User Guide

# Quick-Neuron™ GABAergic- Human iPSC-derived Neurons

Catalog Numbers: GA-mRNA-CW50065, GA-mRNA-CW10149, GA-mRNA-CW20300, GA-mRNA-CW50023, GA-mRNA-CW70067, GA-mRNA-CW50025, GA-mRNA-CW50113, GA-mRNA-CW50114, GA-mRNA-CW50115, GA-mRNA-CW50137, GA-mRNA-CW50147, GA-mRNA-CW60130, GA-mRNA-CW60231, GA-mRNA-CW60236, GA-mRNA-CW20026, GA-mRNA-CW20090, or GA-mRNA-CW10130

## Introduction

Elixirgen Scientific's proprietary transcription factor-based stem cell differentiation method uses synthetic mRNAs to produce highly pure populations of neurons without a genetic footprint. Our Quick-Neuron™ GABAergic - Human iPSC-derived Neurons 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 GABAergic marker glutamic acid decarboxylase 1 (GAD1). 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:** Quick-Neuron™ GABAergic - Human iPSC-derived Neurons are available in two sizes: (Small) 1 million viable cryopreserved cells and (Large) 5 x 1 million viable cryopreserved cells. The instructions outlined in this user guide are for seeding 1 million viable cells at approximately 5 x $10^4$ cells/$cm^2$ into 2 wells of a 6-well plate (5 x $10^5$ cells/well), 10 wells of a 24-well plate (1 x $10^5$ cells/well), or 62 wells of a 96-well plate (1.6 x $10^4$ cells/well).

**Related Products:** Quick-Neuron™ GABAergic - mRNA Kit, Catalog Number: GA-mRNA
Quick-Neuron™ GABAergic - Maintenance Medium, Catalog Number: GA-MM

## Contents

Upon receipt, immediately store the items at the indicated temperatures. Be especially careful to keep the frozen cells on dry ice until placing them in liquid nitrogen and avoid any temperature fluctuation and slight thawing.

| Contents | Amount (Small Size) | Amount (Large Size) | Storage | Thaw |
|---|---|---|---|---|
| Cryopreserved cells | >1 million viable cells, (1 vial, 500 µl) | 5 x >1 million viable cells, (5 vials, 5 x 500 µl) | Liquid nitrogen | 37°C |
| Component N | 2 x 840 µl | 8 x 840 µl | -20°C or -80°C | On ice or 4°C |
| Component G1 | 20 µl | 4 x 20 µl | -20°C or -80°C | On ice or 4°C |
| Component G2 | 60 µl | 3 x 60 µl | -20°C or -80°C | On ice or 4°C |
| Component P | 50 µl | 4 x 50 µl | -20°C or -80°C | Room temperature |

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

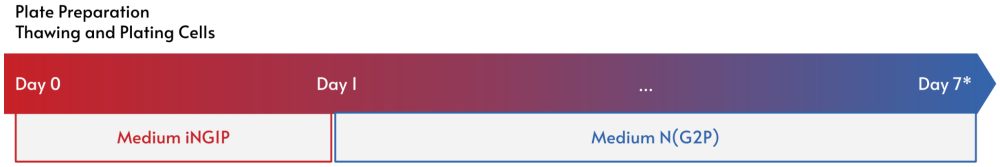
## Required Consumables

| Item | Vendor | Catalog Number |
|---|---|---|
| (Optional) 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., Corning Costar Flat Bottom Cell Culture Plates) | Fisher Scientific | 12-566-70 |
| DMEM/F12 | ThermoFisher | 21331020 |
| Neurobasal Medium | ThermoFisher | 21103049 |
| Glutamax (100x) | ThermoFisher | 35050061 |
| Penicillin-Streptomycin | ThermoFisher | 15140122 |
| 0.01% Poly-L-Ornithine | Sigma-Aldrich | P4957-50ML |
| Extracellular Matrix such as<br>- Laminin Mouse Protein, Natural, or<br>- Geltrex Basement Membrane Matrix | ThermoFisher | 23017015 or<br>A15696-01 |
| Phosphate-buffered saline (without $Ca^{++}$ $Mg^{++}$)* | ThermoFisher | 20012050 |
| ROCK inhibitor Y27632 | Selleckchem | S1049 |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich | D2650 |

*PBS should be used at room temperature unless otherwise specified.

