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Gibco Neurobiology Protocol Handbook

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Introduction

Neural development and neural stem cells

Overview

It has long been thought that the adult mammalian nervous system was incapable of regeneration after injury. However, recent advances in our understanding of stem cell biology and neuroscience have opened up new avenues of research for developing potential treatments for incurable neurodegenerative diseases and neuronal injuries. Because stem cells have the capacity to self-renew and generate differentiated cells, stem cell replacement therapy for central and peripheral nervous system disorders and injuries strives to repopulate the affected neural tissue with neurons and other neural cells. One of the main strategies towards this end aims to recapitulate the normal development of the nervous system by activating the endogenous regenerative capacity of neural stem cells or by transplanting neural or embryonic cells.

This chapter defines the key concepts in stem cell biology with respect to the nervous system, presents an overview of neural development, and summarizes the involvement of neural cell types in specific neural diseases.

Stem Cells

The classical definition of a stem cell requires that it has the capacity to self-renew and that it possesses potency. **Self-renewal** is defined as the ability of the stem cell to go through multiple cycles of cell division while maintaining its undifferentiated state (i.e., to generate daughter cells that are identical to their mother). **Potency** is the ability of the stem cell to differentiate into specialized cell types.

Pluripotent vs. adult stem cells

A stem cell can divide to generate one daughter cell that is a stem cell, maintaining its capacity for self-renewal and potency, and another daughter cell that can further produce differentiated cells. Some **pluripotent stem cells**, including embryonic stem cells (ESC) and induced

pluripotent stem cells (iPSCs), have the capacity for multilineage differentiation, enabling construction of a complete, viable organism (i.e., they are pluripotent). By contrast, **adult stem cells** can generate only one specific lineage of differentiated cells to reconstitute tissues or organs.

Neural stem cells

Neural stem cells (NSCs) are stem cells in the nervous system that can self-renew and give rise to differentiated progenitor cells that generate lineages of neurons as well as glia, such as astrocytes and oligodendrocytes. This characteristic is known as **multipotency**. NSCs and neural progenitor cells are present throughout development and persist in the adult nervous system. Multiple classes of NSCs have been identified that differ from each other in their differentiation abilities, their cytokine responses, and their surface antigen characteristics.

Rationale for studying neural stem cells

Neurological disorders, especially neurodegenerative disorders, are at the top of the list of diseases that have been suggested as targets for stem cell therapy. A thorough characterization of NSCs, a better understanding of neural patterning, and the generation of all three major cell types that constitute the central nervous system (i.e., neurons, astrocytes, and oligodendrocytes), as well as the microenvironments that can support them, is crucial to increase the likelihood of clinical success for the use of stem cells in neurological disorders.

Stem cells and cancer

The discovery that many cancers may be propagated by a small number of stem cells present in the tumor mass is an extremely exciting finding. This was first described in breast cancers and subsequently in a variety of solid tumors. Several reports have suggested that cancer stem cells can be identified in the nervous system as well, and that these cells bear a remarkable similarity to neural stem cells present in early development. Likewise, cells resembling

glial progenitors have been isolated from some glial tumors suggesting an intriguing link between developmental and cancer biology.

Neural Development

Establishment of the central nervous system (CNS) is initiated early in development by the induction of NSCs and neural progenitor cells; this stage in development is called **neural ectoderm induction**. By studying **neural induction** and neural development, we can determine the various factors that stimulate or inhibit the differentiation of NSCs and the requirements of these NSCs and their offspring for survival and proper function.

Stages of neural development

The nervous system is one of the earliest organ systems that differentiate from the blastula-stage embryo. This differentiation can be mimicked in culture, and NSCs can be derived from human ESC cultures over a period of 1–2 weeks. *In vivo*, the primitive neural tube forms by approximately the fourth week of gestation via a process termed primary **neurulation**. Neurogenesis commences by the fifth week of development in humans.

Separation of PNS and CNS

During neurulation, the neuroectoderm segregates from the ectoderm, and the initially formed neural plate undergoes a stereotypic set of morphogenetic movements to form a hollow tube. Neural crest stem cells generate the peripheral nervous system (PNS), which segregates from the CNS at this stage. The neural crest stem cell generates the sympathetic and parasympathetic systems, the dorsal root ganglia and the cranial nerves, as well as the peripheral glia including Schwann cells and enteric glia. In addition to neural derivatives, the cranial crest generates craniofacial mesenchyme that include bone, cartilage, teeth, and smooth muscle, while both cranial and caudal crests generate melanocytes. Placodes, which will form a subset

of the peripheral nervous system and the cranial nerves, arise at this stage as well. These populations, which are distinct from CNS stem cells, can use similar media and culture conditions for propagation over limited time periods.

