User Guide

ELIXIRGEN SCIENTIFIC

Quick-Neuron™ GABAergic - mRNA Kit (Small)

Catalog Number: GA-mRNA-S

Introduction

The Quick-Neuron™ GABAergic - mRNA Kit (Small) facilitates rapid and efficient differentiation of human iPS or ES cells into a population of primarily GABAergic neurons in just 10 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™ GABAergic differentiated cell cultures display typical neurite outgrowth and express a neuronal markers, such as the pan-neuronal marker tubulin beta 3 class III (TUBB3) and the GABAergic marker glutamic acid decarboxylase (GAD67). When handled and maintained according to the instructions in this user guide, GABAergic neurons are viable long-term and are suitable for a variety of characterization and neurotoxicity assays.

Scale: The Quick-Neuron™ GABAergic - mRNA Kit (Small) contains a set of reagents for use with a total 4 wells of a 24-well plate.

Related Products: Quick-Neuron™ GABAergic - Human iPSC-derived Neurons, Catalog Number: GA-mRNA-CW
Quick-Neuron™ GABAergic - Maintenance Medium, Catalog Number: GA-MM

Kit Contents

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

Reagents	Volume	Storage
QNG1-mRNA	9 µl (3.38 µg) x 2	-80°C
QNG2-mRNA	9 µl (2.25 µg) x 2	-80°C
Component N	840 µl	-20°C or -80°C
Component P	14 µl	-20°C or -80°C
Component G1	10 µl	-20°C or -80°C
Component G2	16 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.).

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 contact us at cs@elixirgensci.com or call +1 (443) 869-5420 (M-F 9 am-5 pm EST).

Last revised: January 22, 2021

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 (100x)	ThermoFisher	35050061
Penicillin-Streptomycin	ThermoFisher	15140122
StemFit Basic04 Complete Type, or StemFit Basic04, or StemFit Basic02, or StemFlex Medium	Elixirgen Scientific Elixirgen Scientific Elixirgen Scientific ThermoFisher	ASB04-C, or ASB04-F, or ASB02, or A3349401
TrypLE Select Enzyme (1X)*	ThermoFisher	12563011
0.02% EDTA in DPBS	Sigma-Aldrich	E8008-100ML
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
12-mm glass coverslips***	VWR	89167-106
100% Ethanol	Multiple vendors	

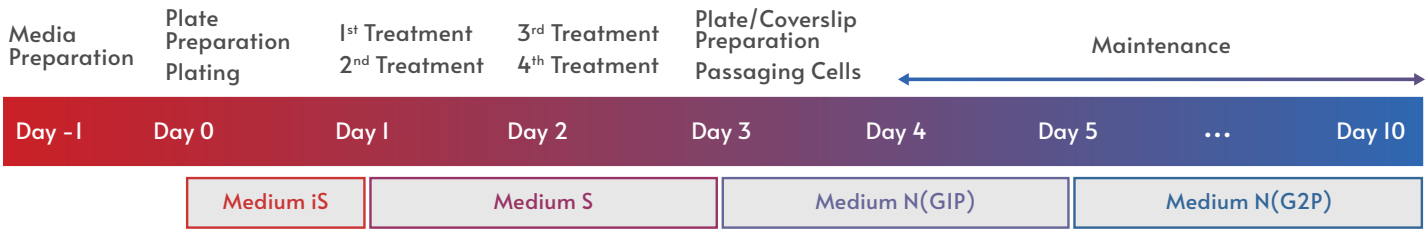
* Can be substituted with our Cell Dissociation Reagent (Solution D1), Catalog Number: CDR.
** Can be substituted with our Neuron Coating Solutions (Coating Materials B and C), Catalog Number: NCS.
*** Glass coverslips from different brands might have different effects on the growth of neural cells. We recommend using glass coverslips from Chemglass for the best results.

Source hPSC Culture Conditions

The Quick-Neuron™ GABAergic - mRNA Kit (Small) gives the best differentiation results when source 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 a 35-mm culture dish or one well of a 6-well plate. If iMatrix-511 silk is routinely used as a coating substrate, prepare one culture dish or well precoated with 0.25 µg/cm² 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, NI511S).
- Differentiation should not be performed until the cells are at least 14 days post-thaw.

