User Guide

ELIXIRGEN SCIENTIFIC

Quick-Neuron™ Cholinergic - SeV Kit (Small)

Catalog Number: CH-SeV-S

Introduction

The Quick-Neuron™ Cholinergic - SeV Kit (Small) facilitates rapid and efficient differentiation of human iPS or ES cells into cholinergic neurons in just 7 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 (Small) contains a set of reagents for use with a total of 4 wells of a 24-well plate.

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

Contents

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

Contents	Volume	Storage	Thaw
QN-SeV-P*	55 µl	-80°C	On ice
Component N1	830 µl	-20°C or -80°C	On ice or 4°C
Component A	80 µl	-20°C or -80°C	Room Temperature
Component P	50 µl	-20°C or -80°C	Room Temperature
Coating Material A	15.7 µl	-20°C or -80°C	Room Temperature

This kit contains iMatrix-511 silk (Nippi, Inc.).

***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.

Condition 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 refer to the FAQ on our website.
You may also contact us at cs@elixirgensci.com or call +1 (443) 869-5420 (M-F 9am-5pm EST).

Required Consumables

Item	Vendor	Catalog Number
24-well tissue-culture-treated polystyrene plate (e.g., Corning Costar Flat Bottom Cell Culture Plates)	Fisher Scientific	07-200-740
DMEM/F12	ThermoFisher	21331020
Neurobasal Medium	ThermoFisher	21103049
GlutaMAX	ThermoFisher	35050061
Penicillin-Streptomycin	ThermoFisher	15140122
TrypLE Select Enzyme (1X)	ThermoFisher	12563011
0.02% EDTA in DPBS	Sigma-Aldrich	E8008-100ML
0.01% 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	D2650
Puromycin (10 mg/ml)	InvivoGen	ant-pr-1
12-mm glass coverslips**	VWR	89167-106
100% Ethanol	Multiple vendors	

*PBS should be used at room temperature unless otherwise specified.
**Glass coverslips from different brands might have different effects on the growth of neural cells. We recommend using glass coverslips from Chemglass for best results.

Source hPSC Culture Conditions

The Quick-Neuron™ Cholinergic- SeV Kit (Small) gives the best differentiation results when source human pluripotent stem cells (hPSCs) have been maintained in StemFit® Basic04, StemFit® AK02N, 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 a 35-mm culture dish or one well of a 6-well plate. If iMatrix-511 silk is routinely used as a coating substrate, prepare one culture dish or well precoated with 0.25 µg/cm^2 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-C, NI511S).
- Differentiation should not be performed until the cells are at least 14 days post-thaw.
- We recommend preparing a minimum of 2.2×10^5 viable hPSC for use with this kit. This is usually obtained by using 1 well of a 6-well plate at 50-70% confluency.
- For optimal differentiation, hPSC confluency should be around 50% to 70%. Do not use wells more than 90% confluent.

Drug Selection

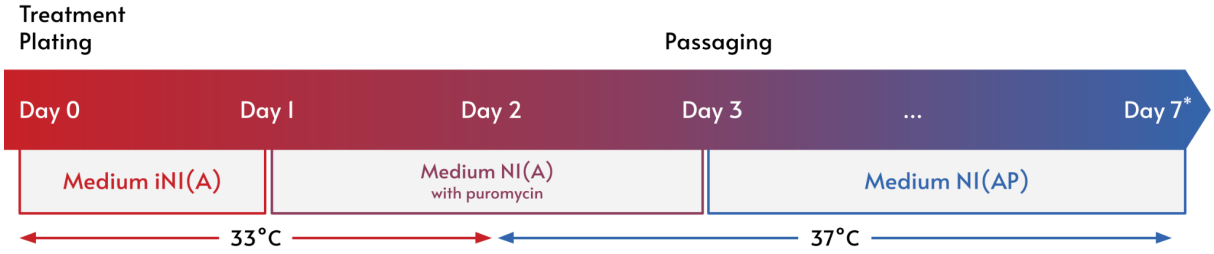
Users should perform a puromycin kill curve for their cells to determine the minimum concentration required to kill all non-treated cells within ~60 hours. Based on Elixirgen Scientific's internal tests, the appropriate concentration ranges between 0.5 and 2 µg/ml. We recommend maintaining 2 wells of untransfected iPSC (with standard StemFit conditions),

alongside the transfected wells, until after puromycin selection is performed. Treat 1 of those wells with puromycin at your selected concentration so as to confirm that the puromycin is effective at killing the untransfected cells in your experiment.

