

1 **2. Materials**

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4 All reagents and materials used must be sterile.

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7 **2.1 Reagents**

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10 1. Dulbecco's modified Eagle's medium (DMEM)/F12 + GlutaMax™ (Life Technologies, cat
11 no. 10565-018)
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16 2. KnockOut™ serum replacement (KSR: Life Technologies, cat no 10828-028)
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19 3. MEM non-essential amino acids (100×) (Life Technologies, cat no. 1140-050,)
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22
23 4. 2-Mercaptoethanol (Wako cat no. 139-06861, Japan)
24
25
26 5. Penicillin Streptomycine, Liquid (100×) (Life Technologies, cat no. 15140-122)
27
28
29 6. Basic fibroblast growth factor (bFGF) (Wako, cat no. 068-04544, Japan)
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31
32 7. DMEM, high glucose, pyruvate (Life Technologies, cat no. 11995-065)
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36 8. Fetal Bovine Serum (Tissue Culture Biologicals, cat no.101, USA)
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38
39 9. PBS (Phosphate Buffered Salts) tablets (TaKaRa, cat no. T900)
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42 10. Mitomycin C (Kyowa Hakko Kirin, Japan)
43
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45 11. 0.05% Trypsin/EDTA (Life Technologies, cat no. 25300-062)
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48 12. Gelatin from porcine skin Type A (Sigma-Aldrich, cat no G1890-100G)
49
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51 13. 2.5% Trypsin (10×), no phenol red (Life Technologies, cat no. 15090-046)
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55 14. Collagenase, Type IV (Life Technologies, cat no. 17104-019)
56
57
58 15. CaCl₂ (Wako cat no. 039-00475, Japan)
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1 16. Y-27632 (WAKO, cat no. 253-00513, Japan)

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

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6
7 18. Pronase/EDTA for Stem™ (Kyokuto Pharmaceutical Industrial, Japan)

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9
10 0.075 mg/mL Pronase and 0.2 mM EDTA in D-PBS (-) (*see Note 1*)

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12
13 19. Freezing medium CP-5E™ (Kyokuto Pharmaceutical Industrial, Japan)

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15
16 6% (w/v) Hydroxyethylstarch, 5% (v/v) dimethyl sulfoxide, and 5% (v/v) ethylene glycol in
17
18 physiological saline

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

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

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26
27 22. Mouse fibroblast SNL76/7 feeder cell line [an STO cell line that expresses both G418
28
29 resistance and leukemia inhibitory factor, European Collection of Cell Culture (ECACC),
30
31 UK]

32 33 34 35 36 37 38 39 40 41 42 **2.2. Reagent setup**

43 44 45 1. Human ES/iPS medium

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47
48 DMEM/F12 containing 20% KSR, 1% non-essential amino acids, 0.2 mM
49
50
51 2-mercaptoethanol, 1× penicillin/streptomycin, and 2 ng/mL bFGF: To prepare 500 mL of
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53
54 this medium, mix 100 mL KSR, 5 mL GlutaMax, 5 mL of the 100× non-essential amino acid
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56
57 solution, 0.5 mL of 0.1 M 2-mercaptoethanol, and 5 mL of 100× penicillin/streptomycin, and
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1 then make up to 500 mL with DMEM/F12. Add 0.5 mL of 5 µg/mL bFGF before use. Store at
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4 4°C for up to a week.
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10 2. SNL medium
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13 DMEM (high glucose, pyruvate, 2 mM L-glutamine) containing 10% Fetal Bovine Serum, 1×
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15 MEM-NEAA, and 1× penicillin/streptomycin: To prepare 500 mL of this medium, mix 50
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17 mL of FBS, 5 mL of 10× MEM-NEAA and 5 mL of 100× penicillin/streptomycin. Store at
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22 4°C for up to a week.
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29 3. Gelatin-coated culture dishes
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31
32 To prepare a 0.1% gelatin solution, dissolve 0.5 g gelatin powder in 500 mL of distilled water,
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34 and sterilize by autoclaving. To coat a culture dish, add a sufficient volume of this solution to
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36 cover the bottom of the culture well. For example, 1 mL is required for a 35-mm (6-well
37
38 plate) surface, while 5 mL is required to coat a 100-mm dish. After coating, incubate the dish
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42 for at least 30 min at 37°C. The excess gelatin solution should be aspirated before use.
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51 4. CTK dissociation solution
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54 D-PBS(-) containing 0.25% trypsin, 1 mg/mL collagenase IV, 20% KSR, and 1 mM CaCl₂.
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1 5. ROCK inhibitor Y-27632