Stem cells in the ventricular zone

Stem cells that will generate the CNS reside in the ventricular zone (VZ) throughout the rostrocaudal axis and appear to be regionally specified. These stem cells proliferate at various rates and express different positional markers. The anterior neural tube undergoes a significant expansion and can be delineated into three primary vesicles: the forebrain (prosencephalon), the midbrain (mesencephalon), and the hindbrain (rhombencephalon). Differential growth and further segregation leads to additional delineation of the prosencephalon into the telencephalon and diencephalon, and delineation of the rhombencephalon into the metencephalon and myelencephalon. The caudal neural tube does not undergo a similar expansion, but increases in size to parallel the growth of the embryo as it undergoes further differentiation and forms the spinal cord. The ventricular zone stem cells appear homogeneous despite the acquisition of rostrocaudal and dorsoventral identity, but differ in their differentiation ability and self-renewal capacity. Specific regions of the brain may have relatively distinct stem cell populations, such as the developing retina and the cerebellum.

Stem cells in subventricular zone

As development proceeds, the ventricular zone is reduced in size and additional zones of mitotically active precursors appear. Mitotically active cells that accumulate adjacent to the ventricular zone are called the subventricular zone (SVZ) cells. The SVZ later becomes the subependymal zone as the ventricular zone is reduced to a single layer of ependymal cells. The SVZ is prominent in the forebrain and can be identified as far back as the fourth ventricle, but it cannot be detected in more caudal regions of the brain; if it exists in these regions, it likely consists of a

very small population of cells. An additional germinal matrix derived from the rhombic lip of the fourth ventricle, called the external granule layer, generates the granule cells of the cerebellum.

Like the VZ, the SVZ can be divided into subdomains that express different rostrocaudal markers and generate phenotypically distinct progeny. Distinct SVZ domains include the cortical SVZ, the medial ganglion eminence, and the lateral ganglion eminence. The proportion of SVZ stem cells declines with development, and multipotent stem cells are likely to be present only in regions of ongoing neurogenesis (e.g., anterior SVZ and the SVZ underlying the hippocampus) in the adult CNS. At this stage, marker expression is relatively heterogeneous. Other relatively less-characterized stem cells have also been described.

Neural precursor cells

Neural stem cells do not generate differentiated progeny directly but rather generate dividing populations of more restricted precursors analogous to the blast cells or restricted progenitors described in hematopoietic lineages. These precursors can divide and self-renew, but they are located in regions distinct from the stem cell population and can be distinguished from them by the expression of cell surface and cytoplasmic markers and their ability to differentiate. Several such classes of precursors have been identified, including neuronal precursors and uni-, bi-, and tri-potential glial precursors that generate astrocytes and oligodendrocytes. Other precursors, such as a neuron-astrocyte precursor may also exist, and the same precursor may have multiple names. Such precursors can be distinguished from stem cells by their marker expression, ability to differentiate, and time of development.

Neural cell types in neurological diseases

The table below lists some of the better-known neurological disorders with experimental models, cell types, growth factors, and markers studied.