Workflow

IMPORTANT! This workflow requires a humidified 33°C, 5% CO_2 incubator. Before starting this protocol, please make sure the temperature is stable at 33°C.

Note: This protocol assumes that Day 0 is a Monday.



* From Day 7, users may maintain differentiated neurons in the medium best suited for their needs, though we recommend Quick-Neuron™ Cholinergic - Maintenance Medium, Catalog Number: CH-MM.

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, hereafter referred to as iROCK, can be stored at -20°C.

0.5X TrypLE Select with EDTA (Solution D1)

1. Mix 1 ml TrypLE Select Enzyme (1X) with 1 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 300 µl 0.01% Poly-L-Ornithine solution and mix it with 1.2 ml PBS.
2. The 0.002% Poly-L-Ornithine solution, hereafter referred to as ornithine, can be stored 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.,15 µl) and store at -20°C.

Medium N1(A)

1. Prepare Medium N1(A) using the reagents listed in the table below.
    - Thaw Components N1 and A for 20-30 minutes at the temperatures indicated in the “Contents” table on page 1.
    - Warm all other reagents at room temperature for 20-30 minutes.
    - Tap each Component tube 3 times and then briefly spin all tubes down before use.
    - Keep Medium N1(A), and any subsequent media made with it, protected from light.

- Store Medium N1(A) for up to 2 weeks at 4°C.
- Leftover Components can be discarded or saved at 4°C for up to two weeks.

Reagents	Volume
DMEM/F12	8.2 ml
Neurobasal Medium	8.2 ml
GlutaMAX	85 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	170 µl
Component N1	510 µl
Component A	17 µl

Medium N1(AP)

1. Prepare Medium N1(AP) using the reagents listed in the table below.
   - Thaw Component P for 20-30 minutes at the temperature indicated in the "Contents" table on page 1.
   - Tap the Component P tube 3 times and then briefly spin it down before use.
   - Store Medium N1(AP) for up to 2 weeks at 4°C.
   - The leftover reagents can be discarded or saved at 4°C for up to two weeks.

Reagents	Volume
Medium N1(A)	7.7 ml
Component P	3.9 µl

Day -3

~1 hour

Note: This protocol assumes that Day 0 is a Monday so Day -3 is Friday.

Plate Preparation

1. Prepare diluted Coating Material A by mixing together the following components in a 15 ml conical tube.
   - Thaw Coating Material A for 20-30 minutes at the temperature indicated in the "Contents" table on page 1.
   - Keep Coating Material A on ice.
   - Make sure chilled PBS is used for this mixture.

Reagents	Volume
Coating Material A	6.0 µl
Chilled PBS	1.8 ml

2. Add 400 µl diluted Coating Material A to each new well of 4 wells.
3. Incubate the plate at 4°C.
   Note: For best results we recommend precoating the plate 1 day or up to a week before use and keeping at 4°C. Alternatively plates can be precoated on Day 0 and placed at 37°C for at least 2 hours before use.

Day 0

~4 hours

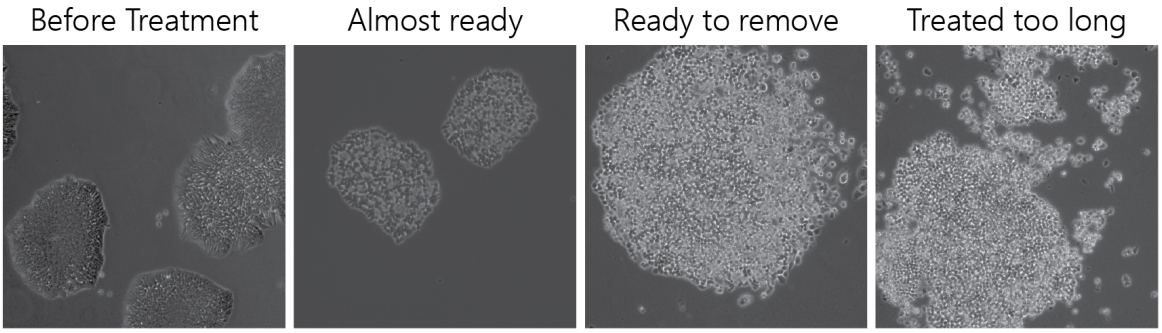
Note: This protocol assumes that Day 0 is a Monday.

