Instruction Manual: Cell Culture with ReproNaïve™ Culture Medium

Cat. No. RCHEMD008
Version 1.0

Overview

This protocol describes proper procedures for transitioning feeder-dependent human induced pluripotent stem cells (hiPSCs) to ReproNaïve™ medium culture, and for routine culture and passaging of the adapted cells in ReproNaïve™ medium. hiPSCs are passaged on SL10 feeder cells (RCHEF001) after transition and cultivated in a hypoxic (5% O_2, 5% CO_2) incubator.

Reading and understanding the entire protocol prior to beginning your experiments is highly recommended. To maintain sterility, all procedures (except as indicated) should be performed in a biological safety cabinet.

Conditions of Use

This product is for research use only. It cannot be used for therapeutic or diagnostic purposes. Sale of this product to a third party, or any commercial use for the product, is prohibited without prior permission from ReproCELL.

Storage

ReproNaïve™ medium should be stored at -20 °C upon receipt. After thawing, store at 2-8 °C and use within two weeks. Avoid repeated freezing and thawing.

Characterization of ReproNaïve™ medium

- Each lot is culture-tested with human iPS cells as described in Takahashi *et a*l., Cell 131:861-72 (2007).
- Each lot is tested for osmolality, pH, sterility, and mycoplasma.
- ReproNaïve™ medium is serum-free.

General notes

- Use of a hypoxic (5% O_2, 5% CO_2) incubator is required for best results.
- **Complete ReproNaïve™ medium** contains ReproNaïve™ Basal medium, ReproNaïve™ Supplement (6.8 µL/mL), and Human Recombinant LIF (10 – 100 ng/mL).
- Minimize the exposure of the Supplement to bright light.
- Our standard protocol uses 20 ng/mL LIF. Some hiPSC lines require titration of LIF concentration for optimal results.
- Warm the medium and Supplement at room temperature. Do not warm the ReproNaïve™ Basal medium or Supplement in a 37 °C bath to maintain best stability.
- Addition of Y27632 (Rock Inhibitor) to 10 µM to the Complete ReproNaïve™ medium for the first 48 hours after passaging is recommended for best results.

Contents

Distributed in Austria, Germany and Switzerland by PELOBIOTECH GmbH

PELOBIOTECH GmbH
Am Klopferspitz 19
82152 Planegg/Germany
(P) +49 (0)89 517 286 59-0 | (F): +49 (0)89 517 286 59-88
Email:info@pelobiotech.com | www.pelobiotech.com

ReproNaïve™

Product Description	Cat. No.	Format	Storage
ReproNaïve™ Basal Medium	RCHEMD008	500 mL	-20 °C
ReproNaïve™ Supplement		0.68 mL	-20 °C

Required Reagents and Equipment

Product Description	Cat. No.	Format	Storage
Stemfactor™ LIF, Human Recombinant	ReproCELL 03-0016	10 µg (1 mL)	4 °C
SL10 Feeder Cells	RCHEFC001	3x10^{6}cell per vial (5 vials)	Liquid nitrogen
Dissociation solution for human ES/iPS cells	RCHETP002	30 mL	-20 °C
ESGRO Complete™ Accutase™	Millipore SF006	100 mL	4 °C
Y27632	Stemgent 04-0012	2 mg	4 °C
ReproCoat™	RCHEOT001	500 mL	RT
DMEM-high glucose	Sigma, Cat.D5796	500 mL	4 °C
Fetal bovine serum (FBS)	GIBCO, Cat.10437	500 mL	4 °C
Sodium Pyruvate	Sigma, Cat.S8636	100 mL	4 °C
PBS (-). Ca^{2+}- and Mg^{2+}-Free	Standard Lab Suppliers	-	-
60 mm Tissue Culture Dish	Standard Lab Suppliers	-	-
5% O_2, 5% CO_2 incubator	Standard Lab Suppliers	-	-
Standard cell culture equipment	Standard Lab Suppliers	-	-

ReproNaïve™ Protocols

Material preparation

Y27632 Stock Solution (10 mM in DMSO)

1. Warm Y27632 powder (2 mg) to room temperature.
2. Add 624.4 µL DMSO to the Y27632 vial.
3. Vortex or warm the vial (37 °C bath) if necessary to get complete dissolution.
4. Divide into smaller aliquots and store aliquots at -20 °C

Preparation of SL10 feeder cells

1. Coat substrate (dish or plate) with ReproCoat or 0.1% gelatin according to the directions on the label on the bottle.
2. Thaw SL10 feeder cells according to the manual for SL10 feeder cells.
3. Plate SL10 feeder cells on ReproCoat-coated dish at 4.5x10^4 cells/cm^2 or 1x10^6 cells in a 60 mm dish. (See Table 1 in the Appendix for appropriate number of cells for different size dishes.)
   Note: This is a higher feeder cell density than is typically used for standard hiPSC culture.
4. Culture the feeder cells for 3-4 days in 5%O_2, 5%CO_2 incubator in 10%FBS/DMEM **without medium changes** prior to use in ReproNaïve™ culture.