Workflow



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

Media Preparation

IMPORTANT! For the best possible delivery of QNG1-mRNA and QNG2-mRNA 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. QNG1-mRNA or QNG2-mRNA 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 is hereafter referred to as iROCK and 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 is hereafter referred to as Medium S and can be stored at -80°C.

*Medium S can be substituted with StemFit Basic02/04 (with bFGF added) or 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 300 µl 0.01% Poly-L-Ornithine solution and mix it with 1.2 ml PBS.
2. Store the resulting 0.002% Poly-L-Ornithine solution (hereafter referred to as ornithine) for up to 2 weeks at 4°C.

*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 N(P)

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

Medium N(P) Reagents	Volume
DMEM/F12	8.6 ml
Neurobasal Medium	8.6 ml
200 mM Glutamax (100x)	89 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	179 µl
Component N	554 µl
Component P	9 µl

Day 0

~8 hr

Plate Preparation

1. Prepare diluted Coating Material A by mixing together the following components in a 15 ml conical tube.
   - Thaw Coating Material A on ice for 20-30 minutes (or at 4°C overnight one day before Day 0).
   - Make sure chilled PBS is used for this mixture.

Diluted Coating Material A Reagents	Volume
Coating Material A	11.9 µl
Chilled PBS	3.6 ml

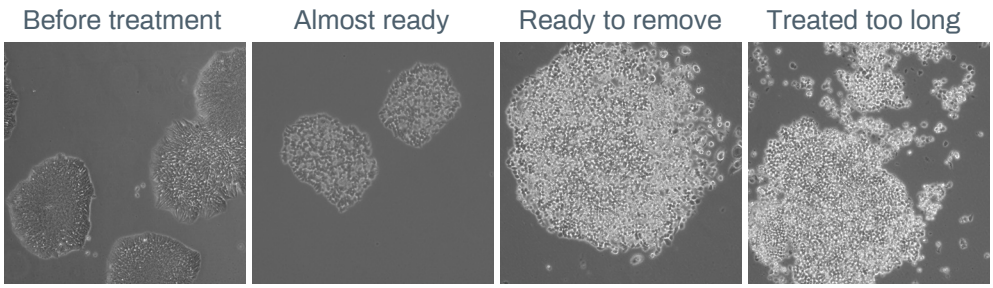
2. Add 400 µl diluted Coating Material A to each new well of 8 wells.
3. Incubate the plate at 37°C, 5% CO_2 for 2 hours (or 4°C overnight one day before Day 0).
4. Aspirate the supernatant from each well and add 500 µl PBS.
5. Incubate the plate at 37°C, 5% CO_2 until the hPSCs are ready for plating.

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. Tap the Solution D1 tube 5 times with a finger and centrifuge at maximum speed for 1 minute.
3. Aspirate old medium from hPSC culture and add 2 ml PBS.
4. 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.
5. Incubate the plate at 37°C, 5% CO_2 for 5 minutes. If all the cells are not rounded under a microscope, incubate at 37°C, 5% CO_2 for up to 5 more minutes in 1-2 minute increments (see images below).



6. Carefully pipet out Solution D1 from the culture using a P1000 pipettor and add 1 ml Medium iS to the well.
7. Disperse the medium over the bottom surface of the well by pipetting 8-15 times to detach cells.
8. Using the same pipette tip, collect the cell suspension in a 1.5 ml tube.

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.

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.
    - 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 200xg for 4 minutes, remove the supernatant, and resuspend the pellet in 500 µl Medium iS.
10. Aspirate PBS from each coated well and add 500 µl cell suspension to each well using a P1000 pipettor.
11. Place the plate in the incubator and rock it front to back and side to side for 15 seconds to make sure that the cells are evenly distributed.
12. Incubate the cultures at 37°C, 5% CO_2 overnight.

Day 1

~8 hr

IMPORTANT! Observe all wells under a microscope and select any 4 wells that show 50-70% confluency for transfections with QNG1-mRNA. If none of the wells falls within the range of confluence, do not proceed.

