gibco

Rat fetal neural stem cells

USER GUIDE

Catalog Numbers N7744-100 and N7744-200

Publication Number MAN0001642

Revision 2.0

ThermoFisher SCIENTIFIC

Life Technologies Corporation | 3175 Staley Road | Grand Island, NY 14072
For descriptions of symbols on product labels or product documents, go to **thermofisher.com/symbols-definition**.

The information in this guide is subject to change without notice.

Revision history: Pub. No. MAN0001642

Revision	Date	Description
2.0	17 February 2020	Changed thawing instructions
1.0	7 April 2009	New document

Contents

Contents and storage

Kit configurations

Catalog no. R7744-100 includes cells only.

Catalog no. R7744-200 includes cells plus media.

Shipping

Rat Fetal Neural Stem Cells, StemPro™ NSC SFM™ Supplement, FGF Basic Recombinant Human, and EGF Recombinant Human are shipped on dry ice.

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

Kit contents and storage

Kit components and storage conditions for R7744-100 and R7744-200 are listed in the table below.

R7744-100	Amount	Storage
Rat Fetal Neural Stem Cells (2×10^6 cells/mL in freezing medium[1])	1 mL	Liquid nitrogen, vapor-phase

[1] **Freezing medium**: DMEM/F-12 containing non-essential amino acids, 2 mM GlutaMax, 0.1 mM β-mercaptoethanol, 100 µg/ml apo-transferrin, 25 µg/ml insulin, 100 µM putrescine, 30 nM sodium selenite, 20 nM progesterone, 10 ng/ml bFGF, plus 10% DMSO.

R7744-200	Amount	Storage
Rat Fetal Neural Stem Cells (2×10^6 cells/mL in freezing medium)	1 mL	Liquid nitrogen, vapor-phase
KnockOut™ DMEM/F-12	500 mL	2 to 8°C, **in the dark**
StemPro™ NSC SFM™ 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

CAUTION! 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 Material Safety Data Sheet (MSDS) before handling. Material Safety Data Sheets (MSDSs) are available on our website.

Important guidelines for thawing and storing cells

- Upon receipt, immediately thaw cells or place into vapor-phase liquid nitrogen storage until ready to use. **Do not store the cells at –80°C**.
- Avoid short-term extreme temperature changes. When storing cells in liquid nitrogen after shipping on dry ice, allow the cells to remain in liquid nitrogen for 3-4 days before thawing.

Additional products

The products listed in this section may be used with Rat Fetal Neural Stem Cells. For more information, refer to our website.

Item	Quantity	Cat. no.
StemPro™ NSC SFM™ (contains KnockOut™ DMEM/F-12, FGF Basic Recombinant Human, EGF Recombinant Human, and StemPro™ NSC SFM™ Supplement)	1 kit	A1050901
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	100 mL 500 mL	16141-061 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

Item	Quantity	Cat. no.
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	10142-01
Geltrex™ Reduced Growth Factor Basement Membrane Matrix™	5 mL	12760-021
Fibronectin, Human Plasma	5 mg	33016-015
StemPro™ Accutase™ Cell Dissociation Reagent	100 mL	A11105-01
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
β-Mercaptoethanol (1,000X), liquid	50 mL	21985-023

The products listed in this section may be used with Rat Fetal Neural Stem Cells. For more information, refer to our website.

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 Rat Fetal Neural Stem Cells, and well as neurons, oligodendrocytes, and astrocytes. In addition to the primary antibodies listed below, we offer a variety of isotype specific secondary antibodies conjugated with enzymatic and fluorescent indicators, as well as antibody sera and diluents. For more information, refer to our website.

Item	Quantity	Cat. no.
Mouse anti-MAP2	100 µg	13-1500
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

Introduction

Rat fetal neural stem cells (NSCs)

Introduction

Rat Fetal Neural Stem Cells (NSCs) are isolated from the cortexes of the fetal (embryonic day 14) Sprague-Dawley™ rats. The cells are isolated under sterile conditions and expanded in N-2/DMEM/F-12 medium supplemented with 10 ng/mL basic fibroblast growth factor (bFGF) before cryopreservation at passage 0 (P0) in 90% N-2/DMEM/F-12 medium and 10% DMSO.

