ELIXIRGEN SCIENTIFIC

Quick-Neuron™ Motor - mRNA Kit (Large)

Catalog Number: MT-mRNA-L

Introduction

The Quick-Neuron™ Motor - mRNA Kit (Large) facilitates rapid and efficient differentiation of human iPS or ES cells into a population of motor neurons in just 9 days. Our proprietary transcription factor-based stem cell differentiation method uses synthetic mRNAs 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), 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 - mRNA Kit (Large) contains a set of reagents for use with a total of 6 wells of a 6-well plate.

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

Kit Contents

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

Contents	Volume	Storage
QN-mRNA-P	4 x 33 µl	-80°C
Component N1	2 x 830 µl	-20°C or -80°C
Component P	2 x 14 µl	-20°C or -80°C
Component A	2 x 38 µl	-20°C or -80°C
Component K	25 µl	-20°C or -80°C

Conditions of Use

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

Technical Support

For technical support please 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
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
Lipofectamine MessengerMAX	ThermoFisher	LMRNA001
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 StemFlex Medium	Elixirgen Scientific ThermoFisher	ASB04-C, or A3349401
iMatrix-511 silk	Elixirgen Scientific	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	D8418
Puromycin (10 mg/ml)	InvivoGen	ant-pr-1
(Optional) STEM-CELLBANKER***	AMSBIO	11890

* Can be substituted with our Cell Dissociation Reagent (Solution D1), Catalog Number: CDR.
** Can be substituted with our Neuron Coating Solutions (Coating Materials B and C), Catalog Number: NCS.
*** This is only required if you intend to cryopreserve the cells after differentiation.

Source hPSC Culture Conditions

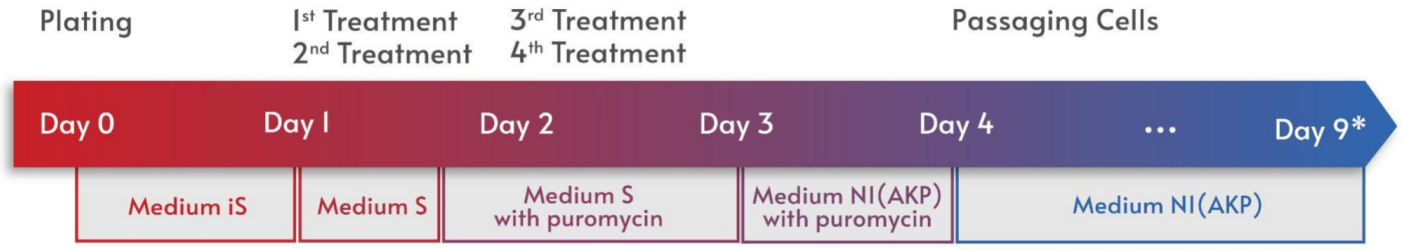
The Quick-Neuron™ Motor - mRNA Kit (Large) gives the best differentiation results when source human pluripotent stem cells (hPSCs) have been maintained in StemFit® Basic04, StemFlex™ Medium, or other similar culture media which enable the maintenance of cultures by single-cell passaging. This protocol also assumes that the source hPSCs are cultured in 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 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.
- 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 0.25 and 3 µg/ml. We achieved successful results using 1 µg/ml puromycin for 16 hours after the second QN treatment, and for 40-50 hours after the fourth QN treatment. 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



*From Day 9, 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.

Media Preparation

Important Note! For the best possible delivery of QN-mRNA-P into cells, we recommend Lipofectamine MessengerMax. If users prefer another transfection reagent, please make sure that the reagent provides a transfection efficiency of ≥80% prior to using this kit. QN-mRNA-P mixed with Lipofectamine MessengerMax must be immediately applied to cultures and cannot be stored.

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 1.5 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.

*Can be substituted with our Cell Dissociation Reagent (Solution D1), Catalog Number: CDR.

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.

