

AXOL

Cortical Excitatory Neurons

User guide

Version 3

Contents

Product Information	3
Process Overview	4
Preparation of Media.....	4
Neural Plating Media	4
Neural Maintenance Media.....	5
Neural Maturation Basal Media.....	5
Preparation of Stock Solutions	5
Preparation of BDNF Growth Factor (ax139800).....	6
Preparation of GDNF Growth Factor (ax139855)	6
Preparation of NeurOne Supplement A & B (ax0674-A & ax0674-B) (Light sensitive).....	6
Coating Cell Culture Vessel	7
PDL.....	7
SureBond-XF	7
Thawing and Plating axoCells Neural Stem Cells (Day 0)	7
Differentiation of axoCells Neural Stem Cells (Day 1-6).....	9
Maturation of young neurons (Day 7-13)	9
Long term culture of axoCells Cortical Excitatory Neurons (after Day 14).....	10

Updates to protocol:

Part	Change	Rationale	Previous Version	New Version
Product information	GDNF (ax139855) and dPBS (-/-) added, storage conditions for media components updated. Available product codes amended.	To align with updates to process	2	3
Differentiation of axoCells Neural Stem Cells	For 96 well plate format volume increased to 200µL from day 1	To align with updates to process	2	3

This protocol and its associated products are for research use only.

Products are not for diagnostic or therapeutic use and are not to be administered to humans or animals.

Product Information

This User Guide is for the following products			
Catalog. No.	Product Name	Format	Storage on Arrival
ax0004/ax0015/ax0219	axoCells Human iPSC-derived Neural Stem Cells	≥1.5 million cells/vial	Vapor phase LN storage

Media Components - Axol					
Catalog. No.	Product Name	Format	Stock Conc.	Storage on Arrival	Thawing Instructions
ax0031	Neural Maintenance Media	500ml	1x	Aliquot & store at -80°C until the given expiry date. Keep in the dark.	Overnight at 4°C and store at 4°C for 2 weeks
ax0033	Neural Plating Media	30ml	1x	Store at -80°C until the given expiry date. Keep in the dark.	Overnight at 4°C and store at 4°C for 2 weeks
ax0674-A	NeurOne Sup. A	1mL	50x	Store at -80°C until date of expiry. Keep in the dark.	Thaw at room temperature in the dark
ax0674-B	NeurOne Sup. B	1mL	50x		
ax139800 (10µg)	Recombinant Human Brain-Derived Neurotrophic Factor (BDNF)	10µg Lyophilized Powder	N/A	-20°C to -80°C	Reconstituted protein should be used immediately or stored in working aliquots at -20°C for 6 months
ax139855 (10µg)	Recombinant Human Glial Derived Neurotrophic Factor (GDNF)	10µg Lyophilized Powder	N/A	-20°C to -80°C	Reconstituted protein should be used immediately or stored in working aliquots at -20°C for 6 months
ax0053	SureBond-XF	1mL	200x	Store at 4°C until date of expiry.	N/A

Lot-specific information such as specifications and quality control details are stated in the Certificate of Analysis. Expiry dates for Axol- supplied components are stated on the label. Consult the manufacturer's guidelines for expiry dates of any additional reagents.

IMPORTANT!

Axol Neural Cell Culture Media DOES NOT contain antibiotics or antifungal agents. Axol does not recommend the use of antimicrobial agents such as penicillin, streptomycin and amphotericin. Antimicrobial agents should not be necessary if proper aseptic techniques are adopted.

Process Overview

	Thawing & Plating	Neuronal Differentiation	Neuronal Maturation	Assay-ready	Long Term Culture
Stages	Day 0	Day 1 - 6	Day 7 - 13	Day 14 -27	>Day 28
Basal Media	Neural Plating Media	Neural Maintenance Media	Neural Maturation Basal Media		
Supplement	Y-27632.2HCl	NeurOne Sup. A SureBond-XF	NeurOne Sup. B BDNF, GDNF, cAMP & Ascorbic acid SureBond-XF	BDNF, GDNF, cAMP & Ascorbic acid BDNF, GDNF, cAMP & Ascorbic acid	
Final Media	Complete Neural Plating Media	Differentiation Media	Complete Maturation Media	Long Term Culture Media	
Coating	PDL & SureBond-XF				

Preparation of Media

Prepare the above chemicals and media in a class II biosafety cabinet under sterile conditions.

Neural Plating Media

- Upon receipt, **Neural Plating Media** (ax0033) should be stored at -80°C protected from light until the given expiry date.
- When ready to use, thaw **Neural Plating Media** (ax0031) overnight at 4°C in the dark.
- Once thawed **Neural Plating Media** can be stored at 4°C for 2 weeks, protected from light.

