User Guide

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Ricoh Biosciences, Inc.

Quick-Neuron™ Motor - SeV Kit

Catalog Number: MT-SeV

Introduction

The Quick-Neuron™ Motor - SeV Kit facilitates rapid and efficient differentiation of human iPS or ES cells into motor 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™ Motor 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), the cholinergic marker choline acetyl-transferase (ChAT), ISL LIM Homeobox 1 (ISL1), and the homeobox transcription factor HB9 expressed in motor neurons. When handled and maintained according to the instructions in this user guide, motor neurons are viable long-term and are suitable for a variety of characterization and neurotoxicity assays.

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

Related Products: Quick-Neuron™ Motor - Human iPSC-derived Neurons, Catalog Number: MT-SeV-HC-CW50065
Quick-Neuron™ Motor - Maintenance Medium, Catalog Number: MT-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 N1	3 x 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
Component K	20 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
DMEM/F12	ThermoFisher	21331020
Neurobasal Medium	ThermoFisher	21103049
GlutaMAX	ThermoFisher	35050061
Penicillin-Streptomycin	ThermoFisher	15140122
StemFit Basic04 Complete Type, or	Amsbio	SF041-00
StemFlex Medium	ThermoFisher	A3349401
iMatrix-511 silk	Ricoh Biosciences	RBI-2040
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™ Motor- 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 for StemFit® Basic04 (Catalog Number: SF041-001) culture conditions and iMatrix-511 silk (Catalog Number: RBI-2040) are available at Ricoh Biosciences.

- 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.

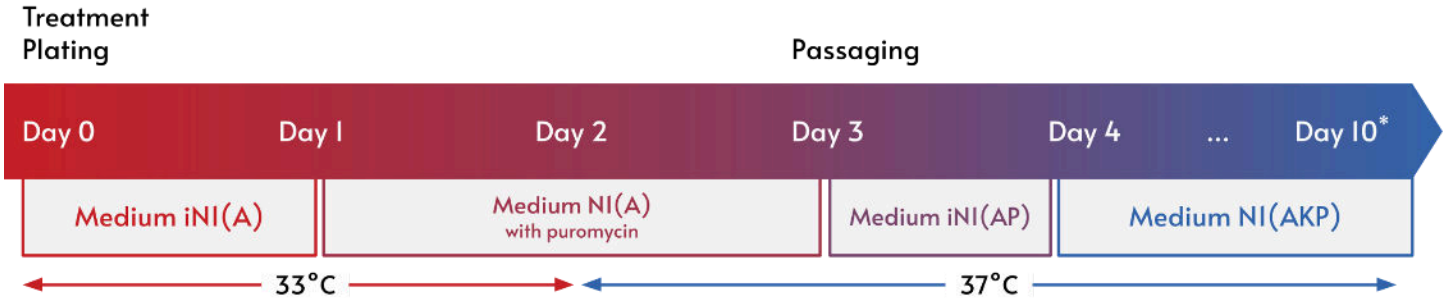
Drug Selection

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™ Motor - Maintenance Medium, Catalog Number: MT-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 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 Component N1 can be discarded or saved at 4°C for up to two weeks.

Reagents	Required Volume	
	Passaging on Day 3	Cryopreserving on Day 3
DMEM/F12	37 ml	17.8 ml
Neurobasal Medium	37 ml	17.8 ml
GlutaMAX	385 µl	185 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	770 µl	370 µl
Component N1	2.31 ml	1.1 ml
Component A	77 µl	37 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 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 new 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.