## Workflow

**Note**: This protocol assumes that Day 0 is a Tuesday.




* From Day 7, 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.

## Experiment Planning

Define the cell culture plate or dish format in advance and calculate the number of wells to be used for each format in advance. For example, you may use only a certain number of wells of a 96-well plate. The following section describes culture condition volumes per well as user needs may vary. When a 96-well plate is used, we recommend filling the edge wells of the plate with an aqueous medium instead of cells and culture medium. This will maintain humidity on the entire plate. If performing an image-based analysis with a 96-well plate, we have found plating approximately 1-1.5 x $10^4$ cells/well to yield the best results. Please refer to the table below for plate formats and corresponding surface area of each well used for calculating reagents in the following sections.

| Plate format | 6-well plate | 24-well plate | 96-well plate |
|---|---|---|---|
| Approximate cell growth surface area per well | 9.5 $cm^2$ | 1.9 $cm^2$ | 0.32 $cm^2$ |

## 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.

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

1. Take 700 µl 0.01% Poly-L-Ornithine solution and mix it with 2.8 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., 35 µl) and store at -20°C.

### Medium N

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

| Reagents | Volume |
|---|---|
| DMEM/F12 | 18.6 ml |
| Neurobasal | 18.6 ml |
| 200 mM Glutamax (100x) | 195 µl |
| Penicillin-Streptomycin (10000 units/ml; 100x) | 390 µl |
| Component N | 1.2 ml |

## Day 0

**5-6 hours**

### Plate Preparation

**IMPORTANT!** Cells can be plated in 6-well, 24-well, and 96-well plates depending on the desired format. Refer to the table at the bottom of this page for the recommended volumes per well.

1. Vortex ornithine briefly and centrifuge it at a maximum speed for 1 minute.
2. Add ornithine to each well in the volume specified in the table.
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 PBS on ice for 20-30 minutes in the volumes calculated in the table. Add 1/100 volume of laminin to chilled PBS. Mix well.

- All PBS washes should be done dropwise and with room temperature PBS. Chilled PBS is only for the coating step.

5. Aspirate the supernatant from each well and add PBS in the volume specified in the table.
6. Repeat Step 5.
7. Aspirate PBS from each well and add diluted laminin in the volume specified in the table.
8. Incubate the plate at 37°C, 5% $CO_2$ for at least 2 hours.
9. While the plate is incubating, Prepare Medium iN(G1P) using the reagents listed in the table below.
    - Thaw Components G1 and P for 20-30 minutes at the temperatures indicated in the "Contents" table on page 1.
    - Thaw iROCK at room temperature for 20-30 minutes.
    - Warm Medium N at room temperature for 20-30 minutes.
    - Tap each Component tube 3 times and then briefly spin all tubes down before use.
    - Store Medium N(G1P) for up to 2 weeks at 4°C.
    - Leftover reagents can be stored at 4°C for up to two weeks.

| Reagents | Volume | | |
|---|---|---|---|
| | 6-well plate | 24-well plate | 96-well plate |
| Medium N | 4.5 ml | 5.6 ml | 6 ml |
| Component G1 | 9 µl | 11.2 µl | 12 µl |
| Component P | 9 µl | 5.6 µl | 6 µl |
| iROCK | 4.5 µl | 5.6 µl | 6 µl |

10. After the laminin incubation, aspirate most, but not all, of the supernatant and add PBS in the volume specified in the table. Add the PBS dropwise to each well.
11. Pipet out most of the PBS, but not all, from each well.
12. Repeat Steps 10-11.
13. Add Medium iN(G1P) to each well in the volume specified in the table.
14. Incubate the plate at 37°C, 5% $CO_2$ until cells are ready for plating.

