3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

Mixed Neuron Culturing Information for iPSC-differentiated neurons

Catalog Numbers **NEUR1001, NEUR1004, NEUR1006, NEUR1007, NEUR1008, NEUR1039, NEUR1041, and NEUR1042**
Storage Temperature –196 °C (Liquid Nitrogen, LN_2)

TECHNICAL BULLETIN

Product Description

These products contain cryo-preserved, pre-differentiated mixed population neurons derived from a footprint-free, karyotype normal human iPSC line. They are designed for customers to generate mature neurons using the optimized maturation medium and supplement. Mature neurons can be obtained within 8 days. The neurons can be seeded on various culture vessel formats including 96 well plates on either glass or plastic surfaces and cultured as adherent cells. Shortly after seeding, the cells proliferate slightly for up to 3 days and show extensive neurite outgrowth and proper neuronal morphology. In general, at 8 days post-seeding, the cell population will contain ≥98% neurons and ≤1% Glial Fibrillary Acidic Protein (GFAP) positive astrocytes.

Reagents and Equipment Required but Not Provided.

Neuronal Medium with Supplement A
(Catalog Numbers NEUR1050 [50 mL of medium] and NEUR1051 [250 ml of medium])

Poly-L-ornithine hydrobromide
(Catalog Number P3655)

Laminin
(Life Technologies Catalog Number 23017-015)

0.4% Trypan Blue solution
(Catalog Number T8154)

Primary antibodies:
Monoclonal anti-β-tubulin III, isotype III
clone SDL.3D10 (Catalog Number T8660)

Monoclonal Anti-Glial Fibrillary Acidic Protein (GFAP)
(Dako Catalog Number M0761)

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The storage conditions for the mixed neurons and the required Neuronal Medium are shown in Table 1.

A vial with cryopreserved mixed neurons is packed in a small Ziploc® bag, which is buried in the dry ice. Upon receiving the product, check the integrity of the package and the presence of dry ice.

Cells can be plated immediately after arrival or transferred into liquid nitrogen. Do not remove the vials from the dry ice during transportation to liquid nitrogen storage units. Transfer the cryopreserved mixed neurons directly to liquid nitrogen storage units from the dry ice, avoiding exposure to room temperature.

Re-freezing the Neuronal Medium and supplement, or cryopreserving cultured mixed neurons is not recommended.

Table 1.
Storage conditions of mixed neuron components

Component	Storage Temperature
Cell vials	–196 °C (Liquid Nitrogen, LN_2)
Neuronal Medium	–20 °C
Supplement A	–20 °C

Neuron Maturation Procedure
This procedure has been extensively tested with the mixed neurons and Neuronal Medium. The user should follow this procedure closely. The user assumes all responsibility for the failure of the experiment should there be any deviation from this procedure.

Coating Cell Culture Vessels with Poly-L-ornithine and Laminin
Cell culture vessels should be coated one day before or on the day of plating the cells.

1. Prepare a stock solution of poly-L-ornithine (10 mg/mL) by dissolving the powder in sterile cell culture grade water. The stock solution should be stored at –20 °C.
2. Thaw a 1 mg bottle of Mouse Laminin solution (Life Technologies Catalog Number 23017-015) on ice.
3. Prepare working solution of poly-L-ornithine in sterile cell culture grade water to a final concentration of 20 μg/mL.
4. Add poly-L-ornithine solution into desired cell culture vessel to entirely cover the growth surface of the vessel (see Table 2).
5. Distribute the solution evenly and incubate vessels in a cell culture incubator for 2 hours (37 °C/ 5% CO_2 with humidity control).
6. Rinse vessels twice with cell culture grade water. Pipette water gently toward the corner of the vessel to avoid mechanical removal of poly-L-ornithine coating.
7. Dilute the Mouse Laminin solution in sterile cell culture grade water to a final working concentration of 10 μg/mL.
8. Aspirate water from the vessels and add laminin working solution to entirely cover the bottom of the vessel. Incubate in the cell culture incubator for 2 hours (37 °C/5% CO_2 with humidity control).
9. It is recommended to use freshly coated vessels. However, if not used immediately, store coated vessels at 2–8 °C in laminin solution (up to 4 days).
10. Pre-warm vessels at 37 °C before use.
11. Aspirate laminin just before seeding dopaminergic neuron precursors. Do not let the surface dry.