Transition from feeder-dependent culture to ReproNaïve™ medium culture

Note: Volumes are based on culture in a 60 mm tissue culture dish. Please scale volumes appropriately for other culture sizes. (See Table 2 in Appendix.)

Note: PSCs cultured in feeder-dependent culture conditions are ready for transition to ReproNaïve™ Medium when they reach greater than 30% confluence (Figure 1).

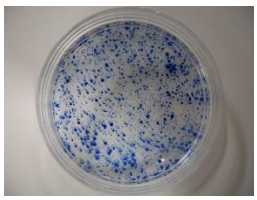


Figure 1. Alkaline Phosphatase staining of the dish at the time of transition.

Preparation of Supplemented Complete ReproNaïve™ medium (+ Y27632)

Note: Prepare complete ReproNaïve™ Medium fresh daily.

1. Aliquot the desired volume of ReproNaïve™ Basal medium and warm to room temperature on the bench top.
2. Equilibrate ReproNaïve™ Supplement to room temperature and vortex gently for 1 minute to mix.
   Note: For best stability of ReproNaïve™ Supplement, protect the stock vial from light during use.
3. Prepare enough Supplemented Complete ReproNaïve™ medium (+ Y27632) for a 60 mm dish according to the following table. Scale volumes appropriately (See Table 2 in Appendix).

ReproNaïve™ Basal Medium:	**5 mL**
ReproNaïve™ Supplement:	**34.0 µL**
LIF (10 µg/mL stock)	**10 µL**
Y27632 (10 mM stock)	**5 µL**
Total:	**5.049 mL**

Initial passage of PSCs into ReproNaïve™ medium (Day 0)

1. Remove the medium from the dish containing the feeder-dependent culture of hiPSCs that are ready for passaging. Wash the cells with 2 mL of PBS (-).
2. Remove the PBS (-). Add 1 mL of dissociation solution for hiPSCs to the dish allowing the solution to cover the entire surface. Incubate for approx. 4 minutes in a 5% CO_2 incubator at 37 °C.
3. Remove the supernatant. Add 2 mL of fresh feeder-dependent medium. Detach all hiPSCs and feeder cells from the dish by 5 mL disposable pipette and transfer them to a 15 mL tube. Pipet repeatedly in the 15 mL tube to gently suspend the cells.
4. Stand for 3 minutes to allow hiPSCs colonies to settle. Remove as much of the supernatant as possible.
5. Add 1 mL of ESGRO Complete ACCUTASE to the 15 mL tube. Incubate for 5 minutes in a 5% CO_2 incubator at 37 °C.
6. Add 1 mL medium used to culture hiPSCs to the 15 mL tube. Centrifuge in 300×g for 5 minutes at room temperature. Remove the supernatant as much as possible.
7. Add 1 mL of **Supplemented Complete ReproNaïve™ medium (+ Y27632)** to the 15 mL tube and gently pipette the cell suspension.
8. Retrieve the previously prepared SL10 feeder cell dish(s) from the incubator and remove the medium. Add 3 mL of **Supplemented Complete ReproNaïve™ medium (+ Y27632)**.
9. Transfer all of the cell suspension to the SL10 feeder cells.
10. Swirl the dish to spread the cells evenly and incubate at 37 °C in a 5% O_2, 5% CO_2 incubator overnight.

First Medium Change (Day 1)

1. Prepare Supplemented Complete ReproNaïve™ medium (+ Y27632) as described above.
2. Remove medium from ReproNaïve™ cultured cells. Replace with 4 mL fresh **Supplemented Complete ReproNaïve™ medium (+ Y27632)**.
3. Incubate at 37 °C in a 5% O_2, 5% CO_2 incubator overnight.