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4 Dissolve 5 mg Y-27632 in 1.48 mL of distilled water to give a 10-mM stock solution. Aliquot
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7 and store at -20°C.
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13 **2.3.Equipment**
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16 1. Cryovial (2-mL tube, AGC Techno Glass, Japan)
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19 2. 15 and 50 mL conical tubes (Thermo Scientific,)
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21
22 3. 6-well culture plate (BD Biosciences, cat no.353046,)
23
24
25 4. 10 and 25 mL plastic disposable pipettes (BD Biosciences)
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29 5. 0.22-µm filter (Millipore)
30
31
32 6. Disposable syringes, 10 and 50 mL (NIPRO, Japan)
33
34
35 7. Centrifuge (TOMY, LC-230, Japan)
36
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38
39 8. Inverted phase-contrast microscope (4, 10, 20, and 40× objectives) (OLYMPUS, IX71, Japan)
40
41
42 9. PCV Clean Bench (HITACHI, Japan)
43
44
45 10. Micropipettes (10, 20, 200, and 1000 µL) (GILSON,)
46
47
48 11. Pipette aid (Drummond Scientific Company,)
49
50
51 12. Tissue culture incubator (Pharmaceutical Incubator, USA) maintained at 37°C and 85%
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53
54 humidity in a 5% CO₂ atmosphere
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57 13. Water bath (Thermal ROBO TR-1A, Iuchi, Japan)
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1 14. Hemocytometer (Cell Science & Technology Institute Inc., Japan)

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

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10 **3. Methods**

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13 **3.1. Passaging of hES/hiPS cells**

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16 Maintain hES/hiPS cells on mitomycin C-treated mouse fibroblast SNL76/7 feeder cells in a
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129 **3.2. Freezing stocks of hES/hiPS cells**

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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

1 pipette.

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3
4 6. Harvest the cell suspension and centrifuge (300 ×g, 3 min).
5
6
7 7. Discard the supernatant and re-suspend the cells in 5 mL of the cold **CP-5E™** freezing
8
9
10 medium (*see Note 3*).
11
12
13 8. Aliquot the cells (0.5-mL aliquots per 2-mL cryovial; a 1/10 split is suitable)
14
15
16 9. Place the vials in the cell-freezing container and store at -80°C overnight.
17
18
19
20 10. Transfer the vials to liquid nitrogen or -150°C for long-term storage the following day (*see*
21
22 **Note 4**).

23 24 25 26 27 28 29 **3.3. Thawing of the hES/hiPS cells**

30 Preparation of a feeder-seeded culture plate (using one 100-mm dish) one day before thawing of hES
31
32
33 and hiPS cells
34
35
36