First Treatment

1. Thaw 1 vial of QNG1-mRNA on ice for 30 minutes and warm Opti-MEM and Medium S at room temperature for 20-30 minutes.
2. Aspirate the old medium from one well at a time and add 500 µl Medium S. Repeat this process for each of the selected 4 wells. Do not let cells dry out during the medium change.
3. Incubate the cultures at 37°C, 5% CO_2 for at least 10 minutes.
4. Prepare QNG1 by the following steps:
    - Prepare two 1.5 ml tubes with 220 µl Opti-MEM each.
    - Add 9 µl Lipofectamine MessengerMax (LMM) to one of the 1.5 ml tubes 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 9 µl QNG1-mRNA 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 QNG1. Leave QNG1 at room temperature for 5 minutes and no longer.

Mix 1 Reagents	Volume
Opti-MEM	220 µl
LMM	9 µl

Mix 2 Reagents	Volume
Opti-MEM	220 µl
QNG1-mRNA	9 µl

5. Add 104 µl QNG1 to each culture by adding QNG1 dropwise with one hand while gently shaking the plate with the other hand.
6. Rock the plate front to back and side to side for 15 seconds to make sure that QNG1 is evenly distributed in the cultures.
7. Incubate the cultures at 37°C, 5% CO_2 for 2.5-3 hours.

Second Treatment

1. Pipet out the medium from each well using a P1000 pipettor and add 500 µl Medium S.
2. Incubate the cultures at 37°C, 5% CO_2 for 2 hours.
    - Put 1 vial of QNG1-mRNA on ice when the incubation above reaches 1.5 hours and leave it for 30 minutes.
3. Repeat Steps 4-7 of the previous "First Treatment" section.
4. Pipet out the medium from each well using a P1000 pipettor and add 500 µl Medium S.
5. Incubate the cultures at 37°C, 5% CO_2 overnight.

Day 2

~8 hr

IMPORTANT! Observe the QNG1-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 to maintain cultures described in Day 3. The protocol will then be accelerated by one day. First time users, who have plated cells at different densities, should proceed to the third and fourth treatments for only two wells with higher plating densities. For the other two wells (with lower densities), 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 for the lower density wells.

Third Treatment

1. Thaw 1 vial of QNG2-mRNA on ice for 30 minutes and warm Opti-MEM and Medium S at room temperature for 20-30 minutes.
2. Aspirate the old medium from one well at a time and add 500 µl Medium S to the well. Repeat this process for the rest of the treated wells. Do not let cells dry out during the medium change.
3. Pipet out old medium from the other 4 wells that have not been treated.
4. Incubate the cultures at 37°C, 5% CO_2 for at least 10 minutes.
5. Prepare QNG2 by the following steps:
    - Prepare two 1.5 ml tubes with 220 µl Opti-MEM each.
    - Add 9 µl LMM to one of the 1.5 ml tubes 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 9 µl QNG2-mRNA in 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 it up and down 8-10 times. This mixture is called QNG2. Leave QNG2 at room temperature for 5 minutes and no longer.

Mix 1 Reagents	Volume
Opti-MEM	220 µl
LMM	9 µl

Mix 2 Reagents	Volume
Opti-MEM	220 µl
QNG2-mRNA	9 µl

6. Add 104 µl QNG2 dropwise to each culture with one hand while gently shaking the plate with the other hand.
7. Rock the plate front to back and side to side for 15 seconds to make sure that QNG2 is evenly distributed in the cultures.
8. Incubate the cultures at 37°C, 5% CO_2 for 2.5-3 hours.

Fourth Treatment

1. Pipet out the medium from each well using a P1000 pipettor and add 500 µl Medium S.
2. Incubate the cultures at 37°C, 5% CO_2 for 2 hours.
    - Put 1 vial of QNG2-mRNA on ice when the incubation above reaches 1.5 hours and leave it for 30 minutes.
3. Repeat Steps 5-8 of the previous "Third Treatment" section.
4. Pipet out the medium from each well using a P1000 pipettor and add 500 µl Medium S.
5. Incubate the cultures at 37°C, 5% CO_2 overnight.

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.