Additional Medium Changes (Days 2 and 3)

1. Aliquot the desired volume of ReproNaïve™ Basal medium and warm to room temperature on the bench top.
2. Equilibrate ReproNaïve™ Supplement to room temperature and vortex gently for 1 minute to mix.
   Note: Protect the stock vial of ReproNaïve™ Supplement from light during use.

3. Prepare **Complete ReproNaïve™ Medium** according to following table.

ReproNaïve™ Basal Medium:	**5 mL**
ReproNaïve™ Supplement:	**34.0 µL**
LIF (10 µg/mL stock)	**10 µL**
Total:	**5.044 mL**

4. Remove medium from ReproNaïve™ cultured cells. Replace with 4 mL fresh **Complete ReproNaïve™ Medium.**
5. Incubate at 37 °C in a 5% O_2, 5% CO_2 incubator overnight.
6. Change medium every day.

   Note: Cells are ready for passaging on Day 3 to 4. (The day of seeding is Day 0).

Passaging adapted PSCs in ReproNaïve™ medium

Note: Volumes are based on culture in a 60 mm tissue culture dish. Please scale volumes appropriately for other culture sizes.

Preparation of Supplemented Complete ReproNaïve™ Medium (+ Y27632)

Note: Prepare Supplemented Complete ReproNaïve™ Medium (+ Y27632) fresh daily.

1. Aliquot the desired volume of ReproNaïve™ Basal medium and warm to room temperature.
2. Equilibrate ReproNaïve™ Supplement to room temperature and vortex gently for 1 minute to mix.
   Note: Protect the stock vial of ReproNaïve™ Supplement from light during use.
3. Prepare enough **Supplemented Complete ReproNaïve™ Medium (+ Y27632)** for a 60 mm dish according to the following table. Scale volumes appropriately.

ReproNaïve™ Basal Medium:	**5 mL**
ReproNaïve™ Supplement	**34.0 µL**
LIF (10 µg/mL stock)	**10 µL**
Y27632 (10 mM stock)	**5 µL**
Total:	**5.049 mL**

Passaging adapted PSCs in ReproNaïve™ Medium (Day 0)

Note: Have on the appropriate number of prepared dishes of SL10 feeder cells.

1. Remove the medium from the dish containing the cells in ReproNaïve medium that are ready for passaging. Wash the cells with 2 mL of PBS (-).
2. Remove the PBS(-). Add 1 mL of ESGRO Complete ACCUTASE to the cells. Incubate for 10 minutes in a 5% O_2,5% CO_2 incubator at 37 °C.
3. Add 0.5 mL **Supplemented Complete ReproNaïve™ Medium (+Y27632)** to the plate. Detach all ES/iPS cells and feeder cells from the dish using a P-1000 pipette and transfer them to a 15 mL tube.
4. Centrifuge at 300×g for 5 minutes at room temperature. Remove as much supernatant as possible.
5. Suspend the cells in 0.3 mL of **Supplemented Complete ReproNaïve Medium (+ Y27632)** by gentle pipetting.
6. Retrieve the previously prepared SL10 feeder cells from the incubator and remove the medium. Add 4 mL of **Supplemented Complete ReproNaïve Medium (+ Y27632)** medium.
7. Transfer 0.1 mL cell suspension to the SL10 feeder cells.
   Note: The first 4 passages should be carried out at a dilution ratio of 1:3 because the cells will be decreased gradually. After the cultivation is stabilized, around passage 5, it is possible to passage at the dilution rate of 1:3 to 1:4.
8. Swirl the dish to spread the cells evenly and incubate at 37 °C in a 5% O_2, 5% CO_2 incubator overnight.

First Medium Change (Day 1)

1. Prepare Supplemented Complete ReproNaïve Medium (+ Y27632) as described above.
2. Remove medium from ReproNaïve cultured cells. Replace with 4 mL fresh **Supplemented Complete ReproNaïve Medium (+ Y27632)**.
3. Incubate at 37 °C in a 5% O_2, 5% CO_2 incubator overnight.

