User Guide

Quick-Neuron™ Cholinergic - mRNA Kit (Small)

Catalog Number: CH-mRNA-S

Introduction

The Quick-Neuron™ Cholinergic - mRNA Kit (Small) facilitates rapid and efficient differentiation of human iPS or ES cells into a population of cholinergic neurons in just 7 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™ 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 - mRNA 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 mRNA Kit (Large), Catalog Number: CH-mRNA-L
Quick-Neuron™ Cholinergic - Human iPSC-derived Neurons, Catalog Number: CH-mRNA-CW
Quick-Neuron™ Cholinergic - Maintenance Medium, Catalog Number: CH-MM

Kit Contents

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

Contents	Volume	Storage
QN-mRNA-P	4 x 9 µl	-80°C
Component N1	830 µl	-20°C or -80°C
Component P	14 µl	-20°C or -80°C
Component A	38 µl	-20°C or -80°C
Coating Material A	15.7 µl	-20°C or -80°C

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

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
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
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
12-mm glass coverslips***	VWR	89167-106
100% Ethanol	Multiple Vendors	

* 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.
*** Glass coverslips from different brands might have different effects on the growth of neural cells. We recommend using glass coverslips from Chemglass for the best results.

Source hPSC Culture Conditions

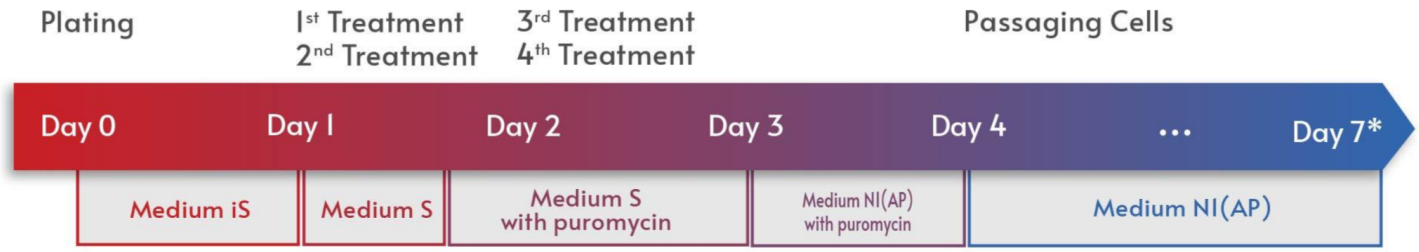
The Quick-Neuron™ Cholinergic - mRNA Kit (Small) gives the best differentiation results when source human pluripotent stem cells (hPSCs) have been maintained in StemFit® Basic04, StemFlex™ Medium, or other similar culture media which enable the maintenance of cultures by single-cell passaging. This protocol 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 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.

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.

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.

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.

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

0.002% Poly-L-Ornithine solution (ornithine)*

1. Take 500 µl 0.01% Poly-L-Ornithine solution and mix it with 2.5 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., 15 µl) and store at -20°C.

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

Medium N1(AP)

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

Medium N1(AP) Reagents	Volume
DMEM/F12	5.8 ml
Neurobasal	5.8 ml
GlutaMAX	60 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	120 µl
Component N1	360 µl
Component A	12 µl
Component P	6 µl

Day 0

~8 hours

Plate Preparation

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

Diluted Coating Material A Reagents	Volume
Coating Material A	11.6 µl
Chilled PBS	3.5 ml

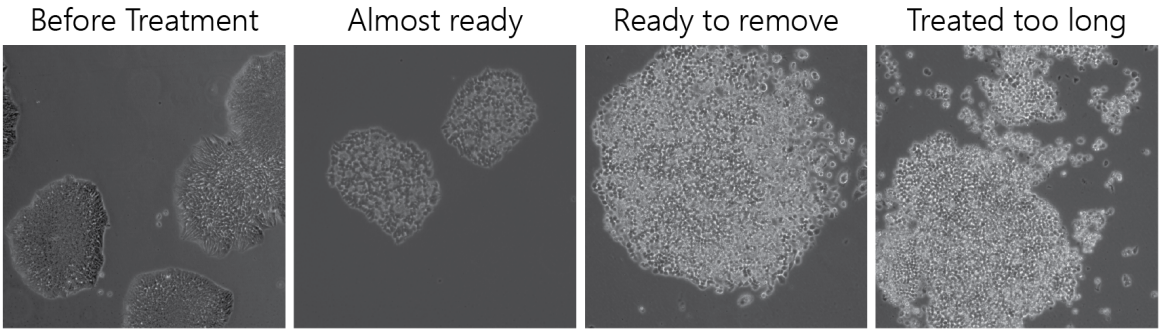
2. Add 400 µl diluted Coating Material A to each new well of 8 wells.
3. Incubate the plate at 37°C, 5% CO_2 for 2 hours (or 4°C overnight one day before Day 0).
4. Aspirate the supernatant from each well and add 500 µl PBS.
5. Incubate the plate at 37°C, 5% CO_2 until the hPSCs are ready for plating.

