User Guide

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Ricoh Biosciences, Inc.

Quick-Neuron™ Excitatory - SeV Kit

Catalog Number: EX-SeV

Introduction

The Quick-Neuron™ Excitatory - SeV Kit facilitates rapid and efficient differentiation of human iPS or ES cells into excitatory neurons in just 10 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™ Excitatory 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 glutamatergic neuron marker vesicular glutamate transporter 1 (vGLUT1). When handled and maintained according to the instructions in this user guide, excitatory neurons are viable long-term and are suitable for a variety of characterization and neurotoxicity assays.

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

Related Products: Quick-Neuron™ Excitatory - Human iPSC-derived Neurons, Catalog Number: EX-SeV-HC-CW50065
Quick-Neuron™ Excitatory - Maintenance Medium, Catalog Number: EX-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 (undiluted)*	100 µl	-80°C	On ice
Component N	3 x 840 µl	-20°C or -80°C	On ice or 4°C
Component G1	3 x 20 µl	-20°C or -80°C	On ice or 4°C
Component G2	60 µl	-20°C or -80°C	On ice or 4°C
Component P	50 µl	-20°C or -80°C	Room Temperature

***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@biosciences.ricoh.com or call +1 (443) 869-5420 (M-F 9am-5pm EST).

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
Opti-MEM I Reduced Serum Medium	ThermoFisher	31985062
DMEM/F12	ThermoFisher	21331020
Neurobasal Medium	ThermoFisher	21103049
GlutaMAX	ThermoFisher	35050061
Penicillin-Streptomycin	ThermoFisher	15140122
StemFit Basic04 Complete Type, or StemFit AK02N, or StemFlex Medium	AMSBio TaKaRa ThermoFisher	SF041-001, AK02N, or A3349401
iMatrix-511 silk	Ricoh Biosciences	NI511S
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
(Optional) STEM-CELLBANKER**	AMSBIO	11890

* PBS should be used at room temperature unless otherwise specified.
** This is only required if you intend to cryopreserve the cells after differentiation.

Source hPSC Culture Conditions

The Quick-Neuron™ Excitatory - SeV Kit 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 two to three 35-mm culture dishes or two to three wells of a 6-well plate. If iMatrix-511 silk is routinely used as a coating substrate, prepare culture dishes or wells precoated with 0.25 µg/cm^2 iMatrix-511 silk diluted in 2 ml chilled PBS per well or dish for this kit.

- The protocols and reagents for StemFit® Basic04 and iMatrix-511 silk culture conditions are available at Ricoh Biosciences(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 3.3 x 10^6 viable hPSC for use with this kit. This is usually obtained by using 3 wells 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.

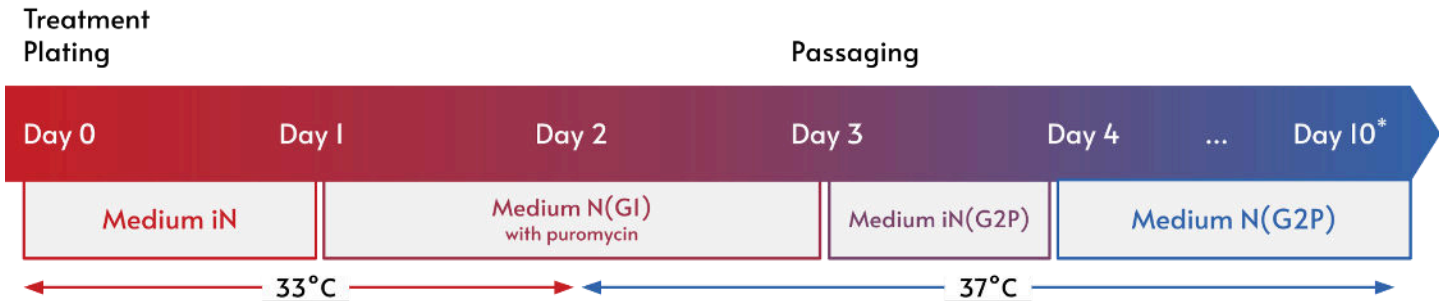
Drug Selection

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 Ricoh Biosciences' 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 (or Tuesday if cells will be cryopreserved instead of passaged on day 3).



