Ncardia
Stem cell experts

Ncyte™ CNS Neuron Kit

Human iPSC-derived central nervous system cells

Manual

Version 1.3 / January 2020

Contents

Getting Started

Please make sure to read the entire Manual carefully before you start thawing and culturing Ncyte™ CNS Neuron Cells.

The Ncyte CNS Neuron Kit is for *in vitro* life science research use only.

The Material Safety Data Sheet (MSDS) for the Ncyte CNS Neuron Kit is available at www.ncardia.com.

Technical Support and Training

Ncardia scientists are ready to help you with any questions you have regarding this Manual or the Ncyte CNS Neuron Kit. On-site training is also available upon request. Visit www.ncardia.com for more information or send an e-mail to support@ncardia.com.

1. General Information

This protocol covers thawing, seeding, and culturing of Ncyte™ CNS Neuron Cells. Please read the entire protocol before you begin your experiment. The most recent version of this Manual can be downloaded at www.ncardia.com.

Ncyte CNS Neuron Cells are produced through a well-defined *in vitro* differentiation process from human induced pluripotent stem cells (iPSC). The iPSC line is generated by introducing defined transcription factors, described by Yamanaka in a human skin fibroblast, using a non-viral system.

Ncyte CNS Neuron Cells are composed of two key cell types of the human central nervous system (CNS): neurons (glutamatergic, GABAergic and dopaminergic) and astrocytes. They express relevant markers GFAP as a marker for astrocytes, beta III tubulin as a pan-neuronal marker, as well as tyrosine hydroxylase, vGlut and vGAT as markers for dopaminergic, glutamatergic and GABAergic neurons, respectively.

2. Safety Information

- The Ncyte™ CNS Neuron Kit is intended for *in vitro* research use only. The kit is not intended for diagnostics, therapeutic or clinical use and are not approved for human *in vivo* applications.
- Ncyte CNS Neuron Cells are genetically modified human cells and therefore genetically modified organisms (GMO). They should be handled according to local directives (Biosafety level 1, US-CDC, or S1, GenTSV, Germany).
- Ncyte CNS Neuron Cells are inactivated by autoclaving at 121°C for 20 minutes.
- Ncyte CNS Neuron Cells must be cultured in a sterile environment.

It is highly recommended that gloves and lab coats are worn when handling all reagents, as some reagents contain chemicals that may be harmful. Please consult the product's Certificate of Analysis (CoA) and Material Safety Data Sheet (MSDS) for further information and safety instructions.

3. Material

3.1 Cells and media provided by Ncardia

Ncyte CNS Neuron Cells are supplied cryopreserved in a vial containing 2 million cells. The complete kit includes cells, medium, and medium supplement (order no. Nc-K-HZ02-2M). The kit contains components for 100 ml of culture medium, which allows cultivation of the cells for 2 weeks when cultivated in a 48- or 96-well format.

The Ncyte™ CNS Neuron culture medium is prepared by adding Ncyte™ CNS Neuron Supplement to Ncyte™ Neuro Basal Medium (see 4.1). It is used for seeding and culturing of Ncyte CNS Neuron Cells. Ncyte Neuro Basal Medium is used for thawing of the cells.

Material	Order number	Container	Content	Storage
Ncyte™ CNS Neuron Cells	Nc-C-HZ02-2M	1 cryo vial	2 million cells	Liquid nitrogen
Ncyte™ Neuro Basal Medium A	Nc-M-NBM250A	1 bottle	250 ml	Liquid, +4 °C
Ncyte™ CNS Supplement	Nc-MS-DCS-C	2 cryo vials	1.25 ml each	Frozen, -80 °C

Table 1: Overview of Ncyte™ CNS Neuron Kit components

Antibiotics may be added to the culture medium if desired. Note that antibiotic use is optional. Cell growth and function is not compromised by long-term culture in the presence of ciprofloxacin. A protocol for creating a ciprofloxacin solution, including a recommended supplier, is available upon request at **support@ncardia.com** or from your local Ncardia representative.