Plating

1. 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	Volume
Medium S	5.5 ml
iROCK	5.5 µl

2. Aspirate old medium from hPSC culture and add 2 ml PBS to each well being harvested.
3. 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.
4. 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).



5. 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.
6. Disperse the medium over the bottom surface of the well by pipetting 8-15 times to detach cells.
7. Using the same pipet tip, collect the cell suspension in a 15 ml tube.
8. Count cells and determine viability.

IMPORTANT! In this protocol, users will plate the hPSCs into 8 wells with 500 µl Medium iS per well. However, if users know the best plating density already, prepare a cell suspension enough to plate 5 wells (4 wells plus 1 extra to account for the pipetting/plating error). We recommend aiming for 50-70% initial cell confluency on Day 1, with 20-50 cells per colony. Our data indicate that cell counts ranging from 1.0 - 2.4 x 10^5 viable cells per well are suitable. Cell count may vary based on cell health and the method used for cell counting.

If the confluency on Day 1 is

- above the target range, the differentiation efficiency will decrease.
- below the target range, more cell death will be observed.
- For first time users, we recommend plating the following numbers of cells into each of 8 wells: 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2 and 2.4 x 10^5 cells. On Day 1, 4 wells showing 50-70% confluency should be selected for transfection.

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9. Take out the volume of the cell suspension needed for each cell density, according to the note above, and place each in a new tube labeled with the corresponding density.
10. Bring the volume of the cell suspension in each tube up to 500 µl with Medium iS.
    - If the volume in the tube exceeds 500 µl, centrifuge the required volume of cell suspension at 200 x g for 4 minutes, remove the supernatant, and resuspend the pellet in 500 µl Medium iS.
11. Aspirate PBS from each coated well and add 500 µl cell suspension to each well using a P1000 pipettor.
12. Leave the plate flat at room temperature for 10 minutes.
13. 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 at least 4 wells are at 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 220 µl Opti-MEM each. Label the 15 ml tube “Mix 1” and the 1.5 ml tube “Mix 2”.
    - Add 4.5 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	220 µl
LMM	4.5 µl

Mix 2 Reagents	Volume
Opti-MEM	220 µl
QN-mRNA-P	~9 µl

3. Add 1.75 ml Medium S to QN (for a final volume of 2.2 ml) 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 500 µl 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 500 µl 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 2 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 500 µl 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 (≥70%). If the cultures are <50% confluent and show significant 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 “Medium Change" section. Note: Some cell death is normally observed due to the puromycin selection.

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 using a P1000 pipettor and add 500 µl 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 2 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 using a P1000 pipettor and add 500 µl 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(AP) at room temperature for 20-30 minutes.
2. Transfer 2 ml Medium N1(AP) into a tube and add puromycin to it at the predetermined optimal concentration (see earlier section on "Drug Selection").
3. Pipet out the old medium from each well using a P1000 pipettor and** add 500 µl Medium N1(AP) with puromycin.
    - **IMPORTANT!** For medium change, 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 500 µl Medium N1(AP) with puromycin.
4. Incubate the culture plate at 37°C, 5% CO_2 overnight.

Day 4

4-6 hours

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 1 and skip Steps 2 and 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. If the well being passaged is over 70% confluent, prepare two coverslips or wells for it.

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 5 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.

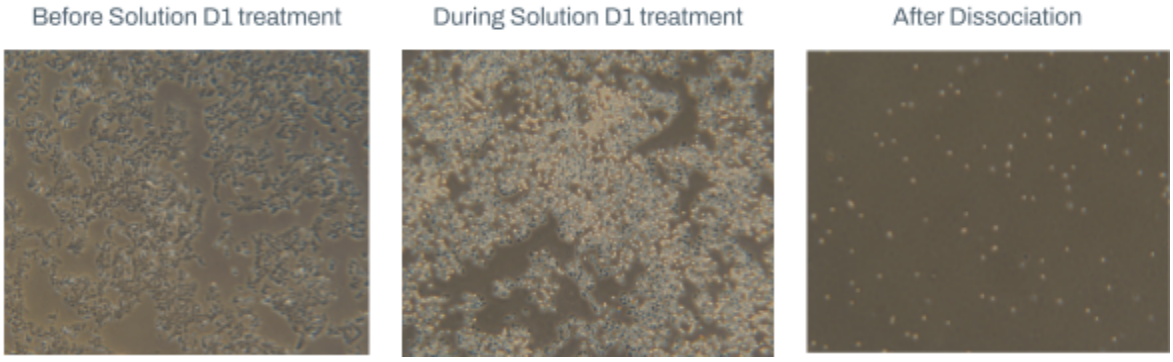
7. Add 15 µl laminin to the chilled PBS. Mix well.
    - All PBS washes should be done dropwise and with room temperature PBS. Chilled PBS is only for the coating step.
8. Aspirate the supernatant from each coverslip/well and add 500 µl PBS.
9. Repeat Step 8.
10. Aspirate PBS from each coverslip/well 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 using a P1000 pipettor.
14. Rinse with 500 µl PBS.
15. Pipet out most of the PBS, but not all, from each well using a P1000 pipettor.
16. Repeat Steps 14-15.
17. Add 600 µl Medium N1(AP) to each well or coverslip using a P1000 pipettor.
18. Incubate the plate at 37°C, 5% CO_2 until cells are ready for plating.