Recommended volumes per well for different plate formats.

| Reagents | Corresponding steps | Recommended volume per well | | |
|---|---|---|---|---|
| | | 6-well plate | 24-well plate | 96-well plate |
| Ornithine | 1, 2 | 1.5 ml | 300 µl | 50 µl |
| PBS for laminin dilution | 4 | 1.65 ml | 330 µl | 55 µl |
| PBS | 5, 6, 10, 11 | 2 ml | 500 µl | 100 µl |
| Diluted laminin | 7 | 1.5 ml | 300 µl | 50 µl |
| Medium iN(G1P) | 13 | 1 ml | 300 µl | 35 µl |

## Thawing Cells

1. Warm Medium N at room temperature for 20-30 minutes.
2. Take out the vial of frozen cells from the liquid nitrogen storage tank.
3. Incubate the cryovial in a water bath set at 37°C (do not submerge the cap) until most of the content is thawed but a small ice crystal remains (~2 min).
4. Wipe the vial with a dry paper towel. Spray the vial with 70% ethanol and place it inside a biosafety cabinet.
5. Transfer 4.5 ml room temperature Medium N to a new 15 ml conical tube.
6. Set a P1000 pipette to 1 ml but take approximately 500 µl Medium N from the 15 ml conical and add it to the cryovial dropwise at 1 drop per 1-2 seconds.

- **IMPORTANT!** Use the same pipette tip for Steps 6-10.

7. Gently pipet the cell suspension up and down once.
8. Gently transfer all of the cell suspension to the 15 ml conical tube prepared in Step 5.
9. Take 1 ml of the cell suspension from the conical tube and add it to the original cryovial and pipet up and down 2-3 times and then transfer the whole contents back to the same 15 ml conical tube.
10. Mix the contents in the conical tube by gently pipetting cell suspension up and down 3 times.
11. Centrifuge the cell suspension in the 15 ml conical tube at 200 x g for 4 minutes.
12. Use an aspirator to remove most of the supernatant from the conical tube, leaving a small volume of the supernatant (<50 µl) to cover the pellet.
13. Tap the side of the conical tube up to 10 times to break up the cell pellet.
14. Add 1 ml room temperature Medium iN(G1P) to the conical tube and pipet up and down no more than 2-3 times.

### Plating Cells

1. Count the cells to determine the volume of cell suspension needed for the chosen number of wells and include 10% extra for cell number and volume (e.g., for a 24-well plate scenario, a total of 1.1 x $10^6$ cells to plate 1 x $10^5$ cells in each of the 10 wells). If the volume of the cell suspension needs to be adjusted, centrifuge the required volume of cell suspension at 200 x g for 4 minutes, remove the supernatant, and resuspend the pellet with Medium iN(G1P) to reach the multiplied volume of cell suspension with the number of wells.
2. Add cell suspension to the center of each well. Since each well already has Medium iN(G1P), the total volume of the medium in each well is indicated in the table below.
3. Incubate at 37°C, 5% $CO_2$ overnight.

| | Recommended amounts | | |
|---|---|---|---|
| | **6-well plate** | **24-well plate** | **96-well plate** |
| Viable cells/well | 5 x $10^5$ cells | 1 x $10^5$ cells | 1.6 x $10^4$ cells |
| Required total volume of cell suspension/well<br>• (Volume of cell suspension/well) + 10% extra | 550 µl | 110 µl | 38.5 µl |
| Volume of cell suspension distributed/well | 500 µl | 100 µl | 35 µl |
| Total volume/well<br>• Medium iN(G1P) + cell suspension | 1.5 ml | 400 µl | 70 µl |

## Day 1

**< 2 hour**

### Medium Change

1. Prepare Medium N(G2P) using the reagents listed in the table below.
   - Warm/Thaw Component G2 and P for 20-30 minutes at the temperatures indicated in the “Contents” table on page 1.
   - Warm Medium N for 20-30 minutes at room temperature.
   - Tap each Component tube 3 times and then briefly spin all tubes down before use.
   - Store Medium N(G2P) for up to 2 weeks at 4°C.

| Reagents | Volume | | |
|---|---|---|---|
| | **6-well plate** | **24-well plate** | **96-well plate** |
| Medium N | 11 ml | 22 ml | 24.6 ml |
| Component G2 | 11 µl | 22 µl | 24.6 µl |
| Component P | 11 µl | 22 µl | 24.6 µl |

