Note: Link to video protocols: Thawing for hNP1™ Cells; Plating for hNP1™ Cells; Subculture Protocol for hNP1™ Cells

hNP1™ Neural Progenitor Expansion Kit (Catalog#: HN60001)

Kit Contents

- 1 vial of hNP1™ Human Neural Progenitor Cells
- 1 500-ml bottle of AB2™ Basal Neural Medium
- 2 x 5-ml vials of ANS™ Neural Supplement

Required but not Supplied

- bFGF (50 µg/ml) – Neuromics: Cat# PR15002CF
- L-Glutamine (200 mM) – Invitrogen: Cat#25030

Recommended but not Supplied

- LIF (10 µg/ml) – Neuromics: Cat# PR16101

Optional but not Supplied

- Penicillin (5,000 U/ml)/ Streptomycin (5,000 µg/ml) - Invitrogen: Cat#15070

Unpacking and Storage Instructions

hNP1™ Human Neural Progenitor Cells

- Cells must be moved from dry ice to liquid nitrogen **IMMEDIATELY**. Temperature fluctuations will have adverse effects on cell health and viability.
- When stored in the recommended storage conditions (liquid nitrogen), **hNP1™ Human Neural Progenitor Cells** can remain stable in excess of 3 years.

Medium and Supplements

- Upon arrival, store the AB2™ Neural Medium at 2-8°C protected from light.
- Upon arrival, store ANS™ Supplement at -20°C.
- After supplements are thawed, use within one month.
- Do not refreeze

Supplementing the AB2™ Basal Medium

1. Decontaminate the external surfaces of all supplement vials and the medium bottle with ethanol or isopropanol.
2. Aseptically open each supplement vial and add the amount indicated below to the basal medium with a pipette.

To make 100 ml of complete medium:	
AB2™ Neural Medium	96 ml
ANS™ Supplement	2 mL
bFGF, 50 µg/ml	40 µl
L-Glutamine	1 mL
Penicillin/Streptomycin (optional)	1 mL
LIF (10 µg/ml) (optional)	100 µl

3. We recommend filtering the supplemented medium through a sterile 0.22µm filter prior to use.

4. Fully supplemented medium should be stored at 2-8°C, protected from light. The medium should be given an expiration date of up to 2 weeks, but not more than 1 month from the thawing of the ANS™ Neural Medium Supplement. Dispense the complete medium into aliquots to avoid repeated heating prior to each use.

Plate Coating Protocol Protocol
Description:

hNP1™ Cells form adherent monolayer cultures when grown on cell culture plates pre-coated with substrate. We recommend pre-coating your plates with Matrigel™ using the following protocol.

Required but not supplied:
BD Matrigel™ Basement Membrane Matrix
Dulbecco's Modified Eagle's Medium
Tissue culture treated polystyrene plate

To coat dishes perform the following steps:

1. Thaw BD Matrigel™ at 2-8°C overnight. Matrix will gel rapidly at 22°C to 35°C. Keep Matrigel™ on ice and use pre-cooled pipettes, plates and tubes when preparing. Gelled Matrigel™ may be re-liquified if placed at 2-8°C on ice for 24 to 48 hours.
2. Handle using aseptic technique in a laminar flow hood.
3. Once BD Matrigel™ Matrix is thawed, swirl vial to be sure that material is evenly dispersed.
4. Place thawed vial of BD Matrigel™ Matrix in sterile area, decontaminate the external surfaces with ethanol or isopropanol and air dry. BD Matrigel™ Matrix may be gently pipetted using a pre-cooled pipette to ensure homogeneity. For long term storage, Matrigel™ can be diluted 1:2 with cooled Dulbecco's Modified Eagle's Medium and refrozen into aliquots. These aliquots can then be thawed as described in step 1 and stored at 4°C for up to 1 week before dilueting further.
5. Dilute Matrigel™ 1:200 with cooled Dulbecco's Modified Eagle's Medium. Keep on ice.
6. Using the chart below, add the corresponding volume of diluted Matrigel™ to the plate size being used. Swirl to ensure the entire surface of the plate or flask is covered with the Matrigel™ solution.
7. Allow dishes to incubate at room temperature for at least 15 minutes, but no longer than 1 hour before use.
8. Remove Matrigel™ and use plates immediately. Freshly prepared Matrigel™ plates should always be used with HNP1 Human Neural Progenitor Cells. To avoid waste, the 1:200 dilution of Matrigel can be stored at 4°C for up to 1 week to coat fresh plates as needed.