Each vial of Rat Fetal NSCs contains 2×10^6 cells/mL that can be expanded in culture for up to three passages. Withdrawal of bFGF allows the cells to differentiate into neurons, astrocytes, and oligodendrocytes (Gage, 2000; Wu *et al*., 2002). Because of their property to generate glial cells and electrically active neurons, Rat Fetal NSCs can be used for neuroscience studies as well as stem cell differentiation, tissue engineering, cell and genetic therapy, and transplantation experiments (Bjorklund & Lindvall, 2000; Gage, 2000; Temple, 2001; Zhao *et al*., 2008).

We recommend that you use StemPro™ NSC SFM™ (see "Rat fetal neural stem cells (NSCs)" on page 9) for optimal growth and expansion as adherent cells on matrix or suspended neurospheres.

Characteristics of rat fetal NSCs

- Isolated from fetal brain cortex of Sprague-Dawley™ rats on day 14 of gestation (E14)
- Capacity for self-renewal
- Ability to differentiate into neurons, oligodendrocytes, and astrocytes
- Stain positive for the neural stem cell-type specific marker nestin (> 75%)
- Stain ≤ 10% for differentiated phenotype markers Dcx, GFAP, and GalC
- Exhibit a doubling time of 20–30 hours, which tends to increase with passage number
- Can be expanded in culture up to three passages without differentiation

Rat fetal NSC culture

Primary cells isolated from the cortical neuroepithelium of the fetal (embryonic day 14) rat can be expanded up to three passages in culture. The image below shows undifferentiated Rat Fetal NSCs maintained as an adherent culture.

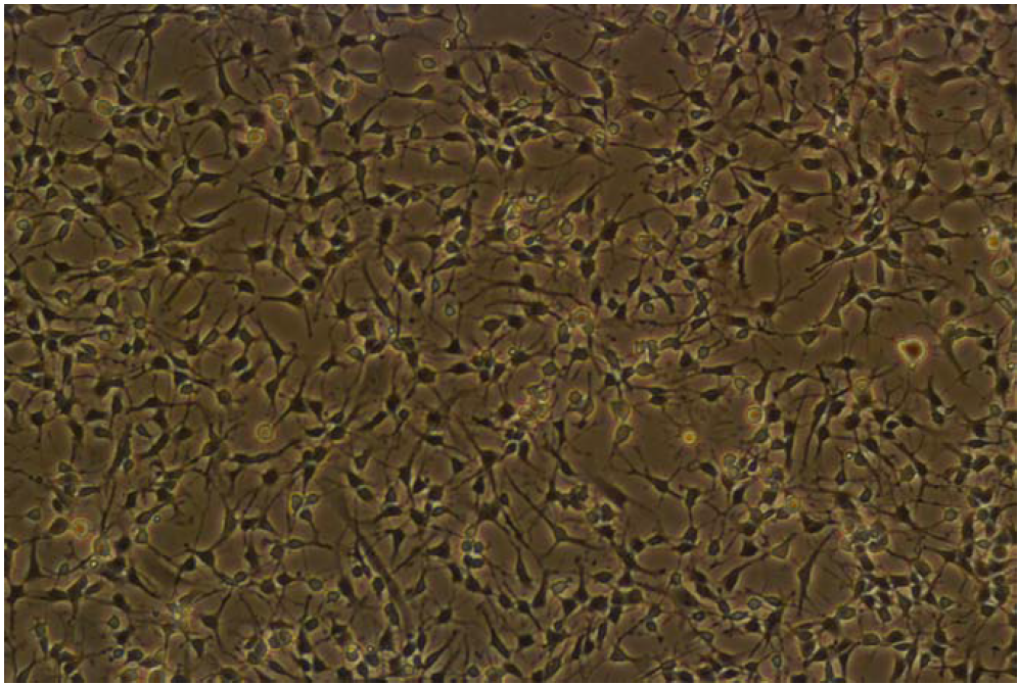

Figure 1 Bright field image of adherent Rat Fetal NSCs at P3 (passage 3) that have been cultured in complete StemPro™ NSC SFM™ for 10 days. The image was captured using 10X objective lens.

