



ENStem-A™ Human Neural Progenitor Expansion Kit

CATALOG NUMBER: SCR055

LOT NUMBER:

QUANTITY: ENStem-A™ Human Neural Progenitor Cells: 1 X 10⁶ viable cells upon thawing (Part No. SCC003).

ENStem-A™ Neural Expansion Medium: 500 mL, including 1 vial of 50 µg FGF, lyophilized (Part No. SCM004).

DESCRIPTION:

ENStem-A™ Human Neural Progenitor Cells (Part No. SCC003):

ENStem-A™ Human Neural Progenitor Cells are derived from NIH approved H9 human embryonic stem cells (hESCs). Using a proprietary method developed by Aruna Biomedical, these hESC derived neural progenitors proliferate as an adherent cell monolayer and can readily differentiate into different neuronal subtypes. Detailed spectral analyses indicate ENStem-A™ cells retained a normal diploid karyotype in culture after ten passages. ENStem-A™ human neural progenitor cells may be used for a variety of research applications such as studies of neurotoxicity, neurogenesis, electrophysiology, neurotransmitter and receptor functions.

ENStem-A™ Neural Expansion Medium (Part No. SCM004):

ENStem-A™ Neural Expansion Medium is a defined serum-free formulation that has been optimized for the culture and expansion of ENStem-A™ Human Neural Progenitor Cells. When used in conjunction with L-Glutamine (not provided) and FGF-2 (provided), the expansion medium will allow for the maintenance and proliferation of ENStem-A™ Human Neural Progenitor Cells.

For Research Use Only; not for use in diagnostic procedures

KIT COMPONENTS:

1. > 1x10⁶ viable ENStem-A™ Human Neural Progenitor Cells: (Part. No. SCC003) derived from NIH approved H9 human embryonic stem cells, cryopreserved. Store in liquid nitrogen.
2. ENStem-A™ Neural Expansion Medium (Part. No. SCM004) contains:
 - a. 500 mL ENStem-A™ Neural Expansion Medium (Part No. SCM004a)
 - b. 50 µg Basic FGF-2, lyophilized (Part No. GF003)

CHARACTERIZATION OF CELLS:

Each lot of ENStem-A™ Human Neural Progenitor Cells has been validated for high levels of expression of Nestin and Sox 2, and low level expression of Oct-4. The ability of ENStem-A™ cells to differentiate into multiple neuronal phenotypes and maintain a normal karyotype after multiple passages has been verified (please refer to datasheet figures). The cells have been confirmed to be negative for mycoplasma.

MATERIALS REQUIRED BUT NOT SUPPLIED:

1. ENStem-A™ Neuronal Differentiation Medium (Part No. SCM017)
2. ENStem-A™ Neural Freezing Medium (Part No. SCM011)
3. Human Neural Stem Cell Characterization Kit (Part No. SCR060)
4. Poly-L-ornithine hydrobromide (Sigma Part No. P3655)
5. Mouse Laminin (Sigma Part No. L-2020)
6. L-Glutamine (Part No. TMS-002-C)
7. Penicillin/Streptomycin Solution (Part No. TMS-AB2-C)
8. Accutase™ (Part No. SCR005)
9. Chamber slides
10. Tissue culture-ware
11. Phosphate-Buffered Saline (1X PBS) (Part No. BSS-1005-B)
12. Fixative (e.g. 4% Paraformaldehyde in 1X PBS)
13. Blocking Solution (5% normal donkey serum, 0.3% Triton X-100 in 1X PBS)
14. 4'-6-Diamidino-2-phenylindole (DAPI) / PBS Solution
15. Anti-fading mounting solution (DABCO/PVA)
16. Hemacytometer
17. Microscope

STORAGE / HANDLING:

ENStem-A™ Human Neural Progenitor Cells: When stored at the recommended storage conditions (liquid nitrogen), ENStem-A™ cells are stable up to five years. Do not expose to elevated temperatures. Discard any remaining reagents after the expiration date. We recommend that the cells be used within ten passages.

