Quick-Neuron™ Mixed SeV Complete Kit

Catalog Number: EXGS-QNMSV

User Manual

This kit (EXGS-QNMSV) contains 1 set of reagents for use with a total of 4 wells of a 24-well plate

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I. Introduction

Thank you for purchasing the Quick-Neuron™ Mixed - SeV Complete Kit. This kit allows users to differentiate human pluripotent stem cells (hPSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), into a population of mixed neurons within 10 days. The population includes tubulin beta 3 class III (TUBB3, a pan-neuronal marker), choline acetyltransferase (ChAT, a cholinergic neuron marker), tyrosine hydroxylase (TH, a dopaminergic neuron marker), parvalbumin (PVALB, a GABAergic neuron marker), vesicular glutamate transporter 1 (vGLUT1, a glutamatergic neuron marker), and tryptophan hydroxylase 2 (TPH2, a serotonergic neuron marker)-positive neurons.

NOTE: EXPERIMENT SCALE

This kit contains enough reagents to differentiate 4 wells of a 24-well plate. If you are unfamiliar with our kits, we recommend differentiating 4 wells at a time; our protocol instructions are written for a 4-well experiment. For the use in a 6-well format, simply multiply the recipe by four.

II. Kit Contents

Upon receipt of this kit, immediately store all reagents at their proper storage temperatures as described in the table below. All reagents are shipped on dry ice.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Amount</th>
<th>Storage Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>QN-SeV</td>
<td>55 µl</td>
<td>-80 °C</td>
</tr>
<tr>
<td>Component N</td>
<td>840 µl</td>
<td>-20 °C or -80 °C</td>
</tr>
<tr>
<td>Component K</td>
<td>25 µl</td>
<td>-20 °C or -80 °C</td>
</tr>
<tr>
<td>Solution D1</td>
<td>1 ml</td>
<td>-20 °C or -80 °C</td>
</tr>
<tr>
<td>Coating Material A</td>
<td>15.7 µl</td>
<td>-20 °C or -80 °C</td>
</tr>
<tr>
<td>Coating Material B</td>
<td>1.3 ml</td>
<td>-20 °C or -80 °C</td>
</tr>
<tr>
<td>Coating Material C</td>
<td>16.5 µl</td>
<td>-20 °C or -80 °C</td>
</tr>
</tbody>
</table>

- This kit contains iMatrix-511 silk (Nippi, Inc.) and Sendai virus vector (ID pharma Co., Ltd.).
- This kit contains Sendai virus (SeV) particles which are active at 33°C and become inactive at 37°C. SeV is non-pathogenic in humans, and humans are not natural hosts of the Sendai Virus; however, Biosafety Level 2 (BSL2) containment is required for its use. Please use a biological safety cabinet, laminar flow hood, and proper personal protective equipment in order to prevent mucosal exposure.
- All plasticware exposed to SeV should be rinsed with bleach solution (or 1% sodium hypochlorite or formaldehyde) before being deposited into a Biohazardous and Regulated Medical Waste Bag. Liquid waste should be collected in a separate bottle containing the bleach solution prior to disposal into a Biohazardous Liquid Waste container. DO NOT use an aspirator to collect liquid waste exposed to SeV. Institutional Biosafety Committee (IBC) registration and approval may be required for the use of SeV. Please make sure that users follow the laboratory biosafety protocols referred to by IBC policy (http://osp.od.nih.gov/office-biotechnology-activities/biosafety/institutional-biosafety-committees).

III. Additional Materials Required

The following materials are needed but not supplied with this kit:
- 24-well tissue-culture-treated polystyrene plate
IV. Pre-Protocol Preparation

NOTE: SOURCE hPSC CULTURE CONDITIONS

- The recipe in the Quick-Neuron™ Mixed - SeV Complete Kit protocol assumes that the undifferentiated hPSCs (starting materials) are cultured in a 35-mm culture dish (or one well of a 6-well plate).

- This protocol requires two CO₂ incubators, at 33°C and 37°C respectively.
- Prepare a neural differentiation medium by mixing following reagents after thawing Component N at 4°C overnight. The medium is called Medium N and stable for up to 2 weeks when stored at 4°C.

