Instruction Manual

ReproFF2™

Cat. # RCHEMD006

Version 2.0

Instructions for using ReproFF2™

- ReproFF2™ should be used with Gentle Cell Dissociation Reagent (GCDR).
- For passage, ensure colony size is not too small during passage; the size should be between 200 and 300 μm.
- Ensure the culture period in ether on feeder culture or feeder-free culture before transition is 7 days, which is longer than usual culture schedule, and size is 500-1000 μm, which is bigger than that used for passaging as usual.
- It may require approximately 4 passages before obtaining cell stability. We recommend to passage in 1:2 ratio.
- As cell state can vary between cell lines, the maintenance of an undifferentiated state can be optimized by adjusting the concentration of basic fibroblast growth factor (bFGF) (between 10 and 30 ng/mL).
- We recommend to culture cells according to the following schedule. (The passage schedule may differ according to cell line.)

	Mon	Tue	Wed	Thu	Fri	Sat	Sun
Week 0					Change to ReproFF2™		
Week 1	Pa.1		MC		Pa.2		
Week 2	MC		MC		Pa.3		
Week 3	MC		MC		Pa.4		

Pa: passage, MC: medium change

Conditions of Use

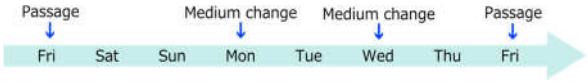
This product is for research use only. It is not for therapeutic or diagnostic purposes. Sale of this product to a third party or commercial use are prohibited without prior permission from ReproCELL.

Storage

This product is shipped frozen. Store at -20°C upon receipt. After thawing, store at 2–8°C and use within two weeks. Avoid repeated freezing and thawing.

Characteristics of the Product

- Human embryonic stem cells (ES) and induced pluripotent stem cells (iPS) can be maintained only 3 days a week of cultivation (e.g., Monday, Wednesday, and Friday).



- Suitable for feeder-free culture of human ES/iPS cells.
- Each lot is culture-tested with human iPS cells as described in Takahashi K, *et al*., *Cell* 2007;131:861–72.
- Lot-to-lot control testing of other critical criteria, including osmolality, pH, sterility, and mycoplasma is performed.
- Serum-free.

*Includes 2-Mercaptoethanol.

Instructions for Use

The procedure for passaging human ES/iPS cells using ReproFF2™ is described below.

Materials Required

- ReproFF2™ supplemented with 5 ng/mL of bFGF (RCHEOT002, 003; hereafter referred to as ReproFF2™). Addition of bFGF may improve the culture of human ES/iPS cells. Concentration differs depending on the cell line used.
- Gentle Cell Dissociation Reagent (GCDR ; STEM CELL TECHNOLOGIES, 07174)
- Vitronectin-N (LIFE TECHNOLOGIES, REFA14700)
- PBS (-): Ca^{++} and Mg^{++}-free PBS.
- 60 mm dish.
- P-1000 pipette and tips.
- Scraper.
- Standard cell culture equipment.

*** GCDR and the medium equilibrate to room temperature before use**

*** Vitronectin-N is strongly recommended to culture with ReproFF2 as coating solution.**

*** The coating materials should store at 4 degrees before use.**

*** Laminin-5 (RCHEOT004) and Corning® Matrigel® hESC-Qualified Matrix (Corning, 354277) are also useful for culturing with ReproFF2. Please see the manuscripts.**

Contents

A: Surface Coating

<Preparation of aliquots of Vitronectin-N >

A1. Thaw a vial of Vitronectin-N at room temperature.

A2. Aliquot 120 µL of Vitronectin-N into each 1.5mL tube.

A3. Store the aliquots at −80 °C.

<Coating (for four 60-mm dishes)>

A4. Thaw an aliquot of Vitronectin-N at room temperature.

A5. Add 8 mL of PBS (−) into a 15 mL tube.

A6. Add 120 µL of Vitronectin-N into the 15 mL tube with PBS (-).

A7. Add 2 mL of diluted Vitronectin-N into a 60 mm dish and spread the solution by swirling gently. Seal the dish with parafilm.

A8. Incubate the dish at room temperature for 1 hour. If not used immediately, store it for up to one week at 4 °C.

B: Transition from feeder-dependent to feeder-free culture

(Each reagent volume is for 60 mm dish)

Note 1: The transition should be performed when human iPS cell colonies on feeder or feeder-free cover more than 30% of the surface area of the culture dish (Fig. 1). Use collective cells from two dishes. If the cell density is too low after transition, the transition will not be successful.

Note 2: Make sure the cultivation period in an on feeder culture or feeder-free culture before transition is 7 days which are longer than usual culture schedule

Note 3: Prepare bigger colony size (500-1000 µm) before transition.

Fig. 1 The density of the colony at the time of transition from feeder-dependent to feeder-free cultivation. ALP staining of the dish at the time of transition.

B1. Prepare the coated dishes in advance as described above in **A**. For Vitronectin-N or Matrigel-coated dishes, remove the coating solution and add 2 mL/dish of ReproFF2™. (Do not dry the coated surface).

B2. Remove the medium from the dish containing the feeder-dependent culture of human ES/iPS cells that are ready for passaging. Wash the cells with 2 mL of PBS (-).