Treatment

1. Prepare Medium iN1(A) by mixing together the following components in a 15 ml conical tube.
   - Warm Medium N1(A), iROCK, and Solution D1 at room temperature for at least 1 hour.
   - The rest of Medium N1(A) should be stored at 4°C for later use.

Reagents	Volume
Medium N1(A)	2.5 ml
iROCK	2.5 µl

2. Thaw QN-SeV-P on ice.
3. Aspirate old medium from hPSC culture and add 2 ml PBS to each well being harvested.
4. Rock the plate 3 times, aspirate PBS from the culture, and add 300 µl of the cell dissociation reagent Solution D1 to each well.
   - Keep the rest of Solution D1 at 4°C for use on Day 3.
5. Incubate the culture plate at 37°C, 5% CO_2 for 5 minutes. If all the cells are not rounded under a microscope, continue to incubate at 37°C, 5% CO_2 in 1-2 minute increments (see images below).



6. Carefully pipet out Solution D1 from the culture and add 1 ml Medium iN1(A) to the well.
7. Disperse the medium over the bottom surface of the well by pipetting 8-15 times to detach cells.
8. Using the same pipet tip, collect the cell suspension in a 1.5 ml tube.

IMPORTANT! In this protocol, users will treat hPSCs with QN-SeV-P in a tube and then plate the cells onto 4 wells with 250 µl Medium iN1(A) (0.5×10^5 cells) per well. However, we recommend preparing a suspension of 1.1 ml to avoid insufficiency. First, QN-SeV-P should be mixed with 110 µl of a dense cell suspension to increase the chance that QN-SeV-P finds its host cells. After 10 minutes incubation at 33°C, the total volume will be brought up to 1.1 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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9. Count cells and determine viability.
10. Take out the volume of the cell suspension needed for 4 wells and include 10% extra (a total of 2.2×10^5 cells to plate 0.5×10^5 cells in each of the 4 wells). Transfer the determined volume of the cell suspension into a 15 ml conical tube.
11. Bring the volume of the cell suspension up to 110 µl with Medium iN1(A).
    - If the volume of the cell suspension needed to get 2.2×10^5 cells exceeds 110 µl, centrifuge the required volume of cell suspension at 200 x g for 4 minutes, remove the supernatant, and resuspend the pellet into 110 µl Medium iN1(A).

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

12. Add 55 µl QN-SeV-P to the hPSCs and mix them by tapping with a finger 2-3 times. Cap the tube loosely to allow gas exchange.
13. 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 1.1 ml with Medium iN1(A).
2. Aspirate diluted Coating Material A from only one coated well at a time and add 250 µl cell suspension to the well.
    - Most of the diluted Coating Material A should be aspirated but not completely to prevent the coated well from drying before adding the cell suspension. The cell suspension should be added to the well immediately after diluted Coating Material A 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 culture plate at 33°C, 5% CO_2 overnight.

Day 1

< 1 hour

Medium Change

1. Transfer 4.4 ml Medium N1(A) into a tube and add puromycin to it at the predetermined optimal concentration (see earlier section on “Drug Selection”)."
2. Pipet out most of the medium from each well and add 500 µl Medium N1(A) with puromycin.
3. Incubate the culture plate at 33°C, 5% CO_2 overnight.

Day 2

< 1 hour

Medium Change and Temperature Shift

Note: This should be performed in the late afternoon.

1. Warm Medium N1(A) with puromycin at room temperature for 20-30 minutes.
2. Pipet out most of the medium from each well and add 500 µl Medium N1(A) with puromycin.
3. Incubate the culture plate at 37°C, 5% CO_2 overnight.

Day 3

4-6 hours

IMPORTANT! Following this protocol, users will encounter cell death in the infected cultures. It will be most noticeable the day after making the temperature shift. When monitoring the health of infected cultures, please refer to the images in the appendix showing the recovery trajectory of a typical hPSC culture subjected to QN-SeV-P mediated differentiation. It is optional, but recommended, to include the PBS wash if cell death/floating cells are observed.