- 37
38
39 1. Coat the culture plate with 0.1% gelatin (10 mL/100-mm dish).
40
41
42 2. Incubate the culture plate at 37°C for 1 h.
43
44
45 3. Rinse the culture plate with PBS (-).
46
47
48 4. Add the mitomycin c-treated SNL76/7 feeder cells suspended (*see Note 5*) in the SNL
49
50
51 medium to the gelatin-coated dish at a density of $5-7 \times 10^3$ cells/cm² (*see Note 6*). Culture
52
53
54 overnight. To maximize the viability of the cultured cells, be sure to warm up the media up to
55
56
57 37°C before use.
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- 1 5. Draw a vial from liquid nitrogen and immediately thaw in a 37°C water bath.
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- 3
- 4 6. Remove the vial from the water bath as soon as the cells are thawed, and spray with 70%
- 5
- 6
- 7 ethanol to sterilize the surface.
- 8
- 9
- 10 7. Transfer the cell suspension to a 15-cm conical tube containing 5 mL of ice-cold human
- 11
- 12
- 13 ES/iPS medium and pellet the cells by centrifugation at 300 × g for 5 min.
- 14
- 15
- 16
- 17 8. Discard the supernatant, and resuspend the hES/hiPS cells in 10 mL fresh medium containing
- 18
- 19 the ROCK inhibitor Y-27632 (10 μM).
- 20
- 21
- 22
- 23 9. After 48 h, remove the media and replace with media containing no ROCK inhibitor (*see*
- 24
- 25
- 26 **Note 7**).
- 27
- 28
- 29 10. Incubate at 37°C, 5% CO₂ until the hES/hiPS colonies grow to an appropriate size. The
- 30
- 31
- 32 medium should be changed every day.
- 33
- 34
- 35
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- 38

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

- 40
- 41
- 42 1. Remove the culture medium
- 43
- 44
- 45 2. Fix the colonies with 4% (w/v) paraformaldehyde in PBS for 1 h at room temperature.
- 46
- 47
- 48
- 49 3. Wash twice with PBS.
- 50
- 51
- 52 4. Start the color reaction using the alkaline phosphatase substrate kit IV, as per the
- 53
- 54 manufacturer's instructions.
- 55
- 56
- 57
- 58 5. Stop the reaction after 1 h by washing twice with PBS.
- 59
- 60
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1 6. Count the stained colonies (**Fig. 3**)
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10 **4. Notes**
11

12
13 1. Pronase is isolated from the growth medium of *Streptomyces griseus* cultures (19). No
14
15 animal-derived components are utilized for the culture of this bacterium.
16
17

18
19 2. The treatment time with **Pronase/EDTA for Stem™** varies and depends on the cell lines and the
20
21 quality of colonies used. Cell colonies should not be disrupted into single cells. Pronase
22
23 dissociates hES/iPS cells by detaching the SNL feeder cells from the hES/hiPS cells, while
24
25 EDTA breaks the hES/hiPS cell colonies into small clumps. We assume that the small cell clump
26
27 size (approximately 2000 μm^2) obtained with the combination of Pronase/EDTA facilitates good
28
29 delivery of the cryopreservatives to individual cells within the cell clumps.
30
31

32
33 3. The freezing medium **CP-5E™** should be equilibrated on ice before use.
34
35
36

37
38 4. Long term storage in a -80°C deep freezer should be avoided, as extended storage at -80°C
39
40 causes a decline in cell recovery.
41
42
43

44
45 5. The feeder cell density should be determined in advance based on the colony morphology of
46
47 hES/iPS cells.
48
49

50
51 6. The mitomycin C-treated SNL dishes should be prepared one day before use. In addition, frozen
52
53 stocks of mitomycin C-treated SNL cells can be prepared using a standard technique and stored
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1 at -80°C or in a liquid nitrogen tank, in the vapor phase. These stocks should be revived in a
2
3
4 gelatin-coated dish or plate within 3 d of preparation.
5
6

7 7. The culture plates should not be moved on the day following the passaging.
8
9

