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Xeno-Free Culture and Differentiation of Neural Stem Cells into Neurons

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Introduction

As the field of neuroscience moves closer to the reality of neural stem cell-derived therapies, the importance of reagent selection early in the development of clinical-grade neural cell lines has become increasingly clear. More specifically, the use of media and supplement combinations that contain xeno-free or only human-origin or recombinant components is critical to safety and traceability of the final product.

Concerns about pathogen cross-transfer from non-human sources or contamination with non-neural cells, which limit the efficiency and specificity of the differentiation protocols, as well as the various challenges faced in maintaining healthy cultures, have lead to the development of xeno-free media systems with optimized conditions for maintaining, differentiating, and expanding neural stem cells (NSCs).

The following protocol is broken in to three parts: Xeno-Free Culture of Neural Stem Cells, Xeno-Free Differentiation of Neural Stem Cells into Neurons, and Xeno-Free Culture of Neurons.

Materials Needed

Cells

- Gibco® Human Neural Stem Cells (H9-Derived) (Cat. no. N7800-100) or an in-house produced cell line

Reagents

- CELLstart™ CTS™ Substrate (Cat. no. 10142-01)
- KnockOut™ DMEM/F12 CTS™ (Cat. no. A13708-01)
- B-27® Supplement XenoFree CTS™ (Cat. no. A14867-01)
- N-2 Supplement CTS™ (Cat. no. A13707-01)
- bFGF, Recombinant Human CTS™ (Cat. no. CTP0261)
- EGF, Recombinant Human (Cat. no. PHG0311)
- GlutaMAX™-I CTS™ Supplement (A12860-01)
- Ascorbic Acid (Sigma, Cat. no. A8960)
- TrypLE™ Select CTS™ Dissociation Enzyme (Cat. no. A12859-01)
- DPBS CTS™ without Calcium Chloride, without Magnesium Chloride (Cat. no. A1285601)
- DPBS CTS™ (with Calcium & Magnesium) (Cat. no. A1285801)
- Human Laminin (Sigma, Cat. no. L6274)
- Neurobasal® Medium CTS™ (Cat. no. A1371201)
- Poly-L-Lysine or Poly-D-Lysine (Sigma, Cat. no. p7280 or p9155)
- Water, distilled (Cat. no. 15230)

This protocol is for research use and not intended for clinical or diagnostic testing.

Preparing Reagents and Media

Ascorbic Acid Stock Solution

1. Resuspend 500 mg of ascorbic acid powder in 8.634 mL of distilled water to make a 200 mM stock solution.

   Note: The molecular weight of ascorbic acid is 289.54.
2. Sterilize the ascorbic acid solution by filtering it through a 0.22-µm filter.
3. Aliquot the ascorbic acid solution into sterile tubes and store at –20°C in the dark for up to 6 months.

bFGF and EGF Stock Solutions

1. To prepare a 20 µg/mL stock solution of bFGF or EGF, resuspend 10 µg of bFGF or EGF in 500 µL of 0.1% human serum albumin in DPBS CTS™ without Calcium Chloride, without Magnesium Chloride.
2. Aliquot the bFGF or EGF stock solution into sterile tubes and store at –20°C in the dark for up to 6 months.

Xeno-Free Proliferation Medium

Complete Xeno-Free Proliferation Medium is used for culturing neural stem cells. It consists of KnockOut™ DMEM-F12 CTS™ medium with GlutaMAX™-I CTS™ supplement, ascorbic acid, bFGF, EGF, N-2 Supplement CTS™, and B-27® Supplement XenoFree CTS™.

1. To prepare 100 mL of complete Xeno-Free Proliferation Medium, aseptically mix the following components:

Component	Final concentration	Amount
KnockOut™ DMEM/F12 CTS™ Medium	1X	96 mL
GlutaMAX™-I CTS™ Supplement	2 mM	1 mL
bFGF (prepared as 20 µg/mL stock)	20 ng/mL	100 µL
EGF (prepared as 20 µg/mL stock)	20 ng/mL	100 µL
B-27® Supplement XenoFree CTS™	2%	2 mL
N-2 Supplement CTS™	1%	1 mL
Ascorbic acid	200 µM	100 µL

2. Complete Xeno-Free Proliferation Medium can be stored at 2–8°C in the dark for up to 2 weeks.

Xeno-Free Differentiation Medium

Xeno-Free Differentiation Medium is used for differentiating neural stem cells into neurons, and it consists of Neurobasal® Medium CTS™ with GlutaMAX™-I CTS™ supplement, ascorbic acid, and B-27® Supplement XenoFree CTS™.

