invitrogen™

GIBCO® Human Neural Stem Cells (H9 hESC-Derived)

Catalog nos. N7800-100, N7800-200

Rev. date: 7 December 2009
Manual part no. *A11592*

MAN0001758

User Manual

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Contents and Storage

Kit Configurations

Catalog no. N7800-100 includes cells only.

Catalog no. N7800-200 includes cells plus media.

Shipping

GIBCO® Human Neural Stem Cells (H9-Derived) and StemPro® Neural Supplement are shipped on dry ice.

FGF Basic Recombinant Human and EGF Recombinant Human are shipped on gel ice.

KnockOut™ DMEM/F-12 is shipped at room temperature.

Kit Contents and Storage

Kit components and storage conditions for N7800-100 and N7800-200 are listed in the table below.

N7800-100	Amount	Storage
GIBCO® Human Neural Stem Cells (H9-Derived) (>1 × 10^6 cells/mL in freezing medium*)	1 mL	Liquid nitrogen

N7800-200	Amount	Storage
GIBCO® Human Neural Stem Cells (H9-Derived) (>1 × 10^6 cells/mL in freezing medium)	1 mL	Liquid nitrogen
KnockOut™ DMEM/F-12	500 mL	2 to 8°C, **in the dark**
StemPro® Neural Supplement	10 mL	–5 to –20°C, **in the dark**
FGF Basic Recombinant Human	10 µg	2 to 8°C
EGF Recombinant Human	10 µg	2 to 8°C

*Composition of the freezing medium is proprietary.

Handle cells as potentially biohazardous material under at least Biosafety Level 1 (BL-1) containment. This product contains Dimethyl Sulfoxide (DMSO), a hazardous material. Review the Safety Data Sheet (SDS) before handling. Safety Data Sheets (SDSs) are available on our website at www.invitrogen.com/sds.

Intended Use

GIBCO® Human Neural Stem Cells (H9-Derived) are for research use only. They are not intended for any animal or human therapeutic or diagnostic use.

GIBCO® Human Neural Stem Cells (H9-Derived)

Neural Stem Cells

Neural stem cells (NSCs) are self-renewing, multipotent stem cells of nervous system, which can differentiate into neurons, oliogdendrocytes, and astrocytes. Multipotent NSCs can be isolated from the fetal or adult central nervous system or derived from embryonic stem cells.

NSCs are an invaluable resource not only for neuroscience and stem cell studies such as regulation of neurogenesis, neurotransmitter and receptor functions, and stem cell differentiation, but also for tissue engineering, cell and genetic therapy, and transplantation experiments to treat neurodegenerative diseases and neurological disorders.

GIBCO® Human Neural Stem Cells (H9-Derived)

GIBCO® Human Neural Stem Cells (H9-Derived) are derived from the NIH approved H9 (WA09) human embryonic stem cells (hESCs). Each vial of GIBCO® hNSCs contains > 1×10^6 viable cells that can be propagated as an adherent culture in complete StemPro® NSC SFM (see page 6 for composition). GIBCO® Human NSCs (hNSCs) retain their normal female human karyotype and their potential to differentiate into neurons and glial cells after multiple passages.

GIBCO® hNSCs remove the complicated isolation or derivation process, and allow the researchers who do not have access to human embryonic stem cells or human tissue the use of the hNSCs.

Characteristics of GIBCO® Human Neural Stem Cells (H9-Derived)

- Derived from the NIH approved H9 (WA09) human embryonic stem cells
- Retain their capacity for self-renewal
- Can differentiate into neurons, oligodendrocytes, and astrocytes
- Stain positive for the neural stem cell-type specific markers nestin and SOX2, and the proliferation marker Ki67 (> 80%)
- Stain ≤ 5% for embryonic stem cell-specific marker Oct4
- Exhibit a doubling time of 40–50 hours
- Retain their proliferation and differentiation potential for at least 3 passages after thawing

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Phenotype Marker Expression of GIBCO® Human NSCs (H9-Derived)

Undifferentiated GIBCO® Human NSCs

The presence of basic fibroblast growth factor (bFGF) in complete StemPro® NSC SFM allows the maintenance of GIBCO® hNSCs in their undifferentiated state. The images below show the phenotype marker expression of undifferentiated human NSCs after three rounds of passaging (P3) in StemPro® NSC SFM.

