Protocol of ReproNeuro™

Cat. No. RCDN002N, RCDN003P, RCDN001N
(also discontinued items RCESDN001, RCESDN002)

Version 2.0

Critical points for cell handling

1. Frozen cell vial should be stored at liquid nitrogen.
2. Frozen cell vials have a risk of explosion. Release the pressure of frozen cell vial by unscrewing the lid (one-quarter turn) of vial before thawing.
3. The thawing process in steps 4.3 to 4.4 must be done in less than 2 min.

Day	-1	0	3	7	14
Plate Coating	●				
Medium Preparation		●			
Thawing cells and plating		●			
Fresh Medium exchange			●	●	●

Table 1. Required reagents

Name	Catalog number	Note
ReproNeuro Culture Medium ReproNeuro MQ Medium	ReproCELL, RCDN101 RCDN102	Store at 4℃
ReproNeuro Coat	ReproCELL, RCDN201	Store at 4℃
Dulbecco's Phosphate Buffered Saline without calcium & magnesium (PBS)	Sigma, Cat. No. D5652	-
Penicillin/Streptomycin	ThermoFisher Corporation (GIBCO), Cat. No. 15140-122	Store at 4℃
0.01% Poly-L-Lysine (PLL) solution	Sigma, Cat. P4832	Store at 4℃
Flat bottom 96well plate	TPP, Cat. No. TPP92096	-

Note 1. ReproNeuro is delivered by dry shipper. Upon receipt, the frozen cell vial should be put in a liquid nitrogen dewar as soon as possible.

＊ Measurement of the extracellular electric potential is enhanced and stabilized by using ReproNeuro MQ medium instead of Culture medium . Previously Culture medium was called Maturation Medium (RCESDN301); the name and catalog number was changed effective Oct.1, 2015.

1. Coating of 96-well plate (The day before plating the cells)

1.1. Thaw the Coating solution at 4°C for at least 2 h.

1.2. Dilute 0.01% PLL in PBS to prepare 5 mL of 0.0033% PLL solution.

1.3. Add 50 µL of 0.0033% PLL solution into each well.

1.4. Incubate the plate at 37°C for 2 h.

1.5. Just prior to the next step, mix 5 mL of PBS and 150 µL of ReproNeuro Coat solution in a 15-mL tube.

1.6. Remove the PLL solution from each well by using a pipette.

1.7. Wash each well with 200 µL of PBS twice.

1.8. Add the previously prepared ReproNeuro Coat solution (50 µL/well) and incubate the plate at 37°C overnight.

2. Preparation of the ReproNeuro Culture medium

2.1. Add 520 µL of Additive A into the Culture medium bottle. Store the Culture medium at 4°C. This solution can be used for approximately 3 weeks.

3. Warming up the medium

3.1. Transfer 10 mL of the Culture medium into a 15-mL conical tube (Tube A).

3.2. Transfer 15 mL of the Culture medium into a 50-mL conical tube (Tube B).

3.3. Warm both tubes in a 37°C water bath for at least 15 min.

3.4. Take tube A alone to a clean bench and open the lid halfway. Place the micropipette in a safety cabinet and set it to 1 mL. Put on a pipette tip in advance so that it is ready for use.

Note 2. Finish all preparations (Steps 2 and 3) before starting step 4.

4. Thawing of frozen cells

4.1. Prepare a transfer box containing liquid nitrogen.

4.2. Take out a frozen cell vial from the liquid nitrogen dewar and immediately place it in the transfer box containing liquid nitrogen.

4.3. Transfer the vial onto the bench and release any pressure by unscrewing the lid of the vial (about one-quarter turn). Do not open the lid of vial completely.

4.4. Close the lid of vial after releasing the pressure.

Note 3. The pressure release process in step 4.3 to 4.4 must be done in less than 1min after removal of the cell cryovial from the liquid nitrogen.

4.5. Dip the frozen cell vial in the 37°C water bath as quickly as possible, and gently swirl it for 90s.

Note 3. The interval in warming the vial in the water bath must be less than 90 s. Otherwise, the cell viability will decrease.

4.6. Immediately after the 90 s-bath immersion, bring the partially thawed vial to the bench, dry the water on the outside of the vial, and decant all contents of the vial to tube A.

Note 4. Steps 4.3 to 4.4 should be completed within 2 min (90 s in water bath and 30 s in the transfer process.).

4.7. Transfer 1 mL of medium from tube A to the emptied vial for a brief rinse, and transfer it back again into tube A.

4.8. Centrifuge tube A at 400 × *g* for 5 min at room temperature. The centrifuge settings must be no less than 400 × g for 5 min.