1. To prepare 100 mL of complete Xeno-Free Differentiation Medium, aseptically mix the following components:

Component	Final concentration	Amount
Neurobasal® Medium CTS™	1X	97 mL
GlutaMAX™-I CTS™ Supplement	2 mM	1 mL
B-27® Supplement XenoFree CTS™	2%	2 mL
Ascorbic acid	200 µM	100 µL

2. Complete Xeno-Free Differentiation Medium can be stored at 2–8°C in the dark for up to 2 weeks.

Xeno-Free Culture Medium

Complete Xeno-Free Culture Medium is used for culturing neurons, and it consists of Neurobasal® Medium CTS™ with GlutaMAX™-I CTS™ supplement, ascorbic acid, and B-27® Supplement XenoFree CTS™.

1. To prepare 100 mL of complete Xeno-Free Culture Medium, aseptically mix the following components:

Component	Final concentration	Amount
Neurobasal® Medium CTS™	1X	97 mL
GlutaMAX™-I CTS™ Supplement	2 mM	1 mL
B-27® Supplement XenoFree CTS™	2%	2 mL
Ascorbic acid	200 µM	100 µL

2. Complete Xeno-Free Culture Medium can be stored at 2–8°C in the dark for up to 2 weeks.

Preparing Matrix

Coating Culture Vessels with CELLstart™ CTS™ Substrate

1. Dilute CELLstart™ CTS™ substrate 1:100 in DPBS CTS™ (with Calcium & Magnesium) (i.e., 50 µL of substrate into 5 mL of DPBS).

   Note: CELLstart™ CTS™ substrate should not be frozen, vortex or exposed to vigorous agitation due to potential gel formation.

2. Coat the surface of the culture vessel with the working solution of CELLstart™ CTS™ substrate (14 mL for T-75, 7 mL for T-25, 3.5 mL for 60-mm dish, 2 mL for 35-mm dish).
3. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO_2 for 1 hour.
4. Remove the vessel from the incubator and store it until use. Remove all CELLstart™ CTS™ substrate solution immediately before use, and fill the vessel with complete Xeno-Free Proliferation Medium.

   Note: You may coat the plates in advance and store them at 4°C, wrapped tightly with Parafilm® laboratory film, for up to 2 weeks. Do not remove CELLstart™ CTS™ solution until just prior to use. Make sure the plates do not dry out.

Coating Culture Vessels with Human Laminin

1. Thaw the laminin slowly at 2–8°C and prepare a 10-µg/mL working solution in sterile, distilled water. Aliquot the working solution into polypropylene tubes, and store the tubes at –20°C until use. Avoid repeated freeze/thaw cycles.

   Note: Laminin may form a gel if thawed too rapidly.

2. Coat the surface of the culture vessel with the laminin working solution (14 mL for T-75, 7 mL for T-25, 3.5 mL for 60-mm dish, 2 mL for 35-mm dish).
3. Incubate the culture vessel overnight at 4°C or for 2 hours at 37°C.
4. Rinse the culture vessel with DPBS CTS™ without Calcium Chloride, without Magnesium Chloride, and store the vessel covered with DPBS until use. Immediately before use, remove all DPBS and replace it with complete medium.

   Note: You may coat the plates in advance and store them at 4°C, wrapped tightly with Parafilm® laboratory film, for up to 2 weeks. Do not remove DPBS until just prior to use. Make sure the plates do not dry out. Do not use the laminin-coated plate if discoloration or spider web formations appear on its surface.

Coating Culture Vessels with Poly-L-Lysine or Poly-D-Lysine

1. Prepare a 10-µg/mL working solution of Poly-L-Lysine or Poly-D-Lysine in sterile, distilled water.
2. Coat the surface of the culture vessel with the working solution of Poly-L-Lysine or Poly-D-Lysine (14 mL for T-75, 7 mL for T-25, 3.5 mL for 60-mm dish, 2 mL for 35-mm dish).
3. Incubate the culture vessel at room temparature for 1 hour.
4. Aspirate the Poly-L-Lysine or Poly-D-Lysine solution from the coated vessel and rinse the surface of the vessel twice with sterile, distilled water.

   Note: Make sure to rinse the culture vessel thoroughly, because excess poly-D-lysine can be toxic to the cells.
5. Leave the coated vessels uncovered in the laminar hood until they have completely dried. You may use the dry plates immediately or store them at 4°C, wrapped tightly with Parafilm® laboratory film, for up to one week.