Neural disease	Experimental model	Cell type	Growth factor	Progenitor cell	Marker	Mature marker	Transplantation	Reference
Spinal cord injury	Transplantation of oligodendrocyte progenitor cells (OPCs) into demyelination model	Oligodendrocyte	EGF, bFGF, PDGF, RA	OPC	OLIG1, A2B5, SOX10, NG2, O4	GalC, MBP, RIP	Yes	Keirstead et al., 2005
Alzheimer's disease	Transgenic models targeting APP, presenilin, tau proteins, tauopathy, atrophy, neural loss in frontal cortex as well as in other regions	Cholinergic neurons	BDNF	Cholinergic neurons, NMDA receptors	A β or tau accumulations in neurons, monitor neurofibrillary tangles or A β peptide	Val66Met polymorphism	No	Giri et al., 2016
Multiple sclerosis	Demyelinated axons, co-cultured with rat hippocampal neurons	Oligodendrocyte	EGF, bFGF, PDGF, RA	OPC	PDGFR, A2B5, NG2	O4, O1, MBP, PLP	No	Kang et al., 2007
	Remyelination models	Oligodendrocyte	RA, EGF, bFGF, Noggin, vitamin C	OPC	PDGFR, NG2, OLIG1/2, SOX10	O4, O1, MBP, PL	Yes	Izrael et al., 2007
Amyotrophic lateral sclerosis and spinal muscular atrophy	Transplantation of motoneuron progeny into the developing chick embryo	Motoneuron	BDNF, GDNF, AA, RA, SHH, Noggin	Motoneuron progenitor	HOXB4, NKX6-1/6-2, OLIG1/2	NKX6-1, OLIG2, NGN2, ISL1, ChAT, VAcHt, HB9, LHX3, HOX	Yes	Lee et al., 2007
	<i>In vitro</i> studies only	Motoneuron	bFGF, RA, SHH, BDNF, GDNF, IGF-1	Motoneuron progenitor	OLIG1/2, NKX6-1/6-2, NGN2	NKX6-1, OLIG2, NGN2, ISL1, ChAT, VAcHt, HB9, synapsin	No	Li et al., 2005

Neural disease	Experimental model	Cell type	Growth factor	Progenitor cell	Marker	Mature marker	Transplantation	Reference
Parkinson's disease	Not applicable	Dopaminergic (DA) neuron	SHH, FGF8, BDNF, AA, TGF β , TGF-3	DA neuron precursor	PAX2, PAX5, LMX, EN1	MAP2, TH, AADC, VMAT, NURR1, PTX3	No	Perrier et al., 2004
	<i>In vitro</i> drug screening	DA neuron	FGF2 or FGF8, SHH, BDNF, GDNF, cAMP, AA	DA neuron precursor	EN1, OTX2, WNT1, PAX2, GBX2	TH, GABA, EN1, AADC	No	Yan et al., 2005
	Transplantation into the neostriata of 6-hydroxy-dopamine-lesioned Parkinsonian rats	DA neuron	FGF2, FGF8, SHH, BDNF, GDNF, FBS	DA neuron precursor	EN1, PAX2, OTX2	TH, TUJ-1	Yes	Roy et al., 2006
	Transplantation into the striatum of hemi-Parkinsonian rats	DA neuron	SHH, FGF8, BDNF, GDNF, AA, IGF-1	DA neuron precursor	PAX2, EN1, NURR1, LMX1B	TH, EN1, AADC	Yes	Park et al., 2005
	Transplantation into the striatum of hemi-Parkinsonian rats and MPTP-treated rhesus monkeys	DA neuron	SHH, dual Smad inhibitor, FGF8, CHIR, BDNF, GDNF, dbcAMP, TGF β 3	Midbrain floor plate cells	FoxA2, LMX1A, OTX2	TUJ-1, TH, NURR1, FoxA2, PTX3	Yes	Kriks et al., 2011
Glial-related diseases	Astrocyte-related disease	Astrocyte	Cyclopamine, human astrocyte medium	—	—	GFAP, S100, GLAST, BDNF, GDNF	No	Lee et al., 2006
CNS and PNS diseases	Peripheral and central nervous system neurons	Peripheral sensory neurons	Noggin, NGF	Neural precursor	NCAM, TUJ-1, SNAIL, dHAND, SOX9	Peripherin, BRN3, TH, TRK-A	No	Brokhman et al., 2008
Macular retinal degeneration	Not applicable	Retinal pigmented epithelium	Noggin, Dickkopf-1, IGF-1	Retinal progenitor	RX, PAX6, LHX2, SIX3	RPE-65	No	Lamba et al., 2006

Neural disease	Experimental model	Cell type	Growth factor	Progenitor cell	Marker	Mature marker	Transplantation	Reference
Huntington's disease	—	Striatal medium spiny neuron specification GABA neurons	—	—	Islet1, DARPP-32, mGluR1, NeuN	—	—	Molero et al., 2009
Hirschsprung's disease	Engraftment and functional rescue in HSCR mouse model	Enteric neurons	LDN, SB, CHIR, RA, FGF2, EGF, GDNF, AA	Enteric nervous system progenitors	Sox10, CD49d, EDNRB, RET, HOXB2, HOXB5	TUJ-1, PHOX2A, TRKC, 5-HT, GABA	Yes	Fattahi et al., 2016