Neural Maintenance Media

- Upon receipt, aliquot the **Neural Maintenance Media** according to your requirements.
- Store the aliquoted **Neural Maintenance Media** at or below -80°C protected from light.
- Frozen **Neural Maintenance Media** can be stored at -80°C until the given expiry date.
- When ready to use, thaw an aliquot of **Neural Maintenance Media** overnight at 4°C in the dark.
- Once thawed **Neural Maintenance Media** can be stored at 4°C for **2 weeks**, protected from light.

Neural Maturation Basal Media

Neural Maturation Basal Media is made according to the recipe below:

Components	Volume
Neurobasal™- A Media	500mL
B-27 supplement	10mL
GlutaMAX™	5mL
2-Mercaptoethanol (50 mM)	250µL

- Store Neural Maturation Basal Media at 4°C and use within two weeks.

Preparation of Stock Solutions

Reagent	Stock Conc.	Reconstitution	Storage after reconstitution
Y-27632.2HCl	10mM (1000x)	<i>Sterile tissue culture- grade water</i>	3 months, -20°C
Ascorbic Acid	20mM (100x)		
Human BDNF (ax139800-10µg)	20µg/mL (2000x)	0.05% HSA in D-PBS filter sterilised (0.2µm)	6 months, -20°C
Human GDNF (ax139855-10µg)			
cAMP	50mM (100x)		6 months, -80°C
NeurOne Supplement A (ax0674-A)	N/A	N/A	On arrival, thaw and aliquot into appropriate volumes, store at -80°C until date of expiry
NeurOne Supplement B (ax0674-B)			

Prepare all chemicals and growth factors in a Class II Biosafety Cabinet under sterile conditions.

- cAMP should be filter sterilized through a 0.22µM PES filter prior to use.

Preparation of BDNF Growth Factor (ax139800)

- Prepare a 0.05% solution of HSA in Dulbecco's Phosphate-Buffered Saline (D-PBS) (without calcium or magnesium). Filter sterilise the HSA prior to use (0.2µm).
- Prepare a stock solution of BDNF by resuspending the lyophilized powder in 0.05% HSA. The recommended concentrations and dilution factors are outlined in the table above.
- We do not recommend repeated freeze-thaws of reconstituted proteins. We would recommend dilution to stock concentration and aliquoting into single-use aliquots of appropriate volumes e.g. BDNF at 10µL of 20µg/mL for 20mL media. The growth factors can be aliquoted and stored at -20°C for up to 6 months.

Preparation of GDNF Growth Factor (ax139855)

- Prepare a 0.05% solution of HSA in Dulbecco's Phosphate-Buffered Saline (D-PBS) (without calcium or magnesium). Filter sterilise the HSA prior to use (0.2µm).
- Prepare a stock solution of GDNF by resuspending the lyophilized powder in 0.05% HSA. The recommended concentrations and dilution factors are outlined in the table above.
- We do not recommend repeated freeze-thaws of reconstituted proteins. We would recommend dilution to stock concentration and aliquoting into single-use aliquots of appropriate volumes e.g. GDNF at 10µL of 20µg/mL for 20mL media. The growth factors can be aliquoted and stored at -20°C for up to 6 months.

Preparation of NeurOne Supplement A & B (ax0674-A & ax0674-B) (Light sensitive)

- Each supplement should be aliquoted, depending on the customer's requirements (each supplement is diluted 1 in 50 when added to the basal medium).
- Thaw each supplement at room temperature in the dark.
- Once each 1mL supplement is thawed, mix the contents to ensure appropriate resuspension of the contents using a 1mL pipette, before aliquoting.
- Aliquots can be stored at -80 °C until date of expiry.

Coating Cell Culture Vessel

A variety of extracellular matrices (ECM) can be used for the cells, such as PEI, Polyornithine, Poly-D-lysine, Laminin or Matrigel.

Recommended well volumes for each plate type: 12-well = 500µL, 48-well = 300µL, 96-well = 100µL.

We would recommend following ECM combination for culturing Neural Stem Cells to Cortical Excitatory Neurons:

PDL

- Dilute the liquid poly-D-lysine 1:1 in D-PBS (-/-) to create a 0.05 mg/ml working solution (0.5x).
- Add 300µL per cm² of 0.5x PDL working solution (0.05mg/mL) to the culture vessel. Incubate for 60 mins at 37°C, remove the solution through aspiration and thoroughly rinse the surface twice with D-PBS (-/-).
- Remove D-PBS (-/-) and allow the surface to dry for at least two hours before proceeding onto **SureBond-XF** coating.

SureBond-XF

- Dilute the **SureBond-XF** stock solution (200x) in D-PBS (-/-) to make 1x working solution, e.g. 30µL in 6mL.
- Coat the culture surface with 300µL per cm² of the **SureBond-XF** 1x working solution.
- Incubate for at least 4 hours at 37°C, preferably overnight.
- Remove the **SureBond-XF** from the culture vessel immediately prior to plating of cells. Do not wash the culture vessel after coating with **SureBond-XF**.
- Do not let the **SureBond-XF** coating dry out before plating the cells.

