User Guide

Quick-Neuron™ Cholinergic - SeV Kit (Large)

Catalog Number: CH-SeV-L

Introduction

The Quick-Neuron™ Cholinergic - SeV Kit (Large) facilitates rapid and efficient differentiation of human iPS or ES cells into cholinergic neurons in just 6 days. Our proprietary transcription factor-based stem cell differentiation method uses Sendai virus to produce highly pure populations of neurons without a genetic footprint. Quick-Neuron™ Cholinergic differentiated cell cultures display typical neurite outgrowth and express a variety of neuronal markers, such as the pan-neuronal marker tubulin beta 3 class III (TUBB3) and the cholinergic marker choline acetyl-transferase (ChAT). When handled and maintained according to the instructions in this user guide, cholinergic neurons are viable long-term and are suitable for a variety of characterization and neurotoxicity assays.

Scale: The Quick-Neuron™ Cholinergic - SeV Kit (Large) contains a set of reagents for use with a total of 6 wells of a 6-well plate.

Related Products:
Quick-Neuron™ Cholinergic - SeV Kit (Small), Catalog Number: CH-SeV-S
Quick-Neuron™ Cholinergic - mRNA Kit, Catalog Number: CH-mRNA
Quick-Neuron™ Cholinergic - Human iPSC-derived Neurons, Catalog Number: CH-SeV-CW
Quick-Neuron™ Cholinergic - Maintenance Medium, Catalog Number: CH-MM

Kit Contents

Upon receipt, store the reagents at the temperatures indicated in the table below. All reagents are shipped on dry ice.

Reagents	Volume	Storage
QN-SeV (undiluted)*	110 µl	-80°C
Component N1	2x 830 µl	-20°C or -80°C
Component A	2x 38 µl	-20°C or -80°C
Component P	2x 14 µl	-20°C or -80°C

***IMPORTANT!** This kit contains Sendai virus (SeV) particles that are active at 33°C and become inactive at 37°C. SeV is non-pathogenic in humans, and humans are not natural hosts of SeV; however, Biosafety Level 2 (BSL-2) containment is required for its use. Please use a biological safety cabinet, laminar flow hood, and proper personal protective equipment in order to prevent mucosal exposure. More information on BSL-2 guidelines can be found at www.cdc.gov/labs/BMBL.html.

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 22, 2021

Required Consumables

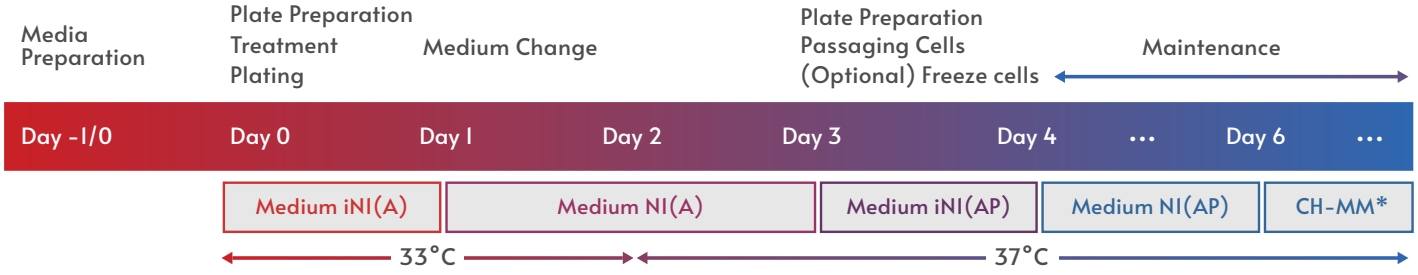
Item	Vendor	Catalog Number
6-well tissue-culture-treated polystyrene plate (e.g., Corning Costar Flat Bottom Cell Culture Plates)	Fisher Scientific	07-200-80
(Optional) 24-well tissue-culture-treated polystyrene plate (e.g., Corning Costar Flat Bottom Cell Culture Plates)	Fisher Scientific	07-200-740
(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
iMatrix-511 silk	Elixirgen Scientific	NI511S
TrypLE Select Enzyme (1X)	ThermoFisher	12563011
0.02% EDTA in DPBS	Sigma-Aldrich	E8008-100ML
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
STEM-CELLBANKER	AMSBIO	11890