1. Warm Medium N(G1P) and thaw ornithine* at room temperature for 20-30 minutes.
2. (Coverslip only) Soak 12-mm glass coverslips and the tips of forceps in 100% ethanol for 3 minutes.
3. (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.
4. Vortex ornithine briefly and centrifuge it at a maximum speed for 1 minute.
5. 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.
6. Incubate the plate at 37°C, 5% CO_2 for at least 2 hours (or at 4°C overnight one day before plating).
7. Thaw laminin* and chill 1.5 mL PBS on ice for 20-30 minutes.
8. 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.
9. Aspirate the supernatant from each coverslip/well and add 500 µl PBS.
10. Repeat Step 9.
11. Aspirate PBS from each coverslip/well and add 300 µl diluted laminin.
12. Incubate the plate at 37°C, 5% CO_2 for at least 2 hours or until cells are ready for plating.
13. While the plate is incubating, prepare Medium N(G1P) using the volumes indicated in the table below.
    - Warm Medium N(P) at room temperature for 20-30 minutes.
    - Thaw Component G1 on ice for 20-30 minutes. Spin down before use.
    - Keep the rest Medium N(P) at 4°C for its use on Day 4.

Medium N(G1P) Reagents	Volume
Medium N(P)	4.5 ml
Component G1	9 µl

14. After the laminin incubation, pipet out most of the supernatant, but not all, from each coverslip or well using a P1000 pipettor.
15. Rinse with 500 µl PBS.
16. Pipet out most of the PBS, but not all, from each well using a P1000 pipettor.
17. Repeat Steps 15-16
18. Add 300 µl Medium N(G1P) to each well or coverslip using a P1000 pipettor.
19. 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.

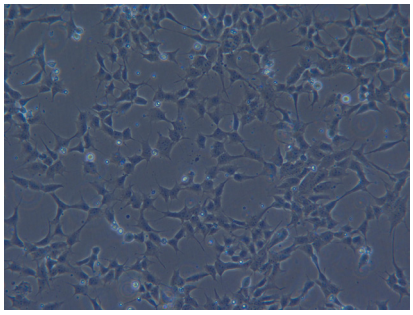
Passaging Cells

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

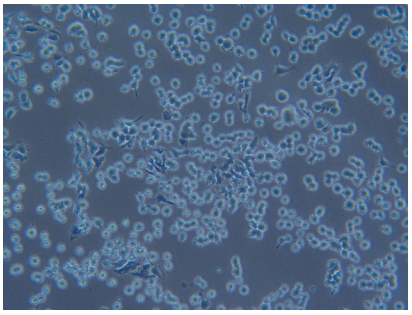
1. Make sure that Solution D1 is at room temperature for at least 1 hour before use.
2. Pipet out the old medium from each well using a P1000 pipettor and add 500 µl PBS.
3. Pipet out the PBS from each well using a P1000 pipettor and add 80 µl Solution D1.
4. Rock the plate 3 times to spread the Solution D1 evenly.
5. Incubate the cultures at 37°C, 5% CO_2 for 3 minutes.
6. Carefully pipet out Solution D1 from each well using a P200 pipettor and add 200 µl Medium N(G1P).

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.

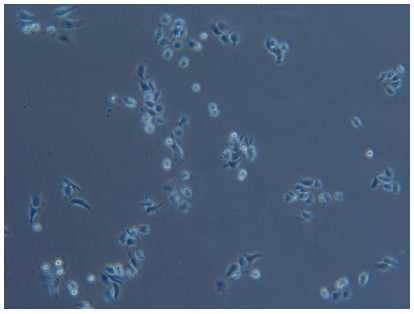
Before Solution DI treatment



During Solution DI treatment



After dissociation



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 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 each 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 4, 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 N(G1P) after the cell suspension is transferred to bring the total volume up to 200 µl. Please keep in mind that this protocol supports the maintenance of up to 5 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 right at the center of the coverslip.
11. Incubate the cultures at 37°C, 5% CO_2 for 1 hour.
12. Observe each well under the microscope to confirm that the cells are attached to the well or glass coverslip.
13. Incubate the cultures at 37°C, 5% CO_2 overnight.

Maintenance (Unpassaged Cultures Only)

1. Pipet out the old medium from each unpassaged well using a P1000 pipettor and add 500 µl Medium N(G1P).
    - **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 cultures at 37°C, 5% CO_2 overnight.

Day 4

<1 hr

IMPORTANT! It is optional, but recommended, to include the below PBS wash if cell death/floating cells are observed.

