilled water, and sterilize by autoclaving. To coat a culture dish, add a sufficient volume of this solution to cover the bottom of the culture well. For example, 1 mL is required for a 35-mm (6-well plate) surface, while 5 mL is required to coat a 100-mm dish. After coating, incubate the dish for at least 30 min at 37°C. The excess gelatin solution should be aspirated before use.

4. CTK dissociation solution

D-PBS(-) containing 0.25% trypsin, 1 mg/mL collagenase IV, 20% KSR, and 1 mM $CaCl_2$.

5. ROCK inhibitor Y-27632

   Dissolve 5 mg Y-27632 in 1.48 mL of distilled water to give a 10-mM stock solution. Aliquot and store at -20°C.

2.3.Equipment

1. Cryovial (2-mL tube, AGC Techno Glass, Japan)
2. 15 and 50 mL conical tubes (Thermo Scientific,)
3. 6-well culture plate (BD Biosciences, cat no.353046,)
4. 10 and 25 mL plastic disposable pipettes (BD Biosciences)
5. 0.22-μm filter (Millipore)
6. Disposable syringes, 10 and 50 mL (NIPRO, Japan)
7. Centrifuge (TOMY, LC-230, Japan)
8. Inverted phase-contrast microscope (4, 10, 20, and 40× objectives) (OLYMPUS, IX71, Japan)
9. PCV Clean Bench (HITACHI, Japan)
10. Micropipettes (10, 20, 200, and 1000 μL) (GILSON,)
11. Pipette aid (Drummond Scientific Company,)
12. Tissue culture incubator (Pharmaceutical Incubator, USA) maintained at 37°C and 85% humidity in a 5% CO_2 atmosphere
13. Water bath (Thermal ROBO TR-1A, Iuchi, Japan)

14. Hemocytometer (Cell Science & Technology Institute Inc., Japan)

15. Freezing container (NALGENE™ Cryo 1°C Freezing Container, Nalgene)

3. Methods

3.1. Passaging of hES/hiPS cells

Maintain hES/hiPS cells on mitomycin C-treated mouse fibroblast SNL76/7 feeder cells in a gelatin-coated 6-well feeder cell plate. Incubate the cells in a 95% humidity 5% CO_2 atmosphere at 37°C, until they become 80–90% confluent. Passage colonies by CTK cell detachment treatment every 4 d. Exchange culture medium every day except the day following the passaging.

3.2. Freezing stocks of hES/hiPS cells

The overall scheme of our protocol is shown in **Fig. 1.**

1. Grow cells to the exponential phase in a 6-well plate.
2. Aspirate the medium and wash the cells twice with 2 mL PBS.
3. Add 1 mL of pre-warmed **Pronase/EDTA for Stem™** and incubate for 2-5 min at RT.(*see* **Note 2**) **(Fig. 2)**
4. Aspirate **Pronase/EDTA for Stem™** with the detached feeder cells, and gently wash the wells with the human ES/iPS medium.
5. Add 1 mL of the human ES/iPS medium to the plate. Scrape the colonies off using the

pipette.

6. Harvest the cell suspension and centrifuge (300 ×*g*, 3 min).

7. Discard the supernatant and re-suspend the cells in 5 mL of the cold **CP-5E™** freezing medium **(***see* **Note 3)**.

8. Aliquot the cells (0.5-mL aliquots per 2-mL cryovial; a 1/10 split is suitable)

9. Place the vials in the cell-freezing container and store at -80°C overnight.

10. Transfer the vials to liquid nitrogen or -150°C for long-term storage the following day **(***see* **Note 4)**.

3.3. Thawing of the hES/hiPS cells

Preparation of a feeder-seeded culture plate (using one 100-mm dish) one day before thawing of hES and hiPS cells

1. Coat the culture plate with 0.1% gelatin (10 mL/100-mm dish).

2. Incubate the culture plate at 37°C for 1 h.

3. Rinse the culture plate with PBS (-).

4. Add the mitomycin c-treated SNL76/7 feeder cells suspended (*see* **Note 5**) in the SNL medium to the gelatin-coated dish at a density of $5–7\times10^3$ cells/cm^2 (*see* **Note 6**). Culture overnight. To maximize the viability of the cultured cells, be sure to warm up the media up to 37°C before use.

