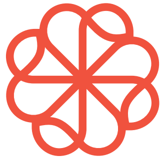

Ncardia
Stem cell experts

CNS.4U®

Human iPSC-derived central nervous system cells

Manual

Version 1.0 / July 2018

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Getting Started

Please make sure to read the entire Manual carefully before you start thawing and culturing CNS.4U® neural cells.

CNS.4U® neural cells are for *in vitro* life science research use only.

A Material Safety Data Sheet (MSDS) for CNS.4U® neural cells is available on our website.

Technical support and training

Our scientists are ready to help you with any questions you may have regarding this Manual or our CNS.4U® neural cells. In addition, in-lab training is available upon request. For further information please visit our website www.ncardia.com, or contact us directly by e-mail (support@ncardia.com)

1. General Information

This protocol covers thawing, seeding, and culturing of CNS.4U® neural cells. Please read the entire protocol before you start your experiment.

Detailed handling guides for specific applications and assays are available and can be downloaded from our website www.ncardia.com.

CNS.4U® neural 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[1] in a human skin fibroblast, using a non-viral system[2].

CNS.4U® neural cells comprise two key cell types of the human central nervous system (CNS): neurons (glutamatergic, GABAergic and dopaminergic) and astrocytes, and express the 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). Based on immunofluorescence data, the composition of the neuronal cultures are 80-90% neurons and 10-20% astrocytes. Of the neurons, 90% are glutamatergic and GABAergic and 10% are dopaminergic neurons.

2. Safety Information

- CNS.4U® neural cells are intended for *in vitro* research use only. The cells are not intended for diagnostics, therapeutic or clinical use and are not approved for human *in vivo* applications.
- CNS.4U® neural 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).
- CNS.4U® neural cells can be inactivated by autoclaving at 121°C for 20 minutes.
- CNS.4U® neural cells should 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 certificate of analysis (CoA) and material safety data sheets (MSDS) for additional safety instructions where applicable.

3. Material

3.1 Cells and media provided by Ncardia

CNS.4U® are available cryopreserved with 2 million cells / vial and are offered as a bundle including cells, medium, and medium supplement (order no. Ax-B-HZ02-2M). The bundle contains ingredients for 100 ml of culture medium and allows cultivation of the cells for two weeks when cultivated in 48- or 96-well format. Bundle ingredients can also be ordered separately (see Table 1 for order numbers).

The medium for CNS.4U®, i.e., CNS.4U® culture medium, is prepared by adding CNS.4U® Supplement to Neuro.4U Basal Medium (see 4.1). Neuro.4U Basal Medium is used for thawing of the cells, while CNS.4U® culture medium is used for seeding and culturing of CNS.4U®.

Supplement C contains Ciprobay (2 mg/ml) and may be added to the culture medium where the use of antibiotics is desired. Note that the use is optional; importantly, cell growth and function is not compromised by long-term culture in the presence of Ciprobay.

Material	Container	Content	Storage	Shelf life
Cryopreserved CNS.4U® (Ax-C-HZ02-2M)	Cryo vial (x 1)	2 million cells	Liquid nitrogen	Max. 1.5 years from issue date on CoA
Neuro.4U Basal Medium (Ax-M-NBM250)	Bottle (x 1)	250 ml	Liquid +4 °C	See expiry date on bottle label
Neuro-Supplement (Ax-M-DCS-DA)	Cryo vial (x 2)	1.25 ml each	Frozen -80 °C	See expiry date on cryo vial label
Supplement C (Ax-M-CB-5)	Cryo vial (x 1)	250 µl	Liquid RT, dark	See expiry date on cryo vial label

Table 1: Overview of CNS.4U® product bundle

3.2 Storage conditions

- **Cryopreserved cells:** Upon receipt of cryopreserved CNS.4U®, transfer the vials directly to the vapor phase of liquid nitrogen for further storage. Do not expose the vials to room temperature and do not store cells at -80 °C, as recrystallization will harm the cells.
- **Medium:** Store Neuro.4U Basal Medium at +4°C. Avoid excessive exposure to light. For storage life see expiry date on bottle.

CNS.4U® culture medium (Neuro.4U Basal Medium with added CNS.4U® 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. Preparations

4.1 Medium

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

Note

- **If less than 50 ml culture medium is needed, aliquot the CNS.4U® Supplement after thawing and refreeze in adequate portions. Do not repeatedly thaw and freeze this supplement!**
- **Do not pre-warm Neuro.4U® Basal Medium and CNS.4U® culture medium to 37 °C to avoid premature degradation of the ingredients.**

4.2 Surfaces

CNS.4U® can be cultured on various surfaces.

Plastic

CNS.4U® adhere best on cell culture-treated plastic surfaces. We recommend using plastic ware from Nunc (Nunclon Delta Surface) or Greiner (Greiner Bio-One).

Glass

The attachment of CNS.4U® 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 / assay purpose. As a standard coating for CNS.4U® cultivation, we recommend using either poly-L-ornithine (PLO) / Laminin (L) or Matrigel.
For electrophysiological assays, e.g. manual patch clamp or MEA recordings, we recommend using Polyethyleneimine (PEI) as coating. Refer to our specific assays protocol for detailed instructions (see 7. Related Documents).

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 h.
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

Note

Do not allow the surface to dry during coating.

5. Cell Culture

5.1 Thawing

Note: Use Neuro.4U 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 Neuro.4U Basal Medium to room temperature.
3. Add 1 ml Neuro.4U Basal Medium to a 50 ml polypropylene tube.
4. Quickly transfer cryopreserved CNS.4U® 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.
9. (Optional: Transfer 20 µl of the cell suspension to a 1.5 ml tube for cell counting before centrifugation.)
10. Add further 8 ml Neuro.4U Basal Medium to the cell suspension in the 50 ml tube and gently mix the cell suspension; you now have a total volume of 10 ml.
11. Pellet CNS.4U® by centrifugation at 260 x g for 8 min at room temperature.
12. Aspirate the supernatant. Gently resuspend the cells in 1 ml CNS.4U® culture medium.
13. Use 20 µl of cell suspension for cell counting (see 5.2) and proceed with cell seeding (see 5.3).