3.2 Storage conditions

- **Cryopreserved cells:** Upon receipt, transfer the vials directly to the vapor phase of liquid nitrogen for storage. Do not expose the vials to room temperature. Do not store cells at -80 °C, as recrystallization will harm the cells.
- **Medium:** Store Ncyte Neuro Basal Medium at +4°C. Avoid excessive exposure to light. For storage life see expiry date on bottle. The Ncyte CNS Neuron culture medium (Ncyte Neuro Basal Medium with added Ncyte CNS Supplement) can be stored at 4 °C for up to 1 week.

3.3 Required consumables

Consumables	Vendor	Cat. No.
Tissue culture flask or multi-well plates	Various	-
Sterile 50 ml polypropylene tubes	Various	-
Poly-L-ornithine (PLO) hydrobromide	Sigma-Aldrich	P3655
Laminin (L) solution	Sigma-Aldrich	L2020
Matrigel hESC-Qualified Matrix	Corning	354277
DMEM/F12 (optional)	Various	-
Trypan blue solution 0.4 %	Sigma-Aldrich	T8154

Table 2: Overview of required consumables

3.4 Required equipment

Item	Vendor
37 °C water bath	Various
Laminar flow hood	Various
Cell culture incubator (37 °C, 95 % humidity, 5 % CO_2)	Various
Neubauer hemocytometer	Various
Centrifuge (swinging bucket rotor)	Various
Inverse microscope	Various
Liquid nitrogen storage	Various

Table 3: Overview of required equipment

4. Preparation

4.1 Medium

1. If use of antibiotics is desired, add 250 µl of a ciprofloxacin solution (2 mg/ml) to 250 ml Ncyte™ Neuro Basal Medium A to final concentration of 2 µg/ml ciprofloxacin.
2. Thaw a vial containing Ncyte CNS™ Supplement on ice.
3. Transfer 50 ml Ncyte Neuro Basal Medium A to a 50 ml polypropylene tube.
4. Homogenize Ncyte CNS Supplement by pipetting; add 1.25 ml to the Neuro Basal Medium A in the 50 ml tube.
5. Mix by pipetting. Ncyte™ CNS Neuron culture medium is ready to use. Store at 4 °C.

Note

- If less than 50 ml culture medium is needed, aliquot the Ncyte CNS Supplement after thawing and refreeze in portions. Do not repeatedly thaw and freeze this supplement!
- Do not pre-warm Ncyte Neuro Basal Medium A and Ncyte CNS Neuron culture medium to 37 °C to avoid premature degradation of the ingredients.

4.2 Surfaces

Ncyte CNS Neuron Cells can be cultured on numerous surfaces.

Plastic: Ncyte CNS Neuron Cells adhere best on cell culture-treated plastic surfaces. Plasticware from Nunc (Nunclon Delta Surface) or Greiner (Greiner Bio-One) are recommended.
Glass: The attachment of Ncyte CNS Neuron Cells on glass surfaces (e.g., cover slips for patch clamp) is not as tight as on plastic ware. Please contact support@ncardia.com for additional information.

4.3 Coating

Choice of coating depends on a specific cell and assay purpose. As a standard coating for Ncyte™ CNS Neuron Cell cultivation, we recommend using poly-L-ornithine (PLO)/Laminin (L) or Matrigel. For electrophysiological assays, e.g. manual patch clamp or MEA recordings, we recommend using Poly-ethyleneimine (PEI) as coating.

Note: Do not allow the surface to dry during coating.

Coating plates with poly-L-ornithine (PLO)/Laminin (L)

1. Dilute sterile poly-L-ornithine (PLO) hydrobromide to 10 µg/ml in PBS without Ca^{2+} and Mg^{2+}.
2. Add a proper volume of PLO solution to cover the bottom of the culture vessel (see Table 4.)
3. Incubate the culture vessel in a cell culture incubator at 37 °C for 1 h.
4. After the incubation period, aspirate PLO and wash 3 times with PBS without Ca^{2+} and Mg^{2+}.
5. Dilute sterile Laminin (L) solution to 10 µg/ml in PBS without Ca^{2+} and Mg^{2+}.
6. Add a proper volume of Laminin solution to cover the bottom of the well.
7. Incubate the culture vessel in a cell culture incubator at 37 °C for 1 h.
8. Aspirate Laminin immediately before seeding.