Maintenance

1. Warm Medium N(G1P) at room temperature for 20-30 minutes.
2. Pipet out the old medium from each well using a P1000 pipettor and* add 500 µl Medium N(G1P).
    - **IMPORTANT!** For the wells that are not passaged, the attachment of cells is weaker and so a small volume of the old medium (i.e., just enough to cover the surface of the well) should be left. The fresh medium should be applied along the wall of the well very slowly.
    - *(Optional) Slowly add 500 µl PBS alongside the wall of each well to avoid lifting attached cells. Gently pipet out PBS before adding 500 µl Medium N(G1P).
3. Incubate the cultures at 37°C, 5% CO_2 overnight.

Day 5

~1 hr

IMPORTANT! It is optional, but recommended, to include the below PBS wash if cell death/floating cells are observed.

Maintenance

1. Prepare Medium N(G2P) by mixing together the following components in a 15 ml conical tube.
   - Warm Medium N(P) at room temperature for 20-30 minutes.
   - Thaw Component G2 on ice for 20-30 minutes.
   - Keep the rest of Medium N(G2P) at 4°C

Medium N(G2P) Reagents	Volume
Medium N(P)	11 ml
Component G2	11 µl

2. Pipet out the old medium from each well using a P1000 pipettor and* add 800 µl Medium N(G2P).
   - **IMPORTANT!** For the wells that are not passaged, the attachment of cells is weaker and so a small volume of the old medium (i.e., just enough to cover the surface of the well) should be left. The fresh medium should be applied along the wall of the well very slowly.
   - *(Optional) Slowly add 500 µl PBS alongside the wall of each well to avoid lifting attached cells. Gently pipet out PBS before adding 800 µl Medium N(G2P).
3. Incubate the cultures at 37°C, 5% CO_2 for 2 days.

Day 7

<1 hr

IMPORTANT! It is optional, but recommended, to include the below PBS wash if cell death/floating cells are observed.

Maintenance

1. Warm Medium N(G2P) at room temperature for 20-30 minutes.
2. Pipet out the old medium from each well using a P1000 pipettor and* add 800 µl Medium N(G2P).
   - **IMPORTANT!** For the wells that are not passaged, the attachment of cells is weaker and so a small volume of the old medium (i.e., just enough to cover the surface of the well) should be left. The fresh medium should be applied along the wall of the well very slowly.
   - *(Optional) Slowly add 500 µl PBS alongside the wall of each well to avoid lifting attached cells. Gently pipet out PBS before adding 800 µl Medium N(G2P).
3. Incubate the cultures at 37°C, 5% CO_2 for 2 days.

Day 9

<1 hr

Maintenance

1. Warm Medium N(G2P) at room temperature for 20-30 minutes.
2. Pipet out the old medium from each well using a P1000 pipettor and add 400 µl Medium N(G2P).
   - **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.
3. Incubate the cultures at 37°C, 5% CO_2 overnight.

Day 10

Assay or Continuous Maturation

Differentiated neurons can be observed on Day 5. 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™ GABAergic - Maintenance Medium, Catalog Number: GA-MM. Differentiation into GABAergic neurons after using the Quick-Neuron™ GABAergic - mRNA Kit can be confirmed with anti-TUBB3 (tubulin beta 3 class III, a global marker for neurons) and anti-GAD67 (glutamic acid decarboxylase, a GABAergic neuron marker) antibodies.