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

1 mg/ml laminin stock solution (laminin)*

1. Thaw Laminin Mouse Protein, Natural and chill PBS at 4°C or on ice.
2. Mix the Laminin Mouse Protein, Natural and PBS to make the 1 mg/ml stock solution, hereafter referred to as laminin.
    - Laminin concentration varies by lot, so use the number specified on the vial or CoA when making your calculations.
3. Make aliquots of a convenient volume (e.g., 90 µl) and store at -20°C.

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

Diluted Component K

1. Thaw Component K at room temperature for 20-30 minutes.
2. Tap the tube of Component K three times and then briefly centrifuge it to bring the content down.
3. Prepare a diluted working solution of Component K by mixing together the following components.

Diluted Component K	Volume
DMSO	154 µl
Component K	12.5 µl

Medium N1(AKP)

1. Prepare Medium N1(AKP) using the reagents listed in the table below. Use the appropriate volume for the plate format you plan to use on Day 4 when passaging the cells.
    - Thaw Component N1 on ice for 20-30 minutes.
    - Warm all other reagents at room temperature for 20-30 minutes.
    - Store Medium N1(AKP) for up to 2 weeks at 4°C. The leftover reagents can be discarded or saved for other uses.

Medium N1(AKP) Reagents	Required Volume for each format	
	6-well plate or 24-well plate	96-well plate
DMEM/F12	21.6 ml	14,9 ml
Neurobasal	21.6 ml	14.9 ml
GlutaMAX	226 µl	155 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	450 µl	310 µl
Component N1	1.35 ml	930 µl
Component A	45.2 µl	31.2 µl
Component P	22.6 µl	15.6 µl
Diluted working solution of Component K	45.2 µl	31.2 µl

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 StemFlex.

Day 0

~8 hours

Note: This protocol assumes that Day 0 is a Monday and that user's hPSCs have already been used with a small size kit (Catalog number: MT-mRNA-S) so that users are familiar with the experimental process and have optimized conditions for their particular cells.

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	43.2 µl
Chilled PBS	13 ml

2. Add 2 ml diluted iMatrix-511 silk to each new well of a 6-well plate.
3. Incubate the plate at 37°C, 5% CO_2 for 2 hours (or 4°C overnight one day before Day 0).
4. Aspirate the supernatant from each well and add 500 µl PBS.
5. Incubate the plate at 37°C, 5% CO_2 until the hPSCs are ready for plating.

Plating

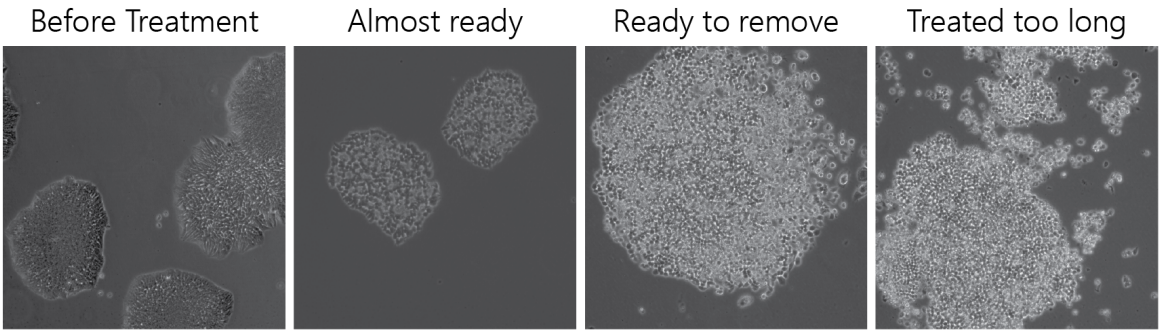
IMPORTANT! Source hPSC wells should be no more than 50-70% confluent thus requiring a minimum of 2 wells to begin differentiation.

1. Determine the number of wells required to get 3.6 x 10^6 cells from the source hPSC 6-well plate.
   NOTE: Cells will be plated in a new 6-well plate at 3 densities (0.5 x 10^6 cells, 0.55 x 10^6 cells, and 0.6 x 10^6 cells), with 2 wells per density.
2. Prepare Medium iS by mixing together the following components in a 15 ml conical tube.
    - Warm Medium S, iROCK, and Solution D1 at room temperature for at least 1 hour protected from light.
    - The rest of Medium S should be stored at 4°C for later use.

