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.

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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.

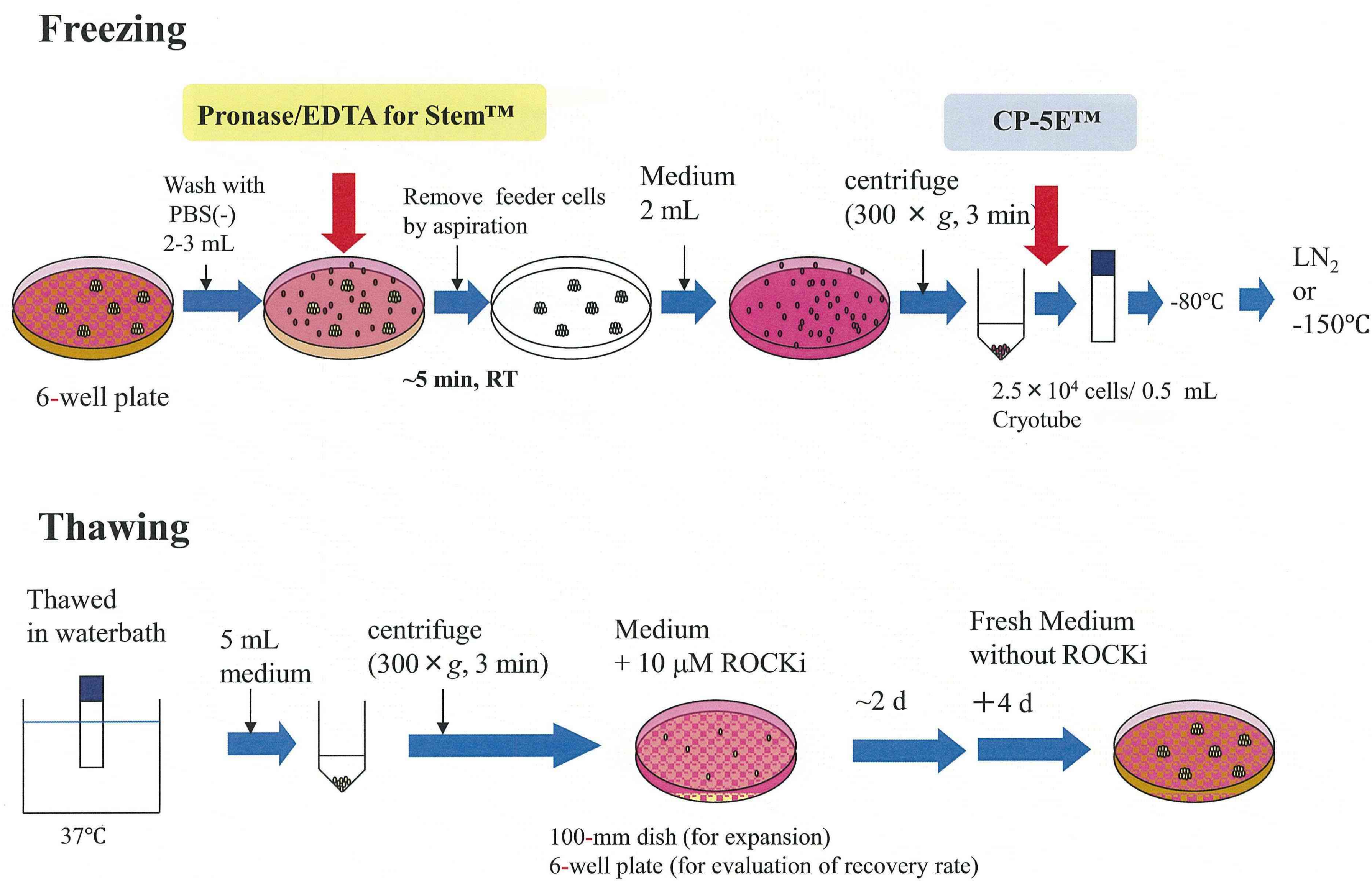
Fig. 2. Detachment of hES/hiPS colonies by Pronase/EDTA for Stem™ Human ES (KhES-1) (top) and human iPS (201B7) (bottom) colonies before (upper and lower left panels) and 3 min after (upper and lower right panels) treatment with **Pronase/EDTA for Stem™**. The right-hand side panels show the detachment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