Source hPSC Culture Conditions

The Quick-Neuron™ Cholinergic - SeV Kit (Large) gives the best differentiation results when source hPSCs have been maintained in StemFit® Basic04, StemFlex™ Medium, or other similar culture media which enable the maintenance of cultures by single-cell passaging. This protocol also assumes that the source hPSCs are cultured in 2-3 35-mm culture dishes or 2-3 wells of a 6-well plate. If iMatrix-511 silk is routinely used as a coating substrate, prepare 2-3 culture dishes or wells precoated with 0.25 µg/cm² iMatrix-511 silk diluted in 2 ml chilled PBS for this kit.

- The protocols and reagents for StemFit® Basic04 and iMatrix-511 silk culture conditions are available at Elixirgen Scientific (Catalog Numbers: ASB04, NI511S).
- Differentiation should not be performed until the cells are at least 14 days post-thaw.
- For optimal differentiation, hPSC confluency should be around 50% to 70%. Do not use wells more than 90% confluent.

Workflow



*From Day 6, 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.

0.5X TrypLE Select with EDTA (Solution D1)

1. Mix 1.5 ml TrypLE Select Enzyme (1X) with 1.5 ml 0.02% EDTA in DPBS.
2. This mixture (hereafter referred to as Solution D1) can be stored at 4°C for 2 weeks.

0.002% Poly-L-Ornithine solution (ornithine)

1. Take 2 ml 0.01% poly-L-ornithine solution and mix it with 8 ml PBS.
2. Store the 0.002% poly-L-ornithine solution (hereafter referred to as ornithine) at 4°C for up to 2 weeks.

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., 90 µl) and store them at -20°C.

Medium N1(A)

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

Medium N1(A) Reagents	Volume
DMEM/F12	24 ml
Neurobasal Medium	24 ml
200 mM Glutamax (100x)	250 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	500 µl
Component N1	1.5 ml
Component A	50.3 µl

Day 0

~5 hr

Plate Preparation

1. Prepare diluted iMatrix-511 silk by mixing together the following components in a 15 ml conical tube.
    - Keep iMatrix-511 silk on ice and make sure chilled PBS is used for this mixture.

Diluted iMatrix-511 silk Reagents	Volume
iMatrix-511 silk	44.6 µl
Chilled PBS	13.5 ml

1. Add 2 ml diluted iMatrix-511 silk to each well of a new 6-well plate.
2. Incubate the plate at 37°C, 5% CO_2 for at least 2 hours (or 4°C overnight one day before Day 0).
3. Aspirate the supernatant from each well and add 2 ml PBS.
4. Incubate the plate at 37°C, 5% CO_2 until hPSCs are ready for plating.

Treatment

IMPORTANT! We do not recommend using only 1 well of source hPSC as harvesting enough cells from 1 well likely means that the well was over confluent and not ideal for differentiation.

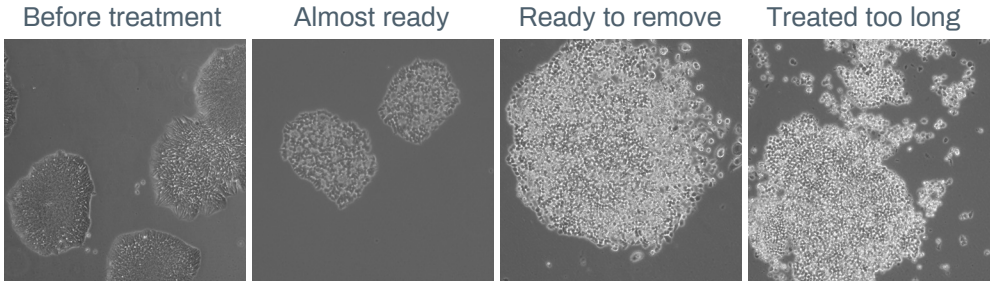
1. Determine the number of wells required to get 3.3 x 10^6 cells from the source hPSC 6-well plate.
2. Prepare Medium iN1(A) by mixing together the following components in a 15 ml conical tube.
    - Warm Medium N1(A) and iROCK at room temperature for 20-30 minutes.
    - The rest of Medium N1(A) should be stored at 4°C for later use.