Medium iS Reagents	Required medium volume based on # of wells of a 6-well plate	
	2 wells	3 wells
Medium S	12.7 ml	15.5 ml
iROCK	12.7 µl	15.5 µl

3. Aspirate old medium from hPSC culture and add 1.5 ml of Medium iS to each well.
4. 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.
5. Aspirate old medium from hPSC culture and add 2 ml PBS to each well being harvested.
6. 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 4.

7. 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).



8. Carefully pipet out Solution D1 from the culture using a P1000 pipettor and add 1 ml Medium iS to the well.
    - Follow Steps 8-10 one well at a time if multiple wells are used.
9. Disperse the medium over the bottom surface of the well by pipetting 8-15 times to detach cells.
10. Using the same pipet tip, collect the cell suspension in a 15 ml tube.
11. Count cells and determine viability.
12. Take out the volume of the cell suspension needed for 2 wells of each cell density, according to the note in step 1, and include an extra 10% as a buffer. Place each in a new tube labeled with the corresponding density.
13. Bring the volume of the cell suspension in each tube up to 2.2 ml with Medium iS.
    - If the volume in the tube exceeds 2.2 ml, centrifuge the required volume of cell suspension at 200 x g for 4 minutes, remove the supernatant, and resuspend the pellet in 2.2 ml Medium iS.
14. Aspirate PBS from each coated well and add 1 ml cell suspension to each well.
15. Leave the plate flat at room temperature for 10 minutes.
16. Incubate the culture plate at 37°C, 5% CO_2 overnight.

Day 1

~8 hours

IMPORTANT! Observe all wells under a microscope and confirm that all 6 wells show 50-70% confluency for transfections with QN-mRNA-P. If there are any wells that do not fall within the range of confluence, do not use them.

First Treatment

1. Thaw 1 vial of QN-mRNA-P on ice for 30 minutes and warm Opti-MEM and Medium S at room temperature for 20-30 minutes.
2. Prepare QN by the following steps:
    - Prepare a 15 ml tube and a 1.5 ml tube with 825 µl Opti-MEM each. Label the 15 ml tube “Mix 1” and the 1.5 ml tube “Mix 2”.
    - Add 16.5 µl Lipofectamine MessengerMax (LMM) to the Mix 1 tube and mix by brief vortexing. Leave it at room temperature for 10 minutes (Mix 1). Keep the rest of LMM at 4°C for later treatments.
    - **IMPORTANT!** Immediately before 10 minutes pass (i.e., around 8 minutes), add the entire contents of the QN-mRNA-P vial to the other 1.5 ml tube with Opti-MEM (Mix 2). Mix by tapping 5 times. Do not vortex.
    - 10 minutes after mixing LMM with Opti-MEM, add Mix 2 into Mix 1, and pipet up and down 8-10 times. This mixture is called QN. Leave QN at room temperature for 5 minutes and no longer.

Mix 1 Reagents	Volume
Opti-MEM	825 µl
LMM	16.5 µl

Mix 2 Reagents	Volume
Opti-MEM	825 µl
QN-mRNA-P	~33 µl

3. Add 6.6 ml Medium S to QN and pipet up and down 2-3 times to mix.
4. Working with up to 2 wells at a time, aspirate the old medium out and add 1.25 ml of QN mixture to each well. Repeat until QN mixture has been added to all wells.

5. Incubate the culture plate at 37°C, 5% CO_2 for 2.5 hours.

Second Treatment

1. Pipet out the medium from each well using a P1000 pipettor and add 1 ml Medium S.
2. Incubate the culture plate at 37°C, 5% CO_2 for 2 hours.
    - Put 1 vial of QN-mRNA-P on ice when the incubation above reaches 1.5 hours and leave it for 30 minutes.
3. Repeat Steps 2-5 of the previous "First Treatment" section.