* From Day 10, users may maintain differentiated neurons in the medium best suited for their needs, though we recommend Quick-Neuron™ Excitatory- Maintenance Medium, Catalog Number: EX-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.

StemFit Basic04 Complete Type (Medium S)*

1. Thaw StemFit Basic04 Complete Type bottle overnight or multiple nights at 4°C.
2. Make aliquots of a convenient volume (e.g., 40 ml).
3. This solution, hereafter referred to as Medium S, can be stored at -80°C. Once thawed, Medium S should be stored at 4°C for up to 2 weeks.
    - After thawing users may choose to add Penicillin-Streptomycin at a 1:200 dilution (e.g., 200 µl in 40 ml of Medium S) before using Medium S.

*Medium S can be substituted with StemFit AK02N or StemFlex.

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. 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., 90 µl) and store at -20°C.

Medium N

1. Prepare Medium N using the reagents listed in the table below.
    - Thaw Component N for 20-30 minutes at the temperature indicated in the “Contents” table on page 1.
    - Warm all other reagents at room temperature for 20-30 minutes.
    - Tap Component N tubes 3 times and then briefly spin all tubes down before use.
    - Keep Medium N, and any subsequent media made with it, protected from light.
    - Store Medium N for up to 2 weeks at 4°C.
    - Leftover Component N can be discarded or saved at 4°C for up to two weeks.

	Required Volume	
Reagents	**Passaging on Day 3**	**Cryopreserving on Day 3**
DMEM/F12	37.2 ml	18.6 ml
Neurobasal Medium	37.2 ml	18.6 ml
GlutaMAX	390 µl	195 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	780 µl	390 µl
Component N	2.4 ml	1.2 ml

Day -3

< 1 hour

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

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.
    - Make sure chilled PBS is used for this mixture.

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

2. Add 2 ml diluted iMatrix-511 silk to each new well of a 6-well plate.
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 (or Tuesday if cells will be cryopreserved instead of passaged on day 3).*

Treatment

IMPORTANT! Source hPSC wells should be no more than 50-70% confluent thus requiring a minimum of 2 wells to begin differentiation.

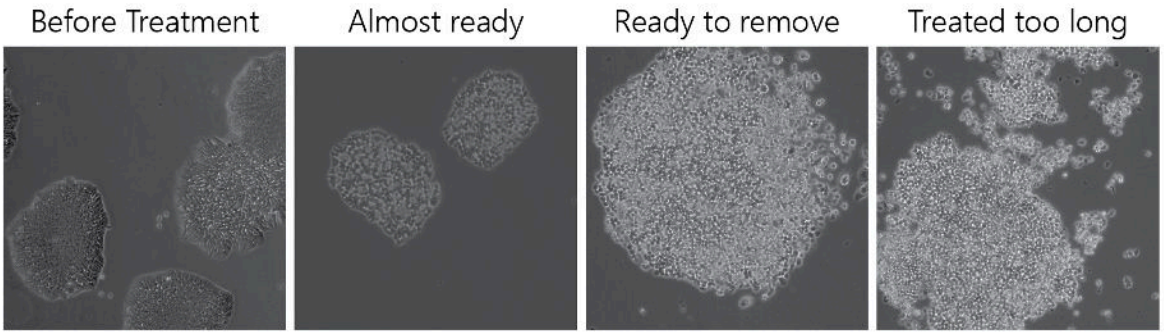
1. Determine the number of wells required to get 3.3 x 10^6 cells from the source hPSC 6-well plate.
2. Prepare Medium iN by mixing together the following components in a 15 ml conical tube.
   - Warm Medium N and iROCK at room temperature for at least 1 hour.
   - The rest of Medium N should be stored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

	Required medium volume based on # of wells of a 6-well plate	
Reagents	2 wells	3 wells
Medium N	9.4 ml	10 ml
iROCK	9.4 µl	10 µl

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

	Required medium volume based on # of wells of a 6-well plate	
Reagents	2 wells	3 wells
Medium S	3.5 ml	5 ml
iROCK	3.5 µl	5 µl

4. Aspirate old medium from hPSC culture and add 1.5 ml of Medium iS 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-P 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 plate 3 times, aspirate PBS from the culture, and add 300 µl of the cell dissociation reagent Solution D1.
   - Keep the rest of Solution D1 at 4°C for use on Day 3.
8. 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).