Plate/Coverslip Preparation

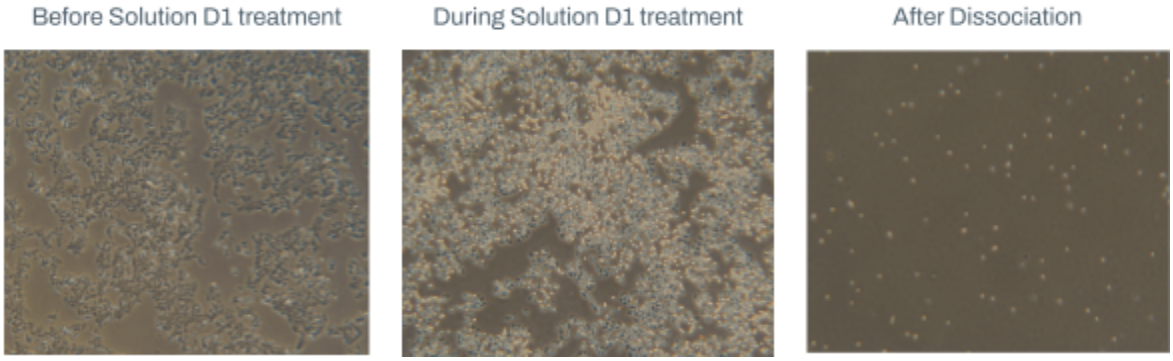
IMPORTANT! Cells can be plated on glass coverslips or in wells of a 24-well plate depending on the desired application. Wells are better suited for functional assays, whereas coverslips work best for immunostaining and imaging. If using glass coverslips, follow all instructions starting at Step 1. If not, start at Step 3. For first time users, we recommend only passaging two of the cultures and maintaining the other cultures (or any cultures less than 40% confluent) without passaging. Prepare one coverslip or well for each well being passaged.

1. (Coverslip only) Soak 12-mm glass coverslips and the tips of forceps in 100% ethanol for 3 minutes.
2. (Coverslip only) One by one, air dry each coverslip for 1 minute or until completely dry and put it into a well of the 24-well plate using the sterilized forceps.
3. Vortex ornithine briefly and centrifuge it at a maximum speed for 1 minute.

4. Add 300 µl ornithine to the surface of each glass coverslip or well.
   IMPORTANT! If using coverslips, ensure they are fully submerged under solutions from Step 4 onwards.
5. Incubate the plate at 37°C, 5% CO_2 for at least 2 hours (or at 4°C overnight one day before plating).
6. Thaw laminin and chill 1.5 mL PBS on ice for 20-30 minutes.
7. Add 15 µl laminin to the chilled PBS. Mix well.
   - All PBS washes should be done dropwise and with room temperature PBS. Chilled PBS is only for the coating step.
8. Aspirate the supernatant from each coverslip/well and add 500 µl PBS.
9. Repeat Step 8.
10. Aspirate PBS from each coverslip/well and add 300 µl diluted laminin.
11. Incubate the plate at 37°C, 5% CO_2 for at least 2 hours.
12. Around 30 minutes before the end of the laminin incubation, warm Medium N1(AP) at room temperature for 20-30 minutes.
13. After the laminin incubation, pipet out most of the supernatant, but not all, from each coverslip or well.
14. Rinse with 500 µl PBS.
15. Pipet out most of the PBS, but not all, from each well.
16. Repeat Steps 14-15.
17. Add 300 µl Medium N1(AP) to each well or coverslip.
18. Incubate the plate at 37°C, 5% CO_2 until cells are ready for plating.

Passaging Cells

IMPORTANT! For the following steps, gently pipet and add solutions. Differentiating cells are delicate and should be handled with great care. Steps 2-10 below are critical. Perform these steps **one well at a time**. Refer to the images below to successfully manage cell treatment and dissociation.



