User Guide

Quick-Neuron™ Cholinergic - Human iPSC-derived Neurons

Catalog Numbers: CH-SeV-CW50065, CH-SeV-CW10149, CH-SeV-CW20300, CH-SeV-CW50023, CH-SeV-CW70067, CH-SeV-CW50025, CH-SeV-CW50113, CH-SeV-CW50114, CH-SeV-CW50115, CH-SeV-CW50137, CH-SeV-CW50147, CH-SeV-CW60130, CH-SeV-CW60231, CH-SeV-CW60236, CH-SeV-CW20026, CH-SeV-CW20090, or CH-SeV-CW10130

Introduction

Elixirgen Scientific's proprietary transcription factor-based technology allows rapid and reproducible differentiation of human iPSCs into neurons without sacrificing the purity of the cells. Our Quick-Neuron™ Cholinergic - Human iPSC-derived Neurons display typical neuronal morphology with outgrowing neurites and express neuronal markers, including the pan-neuronal marker tubulin beta 3 class III (TUBB3) and cholinergic marker choline acetyl-transferase (ChAT). When handled and maintained according to the instructions in this user guide, the iPSC-derived cholinergic neurons are viable long-term and are suitable for a variety of characterization and neurotoxicity assays.

Scale: Quick-Neuron™ Cholinergic - Human iPSC-derived Neurons are available in two sizes: (Small) 1 million viable cryopreserved cells and (Large) 5 x 1 million viable cryopreserved cells. The instructions outlined in this user guide are for seeding 1 million viable cells at approximately 5 x 10^4 cells/cm^2 into 2 wells of a 6-well plate (5 x 10^5 cells/well), 10 wells of a 24-well plate (1 x 10^5 cells/well), or 62 wells of a 96-well plate (1.6 x 10^4 cells/well).

Related Products: Quick-Neuron™ Cholinergic - mRNA Kit, Catalog Number: CH-mRNA
Quick-Neuron™ Cholinergic - SeV Kit, Catalog Number: CH-SeV
Quick-Neuron™ Cholinergic - Maintenance Medium, Catalog Number: CH-MM

Kit Contents

Upon receipt, immediately store the items at the indicated temperatures. Be especially careful to keep the frozen cells on dry ice until placing them in liquid nitrogen and avoid any temperature fluctuation and slight thawing.

Contents	Amount (Small Size)	Amount (Large Size)	Storage
Cryopreserved cells	>1 million viable cells, (1 vial, 500 µl)	5 x >1 million viable cells, (5 vials, 5 x 500 µl)	Liquid nitrogen
Component N1	2 x 830 µl	10 x 830 µl	-20°C or -80°C
Component A	38 µl	5 x 38 µl	-20°C or -80°C
Component P	14 µl	5 x 14 µl	-20°C or -80°C

Conditions of Use

This product is for research use only. It is not approved for use in humans or for therapeutic or diagnostic use.

Technical Support

For technical support, please contact us at cs@elixirgensci.com or call +1 (443) 869-5420 (M-F 9am-5pm EST).

