Human iPSC-Derived Neural Stem Cells

System A, B, C and D
Table of Contents

Human iPSC-Derived Neural Cell Culture System  2

What’s the Best System for Your Discovery?  3

Product Information  4

Preparation of Reagents  5
Neural Expansion-XF Medium (required for systems A and C)  5
Neural Maintenance-XF Medium (required for all systems)  6
Neural Plating–XF Medium (required for all systems)  6
Neural Differentiation-XF Medium (required for systems A and B)  6
Unlock (required for systems A and C)  6

Preparation of Coating Reagents and Coating the Culture Vessel  7
SureBond Coating Solution (required for systems A and C)  7
SureBond-XF Coating Solution (required for all systems for final plating on plastic or a fully xeno-free system in systems A and C)  7
SureBond+ReadySet Coating Solution (required for all systems for final plating on glass)  8

System A  9
Expansion and Synchronous Differentiation  9
Thawing and Plating of Human iPSC-Derived Neural Stem Cells  9
Expansion of Human iPSC-Derived Neural Stem Cells  11
Synchronous Differentiation of Human iPSC-Derived Neural Stem Cells  12
Maintenance of Human iPSC-Derived Cortical Neurons  13

System B  14
Synchronous Differentiation  14
Thawing and Plating of Human iPSC-Derived Neural Stem Cells  14
Synchronous Differentiation of Human iPSC-Derived Neural Stem Cells  15
Maintenance of Human iPSC-Derived Cortical Neurons  16

System C  17
Expansion and Spontaneous Differentiation  17
Thawing and Plating of Human iPSC-Derived Neural Stem Cells  17
Expansion of Human iPSC-Derived Neural Stem Cells  19
Spontaneous Differentiation and Maintenance of Human iPSC-Derived Neural Stem Cells  20

System D  21
Spontaneous Differentiation  21
Thawing, Plating, Spontaneous Differentiation and Maintenance of Human iPSC-Derived Neural Stem Cells  21
Human iPSC-Derived Neural Cell Culture System

**SureBond**
- Coating solution providing an optimal surface for feeder-free growth & adherence
- Neural Expansion-XF Medium
  - Fully defined cell culture basal medium optimized for NSC expansion (up to 5 passages)
  - +SureGrowth (FGF2)
  - +SureGrowthX (EGF)
- Neural Differentiation-XF Medium
  - Fully defined medium that supports high purity neuronal cultures without compromising cell viability

**SureBond-XF**
- For long-term culture on plastic
- Neural Expansion-XF Medium
- +SureGrowth (FGF2)
- +SureGrowthX (EGF)
- Neural Differentiation-XF Medium
- +SureGrowth (FGF2)
- +SureGrowthX (EGF)

**SureBond+ReadySet**
- For long-term culture on glass
- Neural Expansion-XF Medium
- +SureGrowth (FGF2)
- +SureGrowthX (EGF)
- Neural Differentiation-XF Medium
- +SureGrowth (FGF2)
- +SureGrowthX (EGF)

**Neural Plating-XF Medium**
- Optimized to support maximum cell recovery after thawing
- Unlock*
  - Fully defined, efficient & gentle cell detachment buffer
- Neural Plating-XF Medium
- Optimized to support maximum cell recovery after thawing

**Neural Maintenance-XF Medium**
- Next-generation neural maintenance medium optimized to support low-density culture & long-term functional maturation

*Axol SureBond-XF & Unlock-XF are also available for a completely xeno-free neural cell culture system

www.axolbio.com
What’s the Best System for Your Discovery?

Systems A and B – Synchronous Differentiation

Synchronous Differentiation
Pure population of cerebral cortical neurons

Following the protocol for systems A and B will result in the generation of a pure population of cerebral cortical neurons.

Using system A, iPSC-Derived Neural Stem Cells are expanded and then the synchronously differentiated into neurons, using Neural Differentiation-XF Medium.

Using system B, iPSC-Derived Neural Stem Cells are plated into the final assay format and then synchronously differentiated, using Neural Differentiation-XF Medium, from iPSC-Derived Neural Stem Cells to neurons.

Systems C and D – Spontaneous Differentiation

Spontaneous Differentiation
Mixed population of neuronal and glial cells

Following the protocol for systems C and D will result in the generation of a mixed population of cerebral cortical neurons and glial cells.

Using system C, iPSC-Derived Neural Stem Cells are expanded and then spontaneously differentiated, using Neural Maintenance-XF Medium, from iPSC-Derived Neural Stem Cells to neuronal and glial cells.

Using system D, iPSC-Derived Neural Stem Cells are plated into the final assay format and then spontaneously differentiated into neuronal and glial cells, using Neural Maintenance-XF Medium.