Table 2.
Recommended volumes of coating reagents for various vessels

Vessel type	poly-L-ornithine	Laminin
96 well plate	50 μL/well	50 μL/well
4 or 24 well plate	250 μL/well	250 μL/well
35 mm dish	1.5 mL	1.5 mL
60 mm dish	2.5 mL	2.5 mL

Thawing and Culturing Cryopreserved Neurons
All steps described must be performed in accordance with aseptic cell culture standards. All media and vessels used for cell culture must be pre-warmed to 37 °C prior to use.

For the entire process of neuron maturation, only the Complete Neuronal Medium is required (Neuronal Medium with Supplement A)

1. One day before thawing precursor cells, store the Neuronal Medium at 2–8 °C overnight. Once thawed, medium can be stored at 2–8 °C for up to 3 weeks.

2. On the day of thawing cryopreserved neurons, transfer 25 mL aliquot of Neuronal Medium into a 50 mL conical tube and add 25 μL of Supplement A (pre-thawed on ice) to make the Complete Neuronal Medium.

3. Add a 5 mL aliquot of Complete Neuronal Medium to a 15 mL conical tube and pre-warm at 37 °C. This aliquot will be used for recovery of precursor cells from frozen stock.

4. Prepare another aliquot of Complete Neuronal Medium according to volumes required for cell culture vessels utilized (see Table 3). Pre-warm at 37 °C only enough Complete Neuronal Medium for use in cell culture that day. Store the remaining Complete Medium A at 2–8 °C.

Table 3.
Recommended volumes of medium in various vessels

Vessel type	Medium volume
96 well plate	100 μL/well
4 or 24 well plate	500 μL/well
35 mm dish	2 mL
60 mm dish	5 mL

5. Shortly before thawing the cells, place pre-warmed medium and vessels in the biosafety cabinet.

6. To thaw cryopreserved neurons, remove one vial from liquid nitrogen and place immediately onto dry ice (the vial must be buried in the dry ice).

7. Bring dry ice container with the vial to a 37 °C water bath and immerse the vial in the bath (up to 2/3rd of the vial) and thaw cells until only a small piece of ice is still visible (~1 minute).
   Note: Do not shake vial during thawing.

8. Immediately bring the vial to the biological cabinet, spraying it thoroughly with 70% ethanol solution and wiping with an autoclaved paper towel.

9. Remove cells from the vial using a micropipette (or serological pipette) and transfer drop-wise while swirling into 15 mL conical tube containing 5 mL of pre-warmed Complete Neuronal Medium (step 3). Wash the vial with 1 mL of medium from the 15 mL conical tube and transfer it back to the tube.
   Note: Do not shake vial during thawing.

10. Centrifuge cells at 400 × *g* for 5 minutes at room temperature.

11. Aspirate the medium very carefully using a vacuum (or pipette if preferred) leaving only a drop of liquid in the tube. Do not remove or disturb the cell pellet during aspiration of medium.

12. Using a micropipette, add 1 mL of the Complete Neuronal Medium (step 4) into the tube and gently re-suspend cells by pipetting up and down 4–6 times.

13. Remove a 10 µL aliquot of cell suspension and mix it with 10 µL of 0.4% Trypan blue solution.

14. Count the cells.

15. Take the appropriate volume of pre-warmed Complete Neuronal Medium and add to the tube with the cells.

16. Aspirate laminin solution from pre-warmed cell culture vessel and seed neurons at a density ranging from 4×10^4 to 8×10^4 live cells/cm^2.

Table 4.
Recommended seeding densities for neurons in various types of cell culture vessels. Range: from low to high.