Phenotype marker expression of rat fetal NSCs

Undifferentiated rat fetal NSCs

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

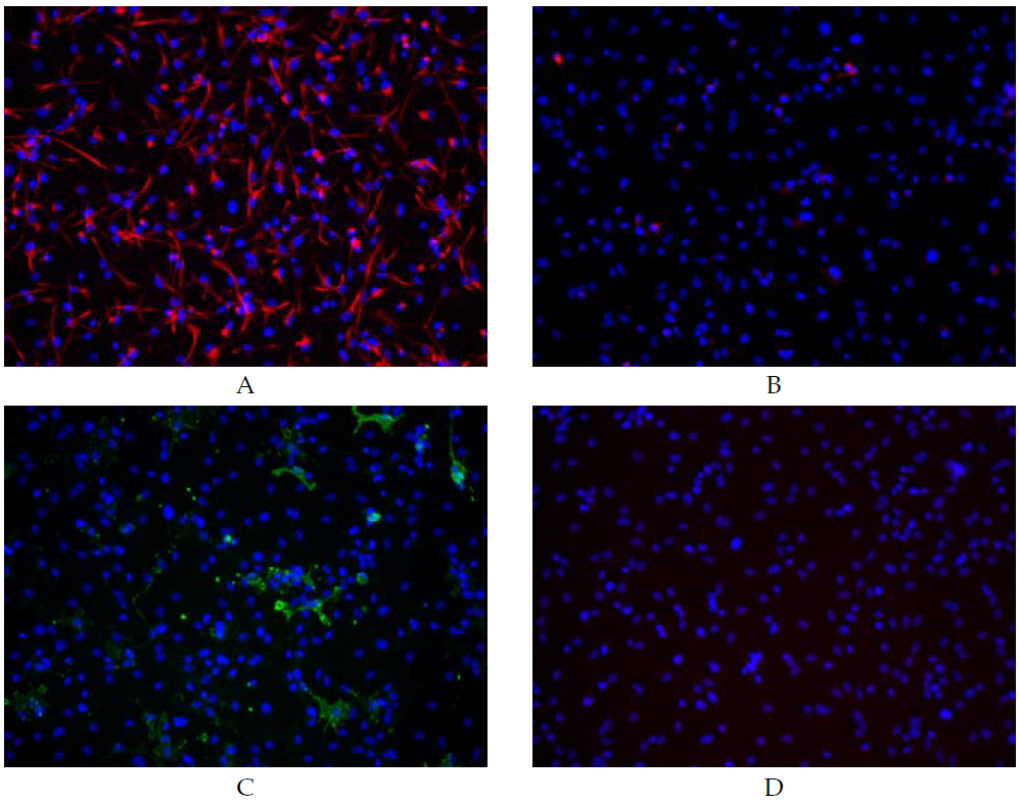

Figure 2 Fluorescence images (20X) of Rat Fetal NSCs at P3 that have been cultured in StemPro™ NSC SFM™ for 10 days and then stained for the appropriate phenotypic marker. Cells were stained for the undifferentiated NSC marker, nestin (red) (panel A), for the neuronal marker, Dcx (red) (panel B), for the oligodendrocyte marker, GalC (green) (panel C), or for the astrocyte marker, GFAP (red) (panel D). The nuclei were stained with DAPI (blue) in all panels. While approximately 90% of the cells stain positive for the undifferentiated NSC marker, nestin, less than 10% of the cells are positive for differentiated cell type markers Dcx, GalC, and GFAP.

Differentiated rat fetal NSCs

Rat Fetal NSCs spontaneously differentiate into neurons, oligodendrocytes, or astrocytes upon withdrawal of bFGF from culture media, or they can be enriched toward a specific lineage upon selection on differentiation medium. The images below show the differentiation potential of Rat Fetal NSCs after three passages in StemPro™ NSC SFM™.