Caution!
Increasing the number of days the iPSC-Derived NSCs are in culture and the more expansions the NSCs undergo will increase the population of glial cells in the final culture. We recommend a maximum of 5 passages for healthy/control lines and a maximum of 3 passages for the disease/patient lines. Expansion must be conducted with caution and the cells should not be pushed to proliferate for too long before differentiation.
## Product Information

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<th>Catalog. No.</th>
<th>Product Name</th>
<th>Format</th>
<th>Stock Conc.</th>
<th>Storage on Arrival</th>
<th>Thawing Instructions</th>
<th>Storage Once Thawed</th>
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<td>ax0015</td>
<td>Human iPSC-Derived Neural Stem Cells (Healthy Donors)</td>
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<td>N/A</td>
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<td>Follow protocol</td>
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<td>ax0030-500</td>
<td>Neural Expansion-XF Medium</td>
<td>500 mL</td>
<td>1x</td>
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<td>Neural Maintenance-XF Medium</td>
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<td>Reconstituted protein should be used immediately or stored in working aliquots at -20°C</td>
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<td>-20°C</td>
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Lot-specific information such as specifications and quality control details are stated in the Certificate of Analysis.
Preparation of Reagents

**Neural Expansion-XF Medium (required for systems A and C)**

- Upon receipt, store **Neural Expansion-XF Medium** at or below -80°C protected from light. Stored at -80°C, medium is stable for 6 months from date of manufacture.
- When ready to use, thaw medium overnight at 4°C in the dark, aliquot and store at -80°C.
- On the day of thawing **Human iPSC-Derived Neural Stem Cells (NSCs)**, prepare growth factors as follows:
  - **SureGrowth Recombinant Human FGF2**
    Prepare 100 µg/mL solution (5000x) of **SureGrowth Recombinant Human FGF2** by resuspending the 100 µg of lyophilized powder in 1 mL of PBS (1x) supplemented with 0.1 % human serum albumin.
  - **SureGrowthX Recombinant Human EGF**
    Prepare 100 µg/mL solution (5000x) of **SureGrowth Recombinant Human EGF** by resuspending the 100 µg of lyophilized powder in 1 mL of PBS (1x) supplemented with 0.1 % human serum albumin.
- Supplement **Neural Expansion-XF Medium** with 100 µg/mL **SureGrowth Recombinant Human FGF2** and **SureGrowth Recombinant Human EGF** to a final concentration of 20 ng/mL of FGF2 and EGF.
- Growth factors should be added fresh each time an aliquot of **Neural Expansion-XF Medium** is thawed.
- A thawed and supplemented aliquot of **Neural Expansion-XF Medium** can be stored at 4°C for 1 week.

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**Important!**

**Axol Neural Cell Culture Media**

DOES NOT contain antibiotics or antifungal agents. Axol Bioscience does not recommend the use of antimicrobial agents such as penicillin, streptomycin and amphotericin. Antimicrobial agents should not be necessary if proper aseptic technique is adopted.
Neural Maintenance-XF Medium (required for all systems)
- Upon receipt aliquot and store Neural Maintenance-XF Medium at or below -80°C protected from light. Stored at -80°C, medium is stable for 6 months from date of manufacture.
- When ready to use, thaw an aliquot of Neural Maintenance-XF Medium overnight at 4°C in the dark.
- A thawed aliquot of Neural Maintenance-XF Medium can be stored at 4°C for 1 week, protected from light.
- Neural Maintenance-XF Medium is fully supplemented and ready to use.

Neural Plating–XF Medium (required for all systems)
- Upon receipt store Neural Plating–XF Medium at or below -80°C protected from light.
- When ready to use, thaw Neural Plating–XF Medium overnight at 4°C in the dark.
- Once thawed, Neural Plating–XF Medium must be used and cannot be refrozen.

Neural Differentiation-XF Medium (required for systems A and B)
- Upon receipt, aliquot and store Neural Differentiation-XF Medium at or below -80°C protected from light. Stored at -80°C, the medium is stable for 6 months from date of manufacture.
- When ready to use, thaw an aliquot of Neural Differentiation-XF Medium overnight at 4°C in the dark.
- A thawed aliquot of Neural Differentiation-XF Medium can be stored at 4°C for 1 week, protected from light.
- Neural Differentiation-XF Medium is fully supplemented and ready-to-use.

Unlock (required for systems A and C)
- Upon receipt aliquot and store Unlock at or below -80°C protected from light. Stored at -80°C, the reagent is stable for 6 months from date of manufacture.
Preparation of Coating Reagents and Coating the Culture Vessel

**SureBond Coating Solution** *(required for systems A and C)*

- Upon receipt store SureBond at or below -80°C.
- Thaw the SureBond coating solution overnight at 4°C.
- Calculate the total surface area that requires coating.
- Dilute the SureBond stock solution (50x) in Dulbecco’s-PBS (1x) (D-PBS, without calcium or magnesium) to make 1x working solution e.g. 120 μL in 6 mL.
- Coat the surface of your culture vessel with the SureBond 1x working solution. We recommend coating at a volume of 200 μL per cm² however, please optimize for your experiments.
- Incubate your culture vessel overnight at 37°C.

**Important!**

Make sure that the coating does not evaporate overnight.
Do not let the SureBond coating dry out before seeding the cells.
DO NOT wash the vessel after coating with SureBond.