Medium Change and Drug Selection

1. Transfer 10 ml Medium S into a tube and add puromycin to it at the predetermined optimal concentration (see earlier section on "Drug Selection").
2. Pipet out the medium from each well and add 1.5 ml Medium S with puromycin.
3. Incubate the culture plate at 37°C, 5% CO_2 overnight.

Day 2

~8 hours

IMPORTANT! Observe the QN-treated cultures to make sure that they are reaching confluency (≥90%). If the cultures are <50% confluent and show signs of cell death (e.g., many floating cells), users should skip the third and fourth treatments on Day 2 and proceed directly to the steps described in Day 3. The protocol will then be accelerated by one day.

Third Treatment

1. Thaw 1 vial of QN-mRNA-P on ice for 30 minutes and warm Opti-MEM and Medium S at room temperature for 20-30 minutes.
2. Repeat Steps 2-5 of the previous "First Treatment" section.

Fourth Treatment

1. Pipet out the medium from each well and add 1 ml Medium S.
2. Incubate the culture plate at 37°C, 5% CO_2 for 2 hours.
    - Put 1 vial of QN-mRNA-P on ice when the incubation above reaches 1.5 hours and leave it for 30 minutes.
3. Repeat Steps 2-5 of the previous "First Treatment" section.

Medium Change and Drug Selection

1. Transfer 10 ml Medium S 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.
2. Pipet out the medium from each well and add 1.5 ml Medium S with puromycin.
3. Incubate the culture plate at 37°C, 5% CO_2 overnight.

Day 3

< 1 hour

Medium Change and Drug Selection

1. Warm Medium N1(AKP) at room temperature for 20-30 minutes.
2. Transfer 10 ml Medium N1(AKP) into a tube and add puromycin to it at the predetermined optimal concentration (see earlier section on "Drug Selection").
3. Pipet out the medium from each well and add 1.5 ml Medium N1(AKP) with puromycin.

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

Day 4

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. Surplus cells can be frozen according to the instructions 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.

*Ornithine and laminin can be substituted with Coating Materials B and C, respectively, from our Neuron Coating Solutions, Catalog Number: NCS.

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 N1(AKP)	2.5 ml	700 µl	85 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 Preparation

1. Warm Medium N1(AKP) at room temperature for 20-30 minutes.
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 N1(AKP) in the volume specified in Table A above.
4. Incubate the plate at 37°C, 5% CO_2 until cells are ready for plating.

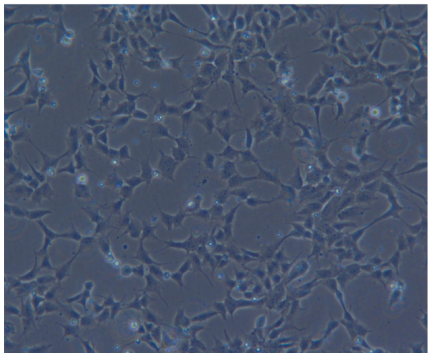
Passaging Cells

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

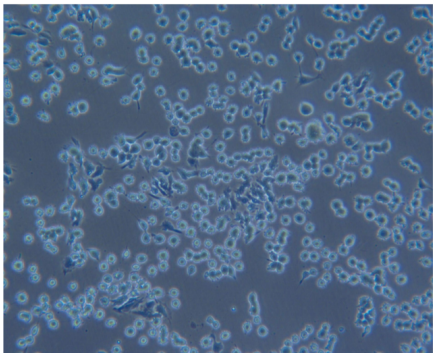
1. Make sure that Solution D1 is at room temperature for at least 1 hour before use.
2. Working one well at a time, pipet out the old medium from each well and add 1 ml PBS to the well.
3. Working one well at a time, pipet out the PBS from each well and add 300 µl Solution D1.
4. Rock the plate 3 times to spread the Solution D1 evenly.
5. Incubate the cultures at 37°C, 5% CO_2 for 3 minutes.
6. Working one well at a time, carefully pipet out Solution D1 from each well and add 750 µl Medium N1(AKP).