Recommended volumes to coat flasks:

Plate/Flask	Working Volume
96 well plate	100 µl/well
35 mm dish	2 mL
6 well plate	2 mL/well

NOTE: IF USING CELLS IN A FORMAT OTHER THAN DESCRIBED ABOVE PLEASE CONTACT TECHNICAL SUPPORT FOR ASSISTANCE.

Cell Thawing Protocol

Protocol Description: hNP1™ Human Neural Progenitor Cells form adherent monolayer cultures when grown on cell culture plates pre-coated with substrate. We recommend thawing your hNP1™ Human Neural Progenitor Cells using the following protocol.

Required but not supplied:
Pre-coated plates (see plate coating protocol)

To plate the cells perform the following steps:

1. Do not thaw the cells until the recommended medium and appropriately coated plasticware and/or glassware are on hand.
2. Remove the vial from liquid nitrogen and incubate in a 37°C water bath. Closely monitor until the cells are completely thawed. Maximum cell viability is dependent on the rapid and complete thawing of frozen cells.

IMPORTANT: Do not vortex the cells. Breaking cells down to single cell suspensions will significantly increase cell death.

3. As soon as the cells are completely thawed, disinfect the outside of the vial with 70% ethanol or isopropanol. Proceed immediately to the next step.
4. In a laminar flow hood, use a 1 or 2 mL pipette to transfer the cells to a sterile 15 mL conical tube. Be careful to not introduce any bubbles during the transfer process.
5. Using a 10 mL pipette, slowly add dropwise 9 mL of fully supplemented AB2™ Neural Medium (pre-warmed to 37°C) to the 15 mL conical tube.

IMPORTANT: Do not add the whole volume of medium at once to the cells. This may result in decreased cell viability due to osmotic shock.

6. Gently mix the cell suspension by slow pipetting up and down twice. Be careful to not introduce any bubbles.

IMPORTANT: Do not vortex the cells. Breaking cells down to single cell suspensions will significantly increase cell death.

7. Centrifuge the tube at room temperature at 200 x g for 4 minutes to pellet the cells.
8. Aspirate as much of the supernatant as possible. Steps 4-7 are necessary to remove residual cryopreservative (DMSO).
9. Resuspend the cells in a total volume of 2 ml of fully supplemented AB2™ Neural Medium (pre-warmed to 37°C).
10. Plate the 1 mL cell suspension of hNP1™ cells onto a Matrigel-coated 35 mm dish. Gently move the plate in a side-to-side and back-and-forth manner to disperse cells in a uniform manner.
11. Incubate the cells at 37°C in a 5% CO_2 humidified incubator.
12. Exchange the medium with fresh fully supplemented AB2™ Neural Medium 24 hours post plating. Exchange with fresh medium every other day thereafter. Use caution not to dislodge the cells; do not pipette media directly onto the cells but rather onto the side of the culture dish.
13. Once the hNP1™ cells reach 100% confluence, they can be dissociated manually for passaging (e.g., by cell scraping or by gentle and slow pipeting up and down to detach the cells). The cells should be maintained at a high density at all times – the recommended passaging ratio is 1:2.

Subculture of hNP1™ Cells

1. Once the hNP1™ cells reach 100% confluence, carefully remove the medium from the 35 mm dish.

2. Apply 2 ml fully supplemented AB2™ Neural Medium (pre-warmed to 37°C) to the cells so that the cells can be harvested in fresh medium.