Coating with Matrigel

Handle Matrigel hESC-Qualified Matrix according to the manufacturer's instructions. Keep Matrigel and dilutions of Matrigel on ice during all steps and use pre-cooled tubes, pipettes, plates, and dishes. Matrigel will solidify rapidly at room temperature.

1. Thaw Matrigel on ice overnight at 4 °C.
2. Dilute Matrigel 1:100 with ice-cold DMEM/F-12.
3. Add diluted Matrigel to plates to be coated. Volume should be sufficient to cover the entire surface (see Table 4.).
4. Incubate at room temperature for 1 hour.
5. Plates not used immediately may be wrapped in parafilm and stored at 4 °C for up to 2 weeks.
6. Before plating neurons, warm up the plates for 1 hour at 37 °C.
7. Aspirate Matrigel immediately before seeding the neurons.

Format	Volume coating (ml)	Volume media (ml)
T75 flask	10	15
T25 flask	5	5
96-well plate	0.05	0.05

Table 4: Suggested coating and media volumes per culturing format

5. Cell Culture

5.1 Thawing

Note: Use Ncyte™ Neuro Basal Medium (without supplements) for thawing of cells.

1. Coat a flask or multi-well plate of desired format with desired coating (see 4.3.)
2. Warm Ncyte Neuro Basal Medium A to room temperature.
3. Add 1 ml Neuro Basal Medium A to a 50 ml polypropylene tube.
4. Quickly transfer cryopreserved Ncyte CNS Neuron Cells from the vapor phase of liquid nitrogen or from a transport dewar with liquid nitrogen directly to a 37 °C water bath.
5. Thaw the vial until the frozen cell suspension detaches from the bottom of the vial and only a small ice clump is visible (2 minutes).
6. Gently resuspend the sedimented cells by carefully swinging the vial back and forth. Avoid repeatedly pipetting the thawed neuronal cells.
7. Transfer the cell suspension to the 50 ml tube using a 1000 µl pipette.
8. Gently mix the cell suspension by carefully swinging the Falcon tube back and forth. Optional: Transfer 20 µl of the cell suspension to a 1.5 ml tube for cell counting before centrifugation.
9. Add 8 ml Ncyte Neuro Basal Medium A to the cell suspension in the 50 ml tube and gently mix the cell suspension to achieve a total volume of 10 ml.
10. Pellet Ncyte CNS Neuron Cells by centrifugation at 260 x g for 8 min at room temperature.
11. Aspirate the supernatant. Gently resuspend the cells in 1 ml Ncyte CNS Neuron culture medium.
12. Use 20 µl of cell suspension for cell counting (see 5.2) and proceed with cell seeding (see 5.3).

Note

- For transport of frozen vials from a liquid nitrogen storage tank to the cell culture room, a dewar filled with liquid nitrogen should be used. Do not use dry ice for the transport because this might affect cell viability.
- Avoid damage of the thawed neuronal cells by repeated pipetting, vigorous shaking or vortexing. Careful mixing of the cells is key to ensuring maximal viability.

5.2 Counting of cells and determination of viability

1. Add 20 µl trypan blue solution to 20 µl cell suspension withdrawn for counting.
2. Apply 10 µl of the 1:1 mixture to a Neubauer hemocytometer and count viable (clear), dead (blue) and total cells.
3. Count the number of cells in each of the four outer squares highlighted in red of figure 1. Calculate the mean number of cells per red square.
4. Calculate the number of cells corrected by chamber factor (1 x 104), dilution factor (2), and total volume of cell solution (e.g., 1 ml).