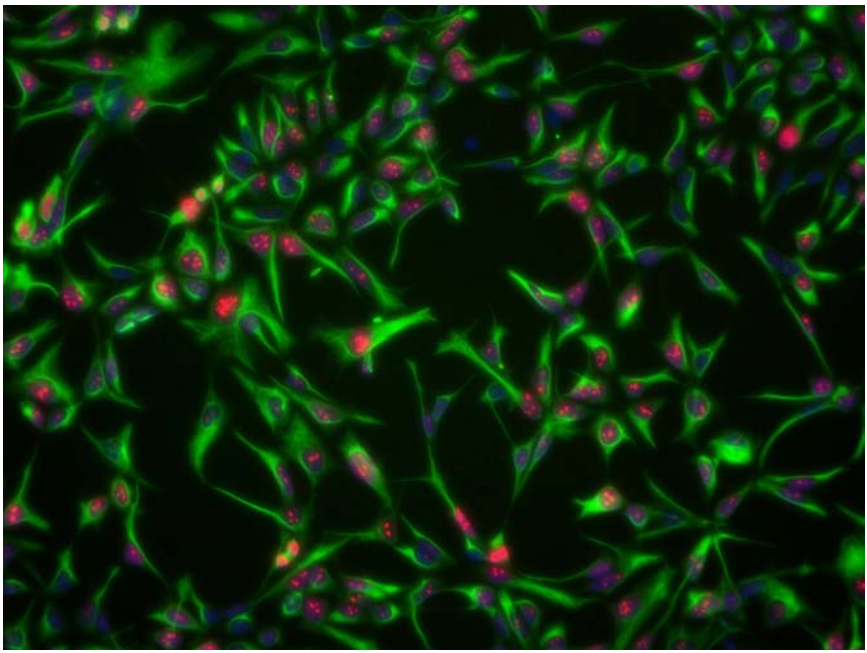

Figure 1. Fluorescence image (20X) of GIBCO® hNSCs at P3 that have been cultured in StemPro® NSC SFM and stained for the NSC phenotype markers nestin (green) and the proliferation marker Ki67 (red). Cell nuclei were counterstained with DAPI (blue). Approximately 90% of the cells stain positive for the undifferentiated NSC marker nestin and the proliferation marker Ki67. Lack of Oct4 staining indicates that there are no remnant hESCs in the culture (data not shown).

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Phenotype Marker Expression of GIBCO® Human NSCs, continued

Differentiation Potential of GIBCO® Human NSCs

GIBCO® hNSCs spontaneously differentiate into neurons, oligodendrocytes, or astrocytes upon withdrawal of bFGF and EGF from culture media. Alternatively, they can be enriched toward a specific lineage upon selection on differentiation medium (see Figure 2, below).

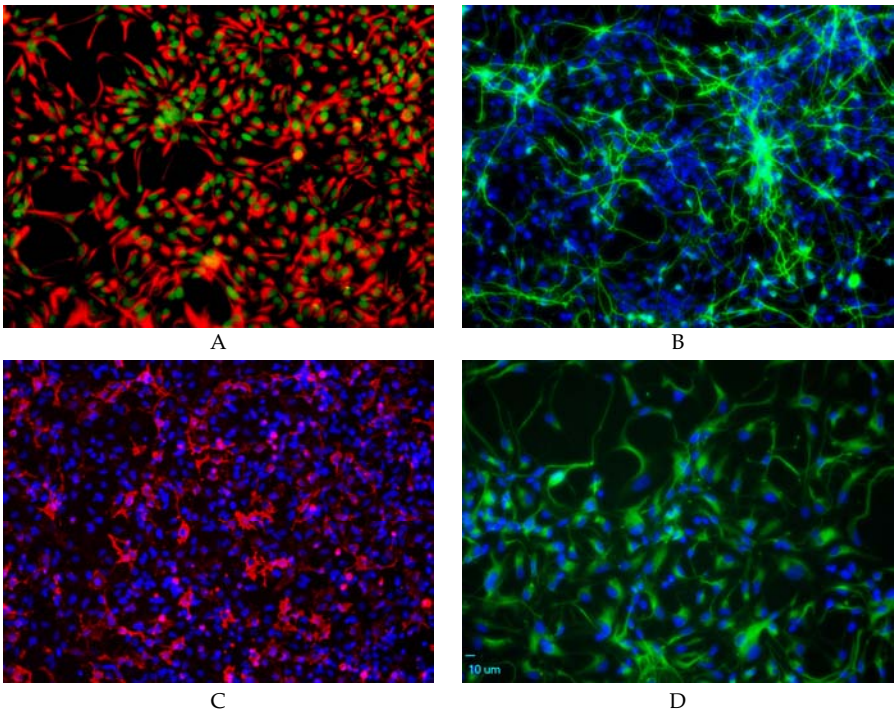

Figure 2. Fluorescence images (20X) of GIBCO® hNSCs that have been cultured in StemPro® NSC SFM for three passages, and then allowed to differentiate into neurons, oligodendrocytes, or astrocytes. Upon directed differentiation, cells start to lose the undifferentiated NSC marker, nestin, but stain positive for the differentiated cell type markers Dcx, GalC, and GFAP. Cells were stained for the undifferentiated NSC markers nestin (red) and SOX2 (green) prior to directed differentiation (**panel A**). Cell were then differentiated into neurons and glial cells, and respectively stained for the neuronal marker Dcx (green) (**panel B**), for the oligodendrocyte marker GalC (red) (**panel C**), or for the astrocyte marker, GFAP (green) (**panel D**). The nuclei were counterstained with DAPI (blue) in panels B–D.

Methods

Handling GIBCO® Human NSCs

As with other mammalian cell lines, handle GIBCO® Human Neural Stem Cells as potentially biohazardous material under at least Biosafety Level 1 (BL-1) containment. For more information on BL-1 guidelines, refer to *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed., published by the Centers for Disease Control, or see the following website: www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm

Guidelines for Culturing GIBCO® Human NSCs

Follow the general guidelines below to grow and maintain GIBCO® hNSCs.

- **All solutions and equipment that come in contact with the cells must be sterile.** Always use proper aseptic technique and work in a laminar flow hood.
- Before starting experiments, make sure that the cells have been established (at least 1 passage).
- For consistent results in your differentiation studies and other experiments, we recommend using cells below passage 3 (P3). If you expand GIBCO® hNSCs beyond P3, we recommend that you perform another round of characterization prior to further experiments.
- When thawing or subculturing cells, transfer cells into pre-warmed medium. Thaw hNSCs rapidly, but resuspend them slowly to avoid osmotic shock.
- For general maintenance of GIBCO® hNSCs in adherent culture, the cells should be approximately 90% confluent prior to subculturing. Passage the cells at a seeding density of 50,000 cells/cm^2.