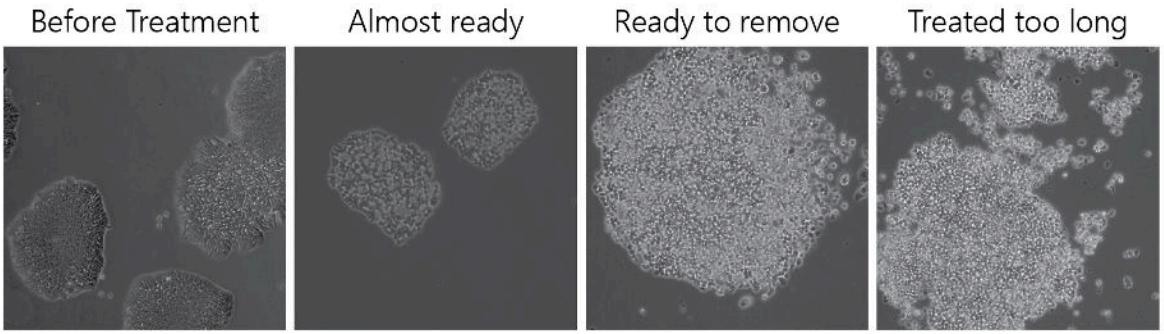
1. Determine the number of wells required to get 3.3 x 10^6 cells from the source hPSC 6-well plate.
2. Prepare Medium iN1(A) by mixing together the following components in a 15 ml conical tube.
   - Warm Medium N1(A), iROCK, and Solution D1 at room temperature for at least 1 hour.
   - The rest of Medium N1(A) should be stored at 4°C for later use.

	Required volume per # of wells of a 6-well plate	
Reagents	**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 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 volume per # of wells of a 6-well plate	
Reagents	**2 wells**	**3 wells**
Medium S	3 ml	4.5 ml
iROCK	3 µl	4.5 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 (undiluted) on ice and warming 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 to each well.
   - 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 iN1(A) 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 (undiluted) in a tube and then plate the cells onto 6 wells with 1 ml Medium iN1(A) (0.5×10^6 cells) per well. However, we recommend preparing a suspension of 6.6 ml to avoid insufficiency. First, QN-SeV-P (undiluted) should be mixed with 580 µl of a dense cell suspension to increase the chance that QN-SeV-P (undiluted) 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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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 iN1(A).
    - 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 200 x g for 4 minutes, remove the supernatant, and resuspend the pellet into 580 µl Medium iN1(A).

IMPORTANT! Before adding QN-SeV-P (undiluted), 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 a 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 iN1(A) and mix 2-3 times with a serological pipet.
2. Aspirate diluted iMatrix-511 silk from only one newly coated well at a time and add 1 ml 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 culture plate at 33°C, 5% CO_2 overnight.

Day 1

< 1 hour

Medium Change

1. Warm Medium N1(A) at room temperature for 20-30 minutes.
2. Transfer 9.5 ml Medium N1(A) 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 medium from each well and add 1.5 ml Medium N1(A) with puromycin.
4. 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) at room temperature for 20-30 minutes.
2. Transfer 9.5 ml Medium N1(A) 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 medium from each well and add 1.5 ml Medium N1(A) with puromycin.
4. Incubate the culture plate at 37°C, 5% CO_2 overnight.

Day 3

4-6 hours

Note: Beginning at Day 3, users may choose to passage the cells or to cryopreserve them by making the media in the volumes indicated under "cryopreserving" in Table B below and then following instructions in Appendix B.

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. Surplus cells can be frozen.

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.

	Required volume per well		
Reagents	**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.

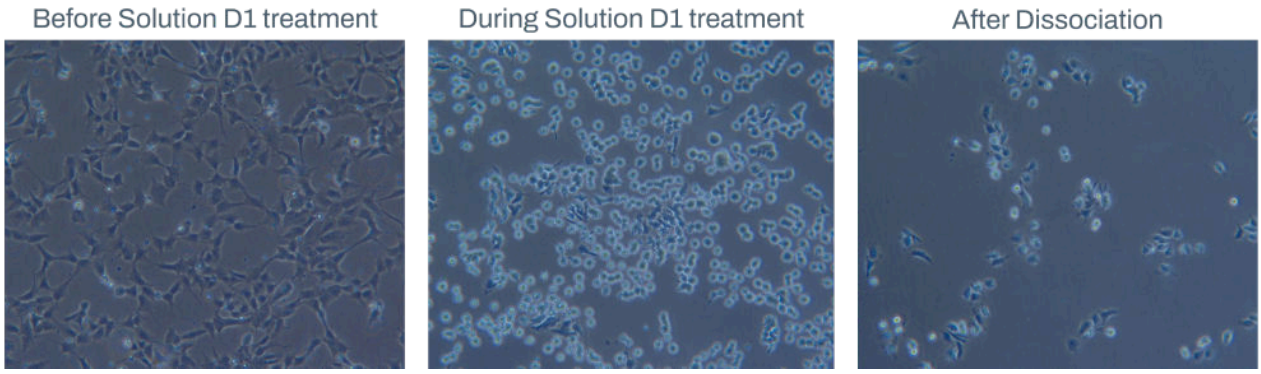
Reagents		Required volume per plate			
		6-well plate	24-well plate	96-well plate	Cryopreserving
Diluted laminin	Laminin	100 µl	80 µl	53 µl	
	Chilled PBS	10 ml	8 ml	5.3 ml	
Medium iN1(AP)	Medium N1(A)	12 ml	13 ml	10.5 ml	5 ml
	Component P	6 µl	6.5 µl	5.3 µl	2.5 µl
	iROCK	12 µl	13 µl	10.5 µl	5 µl