<table>
<thead>
<tr>
<th>No.</th>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMEM/F12</td>
<td>12 ml</td>
</tr>
<tr>
<td>2</td>
<td>Neurobasal</td>
<td>12 ml</td>
</tr>
<tr>
<td>3</td>
<td>200 mM Glutamax (100x)</td>
<td>125 µl</td>
</tr>
<tr>
<td>4</td>
<td>Penicillin-Streptomycin (10000 units/ml; 100x)</td>
<td>250 µl</td>
</tr>
<tr>
<td>5</td>
<td>Component N</td>
<td>775 µl</td>
</tr>
</tbody>
</table>

- Prepare a 10 mM ROCK inhibitor Y27632 stock solution in DMSO to prepare for Medium iN on Day 0. Preparation steps are as follows:
  - Dissolve 10 mg ROCK inhibitor Y27632 in 3.1225 ml DMSO.
  - Make aliquots of a convenient volume (e.g., 100 µl) and store at -20°C.
- We do not recommend additional freeze-thaw cycles of any reagents.
- Taking 4x and/or 10x images of cultures every day (or even after every medium change) is a good way to monitor your experiment.
- Customer service on the phone (+1-443-869-5420) and through email (cs@elixirqenscientific.com) is available to assist users in troubleshooting and interpretation of results.
V. Protocol

Day 0 - Plating and Treatment

New Plate Preparation

1. Start thawing Solution D1 and warm Medium N and 10 mM ROCK inhibitor Y27632 at room temperature. Make sure that Solution D1, Medium N, and 10mM ROCK inhibitor Y27632 are at room temperature for at least 1 hour before use.
2. Thaw Coating Material A on ice for 20-30 minutes (or at 4°C overnight one day before Day 0).
3. Take 2 ml ice-cold PBS into a new 15 ml conical tube and add 6.6 µl Coating Material A to it. Mix them well.
4. Add 400 µl diluted Coating Material A to each new well.
5. Incubate the plate at 37°C, 5% CO₂ for 2 hours (or 4°C overnight one day before Day 0).
6. Aspirate the supernatant from each well and add 500 µl PBS to it.
7. Incubate the plate at 37°C, 5% CO₂ until user’s hPSCs are ready for plating.

Treatment

1. (If adding retinoic acid is desired) thaw Component K at room temperature for 20-30 minutes. Add 24.5 µl Component K to 24.5 ml Medium N. This medium is referred to as Medium N(K) and stable for up to 2 weeks when stored at 4°C.
2. Take 2.5 ml Medium N or Medium N(K) into a new 15 ml conical tube and add 2.5 µl 10 mM ROCK inhibitor Y27632 to it. Mix them well. This medium is referred to as Medium iN or Medium iN(K). Keep the rest of Medium N or Medium N(K) at 4°C for its later use.
3. Start thawing QN-SeV on ice.
4. Aspirate old medium from user’s hPSC culture and add 2 ml PBS to it.
5. Rock the plate 3 times, aspirate PBS from the culture, and add 300 µl Solution D1 to it. Keep the rest of Solution D1 at 4°C for its use on Day 3.
6. Incubate the culture at 37°C, 5% CO₂ for 5-7 minutes.
7. Carefully pipet out Solution D1 from the culture using a P1000 pipettor and add 1 ml Medium iN or Medium iN(K) to it.
8. Disperse the medium over the well bottom surface by pipetting 8-15 times to detach cells.
9. Collect the cell suspension in a tube.
10. Count cells to determine the volume of cell suspension needed for 4.4 wells (a total of $2.2 \times 10^5$ cells). Adjust the volume to 110 µl with Medium iN or Medium iN(K).

NOTE: CELL DENSITY
• In this protocol, users will treat hPSCs with QN-SeV in a tube and then plate the cells onto 4 wells with 250 µl Medium iN or Medium iN(K) ($0.5 \times 10^5$ cells) per well. However, we recommend preparing a suspension 1.1 ml to avoid insufficiency.
• Cell count may vary based on cell health, the method, and instrument used for cell counting.

NOTE: VIRUS IS DELICATE
• Before adding QN-SeV, do not centrifuge, vortex, or mix it with a pipettor; it is highly sensitive to physical stress.

11. By then, QN-SeV on ice should be thawed (see “Note: virus is delicate” above).
12. Take out the determined volume of the cell suspension from the previous step 10 in a 2.0 ml freezing vial or a 15 ml conical tube and add 55 µl QN-SeV to the hPSCs and mix them by tapping with finger 2-3 times. Cap the tube loosely to allow gas exchange.
13. Incubate the cell suspension at 33°C, 5% CO₂ for 10 minutes with intermittent mixing of the cells every 2 minutes.