  Note: Passaging hNSCs at a lower density decreases their proliferation efficiency.
- You may culture GIBCO® hNSCs on tissue-culture vessels coated with CELLStart™, Geltrex™, fibronectin, or a double coating of poly-L-ornithine and laminin.

  Note: The attachment strength of GIBCO® hNSCs is greatest for CELLStart™, followed by fibronectin, and is weakest for poly-L-ornithine.
- Standard physical conditions for GIBCO® hNSCs grown in StemPro® NSC SFM are 36 to 38°C in a humidified atmosphere of 4 to 6% CO_2 in air.

Media Requirements

Important

It is very important to strictly follow the guidelines for culturing GIBCO® hNSCs in this manual to keep them undifferentiated.

Media Requirements

We recommend using complete StemPro® NSC SFM for optimal growth and expansion of GIBCO® hNSCs, and to keep the NSCs undifferentiated. StemPro® NSC SFM is designed to support growth of neural stem cells derived from embryonic stem cells or isolated from fetal tissue as adherent culture on CELLStart™, Geltrex™, fibronectin, or poly-L-ornithine/laminin coated tissue culture-treated vessels (see page 23 for ordering information).

- Prepare your growth medium prior to use.
- To maintain undifferentiated NSCs, supplement the medium every day with bFGF to 10 ng/mL.

  Note: If you are using complete StemPro® NSC SFM to culture your cells, you do not need to supplement the medium with bFGF.
- When thawing or subculturing NSCs, transfer them into **pre-warmed** medium at 37°C.
- We recommend that you aliquot complete growth medium into required working amounts to avoid exposing the medium to 37°C multiple times.
- You may store the complete StemPro® NSC SFM **in the dark** at 4°C for up to four weeks. Do **not** freeze complete StemPro® NSC SFM.
- You may refreeze unused StemPro® Neural Supplement; however, avoid repeated freeze-thaw cycles.

Preparing Complete StemPro® NSC SFM

Preparing Complete StemPro® NSC SFM

StemPro® NSC SFM complete medium consists of KnockOut™ D-MEM/F-12 with StemPro® Neural Supplement, EGF, bFGF, and GlutaMAX™-I. Complete medium is stable for up to 4 weeks when stored **in the dark** at 4°C.

To make 100 mL of complete StemPro® NSC SFM, aseptically mix the following components:

Component	Concentration	Amount
KnockOut™ D-MEM/F-12	1X	97 mL
GlutaMAX™-I Supplement	2 mM	1 mL
bFGF	20 ng/mL	2 µg
EGF	20 ng/mL	2 µg
StemPro® Neural Supplement	2%	2 mL

Note: You may observe a white precipitate when thawing StemPro® Neural Supplement. This precipitate will disappear when the supplement is completely thawed or dissolved.

Preparing Matrix for Adherent Cell Culture

Coating Culture Vessels with CELLStart™

1. Dilute CELLStart™ 1:100 in D-PBS with calcium and magnesium (i.e., 50 µL of CELLStart™ into 5 mL of D-PBS) (see page 23).
2. Coat the surface of the culture vessel with the working solution of CELLStart™ (14 mL for T75, 7 mL for T25, 3.5 mL for 60-mm dish, 2 mL for 35-mm dish).
3. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO_2 in air for 1 hour.
4. Remove the vessel from the incubator and store it until use. Immediately before use, remove all CELLStart™ solution and replace it with complete StemPro® NSC SFM.

   Note: You may coat the plates in advance and store them at 4°C, wrapped tightly with Parafilm, for up to 2 weeks. Do **not** remove CELLStart™ solution until just prior to use. Make sure the plates do **not** dry out.

Coating Culture Vessels with Geltrex™

1. Thaw the Geltrex™ bottle at 4°C overnight to prevent polymerization. The next day, dilute Geltrex™ 1:2 with D-MEM/F-12 at 4°C to make 100X stock solution, using an ice bucket to keep the bottles cold. Quickly prepare 0.5 mL aliquots in 50-mL conical tubes (pre-chilled on ice), and store the tubes at –20°C.
2. Thaw 1 tube of Geltrex™ (0.5 mL, aliquoted as above) slowly at 4°C, and add 49.5 mL of cold D-MEM/F-12 (1:100 dilution). Mix gently.
3. Cover the whole surface of each culture plate with the Geltrex™ solution (1.5 mL for a 35-mm dish, 3 mL for 60-mm dish, 5 mL for a T25 culture flask).
4. Seal each dish with parafilm to prevent drying, and incubate 1 hour at room temperature in a laminar flow hood.
5. Remove the vessel from the incubator and store it until use. Immediately before use, remove all Geltrex™ solution, wash once with D-PBS with calcium and magnesium, and replace pre-warmed complete medium.

   Note: You may store the Geltrex™-treated dish at 4°C for up to 1 month. Do **not** remove Geltrex™ solution until just prior to use.

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Coating Culture Vessels with Poly-L-Ornithine and Laminin

1. Dissolve poly-L-ornithine (Sigma, Cat. no. P3655) in cell culture-grade distilled water to make 10 mg/mL stock solution (500X). Aliquot the solution and store it at –20°C until use.
2. Thaw the laminin slowly at 2–8°C and prepare 10 µg/mL working solution in cell culture-grade distilled water. Aliquot the working solution into polypropylene tubes, and store the tubes at –20°C until use. Avoid repeated freeze/thaw cycles.