**SureBond-XF Coating Solution** *(required for all systems for final plating on plastic or a fully xeno-free system in systems A and C)*

- Upon receipt store SureBond-XF at 4°C.
- Calculate the total surface area that requires coating.
- Dilute the SureBond-XF stock solution (200x) in D-PBS (1x) (without calcium or magnesium) to make 1x working solution e.g. 30 μL in 6 mL.
- Coat the surface of your culture vessel with the SureBond-XF 1x working solution. We recommend coating at a volume of 200 μL per cm² however, please optimize for your experiments.
- For final culture in a 96-well plate format see Protocol Supplement (version 1.0): Culture of Human iPSC-Derived Neural Stem Cells in a 96-well Plate Format.

**Important!**

Make sure that the coating does not evaporate.
Do not let the SureBond-XF coating dry out before seeding the cells.
DO NOT wash the vessel after coating with SureBond-XF.
SureBond+ReadySet Coating Solution (required for all systems for final plating on glass)

- Upon receipt store SureBond at or below -80°C and store ReadySet at 4°C.
- Thaw the SureBond coating solution overnight at 4°C.
- Calculate the total surface area that requires coating.
- Pre-coat your culture vessel with ReadySet at a volume of 250 μL per cm².
- Incubate at 37°C for 45 minutes.
- Wash the plate thoroughly four times using an equal volume of sterile distilled H₂O (e.g. if 250 μL of ReadySet, use 250 μL sterile distilled H₂O). During each wash rock the dish to ensure thorough washing.
- Do not let the ReadySet dry out following washing, proceed straight to coating with SureBond.
- Dilute the SureBond stock solution (50x) in D-PBS (1x) (without calcium or magnesium) to make 1x working solution e.g. 120 μL in 6 mL.
- Coat the surface of your culture vessel with the SureBond 1x working solution. We recommend coating at a volume of 200 μL per cm².
- Incubate for 1 hour at 37°C.
- For final culture in a 96-well plate format see Protocol Supplement (version 1.0): Culture of Human iPSC-Derived Neural Stem Cells in a 96-well Plate Format.

Important!

Do not let the SureBond coating dry out before seeding the cells.
DO NOT wash the vessel after coating with SureBond.
System A
Expansion and Synchronous Differentiation

The expansion of Human iPSC-Derived Neural Stem Cells followed by the generation of a pure population of cortical neurons

Following the protocol for system A will result in the generation of a pure population of cerebral cortical neurons. System A includes the expansion of iPSC-Derived Neural Stem Cells and then the synchronized differentiation of iPSC-Derived Neural Stem Cells to neurons.

Thawing and Plating of Human iPSC-Derived Neural Stem Cells

The day before thawing Human iPSC-Derived NSCs:
- Thaw Neural Plating–XF Medium overnight at 4°C.
- Prepare culture vessels with SureBond and incubate overnight at 37°C (if using a completely xeno-free system pre-coat the culture vessels with SureBond-XF 4 hours prior to thawing cells). Axol recommends: coating and seeding into 2 wells of a 6-well plate for initial plating post-thaw.

On the day of thawing Human iPSC-Derived NSCs:
- Pre-warm all media and culture vessels to 37°C before use.
- To thaw cells – transfer the cells from storage by transporting cells buried in dry ice. Remove the cells from dry ice and transfer the cells to a 37°C water bath.
- Quickly thaw the vial of cells in a 37°C water bath. Do not completely submerge the vial (only up to 2/3rd of the vial). Remove the vial before the last bit of ice has melted, after ~1-2 minutes.
- Do not shake the vial during thawing.
- Take the vial of cells to a biological safety cabinet, spraying the vial and hood thoroughly with 70% ethanol and wiping with an autoclaved paper towel before placing the vial in the hood.

Important!

Do not mix the cells vigorously. Avoid generating bubbles.
Once thawed, use a P1000 pipette to transfer the cells into a 15 mL sterile conical tube drop-wise add 10 mL of pre-warmed, 37°C, Neural Expansion-XF Medium. Gently wash the cryogenic vial with 1 mL of Neural Expansion-XF Medium. Transfer this to the 15 mL sterile conical tube containing the cells.

Centrifuge cells at 200 x g for 5 minutes at room temperature.

During the centrifugation step, remove the coating solution.

**Axol Recommends**: Once the coating solution has been removed add 1.95 mL of Neural Plating–XF Medium into 2 wells of the pre-coated 6-well plate, ensuring that the culture vessel does not dry. Return the plate containing the Neural Plating–XF Medium to the incubator until ready for cell seeding.

Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet in 1 mL of Neural Plating–XF Medium until they are in a single cell suspension.

**Perform a cell count to ensure optimal seeding density.**

Plate the resuspended cells drop-wise and evenly at a density ranging from 70,000 - 200,000 cells/cm² on SureBond coated culture vessel.

To ensure an even plating of Human iPSC-Derived NSCs, gently rock the culture vessel back and forth and side to side several times.

Incubate the cells at 37°C, 5% CO₂.

The day after plating, replace the medium with fresh pre-warmed, 37°C, Neural Expansion-XF Medium (freshly supplemented with EGF (20 ng/mL) and FGF2 (20 ng/mL)), to remove any dead cells/debris and to promote proliferation.