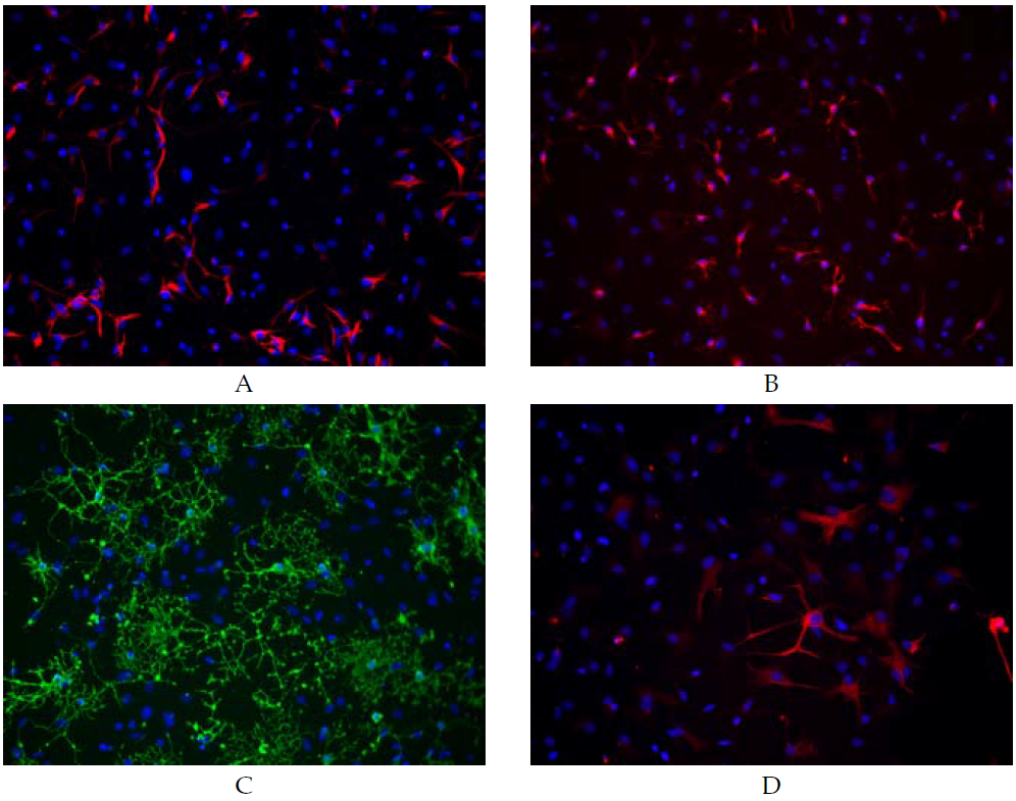

Figure 3 Fluorescence images (20X) of Rat Fetal NSCs that have been cultured in StemPro™ NSC SFM™ for three passages, and then allowed to differentiate into neurons, oligodendrocytes, or astrocytes. Upon differentiation, cells start to lose undifferentiated NSC marker, nestin, but stain positive for differentiated cell type markers Dcx, GalC, and GFAP. Cells were stained for the undifferentiated NSC marker, nestin (red) (panel A), for the neuronal marker, Dcx (red) (panel B), for the oligodendrocyte marker, GalC (green) (panel C), or for the astrocyte marker, GFAP (red) (panel D). The nuclei were stained with DAPI (blue) in all panels.

Methods

Handling rat fetal NSCs

CAUTION! As with other mammalian cell lines, when working with Rat Fetal NSCs, handle 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 rat fetal NSCs

Follow the general guidelines below to grow and maintain Rat Fetal NSCs.

- **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 Rat Fetal NSCs beyond P3, we recommend that you perform another round of characterization prior to further experiments.
- For general maintenance of Rat Fetal NSCs in adherent culture, the growth rate should be in mid-logarithmic phase with 75–90% confluency prior to subculturing. Passage cells at a seeding density of 50,000 cells/cm^2.

  Note: Passaging Rat Fetal NSCs at a lower density causes the cells to differentiate.
- In suspension culture as neurospheres, Rat Fetal NSCs can be passaged when neurospheres are larger than 3.5 mm in diameter.
- When thawing or subculturing cells, transfer cells into pre-warmed medium.
- Standard physical conditions for Rat Fetal NSCs 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 Rat Fetal Neural Stem Cells in this manual to keep them undifferentiated.