3. Using a pipette, manually detach the cells from the dish by slow pipetting up and down the dish. Be careful to avoid introducing any bubbles. We recommend using a 200 µl or 1000 µl manual pipette to dislodge the attached cells. Alternatively, cells can be dislodged with a sterile cell scraper.

*IMPORTANT: We do **NOT** recommend enzymatic methods for passaging the hNP1™ cells. Doing so reduces the long term viability of the cells and can cause karyotypic abnormalities.*

4. Plates should be observed to ensure that all cells have been removed. This is most easily accomplished by working under a dissection microscope within a laminar flow hood, but can also be achieved by frequent observation under a bright field or phase contrast microscope.

5. Transfer the dissociated cells to a 15 mL conical tube. Inspect the plate to ensure that all the cells have been removed.

6. If necessary, count the cells and calculate the cell concentration. Cells can be centrifuged at 200 x g for 4 minutes in order to concentrate the cell suspension for higher plating densities.

7. Plate the cells at the desired density into the appropriately coated flasks, plates or wells (see plate coating protocol) in fully supplemented AB2™ Neural Medium. We recommend keeping the cells at a high cell density by passaging 1:2 – 1:3.

8. Incubate the cells at 37°C in a 5% CO_2 humidified incubator.

9. Exchange the medium with fresh fully supplemented AB2™ Neural Medium 24 hours post plating. Exchange with fresh medium every other day thereafter. Use caution not to dislodge the cells; do not pipette media directly onto the cells but rather onto the side of the culture dish.

Patent US 6,200,806
Patent US 7,531,354,B2

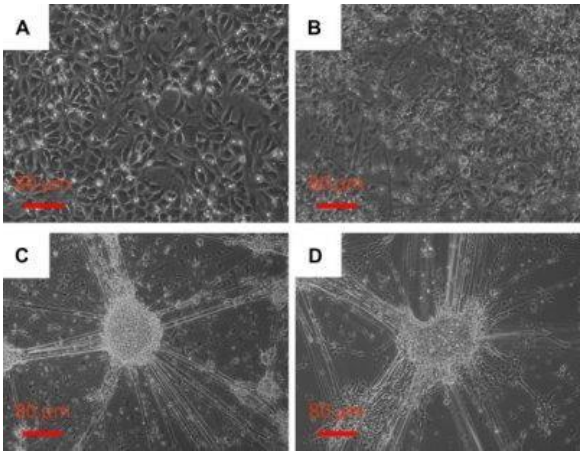
Publications

Xiufang Guo, Severo Spradling, Maria Stancescu, Stephen Lambert, James J. Hickman. Derivation of sensory neurons and neural crest stem cells from human neural progenitor hNP1. Biomaterials, In Press, Corrected Proof,Mar 2013.doi:10.1016/j.biomaterials.2013.02.061

Mahesh C. Dodla, Amber Young, Alison Venable, Kowser Hasneen1, Raj R. Rao, David W. Machacek, Steven L. Stice. Differing Lectin Binding Profiles among Human Embryonic Stem Cells and Derivatives Aid in the Isolation of Neural Progenitor Cells. PLoS ONE 6(8): e23266. doi:10.1371/journal.pone.0023266

A. Young, D.W. Machacek, S.K. Dhara, P.R. MacLeish, M. Benveniste, M.C. Dodla, C.D. Sturkie and S.L. Stice. Ion channels and ionotrophic receptors in a human embryonic stem cell derived neural progenitors.doi:10.1016/j.neuroscience.2011.04.039. ...mouse nonoclonal anti nestin (neuromics), mouse monoclonal anti tuj1 (neuromics)...

Adam D. Bachstetter, Jennifer Jernberg, Andrea Schlunk, Jennifer L. Vila, Charles Hudson, Michael J. Cole, R. Douglas Shytle, Jun Tan, Paul R. Sanberg, Cyndy D. Sanberg, Cesario Borlongan, Yuji Kaneko, Naoki Tajiri, Carmelina Gemma, Paula C. Bickford. Spirulina Promotes Stem Cell Genesis and Protects against LPS Induced Declines in Neural Stem Cell Proliferation. PLoS ONE 5(5): e10496. doi:10.1371/journal.pone.0010496.