4.9. While centrifuging tube A, transfer tube B containing the Culture medium from the water bath to the bench. Spray it with 70% ethanol and wipe the outer side of tube B before placing it in the safety cabinet.

4.10. After centrifuging, place tube A in the safety cabinet. Aspirate all of the supernatant carefully and leave the cell pellet in tube A.

Note 5. Be careful not to aspirate the pellet itself (it may be difficult to see).

4.11. Transfer another 2 mL of the Culture medium from tube B into tube A and mix the contents by gently pipetting as you did before.

4.12. Transfer 13 mL of Culture medium from tube B to tube A. The cell concentration is 2.0×10^5 cells/mL.

5. Seeding the cells (96-well plate)

*See table 2 if other multi-well plates are used.

5.1. Gently pipette the cell suspension 4 times using a 10-mL pipette (5 sec per repetition). Avoid making bubbles due to intense pipetting.

5.2. Transfer the pre-coated 96-well plate from the 37°C incubator to the safety cabinet.

5.3. Remove the Coating solution from 1 row of wells (12-well) by using an aspirator.

5.4. Immediately add 150 µL/well (for a 96-well plate) of the cell suspension from tube A to the emptied wells within 30 sec.

5.5. Re-suspend the cell suspension in tube A very gently and then repeat step 5.3 and 5.4.

5.6. Place the seeded plate in a 37°C, 5% CO_2 incubator and start the culture.

Note 6. The interval from step 4.1 to 5.6 must not exceed 30 min.

Table 2. Cell numbers required in various multi-well plates

Multi-well plate format	Volume of medium per well for cell seeding (µL)	Number of cells/well
12-well plate	1,400	2.8×10^5
24-well plate	750	1.5×10^5
48-well plate	280	5.6×10^4
96-well plate	150	3.0×10^4

6. Changing the culture medium

*Replace half of the volume of the Culture medium on days 3, 7, and 14.

6.1. Warm-up the fresh Cuture medium in a 37°C water bath at least 15 min before use and then place it in a safety cabinet.

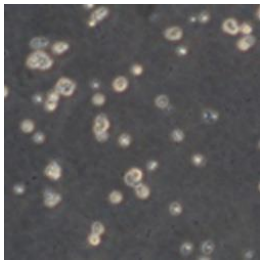
6.2. Place the seeded plate in the safety cabinet and remove half of the volume (75 µL) of the culture medium from the wells using a multichannel pipette.

6.3. Immediately add 75 µL/well (for a 96-well plate) of fresh pre-warmed Culture medium. Add the medium gently to the side of the wall (not directly to the cells). Medium replacement should be completed within 15 min.

6.4. Place the seeded plate in a 37°C, 5% CO2 incubator.

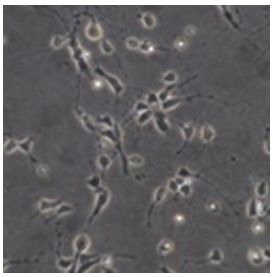
The morphology of the cells after seeding

Most cells are single. Some aggregations are observed.

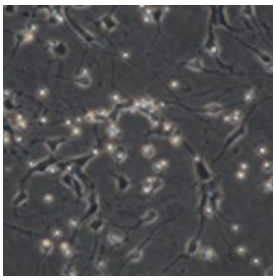


Day 1

The cells adhere to the plate in 30 min and begin to grow.

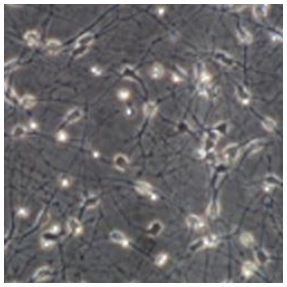


Day 7



Day 14

The cells are now ready for use in various types of assays.



Frequently Asked Questions (FAQs)

Q1. What happens when cell thawing takes more than 90 seconds?

A1. If the cells are completely thawed in a vial, their viability decreases significantly.

Q2. Before seeding, cell aggregation may occur in the cell suspension. Is it necessary to pipette for generating a single-cell suspension?

A2. It is not recommended that pipetting is repeated. Excessive pipetting reduces cell viability. Some aggregation is not influenced by the cell culture procedure.

Conditions for Use

This product is for research use only and not for therapeutic or diagnostic purposes. The sale of this product to a third party or its use for commercial purposes is not allowed without prior permission from ReproCELL.

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