Medium iN1(A) Reagents	Req medium vol based on # of wells of a 6-well plate	
	2 wells	3 wells
Medium N1(A)	9.4 ml	10.5 ml
iROCK	9.4 µl	10.5 µl

3. Referring to the table below, prepare the required volume of hPSC maintenance medium with iROCK in a new 15 ml conical tube. Mix well and allow to warm at room temperature for 20-30 minutes.

Reagents for hPSC treatment	Req medium vol based on # of wells of a 6-well plate	
	2 wells	3 wells
hPSC maintenance medium	3 ml	4.5 ml
iROCK	3 µl	4.5 µl

4. Aspirate old medium from hPSC culture and add 1.5 ml of hPSC maintenance medium with iROCK to each well.
5. Incubate the culture at 37°C, 5% CO_2 for 1 hour before harvesting cells.
    - This is to decrease cell death on Day 1 and minimize the loss of cells.
    - During the incubation, start thawing QN-SeV on ice and Solution D1 at room temperature.
6. Aspirate old medium from hPSC culture and add 2 ml PBS to each well being harvested.
7. Rock the dish/plate 3 times, aspirate PBS from the culture, and add 300 µl Solution D1 to each well to begin cell dissociation treatment.
    - Keep the rest of Solution D1 at 4°C for its use on Day 3.
8. Incubate the culture at 37°C, 5% CO_2 for 5 minutes. If all the cells are not rounded under a microscope, incubate at 37°C, 5% CO_2 for up to 5 more minutes in 1-2 minute increments (see images on next page).



10. Carefully pipet out Solution D1 from the culture using a P1000 pipettor and add 1 ml Medium iN1(A) to each well.
    - Follow Steps 10-12 one well at a time if multiple wells are used.
11. Disperse the medium over the bottom surface of the well by pipetting 8-15 times to detach cells.
12. Using the same pipette tip, collect the cell suspension in a 15 ml conical tube.

IMPORTANT! In this protocol, users will treat hPSCs with QN-SeV in a tube and then plate the cells onto 6 wells with 1 ml Medium iN1(A) (0.5 x 10^6 cells) per well. However, we recommend preparing a suspension of 6.6 ml to avoid insufficiency. First, QN-SeV should be mixed with 580 µl of a dense cell suspension to increase the chance that QN-SeV finds its host cells. After 10 minutes incubation at 33°C, the total volume will be brought up to 6.6 ml with Medium iN1(A). Cell count may vary based on cell health, the method, and instrument used for cell counting.

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13. Count cells to determine the volume of cell suspension needed for 6 wells and include a 10% buffer (a total of 3.3 x 10^6 cells to plate 0.5 x 10^6 cells in each of the 6 wells). Transfer the determined volume of the cell suspension into a 15 ml conical tube. Adjust the volume to 580 µl with Medium iN1(A). If the volume of the cell suspension needed to get 3.3 x 10^6 cells exceeds 580 µl, centrifuge the required volume of cell suspension at 200xg for 4 minutes, remove the supernatant, and resuspend the pellet into 580 µl Medium iN1(A).

IMPORTANT! Before adding QN-SeV, ensure that it is fully thawed. Do not centrifuge, vortex, or mix SeV with a pipettor; SeV is highly sensitive to physical stress.

14. Add 105 µl QN-SeV to the hPSCs and mix them by tapping with finger 2-3 times. Cap the tube loosely to allow gas exchange.
15. Incubate the cell suspension at 33°C, 5% CO_2 for 10 minutes with intermittent mixing, by finger tapping, every 2 minutes.

Plating

1. Bring up the volume of cell suspension to 6.6 ml with Medium iN1(A) and mix 2-3 times with serological pipet.
2. Aspirate PBS from only one coated well at a time and add 1 ml of cell suspension to each well. Most of the PBS should be aspirated but not completely to prevent the coated wells from drying before adding the cell suspension. Likewise, the cell suspension should be added to the well immediately after PBS is removed. Handle one well after another.
3. Move the plate in 5 cycles of quick back-and-forth and side-to-side motions to evenly distribute treated cells in the cultures.
4. Incubate the cultures at 33°C, 5% CO_2 overnight.