B3. Remove the PBS (-). Add 1 mL of GCDR to the dish allowing the solution to cover the entire surface. Incubate for approx. 3 minutes in a 5%CO_2 incubator at 37°C. The incubation time should be adjusted to cell-line or condition.

B4. Periodically observe the cells under a microscope until more than half of the colonies are about to detach from the dish.

B5. Remove the GCDR. Add 2 mL of PBS (-) allowing the solution to cover the entire surface. Remove the PBS (-).

B6. Add 2 mL of fresh ReproFF2™. Detach all ES/iPS cells and feeder cells from the dish by scraper and transfer them to a 15 mL tube.

B7. Add 2 mL of fresh ReproFF2™ again. Detach residual ES/iPS cells and feeder cells from the dish by scraper and transfer them to a 15 mL tube.

B8. Draw the cell suspension at a rate of 2 mL/second using a 5 mL-disposable pipette. Lightly press the tip of the disposable pipette against the bottom of the tube and eject the cell suspension over 1 second. **Perform this procedure once.** It is important that the size of the colony after pipetting remain **between 200 and 300 µm** (Fig. 2). Make sure the size is larger than that used for passaging in an on-feeder cultivation.

Note 4: If this procedure is performed twice or more, colonies may become too small and will not cultivate well.

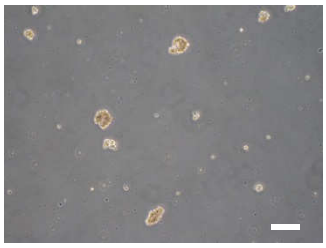


Fig. 2 Suitably sized colonies (200-300 µm)

Scale bar: 250 µm

B9. Transfer all the cell suspension obtained in step B8 into the coated dish prepared in step B1.

B10. Swirl the dish to spread the cells evenly and incubate at 37°C in a 5%CO_2 incubator.

B11. Cultivate the cells according to the following the schedule.

	Mon	Tue	Wed	Thu	Fri	Sat	Sun
Week 0					Change to ReproFF2™		
Week 1	Pa.1		MC		Pa.2		
Week 2	MC		MC		Pa.3		
Week 3	MC		MC		Pa.4		

For Pa.1, colony size will be around 1~2 mm at this point.

Pa; passage, MC; medium change

C: Passaging the established feeder-free culture

(Each reagent volume is for 60 mm dish)

C1. Prepare coated dishes in advance as described in step A, and add 2 mL of ReproFF2™. (Do not dry the coated surface).

C2. Remove the medium from the dish containing the cells that are ready for passaging and wash the cells with 2 mL of PBS (-).

C3. Remove the PBS (-). Add 1 mL of GCDR to the cells allowing the solution to cover the entire surface. Incubate for approx. 3 minutes in a 5%CO_2 incubator at 37°C. The incubation time should be adjusted to cell-line or condition.

C4. Observe the cells under a microscope to confirm that more than half of the colonies are about to detach from the dish.

C5. Remove the GCDR. Add 2 mL of PBS (-) allowing the solution to cover the entire surface. Remove the PBS (-).

C6. Add 2 mL of fresh ReproFF2™. Detach residual ES/iPS cells and feeder cells from the dish by scraper and transfer them to a 15 mL tube.

C7. Add 2 mL of fresh ReproFF2™ again. Detach all ES/iPS cells and feeder cells from the dish by scraper and transfer them to a 15 mL tube.

C8. Draw the cell suspension at a rate of 2 mL/second using a 5

mL-disposable pipette. Lightly press the tip of the disposable pipette against the bottom of the tube and eject the cell suspension over 1 second. It is important that the size of the colony after pipetting remains at **between 200 and 300 µm** (Fig. 2). Make sure the size is larger than that used for passaging in an on-feeder cultivation.

Note 5: If this procedure is performed twice or more, colonies may become too small and will not cultivate well.

C9. Transfer half volume of cell suspension into the coated dish prepared in step C1. After transition from an on-feeder culture to the coated dish, the first 4 passages should be carried out at a dilution ratio of 1:2. After the cultivation is stabilized, around passage 4, it is possible to passage at the dilution rate of 1:3 to 1:4.

C10. Swirl the dish to spread cells evenly and incubate at 37°C in a 5% CO_2 incubator.

C11. Change medium every two days.

D: Q&A

Q1. What should I do if human iPS cells cover 85% or more of the surface area of the culture dish within 4 days after transition from feeder-dependent cultivation and passaging?
A1. When the cell colonies cover more than 85% of the surface area of the culture dish, passage the cells regardless of the culture schedule.

Q2. What should I do if the colonies are small and their size does not increase after passage?
A2. You can continue to culture the cells. This finding could be attributable to technical issues during passage. For example, the tip of the disposable pipette should not be pressed even lightly against the bottom of the tube in step B8 or C8.

Q3. Is it fine if feeder cells are carried over at the time of transition from feeder-dependent cultivation?
A3. This is not a problem as there will be no feeder cells after 4 passages.

Q4. What should I do if the numbers of colonies gradually decrease after every passage?
A4. Continue with the culture schedule. In addition, change the dilution ratio to 1:1. Alternatively, you might use collective cells from three dishes during transition.

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