Media requirements

We recommend using complete StemPro™ NSC SFM™ for optimal growth and expansion of Rat Fetal NSCs, and to keep them undifferentiated. StemPro™ NSC SFM™ is designed to support growth of neural stem cells derived from embryonic stem cells or isolated from fetal tissue, which can be grown either as suspended neurospheres or as adherent culture on CELLStart™, fibronectin, or poly-L-ornithine coated tissue culture treated vessels (see “Additional products” on page 6 for ordering information).

Note: If cultured in serum containing medium, the cells might require adaptation to serum-free medium (see “Adapting NSCs to StemPro™ NSC SFM™” on page 18).

- 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 cells, transfer cells into pre-warmed medium at 37℃.
- We recommend that you aliquot complete growth medium into required working amounts to avoid exposing it to 37℃ multiple times.
- You may store the complete StemPro™ NSC SFM™ **in the dark** at 4℃ for up to four weeks. Do **not** freeze complete StemPro™ NSC SFM™.
- You may refreeze unused StemPro™ NSC SFM™ 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™ NSC SFM™ 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™ NSC SFM™ Supplement	2%	2 mL

Note: You may observe a white precipitate when thawing StemPro™ NSC SFM™, which will disappear when it is completely thawed or dissolved.

Preparing Matrix™ for adherent cell culture

Coating culture vessels for adherent cell culture

If you prefer to maintain your Rat Fetal NSCs as an adherent culture, you may use CELLStart™, fibronectin, or poly-L-ornithine. The attachment strength of Rat Fetal NSCs is greatest for CELLStart™, followed by fibronectin, and is weakest for poly-L-ornithine.

Using CELLStart™ as matrix:

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 “Additional products” on page 6).
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 until use. Immediately before use, remove all CELLStart™ solution and replace with complete StemPro™ NSC SFM™.

   Note: You may coat the plates in advance, and store 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.

Using fibronectin as matrix:

1. Dilute fibronectin (see "Additional products" on page 6) in distilled water to make 1 mg/mL stock solution. Store at –20°C.
2. Dilute fibronectin stock solution 1:50 in PBS (see "Additional products" on page 6) to make 20 µg/mL working solution. Store 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 until use. Immediately before use, remove all fibronectin solution and replace with complete StemPro™ NSC SFM™.

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

Using poly-L-ornithine as matrix:

1. Dissolve poly-L-ornithine (Sigma, Cat. no. P3655) in distilled water to make 10 mg/mL stock solution (500X). Aliquot and store at –20°C until use.
2. Dilute poly-L-ornithine stock solution 1:500 in cell culture grade distilled water make 20 µg/mL working solution.
3. Coat the surface of the culture vessel (with or without cover slips) with 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).
4. Incubate the culture vessel overnight at room temperature.
5. Rinse the culture vessel twice with D-PBS without Ca^{2+} and Mg^{2+} (see "Additional products" on page 6), and store covered with D-PBS until use. Immediately before use, remove all D-PBS and replace with complete StemPro™ NSC SFM™.

   Note: You may coat the plates in advance, and store 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.

Thawing and establishing cells

Materials needed

The following materials are required (see "Additional products" on page 6 for ordering information).

- Rat Fetal NSCs, stored in liquid nitrogen
- Ethanol or 70% isopropanol
- Complete StemPro™ NSC SFM™ (see "Preparing complete StemPro™ NSC SFM™" on page 15); pre-warmed to 37°C
- Disposable, sterile 15-mL tubes
- 37°C water bath

- 37°C incubator with a humidified atmosphere of 5% CO_2
- Microcentrifuge
- CELLStart™, fibronectin, or poly-L-ornithine coated, tissue-culture treated flasks, plates, or Petri dishes (see “Coating culture vessels for adherent cell culture” on page 15)
- Hemacytometer, cell counter and Trypan Blue, LIVE/DEAD™ Cell Vitality Assay Kit, 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 “Additional products” on page 6 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 is compatible with a wide variety of eukaryotic cells.