ENStem-A™ Neural Expansion Medium: ENStem-A™ Neural Expansion Medium should be stored at -20°C until ready to use. Upon thawing, fresh L-Glutamine should be added for a final concentration of 2 mM to the expansion medium. Thawed medium should be stored at 2-8°C and given a 1-month expiration dating. Dispense into aliquots to avoid repeated heating prior to each use.

Basic FGF-2 (50 µg, lyophilized) should be reconstituted with 5 mM Tris-HCL, pH 7.6 for a final concentration of 100 µg/mL. Dispense into aliquots to avoid repeated thawing. Store at -20°C.

PREPARATION OF COATED FLASKS:

We recommend coating tissue culture plastic- or glasswares that are used to culture ENStem-A™ cells with poly-L-ornithine and laminin. Poly-L-ornithine and laminin provide optimal matrix for adhesion and growth of the cells. The following procedure is recommended:

1. Prepare stock solutions of poly-L-ornithine (10 mg/mL) by dissolving poly-L-ornithine in sterile water. The stock solution should be stored at -20°C or -80°C.
2. Dilute poly-L-ornithine with water from the stock concentration (10 mg/mL) to yield:
 - a. 20 µg/mL for polystyrene plates
 - b. 50 µg/mL for glass plates
3. Add enough of the poly-L-ornithine solution to cover the whole surface of the tissue culture-ware. Use 2 mL volume for 3.5 cm plates, 5 mL volume for 6-cm plates and 10 mL volume for 10-cm plates and T75 flasks. Incubate in a humidified 37°C incubator for at least one hour.
4. Remove the poly-L-ornithine solution and rinse once with sterile water. Aspirate after the rinse.

5. Depending upon the application, dilute the laminin to the following final concentrations using sterile 1X PBS:
 - a. **For the culture, propagation and one week differentiation of ENStem-A™ cells**, dilute laminin to final concentration of 5 µg/mL for both glass and polystyrene tissue culture-wares. Use 2 mL volume for 3.5 cm plates, 3-5 mL volume for 6 cm plates and 7-10 mL volume for 10-cm plates and T75 flasks. Incubate in a humidified 37°C incubator for at least 1 hour. Coated plates and flasks can be stored in the laminin solution at 2-8°C for 3 weeks or at -20°C for 6-8 months. Just before use, bring the coated plates or flasks up to room temperature and aspirate the laminin solution. Rinse the plates once with 1X PBS before use.
 - b. **For the differentiation of ENStem-A™ cells beyond one week (i.e. two-week differentiation)**; we recommend that a 1 mg/mL laminin solution be used to coat the 8-well chamber slides used for differentiation. Laminin coating should occur approximately 15-45 minutes before plating of the cells. For each well of an 8-well chamber slide, use 6 µL of laminin solution (1 mg/mL) and cover the surface of the well using a p20 pipette tip. Incubate at room temperature for at least 15-45 minutes before using.

THAWING OF CELLS:

1. Do not thaw the cells until the recommended medium and appropriately coated plasticware and/or glassware are on hand.
2. Remove the vial of ENStem-A™ Human Neural Progenitor Cells 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.**
3. As soon as the cells are completely thawed, disinfect the outside of the vial with 70% ethanol. 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 ENStem-A™ Neural Expansion Medium (Part No. SCM004) (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 pipeting up and down twice. Be careful to not introduce any bubbles. **IMPORTANT: Do not vortex the cells.**
7. Centrifuge the tube at room temperature at 200 x g for 3-5 minutes to pellet the cells.
8. Decant as much of the supernatant as possible. Steps 4-8 are necessary to remove residual cryopreservative (DMSO).
9. Resuspend the cells in a total volume of 2 mL of ENStem-A™ Neural Expansion Medium (Part No. SCM004) (pre-warmed to 37°C) containing freshly added L-Glutamine (2 mM) and FGF-2 (20 ng/mL).
Note: FGF-2 should always be added fresh to the ENStem-A™ Neural Expansion Medium.
10. Plate the cell mixture onto a poly-L-ornithine and laminin-coated 3.5-cm tissue culture plate. **IMPORTANT: For optimal growth, ENStem-A™ cells should be maintained at high cell density at all times. Thus we do not recommend thawing the cells on tissue culture plates that are larger than a 3.5-cm tissue culture plate.**
11. Incubate the cells at 37°C in a 5% CO₂ humidified incubator.
12. The next day, exchange the medium with fresh ENStem-A™ Neural Expansion Medium (Part No. SCM004) (pre-warmed to 37°C) containing L-Glutamine (2 mM) and FGF-2 (20 ng/mL). Exchange with fresh medium containing L-Glutamine and FGF-2 every other day thereafter.
13. When the cells are approximately 90 - 100% confluent, they can be dissociated manually (preferred method) or enzymatically* with Accutase™ (Part. No. SCR005) and passaged or alternatively frozen for later use. The cells

should be maintained at high cell density at all times and thus the recommended passaging is at 1:2 (refer to Figure 1A).

SUBCULTURING VIA MANUAL DISSOCIATION (Preferred method for maximum viability and proliferation rates):

1. Once the ENStem-A™ cells reach 90-100% confluence, carefully remove the medium from the poly-L-ornithine and laminin-coated 3.5-cm tissue culture plate.
2. Apply 2 mL ENStem-A™ Neural Expansion Medium (Part No. SCM004) (pre-warmed to 37°C) to the cells so that they can be harvested in fresh medium.
3. Using a 5 mL pipette, manually detach the cells from the dish by slow pipeting up and down the dish. Be careful to avoid introducing any bubbles.
4. Transfer the dissociated cells to a 15 mL conical tube. Inspect the plate to ensure that all the cells have been removed.
5. Centrifuge the tube at 200 x g for 3-5 minutes to pellet the cells.
6. Discard the supernatant.
7. Apply 2 mL of ENStem-A™ Neural Expansion Medium (Part No. SCM004) containing L-Glutamine (2 mM) and FGF-2 (20 ng/mL) to the conical tube and using a 2 mL pipette, triturate the cells into a single cell suspension. **Note:** Do not vortex the cells.
8. Count the number of cells using a hemacytometer.
9. Plate the cells to the desired density into the appropriate poly-L-ornithine and laminin-coated flasks, plates or wells in ENStem-A™ Neural Expansion Medium (Part No. SCM004) containing L-Glutamine (2 mM) and FGF-2 (20 ng/mL). We recommend keeping the cells at a high cell density by passaging 1:2 – 1:3 (refer to Figure 1A).

SUBCULTURING VIA ENZYMATIC DISSOCIATION* (Please see note at end of section)

1. Once the ENStem-A™ cells reach 90-100% confluence, carefully remove the medium from the poly-L-ornithine and laminin-coated 3.5-cm tissue culture plate.
2. Apply 1 mL of Accutase™ (Part No. SCR005) and incubate in a 37°C incubator for 3-5 minutes.
3. Inspect the plate and ensure the complete detachment of cells by gently tapping the side of the plate with the palm of your hand.
4. Apply 1 mL of ENStem-A™ Neural Expansion Medium (Part No. SCM004) (pre-warmed to 37°C) to the plate.
5. Gently rotate the plate to mix the cell suspension. Transfer the dissociated cells to a 15 mL conical tube.
6. Centrifuge the tube at 200 x g for 3-5 minutes to pellet the cells.
7. Discard the supernatant.
8. Apply 2 mL of ENStem-A™ Neural Expansion Medium (Part No. SCM004) containing L-Glutamine (2 mM) and FGF-2 (20 ng/mL) to the conical tube and resuspend the cells thoroughly. **Note:** Do not vortex the cells.
9. Count the number of cells using a hemacytometer.
10. Plate the cells to the desired density into the appropriate poly-L-ornithine and laminin-coated flasks, plates or wells in ENStem-A™ Neural Expansion Medium (Part No. SCM004) containing L-Glutamine (2 mM) and FGF-2 (20 ng/mL). We recommend keeping the cells at a high cell density by passaging 1:2 – 1:3 (refer to Figure 1A).