**Caution!**

Increasing the number of days the iPSC-Derived NSCs are in culture and the more expansions the NSCs undergo will increase the population of glial cells in the final culture. We recommend a maximum of 5 passages for healthy/control lines and a maximum of 3 passages for the disease/patient lines. Expansion must be conducted with caution and the cells should not be pushed to proliferate for too long before differentiation.
Expansion of Human iPSC-Derived Neural Stem Cells

- **Every 2 days**, replace all of the medium with fresh, pre-warmed, 37°C, Neural Expansion-XF Medium (freshly supplemented with EGF (20 ng/mL) and FGF2 (20 ng/mL)).
- When the culture is 70-80% confluent, it is ready to undergo passaging for expansion.
- **Pre-coat culture vessels**: Make sure to pre-coat the surface of your culture vessel that the cells will be passaged into. If continuing to expand Human iPSC-Derived NSCs, pre-coat culture vessels with SureBond.
- Thaw an aliquot of Unlock, Neural Expansion-XF Medium and Neural Plating–XF Medium overnight at 4°C before use and store at 4°C.
- Pre-warm all media and culture vessels to 37°C before use.
- Remove all spent medium from cell culture vessels.
- Gently rinse the surface of the cell layer once with the Dulbecco's-PBS (1x) (D-PBS, without calcium or magnesium, 2 mL D-PBS (1x) per 10 cm² culture surface area).
- Discard the D-PBS.
- To detach the cells from a coating of SureBond use Unlock.
- Add 1 mL per 10cm² of culture surface area of cold/room temperature Unlock. Evenly distribute it over the entire cell layer. Incubate the cells for 5 minutes at 37°C.
- Use a P1000 pipette to transfer the cells drop-wise into a 15 mL sterile conical tube add gently four volumes of pre-warmed, 37°C, Neural Expansion-XF Medium. (For example if 1 mL of Unlock is used, then add 4 mL of the medium to stop the reaction). Gently pipette up and down a few times to disperse the medium.
- Centrifuge cells at 200 x g for 5 minutes at room temperature.
- During the centrifugation step, remove the coating solution.
- Once coating solution has been removed add Neural Plating–XF Medium to the empty well. Add ~65% of the total well volume so that the culture vessel does not dry out (e.g. 6-well plate maximal volume is < 3 mL, add 1.95 mL of Neural Plating–XF Medium). Return the plate containing the Neural Plating–XF Medium to the incubator until ready for cell seeding.
- Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet in 1 mL of Neural Plating–XF Medium until they are in a single cell suspension.
- **Perform a cell count to ensure optimal seeding density**.
- Plate the resuspended cells drop-wise and evenly at a density ranging from 70,000 - 200,000 cells/cm² on SureBond coated culture vessel.
- To ensure an even plating of Human iPSC-Derived NSCs, gently rock the culture vessel back and forth and side to side several times.
- Incubate the cells at 37°C, 5% CO₂.
- The day after plating, replace the medium with fresh pre-warmed, 37°C, Neural Expansion-XF Medium (freshly supplemented with EGF (20 ng/mL) and FGF2 (20 ng/mL)), to remove any dead cells/debris and to promote proliferation.
- **Every 2 days**, replace all of the medium with fresh, pre-warmed, 37°C, Neural Expansion-XF Medium (freshly supplemented with EGF (20 ng/mL) and FGF2 (20 ng/mL)).
- When the culture is 70-80% confluent, it is ready to undergo another passage for further expansion or seeded for differentiation.
Synchronous Differentiation of Human iPSC-Derived Neural Stem Cells

When you have expanded the cells to your desired passage/cell quantity and are ready to start the differentiation carry out the following procedure:

**Note:**
Differentiation will take a minimum of 3 days and can take up to 10 days depending on the confluency of the Human iPSC-Derived NSCs. The optimal confluency for synchronous differentiation of Human iPSC-Derived NSCs to neurons is 60%.

- **Pre-coat culture vessels:** Make sure to pre-coat the surface of your culture vessel that the cells will be passaged into. If seeding cells onto plastic culture vessels for the final assay format, then pre-coat the vessels with SureBond-XF. If seeding cells onto glass culture vessels for the final assay format, then pre-coat the vessels with SureBond+ReadySet.
- Thaw an aliquot of Unlock, Neural Expansion-XF Medium and Neural Plating–XF Medium overnight at 4°C before use and store at 4°C.
- Pre-warm all media and culture vessels to 37°C before use.
- Remove all spent medium from cell culture vessel.
- Gently rinse the surface of the cell layer once with the Dulbecco’s-PBS (1x) (D-PBS, without calcium or magnesium, 2 mL D-PBS (1x) per 10 cm² culture surface area).
- Discard the D-PBS.
- To detach the cells from a coating of SureBond use Unlock.
- Add 1 mL per 10cm² of culture surface area of cold/room temperature Unlock. Evenly distribute it over the entire cell layer. Incubate the cells for 5 minutes at 37°C.
- Use a P1000 pipette to transfer the cells drop-wise into a 15 mL sterile conical tube add gently four volumes of pre-warmed, 37°C, Neural Expansion-XF Medium. (For example if 1 mL of Unlock is used, then add 4 mL of the medium to stop the reaction). Gently pipette up and down a few times to disperse the medium.
- Centrifuge cells at 200 x g for 5 minutes at room temperature.
- During the centrifugation step, remove the coating solution.
- Once coating solution has been removed add Neural Plating–XF Medium to the empty well. Add ~65% of the total well volume so that the culture vessel does not dry out (e.g. 6-well plate maximal volume is < 3 mL, add 1.95 mL of Neural Plating–XF Medium). Return the plate containing the Neural Plating–XF Medium to the incubator until ready for cell seeding.
- Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet with 1 mL of Neural Plating–XF Medium until they are in a homogeneous single cell suspension.
- **Perform a cell count to ensure optimal seeding density.**
Plate the resuspended cells drop-wise and evenly at a density ranging from 70,000 - 200,000 cells/cm² on pre-coated culture vessels.