Medium Preparation

1. While the plate is incubating, prepare Medium iN1(AP) using the volumes indicated in Table B.
    - Thaw Component P for 20-30 minutes at the temperature indicated in the "Contents" table on page 1.
    - Warm Medium N1(A) at room temperature for 20-30 minutes.
    - Tap the Component P tube 3 times and then briefly spin it down before use.
    - Keep the rest of Medium N1(A) and Component P at 4°C for later use.
2. After the laminin incubation, aspirate most, but not all, of the supernatant from each well of the new plate and add PBS in the volume specified in Table A above. Add the PBS dropwise to each well.
3. Aspirate most, but not all of the PBS and add Medium iN1(AP) in the volume specified in Table A above.
4. 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 iN1(AP) 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 the 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 iN1(AP) based on the table below.
    - If there are leftover cells, freeze the cells down by following instructions 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 iN1(AP), 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
Req vol 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 iN1(AP) + cell suspension	1 ml	300 µl	50 µl

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

Day 4

< 2 hours

Medium Preparation

1. Prepare Diluted Component K as follows:
    - Thaw Component K for 20-30 minutes at the temperature indicated in the "Contents" table on page 1.
    - Tap the tube of Component K 3 times and then briefly spin it down before use.
    - Prepare a diluted working solution of Component K by mixing together the following components.

Reagents	Volume
DMSO	154 µl
Component K	12.5 µl

2. Prepare Medium N1(AKP) using the volumes in the table below.
    - Thaw Component P for 20-30 minutes at the temperature indicated in the "Contents" table on page 1.
    - Warm Medium N1(A) at room temperature for 20-30 minutes.
    - Tap each Component tube 3 times and then briefly spin all tubes down before use.

	Required volume for each format		
Reagents	**6-well plate**	**24-well plate**	**96-well plate**
Medium N1(A)	28 ml	32 ml	24 ml
Component P	14 µl	16 µl	12 µl
Diluted Component K	28 µl	32 µl	24 µl

Medium Change

1. Pipet out the old medium from each well and add* Medium N1(AKP) according to the following table.

Reagents	Required volume per well		
	6-well plate	24-well plate	96-well plate
Medium N1(AKP)	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 N1(AKP).

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

Day 7

< 1 hour

Medium Change

1. Warm Medium N1(AKP) at room temperature for 20-30 minutes.
2. Pipet out half the original volume of the medium from each well and add Medium N1(AKP) according to the following table.

Reagents	Required volume per well		
	6-well plate	24-well plate	96-well plate
Medium N1(AKP)	1 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

- HB9-positive cells begin to appear as early as day 7 but the proportion of the cells that are HB9-positive will increase when the culture is maintained until day 10 at which point nearly all cells will be HB9-positive. From Day 10, users may maintain differentiated neurons in the medium best suited for their needs, though we recommend Quick-Neuron™ Motor Maintenance Medium, Catalog Number: MT-MM.
- Differentiation into Motor neurons after using Quick-Neuron™ Motor - SeV Kit can be confirmed with the markers TUBB3, ChAT, and HB9.