5. Draw a vial from liquid nitrogen and immediately thaw in a 37°C water bath.
6. Remove the vial from the water bath as soon as the cells are thawed, and spray with 70% ethanol to sterilize the surface.
7. Transfer the cell suspension to a 15-cm conical tube containing 5 mL of ice-cold human ES/iPS medium and pellet the cells by centrifugation at 300 × g for 5 min.
8. Discard the supernatant, and resuspend the hES/hiPS cells in 10 mL fresh medium containing the ROCK inhibitor Y-27632 (10 μM).
9. After 48 h, remove the media and replace with media containing no ROCK inhibitor (*see* **Note 7**).
10. Incubate at 37°C, 5% CO2 until the hES/hiPS colonies grow to an appropriate size. The medium should be changed every day.

Optional: Alkaline phosphatase staining to verify the growth of hES/iPS cells

1. Remove the culture medium
2. Fix the colonies with 4% (w/v) paraformaldehyde in PBS for 1 h at room temperature.
3. Wash twice with PBS.
4. Start the color reaction using the alkaline phosphatase substrate kit IV, as per the manufacturer's instructions.
5. Stop the reaction after 1 h by washing twice with PBS.

6. Count the stained colonies (**Fig. 3**)

4. Notes

1. Pronase is isolated from the growth medium of *Streptomyces griseus* cultures *(19).* No animal-derived components are utilized for the culture of this bacterium.

2. The treatment time with **Pronase/EDTA for Stem™** varies and depends on the cell lines and the quality of colonies used. Cell colonies should not be disrupted into single cells. Pronase dissociates hES/iPS cells by detaching the SNL feeder cells from the hES/hiPS cells, while EDTA breaks the hES/hiPS cell colonies into small clumps. We assume that the small cell clump size (approximately 2000 μm^2) obtained with the combination of Pronase/EDTA facilitates good delivery of the cryopreservatives to individual cells within the cell clumps.

3. The freezing medium **CP-5E™** should be equilibrated on ice before use.

4. Long term storage in a -80°C deep freezer should be avoided, as extended storage at -80°C causes a decline in cell recovery.

5. The feeder cell density should be determined in advance based on the colony morphology of hES/iPS cells.

6. The mitomycin C-treated SNL dishes should be prepared one day before use. In addition, frozen stocks of mitomycin C-treated SNL cells can be prepared using a standard technique and stored

at -80°C or in a liquid nitrogen tank, in the vapor phase. These stocks should be revived in a gelatin-coated dish or plate within 3 d of preparation.

7. The culture plates should not be moved on the day following the passaging.

Acknowledgements

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References

1. Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., et al. (1998) Embryonic stem cell lines derived from human blastocysts. *Science* 282: 1145-1157.

2. Takahashi, K., Tanabe, K., Ohnuki, M., Natirta, M., Ichisaka, T., et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131: 861-872.

3. Nishikawa, S., Goldstein, R.A., and Nierras, C.R. (2008) The promise of human induced pluripotent stem cells for research and therapy. *Nat Rev Mol Cell Biol* 9: 725-729.

4. Inoue, H., Nagata, N., Kurokawa, H., and Yamanaka, S. (2014) iPS cells: a game changer for future medicine. *EMBO J.* 33:409-417.

5. Hunt, C.J. (2007) The banking and cryopreservation of human embryonic stem cells. *Transfus Med Hemother.* 34: 293–304.

6. Hunt, C.J. (2011) Cryopreservation of human stem cells for clinical application: A review.*Transfus Med Hemother.* 38: 107–123.

7. Li, T., Mai, Q., Gao, J., and Zhou, C. (2010) Cryopreservation of human embryonic stem cells with a new bulk vitrification method. *Biol.of Reprod.* 82: 848–853.

8. Reubinoff, B.E., Pera, M.F., Vajta, G., and Trounson, A.O. (2001) Effective cryopreservation of human embryonic stem cells by the open pulled straw vitrification method. *Hum. Reprod.* 16: 2187–2194.

9. Richards, M., Fong, C.Y., Tan, S., Chan, W.K. and Bongso, A. (2004) An efficient and safe xeno-free cryopreservation method for the storage of human embryonic stem cells. *Stem Cells* 22: 779–789.

10. Zhou, C.Q., Mai, Q.Y., Li, T. and Zhuang, G.L. (2004) Cryopreservation of human embryonic stem cells by vitrification. *Chin. Med. J.* (Engl.) 117: 1050–1055.

11. Li, T., Zhou, C., Liu, C., Mai, Q. and Zhuang, G. (2008) Bulk vitrification of human embryonic stem cells. *Hum. Reprod.* 23: 358–364.