Xeno-Free Culture of Neural Stem Cells

Thawing Neural Stem Cells

1. Pre-warm 10 mL of complete Xeno-Free Proliferation Medium to 37°C.
2. Transfer a vial of frozen NSCs from liquid nitrogen storage to a 37°C water bath. It is important to make the transfer immediately to prevent crystal formation.
3. Immerse the vial in the 37°C water bath without submerging the cap to thaw the cells. Swirl the vial gently while thawing.
4. When only a small crystal of ice remains, remove the vial from the water bath and sterilize the outside of it with 95% ethanol. Allow the ethanol to evaporate before opening the vial.
5. Pipet the thawed cells gently into a sterile 15-mL tube and slowly add pre-warmed complete medium to a total volume of 5 mL.

   Note: While adding the medium, gently move the tube back and forth to mix the cells. This reduces osmotic shock to the cells.
6. Centrifuge the cells at 300 × *g* for 4 minutes. Aspirate and discard the supernatant.
7. Resuspend the cells in 5 mL of pre-warmed complete Xeno-Free Proliferation Medium, and determine the total number of cells and percent viability.
8. Inoculate a CELLstart™ CTS™ substrate-coated culture vessel with the thawed cells at a final seeding density of 1×10^5 cells/cm^2.

   Note: Aspirate the coating solution from the culture vessel immediately before using the vessel.
9. Incubate the cells at 37°C in a humidified atmosphere (90%) of 5% CO_2 in air.
10. The next day, replace the spent medium with fresh Xeno-Free Proliferation Medium, and change the medium every other day thereafter.
11. Once the culture has reached full confluency, passage the cells at a split ratio of 1:4 or at a seeding density of 1×10^5 cells/cm^2. You may also freeze the cells for long-term storage in liquid nitrogen.

Culture and Propagation of Neural Stem Cells

1. Prepare a CELLstart™ CTS™ substrate-coated culture vessel and pre-warm the Xeno-Free Proliferation Medium to 37°C.
2. Dilute the TrypLE™ Select CTS™ dissociation enzyme to 0.5X in DPBS CTS™ without Calcium Chloride, without Magnesium Chloride.
3. Rinse the cells to be passaged once with DPBS CTS™ without Calcium Chloride, without Magnesium Chloride, using approximately 2 mL DPBS per 10 cm^2 of culture surface area.
4. Add 0.5X TrypLE™ Select CTS™ solution to the cells at approximately 1 mL per 10 cm^2 of culture surface area and incubate for 3 minutes at room temperature or in a 37°C incubator. If the culture is dense, increase the incubation time until the cells start to separate and round up.
5. Gently rinse the culture vessel by pipetting the TrypLE™ Select CTS™ solution up and down onto the cells to detach them from the vessel. Avoid creating bubbles.
6. Collect the cells and transfer to a 15-mL conical tube.
7. Stop the cell dissociation reaction by adding complete Xeno-Free Proliferation Medium at 4X the volume of TrypLE™ Select CTS™ solution in the vessel (approximately 4 mL of medium per 10 cm^2 of culture surface area). Disperse the medium by pipetting it over the cell layer surface several times.
8. Centrifuge the cells at 300 × *g* for 4 minutes. Aspirate and discard the supernatant.
9. Resuspend cells in complete Xeno-Free Proliferation Medium to a final concentration of 1×10^4 cells/µL.
10. Inoculate the CELLstart™ CTS™ substrate-coated culture vessel with the thawed cells at a final seeding density of 1×10^5 cells/cm^2 and place it in a 37°C incubator with a humidified atmosphere of 5% CO_2 in air
11. The next day, replace the spent medium with fresh Xeno-Free Proliferation Medium, and change the medium every other day thereafter.