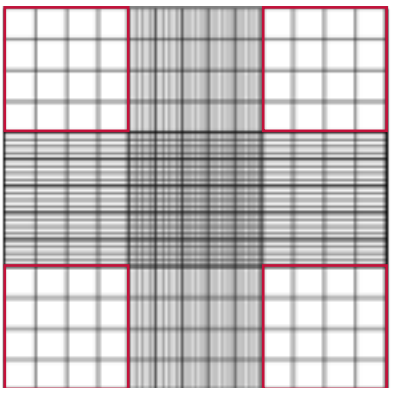

Fig. 1: Neubauer hemocytometer

Calculation example

Mean number of viable cells per square = 100

100 x 10,000 x 2 x 1 = 2,000,000

2 million living cells in the cell suspension

5.3 Seeding

1. Adjust the cell suspension adequately with culture medium depending on the suggested assay conditions (see Table 5); mix the cells carefully by gently agitating the tube.
2. Transfer the coated plates and the cell suspension to the laminar hood.
3. Remove coating solution from the plates by aspiration; do not let the coating dry.
4. Carefully mix the cells again and plate them into the wells. We recommend using a 8-channel pipette when using 96-well formats. Carefully mix the cells regularly during plating, e.g., after pipetting 3 rows each when using 96-well plates.
5. Leave plates for 20 minutes under the hood to achieve an even distribution of the cells in the wells. Incubate cells at 37 °C, 5 % CO_2 in a humidified atmosphere.

Note: Let plates stand horizontally during seeding to avoid an uneven distribution of cells. Keep the tips of the pipette close to the bottom of the plate when seeding to reduce the risk of formation of air bubbles.

Assay and format	No. of cells per surface area	Volume of cell suspension
Immunostaining (96-well format)	150,000/cm²	200 µl/well
Manual patch clamp	150,000/cm²	100 µl/cover slip
Maestro MEA (96-, 48-well format)	36,000/well	Droplet, 3 µl/well

Table 5: Overview of seeding densities according to assay type

5.4 Maintenance

We recommend changing the culture medium 3 times a week during subsequent culture using Ncyte CNS Neuron culture medium at room temperature. Remove 50 % of the medium and add a corresponding amount of fresh medium. Repeat this process once.

Note: Cells are very sensitive to 100 % medium changes, which is why partial (50 %) medium changes are recommended.

6. Ncardia Limited Label Use License

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C. Other patents

Ncardia products which were derived from iPS cells are covered by patents in patent family EP1970446 and US8048999 licensed from iPS Academia (Kyoto University). Additionally, GFP and RFP positive products are covered by patents owned by Evrogen. The GFP and RFP positive products are for internal, non-commercial research use only. The right to use a GFP positive product specifically excludes the right to validate or screen compounds.

7. Appendix

7.1 Ncyte™ CNS Neuron Cell morphology in culture

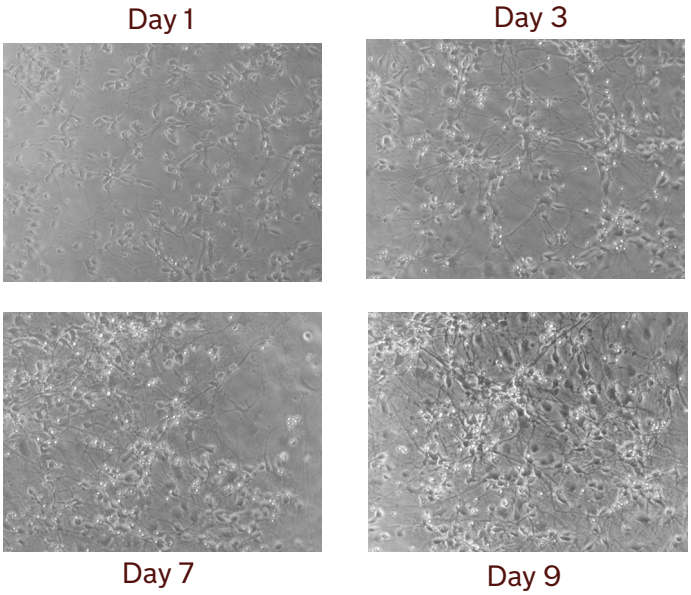

Fig. 2: Ncyte CNS Neuron Cell morphology at different times after seeding. Cells were seeded at 150,000 cells per cm^2 on a Poly-L-ornithine/Laminin coated plate. Magnification 200x.

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