***Note:** Enzymatic dissociation may affect the karyotypic stability of the cells. *Nat. Biotechnol.* 2005 Jan; 23(1): 19-20. Viability may be inconsistent and subsequent proliferation rates may be reduced when enzymatically passaging ENStem-A cells.

DIFFERENTIATION (FOR 8-WELL CHAMBER SLIDES):

1. The 8-well chamber slides should be coated with 50 µg/mL poly-L-ornithine and laminin (please refer to the section on Preparation of Coated Flasks for the appropriate concentration of laminin to use).
2. Plate out 50,000 cells per well into an appropriately coated 8-well chamber slide in ENStem-A™ Neural Expansion Medium (Part No. SCM004) containing L-Glutamine (2 mM) and FGF-2 (20 ng/mL). Total volume per well = 0.5 – 0.75 mL. At this density the cells should be ~50% - 60% confluent by the next day.
3. Once the cells reach 90% confluence, initiate differentiation by removing the medium from each well and replace with ENStem-A™ Neuronal Differentiation Medium (Part No. SCM017) (pre-warmed to 37°C) containing freshly added L-Glutamine (2 mM). Total volume per well = 0.5 – 0.75 mL.
4. Replace with fresh ENStem-A™ Neuronal Differentiation Medium (Part No. SCM017) containing L-Glutamine (2 mM) every 2-3 days for one to two weeks. Cells will have increased βIII-tubulin (a neuronal marker) expression within one week of differentiation. Optimal βIII-tubulin expression is obtained after two weeks of differentiation.

Note: It is important that the section on Preparation of Coated Flasks be strictly adhered to for the one and two week differentiation protocol. If plates or flasks are coated inappropriately with diluted laminin, cells will not adhere for the whole duration of the two week differentiation protocol.

5. After one or two weeks, the cells can be fixed with 4% paraformaldehyde and stained with the desired antibodies.

Note: After two weeks of differentiation, the distribution of the cells will become uneven; with clustering in some regions of the plate (refer to figure 3A). There will be considerable cell death throughout and at the end of the differentiation protocol. Neurons may require additional neurotrophic factors for support. The cell death will not hinder immunocytochemical staining of the remaining neuron population.

CHARACTERIZATION OF ENSTEM-A™ HUMAN NEURAL PROGENITOR CELLS (SCC003):