Appendix A

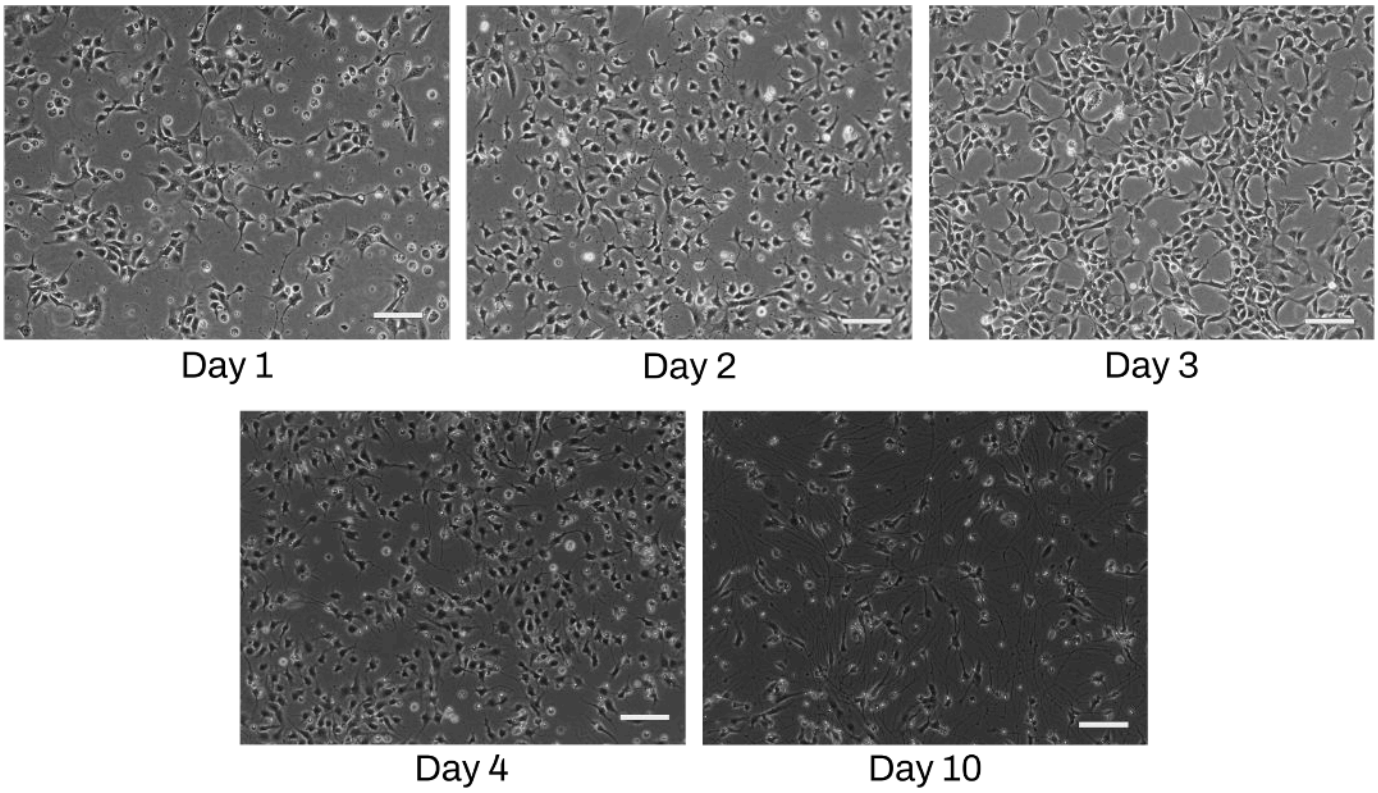


Figure 1. Representative phase contrast images of Quick-Neuron™ Motor - SeV cell cultures on days 1-10 post-differentiation (scale bars = 100 µm).