12. Fujioka, T., Yasuchika, K., Nakamura, Y., Nakatsuji, N. and Suemori, H. (2004) A simple and efficient cryopreservation method for primate embryonic stem cells. *Int. J. Dev. Biol.* 48: 1149–1154

13. Ji, L., de Pablo, J.J. and Palecek, S.P. (2004) Cryopreservation of adherent human embryonic stem cells. *Biotechnol. Bioeng.* 88: 299–312

14. Heng, B.C., Kuleshova, L.L., Bested, S.M., Liu, H. and Cao, T. (2005) The cryopreservation of human embryonic stem cells. *Biotechnol. Appl. Biochem.* 41: 97–104.

15. Ware, C.B., Nelson, A.M. and Blau, C.A. (2005) Controlled-rate freezing of human ES cells. *Biotechniques* 38: 879–880, 882–883.

16. Martin-Ibanez, R., Hovatta, O., Canals, J.M. (2012) Current Frontiers in Cryobiology：Cryopreservation of human pluripotent stem cells: are we going to the right direction? In Katkov II, editor. *InTech* pp139-166.

17. Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., et al. (2007) A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat Biotechnol* 25: 681-686.

18. Imaizumi, K., Nishishita, N., Muramatsu, M., Yamamoto, T., Takenaka, C., et al. (2014) A simple and highly effective method for slow-freezing human pluripotent stem cells using dimethyl sulfoxide, hydroxyethyl starch and ethylene glycol. *PLoS One*. 9: e88696.

19.Nomoto, M., Narahashi, T., and Murakami, M. (1960) A Protelytic enzyme of *Streptomyces griseus*: VI. Hydrolysis of protein by *Streptomyces griseus* protease. *J.Biochemistry* 48: 593-602.

20. Stolzing, A., Naaldijk, Y., Fedorova, V., and Sethe, S. (2012) Hydroxyethyl starch in cryopreservation – Mechanisms, benefits and problems. *Transfusion and Apheresis Science* 46: 137-147.

Fig. 1. Schematic showing our cryopreservation protocol, which is based on a conventional slow-freezing method that uses **Pronase/EDTA for Stem™** and **CP-5E™.** The recovery rate of the cells cryopreserved by this method is more than 80%, for several hES/hiPS cell lines.

Fig. 2. Detachment of hES/hiPS colonies by Pronase/EDTA for Stem™ Human ES (KhES-1) (top) and human iPS (201B7) (bottom) colonies before (upper and lower left panels) and 3 min after (upper and lower right panels) treatment with **Pronase/EDTA for Stem™**. The right-hand side panels show the detachment of the SNL feeder cells and the dissociation of the hES/hiPS colonies. Scale bars indicate 500 μm.

Fig. 3. Colony formation before freezing and after thawing

Alkaline phosphatase staining of hiPS 201B7 colonies maintained for 5 d after passage (left: post-plating, non-frozen control) and 5 d after thaw (right: post-thawing, dissociated using **Pronase/EDTA for Stem™** and cryopreserved using **CP-5E™**). Magnified photos are attached. Scale bars indicate 500 μm.