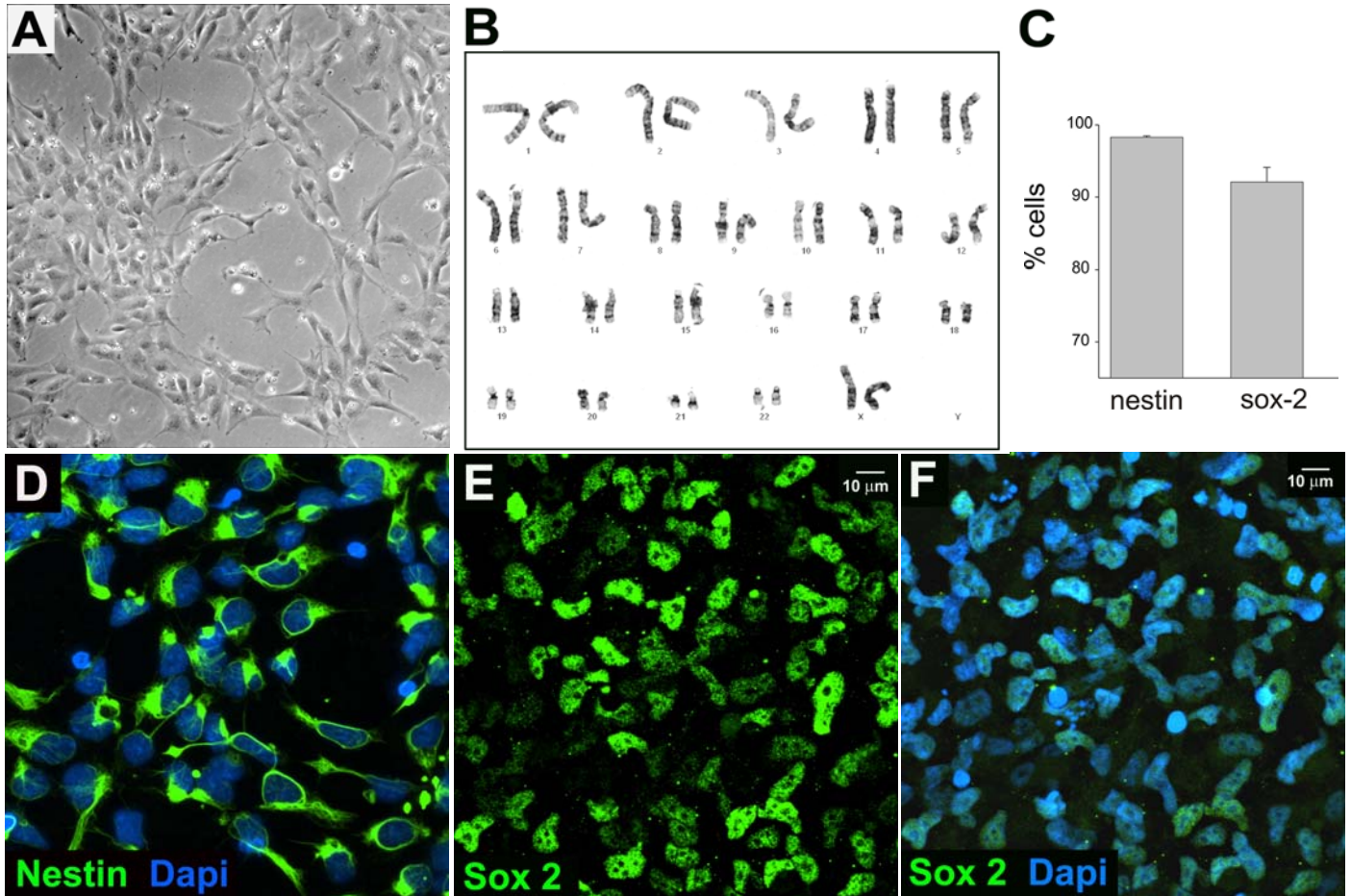


Figure 1. ENStem-A™ Human Neural Progenitor Cells (Part. No. SCC003) are grown as monolayers (A), are karyotypically normal (B) and express NSC markers, Nestin (C, D, green; Part No. MAB5326, 1:500) and Sox-2 (C, E, green; Part No. AB5603, 1:1000). Nuclei of the cells were visualized with DAPI (blue). The Sox-2 transcription factor is co-localized with the DAPI (blue) staining in the nucleus (F).