Plating
1. Bring up the volume to 1.1 ml with Medium iN or Medium iN(K).
2. Aspirate PBS from only one coated well at a time and add 250 µl cell suspension to it. Most of the PBS should be aspirated but not completely to prevent the coated wells from drying before adding the cell suspension. Likewise, the cell suspension should be added to the well immediately after PBS is removed. Handle one well after another.
3. Move the plate by 5 cycles of quick back-and-forth and side-to-side motions to evenly distribute treated cells in the cultures.
4. Incubate the cultures at 33°C, 5% CO₂ overnight.

Day 1 - Feeding
1. Warm Medium N or Medium N(K) at room temperature for 20-30 minutes.
2. Pipet out the old medium with QN-SeV from each well using a P1000 pipettor and add 500 µl Medium N or Medium N(K) to it.
3. Incubate the cultures at 33°C, 5% CO₂ overnight.

Day 2 - Feeding
1. Warm Medium N or Medium N(K) 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 or Medium N(K) to it.
3. Incubate the cultures at 33°C, 5% CO₂ overnight.

Day 3 - Feeding or Passaging
1. Warm Medium N or Medium N(K) at room temperature for 20-30 minutes..
NOTE: PASSAGING CELLS TO WELLS OR GLASS COVERSLEIPS

- Day 3 involves an optional passaging step. Cells can be passaged to either wells or glass coverslips depending on user’s preferences and needs. Wells can be used for functional assays and post-kit work, while coverslips will provide a better environment for immunostaining and imaging.
- Upon user’s choice, select one of the following instructions on Day 3.
  - If you do not intend to passage cells, please complete "A. No Passaging".
  - If you intend to passage cells to new wells, please complete “B1. New Well Preparation” and “C. Passaging Cells”.
  - If you intend to passage cells to glass coverslips, please complete “B2. Glass Coverslip Preparation” and “C. Passaging Cells”

A. No passaging
1. Pipet out the old medium from each well using a P1000 pipettor and add 500 μl Medium N or Medium N(K) to it.
2. Incubate the cultures at 37°C, 5% CO₂ overnight.

B. Passaging (Optional)
B1. Well Preparation
1. Thaw Coating Material B at room temperature for 20-30 minutes.
2. Add 300 μl Coating Material B to each well in a new 24-well plate.
3. Incubate the plate at 37°C, 5% CO₂ for at least 2 hours (or at 4°C overnight one day before Day 3).
4. Thaw Coating Material C on ice for 20-30 minutes. Do not thaw it at room temperature.
5. Take 1.5 ml ice-cold PBS into a tube and add 15 μl Coating Material C to it. Mix them well.
6. Aspirate the supernatant from each well and add 500 μl PBS to it.
7. Repeat step 6.
8. Aspirate PBS from each well and add 300 μl diluted Coating Material C to it.
9. Incubate the plate at 37°C, 5% CO₂ for at least 2 hours.
10. Repeat step 6 twice.
11. Aspirate PBS from each well and add 250 μl Medium N or Medium N(K) to it.

B2. Glass Coverslip Preparation
1. Thaw Coating Material B at room temperature for 20-30 minutes to room temperature.
2. Soak 12-mm glass coverslips in 100% ethanol for 5 minutes.
3. Dry the coverslips with air and place one glass coverslip to each well in a new 24-well plate with sterilized forceps.
   Note: Coverslips should be fully submerged under solutions from step 4 and onwards.
4. Add 150 μl Coating Material B to the surface of each glass coverslip.
5. Incubate the plate at 37°C, 5% CO₂ for at least 2 hours (or at 4°C overnight one day before Day 3).
6. Thaw Coating Material C on ice for 20-30 minutes. Do not thaw it at room temperature.
7. Take 1.5 ml ice-cold PBS into a tube and add 15 μl Coating Material C to it. Mix them well.
8. Aspirate the supernatant from each well and add 500 μl PBS to it.
9. Repeat step 8.
10. Aspirate PBS from each well and add 300 μl diluted Coating Material C to the surface of each glass coverslip.
11. Incubate the plate at 37°C, 5% CO₂ for at least 2 hours.
12. Repeat step 8 twice.
13. Aspirate PBS from each well and add 250 μl Medium N or Medium N(K) to it.

**NOTE: DIFFERENTIATING CELLS ARE DELICATE**

- For the following steps in “C. Passaging Cells”, please pipet and add solutions gently. Differentiating cells are delicate, and should be handled with great care.

**C. Passaging Cells**

1. Warm Solution D1 at room temperature for 20-30 minutes.
2. Pipet out the old medium from each well using a P1000 pipettor and add 500 μl PBS to it.
3. Pipet out PBS from each well using a P1000 pipettor and add 80 μl Solution D1 to each well.
4. Incubate the cultures at 37°C, 5% CO₂ for 3 minutes.
5. Carefully pipet out Solution D1 from each well using a P200 pipettor and add 250 µl Medium N or Medium N(K) to it.