   Note: Laminin may form a gel if thawed too rapidly.
3. Dilute the poly-L-ornithine stock solution 1:500 in cell culture-grade distilled water to make 20 µg/mL working solution.
4. Coat the surface of the culture vessel (with or without cover slips) with the poly-L-ornithine working solution (14 mL for T75, 7 mL for T25, 3.5 mL for 60-mm dish, 2 mL for 35-mm dish).
5. Incubate the culture vessel overnight at 4°C or for 1 hour at 37°C.
6. Rinse the culture vessel twice with sterile water.
7. Coat the surface of the culture vessel (with or without cover slips) with the laminin working solution (14 mL for T75, 7 mL for T25, 3.5 mL for 60-mm dish, 2 mL for 35-mm dish).
8. Incubate the culture vessel overnight at 4°C or for 2 hours at 37°C.
9. Rinse the culture vessel with D-PBS without calcium or magnesium (see page 23), and store the vessel covered with D-PBS until use. Immediately before use, remove all D-PBS and replace it with complete StemPro® NSC SFM.

   Note: You may coat the plates in advance and store them at room temperature, wrapped tightly with Parafilm, for up to 1 week. Do **not** remove D-PBS until just prior to use. Make sure the plates do **not** dry out.

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Preparing Matrix for Adherent Cell Culture, continued

Coating Culture Vessels with Fibronectin

Using fibronectin as a matrix:

1. Dilute fibronectin (see page 23) in distilled water to make 1 mg/mL stock solution. Store the solution at –20°C.
2. Dilute fibronectin stock solution 1:50 in PBS (see page 23) to make 20 µg/mL working solution. Store the solution at –20°C until use.
3. Coat the surface of the culture vessel with the working solution of fibronectin (14 mL for T75, 7 mL for T25, 3.5 mL for 60-mm dish, 2 mL for 35-mm dish).
4. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO_2 in air for 1 hour.
5. Remove the vessel from the incubator and store it until use. Immediately before use, remove all fibronectin solution and replace it with complete StemPro® NSC SFM.

   Note: You may coat the plates in advance and store them at 4°C, wrapped tightly with Parafilm, for up to 2 weeks. Do **not** remove the fibronectin solution until just prior to use. Make sure the plates do **not** dry out.

Thawing and Establishing GIBCO® Human NSCs

Materials Needed

- GIBCO® hNSCs, stored in liquid nitrogen
- Ethanol or 70% isopropanol
- Complete StemPro® NSC SFM (see page 6); **pre-warmed** to 37°C
- Disposable, sterile 50-mL tubes
- 37°C water bath
- 37°C incubator with a humidified atmosphere of 5% CO_2
- Microcentrifuge
- 35-mm tissue-culture treated plate, coated with CELLStart™, Geltrex™, fibronectin, or poly-L-ornithine and laminin (see pages 7–9)

  Note: We recommend thawing GIBCO® hNSCs onto 35-mm plates to maximize the efficiency of recovery.
- Hemocytometer, cell counter and Trypan Blue, or the Countess™ Automated Cell Counter

Note

The Countess™ Automated Cell Counter is a benchtop instrument designed to measure cell count and viability (live, dead, and total cells) accurately and precisely in less than a minute per sample, using the standard Trypan Blue technique (see page 24 for ordering information).

Using the same amount of sample that you currently use with the hemocytometer, the Countess™ Automated Cell Counter takes less than a minute per sample for a typical cell count, and it is compatible with a wide variety of eukaryotic cells.

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Thawing and Establishing GIBCO® Human NSCs, continued

Thawing Procedure

1. Remove the cells from liquid nitrogen storage, and **immediately** transfer the cells to a 37°C water bath to prevent crystal formation.
2. Quickly thaw the vial of cells by swirling it in the 37°C water bath and removing it when the last bit of ice has melted, typically < 2 minutes. Do not submerge the vial completely. **Do not** thaw the cells for longer than 2 minutes.
3. When thawed, immediately transfer the cells into a 50-mL sterile tube, and carefully add 4 mL of **pre-warmed** complete StemPro® NSC SFM **dropwise** (approximately 1 drop per second) while swirling the tube.
4. Add an additional 5 mL of **pre-warmed** complete StemPro® NSC SFM to the same tube.
5. To remove the cryoprotectant, centrifuge the cells for 4 minutes at 200 × *g*, and aspirate the supernatant.
6. Resuspend the cells in 2 mL of complete StemPro® NSC SFM.
7. Determine the viable cell count using your method of choice. The total number of viable cells should be $> 1 \times 10^6$.
8. Plate the resuspended cells at a seeding density of **1.0×10^5 cells per cm^2** on a CELLStart™, Geltrex™, fibronectin, or poly-L-ornithine/laminin coated, tissue-culture treated culture plate.
9. Incubate the plate at 37°C, 5% CO_2, and 90% humidity and allow cells to adhere for at least 24 hours.
10. The next day, replace the medium with an equal volume of fresh, pre-warmed complete StemPro® NSC SFM.
11. In 4–7 days, when the culture is 90% confluent, you may proceed to passage your GIBCO® hNSCs.

Important

If you are culturing GIBCO® hNSCs in growth medium other than complete StemPro® NSC SFM, make sure to supplement the medium every day with bFGF to 20 ng/mL to maintain your cells undifferentiated.

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Expected Results

The total number of viable GIBCO® hNSCs should be $> 1 \times 10^6$ after thawing.