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

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 using a P1000 pipettor and add 500 µl PBS.
3. Pipet out the PBS from the well using a P1000 pipettor 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 800 µl. Please keep in mind that this protocol supports the maintenance of up to 8 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 for 3 days.

Medium Change (Unpassaged Cultures Only)

1. Pipet out the old medium from each unpassaged well using a P1000 pipettor 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.
2. 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 - mRNA Kit can be confirmed with the markers TUBB3 and ChAT.

Appendix

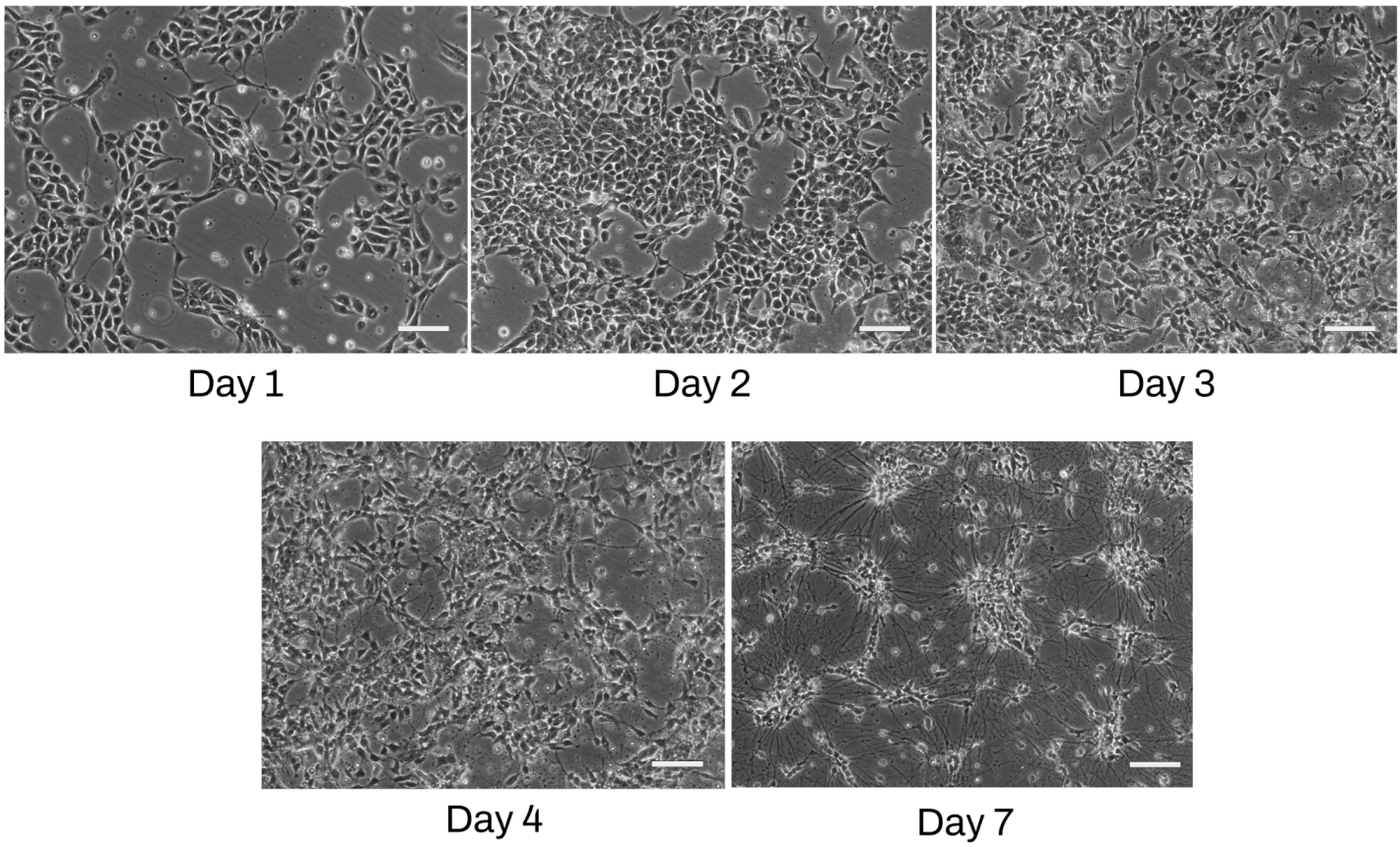


Figure 1. Representative phase contrast images of Quick-Neuron™ Cholinergic - mRNA cell cultures on days 1-7 post-differentiation. 2.0 x 10^5 cells were plated in this well on day 0 (scale bars = 100 µm).

Figure 2. Immunofluorescent staining of Quick-Neuron™ Cholinergic - mRNA cell culture shows typical neurite growth and expression of the pan-neuronal marker TUBB3 and CHAT on day 7 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-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.