Day 1

<1 hr

Medium Change

1. Warm Medium N1(A) at room temperature for 20-30 minutes.
2. Pipet out most of the old medium from each well using a P1000 pipettor and add 1 ml Medium N1(A).
3. Incubate the cultures at 33°C, 5% CO_2 overnight.

Day 2

<1 hr

Medium Change and Temperature Shift

1. Warm Medium N1(A) at room temperature for 20-30 minutes.
2. Pipet out most of the old medium from each well using a P1000 pipettor and add 1 ml Medium N1(A).
3. Incubate the cultures at 37°C, 5% CO_2 overnight.

New Plate Preparation

IMPORTANT! Cells can be plated on 6-well, 24-well, or 96-well plates depending on the desired format. This kit can accommodate replating to all wells of either a 6-well, a 24-well, or a 96-well plate. Refer to the tables at the bottom of this page for the recommended volumes. Please note that the volumes are per well in Table A and per plate in Table B. Surplus cells can be frozen following the instructions in the Appendix.

1. Thaw ornithine at room temperature for 20-30 minutes.
2. Vortex ornithine briefly and centrifuge it at a maximum speed for a few seconds.
3. Add ornithine to each well of a new plate in the volume specified in Table A.
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 specified amounts of PBS on ice for 20-30 minutes. Add laminin to chilled PBS in the volume specified in Table B. 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 Table A.
7. Repeat Step 6.
8. Aspirate PBS from each well and add diluted laminin according to Table A.
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 volumes indicated in Table B.
    - Thaw/warm Medium N1(A) and iROCK at room temperature for 20-30 minutes.
    - Thaw Component P on ice for 20-30 minutes.
    - Keep the rest of Medium N1(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 Table A. Add the PBS dropwise to each well.
12. Aspirate most, but not all of, the PBS and add Medium iN1(AP) in the volume specified in Table A.
13. Incubate the plate at 37°C, 5% CO_2 until cells are ready for plating.

Table A. Recommended volumes per well for different plate formats.

Reagents	Required volume per well		
	6-well plate	24-well plate	96-well plate
Ornithine	1.5 ml	300 µl	50 µl
PBS	2 ml	500 µl	100 µl
Diluted laminin	1.5 ml	300 µl	50 µl
Medium iN1(AP)	500 µl	200 µl	35 µl

Table B. Recommended volumes per plate for different plate formats.

	Reagents	Required volume per plate		
		6-well plate	24-well plate	96-well plate
Diluted laminin	Laminin	100 µl	80 µl	53 µl
	Chilled PBS	10 ml	8 ml	5.3 ml
Medium iN1(AP)	Medium N1(A)	15.2 ml	13 ml	12 ml
	Component P	7.6 µl	6.5 µl	6 µl
	iROCK	15.2 µl	13 µl	12 µl

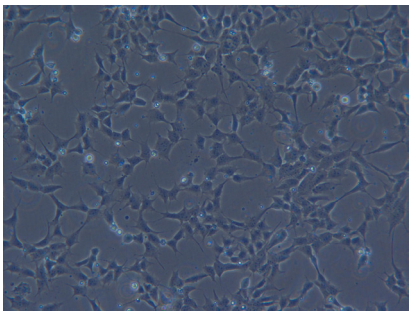
Passaging Cells

IMPORTANT! For the following steps, gently pipet and add solutions. Differentiating cells are delicate and should be handled with great care.

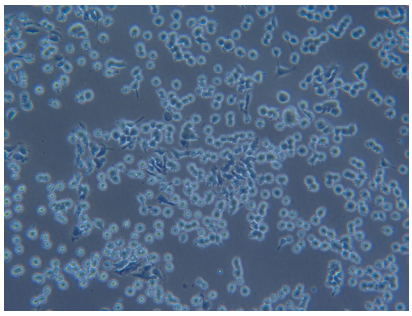
1. Warm Solution D1 at room temperature for at least 1 hour before use.
2. Working one well at a time, pipet out the old medium from each well using a P1000 pipettor and add 1 ml PBS and gently rock the plate.
3. Working one well at a time, pipet out the PBS from each well using a P1000 pipettor and add 300 µl Solution D1.
4. Rock the plate 3 times to spread the Solution D1 evenly.
5. Incubate the cultures at 37°C, 5% CO_2 for 3 minutes.
6. Working one well at a time, gently pipet out Solution D1 from each well using a P200 pipettor and add 750 µl Medium iN1(AP) to each well along the wall of the well.