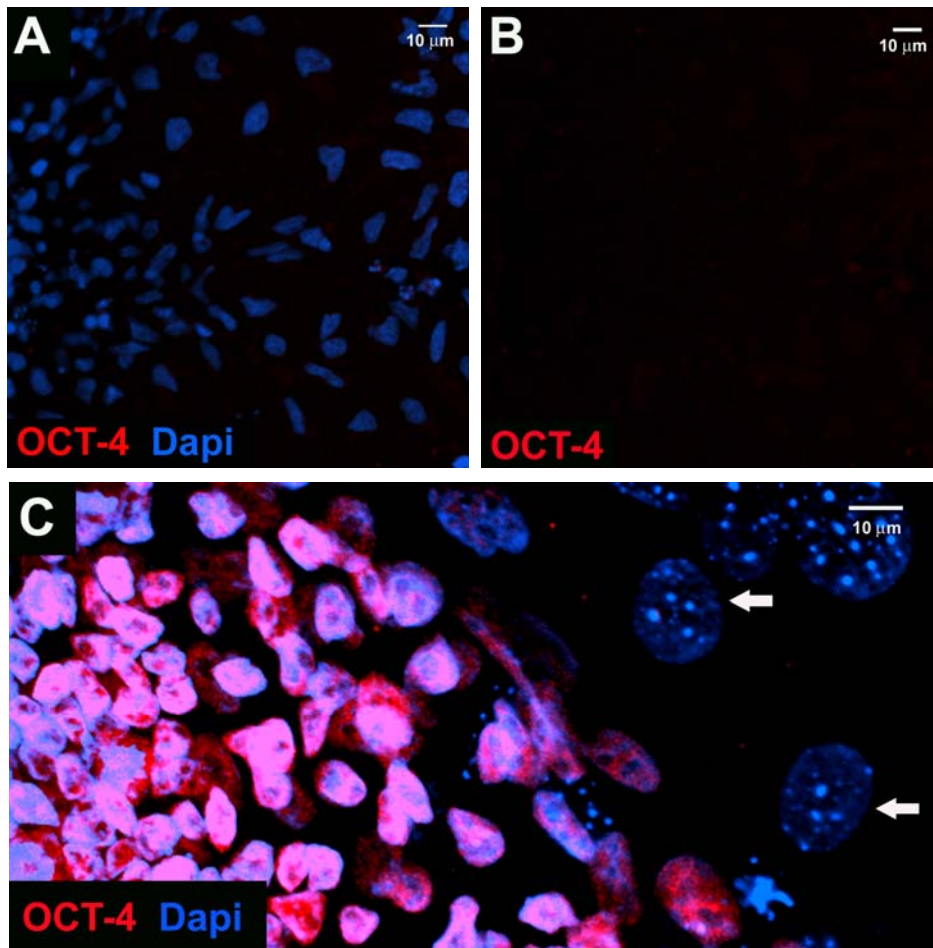


Figure 2. ENStem-A™ Human Neural Progenitor Cells (Part No. SCC003) are negative for Oct-4 staining (**A, B**) while control H9 human embryonic stem cells are positive for Oct-4 staining (**C**; Part No. MAB4401). Mouse feeder cells are also negative for Oct-4 staining (arrows).