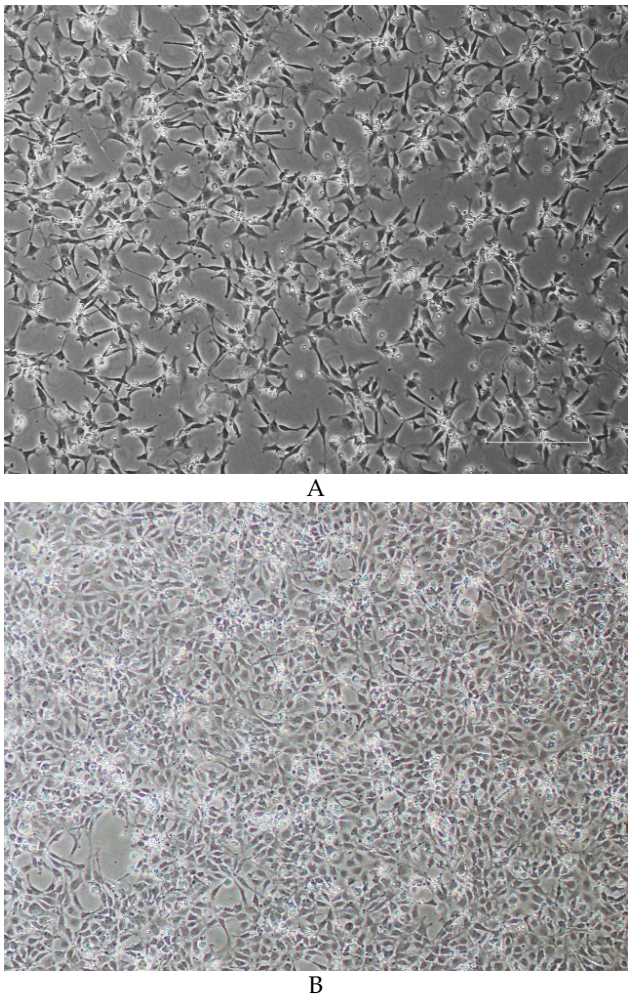

Figure 3. Phase contrast images (10X) of GIBCO® hNSCs cultured in StemPro® NSC SFM at day 1 (**panel A**) and at day 3 (**panel B**) after thawing.

Subculturing GIBCO® Human NSCs

Materials Needed

- Culture vessels containing GIBCO® hNSCs (90% confluent)
- CELLStart™, Geltrex™, fibronectin, or poly-L-ornithine and laminin coated, tissue-culture treated flasks, plates or dishes (see pages 7–9)
- Complete StemPro® NSC SFM, **pre-warmed** to 37°C (see page 6)
- Disposable, sterile 15-mL or 50-mL conical tubes
- 37°C incubator with humidified atmosphere of 5% CO_2
- Dulbecco's Phosphate Buffered Saline (D-PBS), containing no calcium, magnesium, or phenol red
- Dissociation reagent (TrypLE™ or StemPro® Accutase®), **pre-warmed** to 37°C
- Hemocytometer, cell counter and Trypan Blue, or the Countess™ Automated Cell Counter

Passaging GIBCO® Human NSCs

Observe the GIBCO® hNSC culture under the microscope to confirm that the cells are ~90% confluent and ready to be passaged. Pre-warm the cell dissociation reagent (TrypLE™ or StemPro® Accutase®) and the complete StemPro® NSC SFM to 37°C before use.

1. Aspirate the spent complete medium from the cells.
2. Rinse the surface of the cell layer with D-PBS without Ca^{2+} and Mg^{2+} (approximately 2 mL D-PBS per 10 cm^2 culture surface area) by adding the D-PBS to the side of the vessel opposite the attached cell layer, and rocking back and forth several times.
3. Aspirate and discard the D-PBS.
4. To detach the cells, add 1 mL of pre-warmed TrypLE™ or StemPro® Accutase® and evenly distribute it over the attached cell layer. Incubate the cells for 2 to 5 minutes at room temperature.
5. Once you observe cell detachment (detached cells will move with tilting of the flask), gently pipet the cells up and down to break the larger clumps into a single cell suspension.

Protocol continued on next page

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Passaging GIBCO® Human NSCs, continued

Protocol continued from previous page

6. Stop the cell dissociation reaction by adding 9 mL of complete StemPro® NSC SFM. Disperse the medium by pipetting it over the cell layer surface several times.
7. Transfer the cells to a 15-mL or a 50-mL conical tube and centrifuge the tube at 200 × *g* for 4 minutes at room temperature. Aspirate and discard the medium.
8. Resuspend the cell pellet in a minimal volume of pre-warmed complete StemPro® NSC SFM and remove a sample for counting.
9. Determine the total number of cells and percent viability using your method of choice.
10. Following the coating procedure (pages 7–9), remove the coating solution from each coated culture vessel and replace the solution with 5 mL of complete StemPro® NSC SFM.
11. Add enough cell suspension to each coated culture vessel to provide 5×10^4 cells per cm^2 (e.g., 1.25×10^6 cells perT25 flask). Ensure an even distribution of the cell suspension.
12. Incubate the cells at 37°C, 5% CO_2 and 90% humidity. For optimal performance and cell growth, re-feed the culture with fresh medium every two days.

Freezing GIBCO® Human NSCs

Materials Needed

- Culture vessels containing GIBCO® Human NSCs
- Complete StemPro® NSC SFM
- DMSO (use a bottle set aside for cell culture; open only in a laminar flow hood)
- Disposable, sterile 15-mL or 50-mL conical tubes.
- D-PBS, containing no calcium, magnesium, or phenol red
- Dissociation reagent (TrypLE™ or StemPro® Accutase®), **pre-warmed** to 37°C
- Hemocytometer, cell counter and Trypan Blue, or the Countess™ Automated Cell Counter
- Sterile freezing vials
- Controlled rate freezing apparatus (e.g., Mr. Frosty)
- Liquid nitrogen storage container

Guidelines

When freezing GIBCO® Human NSCs, we recommend the following:

- Freeze cells at a density of 1×10^6 viable cells/mL and a volume of 1 mL/vial.
- Use a 2X freezing medium composed of 80% complete StemPro® NSC SFM and 20% DMSO.
- Bring the cells into the freezing medium in two steps, as described in this section.