Note

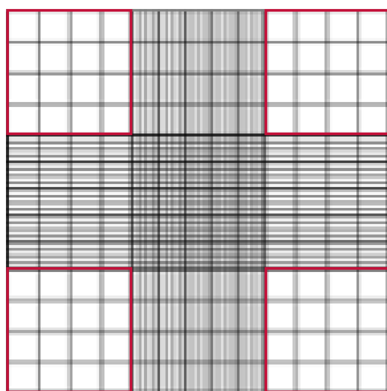
- **For transport of frozen vials from a liquid nitrogen storage tank to the cell culture room, a dewar filled with liquid nitrogen should be used. Do not use dry ice for the transport because this might affect cell viability.**
- **Avoid damage of the thawed neuronal cells by repeated pipetting, vigorous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

The procedure below applies for thawing of vials containing 4 million Cor.4U® and pre-cultivation in flasks.

1. Coat a T75 flask with fibronectin (see 4.2.).
2. Transfer 4 ml Cor.4U® Culture Medium into a 50 ml tube and pre-warm to 37 °C.
3. Prepare a reaction tube with 20 µl trypan blue solution.
4. Quickly transfer the cells from liquid nitrogen directly to a 37 °C water bath. Thaw the vial until the frozen cell suspension detaches from the bottom of the vial and only a small ice clump is visible

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).



Calculation example
E.g.: 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

Fig. 1: Neubauer hemocytometer

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	Volume of cell suspension
Immunostaining (96-well format)	150,000 / cm^2	200 µl / well
Manual patch clamp	150,000 / cm^2	100 µl / cover slip
Maestro MEA 96-well, 48-well	36,000/ well	Droplet 3 µl / well

Table 5: Overview of CNS.4U® seeding densities according to assay type

5.4 Maintenance

We recommend changing the culture medium three times a week during subsequent culture. For this, use CNS.4U® culture medium at room temperature. Remove 50 % of medium and add 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. References

1. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S. (2007) Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell. 2007 Nov 30;131(5):861-72.
2. Axiogenesis AG Patent WO2012098260 A1, A non-viral system for generation of induced pluripotent stem (iPS) cells.

7. Related Documents

1. CNS.4U® in Maestro microelectrode array (MEA) assay

8. Ncardia Limited Label Use License

A. Ncardia Intellectual Property Rights

This product is covered by patent families including, but not limited to, EP1348019; EP1002080; EP1745144; EP1644485; JP4904153; JP4159358; JP3956154; JP4814875; DE10136702 and other families of patent applications ("Ncardia Intellectual Property"). Purchase of the product does not transfer any rights other than those outlined below.

The purchase of this product conveys to the buyer the non-exclusive, non-transferable right to use the purchased amount of the product and the associated Ncardia Intellectual Property for (i) for non-profit internal research conducted by the buyer and (ii) certain for-profit activities, including lead discovery, testing and/or research and development of other products. The use in disease modeling and tissue modeling is expressly excluded in this license. Please contact Ncardia for a license for disease and tissue models at patent@ncardia.com.

B. Use restrictions

This product is not suitable for any clinical, therapeutic (including cell therapy, transplantation, and regenerative medicine), or clinical diagnostic applications. The purchaser shall not use the product in any way that contravenes applicable laws or regulations. The product should be used according to the User Guide. Failure to comply with any provisions in section A, B, or C will make any warranty claims invalid. No rights are conveyed to modify, reproduce, or clone any part of this product or to use Ncardia Intellectual Property in any way that is separate from the purchased product.

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.

9. Appendix

9.1 CNS.4U® morphology in culture

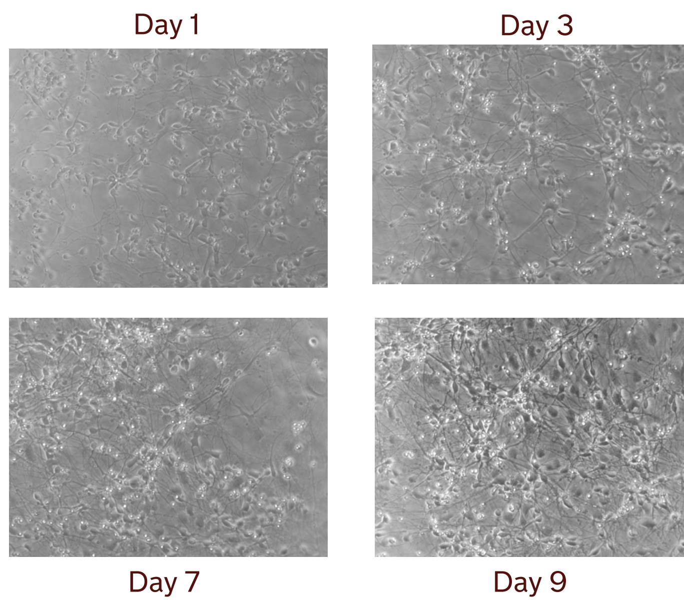
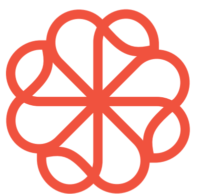


Fig. 2: CNS.4U® morphology at different times after seeding. CNS.4U® were seeded at 150,000 cells per cm2 on a Poly-L-ornithine / Laminin coated plate. Magnification 200x.



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