Additional Medium Changes (Days 2 and 3)

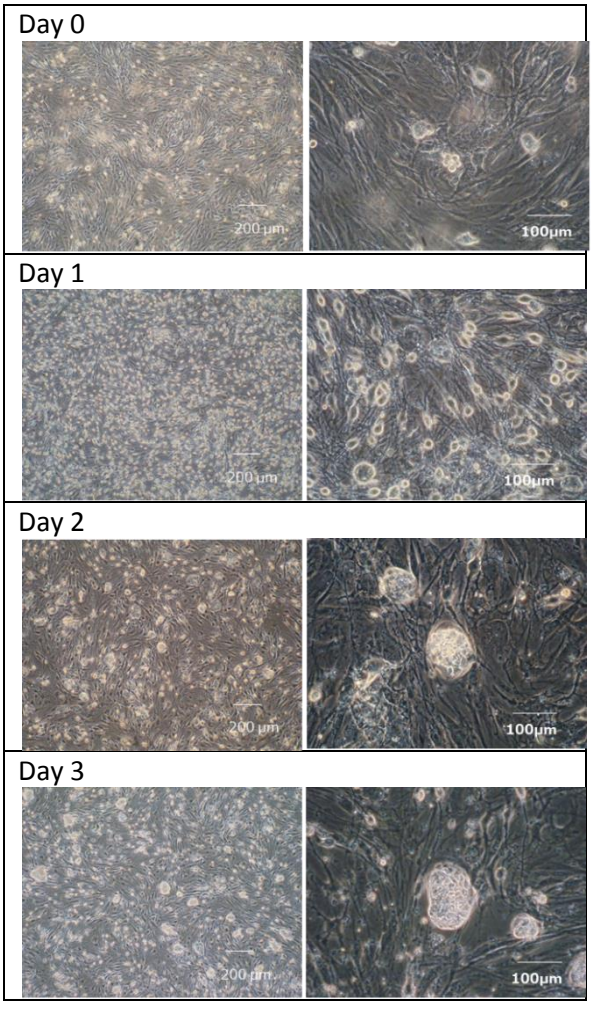
1. Aliquot the desired volume of ReproNaïve Basal medium and warm to room temperature.
2. Equilibrate ReproNaïve Supplement to room temperature and vortex gently for 1 min to mix.
   Note: Protect the stock vial of ReproNaïve Supplement from light during use.
3. Prepare **Complete ReproNaïve™ Medium** according to following table.

ReproNaïve™ Basal Medium:	**5 mL**
ReproNaïve™ Supplement:	**34.0 µL**
LIF (10 µg/mL stock):	**10 µL**
Total:	**5.044 mL**

4. Remove medium from ReproNaïve™ cultured cells. Replace with 4 mL fresh **Complete ReproNaïve™ Medium.**
5. Incubate at 37 °C in a 5% O_2, 5% CO_2 incubator overnight.
6. Change medium every day.

   Note: Cells are ready for passaging on Day 3 to 4. (The day of seeding is Day 0).

Morphology of human iPS cells cultured on SL10 feeder cells in ReproNaïve™ medium



Frequently Asked Questions

Q**. Can Complete ReproNaïve™ medium (with Supplement and LIF) be prepared in advance and stored?**
A. We recommend adding LIF and ReproNaïve™ Supplement to ReproNaïve™ Basal medium fresh every day.

Q. **Can SL10 feeder cells be used immediately after plating?**
A. Cells cultured in ReproNaïve™ medium do not attach well to SL10 feeder cells at Day 1. We recommend using SL10 feeder cells at Day 3 or Day 4 for best results.

Q. **What should I do if the numbers of colonies during transition between passage 1 and 4 gradually decrease after every passage?**
A. Continue culture for at least five passages because ReproNaïve™ medium can cause a temporary lag in cell growth.
Alternatively, you can perform one or both of the following steps.
1) Change the passage ratio to 1:1 or 1:2.
2) Extend the period of culture with Y27632.

Appendix

Table 1. Cell density of SL10 feeder cells and cultivation area

Plate/dish format	Cells per well/dish	Surface area (cm^2)
96 well plate	15,000	0.34
48 well plate	29,000	0.65
24 well plate	86,000	1.90
12 well plate	160,000	3.60
6 well plate	410,000	8.96
35 mm dish	420,000	9.20
60 mm dish	1,000,000	22.10
100 mm dish	2,720,000	60.10

Table 2. Volume of ReproNaïve™ Supplement and LIF for Complete ReproNaïve™ Medium

ReproNaïve™ Basal medium (mL)	ReproNaïve™ Supplement (µL)	LIF (10 µg/mL) (µL)
1	6.8	2
2	13.6	4
3	20.4	6
4	27.2	8
5	34.0	10
6	40.8	12
7	47.6	14
8	54.4	16
9	61.2	18
10	68.0	20
11	74.8	22
12	81.6	24
24	163.2	48
36	244.8	72
48	326.4	96

ReproCELL Inc.
https://www.reprocell.com/en/
E-mail: info_en@reprocell.com