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Freezing Procedure

1. Prepare 2X freezing medium of 80% complete StemPro® NSC SFM and 20% DMSO. Keep the freezing medium on ice until use.
2. Aspirate the spent complete StemPro® NSC SFM from the culture vessel.
3. Wash the cells with D-PBS without Ca^{2+} and Mg^{2+}. Aspirate and discard the D-PBS.
4. Detach the cells following steps 4–6 of the subculture procedure (pages 13–14). Transfer the cells into a 15-mL or 50-mL sterile, conical tube.
5. Centrifuge the cells at 200 × *g* for 4 minutes at room temperature. Aspirate the medium and discard.
6. Resuspend the cell pellet in a minimal volume of pre-warmed complete StemPro® NSC SFM and remove a sample for counting.
7. Determine the viable cell density and calculate the required volume of freezing medium to give the cells the desired final freezing cell density (i.e., 1.0×10^6 cells/mL; 1 mL/vial).
8. Centrifuge the cells at 200 × *g* for 4 minutes at room temperature. Gently aspirate the medium and discard
9. Resuspend the pellet using complete StemPro® NSC SFM to half the final freezing volume.

   Note: The cell concentration is 2×10^6 cells/mL at this stage of the freezing procedure.
10. Add the same amount of 2X freezing medium to the resuspended cells in a **drop-wise** manner.

    Note: The final concentration of DMSO in 1X freezing medium is 10%, and the final cell concentration is 1×10^6 cells/mL.
11. Transfer 1 mL (1×10^6 cells) aliquots of the cell suspension into cryovials. Achieve cryopreservation overnight in a controlled-rate freezing apparatus following standard procedures (1°C decrease per minute).
12. The next day, transfer the frozen vials to a liquid nitrogen tank (vapor phase) for long-term storage.

    Note: You may check the viability and recovery of frozen cells 24 hours after storing cryovials in liquid nitrogen by following the procedure outlined in **Thawing and Establishing GIBCO® Human NSCs,** page 10.

Differentiating GIBCO® Human NSCs

Introduction

One critical hallmark of NSCs is their ability to differentiate into neurons and glial cells. Traditional and modern bioassays are used to demonstrate the multipotency of GIBCO® hNSCs to differentiate along these lineages. This section provides guidelines for spontaneously differentiating GIBCO® hNSCs.

Materials Needed

In addition to materials for passaging GIBCO® hNSCs (see page 13), the following materials are required:

- StemPro® NSC SFM without the growth factors (i.e., without bFGF and EGF)
- CELLStart™, fibronectin, or poly-L-ornithine/laminin coated, tissue-culture treated plate (see pages 7–9)

Spontaneous Differentiation Protocol

To spontaneously differentiate GIBCO® hNSCs into neurons, oligodendrocytes, and astrocytes:

1. Plate GIBCO® hNSCs on a CELLStart™, fibronectin, or poly-L-ornithine coated, tissue culture-treated plate at 2.5×10^4 cells/cm^2 following the protocol for passaging GIBCO® hNSCs (see pages 13–14).
2. After 2 days, change medium to StemPro® NSC SFM without the growth factors (i.e., withdraw growth factors from cell culture), and replace the medium with fresh medium every 2 to 3 days.

Important

Do not expose cells to air at any time after they have differentiated into neurons.

Directed Differentiation Protocol

You may also induce GIBCO® hNSCs to differentiate into neurons, oligodendrocytes, and astrocytes by exposing the cells to specific factors. For more information on how to enrich GIBCO® hNSCs toward neurons, oligodendrocytes, and astrocytes upon selection on the appropriate differentiation medium, contact Technical Support (page 25).

Characterizing the Phenotype of GIBCO® Human NSCs

Introduction

This section provides information on phenotypic marker expression of GIBCO® hNSCs in their undifferentiated state, and after their differentiation into neurons, oligodendrocytes, and astrocytes.

Phenotypic Markers

The following table lists the primary antibodies used for classifying undifferentiated GIBCO® hNSCs, neurons, oligodendrocytes, and astrocytes. See page 24 for ordering information.

	Antigen	Dilution ratio	Antibody type
Undifferentiated GIBCO® hNSCs	Nestin (Abcam, Cat. no. Ab5968)	1:1,000	Rabbit IgG
	SOX2 (R&D Systems, Cat. no. MAB2018)	1:200	Mouse IgG
Neurons	MAP2	1:200	IgG_1, kappa
	Dcx	1:400	Rabbit IgG
Oligodendrocytes	GalC (Millipore, Cat. no. MAB342)	1:200	Mouse IgG
	A2B5	1:100	Mouse IgM
Astrocytes	CD44	1:50	Mouse IgG_{2b}
	GFAP	1:200	Rabbit IgG

Note

See Figures 1 and 2 on pages 2–3 for examples of fluorescent images showing phenotypic marker expression of GIBCO® hNSCs in their undifferentiated state, and after their differentiation into neurons, oligodendrocytes, and astrocytes.