To ensure an even plating of Human iPSC-Derived NSCs gently rock the culture vessel back and forth and side to side several times.

Incubate the cells at 37°C, 5% CO₂.

The day after plating, conduct a full medium change with fresh, pre-warmed, 37°C, Neural Expansion-XF Medium WITHOUT EGF or FGF2.

After a further 24 hours, conduct a full medium change with fresh, pre-warmed, 37°C, Neural Differentiation-XF Medium.

Replace half the volume of medium with fresh pre-warmed, 37°C, Neural Differentiation-XF Medium every 3 days.

Detection of a pure population of neurons is visual. Neurite formation will begin to occur and you will see an increase in neuronal cell types. Check the culture each day to observe neuronal development. When the cells are 60% confluent before starting differentiation, a pure neuronal population will take at least 3 days to develop. If the culture is >60% confluent it may take longer (up to 10 days). If the culture is fully confluent, a pure population of neurons will be difficult to obtain.

**MAINTENANCE**

**Maintenance of Human iPSC-Derived Cortical Neurons**

- Once the culture has reached a pure population of neurons, replace half the volume of medium with fresh, pre-warmed, 37°C, Neural Maintenance-XF Medium.

  - 24 hours after the half medium change to Neural Maintenance-XF Medium, replace half the volume of medium with fresh, pre-warmed, 37°C, Neural Maintenance-XF Medium.

  - To maintain a healthy neuronal culture, replace half the volume of medium with fresh, pre-warmed, 37°C, Neural Maintenance-XF Medium every 3 days.

  - It is important that the medium is pre-warmed and that the medium changes are conducted gently by pipetting to the side of the culture dish.

  - Terminally differentiated neurons can now be maintained in culture until ready for experimental assays.
System B
Synchronous Differentiation

The generation of a pure population of cortical neurons without the expansion of Human iPSC-Derived Neural Stem Cells

Using system B, iPSC-Derived Neural Stem Cells are plated into the final assay format and then synchronously differentiated from iPSC-Derived Neural Stem Cells to neurons, using Neural Differentiation-XF Medium.

Thawing and Plating of Human iPSC-Derived Neural Stem Cells

The day before thawing Human iPSC-Derived NSCs:

- Thaw Neural Plating–XF Medium overnight at 4°C.
- Prepare culture vessels: If seeding cells onto plastic culture vessels for the final assay format, then pre-coat the vessels with SureBond-XF. If seeding cells onto glass culture vessels for the final assay format, then pre-coat the vessels with SureBond+ReadySet.

On the day of thawing Human iPSC-Derived NSCs:

- Pre-warm all media and culture vessels to 37°C before use.
- To thaw cells – transfer the cells from storage by transporting the cells buried in dry ice. Remove the cells from dry ice and transfer the cells to a 37°C water bath.
- Quickly thaw the vial of cells in a 37°C water bath. Do not completely submerge the vial (only up to 2/3rd of the vial). Remove the vial before the last bit of ice has melted, after ~1-2 minutes.
- Do not shake the vial during thawing.
- Take the vial of cells to a biological safety cabinet, spraying the vial and hood thoroughly with 70% ethanol and wiping with an autoclaved paper towel before placing the vial in the hood.
- Once thawed, use a P1000 pipette to transfer the cells into a 15 mL sterile conical tube drop-wise add 10 mL of pre-warmed, 37°C, Neural Expansion-XF Medium. Gently wash the cryogenic vial with 1 mL of Neural Expansion-XF Medium. Transfer this to the 15 mL sterile conical tube containing the cells.

Important!
Do not mix the cells vigorously. Avoid generating bubbles.
Centrifuge cells at 200 × g for 5 minutes at room temperature.

During the centrifugation step, remove the coating solution.

Once coating solution has been removed add Neural Plating–XF Medium to the empty well. Add ~65% of the total well volume so that the culture vessel does not dry out (e.g. 6-well plate maximal volume is < 3 mL, add 1.95 mL of Neural Plating–XF Medium). Return the plate containing the Neural Plating–XF Medium to the incubator until ready for cell seeding.

Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet with 1 mL of Neural Plating–XF Medium until they are in a single cell suspension.

Perform a cell count to ensure optimal seeding density.

Plate the resuspended cells drop-wise and evenly at a density ranging from 70,000 - 200,000 cells/cm² on pre-coated culture vessels.