Thawing procedure

To thaw and establish Rat Fetal NSCs:

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 cells into a 15-mL sterile tube, and add pre-warmed complete StemPro™ NSC SFM™ dropwise up to 10 mL.
4. Centrifuge cells for 4 minutes at 300 × g.
5. Aspirate supernatant and resuspend cells in 2 mL of complete StemPro™ NSC SFM™.
6. Determine the viable cell count using your method of choice. The viability of thawed cells should be >50%, and the total live cell number should be $>2 \times 10^6$.
7. Plate the resuspended cells at a seeding density of 0.5×10^5 cells per cm^2 on a CELLStart™, fibronectin, or poly-L-ornithine coated, tissue-culture treated culture dish. If necessary, add complete StemPro™ NSC SFM™ to the cells to achieve the desired cell concentration and recount the cells.
8. Incubate at 37°C, 5% CO_2, and 90% humidity and allow cells to adhere for at least 24 hours.

9. The next day, replace the medium with an equal volume of fresh, pre-warmed complete StemPro™ NSC SFM™.

10. In 3–4 days, when the culture is 75–90% confluent, you may proceed to passage your Rat Fetal NSCs.

IMPORTANT! If you are culturing Rat Fetal NSCs in growth medium other than complete StemPro™ NSC SFM™, make sure to supplement the medium every day with bFGF to 10 ng/mL to maintain your cells undifferentiated.

Guidelines for subculturing cells

Introduction

You may maintain Rat Fetal NSCs as an adherent culture on CELLStart™, fibronectin, or poly-L-ornithine coated, tissue-culture treated flasks, plates or dishes, or culture them as suspension neurospheres. Subculture adherent cells when 75–90% confluent, before colonies start contacting each other, and cells in suspension when neurospheres are larger than 3.5 mm in diameter.

Materials needed

The following materials are required for passaging Rat Fetal NSCs ("Additional products" on page 6 for ordering information).

- Culture vessels containing Rat Fetal NSCs (75–90% confluent if adherent culture; >3.5 mm in diameter if neurospheres in suspension culture)
- **For adherent culture**: CELLStart™, fibronectin, or poly-L-ornithine coated, tissue-culture treated flasks, plates, or Petri dishes (see "Coating culture vessels for adherent cell culture" on page 15)
- **For suspension culture**: Uncoated, tissue-culture treated flasks, plates, or Petri dishes
- Complete StemPro™ NSC SFM™, pre-warmed to 37°C
- 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
- StemPro™ Accutase™, pre-warmed to 37°C
- Hemacytometer, cell counter and Trypan Blue, LIVE/DEAD™ Cell Vitality Assay Kit, or the Countess™ Automated Cell Counter

Adapting NSCs to StemPro™ NSC SFM™

If you have cultured Rat Fetal NSCs in serum containing medium, you may adapt your cells to StemPro™ NSC SFM™ by first passaging them into 50% parent medium (*i.e.*, serum-containing medium) and 50% complete StemPro™ NSC SFM™, before completely switching to 100% complete StemPro™ NSC SFM™.

Subculturing cells (Adherent culture)

Passaging cells (Adherent culture)

1. Aspirate the complete StemPro™ NSC SFM™ 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 the D-PBS and discard.
4. To detach the cells, add 1 mL of pre-warmed StemPro™ Accutase™. Cells will be lifted off from the culture dish right after the application of StemPro™ Accutase™ (within approximately 30 seconds).
5. Once you observe cell detachment, gently pipette up and down break clumps into a single cell suspension. Stop the cell dissociation reaction by adding 4 mL of complete StemPro™ NSC SFM™. Disperse the medium by pipetting over the cell layer surface several times
6. Transfer the cells to a 15-mL or a 50-mL conical tube and centrifuge at 300 × *g* for 4 minutes at room temperature. Aspirate and discard the medium.
7. Resuspend the cell pellet in a minimal volume of pre-warmed complete StemPro™ NSC SFM™ and remove a sample for counting.
8. Determine the total number of cells and percent viability using your method of choice. If necessary, add complete StemPro™ NSC SFM™ to the cells to achieve the desired cell concentration and recount the cells.
9. Add complete StemPro™ NSC SFM™ to each tube containing cells so that the final viable cell concentration is 5×10^4 cells per cm^2.
10. Add the appropriate volume of cells to each culture vessel and incubate at 37°C, 5% CO_2 and 90% humidity.
11. When cells reach 80–90% confluency (3–4 days after seeding), completely remove the medium, and replace with an equal volume of complete StemPro™ NSC SFM™.