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Immunocyto-chemistry

Fixing Cells:

1. Remove culture medium and gently rinse the cells once with D-PBS, without dislodging the cells.
2. Fix the cells with 4% fresh **Paraformaldehyde Fixing Solution** (PFA; see **Appendix**, page 22 for recipe) at room temperature for 15 minutes.
3. Rinse 3X with D-PBS containing Ca^{2+} and Mg^{2+}.
4. Check for the presence of cells after fixing.
5. Proceed to staining, described below. You may store slides for up to 3–4 weeks in D-PBS at 4°C before staining. **Do not** allow slides to dry.

Staining Cells:

1. Incubate cells for 30–60 minutes in blocking buffer (5% serum of the secondary antibody host species, 1% BSA, 0.1% Triton-X in D-PBS with Ca^{2+} and Mg^{2+}).

   Note: If you are using a surface antigen such as GalC, omit Triton-X from the blocking buffer.
2. Remove the blocking buffer and incubate the cells overnight at 4°C with primary antibody diluted in 5% serum. Ensure that the cell surfaces are covered uniformly with the antibody solution.
3. Wash the cells 3X for 5 minutes with D-PBS containing Ca^{2+} and Mg^{2+} (if using a slide, use a staining dish with a magnetic stirrer).
4. Incubate the cells with fluorescence-labeled secondary antibody (5% serum in D-PBS with Ca^{2+} and Mg^{2+}) **in the dark** at 37°C for 30–45 minutes.
5. Wash the cells 3X with D-PBS containing Ca^{2+} and Mg^{2+}, and in the last wash, counter stain the cells with DAPI solution (3 ng/mL) for 5 minutes, and rinse with D-PBS.
6. If desired, mount using 3 drops of ProLong® Gold antifade reagent per slide and seal with the cover slip (see page 24 for ordering information). You may store the slides **in the dark** at 4°C.

Troubleshooting

Culturing Cells

The table below lists some potential problems and solutions that help you troubleshoot your cell culture problems.

Problem	Cause	Solution
No viable cells after thawing stock	Stock not stored correctly	Order new stock and store in liquid nitrogen. Keep in liquid nitrogen until thawing.
	Home-made stock not viable	Freeze cells at a density of 1×10^6 viable cells/mL.
		Use low-passage cells to make your own stocks.
		Follow procedures in **Thawing and Establishing GIBCO® Human NSCs** (page 11) and **Freezing GIBCO® Human NSCs** (page 16) exactly. Slow freezing and fast thawing is the key. Add Freezing Medium in drop-wise manner (slowly). At time of thawing, thaw quickly and do not expose vial to the air but quickly change from nitrogen tank to 37°C water bath.
		Obtain new GIBCO® Human NSCs.
	Thawing medium not correct	Use pre-warmed complete StemPro® NSC SFM, prepared as described on page 6.
	Cells too diluted	Generally we recommend a high density culture of 1×10^5 cells per cm^2 at the time of recovery.
	Cell not handled gently.	GIBCO® Human NSCs are fragile; treat your cells gently, do not vortex, bang the flasks to dislodge the cells, or centrifuge the cells at high speeds. Do **not** expose neurons to air.
	Poly-L-ornithine incompletely removed from culture vessel	Poly-L-ornithine is toxic to cells. Completely remove poly-L-ornithine from the culture vessel by washing the vessel twice with PBS without Ca^{2+} and Mg^{2+}.
Cells grow slowly	Growth medium not correct	Use pre-warmed complete StemPro® NSC SFM.
	Cells passaged >3 times	Use healthy NSCs, under passage 4 (i.e., 3 passages after thawing); do not overgrow.

Continued on next page

Culturing Cells, continued

Problem	Cause	Solution
Cells differentiated	Culture conditions not correct	Thaw and culture fresh vial of new GIBCO® hNSCs Follow thawing instructions (page 11) and subculture procedures (pages 13–14) exactly. Do not omit bFGF from the medium.
	Cell seeding density at the time of plating is too low or too high.	Cells passaged too sparsely or cells allowed to get too confluent can cause differentiation. Seed cells at a density of 0.5×10^5 cells/cm^2 for adherent cultures.
Cells not adherent after initial thaw	Used D-PBS without Ca^{2+} and Mg^{2+} for CELLStart™	Be sure to prepare CELLStart™-coated culture vessels using D-PBS containing Ca^{2+} and Mg^{2+} (see page 23 for ordering information).
	CELLStart™ too dilute	You may increase the concentration of CELLStart™ up to 1:50 for better adhesion.
	Incubation for poly-L-ornithine too short	Make sure you incubate your culture vessel overnight at room temperature after coating it with poly-L-ornithine.

Differentiating Cells

The table below lists some potential problems and solutions that help you troubleshoot your differentiation problems.

Problem	Cause	Solution
Cells fail to differentiate	Culture medium contains bFGF	Remove bFGF from culture medium.
	Cell density too high and endogenous bFGF is preventing differentiation.	Reduce cell density.
	Cells have been passaged too many times.	Obtain new GIBCO® hNSCs.

Appendix

Recipes

Para-formaldehyde Solution

To prepare 20% paraformaldehyde (PFA) stock solution:

1. Add PBS to 20 g of EM grade paraformaldehyde (Electron Microscopy Services, Cat. no. 19208), and bring the volume up to 100 mL.
2. Add 0.25 mL of 10 N NaOH and heat the solution at 60°C using a magnetic stirrer until the solution is completely dissolved.
3. Filter the solution through a 0.22 micron filter, and cool on ice. Make sure the pH is 7.5–8.0.
4. Aliquot 2 mL in 15-mL tubes, freeze the tubes on dry ice, and store them at –20°C.

To prepare 4% PFA for fixing:

1. Add 8 mL PBS into each 15-mL tube containing 2 mL of 20% PFA, and thaw each tube in a 37°C water bath.
2. Once the solution has dissolved, the tubes cool on ice.