To ensure an even plating of Human iPSC-Derived NSCs gently rock the culture vessel back and forth and side to side several times.

Incubate the cells for 24 hours at 37°C, 5% CO₂.

24 hours after plating, replace half the volume of medium with fresh, pre-warmed, 37°C, Neural Maintenance-XF Medium. This will allow the cells to recover after thawing.

After a further 24 hours, replace the medium fully with fresh pre-warmed, 37°C, Neural Differentiation-XF Medium.

Replace half the volume of medium with fresh, pre-warmed, 37°C, Neural Differentiation-XF Medium every 3 days.

Detection of a pure population of neurons is visual. Neurite formation will begin to occur and you will see an increase in neuronal cell types. Check the culture each day to observe neuronal development. When the cells are 60% confluent before starting differentiation, a pure neuronal population will take at least 3 days to develop. If the culture is >60% confluent it may take longer (up to 10 days). If the culture is fully confluent, a pure population of neurons will be difficult to obtain.

Synchronous Differentiation of Human iPSC-Derived Neural Stem Cells

Note:

Differentiation will take a minimum of 3 days and can take up to 10 days depending on the confluency of the Human iPSC-Derived NSCs. The optimal density for synchronous differentiation of Human iPSC-Derived NSCs to neurons is 60%.
Maintenance of Human iPSC-Derived Cortical Neurons

- Once the culture has reached a pure population of neurons, replace half the volume of medium with fresh, pre-warmed, 37°C, Neural Maintenance-XF Medium.
- 24 hours after the half medium change to Neural Maintenance-XF Medium, conduct another half medium change with fresh, pre-warmed, 37°C, Neural Maintenance-XF Medium.
- To maintain a healthy neuronal culture, replace half the volume of medium with fresh, pre-warmed, 37°C, Neural Maintenance-XF Medium every 3 days.
- It is important that the medium is pre-warmed and that the medium changes are conducted gently by pipetting to the side of the culture dish.
- Terminally differentiated neurons can now be maintained in culture until ready for experimental assays.
System C
Expansion and Spontaneous Differentiation

The expansion of Human iPSC-Derived Neural Stem Cells followed by the generation of a mixed population of cortical neurons and glia

System C includes the expansion of iPSC-Derived Neural Stem Cells and then spontaneous differentiation of iPSC-Derived Neural Stem Cells to neuronal and glial cells, using Neural Maintenance-XF Medium.

Thawing and Plating of Human iPSC-Derived Neural Stem Cells

The day before thawing Human iPSC-Derived NSCs:
- Thaw Neural Plating–XF Medium overnight at 4°C.
- Prepare culture vessels with SureBond and incubate overnight at 37°C (if using a completely XF system pre-coat the culture vessels with SureBond-XF 4 hours prior to thawing cells). Axol recommends: coating and seeding into 2 wells of a 6-well plate for initial seeding post-thaw.

On the day of thawing Human iPSC-Derived NSCs:
- Pre-warm all media and culture vessels to 37°C before use.
- To thaw cells – transfer the cells from storage by transporting the cells buried in dry ice. Remove the cells from dry ice and transfer the cells to a 37°C water bath.
- Quickly thaw the vial of cells in a 37°C water bath. Do not completely submerge the vial (only up to 2/3rd of the vial). Remove the vial before the last bit of ice has melted, after ~1-2 minutes.
- Do not shake the vial during thawing.
- Take the vial of cells to a biological safety cabinet, spraying the vial and hood thoroughly with 70% ethanol and wiping with an autoclaved paper towel before placing the vial in the hood.

Important!

Do not mix the cells vigorously. Avoid generating bubbles.
Once thawed, use a P1000 pipette to transfer the cells into a 15 mL sterile conical tube drop-wise add 10 mL of pre-warmed, 37°C, Neural Expansion-XF Medium. Gently wash the cryogenic vial with 1 mL of Neural Expansion-XF Medium. Transfer this to the 15 mL sterile conical tube containing the cells.

Centrifuge cells at 200 x g for 5 minutes at room temperature.

During the centrifugation step, remove the coating solution.

Once coating solution has been removed add Neural Plating–XF Medium to the empty well. Add ~65% of the total well volume so that the culture vessel does not dry out (e.g. 6-well plate maximal volume is < 3 mL, add 1.95 mL of Neural Plating–XF Medium). Return the plate containing the Neural Plating–XF Medium to the incubator until ready for cell seeding.

Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet with 1 mL of Neural Plating–XF Medium until they are in a single cell suspension.

Perform a cell count to ensure optimal seeding density.

Plate the resuspended cells drop-wise and evenly at a density ranging from 70,000 - 200,000 cells/cm² on SureBond coated culture vessels.

To ensure an even plating of Human iPSC-Derived NSCs gently rock the culture vessel back and forth and side to side several times.

Incubate the cells at 37°C, 5% CO₂.

The day after plating, replace the medium with fresh pre-warmed, 37°C, Neural Expansion-XF Medium (freshly supplemented with EGF (20 ng/mL) and FGF2 (20 ng/mL)), to remove any dead cells/debris and to promote proliferation.