Subculturing cells (Suspension culture)

Passaging cells (Suspension culture as neurospheres)

1. Transfer medium with neurospheres into a 15-mL or 50-mL sterile conical tube.
2. Leave the tube at room temperature to let the neurospheres settle at the bottom of the tube by gravity. Alternatively, you may centrifuge the neurospheres at 200 × *g* for 2 minutes.
3. Aspirate the medium carefully to leave the neurospheres in a minimal volume of medium
4. Wash the neurospheres with 10 mL of D-PBS without Ca^{2+} and Mg^{2+}, and leave a minimal volume of D-PBS.
5. Add 1 mL of pre-warmed StemPro™ Accutase™ to the neurospheres, and incubate for 10 minutes at room temperature.
6. After incubation, gently pipette the cells up and down to get a single cell suspension.
7. Stop the StemPro™ Accutase™ treatment by adding 4 mL of complete StemPro™ NSC SFM™.
8. Centrifuge cells at 300 × *g* for 4 minutes at room temperature. Aspirate and discard the medium.
9. Resuspend the cell pellet in a minimal volume of pre-warmed complete StemPro™ NSC SFM™ and remove a sample for counting.
10. Determine the total number of cells and percent viability using your method of choice. If necessary, add complete StemPro™ NSC SFM™ to the cells to achieve the desired cell concentration and recount the cells.
11. Seed the cells in fresh complete StemPro™ NSC SFM™ at 2 × 10^5 viable cells per cm^2 in a suspension dish or a non-coated flask or Petri dish.
12. Incubate the cells at 37℃, 5% CO_2, and 90% humidity.

Freezing cells

Materials needed

The following materials are required (see “Additional products” on page 6 for ordering information).

- Culture vessels containing Rat Fetal 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

- StemPro™ Accutase™
- Hemacytometer, cell counter and Trypan Blue, LIVE/DEAD™ Cell Vitality Assay Kit, or the Countess™ Automated Cell Counter
- Sterile freezing vials

Guidelines

When freezing Rat Fetal NSCs, we recommend the following:

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

Freezing cells procedure

1. Aspirate complete StemPro™ NSC SFM™ from the flask, well, or dish.
2. Wash the cells with D-PBS without Ca^{2+} and Mg^{2+}. Aspirate the D-PBS and discard.
3. Add 1 mL of pre-warmed StemPro™ Accutase™ to the cells, and gently pipette up and down to get a single cell suspension.
4. Stop the StemPro™ Accutase™ treatment by adding 4 mL of complete StemPro™ NSC SFM™. Transfer the cells into a 15-mL or 50-mL sterile, conical tube.
5. Centrifuge cells at 300 × *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 total number of cells using your method of choice.
8. Prepare 2X freezing medium of 80% complete StemPro™ NSC SFM™ and 20% DMSO. Keep on ice until use.
9. Gently aspirate the medium from the conical tube and resuspend the cells to a concentration of 2×10^6 cells/mL in complete StemPro™ NSC SFM™.
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%.
11. Transfer 1 mL (1×10^6) of cells into each cryovial, and store at –80°C overnight in an isopropanol chamber.
12. The next day, transfer the frozen vials to a liquid nitrogen tank 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 cells” on page 16.

Differentiating rat fetal NSCs

Introduction

One critical hallmark of NSCs is their ability to differentiate into neurons, oligodendrocytes, and astrocytes. Traditional and modern bioassays are used to demonstrate the multipotency of Rat Fetal NSCs to differentiate along these lineages. This section provides guidelines for spontaneously differentiating Rat Fetal NSCs.

Materials needed

In addition to materials for passaging adherent Rat Fetal NSCs (see "Adapting NSCs to StemPro™ NSC SFM™" on page 18), the following materials are required:

- StemPro™ NSC SFM™ without the growth factors (*i.e.*, without bFGF and EGF)
- CELLStart™, fibronectin, or poly-L-ornithine coated, tissue-culture treated plate

Spontaneous differentiation protocol

To spontaneously differentiate Rat Fetal NSCs into neurons, oligodendrocytes, and astrocytes, follow the protocol below.