Vessel	Surface/well	Seeding
96 well plate	0.33 cm^2	$1.3 \times 10^4 – 2.6 \times 10^4$
4 well plate	2 cm^2	$8 \times 10^4 – 1.6 \times 10^5$
35 mm dish	10 cm^2	$4 \times 10^5 – 8 \times 10^5$
60 mm dish	20 cm^2	$8 \times 10^5 – 1.6 \times 10^6$

17. Distribute cells evenly and place vessels in the cell culture incubator (37 °C/5% CO_2 with humidity control). Medium should be changed every other day.

18. Monitor cell survival the following day.

19. Change Complete Neuronal Medium at day 3 post-seeding. Medium change should be done slowly (drop wise) pointing the pipette tip toward the wall of cell culture vessel.

20. Continue maturation of neurons for 8 days. Change medium every other day.
    Note: Cells can be differentiated for up to 5 weeks post-seeding. However, prolonged culture will have increased population of GFAP positive astrocytes.

Results

Characterization of Mature Neurons

The maturation of neurons can be assessed by their morphology, see Figure 1, and by immunostaining of the Tuj-1 marker (neuronal class III-β tubulin), see Figure 2. Percentage of neurons can be determined by a count of Tuj-1 positive neurons divided by the total number of cells (DAPI staining of nuclei).

Figure 1.
Example of neuron morphology at different time points post-seeding. Neurons were seeded at 5×10^4 live cells/cm^2.

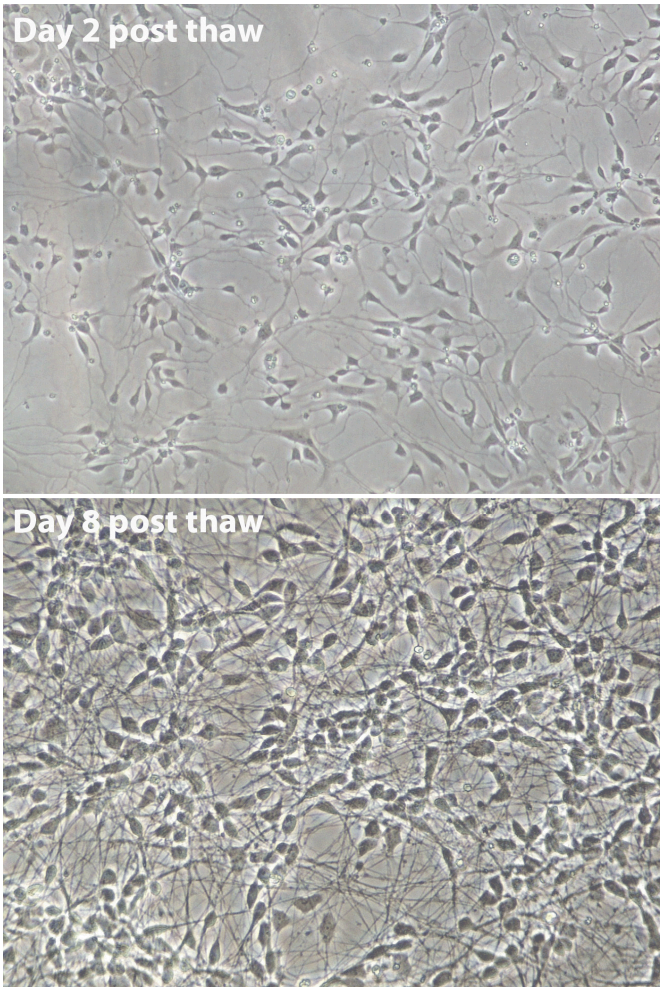

Figure 2.
Example of mature neurons. Immunostaining at 10 days post-seeding, showing ≥98% of total cells expressed the Tuj-1 marker (neuronal class β-III tubulin, green) and ≥40% expressed the GABAergic neuronal marker (red). Total nuclei count (blue) is used as the total number of cells.