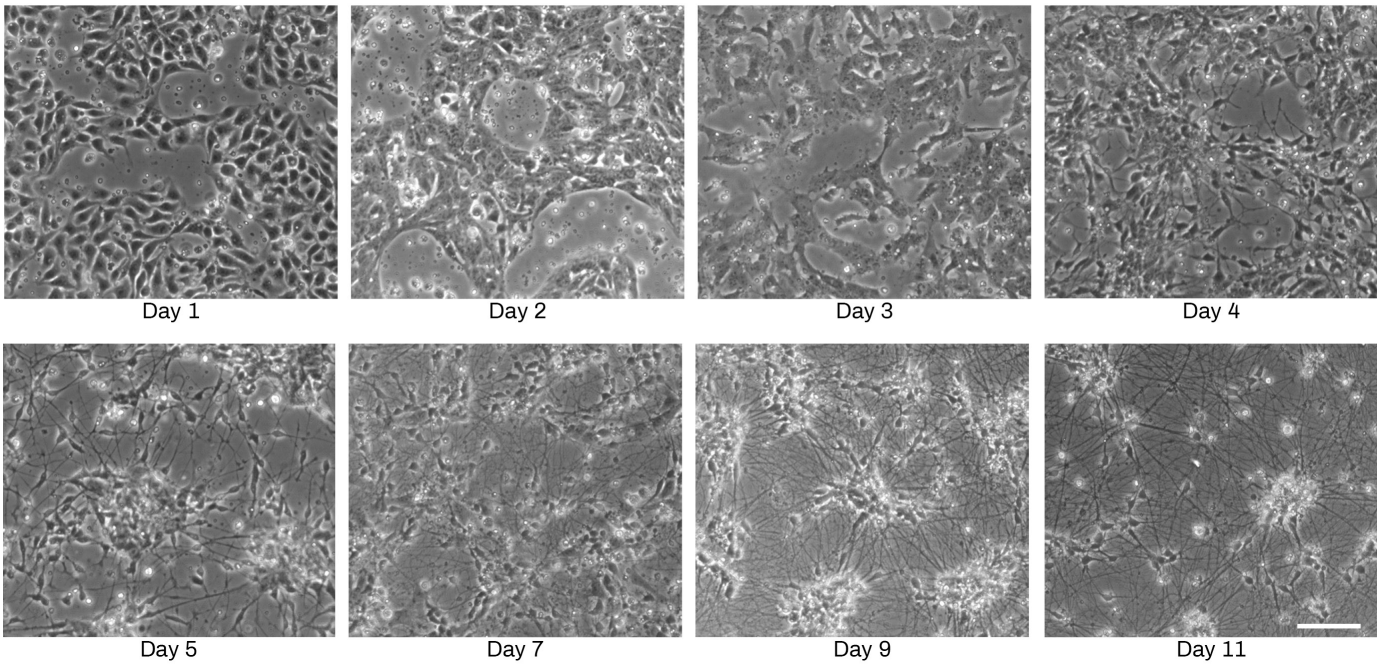


Figure 1. Representative images of Quick-Neuron™ GABAergic - mRNA Kit cell cultures on days 1-11 post-differentiation (scale bar = 100 µm).

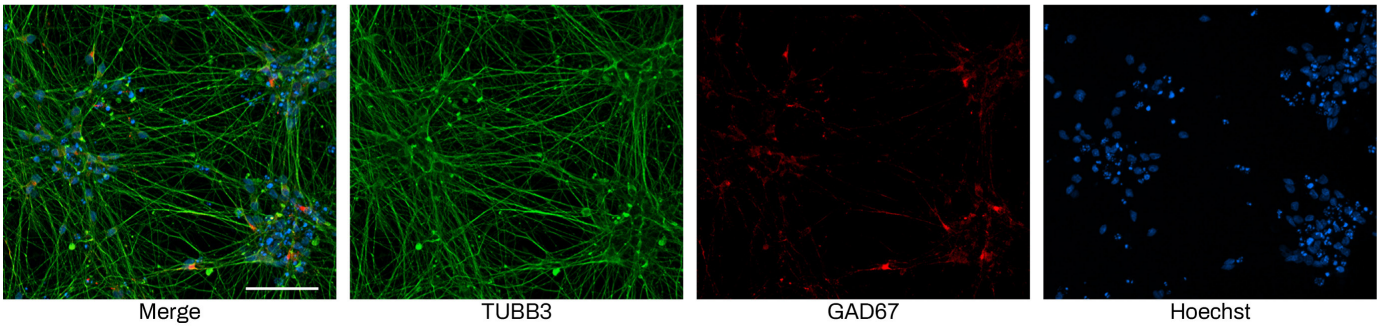


Figure 2. Immunofluorescent staining of Quick-Neuron™ GABAergic - mRNA Kit cell cultures shows typical neurite growth and expression of the pan-neuronal marker TUBB3 and the GABAergic neuron-specific marker GAD67 on day 7 post-differentiation (scale bar = 100 µm). Staining conditions: Anti-β-III tubulin 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-GAD65/GAD67 primary antibody (Fisher Scientific, Catalog Number: PA5-36080, 1:400 dilution) in combination with a secondary antibody (Invitrogen, Catalog Number: A11037 Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594, 1:500 dilution). Nuclei were counterstained with Hoechst 33324.