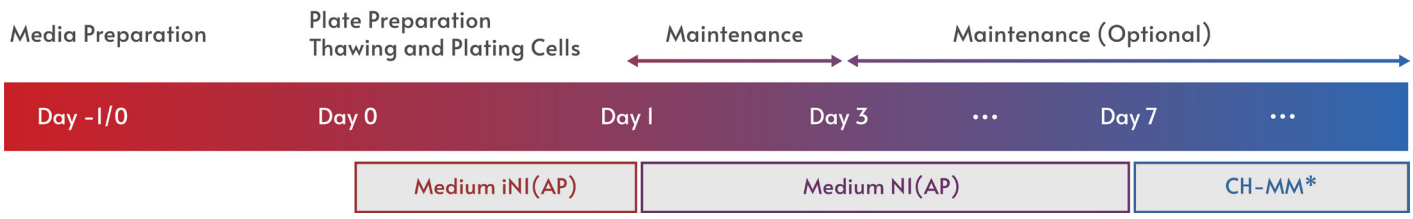
Last revised: January 26, 2021

Required Consumables

Item	Vendor	Catalog Number
(Optional) 6-well tissue-culture-treated polystyrene plate (e.g., Corning Costar Flat Bottom Cell Culture Plates)	Fisher Scientific	07-200-80
(Optional) 24-well tissue-culture-treated polystyrene plate (e.g., Corning Costar Flat Bottom Cell Culture Plates)	Fisher Scientific	07-200-740
(Optional) 96-well tissue-culture-treated polystyrene plate (e.g., Thermo Scientific™ 96 Well Black/Clear Bottom Plate)	Fisher Scientific	12-566-70
DMEM/F12	ThermoFisher	21331020
Neurobasal Medium	ThermoFisher	21103049
Glutamax (100x)	ThermoFisher	35050061
Penicillin-Streptomycin	ThermoFisher	15140122
Poly-L-Ornithine*	Sigma-Aldrich	P4957-50ML
Extracellular Matrix such as* • Laminin Mouse Protein, Natural, or • Geltrex Basement Membrane Matrix	ThermoFisher	23017015 or A15696-01
Phosphate-buffered saline (without Ca^{++} Mg^{++})	ThermoFisher	20012050
ROCK inhibitor Y27632	Selleckchem	S1049
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D8418

* Can be substituted with our Neuron Coating Solutions (Coating Materials B and C), Catalog Number: NCS.

Workflow



*Neurons are ready to be assayed by Day 2/3. However, there is enough media provided to maintain cultures until Day 7. From Day 7, users may maintain differentiated neurons in the maintenance medium best suited for their needs, though we recommend Quick-Neuron™ Cholinergic - Maintenance Medium, Catalog Number: CH-MM.

Media Preparation

10 mM ROCK inhibitor Y27632 (iROCK)

1. Dissolve 10 mg ROCK inhibitor Y27632 in 3.12 ml DMSO.
2. Make aliquots of a convenient volume (e.g., 100 µl).
3. This solution is hereafter referred to as iROCK and can be stored at -20°C.

0.002% Poly-L-Ornithine solution (ornithine)*

1. Take 700 µl 0.01% Poly-L-Ornithine solution and mix it with 2.8 ml PBS.
2. Store the resulting 0.002% Poly-L-Ornithine solution (hereafter referred to as ornithine) for up to 2 weeks at 4°C.

*Ornithine can be substituted with Coating Material B from our Neuron Coating Solutions, Catalog Number: NCS.

1 mg/ml laminin stock solution (laminin)*

1. Thaw Laminin Mouse Protein, Natural and chill PBS at 4°C or on ice.
2. Mix the Laminin Mouse Protein, Natural and PBS to make the 1 mg/ml stock solution (hereafter referred to as laminin).
    - Laminin concentration varies by lot, so use the number specified on the vial or CoA when making your calculations.
3. Make aliquots of a convenient volume (e.g., 35 µl) and store at -20°C.

*Laminin can be substituted with Coating Material C from our Neuron Coating Solutions, Catalog Number: NCS.

Medium N1

1. Prepare Medium N1 using the reagents listed in the table below.
    - Thaw Component N1 on ice for 20-30 minutes.
    - Thaw all other reagents at room temperature for 20-30 minutes.
    - Store Medium N1 for up to 2 weeks at 4°C. The leftover reagents can be discarded or saved for other uses.

Medium N1 Reagents	Volume
DMEM/F12	16 ml
Neurobasal Medium	16 ml
200 mM Glutamax (100x)	167 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	333 µl
Component N1	1 ml

Experiment Planning

Define the cell culture plate or dish format in advance and calculate the number of wells to be used for each format in advance. For example, you may use only a certain number of wells of a 96-well plate. The following section describes culture condition volumes per well as user needs may vary. When a 96-well plate is used, we recommend filling the edge wells of the plate with an aqueous medium instead of cells and culture medium. This will maintain humidity on the entire plate. If performing an image-based analysis with a 96-well plate, we have found plating approximately 1-1.5 x 10^4 cells/well to yield the best results. Please refer to the table below for plate formats and corresponding surface area of each well used for calculating reagents in the following sections.

