User Guide

RICOH imagine. change.

Ricoh Biosciences, Inc.

Quick-Neuron™ Dopaminergic - Human iPSC-Derived Neurons

Catalog Numbers: DA-mRNA-HC-CW50065

Introduction

Ricoh Bioscience's proprietary transcription factor-based stem cell differentiation method uses synthetic mRNAs to produce highly pure populations of neurons without a genetic footprint. Quick-Neuron™ Dopaminergic 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 dopaminergic markers tyrosine hydroxylase (TH) and dopamine (DA). When handled and maintained according to the instructions in this user guide, the iPSC-derived dopaminergic neurons are viable long-term and are suitable for a variety of characterization and neurotoxicity assays.

Scale: Each vial of Quick-Neuron™ Dopaminergic - Human iPSC-derived Neurons is expected to have 1 million viable cryopreserved cells. The instructions outlined in this user guide are for seeding 1 million viable cells at approximately 5 x 104 cells/cm2 into 2 wells of a 6-well plate (5 x 105 cells/well), 10 wells of a 24-well plate (1 x 105 cells/well), or 62 wells of a 96-well plate (1.6 x 104 cells/well).

Related Products: Quick-Neuron™ Dopaminergic - mRNA Kit, Catalog Number: DA-mRNA
Quick-Neuron™ Dopaminergic - Maintenance Medium, Catalog Number: DA-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	Storage	Thaw
Cryopreserved cells	>1 million viable cells, (1 vial, 500 µl)	Liquid nitrogen	37°C
Component N	840 µl	-20°C or -80°C	On ice or 4°C
Component P	50 µl	-20°C or -80°C	Room temperature
Component D4	2 x 20 µl	-20°C or -80°C	On ice or 4°C
Component D5	90 µl	-20°C or -80°C	Room temperature
Component D6	38 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 refer to the FAQ on our website.
You may also contact us at cs@biosciences.ricoh.com or call +1 (443) 869-5420 (M-F 9am-5pm EST).

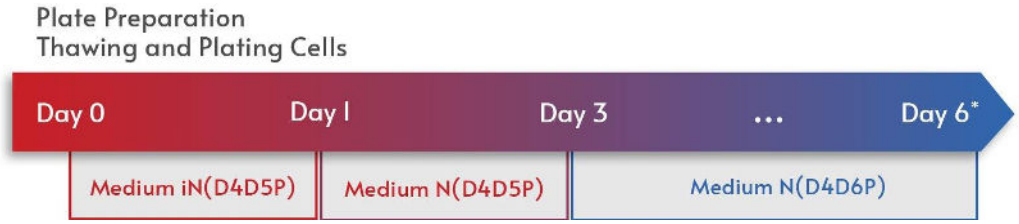
Required Consumables

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	ThermoFisher	35050061
Penicillin-Streptomycin	ThermoFisher	15140122
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	D2650

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

Workflow

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



* From Day 6, users may maintain differentiated neurons in the maintenance medium best suited for their needs, though we recommend Quick-Neuron™ Dopaminergic - Maintenance Medium, Catalog Number: DA-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 1.5-2 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 them at -20°C.

Medium N(P)

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

Reagents	Volume
DMEM/F12	11.9 ml
Neurobasal	11.9 ml
GlutaMAX	125 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	250 µl
Component N	775 µl
Component P	25 µl

Medium N(D4D5P)

1. Prepare Medium N(D4D5P) using the reagents listed in the table below.
    - Thaw Components D4 and D5 for 20-30 minutes at the temperatures indicated in the “Contents” table on page 1.
    - Warm Medium N(P) 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(D4D5P) for up to 2 weeks at 4°C.

Reagents	Volume
Medium N(P)	11 ml
Component D4	11 µl
Component D5	22 µl

Medium N(D4D6P)

1. Prepare Medium N(D4D6P) using the reagents listed in the table below.
    - Thaw Components D4 and D6 for 20-30 minutes at the temperatures indicated in the “Contents” table on page 1.
    - Warm Medium N(P) 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(D4D6P) for up to 2 weeks at 4°C.

Reagents	Volume
Medium N(P)	8 ml
Component D4	8 µl
Component D6	8 µl

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(D4D5P) using the volume of Medium N(D4D5P) indicated in the table calculated for the number of wells in use plus 1.1 ml for resuspension (i.e., 2 wells of a 6-well plate needs 3.3 ml Medium N(D4D5P) (1.1 x 2 + 1.1), 10 wells of a 24-well plate needs 4.4 ml Medium N(D4D5P) (0.33 x 10 + 1.1) for this step).
    - Thaw/warm Medium N(D4D5P), iROCK at room temperature for 20-30 minutes.

- Add 1/1000 of iRock to Medium N(D4D5P).
- Keep the rest of Medium N(D4D5P) at 4°C for later use.

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. Add PBS dropwise in the volume specified in the table.
13. Repeat Step 11.
14. Add Medium iN(D4D5P) to each well in the volume specified in the table.
15. 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 N(D4D5P) for Medium iN(D4D5P)	9	1.7 ml	450 µl	80 µl
Medium iN(D4D5P)	13	1 ml	300 µl	35 µl

Thawing Cells

1. Warm Medium N(P) 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(P) to a new 15 ml conical tube.
6. Set a P1000 pipette to 1 ml but take approximately 500 µl Medium N(P) 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 entire 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(D4D5P) 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(D4D5P) 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(D4D5P), 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 • (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 • Medium iN(D4D5P) + cell suspension	1.5 ml	400 µl	70 µl

Day 1

< 1 hour

Maintenance

1. Warm Medium N(D4D5P) at room temperature for 30 minutes.
2. Pipet out the old medium from each well and add PBS 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.