Additional Products

Additional Products

The products listed in this section may be used with GIBCO® hNSCs. For more information, refer to our website (www.invitrogen.com) or contact Technical Support (see page 25).

Item	Quantity	Cat. no.
StemPro® NSC SFM (contains KnockOut™ DMEM/F-12, FGF Basic Recombinant Human, EGF Recombinant Human, and StemPro® Neural Supplement)	1 kit	A1050901
StemPro® Neural Supplement	10 mL	A10508-01
GlutaMAX™-I Supplement	100 mL	35050-061
KnockOut™ DMEM/F-12	500 mL	12660-012
FGF Basic Recombinant Human (bFGF)	10 µg	PHG0024
EGF Recombinant Human	10 µg	PHG0314
Fetal Bovine Serum (FBS), ES Cell-Qualified	500 mL	16141-079
BSA, 10% Stock Solution	25 mL	P2489
Dulbecco's Phosphate Buffered Saline (D-PBS), containing no calcium, magnesium, or phenol red	500 mL	14190-144
Dulbecco's Phosphate Buffered Saline (D-PBS), containing calcium and magnesium, but no phenol red	500 mL	14040-133
Dulbecco's Modified Eagle Medium (D-MEM) (1X), liquid (high glucose)	1000 mL	11995-040
CELLStart™ Defined, Humanized Substrate for Cell Culture	2 mL	A1014201
Geltrex™ Reduced Growth Factor Basement Membrane Matrix	5 mL	12760-021
Fibronectin, Human Plasma	5 mg	33016-015
Laminin, Natural Mouse	1 mg	23017-015
StemPro® Accutase® Cell Dissociation Reagent	100 mL	A11105-01
TrypLE™ Express (1X), liquid without phenol red	100 mL	12604-013
Neurobasal™ Medium (1X), liquid	500 mL	21103-049
B-27 Serum-Free Supplement (50X), liquid	10 mL	17504-044
N-2 Supplement (100X), liquid	5 mL	17502-048
Antibiotic-Antimycotic (100X), liquid	100 mL	15240-062

Continued on next page

Additional Products, continued

The products listed in this section may be used with GIBCO® hNSCs. For more information, refer to our website (www.invitrogen.com) or contact Technical Support (see page 25).

Item	Quantity	Cat. no.
Trypan Blue Stain	100 mL	15250-061
LIVE/DEAD® Cell Vitality Assay Kit	1000 assays	L34951
Countess™ Automated Cell Counter (includes 50 Countess™ cell counting chamber slides and 2 mL of Trypan Blue Stain)	1 unit	C10227
Water, distilled	20 × 100 mL	15230-196

Products for Marker Analysis

The products listed below may be used for analyzing the phenotype of undifferentiated GIBCO® hNSCs, neurons, oligodendrocytes, and astrocytes. In addition to the primary antibodies listed below, Invitrogen offers a variety of isotype specific secondary antibodies conjugated with enzymatic and fluorescent indicators, and antibody sera and diluents. For more information, refer to www.invitrogen.com or contact Technical Support (see page 25).

Item	Quantity	Cat. no.
Mouse anti-MAP2	100 µg	13-1500
Mouse anti-human CD44	0.5 mL	MCHD4400
Rabbit anti-Doublecortin (Dcx)	100 µg	48-1200
Mouse anti-A2B5 (105)	100 µg	433110
Rabbit anti-GFAP (Glial Fibrillary Acid Protein) - concentrate	1 mL	18-0063
DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride)	10 mg	D1306
ProLong® Gold Antifade Reagent	10 mL	P36930
ProLong® Gold Antifade Reagent with DAPI	10 mL	P36931

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, SDSs, FAQs, formulations, citations, handbooks, etc.
- Complete Technical Support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

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References

Bjorklund, A., and Lindvall, O. (2000) Cell replacement therapies for central nervous system disorders. Nat Neurosci *3*, 537-544

Gage, F. H. (2000) Mammalian neural stem cells. Science *287*, 1433-1438

Shin, S., and Vemuri, M. (2010) Culture and Differentiation of Human Neural Stem Cells. In *Protocols for Neural Cell Culture (Springer Protocols Handbooks)*, 4th edn., Doering, Laurie C. ed. (Humana Press), pp.51-74.

Shin S, Sun Y, Liu Y, Khaner H, Svant S, Cai J, Xu Q. X., Davidson B. P., Stice S. L., Smith A. K., Goldman S. A., Reubinoff B. E., Zhan M, Rao M. S., Chesnut J. D. (2007) Whole genome analysis of human neural stem cells derived from embryonic stem cells and stem and progenitor cells isolated from fetal tissue. Stem Cells *25*, 1298-1306.

Shin S, Mitalipova M, Noggle S, Tibbitts D, Venable A, Rao R, Stice S. L. (2006) Long-term proliferation of human embryonic stem cell-derived neuroepithelial cells using defined adherent culture conditions. Stem Cells *24*, 125-138.

Shin S, Rao M. S. (2006) Large-scale analysis of neural stem cells and progenitor cells. Neurodegener Dis. *3*, 106-111.

Temple, S. (2001) The development of neural stem cells. Nature *414*, 112-117

Wu, Y. Y., Mujtaba, T., and Rao, M. S. (2002) Isolation of stem and precursor cells from fetal tissue. Methods Mol Biol *198*, 29-40

Zhao, C., Deng, W., and Gage, F. H. (2008) Mechanisms and functional implications of adult neurogenesis. Cell *132*, 645-660

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