1. Plate Rat Fetal NSCs on a CELLStart™, fibronectin, or poly-L-ornithine coated, tissue culture-treated plate at 5×10^4 cells/cm^2 following the protocol for passaging adherent Rat Fetal NSCs (see "Adapting NSCs to StemPro™ NSC SFM™" on page 18).
2. After 2 days, change medium to StemPro™ NSC SFM™ without the growth factors (*i.e.*, withdraw growth factors from cell culture), and replace with fresh medium every 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 Rat Fetal NSCs to differentiate into neurons, oligodendrocytes, and astrocytes by exposing the cells to specific factors. For more information on how to directly differentiate Rat Fetal NSCs into these lineages, contact Technical Support ().

Characterizing phenotype of rat fetal NSCs

Introduction

This section provides information on phenotypic marker expression of Rat Fetal NSCs 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 Rat Fetal NSCs as well as neurons, oligodendrocytes, and astrocytes. See "Products for marker analysis" on page 8 for ordering information.

	Antigen	Dilution ratio	Antibody type
Undifferentiated Rat Fetal NSCs	Nestin (Abcam, Cat. no. Ab5968)	1:1,000	Rabbit 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	GFAP	1:200	Rabbit IgG

Note: See Figure 2 and Figure 3 for examples of fluorescent images showing phenotypic marker expression of Rat Fetal NSCs in their undifferentiated state, and after their differentiation into neurons, oligodendrocytes, and astrocytes.

Immunocytochemistry

Fixing Cells:

1. Remove culture medium and gently rinse the cells once with D-PBS without dislodging.
2. Fix the cells with 4% fresh **Paraformaldehyde Fixing Solution** (PFA; see Appendix A, "Appendix", for recipe) at room temperature for 15 minutes.
3. Rinse 3X with D-PBS containing Ca^{2+} and Mg^{2+}.
4. Check for presence of cells after fixing.
5. Proceed to staining on the next page. You may also store slides for up to 3–4 weeks in D-PBS at 4°C. **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™ in blocking buffer.
2. Remove the blocking buffer and incubate 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 with DAPI solution (3 ng/mL) for 5 minutes, and rinse with D-PBS.
6. If desired, mount with 3 drops of ProLong™ Gold antifade reagent per slide and seal with the cover slip (see "Products for marker analysis" on page 8 for ordering information). You may store the slides **in the dark** at 4℃.

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 Cells** ("Thawing and establishing cells" on page 16) and **Freezing Cells** ("Freezing cells" on page 20) 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℃ water bath.
		Obtain new Rat Fetal NSCs.
	Thawing medium not correct	Use pre-warmed complete StemPro™ NSC SFM™, prepared as described on "Preparing complete StemPro™ NSC SFM™" on page 15.

Problem	Cause	Solution
No viable cells after thawing stock	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.	Rat Fetal 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 prewarmed complete StemPro™ NSC SFM™.
	Cells passaged >3 times	Use healthy NSCs, under passage 4 (*i.e.*, 3 passages after thawing); do not overgrow.

Culturing cells

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

Problem	Cause	Solution
Cells differentiated	Culture conditions not correct	Thaw and culture fresh vial of new Rat Fetal NSCs. Follow thawing instructions ("Coating culture vessels for adherent cell culture" on page 15) and subculture procedures ("Guidelines for subculturing cells" on page 18) 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, or 2×10^5 cells/cm^2 for suspension 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 "Additional products" on page 6 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 cell culture problems.

Problem	Cause	Solution
Cells fail to differentiate	Culture medium contains bFGF	Remove bFGF from culture medium.
	Cell density too high, and endogeneous bFGF is preventing differentiation.	Reduce cell density.
	Cells have been passaged too many times.	Obtain new, P0 Rat Fetal NSCs.

Appendix

Paraformaldehyde 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 at 60℃ using a magnetic stirrer until completely dissolved.
3. Filter through 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 on dry ice, and store at –20℃.

To prepare 4% PFA for fixing:

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

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 Vermuri, M. Protocols for Neural Cell Culture, 4th edn. (ed. Laurie Doering, in press, 2009)

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

B

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

Documentation and support

Customer and technical support

Visit **thermofisher.com/support** for the latest service and support information.

- Worldwide contact telephone numbers
- Product support information
  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.