Culture of Human iPSC-Derived Neural Stem Cells in a 96-Well Plate Format

Protocol Supplement

This protocol supplement is to be used in addition to the **Neural Stem Cell Culture Protocol** for the culture and differentiation of **Human iPSC-Derived Neural Stem Cells** in 96-well plates. This protocol should be referenced after expansion and during preparation for the final assay format.

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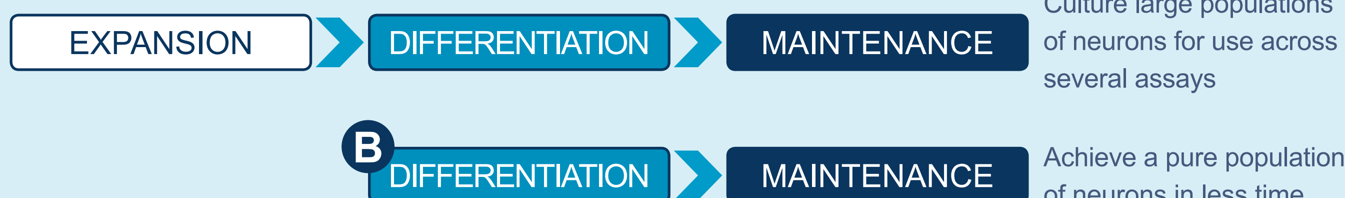
What's the Best System for Your Discovery?

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.

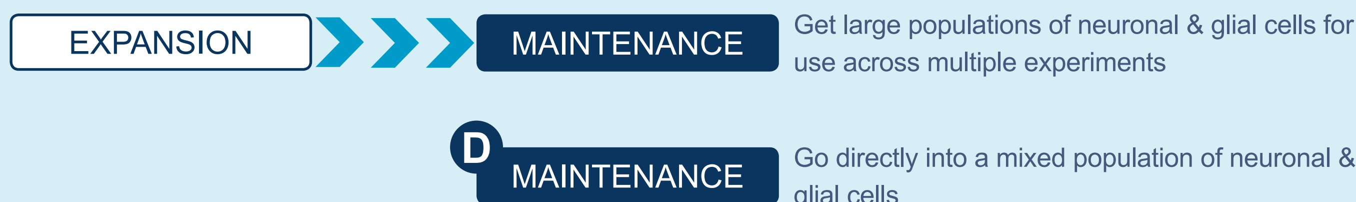
Using **system A**, **iPSC-Derived Neural Stem Cells** are expanded and then the synchronously differentiated into neurons, using **Neural Differentiation-XF Medium**.

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.

Preparation of Coating Reagents and Coating the 96-Well Plate

SureBond-XF Coating Solution (required for all systems for final plating on plastic and cultures >50,000/cm²)

- Upon receipt store **SureBond-XF** at **4°C**.
- Dilute the **SureBond-XF** stock solution (200x) 1:100 in **Neural Plating-XF Medium** to make a **2x working solution** e.g. **60 µL** in **6 mL**.
- Coat the surface of the 96-well plate with the **SureBond-XF** 2x working solution. We recommend coating 96-well plates at a volume of **100-150 µL per well** however, please optimize for your experiments.

SureBond+ReadySet Coating Solution (required for all systems for final plating on glass and for low density cultures (<50,000/cm²)):

- Upon receipt store **SureBond** at or below **-80°C** and store **ReadySet** at **4°C**.
- Thaw the **SureBond** coating solution overnight at **4°C**.
- Coat the surface of the 96-well plate with the **ReadySet** at a volume of 150 µL per well.
- Incubate at **37ºC** for **45 minutes**.
- Wash the plate thoroughly **four times** using an equal volume of sterile distilled H_2O (e.g. if 250 µL of **ReadySet**, use 250 µL sterile distilled H_2O). 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) 1:50 in **Neural Plating-XF Medium** to make 1x working solution e.g. **120 µL in 6 mL**.
- Coat the surface of the 96-well plate with the **SureBond** 1x working solution. We recommend coating at a volume of **150 µL per well**.
- Incubate for **1 hour** at **37ºC**.

Important!

Do not let the **SureBond** or **SureBond-XF** coating dry out before seeding the cells.