2. Pipet out the old medium from each well and add Medium N(G2P) to each well along its wall according to the table below.
    - **IMPORTANT!** To avoid lifting cells, leave a small volume of the old medium (i.e., just enough to cover the surface of the well). PBS should be applied along the wall of the well very slowly.

| | Required volume per well | | |
|---|---|---|---|
| **Reagents** | **6-well plate** | **24-well plate** | **96-well plate** |
| Medium N(G2P) | 2 ml | 800 µl | 150 µl |

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

## Day 3

< 1 hour

### Medium Change

1. Warm Medium N(G2P) at room temperature for 20-30 minutes.
2. Pipet out the old medium from each well and add Medium N(G2P) to each well along its wall according to the table below.
    - **IMPORTANT!** To avoid lifting cells, leave a small volume of the old medium (i.e., just enough to cover the surface of the well). PBS should be applied along the wall of the well very slowly.

| | Required volume per well | | |
|---|---|---|---|
| **Reagents** | **6-well plate** | **24-well plate** | **96-well plate** |
| Medium N(G2P) | 2 ml | 800 µl | 150 µl |

3. Incubate at 37°C, 5% $CO_2$ for 3 days.

## Day 6

< 1 hour

### Medium Change

4. Warm Medium N(G2P) at room temperature for 20-30 minutes.
5. Pipet out half the old medium from each well and add Medium N(G2P) to each well along its wall according to the table below.

| | Required volume per well | | |
|---|---|---|---|
| **Reagents** | **6-well plate** | **24-well plate** | **96-well plate** |
| Medium N(G2P) | 1 ml | 400 µl | 75 µl |

6. Incubate at 37°C, 5% $CO_2$.

## Day 7

< 1 hour

### Assay or Continuous Maturation

- Differentiated neurons can be observed on Day 1-2. For more mature neurons, we recommend culturing cells until Day 7. From Day 7, 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 can be confirmed with the markers TUBB3, PVALB, and GAD1.

## Appendix

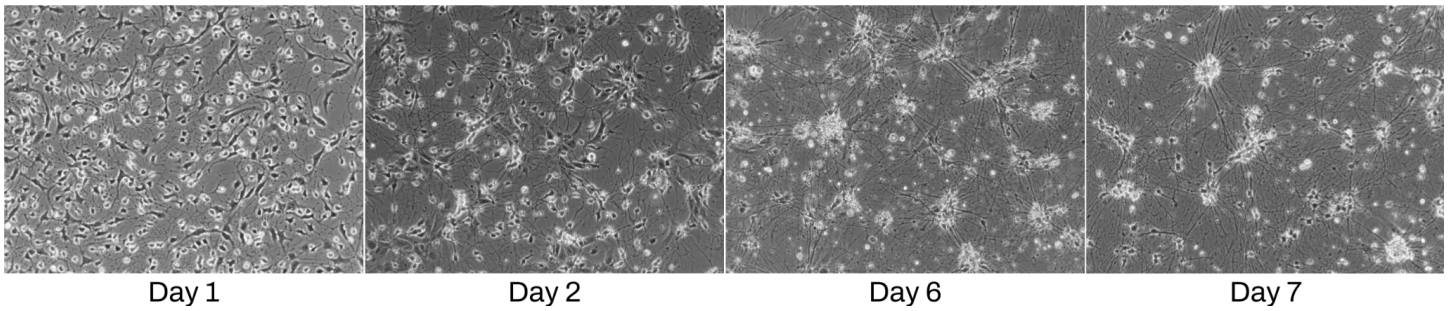



**Figure 1.** Representative phase contrast images of Quick-Neuron™ GABAergic - Human iPSC-derived Neurons cell cultures on days 1, 2, 5 and 7 post-thaw (scale bar = 100 µm).