	6-well plate	24-well plate	96-well plate
Approximate cell growth surface area per well	9.5 cm^2	1.9 cm^2	0.32 cm^2

Day 0

5-6 hr

Plate Preparation

IMPORTANT! Cells can be plated in 6-well, 24-well, and 96-well plates depending on the desired format. Refer to the table on the next page for the recommended volumes per well.

1. Thaw ornithine at room temperature for 20-30 minutes.
2. Vortex ornithine briefly and centrifuge it at a maximum speed for 1 minute.
3. Add ornithine to each well in the volume specified in the table.
4. Incubate the plate at 37°C, 5% CO_2 for at least 2 hours (or at 4°C overnight one day before plating).
5. Thaw laminin and chill PBS on ice for 20-30 minutes in the volumes calculated in the table. Add 1/100 volume of laminin to chilled PBS. Mix well.
    - All PBS washes should be done dropwise and with room temperature PBS. Chilled PBS is only for the coating step.
6. Aspirate the supernatant from each well and add PBS in the volume specified in the table.
7. Repeat Step 6.
8. Aspirate PBS from each well and add diluted laminin in the volume specified in the table.
9. Incubate the plate at 37°C, 5% CO_2 for at least 2 hours or until cells are ready for plating.

10. While the plate is incubating, prepare Medium iN1(AP) using the volume of Medium N1 indicated in the table calculated for the number of wells in use plus 1.1 ml for resuspension (i.e., 2 wells of a 6-well plate needs 3.3 ml Medium N1 (1.1 x 2 + 1.1), 10 wells of a 24-well plate needs 4.4 ml Medium N1 (0.33 x 10 + 1.1) for this step).
    - Thaw/warm Medium N1, Component A, Component P, and iROCK at room temperature for 20-30 minutes.
    - Add 1/1000 of Component A, 1/2000 of Component P and 1/1000 of iRock to Medium N1.
    - Keep the rest of Medium N1, Component A, and Component P at 4°C for later use.
11. After the laminin incubation, aspirate most, but not all, of the supernatant and add PBS in the volume specified in the table. Add the PBS dropwise to each well.
12. Pipet out most of the PBS, but not all, from each well.
13. Add PBS dropwise in the volume specified in the table.
14. Repeat step 12.
15. Add Medium iN1(AP) to each well in the volume specified in the table.
16. Incubate the plate at 37°C, 5% CO_2 until cells are ready for plating.

Reagents	Corresponding Steps	Required volume per well		
		6-well plate	24-well plate	96-well plate
Ornithine	1, 3	1.5 ml	300 µl	50 µl
PBS for laminin dilution	5	1.65 ml	330 µl	55 µl
PBS	6, 7, 11, 13	2 ml	500 µl	100 µl
Diluted laminin	8	1.5 ml	300 µl	50 µl
Medium N1 for Medium iN1(AP)	10	1.1 ml	330 µl	55 µl
Medium iN1(AP)	15	500 µl	200 µl	33 µl

Thawing Cells

1. Warm Medium N1 at room temperature for 20-30 minutes.
2. Take out the vial of frozen cells from the liquid nitrogen storage tank.
3. Incubate the cryovial in a water bath set at 37°C (do not submerge the cap) until most of the content is thawed but a small ice crystal remains (~2 minutes).
4. Wipe the vial with a dry paper towel. Spray the vial with 70% ethanol and place it inside a biosafety cabinet.
5. Transfer 4.5 ml room temperature Medium N1 to a new 15 ml conical tube.
6. Set a P1000 pipette to 1 ml but take approximately 500 µl Medium N1 from the 15 ml conical and add it to the cryovial dropwise at 1 drop per 1-2 seconds.
    - **IMPORTANT!** Use the same pipette tip for Steps 6-10.
7. Gently pipet the cell suspension up and down once.
8. Gently transfer all of the cell suspension to the 15 ml conical tube prepared in Step 5.
9. Take 1 ml of the cell suspension from the conical tube and add it to the original cryovial and pipet up and down 2-3 times and then transfer the whole contents back to the same 15 ml conical tube.
10. Mix the contents in the conical tube by gently pipetting cell suspension up and down 3 times.
11. Centrifuge the cell suspension in the 15 ml conical tube at 200xg for 4 minutes.
12. Use an aspirator to remove most of the supernatant from the conical tube, leaving a small volume of the supernatant (<50 µl) to cover the pellet.
13. Tap the side of the conical tube up to 10 times to break up the cell pellet.
14. Add 1 ml room temperature Medium iN1(AP) to the conical tube using a P1000 pipettor and pipet up and down no more than 2-3 times.