9. Carefully pipet out Solution D1 from the culture and add 1 ml Medium iN to the well.
    - Follow Steps 9-11 one well at a time if multiple wells are used.
10. Disperse the medium over the bottom surface of the well by pipetting 8-15 times to detach cells.
11. Using the same pipet tip, collect the cell suspension in a 15 ml tube.

IMPORTANT! In this protocol, users will treat hPSCs with QN-SeV-P in a tube and then plate the cells onto 6 wells with 1 ml Medium iN (0.5×10^6 cells) per well. However, we recommend preparing a suspension of 6.6 ml to avoid insufficiency. First, QN-SeV-P should be mixed with 580 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 6.6 ml with Medium iN. Cell count may vary based on cell health, the method, and instrument used for cell counting.

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12. Count cells and determine viability.
13. Determine the volume of cell suspension needed for 6 wells and include 10% extra (a total of 3.3×10^6 cells to plate 0.5×10^6 cells in each of the 6 wells). Transfer the determined volume of the cell suspension into a 15 ml conical tube.
14. Adjust the volume to 580 µl with Medium iN.
    - If the volume of the cell suspension needed to get 3.3×10^6 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 iN.

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.

15. Add all the contents of the QN-SeV-P tube to the hPSCs and mix them by tapping with finger 2-3 times. Cap the tube loosely to allow gas exchange.
16. 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 iN and mix 2-3 times with serological pipet.
2. Aspirate diluted iMatrix-511 silk from only one coated well at a time and add 1 ml of cell suspension to each well.
    - Most of the diluted iMatrix-511 silk 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 the diluted iMatrix-511 silk 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

~2 hours

Medium Change

1. Prepare Medium N(G1) by mixing together the following components in a 15 ml conical tube.
    - Thaw Component G1 for 20-30 minutes at the temperature indicated in the “Contents” table on page 1.
    - Warm Medium N at room temperature for 20-30 minutes.

- Tap the Component G1 tube 3 times and then briefly spin it down before use.
- Keep the rest of Medium N, Component G1, and Medium N(G1) at 4°C for subsequent use.

Reagents	Volume
Medium N	20 ml
Component G1	40 µl

2. Transfer 9.5 ml Medium N(G1) into a tube and add puromycin to it at the predetermined optimal concentration (see earlier section on "Drug Selection").
3. Pipet out most of the old medium from each well and add 1.5 ml Medium N(G1) with puromycin. Incubate the cultures 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 N(G1) at room temperature for 20-30 minutes.
2. Transfer 9.5 ml Medium N(G1) into a tube and add puromycin to it at the predetermined optimal concentration (see earlier section on "Drug Selection").
    - If more than 90% of the cells show resistance to puromycin at the concentration used on Day 1, consider increasing its concentration.
3. Pipet out most of the old medium from each well and add 1.5 ml N(G1) with puromycin.
4. Incubate the cultures at 37°C, 5% CO_2 overnight.

Day 3

4-6 hours

New Plate Preparation

IMPORTANT! 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 for the recommended volumes. Please note that the volumes are per well in Table A and per plate in Table B. Cells can be cryopreserved instead of passaging by skipping to Appendix B at this point. Alternatively, cells can be live-passaged as per the instructions below and any surplus cells can be cryopreserved following the instructions beginning at step 3 in Appendix B.

1. Vortex ornithine briefly and centrifuge it at a maximum speed for 1 minute.
2. Add ornithine to each well of a new plate in the volume specified in Table A.
3. Incubate the plate at 37°C, 5% CO_2 for at least 2 hours (or at 4°C overnight one day before plating).
4. Thaw laminin and chill specified amounts of PBS on ice for 20-30 minutes.
5. 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.