10 11 12 13 **Acknowledgements** 14

15
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17
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1 **Fig. 1.** Schematic showing our cryopreservation protocol, which is based on a conventional
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4 slow-freezing method that uses **Pronase/EDTA for Stem™** and **CP-5E™**. The recovery rate of the
5
6
7 cells cryopreserved by this method is more than 80%, for several hES/hiPS cell lines.
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16 **Fig. 2. Detachment of hES/hiPS colonies by Pronase/EDTA for Stem™** Human ES (KhES-1)
17
18 (top) and human iPS (201B7) (bottom) colonies before (upper and lower left panels) and 3 min after
19
20 (upper and lower right panels) treatment with **Pronase/EDTA for Stem™**. The right-hand side
21
22
23 panels show the detachment of the SNL feeder cells and the dissociation of the hES/hiPS colonies.
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29 Scale bars indicate 500 μm .
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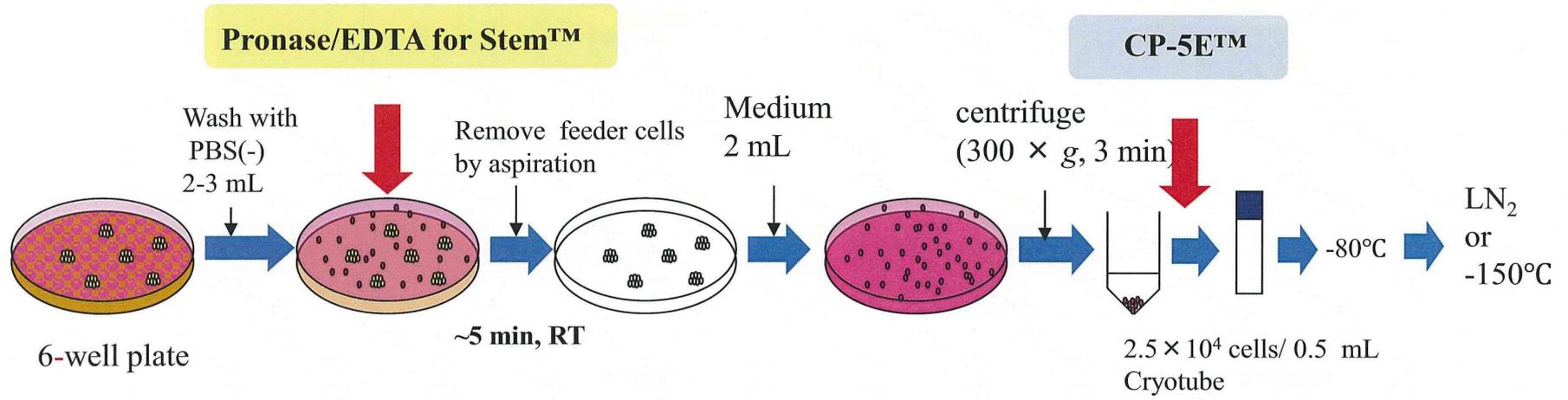
39 **Fig. 3. Colony formation before freezing and after thawing**
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41

42 Alkaline phosphatase staining of hiPS 201B7 colonies maintained for 5 d after passage (left:
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44 post-plating, non-frozen control) and 5 d after thaw (right: post-thawing, dissociated using
45
46
47 **Pronase/EDTA for Stem™** and cryopreserved using **CP-5E™**). Magnified photos are attached.
48
49
50