Plating Cells

1. Count the cells to determine the volume of cell suspension needed for the chosen number of wells and include a 10% buffer for cell number and volume (e.g., for a 24-well plate scenario, a total of 1.1 x 10^6 cells to plate 1 x 10^5 cells in each of the 10 wells). If the volume of the cell suspension needs to be adjusted, centrifuge the required volume of cell suspension at 200xg for 4 minutes, remove the supernatant, and resuspend the pellet with Medium iN1(AP) to reach the multiplied volume of cell suspension with the number of wells.
2. Add cell suspension to the center of each well. Since each well already has Medium iN1(AP), the total volume of the medium in each well is indicated in the table at the top of the next page.

	Recommended amounts		
	6-well plate	24-well plate	96-well plate
Viable cells/well	5 x 10^5 cells	1 x 10^5 cells	1.6 x 10^4 cells
Required volume of cell suspension • (Volume of cell suspension/well) + 10% buffer	550 µl	110 µl	19 µl
Volume of cell suspension distributed/well	500 µl	100 µl	17 µl
Total volume/well • Medium iN1(AP) + cell suspension	1 ml	300 µl	50 µl

3. Incubate the plate at 37°C, 5% CO_2 overnight.

Day 1

<1 hr

Maintenance

1. Warm Medium N1(AP) using the volumes calculated for the number of wells indicated in the table below.
   - Warm Medium N1, Component A, and Component P at room temperature for 20-30 minutes.
   - This will produce enough Medium N1(AP) for 1 week of culture. Store leftover Medium N1(AP) at 4°C for subsequent medium changes throughout the week (e.g., on days 3 and 5).

Medium N1(AP) Reagents	Required volume per well		
	6-well plate	24-well plate	96-well plate
Medium N1	4.4 ml	1.76 ml	330 µl
Component A	4.4 µl	1.76 µl	0.33 µl
Component P	2.2 µl	0.88 µl	0.165 µl

2. Pipet out the old medium from each well and add PBS to each well along its wall according to the table below.
   - **IMPORTANT!** To avoid lifting cells, leave a small volume of the old medium (i.e., just enough to cover the surface of the well). PBS should be applied along the wall of the well very slowly.

	Required volume per well		
	6-well plate	24-well plate	96-well plate
PBS	2 ml	500 µl	100 µl

3. Pipet out the PBS from each well and add Medium N1(AP) to each well along its wall according to the table below.

	Required volume per well		
	6-well plate	24-well plate	96-well plate
Medium N1(AP)	2 ml	800 µl	150 µl

4. Incubate the culture at 37°C, 5% CO_2 for 2 days.

Day 3+

<1 hr

Maintenance (Optional)

IMPORTANT! The neurons are ready to be assayed as early as Day 2/3. However, if you wish to maintain the cultures longer for more mature neurons there is enough media provided to culture until Day 7.

1. Warm Medium N1(AP) at room temperature for 30 minutes.
2. Pipet out half of the old medium from each well.
3. Slowly add fresh Medium N1(AP) equivalent to the volume removed.
4. Incubate the cultures at 37°C, 5% CO_2 for 2-3 days.
5. Repeat Steps 1-4 until Day 7 or until ready to assay.