Figure

Fig. 1

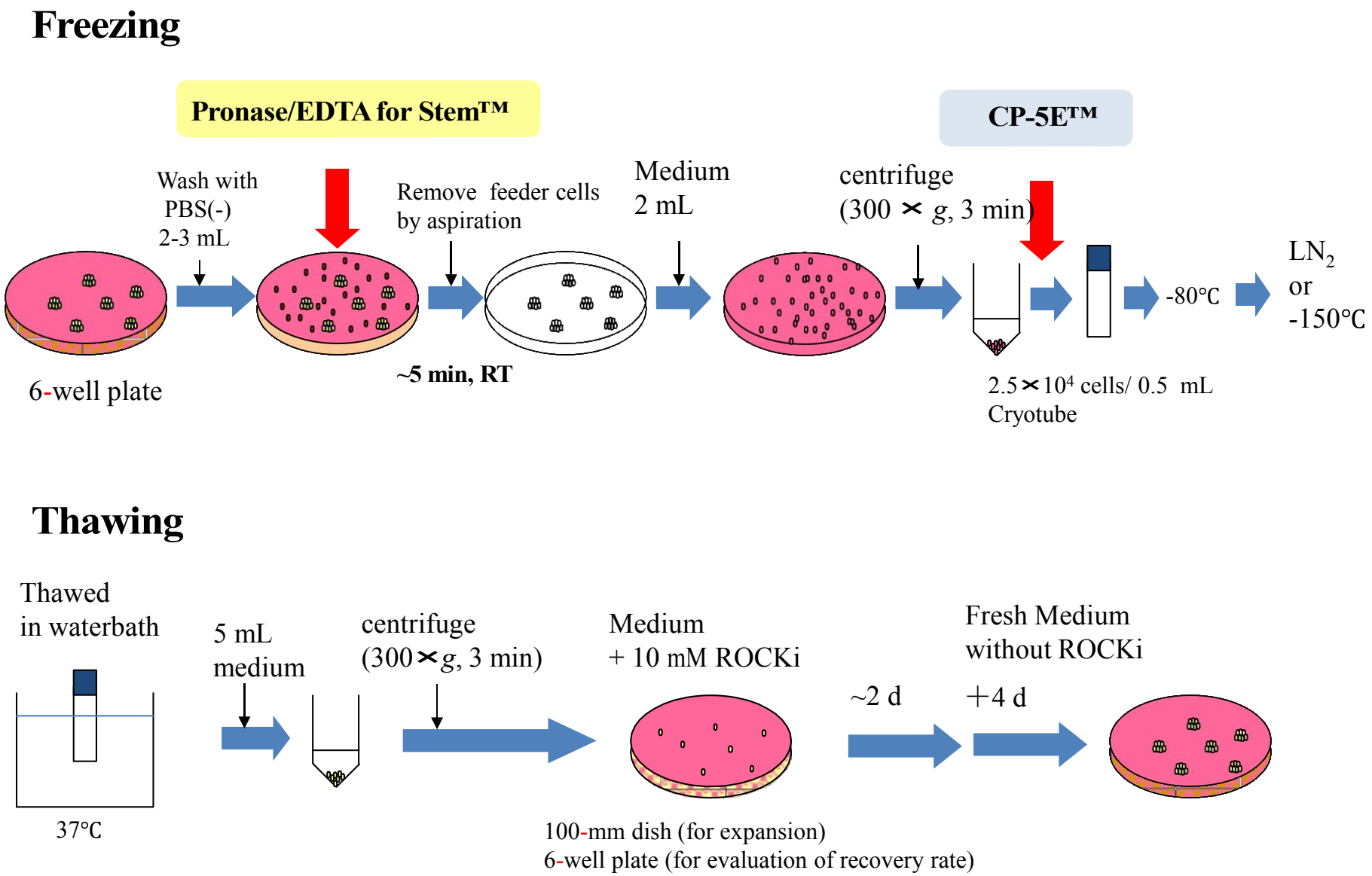

Freezing
Pronase/EDTA for Stem™
CP-5E™
Wash with
PBS(-)
2-3 mL
Remove feeder cells
by aspiration
Medium
2 mL
centrifuge
(300 × g, 3 min)
-80°C
LN2
or
-150°C
6-well plate
~5 min, RT
2.5×10⁴ cells/ 0.5 mL
Cryotube
Thawing
Thawed
in waterbath
37°C
5 mL
medium
centrifuge
(300×g, 3 min)
Medium
+ 10 mM ROCKi
~2 d
Fresh Medium
without ROCKi
+4 d
100-mm dish (for expansion)
6-well plate (for evaluation of recovery rate)