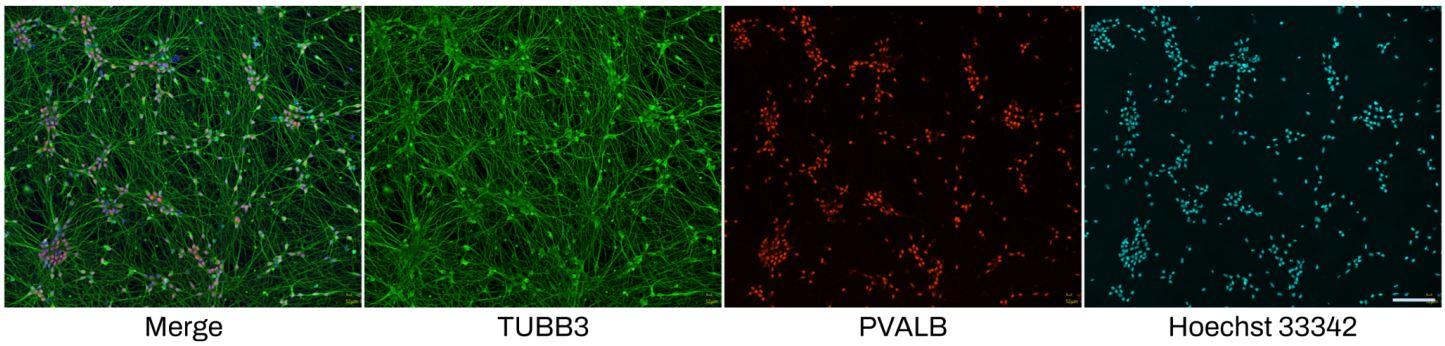



**Figure 2.** Immunofluorescent staining of Quick-Neuron™ GABAergic - mRNA Kit cell cultures shows typical neurite growth and expression of the pan-neuronal marker TUBB3 and the GABAergic neuron-specific marker PVALB on day 10 post-differentiation (scale bar = 100 µm). Staining conditions: Anti-β-III 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-PVALB primary antibody (Novus Biologicals, Catalog Number: NB120-11427 , 1:1000 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 33342.

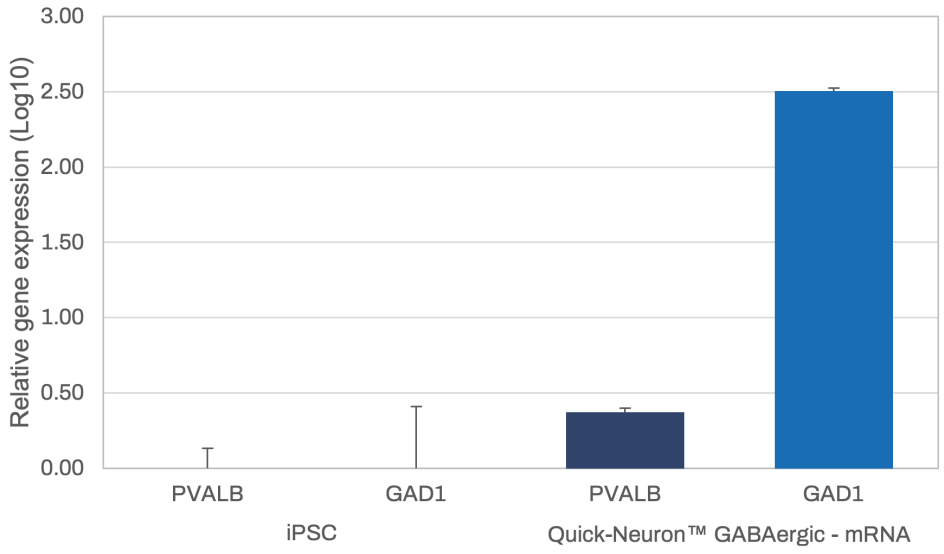



**Figure 3**. Real-time quantitative PCR analysis of expression levels of GABAergic neuron-associated genes *PVALB* and *GAD1* were examined for Quick-Neuron™ - GABAergic - mRNA Culture on day 10. The relative gene expression is normalized to phosphoglycerate

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 |
|---|---|---|---|
| *PVALB* | TCGACCACAAAAAGTTCTTC | TTTAGGATGAATCCCAGCTC | 250 nM |
| *GAD1* | GTCGAGGACTCTGGACAGTA | GGAAGCAGATCTCTAGCAAA | 250 nM |
| *PGK1* | GTATGCTGAGGCTGTCACTCG | CCTTCCAGGAGCTCCAAACTGG | 250 nM |