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

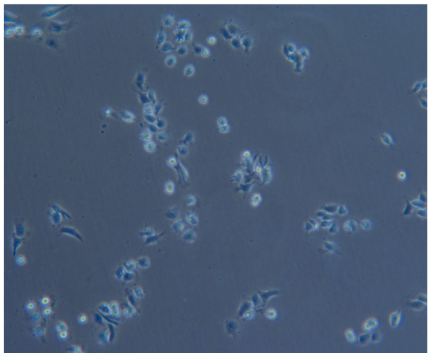
Before Solution D1 treatment



During Solution D1 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. 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. Gently pipet the cell suspension up and down up to 5 times to break the cell aggregates using a P1000 pipettor. Excessive pipetting can damage the already-suspended neuronal cells.
11. Count cells and determine viability.
12. Prepare specified amounts of a 1 x 10^6 live cells/ml cell suspension using Medium N1(AKP) 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.
13. Add cell suspension to the center of each well. Since each well already has Medium N1(AKP), 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% buffer	3.3 ml	2.64 ml	1.6 ml
Volume of cell suspension/well	500 µl	100 µl	15 µl
Total volume/well • Medium N1(AKP) + cell suspension	3 ml	800 µl	100 µl

14. Incubate the culture plate 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 using a P200 or P1000 pipettor 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)	1.5 ml	400 µl	50 µl

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

Day 9

Assay or Continuous Maturation

- From Day 9, 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 - mRNA Kit can be confirmed with the markers TUBB3. ChAT, and HB9.