Fig.2

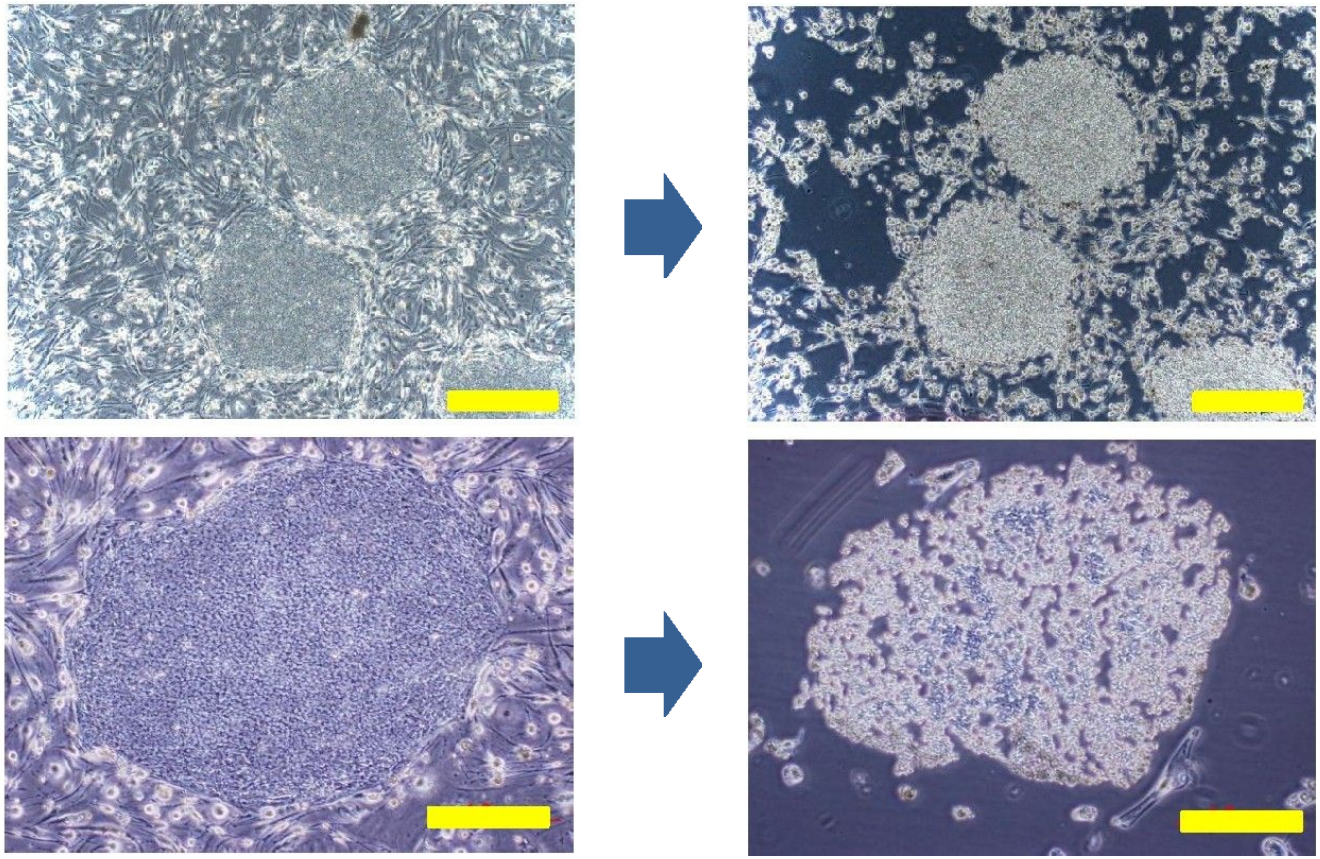
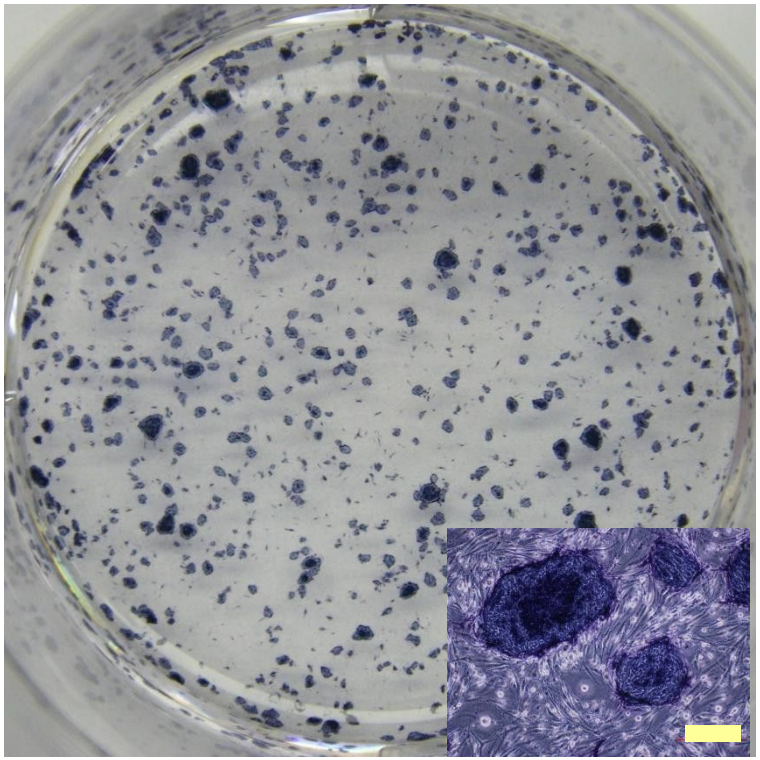


Fig. 3

Before freezing



After thawing