Caution!

Increasing the number of days the iPSC-Derived NSCs are in culture and the more expansions the NSCs undergo will increase the population of glial cells in the final culture. We recommend a maximum of 5 passages for healthy/control lines and a maximum of 3 passages for the disease/patient lines. Expansion must be conducted with caution and the cells should not be pushed to proliferate for too long before differentiation.
Expansion of Human iPSC-Derived Neural Stem Cells

- **Every 2 days**, replace all of the medium with fresh, pre-warmed, 37°C, Neural Expansion-XF Medium (freshly supplemented with EGF (20 ng/mL) and FGF2 (20 ng/mL)).
- When the culture is 70-80% confluent, it is ready to undergo passaging for expansion.
- **Pre-coat culture vessels**: Make sure to pre-coat the surface of your culture vessel that the cells will be passaged into. If continuing to expand Human iPSC-Derived NSCs pre-coat culture vessels with SureBond.
- Thaw an aliquot of Unlock, Neural Expansion-XF Medium and Neural Plating–XF Medium overnight at 4°C before use and store at 4°C.
- Pre-warm all media and culture vessels to 37°C before use.
- Remove all medium from cell culture vessels.
- Gently rinse the surface of the cell layer once with the Dulbecco's-PBS (1x) (D-PBS, without calcium or magnesium, 2 mL D-PBS (1x) per 10 cm² culture surface area).
- Discard the D-PBS.
- To detach the cells from a coating of SureBond use Unlock.
- Add 1 mL per 10cm² culture surface area. of cold/room temperature Unlock. Evenly distribute it over the entire cell layer. Incubate the cells for 5 minutes at 37°C.
- Use a P1000 pipette to transfer the cells drop-wise into a 15 mL sterile conical tube add gently four volumes of pre-warmed, 37°C, Neural Expansion-XF Medium. (For example if 1 mL of Unlock is used, then add 4 mL of the medium to stop the reaction). Gently pipette up and down a few times to disperse the medium.
- Centrifuge cells at 200 x g for 5 minutes at room temperature.
- During the centrifugation step, remove the coating solution.
- Once coating solution has been removed add Neural Plating–XF Medium to the empty well. Add ~65% of the total well volume so that the culture vessel does not dry out (e.g. 6-well plate maximal volume is < 3 mL, add 1.95 mL of Neural Plating–XF Medium). Return the plate containing the Neural Plating–XF Medium to the incubator until ready for cell seeding.
- Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet with 1 mL of Neural Plating–XF Medium until they are in a single cell suspension.
- **Perform a cell count to ensure optimal seeding density**.
- Plate the resuspended cells drop-wise and evenly at a density ranging from 70,000 - 200,000 cells/cm² on SureBond coated culture vessels.
- To ensure an even plating of Human iPSC-Derived NSCs gently rock the culture vessel back and forth and side to side several times.
- Incubate the cells at 37°C, 5% CO₂.
- The day after plating, replace the medium with fresh pre-warmed, 37°C, Neural Expansion-XF Medium (freshly supplemented with EGF (20 ng/mL) and FGF2 (20 ng/mL)), to remove any dead cells/debris and to promote proliferation.
- **Every 2 days**, replace all of the medium with fresh, pre-warmed, 37°C, Neural Expansion-XF Medium (freshly supplemented with EGF (20 ng/mL) and FGF2 (20 ng/mL)).
- When the culture is 70-80% confluent, it is ready to undergo another passage for further expansion or seeding for differentiation.
Spontaneous Differentiation and Maintenance of Human iPSC-Derived Neural Stem Cells

When you have expanded your cells to the desired passage or cell quantity and are ready to start the spontaneous differentiation carry out the following procedure:

- **Pre-coat culture vessels**: Make sure to pre-coat the surface of your culture vessel that the cells will be passaged into. If seeding cells onto **plastic** culture vessels for the final assay format, then pre-coat the vessels with SureBond-XF. If seeding cells onto **glass** culture vessels for the final assay format, then pre-coat the vessels with SureBond+ReadySet.
- Thaw an aliquot of **Unlock**, ** Neural Expansion-XF Medium** and **Neural Plating–XF Medium** overnight at 4°C before use and store at 4°C.
- Pre-warm all media and culture vessels to 37°C before use.
- Remove all medium from cell culture vessel.
- Gently rinse the surface of the cell layer once with the Dulbecco’s-PBS (1x) (D-PBS without calcium or magnesium, 2 mL D-PBS (1x) per 10 cm² culture surface area).
- Discard the D-PBS.
- To detach the cells from a coating of SureBond use Unlock.
- Add 1 mL per 10cm² culture surface area of cold/room temperature Unlock. Evenly distribute it over the entire cell layer. Incubate the cells for 5 minutes at 37°C.
- Use a P1000 pipette to transfer the cells drop-wise into a 15 mL sterile conical tube add gently four volumes of pre-warmed, 37°C, **Neural Expansion-XF Medium**. (For example if 1 mL of Unlock is used, then add 4 mL of the medium to stop the reaction). Gently pipette up and down a few times to disperse the medium.
- Centrifuge cells at 200 x g for 5 minutes at room temperature.
- During the centrifugation step, remove the coating solution.
- Once coating solution has been removed add **Neural Plating–XF Medium** to the empty well. Add ~65% of the total well volume so that the culture vessel does not dry out (e.g. 6-well plate maximal volume is < 3 mL, add 1.95 mL of **Neural Plating–XF Medium**). Return the plate containing the **Neural Plating–XF Medium** to the incubator until ready for cell seeding.
- Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet with 1 mL of **Neural Plating–XF Medium** until they are in a single cell suspension.
- **Perform a cell count to ensure optimal seeding density**.
- Plate the resuspended cells drop-wise and evenly at a density ranging from 70,000 - 200,000 cells/cm² on pre-coated culture vessels.
- To ensure an even plating of **Human iPSC-Derived NSCs** gently rock the culture vessel back and forth and side to side several times.
- Incubate the cells at 37°C, 5% CO₂.
- **24 hours** after plating, replace half the volume of medium with fresh, pre-warmed, 37°C, **Neural Maintenance-XF Medium**.
- After a further 24 hours, again replace the medium fully with fresh, pre-warmed, 37°C, **Neural Maintenance-XF Medium**.
- To maintain a healthy neuronal and glial culture, replace half the volume of medium with fresh, pre-warmed, 37°C, **Neural Maintenance-XF Medium** every 3 days.
- It is important that the medium is pre-warmed and that the medium changes are conducted gently by pipetting to the side of the culture dish.
- Terminally differentiated neurons and glia can be maintained in culture until ready for experimentation.
System D
Spontaneous Differentiation

The generation of a mixed population of cortical neurons and glia without expansion of Human iPSC-Derived Neural Stem Cells

Using system D, iPSC-Derived Neural Stem Cells are plated into the final assay format and then spontaneously differentiated, using Neural Maintenance-XF Medium, from iPSC-Derived Neural Stem Cells to neuronal and glial cells.

Thawing, Plating, Spontaneous Differentiation and Maintenance of Human iPSC-Derived Neural Stem Cells

The day before thawing Human iPSC-Derived NSCs:

- Thaw Neural Plating–XF Medium overnight at 4°C.
- Prepare culture vessels: If seeding cells onto plastic culture vessels for the final assay format, then pre-coat the vessels with SureBond-XF. If seeding cells onto glass culture vessels for the final assay format, then pre-coat the vessels with SureBond+ReadySet.

On the day of thawing Human iPSC-Derived NSCs:

- Pre-warm all media and culture vessels to 37°C before use.
- To thaw cells – transfer the cells from storage by transporting the cells buried in dry ice. Remove the cells from dry ice and transfer the cells to a 37°C water bath.
- Quickly thaw the vial of cells in a 37°C water bath. Do not completely submerge the vial (only up to 2/3rd of the vial). Remove the vial before the last bit of ice has melted, after ~1-2 minutes.
- Do not shake the vial during thawing.
- Take the vial of cells to a biological safety cabinet, spraying the vial and hood thoroughly with 70% ethanol and wiping with an autoclaved paper towel before placing the vial in the hood.
- Once thawed, use a P1000 pipette to transfer the cells into a 15 mL sterile conical tube drop-wise add 10 mL of pre-warmed, 37°C, Neural Expansion-XF Medium. Gently wash the cryogenic vial with 1 mL of Neural Expansion-XF Medium. Transfer this to the 15 mL sterile conical tube containing the cells.
- Centrifuge cells at 200 x g for 5 minutes at room temperature.

**Important!**
Do not mix the cells vigorously. Avoid generating bubbles.
During the centrifugation step, remove the coating solution. Once the coating solution has been removed, add Neural Plating–XF Medium to the empty well. Add ~65% of the total well volume so that the culture vessel does not dry out (e.g., 6-well plate maximal volume is < 3 mL, add 1.95 mL of Neural Plating–XF Medium). Return the plate containing the Neural Plating–XF Medium to the incubator until ready for cell seeding.

Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet with 1 mL of Neural Plating–XF Medium until they are in a single cell suspension.

Perform a cell count to ensure optimal seeding density.

Plate the resuspended cells drop-wise and evenly at a density ranging from 70,000 - 200,000 cells/cm² on pre-coated culture vessels. To ensure an even plating of Human iPSC-Derived NSCs, gently rock the culture vessel back and forth and side to side several times.

Incubate the cells for 24 hours at 37°C, 5% CO₂.

24 hours after plating, replace half the volume of medium with fresh, pre-warmed, 37°C, Neural Maintenance-XF Medium. This will allow the cells to recover after thawing.

After a further 24 hours, again replace the medium fully with fresh, pre-warmed, 37°C, Neural Maintenance-XF Medium.

To maintain a healthy neuronal and glial culture, replace half the volume of medium with fresh, pre-warmed, 37°C, Neural Maintenance-XF Medium every 3 days.

It is important that the medium is pre-warmed and that the medium changes are conducted gently by pipetting to the side of the culture dish.

Terminally differentiated neurons and glia can now be maintained in culture until ready for experimental assays.

Got any questions? Need help with the protocol?
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