DO NOT wash the vessel after coating with **SureBond** or **SureBond-XF**

Systems A and C

Plating Human iPSC-Derived Neural Stem Cells onto a 96-Well Plate

- Pre-coat 96-well plate with **SureBond-XF** or **SureBond+ReadySet**.
- Thaw an aliquot of **Unlock**, **Neural Expansion-XF Medium** and **Neural Plating–XF Medium** overnight at **4ºC** before use and store at **4ºC**.
- Pre-warm all media and culture vessels to **37ºC** 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** 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 **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.
- Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet in **Neural Plating–XF Medium** until they are in a single cell suspension.
- **Perform a cell count to ensure optimal seeding density**.
- Quickly remove the coating solution (**SureBond-XF** or **SureBond+ReadySet**) from the pre-coated wells before plating the resuspended cells. It is recommended that approximately 50 µL of the **SureBond-XF** or **SureBond** is left in the well to ensure that the wells do not dry out.
- Plate the resuspended cells at **100 µL per well** (totally approximately 150 µL in each well) drop-wise and evenly at **no less than 50,000 cells/cm²** for healthy **Human iPSC-Derived NSCs** (ax0015, ax0016, ax0018 and ax0019) and **no less than 70,000 cells/cm²** for all other **Human iPSC-Derived NSCs**.
- 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% CO_2**.
- The day after plating follow either **system A** or **C**:

DIFFERENTIATION

System A – Synchronous Differentiation After Expansion

- **24 hours** after plating, replace **100 µL** of spent medium with fresh, pre-warmed, **37°C**, **Neural Expansion-XF Medium WITHOUT EGF** or **FGF2**.
- After a further **24 hours**, replace **125 µL** of the medium with **125 µL** of 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.
- 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**.
- Every **3 days** replace half the volume of medium with the same volume of **Neural Maintenance-XF Medium**.

System C - Spontaneous Differentiation After Expansion

- **24 hours** after plating, replace **100 µL** of spent medium with fresh, pre-warmed, **37°C**, **Neural Maintenance-XF Medium**.
- After a further **24 hours**, replace **75 µL** of the medium with **75 µL** with fresh, pre-warmed, **37°C**, **Neural Maintenance-XF Medium**.

MAINTENANCE

- 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.

Systems B and D

Thawing and Plating Human iPSC-Derived Neural Stem Cells onto a 96-Well Plate

The day before thawing **Human iPSC-Derived NSCs**:

- Thaw **Neural Plating–XF Medium** overnight at **4ºC**.
- Pre-coat 96-well plate with **SureBond-XF** or **SureBond+ReadySet**.

On the day of thawing **Human iPSC-Derived NSCs**:

- Pre-warm all media and culture vessels to **37ºC** 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 drop-wise into a 15 mL sterile conical tube containing **10 mL** of pre-warmed, **37°C**, **Neural Plating–XF Medium**. Gently wash the vial with **1 mL** of **Neural Plating–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 x *g*** for **5 minutes** at room temperature.
- Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet in **Neural Plating–XF Medium** until they are in a single cell suspension.
- **Perform a cell count to ensure optimal seeding density**.
- Quickly remove the coating solution (**SureBond-XF** or **SureBond+ReadySet**) from the pre-coated wells before plating the resuspended cells. It is recommended that approximately **30 µL** of the **SureBond-XF** or **SureBond** is left in the well to ensure that the wells do not dry out.
- Plate the resuspended cells at **120 µL per well** (totally approximately 150 µL in each well) drop-wise and evenly at **no less than 50,000 cells/cm^2** for healthy **Human iPSC-Derived NSCs** (ax0015, ax0016, ax0018 and ax0019) and **no less than 70,000 cells/cm^2** for all other **Human iPSC-Derived NSCs**.
- 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%**, **CO_2**.
- The day after plating follow either **system B** or **D**:

DIFFERENTIATION

System B – Synchronous Differentiation

- **24 hours** after plating, replace **100 µL** of spent 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 **125 µL** of the medium with **125 µL** of 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 **4 days**.
- Detection of a pure population of neurons is visual. Check the culture each day to observe neuronal development. A pure population will take at least 3 days to develop at 60% confluency, if the culture is very dense it may take longer. If the culture is too confluent a pure population will be difficult to obtain.
- 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**.

System D – Spontaneous Differentiation

- **24 hours** after plating, replace **100 µL** of spent 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 **75 µL** of the medium with **75 µL** fresh, pre-warmed, **37°C**, **Neural Maintenance-XF Medium**.

MAINTENANCE

- 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.

Got any questions? Need help with the protocol?
Contact Axol Technical Support at **support@axolbio.com**
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Notes

Notes

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