59

Fig. 2

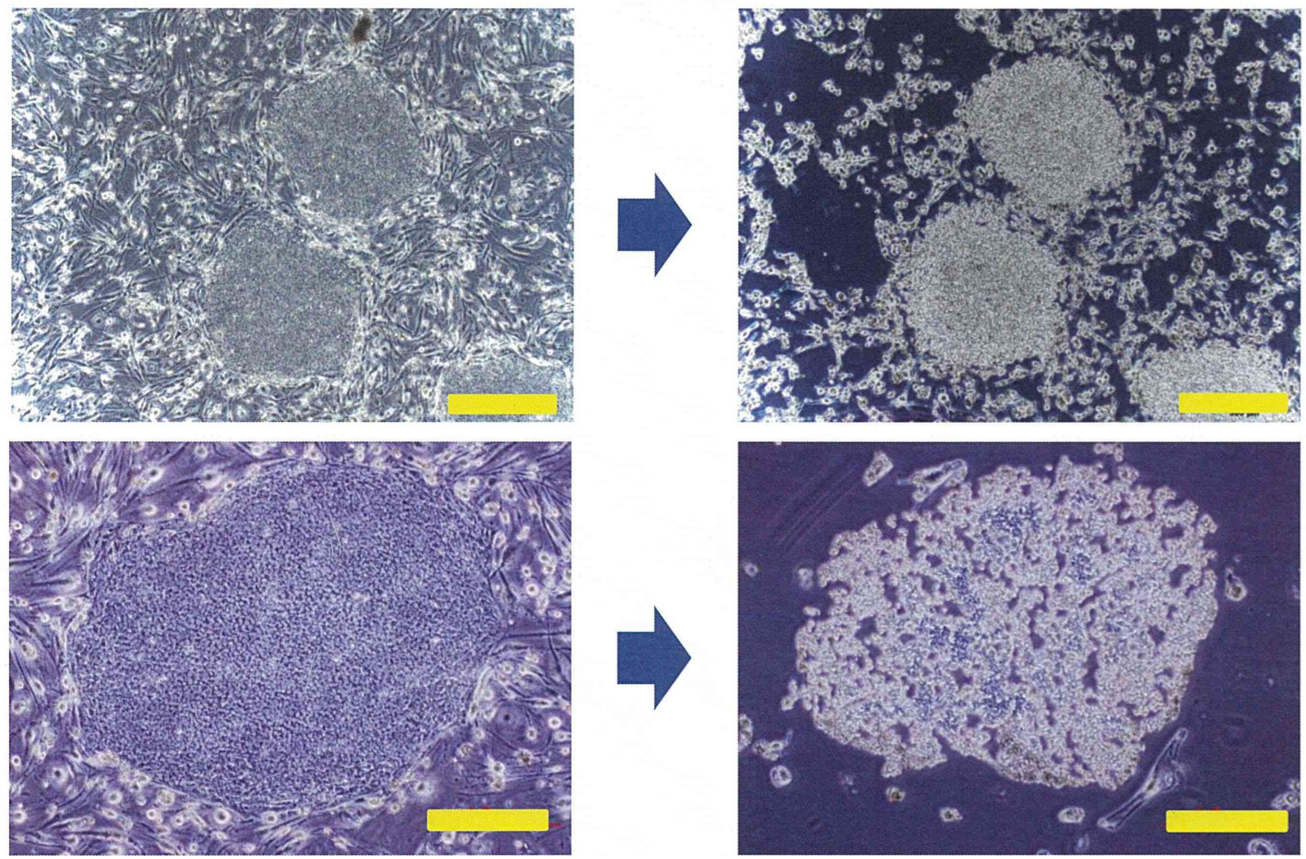
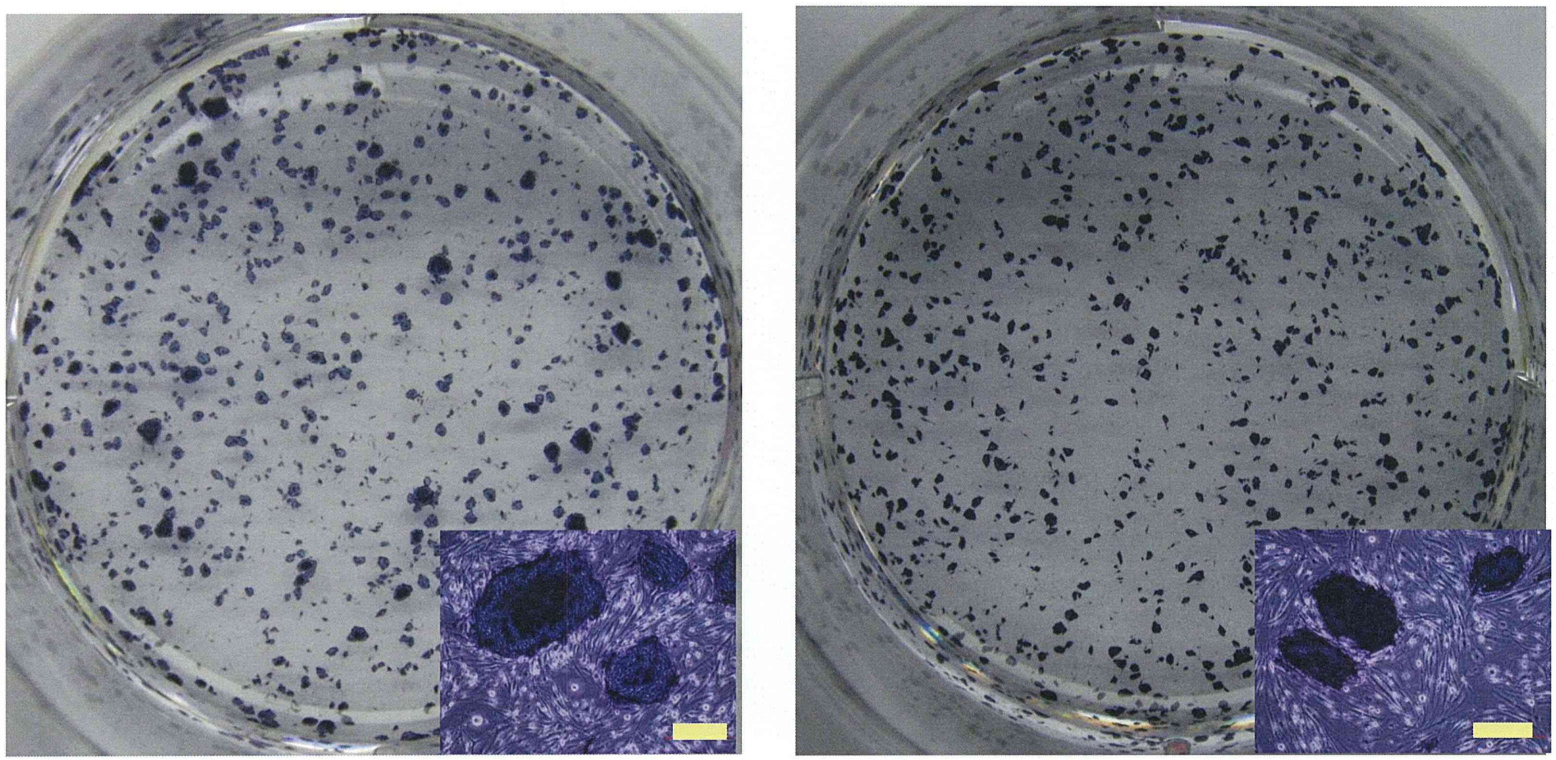


Fig. 3

Before freezing After thawing



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