	Recommended volume per well		
Reagents	**6-well plate**	**24-well plate**	**96-well plate**
PBS	2 ml	500 µl	100 µl

3. Pipet out the PBS from each well and add Medium N(D4D5P) to each well along its wall according to the table below.

	Recommended volume per well		
Reagents	**6-well plate**	**24-well plate**	**96-well plate**
Medium N(D4D5P)	2 ml	500 µl	100 µl

4. Incubate at 37°C, 5% CO_2 for 2 days.

Day 3

< 1 hour

Maintenance

1. Warm Medium N(D4D6P) at room temperature for 20-30 minutes.
2. Pipet out the old medium from each well, leaving a small volume of the old medium (i.e., just enough to cover the surface of the well). Add Medium N(D4D6P) according to the table below and take care to apply the medium slowly along the wall of each well.

	Recommended volume per well		
Reagents	**6-well plate**	**24-well plate**	**96-well plate**
Medium N(D4D6P)	2.5 ml	750 µl	125 µl

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

Day 6

< 1 hour

Assay or Continuous Maturation

- Differentiated neurons can be observed on Day 1. For more mature neurons, we recommend culturing cells until Day 6. From Day 6, users may maintain differentiated neurons in the medium best suited for their needs, though we recommend Quick-Neuron™ Dopaminergic Maintenance Medium, Catalog Number: DA-MM
- Differentiation into Dopaminergic neurons after using Quick-Neuron™ Dopaminergic neurons can be confirmed with the markers TUBB3, TH, and DA.

Appendix

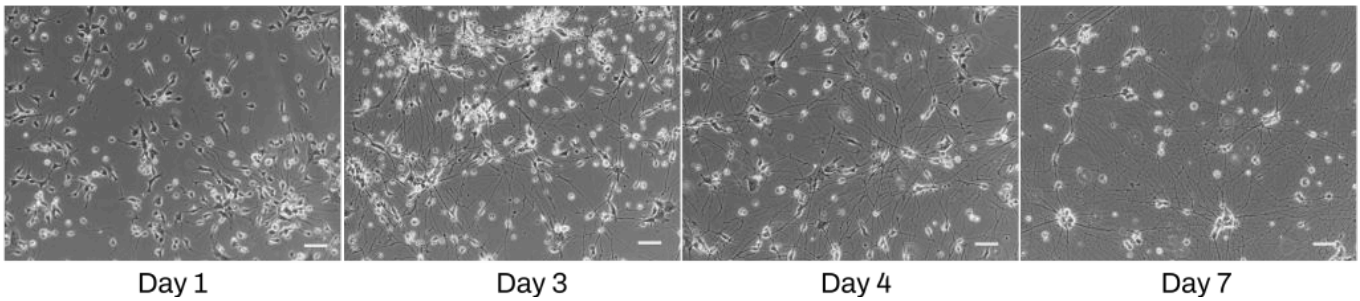


Figure 1. Representative phase contrast images of Quick-Neuron™ Dopaminergic - mRNA cell cultures on days 1, 3, 4, and 7 post-thaw (scale bar = 100 µm).

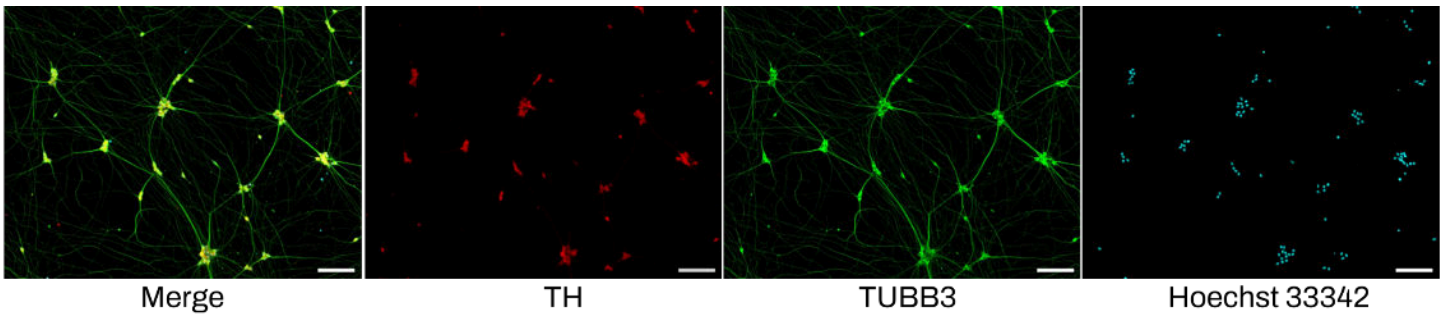


Figure 2. Immunofluorescent staining of Quick-Neuron™ Dopaminergic - mRNA cell cultures shows typical neurite growth and expression of TUBB3 and TH on day 10 post-differentiation (scale bar = 100 µm). Staining conditions: Anti-β-III tubulin monoclonal antibody (R&D Systems, Catalog Number: MAB1195, 1:250 dilution) in combination with a secondary antibody (Invitrogen, Catalog Number: A32723 Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488, 1:500 dilution). Anti-TH polyclonal antibody (Abcam, Catalog Number: Ab6211, 1:2000 dilution) in combination with a secondary antibody (Invitrogen, Catalog Number: A-11037, Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594, 1:1000 dilution). Nuclei were counterstained with Hoechst 33342.