IMPORTANT! Steps 7-9 are critical. Perform these steps one well at a time. Refer to the images below to successfully manage cell treatment and dissociation.

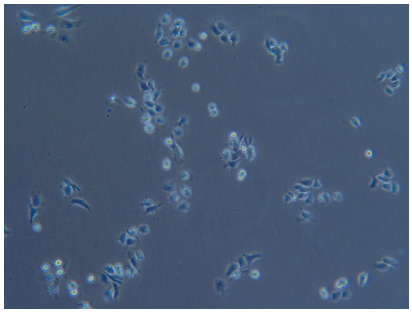
Before Solution DI treatment



During Solution DI treatment



After dissociation



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7. Working one well at a time, disperse the medium quickly over the bottom surface of the well by pipetting 6-8 times to detach cells using a P1000 pipettor.
8. Observe cells and/or cell aggregates floating in the well under a microscope. It is normal that 10-20% of cells remain attached to the well bottom after pipetting. The clusters of cells are not supposed to be lifted. Do not attempt to detach all of the cells remaining on the well bottom.
9. Gently pipet the cell suspension up and down in the well up to 5 times to break the cell aggregates using a P1000 pipettor. Excessive pipetting can damage the already-suspended neuronal cells.
10. Collect all of the cell suspension from each well in a tube using the same P1000 pipette tip.
11. Count cells and determine viability.
12. Prepare specified amounts of a 1 x 10^6 live cells/ml cell suspension using Medium iN1(AP) based on the table below.
    - If there are leftover cells, freeze the cells down by following instructions in the Appendix after plating cell suspensions to the new plate. Keep the leftover cells on ice until freezing.
13. Add cell suspension to the center of each well. Since each well already has Medium iN1(AP), the total volume of medium each well is indicated in the table below.

	Recommended amounts		
	6-well plate	**24-well plate**	**96-well plate**
Viable cells/well	5 x 10^5 cells	1 x 10^5 cells	1.5 x 10^4 cells
Req vol of cell suspension (1 x 10^6 viable cells/ml) • (Vol of cell suspension/well x # of wells) + 10% buffer	3.3 ml	2.64 ml	1.6 ml
Vol of cell suspension/well	500 µl	100 µl	15 µl
Total volume/well • Medium iN1(AP) + cell suspension	1 ml	300 µl	50 µl

14. Incubate the cultures at 37°C, 5% CO_2 for 1 hour.
15. Observe each well under the microscope to make sure that the cells are attached to the well.
16. Incubate the cultures at 37°C, 5% CO_2 overnight.

Days 4

~1 hr

Maintenance

1. Prepare Medium N1(AP) using the volumes indicated in the table below.
   - Warm Medium N1(A) at room temperature for 20-30 minutes.
   - Keep Component P on ice for 20-30 minutes.

Reagents	Required volume for each format		
	6-well plate	24-well plate	96-well plate
Medium N1(A)	26.4 ml	42 ml	32 ml
Component P	13.2 µl	21 µl	16 µl

2. Pipet out the old medium from each well using a P1000 pipettor and add Medium N1(AP) according to the table below.

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

3. Incubate the cultures at 37°C, 5% CO_2 for 2 days.

Day 6

Assay or Continuous Maturation

Differentiated neurons can be observed on Day 4. For more mature neurons, we recommend culturing cells until Day 6. From Day 6, 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. Differentiation into cholinergic neurons after using the Quick-Neuron™ Cholinergic - SeV Kit can be confirmed with anti-TUBB3 (tubulin beta 3 class III, a global marker for neurons) and anti-ChAT (choline acetyltransferase, a cholinergic neuron marker, a cholinergic neuron marker) antibodies.