**NOTE: CRITICAL PASSAGING STEPS**

- Steps 6-8 below are critical. Perform these steps for one well at a time.
- Refer images below to successfully manage cell treatment and dissociation.

<table>
<thead>
<tr>
<th>Before Solution D1 treatment</th>
<th>During Solution D1 treatment</th>
<th>Cell leftover on original wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clusters of cells could remain on the original well after cell treatment and dissociation</td>
<td>The clusters could be identified on the well and attached to the plate stronger than individualized cells</td>
<td>After optimal cell treatment and dissociation, there are some cell leftovers mainly from the clusters of cells</td>
</tr>
</tbody>
</table>

6. Disperse the medium quickly over the well bottom surface by pipetting 6-8 times to detach cells using a P200 pipettor.
7. Observe cells and/or cell aggregates floating in the well under a microscope. It is normal that 10-20% of the cells remain attached to the well bottom after pipetting. The clusters of cells are not supposed to be lifted. Do not attempt to detach all of the cells remaining on the well bottom.
8. Gently pipet up and down the cell suspension in the well up to 5 times to break the cell aggregates using a P200 pipettor. Excessive pipetting can damage the already-suspended cells.
9. Collect 250 μl cell suspension from each well with a P1000 pipettor and transfer it to each new well or glass coverslip coated with Coating Material B and Coating Material C.

Note: Transferring to glass coverslips
- When transferring the cell suspension to a coverslip, bring the tip very close to the coverslip and pipet slowly to mount cells right on the coverslip.
- If users find the cultures are more than 70% confluent on Day 3, we recommend to transfer only half of the cell suspension (125 μl) to each new well or glass coverslip coated with Coating Material B and Coating Material C to avoid excessively high cell density. Add 125 μl more Medium N or Medium N(K) to each well after half of the cell suspension is transferred.
10. Incubate the cultures at 37°C, 5% CO₂ for 1 hour.
11. Observe each well under the microscope to make sure that the cells are attached to the well or glass coverslip.
12. Incubate the cultures at 37°C, 5% CO₂ overnight.

Day 4-9 - Feeding
1. Warm Medium N or Medium N(K) 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 or Medium N(K) to it.
3. Incubate the cultures at 37°C, 5% CO₂ for 2 days.
4. Repeat steps 1-3 until Day 10.

Day 10 - Ready for Assay

NOTE: OBSERVING NEURONS AND ASSAYING

- Differentiated neurons can be observed on Day 4 under the microscope. 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™ Mixed Maintenance Medium, available at [https://elixirgenscientific.com/store/](https://elixirgenscientific.com/store/) (Catalog Number: EXGS-QNMM).

- Differentiation into multiple types of neurons after using Quick-Neuron™ Mixed - SeV Complete Kit can be confirmed with anti-TUBB3 (tubulin beta 3 class III, a global marker for neurons), anti-ChAT (choline acetyltransferase, a cholinergic neuron marker), anti-TH (Tyrosine hydroxylase, a dopaminergic neuron marker), anti-PVALB (parvalbumin, a GABAergic neuron marker), anti-vGLUT1 (vesicular glutamate transporter 1, a glutamatergic neuron marker), and anti-TPH2 (Tryptophan hydroxylase 2, a serotonergic neuron marker) antibodies. Typical immunostaining images and gene expression results of TUBB3-positive, TH-positive, PVALB-positive, vGLUT1-positive, and TPH2-positive neurons on Day 10 following Quick-Neuron™ Mixed - SeV Complete Kit treatment are shown in Appendix A.
VI. Appendix A: Reference Pictures and Figures

With Component K

Without Component K

Figure 1. Phase contrast images show typical appearance of cultures with (top two rows) or without (bottom two rows) Component K on each day. Scale bars, 100 µm.
Figure 2. Multiple subtype-specific markers were examined by immunocytochemistry (ICC) and quantitative RT-PCR (qRT-PCR) to validate the presence of diverse subtypes of neurons in the cultures on Day 10 with Component K (Figure 2) or without Component K (Figure 3). For ICC, antibodies against cholinergic- (anti-ChAT, 1:200), glutamatergic- (anti-vGLUT1, 1:200), dopaminergic- (anti-TH, 1:300), serotonergic- (anti-TPH1, 1:200), and GABAergic (anti-PVALB, 1:1000) neurons as well as an antibody against TUBB3 (1:10,000), a pan-neuronal marker, were used. Nuclei were counterstained with Hoechst33342. Scale bars are 100 µm. For qRT-PCR analysis, expression levels of tubulin beta 3 class III (TUBB3), choline acetyltransferase (ChAT), tyrosine hydroxylase (TH), G-protein-regulated inward-rectifier potassium channel 2 (GIRK2), glutamate decarboxylase 1 (GAD1), vesicular glutamate transporter 1 (vGLUT1), glutamate receptor, ionotropic, NMDA1 (zeta1) (GRIN1), and tryptophan hydroxylase 1 (TPH1) were examined. The results indicate that the neurons are differentiating into diverse subtypes using the Quick-Neuron™ Mixed - SeV Complete Kit. The relative gene expression is normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and then calculated as a fold induction relative to undifferentiated hPSCs as a control. Primers used in real-time PCR experiments were listed below in Table 1.