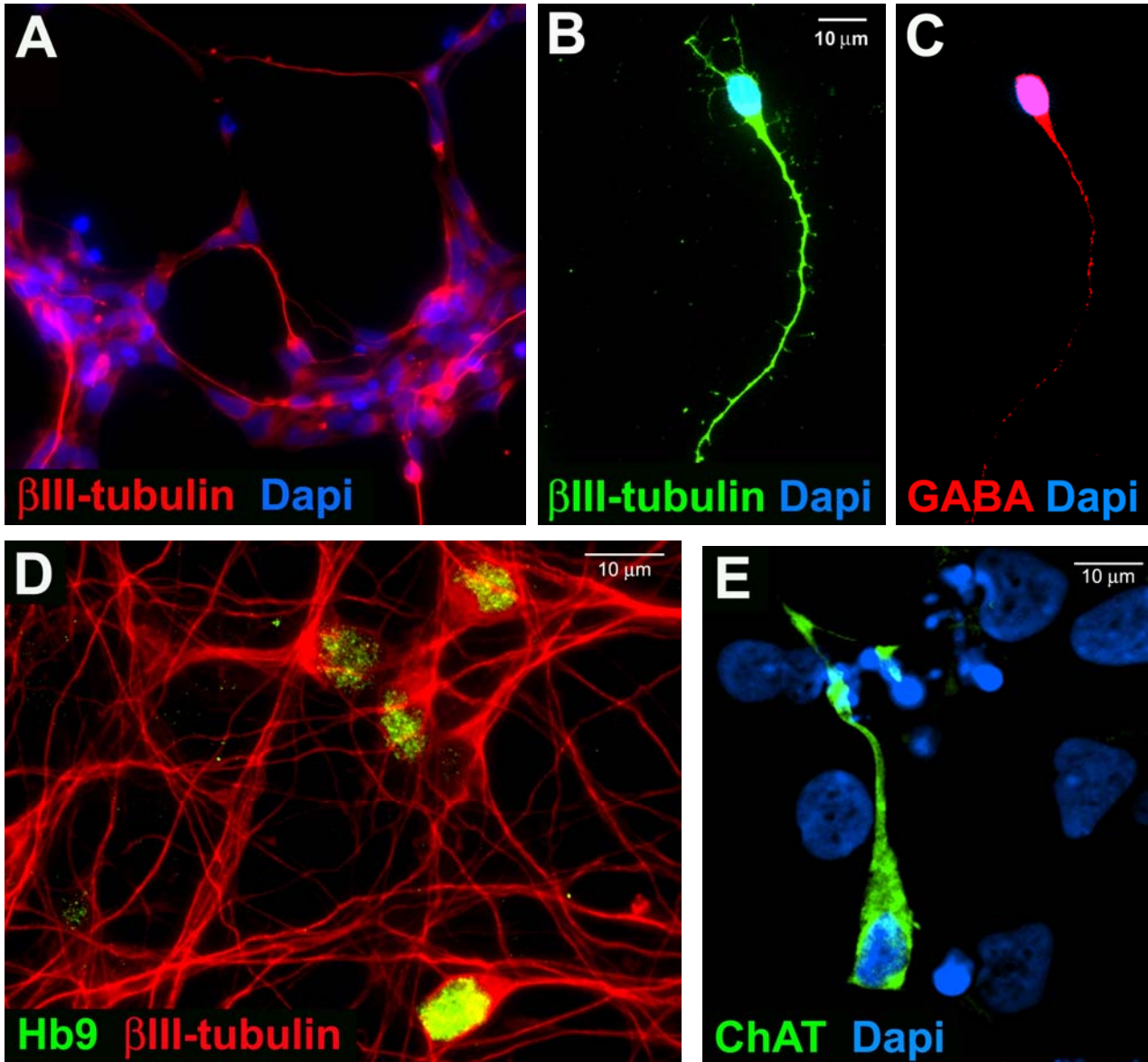


Figure 3. ENStem-A™ Human Neural Progenitor Cells can differentiate into multiple neuronal subtypes. Using ENStem-A™ Neuronal Differentiation Medium (Part No. SCM017), a majority of the cells exhibit a neuronal phenotype (**A**, β III-tubulin, red; Part No. MAB1637, 1:1000). Cultures contain some GABAergic (**C**, GABA, red,) cells that express the transcription factor HB9 (**D**; Part No. AB5963, 1:50) and also cholinergic cells (**E**, ChAT, green; Part No. AB144P, 1:100). GABAergic cells colabel with the neuronal marker β III-tubulin (**B**, **D**). Cell nuclei were visualized with DAPI (blue).

RELATED PRODUCTS:

The following stem cell products are available as separate items.