Appendix

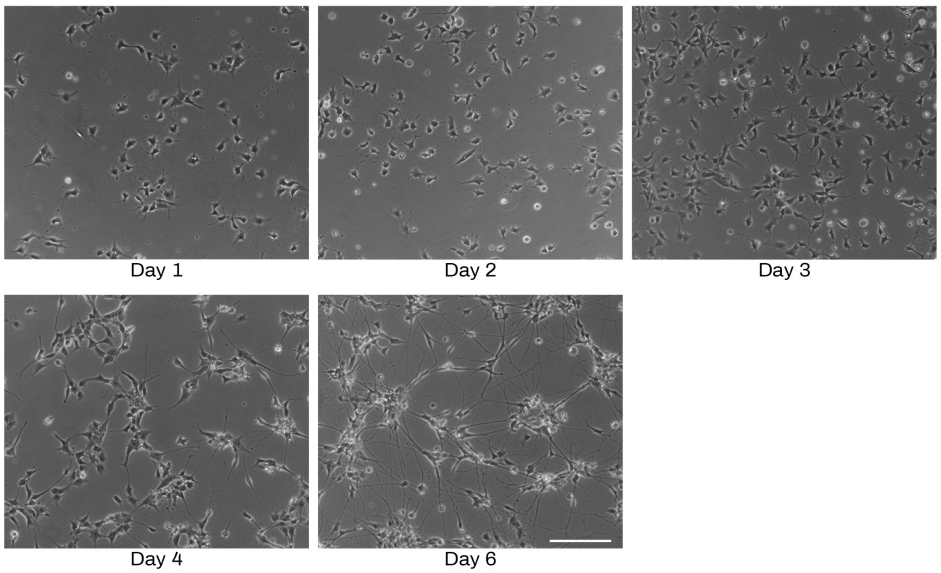

Figure 1. Representative images of Quick-Neuron™ Cholinergic - SeV Kit cell cultures on days 1-6 post-differentiation (scale bar = 100 µm).

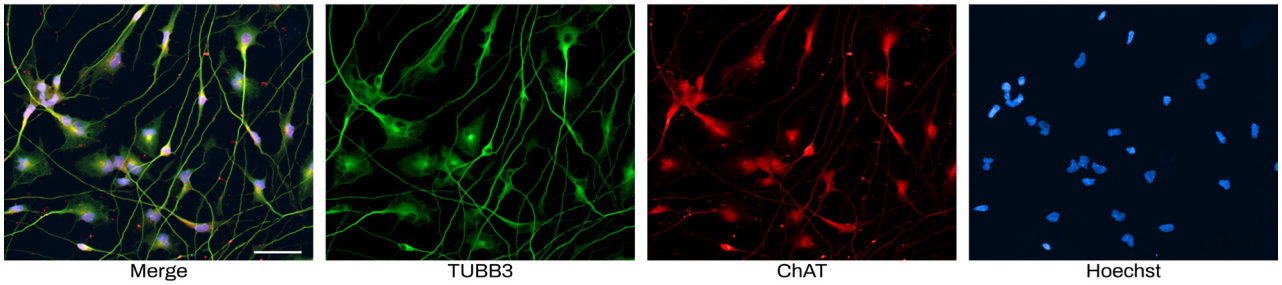

Figure 2. Immunofluorescent staining of Quick-Neuron™ Cholinergic - SeV Kit cell cultures shows typical neurite growth and expression of the pan-neuronal marker TUBB3 and the cholinergic neuron-specific marker ChAT on day 6 post-differentiation (scale bar = 100 µm). Staining conditions: Anti-β-III tubulin 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.

Freezing cells down on Day 3

Note. After thawing frozen cells, approximately 50% of viable cells will be recovered.

1. Determine the volume of the cell suspension and number of cryovials needed to freeze 0.1 ~ 2 x 10^6 cells per cryovial.
2. Centrifuge at 200 xg for 4 min.
3. While waiting for the centrifugation, label each cryovial. We recommend writing the name of the iPSC line used, the type of neurons, harvesting day and date, and the number of cells in the vial.
4. Aspirate the supernatant and resuspend the pellet with 500 µl/vial STEM-CELLBANKER..
5. Distribute 500 µl of the suspension to each cryovial.
6. Make sure that the caps are closed tightly and transfer the cryovials into a Mr. Frosty Freezing Container. Make sure that Mr. Frosty contains 250 ml isopropanol.
7. Loosely close the lid of Mr. Frosty with cryovials, put it into a -80°C freezer and leave it overnight or a few days.
8. Transfer the cryovials into a liquid nitrogen storage tank.
9. Follow the thawing process in the user guide of Quick-Neuron™ Cholinergic - Human iPSC-derived Neurons, Catalog Number: CH-SeV-CW.