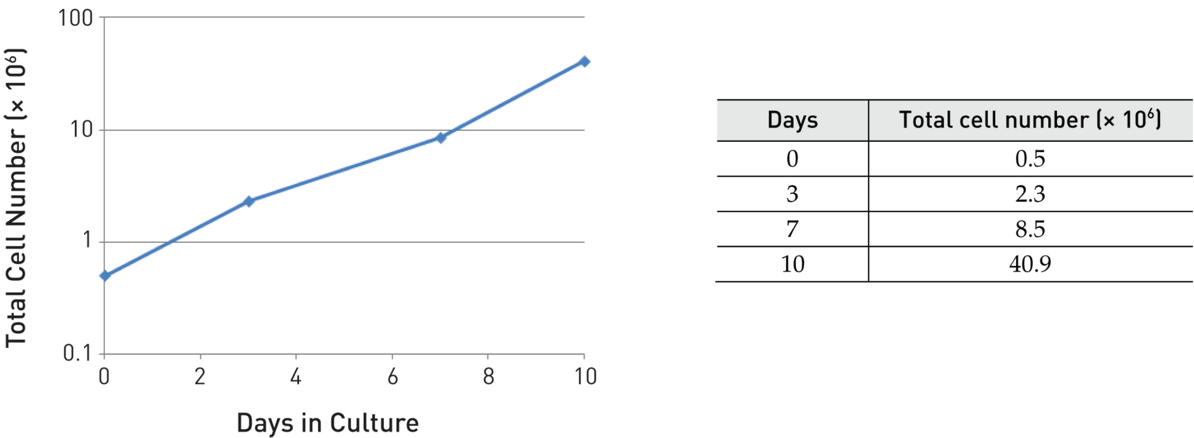
Freezing Neural Stem Cells

1. Prepare 2X freezing solution consisting of 20% DMSO and 80% Xeno-Free Proliferation Medium. Keep the freezing medium on ice until use.
2. Harvest the cells as described in **Culture and Propagation of Neural Stem Cells**, above.
3. Resuspend the cells in complete Xeno-Free Proliferation Medium at a concentration of 2×10^6 cells/mL.
4. Add the same amount of 2X freezing medium to the resuspended cells as was used for resuspending them in a drop-wise manner.

   Note: The final concentration of DMSO in 1X freezing medium is 10%, and the final cell concentration is 1×10^6 cells/mL.
5. Prepare 1 mL aliquots (1×10^6 cells) in cryovials, and place the vials in an isopropanol chamber. Place the isopropanol chamber with the cryovials at –80°C overnight.
6. The next day, transfer the frozen cryovials to a liquid nitrogen tank (vapor phase) for long-term storage.

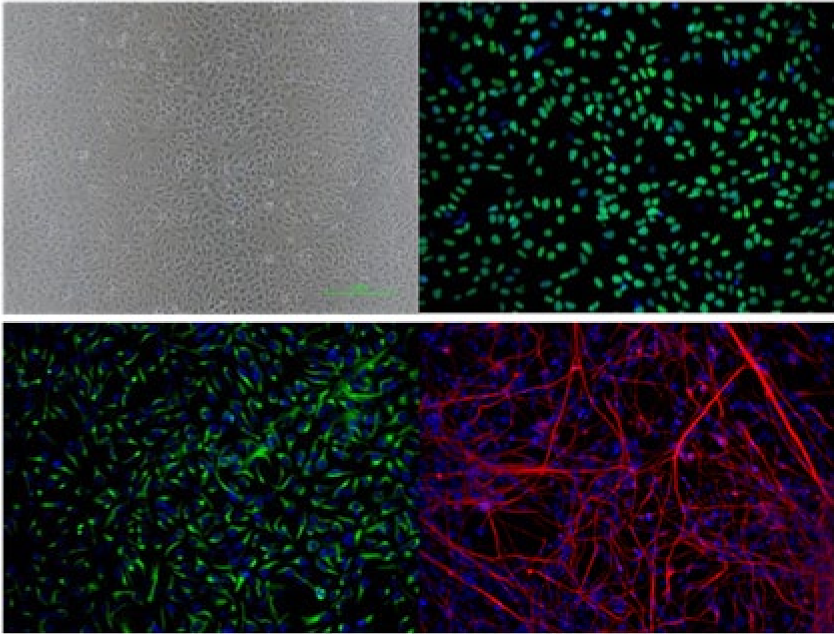
   Note: You may check the viability and recovery of frozen cells 24 hours after storing cryovials in liquid nitrogen by following the procedure outlined in **Thawing Neural Stem Cells**, above.

Figure 1 Culture kinetics of neural stem cells in Xeno-Free Proliferation Medium prepared with B-27® Supplement XenoFree CTS™. After 10 days, the initial culture seeded with 0.5×10^6 cells produced 4.0×10^7 cells.



