Protocol of ReproNeuro™

Cat. No. RCDN002N,RCDN003P

Ver1.0

Critical points for cell handling

1. Frozen cell vial should be stored at liquid N_2.
2. Frozen cell vial have a risk of explosion. Release the pressure of frozen cell vial by opening the lid 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
Coating of plate	●				
Preparation of medium		●			
Thawing and plating		●			
Medium change			●	●	●

Table 1. Required reagents

Name	Catalog number	Note
ReproNeuro Maturation Medium ™ /ReproNeuro MQ medium	ReproCELL, RCESDN301 RCDN102	Store at 4℃
ReproNeuro Thawing medium	ReproCELL, RCESDN303	Store at 4℃
ReproNeuro Coat	ReproCELL, RCESDN304	Store at 4℃
Dulbecco's Phosphate Buffered Saline without calcium and magnesium (PBS)	Sigma, Cat. No. D5652-	-
Penicillin/Streptomycin	Life Technologies Corporation (Gibco), Cat. No. 1514015140	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 N_2 dewar as soon as possible.

＊ Measurement of the extracellular electric potential is stabilized by using ReproNeuro MQ medium (RDCN102) instead of Maturation medium.

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. Mix 5 mL of PBS and 150 µL of Coating 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 Coating solution (50 µL/well) and incubate the plate at 37°C overnight.

2. Preparation of the Maturation medium

2.1. Add 520 µL of Additive A into the Maturation medium bottle. Store the maturation 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 Thawing medium into a 15-mL conical tube (Tube A).

3.2. Transfer 15 mL of the Maturation 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 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 an transfer box poured liquid N_2.

4.2. Take out a frozen cell vial from the liquid N_2 dewar and immediately place it in transfer box poured liquid N_2.

4.3. Transfer the vial into bench and release the pressure by opening the lid of vial. Do not open the lid of vial completely.

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

Note 3. The pressure release process in step 4.3 to 4.4 must be done in less than 1min.

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 90 s-thawing, bring the partially thawed vial to the bench, rub water around 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, and transfer the remaining content of the vial to tube A.

4.8. Centrifuge tube A at 350 × *g* for 5 min at room temperature. The centrifuging condition must be no less than 350 × g for 5 min.

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

4.10. After centrifuging, place tube A on the bench. Aspirate all of the supernatant carefully and leave the pellet in tube A.

Note 5. Be careful not to aspirate the pellet itself, because it is less visible.

4.11. Transfer 2 mL of the Maturation medium from tube B to tube A and mix the contents very gently by pipetting 4 times using a micropipette (at a speed of 1 pipetting per 3 s.).

4.12. Transfer 13 mL of Maturation medium from tube B to tube A. The cell concentration is $2.0x10^5$ 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 (at a speed of 1 pipetting per 5 s.). Avoid making bubbles due to intense pipetting.

5.2. Transfer the coated 96-well plate from incubator to 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 Maturation medium on days 3, 7, and 14.

6.1. Warm-up the Maturation 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.

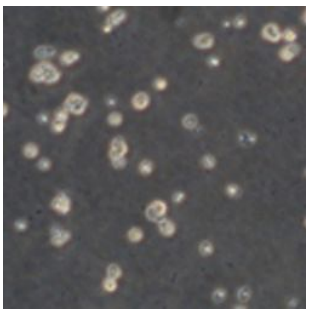
6.3. Immediately add 75 µL/well (for a 96-well plate) of warmed maturation medium. Add the medium gently to the side of the wall (not directly to the cells). Medium replacement should be completed within 15 min.

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

The morphology of the cells after seeding

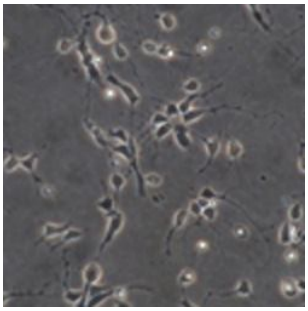
After seeding

Most cells are single. Some aggregations are observed.

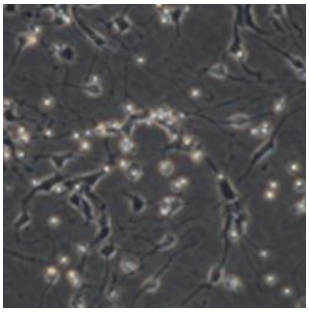


Day 1

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

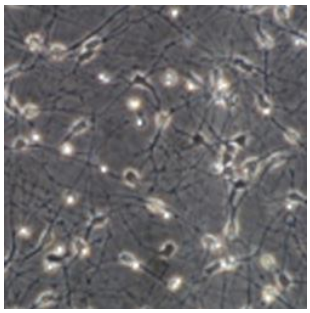


Day 7



Day 14

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



Frequently Asked Questions (FAQs)

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

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

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