1. ENStem-A™ Neural Expansion Medium (Part No. SCM004)
2. ENStem-A™ Neuronal Differentiation Medium (Part No. SCM017)
3. ENStem-A™ Neural Freezing Medium (Part No. SCM011)
4. Human Neural Stem Cell Characterization Kit (Part No. SCR060)
5. Accutase™ (Part No. SCR005)
6. Mouse anti-Nestin (human-specific) (Part No. MAB5326)
7. Rabbit anti-Sox 2 (Part No. AB5603)
8. Mouse anti-Oct-4 (Part No. MAB4401)
9. Mouse anti-βIII-tubulin (Part No. MAB1637)
10. Rabbit anti-HB9 (Part. No. AB5963)
11. Goat anti-ChAT (Part. No. AB144P)

TROUBLESHOOTING:

Common questions:

1. **There is a lot of cell death/floating cells in my culture. Is this normal?**

This is a loosely adherent cell culture, and it is not uncommon to have cells and cell debris floating in the culture media. As long as the adherent cells on the plate are growing nicely in a monolayer, do not worry about the floating cells and they will be washed away during media changes.

2. **Do I have to plate these cells at such a high density or can I split them 1:3 or 1:4?**

Unlike most cell culture systems, these cells need to be plated densely to be highly proliferative. Proliferation is slowed in lower density cultures presumably due to a loss of cell to cell contact. Passaging the cells at a 1:2 ratio is ideal, however if the cells are growing nicely it is possible to split the cells 1:3.

3. **How do you differentiate the cells into specific neural phenotypes?**

The differentiation protocol/media provided is intended as a basal system. For specific cell types, differentiation protocols including additional neurotropic factors can be developed as determined by the end user's interests.

TROUBLESHOOTING GUIDE

Issue	Symptom	Problem	Resolution
Low Viability	Very few cells attach after thawing.	During the thawing process, osmotic shock occurred from adding prewarmed media too rapidly.	Give the culture time, change the media everyday and the surviving cells will proliferate. If not enough cells survived, it may be necessary to start over with a new vial.
Cell Attachment/Plate Coating	<ol style="list-style-type: none"> 1. Cells do not adhere to the plate and grow in suspension as floating cell aggregates. 2. Cells clump together on the plate. They are attached, but do not grow as a monolayer. 3. Cells attach to the plate, but seem to avoid certain areas of the dish 	<p>The cells are viable, however preferentially attach to each other over the plate due to the following issues:</p> <ol style="list-style-type: none"> 1. The plates were not properly coated. 2. The plates were not stored appropriately. 3. The plates were allowed to dry out. 	<ol style="list-style-type: none"> 1. Recoat plates. 2. Avoid freeze/thaw cycles of poly-L-ornithine and laminin stocks. 3. Thaw the laminin stock slowly at 4°C. Keep all coating solutions cold until applied to the plates and incubate the plates for the appropriate times. 4. Before use, check the plates to make sure that dry spots have not developed due to the evaporation of the laminin solution during storage. 5. Between rinsing the plates and applying media, do not allow the coated plates to air dry for extended periods of time.
Slow Proliferation	Cells grow very slow and appear to be unhealthy.	Cell proliferation will slow during the differentiation process, but not stop completely.	<ol style="list-style-type: none"> 1. Passage the cells down to a smaller dish to achieve a density of 60-95% confluence. Change the media everyday until the cells recover. 2. Always wait to passage until the culture plate is 90-100% confluent and split at a ratio of 1:2.
		Reagents/media are old or expired.	<ol style="list-style-type: none"> 1. Make up fresh media. Protect media and reagents from wide swings in temperature and from light.
Differentiation	Cells become confluent and begin to peel off the plate.	Cell proliferation will slow during the differentiation process, but not stop completely.	Depending on the density of the culture at the beginning of differentiation, it may be necessary to split the culture a couple of times during the 2 week process. It may also be useful to start differentiating cells at a density of 50% confluence to allow for continued proliferation.

Important Note: *During shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. For products with volumes of 200 μ L or less, we recommend gently tapping the vial on a hard surface or briefly centrifuging the vial in a tabletop centrifuge to dislodge any liquid in the container's cap.*