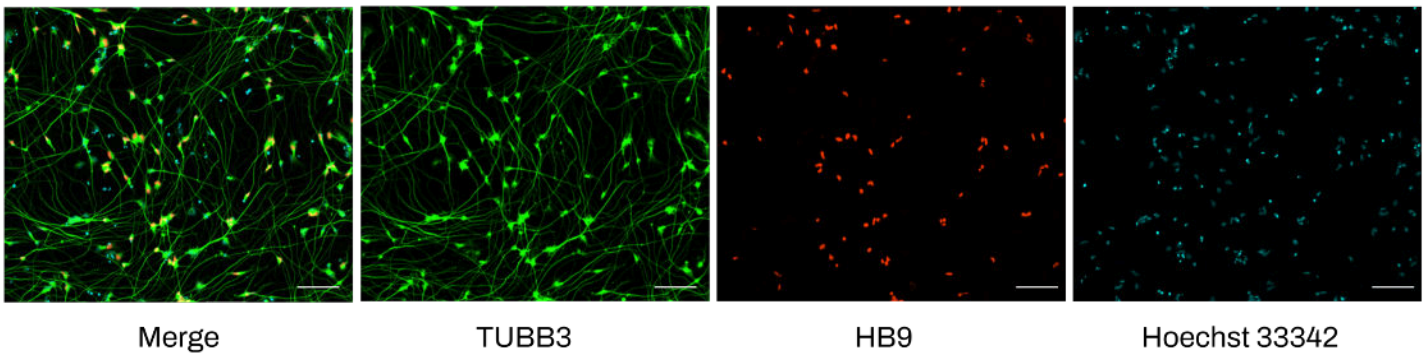


Figure 2. Immunofluorescent staining of Quick-Neuron™ Motor - SeV cell culture shows typical neurite growth and expression of the pan-neuronal marker TUBB3 as well as HB9 on day 10 post-differentiation (scale bars = 100 µm). Staining conditions: Anti- ß-III tubulin monoclonal antibody (Cell Signaling 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-HB9 primary antibody (Santa Cruz Biotechnology, Catalog Number: sc-515769, 1:100 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.

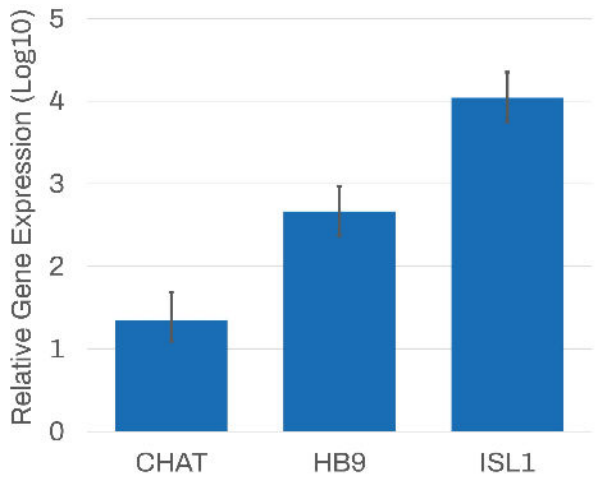


Figure 3. Real-time quantitative PCR analysis of expression levels of genes CHAT, HB9, and ISL1 were examined. The graph shows gene expression in Quick-Neuron™ Motor - SeV culture on day 10 post-differentiation.The relative gene expression is normalized to phosphoglycerate kinase 1 (PGK1), and then calculated as a fold induction relative to undifferentiated hPSCs as a control. Error bars show standard deviation. Primers used are listed in Table 1.

Table 1. List of PCR primers used in Figure 3

Gene	Forward primer	Reverse Primer	Primer Concentration
CHAT	TCATTAATTTCCGCCGTCTC	GAGTCCCGGTTGGTGGAGT	250 nM
HB9	CAAGCTCAACAAGTACCTGTCGC	CTTCTGTTTCTCCGCTTCCTGC	250 nM
ISL1	CAGGTTGTACGGGATCAAATGC	CACACAGCGGAAACACTCGAT	250 nM
PGK1	GTATGCTGAGGCTGTCACTCG	CCTTCCAGGAGCTCCAAACTGG	250 nM

Appendix B

Freezing cells down on Day 3

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

1. Follow steps 1-12 in “Passaging Cells” to harvest the cells.
2. Determine the volume of the cell suspension and number of cryovials needed to freeze 0.1 ~ 2 x 10^6 cells per cryovial.
3. Centrifuge at 310 x g for 4 minutes.
4. 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.
5. Aspirate the supernatant and resuspend the pellet with 0.5 ml / vial STEM-CELLBANKER.
6. Distribute 0.5 ml of the suspension to each cryovial.
7. 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.
8. 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.
9. Transfer the cryovials into a liquid nitrogen storage tank.
10. Follow the thawing process in the user guide of Quick-Neuron™ Motor - Human iPSC-derived Neurons, Catalog Number: MT-SeV-CW.