Appendix

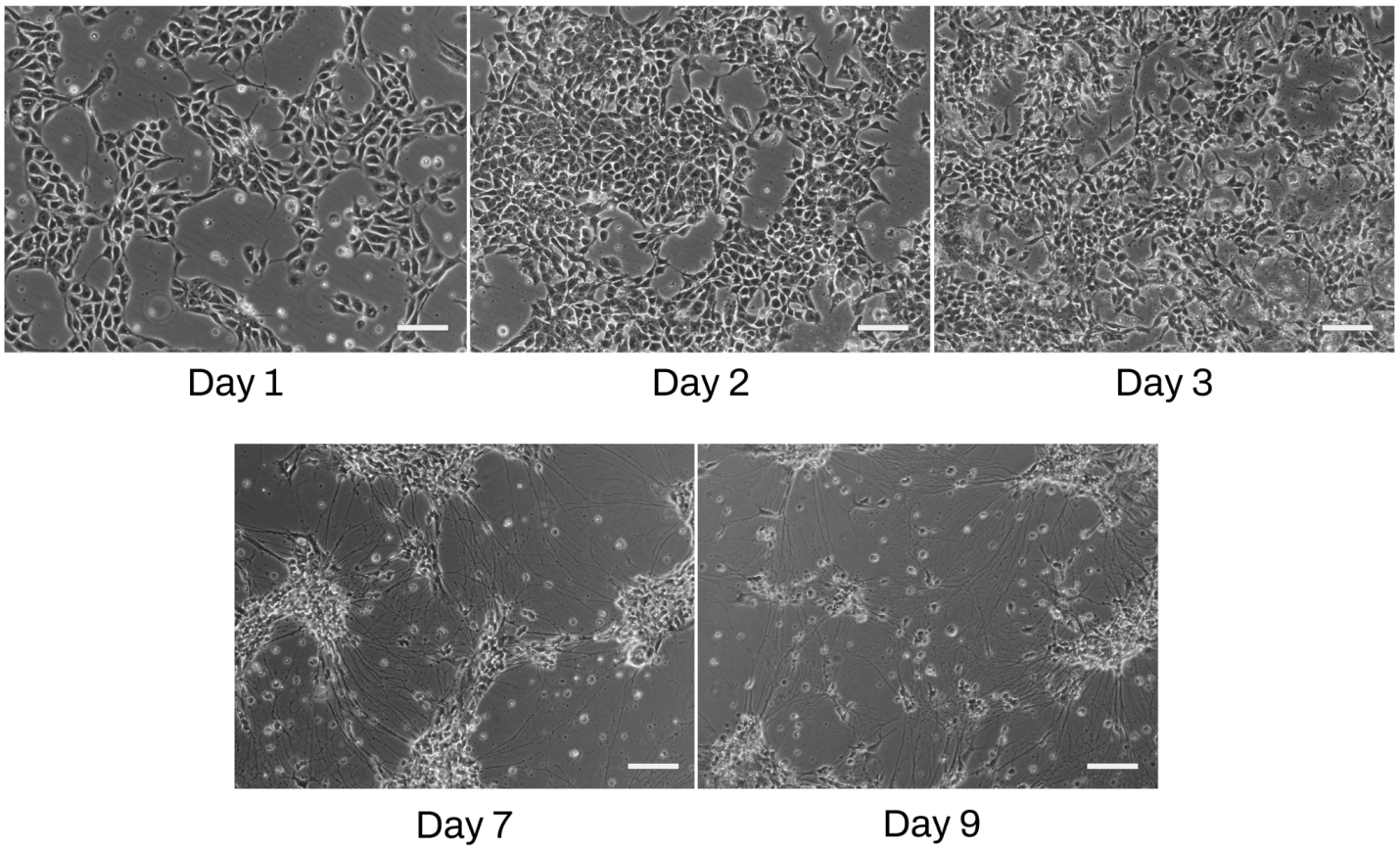

Figure 1. Representative phase contrast images of Quick-Neuron™ Motor - mRNA cell cultures on days 1-9 post-differentiation (scale bar = 100 µm).

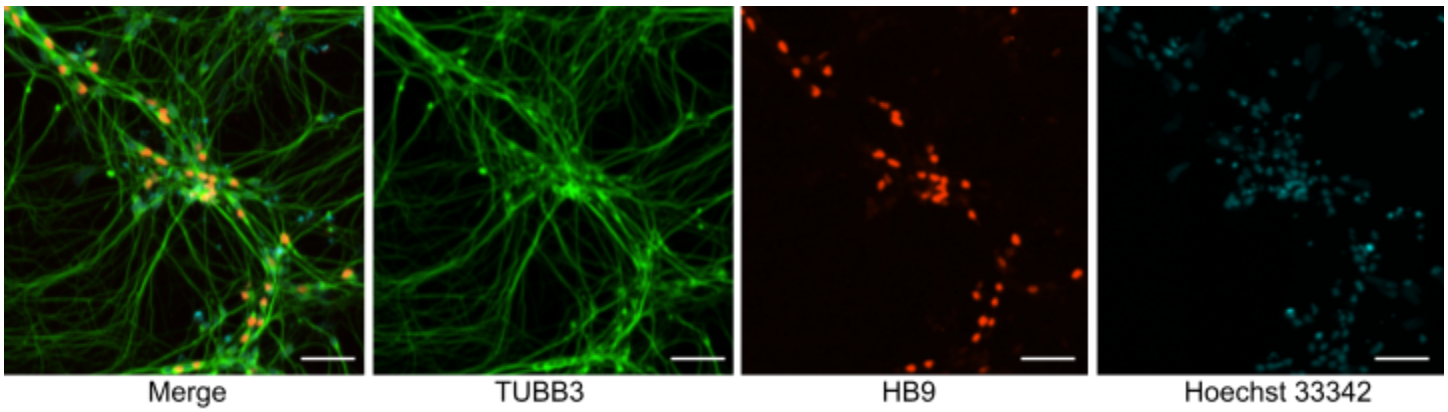

Figure 2. Immunofluorescent staining of Quick-Neuron™ Motor - mRNA cell culture shows typical neurite growth and expression of the pan-neuronal marker TUBB3 as well as HB9 on day 9 post-differentiation (scale bars = 50 µm). Staining conditions: Anti- ß-III tubulin monoclonal antibody (Cell Signaling 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 (Developmental Studies Hybridoma Bank, Catalog Number: 81.5C10, 1:50 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.