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

Reagents	Recommended 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 iN(G2P)	500 µl	200 µl	35 µl

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

Reagents		Recommended 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 N(G2P)	Medium N	41 ml	45 ml	34 ml
	Component G2	41 µl	45 µl	34 µl
	Component P	20.5 µl	22.5 µl	17 µl

Medium Preparation

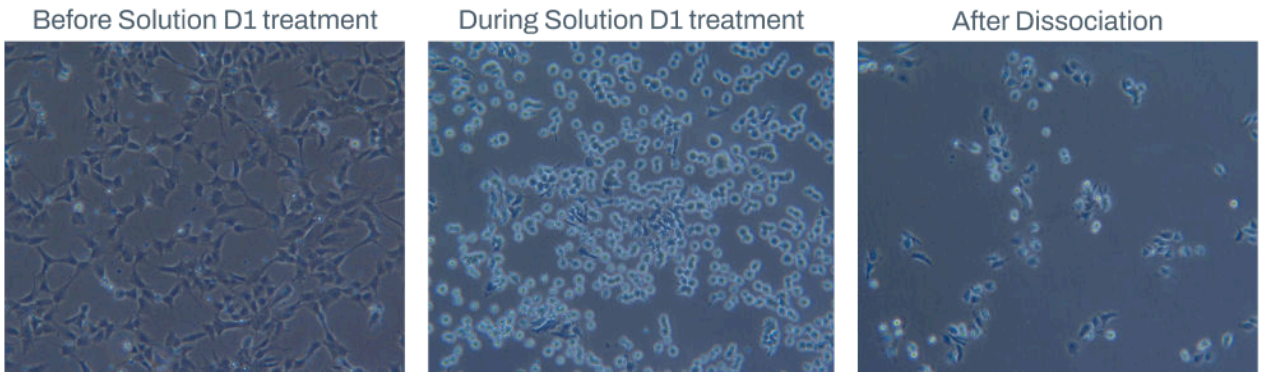
1. While the plate is incubating, prepare Medium N(G2P) using the volumes indicated in Table B.
    - Thaw Components G2 and Component P for 20-30 minutes at the temperature indicated in the "Contents" table on page 1.
    - Warm Medium N at room temperature for 20-30 minutes.
    - Tap each Component tube 3 times and then briefly spin all tubes down before use.
    - Keep the rest of Medium N at 4°C for later use.
2. Prepare Medium iN(G2P)
    - Warm iROCK at room temperature for 20-30 minutes.
    - The rest of Medium N(G2P) should be stored at 4°C for later use.

Reagents	Required medium volume per plate		
	6-well plate	24-well plate	96-well plate
Medium N(G2P)	11.5 ml	13 ml	10 ml
iROCK	11.5 µl	13 µl	10 µl

3. 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.
4. Aspirate most, but not all of, the PBS and add Medium iN(G2P) in the volume specified in Table A.
5. 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 2-9 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 1 ml PBS to the well.
3. Pipet out the PBS from the well 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. Carefully pipet out Solution D1 from the well and add 750 µl Medium iN(G2P) along the wall of the well.
7. Disperse the medium quickly over the bottom surface of the well by pipetting 6-8 times to detach cells.
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. Collect 750 µl cell suspension from each well and transfer to a tube.
10. Repeat steps 2-9 for the rest of the wells.
11. Gently pipet the cell suspension up and down up to 5 times to break the cell aggregates. Excessive pipetting can damage the already-suspended neuronal cells.
12. Count cells and determine viability.
13. Prepare specified amounts of a 1 x 10^6 live cells/ml cell suspension using Medium iN(G2P) based on the table below.
    - If there are leftover cells, freeze the cells down by following instructions beginning at step 3 in Appendix B after plating cell suspensions on the new plate. Keep the leftover cells on ice until freezing.
14. Add cell suspension to the center of each well. Since each well already has Medium iN(G2P), the total volume of the medium in 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
Required volume of cell suspension (1 x 10^6 viable cells/ml) • (Vol of cell suspension/well x # of wells) + 10% extra	3.3 ml	2.64 ml	1.6 ml
Volume of cell suspension/well	500 µl	100 µl	15 µl
Total volume/well • Medium iN(G2P) + cell suspension	1 ml	300 µl	50 µl

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

Day 4

<1 hours

Medium Change

1. Warm Medium N(G2P) at room temperature for 20-30 minutes.
2. Pipet out the old medium from each well and add* room temperature Medium N(G2P) according to the table below.