1. Make sure that Solution D1 is at room temperature for at least 1 hour before use.
2. Pipet out the old medium from one well and add 500 µl PBS.
3. Pipet out the PBS from the well and add 80 µl Solution D1.
4. Rock the plate 3 times to spread the Solution D1 evenly.
5. Incubate the culture plate at 37°C, 5% CO_2 for 3 minutes.
6. Carefully pipet out Solution D1 from the well using a P200 pipettor and add 200 µl Medium N1(AP).
7. Disperse the medium quickly over the bottom surface of the well by pipetting 6-8 times to detach cells using a P200 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. These 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 P200 pipettor. Excessive pipetting can damage the already-suspended neuronal cells.
10. Collect 200 µl cell suspension from the well with a P200 pipettor set to 100 µl and transfer all the cells to a new well or glass coverslip coated with ornithine and laminin 100 µl at a time.
    - **IMPORTANT**! If cultures are >70% confluent on Day 3, transfer only half of the cell suspension (100 µl) to each new well or glass coverslip to avoid excessively high cell density. Add 100 µl Medium N1(AP) after cell

suspension is transferred to bring the total volume up to 500 µl. Please keep in mind that this protocol supports the maintenance of up to 4 newly prepared wells.
    - When transferring the cell suspension to a coverslip, bring the tip very close to the coverslip and pipet slowly to mount cells on the coverslip.
11. Repeat Steps 2 - 10 for the rest of the wells.
12. Incubate the culture plate at 37°C, 5% CO_2 overnight.

Medium Change (Unpassaged Cultures Only)

1. Pipet out the old medium from each unpassaged well and add 500 µl Medium N1(AP).
    - **IMPORTANT!** For the wells that are not passaged, the attachment of cells is weaker and so a small volume of the old medium (i.e., just enough to cover the surface of the well) should be left. The fresh medium should be applied along the wall of the well very slowly.
2. Incubate the culture plate at 37°C, 5% CO_2 overnight.

Day 4

< 1 hour

Medium Change

1. Warm Medium N1(AP) at room temperature for 20-30 minutes.
2. Pipet out most of the medium from each well and** add 800 µl Medium N1(AP).
    - **IMPORTANT!** For the wells that are not passaged, the attachment of cells is weaker and so a small volume of the old medium (i.e., just enough to cover the surface of the well) should be left. The fresh medium should be applied along the wall of the well very slowly.
    - **(Optional) Slowly add 500 µl PBS alongside the wall of each well to avoid lifting attached cells. Gently pipet out PBS before adding 800 µl Medium N1(AP)*.
3. Incubate the culture plate at 37°C, 5% CO_2 for 3 days.

Day 7

Assay or Continuous Maturation

- Differentiated neurons can be observed on Day 4. For more mature neurons, we recommend culturing cells until Day 7. From Day 7, users may maintain differentiated neurons in the medium best suited for their needs, though we recommend Quick-Neuron™ Cholinergic Maintenance Medium, Catalog Number: CH-MM.

- Differentiation into Cholinergic neurons after using Quick-Neuron™ Cholinergic - SeV Kit can be confirmed with the markers TUBB3, ISL1, and ChAT.