51 Scale bars indicate 500 μm .
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Figure
Fig. 1

Freezing



Thawing

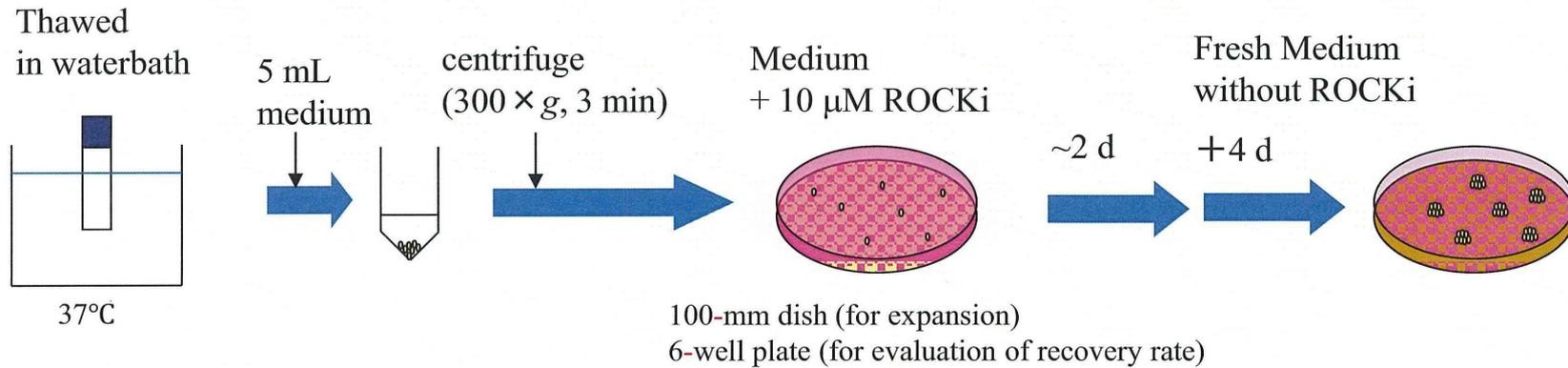


Fig. 2

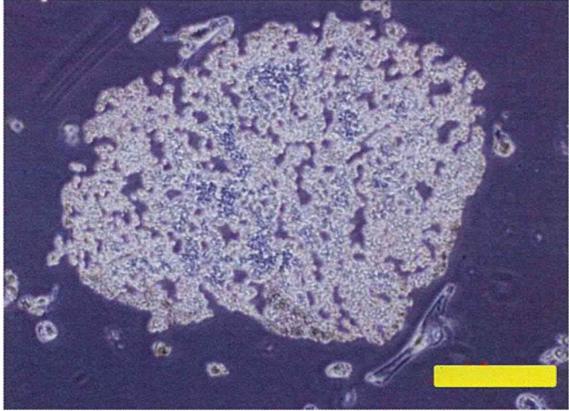
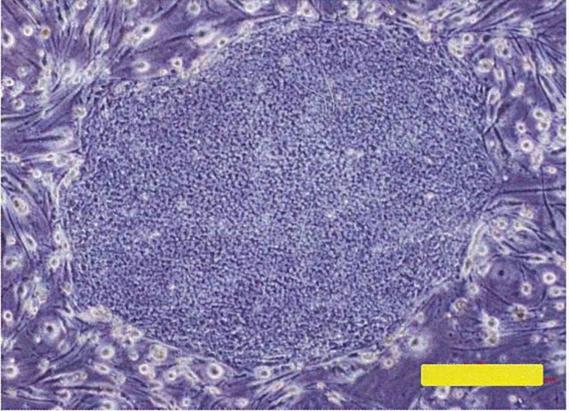
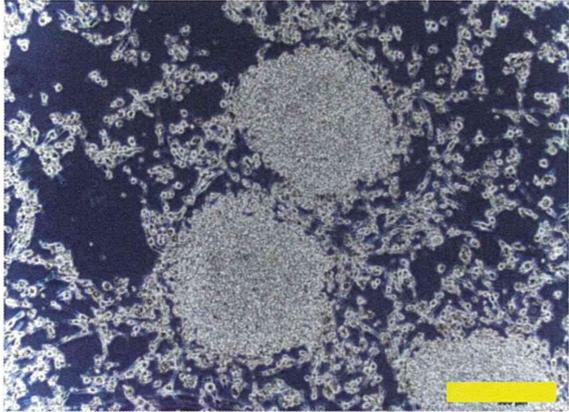