	Recommended volume per well		
Reagents	6-well plate	24-well plate	96-well plate
Medium N(G2P)	3 ml	800 µl	150 µl

*(Optional) Slowly add PBS according to the volumes in the table above alongside the wall of each well to avoid lifting attached cells. Gently pipet out PBS before adding Medium N(G2P).

3. Incubate the culture plate at 37°C, 5% CO_2 for 3 days.

Day 7

<1 hours

Medium Change

1. Warm Medium N(G2P) at room temperature for 20-30 minutes.
2. Pipet out half the original volume of the medium from each well and add Medium N(G2P) according to the following table.

	Recommended volume per well		
Reagents	6-well plate	24-well plate	96-well plate
Medium N(G2P)	1.5 ml	400 µl	75 µl

3. Incubate the culture plate at 37°C, 5% CO_2 for 3 days.

Day 10

Assay or Continuous Maturation

- Differentiated neurons can be observed on Day 4. For more mature neurons, we recommend culturing cells until Day 10. From Day 10, users may maintain differentiated neurons in the maintenance medium best suited for their needs, though we recommend Quick-Neuron™ Excitatory - Maintenance Medium, Catalog Number: EX-MM.
- Differentiation into excitatory neurons after using the Quick-Neuron™ Excitatory - SeV Kit can be confirmed with anti-TUBB3 and anti-vGLUT1 antibodies.

Appendix A

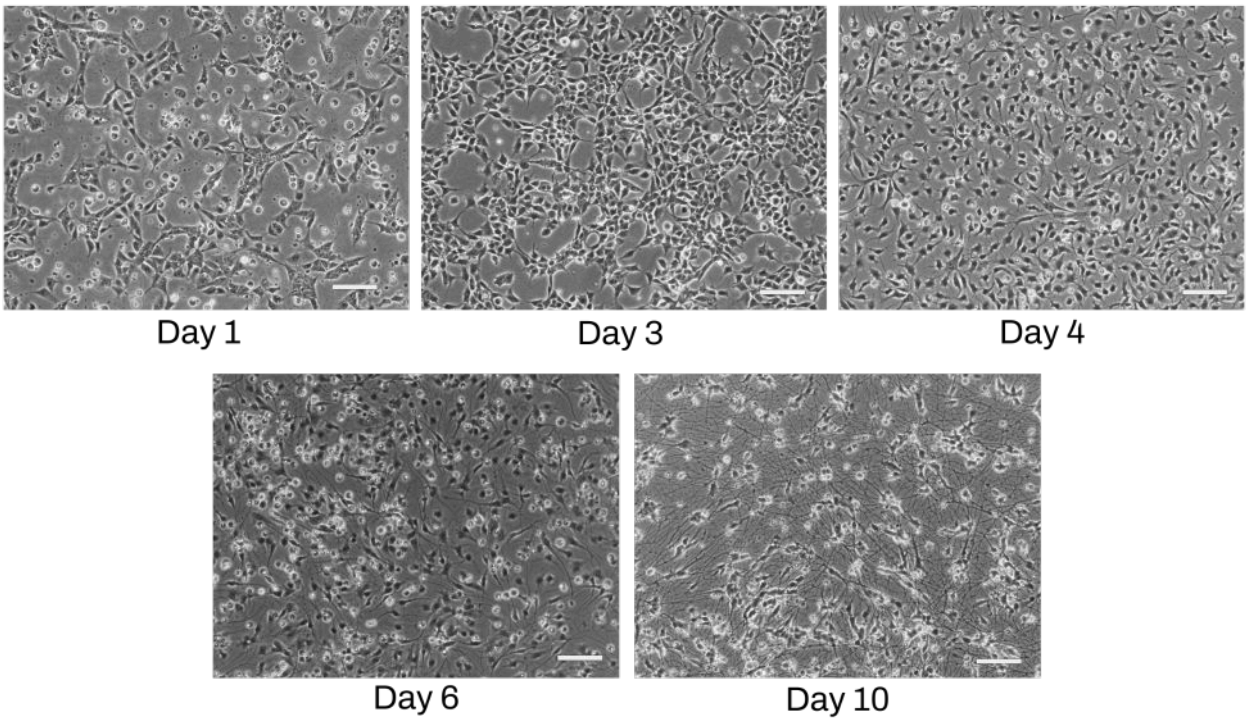


Figure 1. Representative phase contrast images of Quick-Neuron™ Excitatory - SeV cell cultures on days 1,3,4,6 and 10 post-differentiation (scale bar = 100 µm).