### Table 1. List of PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Primer concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUBB3</td>
<td>TTTGGACATCTCTTTCAAGCCTGACA</td>
<td>AGCGAGTGGGTCAGCTGGAAGC</td>
<td>250 nM</td>
</tr>
<tr>
<td>ChAT</td>
<td>TCATTAATTTCCGCGCTCTC</td>
<td>GAGTCCCGGTTGGGGAGT</td>
<td>250 nM</td>
</tr>
<tr>
<td>TH</td>
<td>TATCACCTGTGTCACCAAGTT</td>
<td>GGTGCGGTCGTCGCTGACT</td>
<td>250 nM</td>
</tr>
<tr>
<td>GIRK2</td>
<td>CACATCAGCGGAGAGCGGAC</td>
<td>GGTAGCGCATAGGCTCCTCAA</td>
<td>250 nM</td>
</tr>
<tr>
<td>GAD1</td>
<td>GTCGAGGACTCTGGACAGTA</td>
<td>GGAAGCAGATCTCTAGAAA</td>
<td>250 nM</td>
</tr>
<tr>
<td>vGLUT1</td>
<td>CGAACGACAGCCTTTGTGTTG</td>
<td>GCCGTAGACGTAAGAAAACAGAG</td>
<td>250 nM</td>
</tr>
<tr>
<td>GRIN1</td>
<td>CTACCACCATCCCGTGCTG</td>
<td>GCATCATCTCAACCCACACGC</td>
<td>250 nM</td>
</tr>
<tr>
<td>TPH1</td>
<td>CCCCTTGATCCCAAGATTAC</td>
<td>CATTACATCGACCTGTTATG</td>
<td>250 nM</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GTCAATCCACAGAAATGACGCT</td>
<td>TGAACCAGCTCCATGCCATC</td>
<td>200 nM</td>
</tr>
</tbody>
</table>
VII. Appendix B: Literature References


3. This kit is based on the technology published in (1) and licensed from Keio University and ID Pharma Co., Ltd.
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<table>
<thead>
<tr>
<th>Category</th>
<th>Product</th>
<th>Size</th>
<th>Request Quote (Catalog number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quick-Tissue™ Series Differentiation Support</td>
<td>Quick-Tissue™ Mesendoderm Booster</td>
<td>8 wells of 24-well plate</td>
<td>$99 (EXGS-QTMB)</td>
</tr>
<tr>
<td>Quick-Tissue™ Adaptation Kit</td>
<td>a 35-mm dish or 1 well of 6-well plate</td>
<td>$169 (EXGS-QTA1)</td>
<td></td>
</tr>
<tr>
<td>Reagents for Maintaining Undifferentiated Stem Cells</td>
<td>Ajinomoto StemFit® Basic02</td>
<td>500 mL</td>
<td>Ask (EXGS-ASB02)</td>
</tr>
<tr>
<td></td>
<td>Nippi iMatrix-511 silk</td>
<td>175 µg x 6 tubes</td>
<td>Ask (EXGS-NIS11S)</td>
</tr>
<tr>
<td></td>
<td>Nippi iMatrix-511</td>
<td>175 µg x 6 tubes</td>
<td>$690 (EXGS-NIS11)</td>
</tr>
</tbody>
</table>

Quick-Tissue™ Stem Cell Differentiation Services

15% off for 3 or more kit purchases per order

Elixirgen Scientific provides pluripotent stem cell differentiation services with the world’s fastest turnaround time. Customers can simply ship live iPES cells in a T-25 flask and will receive live or frozen cells in a week (express service) or two weeks (regular service). Contact services@elixirgenscientific.com for more details to customize for your project.

Currently Elixirgen Scientific offers all tissue types from kits for cell differentiation services. More tissue types are coming soon!