Days	Total cell number ($\times 10^6$)
0	0.5
3	2.3
7	8.5
10	40.9

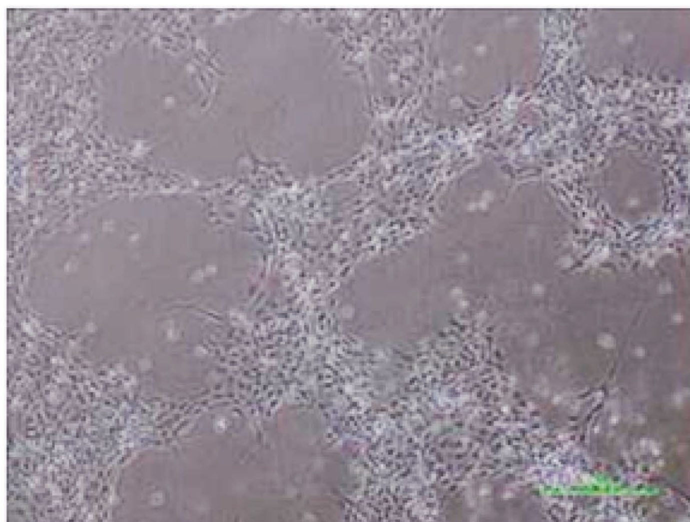
Figure 2 NSC proliferated (p3, upper left) using B-27® Supplement XenoFree CTS™ were characterized with NSC phenotype marker Sox1 (upper right, green) and Nestin (lower left, green), and further differentiated to neurons (lower right, red).



Xeno-Free Differentiation of Neural Stem Cells to Neurons

1. Prepare a laminin-coated culture vessel and pre-warm the Xeno-Free Differentiation Medium to 37°C.
2. Dilute the TrypLE™ Select CTS™ dissociation enzyme to 0.5X in DPBS CTS™ without Calcium Chloride, without Magnesium Chloride.
3. Rinse the neural stem cells once with DPBS CTS™ without Calcium Chloride, without Magnesium Chloride, using approximately 2 mL DPBS per 10 cm^2 of culture surface area.
4. Add 0.5X TrypLE™ Select CTS™ solution to the cells at approximately 1 mL per 10 cm^2 of culture surface area and incubate for 3 minutes at room temperature or in a 37°C incubator. If the culture is dense, increase the incubation time until the cells start to separate and round up.
5. Gently rinse the culture vessel by pipetting the TrypLE™ Select CTS™ solution up and down onto the cells to detach them from the vessel. Avoid creating bubbles.
6. Collect the cells and transfer to a 15-mL conical tube.
7. Stop the cell dissociation reaction by adding complete Xeno-Free Proliferation Medium at 4X the volume of TrypLE™ Select CTS™ solution in the vessel (approximately 4 mL of medium per 10 cm^2 of culture surface area). Disperse the medium by pipetting it over the cell layer surface several times.
8. Centrifuge the cells at 300 × *g* for 4 minutes. Aspirate and discard the supernatant.
9. Aspirate the laminin solution from the laminin-coated culture vessel and immediately add an appropriate amount of Xeno-Free Differentiation Medium to prevent the culture vessel from drying.
10. Inoculate the laminin-coated culture vessel with the neural stem cells at a final seeding density of 1×10^5 cells/cm^2 and place it in a 37°C incubator with a humidified atmosphere of 5% CO_2 in air
11. The next day, change the medium to fresh Xeno-Free Differentiation Medium, and replace half of the medium with fresh Xeno-Free Differentiation Medium every 2–3 days thereafter.

Figure 3 Human NSCs were differentiated for 7 days. Neurite extension was observed (left, phase-contrast image; scale bar = 200 µm) and up-regulation in the expression of neurofilament was examined by qPCR (right; expression was normalized by beta-actin).