Appendix

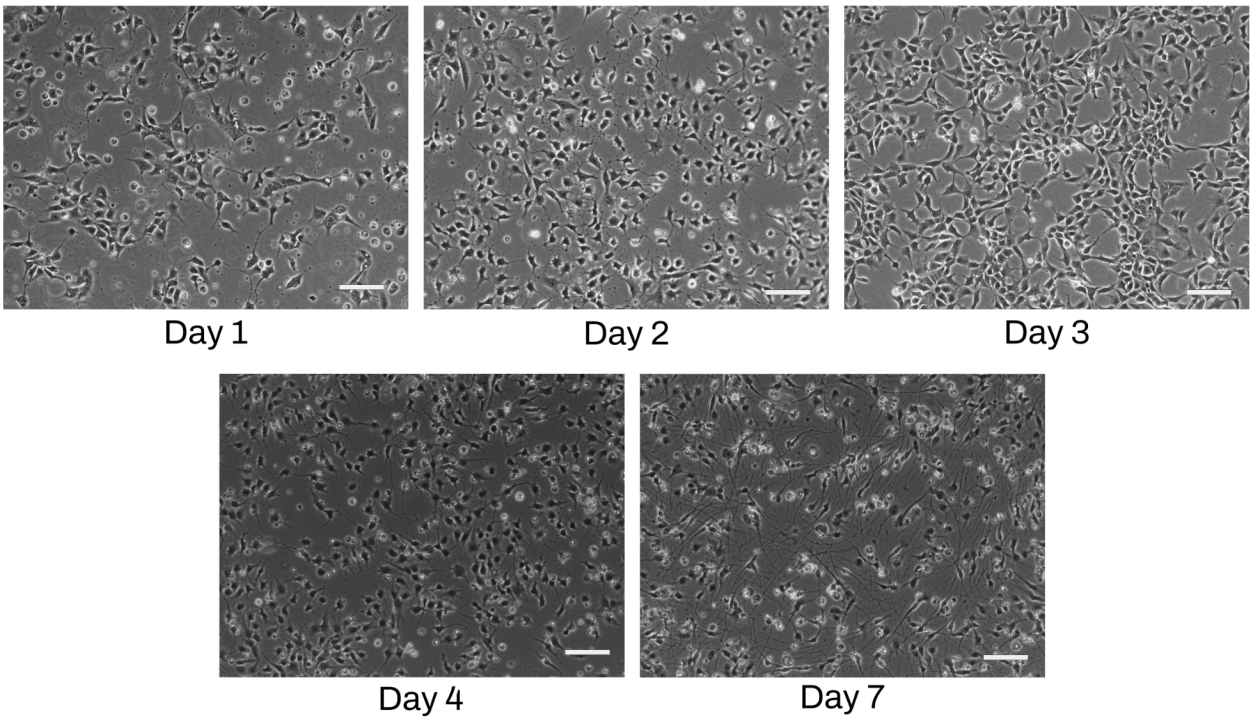


Figure 1. Representative phase contrast images of Quick-Neuron™ Cholinergic - SeV cell cultures on days 1-7 post-differentiation. (scale bars = 100 µm).

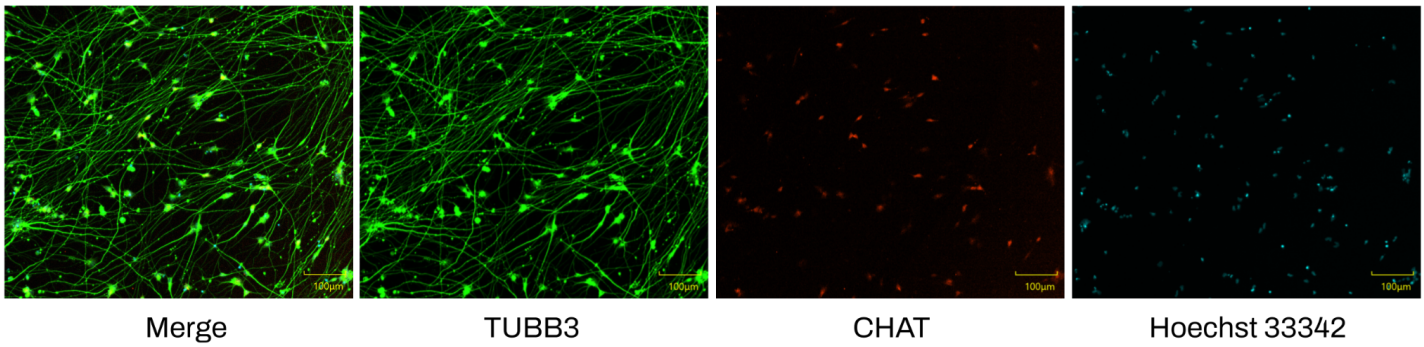


Figure 2. Immunofluorescent staining of Quick-Neuron™ Cholinergic - SeV cell culture shows typical neurite growth and expression of the pan-neuronal marker TUBB3 and CHAT on day 7 post-differentiation (scale bars = 100 µm). Staining conditions: Anti- ß-III tubulin monoclonal antibody (Cell Signaling Technology, Catalog Number: 5568, 1:250 dilution) was used in combination with a secondary antibody (ThermoFisher, Catalog Number: A32731, Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, 1:500 dilution). Anti-CHAT primary antibody (Millipore Sigma, Catalog Number: AMAB91130, 1:500 dilution) was used in combination with a secondary antibody (ThermoFisher, Catalog Number: PIA32742 Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, AlexaFluor Plus 594 1:500 dilution). Nuclei were counterstained with Hoechst 33342.