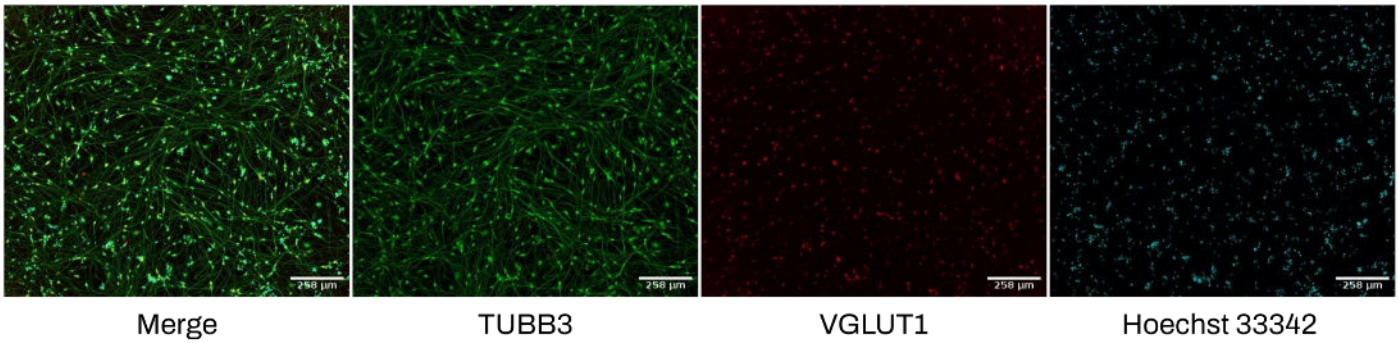


Figure 2. Immunofluorescent staining of Quick-Neuron™ Excitatory - SeV Kit cell cultures shows typical neurite growth and expression of the pan-neuronal marker TUBB3 and the glutamatergic neuron-specific marker vGLUT1 on day 10 post-differentiation (scale bar = 258 µm). Staining conditions: Anti-β-III 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-VGLUT1 Rabbit Polyclonal primary antibody (Synaptic Systems, Catalog Number: 135 303, 1:100 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 33342.

Appendix B

Freezing cells down on Day 3

Note: After thawing frozen cells, over 50% of the cells will be viable.

Required volume

1. Prepare Medium iN by mixing together the following components in a 15 ml conical tube.
    - Warm Medium N and iROCK for 20-30 minutes at room temperature.
    - Keep the rest of Medium N at 4°C for later use.

Reagents	Volume
Medium N	5 ml
iRock	5 µl

2. Harvest cells as per the instructions in steps 1-12 of "passaging cells" on day 3 using Medium iN instead of iN(G2P).
3. Determine the volume of the cell suspension and number of cryovials needed to freeze 0.1 ~ 2 x 10^6 cells per cryovial.
4. Centrifuge at 310 x g for 4 minutes.
5. While waiting for the centrifugation, label each cryovial. We recommend writing the name of the PSC line used, the type of neurons, harvesting day and date, and the number of cells in the vial.
6. Aspirate the supernatant and resuspend the pellet with 0.5 ml / vial STEM-CELLBANKER.
7. Distribute 0.5 ml of the suspension to each cryovial.
8. 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.
9. Loosely close the lid of Mr. Frosty with cryovials, put it into a -80°C freezer and leave it overnight or up to a few days.
10. Transfer the cryovials into a liquid nitrogen storage tank.
11. Follow the thawing process in the user guide of Quick-Neuron™ Excitatory - Human iPSC-derived Neurons, Catalog Number: EX-SeV-HC-CW50065.