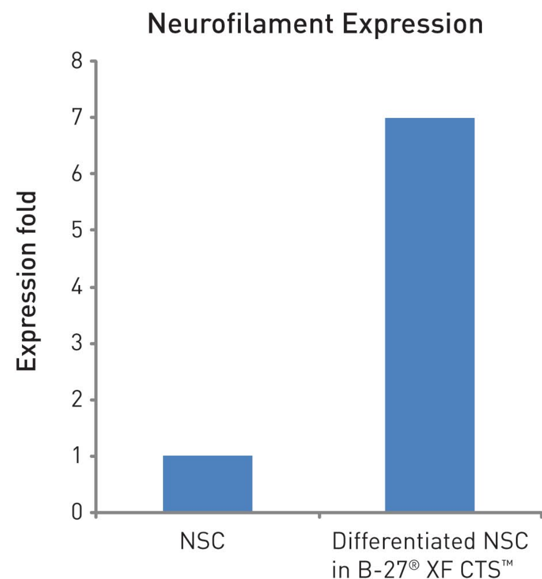
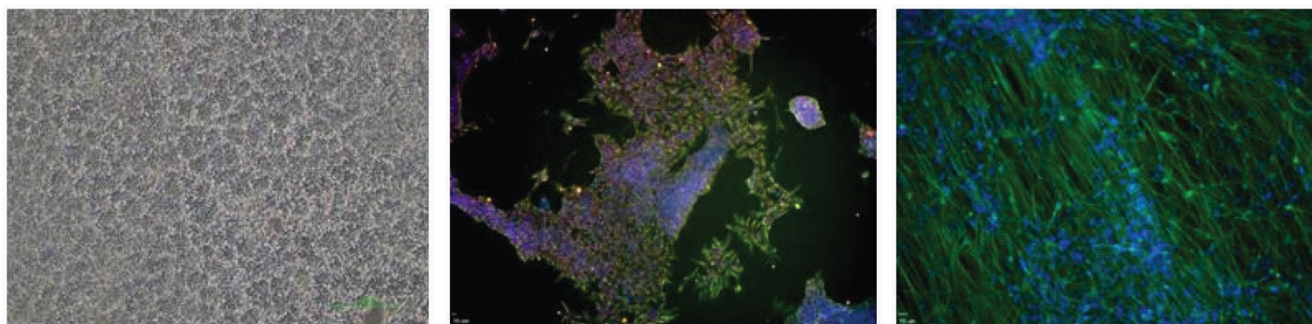


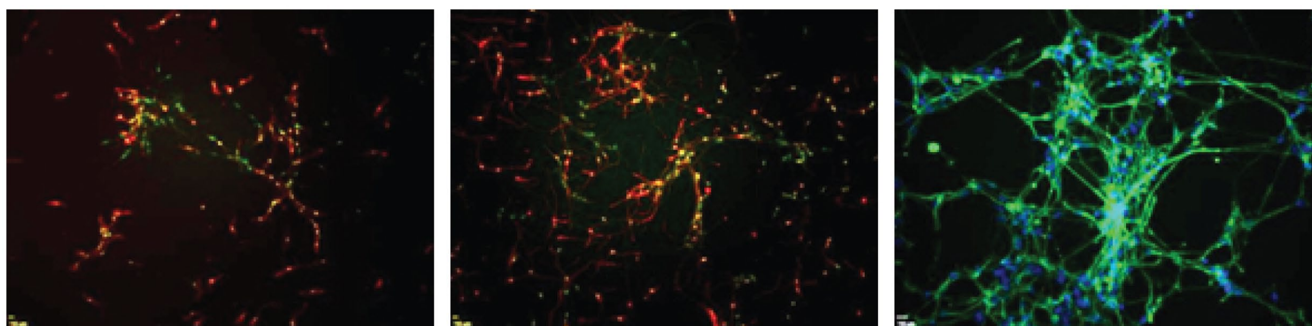
Figure 4 Human induced pluripotent stem cells were differentiated into NSCs using B-27® Supplement XenoFree CTS™, and the derived NSCs were further differentiated in complete XenoFree Differentiation Medium (containing Neurobasal® Medium CTS™ and B-27® Supplement XenoFree CTS™) into neurons. Phase-contrast image of derived NSCs (left), phenotype marker expression of derived NSCs (middle; red = Sox1, green = Nestin), and NSCs differentiated into neurons for 2 weeks, whose neurites were stained (right; beta III tubulin = green)



Xeno-Free Culture of Neurons

1. Pre-warm 10 mL of complete Xeno-Free Culture Medium to 37°C.
2. Rinse the Poly-L-Lysine- or Poly-D-Lysine-coated culture vessel with sterile, distilled water twice, and add an appropriate volume of pre-warmed complete Xeno-Free Culture Medium.
3. Inoculate the Poly-L-Lysine- or Poly-D-Lysine-coated culture vessel with the cells at a final seeding density of 2×10^4–5×10^4 cells/cm^2 and incubate at 37°C overnight.
4. The next day, change the medium to fresh Xeno-Free Culture Medium, and replace half of the medium with fresh Xeno-Free Culture Medium every 2–3 days thereafter.

Figure 5 Culture of human hippocampal neurons, cortical neurons, and Gibco® Episomal iPSC-derived neurons (right). After the hippocampal neurons (left) and cortical neurons (middle) were cultured for a week, live cells were stained with Calcein AM (green) and neurons were stained with Dcx (red). After Gibco® Episomal iPSC-derived neurons (right) were cultured for a week, the cells were stained with Dcx (green) and the nuclei were counterstained with DAPI (blue).



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