Thawing and Plating axoCells Neural Stem Cells (Day 0)

Prepare **Complete Neural Plating Media** as per table below:

Reagent	Stock Concentration	Dilution	Final Concentration
Y-27632.2HCl	10mM	1:1000	10µM
Neural Plating Media (ax0033)	1x	N/A	1x

1. Pre-warm the **Complete Neural Plating Media** to 37°C and then add 8mL to a sterile 15mL conical tube.
2. To thaw the axoCells neural stem cells, transfer the vial of cells from nitrogen storage with the vial buried in dry ice.

3. Remove the vial from dry ice and transfer it immediately to 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.
4. Do not shake the vial during thawing.
5. Spray the vial with 70% ethanol before placing it in the cell culture hood.
6. Once thawed completely, use a P1000 pipette to gently transfer the cells into the 15mL tube containing the 8mL plating media from step 2.
7. Gently wash the cryovial with 1mL of the warm **Complete Neural Plating Media** and transfer this to the 15mL tube containing the cell suspension (10mL total).

IMPORTANT!

Do not mix the cells vigorously. Avoid generating bubbles while pipetting.

8. Centrifuge cells at **200 x g** for **5** minutes at room temperature.
9. Aspirate the supernatant carefully and gently resuspend the cell pellet in **1mL** of **Complete Neural Plating Media** until they become mostly a single-cell suspension.
10. Perform a cell count. We recommend seeding axoCells Cortical Excitatory Neurons at a density range between **100,000 – 200,000 cells per cm²** for mono-culture.
11. Prepare cell suspension in Complete Neural Plating Media.
12. Remove **SureBond-XF** coating solution from the culture vessel.

Timing is critical in this step. NSC attachment is compromised if the SureBond-XF is allowed to dry. Under typical conditions, the well will begin to dry within a few minutes after aspiration of the excess SureBond-XF solution.

13. Plate the resuspended cells dropwise and evenly.
14. Gently rock the culture vessel back and forth to ensure an even seeding density.
15. Incubate the cells in a humidified incubator at 37°C, 5% CO₂.

Differentiation of axoCells Neural Stem Cells (Day 1-6)

1. On day 1, prepare **Differentiation Media** as per the table below and use immediately.

Reagent	Stock Concentration	Dilution	Final Concentration
NeurOne Supplement A (ax0674-A)	50x	1:50	1x
Neural Maintenance Medium (ax0031)	1x	N/A	1x
Filter sterilise 0.22µM PES filter prior to the addition of Surebond-XF			
Surebond-XF	200x	1:400	0.5x

2. Pre-warm the **Differentiation Media** to 37°C.
3. Remove 90% of the spent plating medium and replace with warm **Differentiation Media**. If culturing cells in a 96-well plate, it is recommended to increase the total well volume to 200µL.
4. Perform a half media change with pre-warmed **Differentiation Media** every other day on days 3 and 5.

Maturation of young neurons (Day 7-13)

1. On day 7 prepare **Complete Maturation Media** as per the table below and use immediately.

Reagent	Stock Conc.	Dilution	Final Conc.
NeurOne Supplement B (ax0674-B)	50x	1:50	1x
Human BDNF (ax139800-10µg)	20µg/mL	1:2000	10ng/mL
Human GDNF (ax139855-10µg)			
cAMP	50mM	1:100	0.5mM
Ascorbic Acid	20mM	1:100	0.2mM
Neural Maturation Basal Medium	1x	N/A	1x
Filter sterilise 0.22µM PES filter prior to the addition of Surebond-XF			
Surebond-XF	200x	1:400	0.5x

2. Pre-warm the **Complete Maturation Media** to 37°C.
3. Replace the spent **Differentiation Media** with warm **Complete Maturation Media** by performing a 90% media change.
4. Perform half media changes with pre-warmed **Complete Maturation Media** every other day on days 9, 11 and 13.

Long term culture of axoCells Cortical Excitatory Neurons (after Day 14)

1. On day 15 prepare **Long Term Culture Media** as per table below:

Reagent	Stock Concentration	Dilution	Final Concentration
Human BDNF (ax139800-10µg)	20µg/mL	1:2000	10ng/mL
Human GDNF (ax139855-10µg)			
cAMP	50mM	1:100	0.5mM
Ascorbic Acid	20mM	1:100	0.2mM
Neural Maturation Basal Medium	1x	N/A	1x
Filter sterilise 0.22µM PES filter			

2. Pre-warm the media to 37°C.
3. Perform a half media change with pre-warmed **Long Term Culture Media**.
4. Perform half media changes with the pre-warmed **Long Term Culture Media** every other day from day 15.
5. The neurons are assay-ready post-day 20 with an assay window of at least 14 more days.

Axol Bioscience

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Contact our technical support team at support@axolbio.com