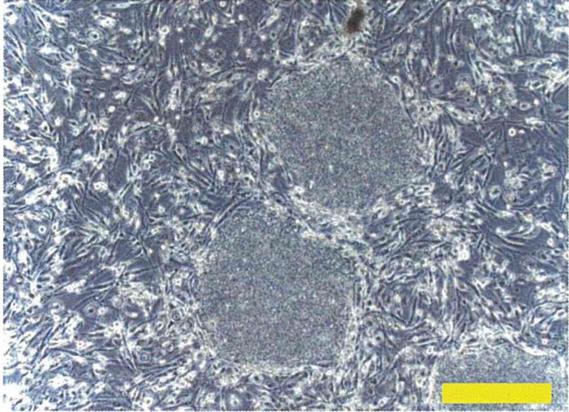
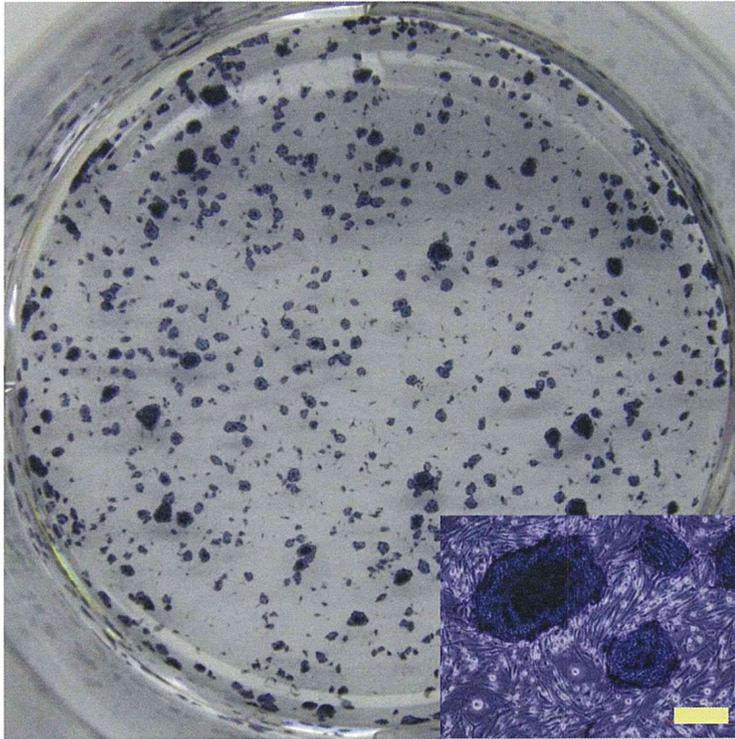
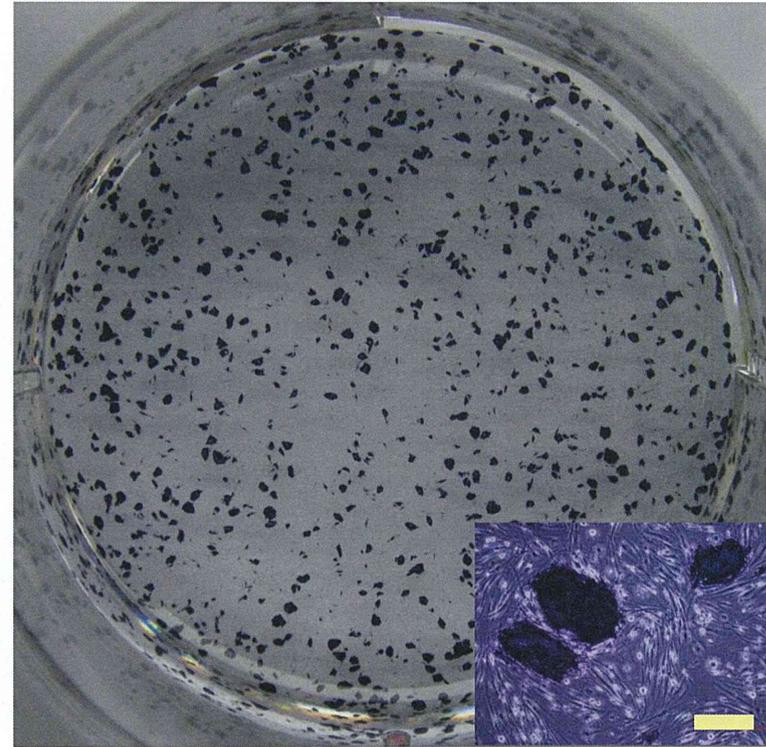


Fig. 3

Before freezing



After thawing



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有害事象発生時の科学的な細胞検証を通じて細胞治療の安全性向上を
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