Images: Phase contrast images of the cultures before and after the sensory neuron induction. A) hNP1 culture before sensory induction. B) hNP1 culture 10 days after sensory induction. C) hNP1 culture 30 days after sensory induction. Neuronal clusters and axonal bundles, which resemble rat DRG cell cultures, were typically observed. D) For comparison, an image of a rat embryonic DRG cell culture at 7 DIV is provided.

doi.org/10.1016/j.biomaterials.2013.02.061

Suggested Components

Component	Vendor	Cat# Number
Matrigel	BD	354234 or 354277
DMEM	Sigma	D6046
L-glutamine	Invitrogen	25030
Penicillin/Streptomycin	Invitrogen	15070
LIF	Neuromics	PR16101
bFGF	Neuromics	PR15002CF
6-well plates	BD Falcon	353046
35mm plates	BD Falcon	353001
96 well plates	Corning	3603
Cell Scraper	Sarstedt or TPP	83.1832 or 83.1830 Or 99002 or 99003

APPENDIX A

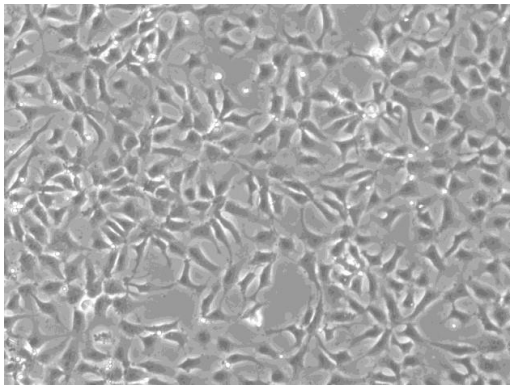
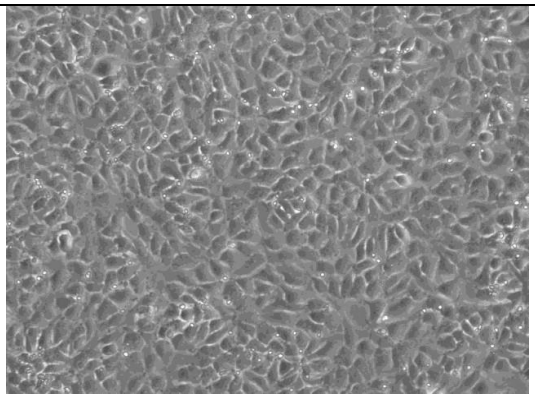
A USER MANUAL SUPPLEMENT

A State of Confluence and Neural Progenitors

Maintenance and passaging of hNP1™Human Neural Progenitor Cells requires finesse. Neural progenitor cells do not thrive well when passaged at 80 or 90% confluence.

The neural progenitors are highly density dependent. Studies suggest that neural progenitors are constantly signaling each other via short range mechanisms linked to purine receptors, which indicates a need for close contact. (Lin, Jane H.C. et al) Purine nucleotides are shown to support self- renewal. Additionally, the use of growth factors in our proliferation media allows the cells to "cross talk", and these purines act as proliferation signals for the neural progenitors. Therefore, if the cells are not at 100% confluence during passage, one runs the risk of the cells seeding too far apart post passage.

Below, we demonstrate key characteristics to look for when working with Neuromics' hNP1™ Neural Progenitor Cells.

	
Picture 1: hNP1™ Neural Progenitor Cells • **85%** confluency (10x magnification) • Cells developing processes • Too few cells in close contact ***Cells <u>are not</u> ready to passage!*** • *Warning*, if you passage now, you passage to a smaller flask/dish	**Picture 2:** hNP1™ Neural Progenitor Cells • **100%** confluency (10x magnification) • Cells present have normal morphology • Cells are in close contact • ***Cells <u>are</u> ready to passage!***

Citation from: Purinergic signaling regulates neural progenitor cell expansion and neurogenesis. Lin, Jane H.C. et al, Dev. Biol. 2007 February 1; 302(1): 356-366