Day 7

Assay or Continuous Maturation

From Day 7, users may maintain differentiated neurons in the maintenence medium best suited for their needs, though we recommend Quick-Neuron™ Cholinergic - Maintenence Medium, Catalog Number: CH-MM. Differentiation into cholinergic neurons can be confirmed with anti-TUBB3 (tubulin beta 3 class III, a global marker for neurons) and anti-ChAT (choline acetyl-transferase, a cholinergic neuron marker) antibodies.

Appendix

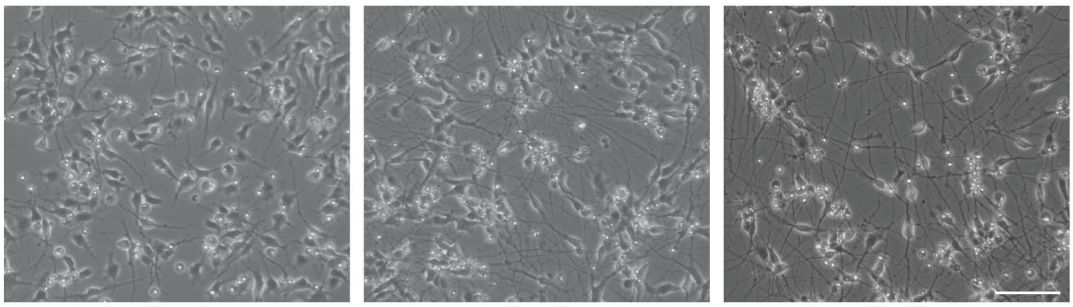


Figure 1. Representative images of Quick-Neuron™ Cholinergic - Human iPSC-derived Neurons on days 1-3 post-thaw (scale bar = 100 µm).

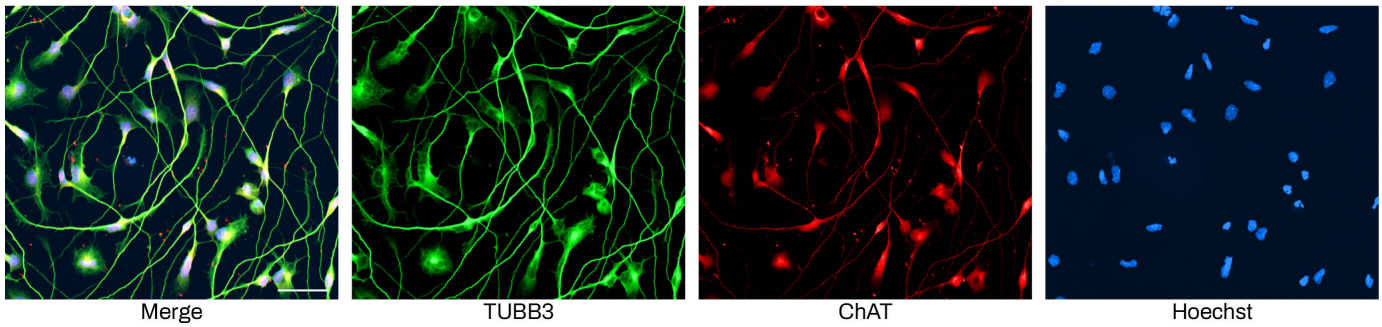


Figure 2. Immunofluorescent staining of Quick-Neuron™ Cholinergic - Human iPSC-derived Neurons shows typical neurite growth and expression of the pan-neuronal marker TUBB3 and the cholinergic neuron-specific marker ChAT on day 3 post-thaw (scale bar = 100 µm). Staining conditions: Anti-β-III tubulin monoclonal antibody (R&D Systems, Catalog Number: MAB1195, 1:250 dilution) in combination with a secondary antibody (Invitrogen, Catalog Number: A32723 Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, AlexaFluor Plus 488, 1:500 dilution). Anti-ChAT primary antibody (EMD Millipore, Catalog Number: AB143, 1:200 dilution) in combination with a secondary antibody (Invitrogen, Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594, 1:500 dilution). Nuclei were counterstained with Hoechst 33324.