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Neural Cell Culture and Differentiation

Isolation, culture, and characterization of cortical and hippocampal neurons

Summary

The ability to culture primary neurons under serum-free conditions facilitates tighter control of neuronal studies. Some serum-free media and supplements allow for the low-density neuronal cultures, which in turn enables the study of individual neurons and synapses. This has not been possible using serum-supplemented media without a feeder layer of glial cells. In serum-supplemented media, glial cells continue to multiply, necessitating the use of cytotoxic mitotic inhibitors. Serum also contains unknown and variable levels of growth factors, hormones, vitamins, and proteins. This chapter details the isolation and culture of neural cells in serum-free media and supplements.

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Required Materials

Isolating rat brain cells

- Gibco™ Hibernate™-E Medium (Cat. No. A1247601)
- Gibco™ B-27™ Supplement (50X), serum free (Cat. No. 17504044)
- Gibco™ GlutaMAX™-I Supplement (Cat. No. 35050061)
- Gibco™ Hibernate™-E Medium, without Ca²⁺ (BrainBits LLC, Cat. No. HE-Ca)
- Papain (Worthington, Cat. No. LS003119)
- Gibco™ Neurobasal™ Medium (Cat. No. 21103049)
- Gibco™ Trypan Blue Solution, 0.4% (Cat. No. 15250061)
- Pasteur pipettes
- Hemocytometer, cell counter, and trypan blue, or the Invitrogen™ Countess™ II Automated Cell Counter (Cat. No. AMQAX1000)
- Conical tubes (15 mL, 50 mL)

Culturing embryonic neurons

- Poly-D-Lysine Hydrobromide (Sigma-Aldrich, Cat. No. P6407)
- 48-well plate or 8-chambered slides
- Gibco™ Distilled Water (Cat. No. 15230162)
- Gibco™ Dulbecco's Phosphate-Buffered Saline (DPBS) with Ca²⁺ and Mg²⁺ (Cat. No. 14040141)
- Gibco Neurobasal Medium (Cat. No. 21103049)
- Gibco B-27 Supplement (50X), serum free (Cat. No. 17504044)
- Gibco GlutaMAX-I Supplement (Cat. No. 35050061)

Immunocytochemistry

- Gibco™ Goat Serum (Cat. No. 16210064)
- Invitrogen™ MAP2 Antibody (M13) (Cat. No. 13-1500)
- Invitrogen™ GFAP Antibody (rabbit, glial fibrillary acidic protein) (Cat. No. PA3-16727)
- Invitrogen™ Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor™ 488 conjugate (Cat. No. A-11029)
- Invitrogen™ Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor™ 594 conjugate (Cat. No. A-11037)
- Invitrogen™ 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Cat. No. D1306)
- Invitrogen™ ProLong™ Gold Antifade Mountant (Cat. No. P36930)
- EM-grade paraformaldehyde (Electron Microscopy Services, Cat. No. 19208)
- Triton™ X-100 surfactant

Preparing Media

Complete Hibernate-E medium

Hibernate-E Medium is a serum-free, nutrient basal medium for the short-term maintenance of cultured rat neurons and long-term storage of viable brain tissue in ambient CO₂ (0.2%) conditions. The complete medium consists of Hibernate-E Medium supplemented with B-27 Supplement (50X), serum free and GlutaMAX-I Supplement. Complete Hibernate-E medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

To prepare 100 mL of complete Hibernate-E medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally.

Component	Final conc.	Amount
Hibernate-E Medium	1X	98 mL
B-27 Supplement (50X), serum free	2%	2 mL
GlutaMAX-I Supplement	0.5 mM	250 µL

Complete Neurobasal medium

Complete Neurobasal medium requires supplementation of Neurobasal Medium with B-27 Supplement (50X), serum free and GlutaMAX-I Supplement. Complete Neurobasal medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

To prepare 100 mL of complete Neurobasal medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally.

Component	Final conc.	Amount
Neurobasal Medium	1X	98 mL
B-27 Supplement (50X), serum free	2%	2 mL
GlutaMAX-I Supplement	0.5 mM	250 µL

For primary hippocampus neuron cultures, complete Neurobasal medium requires additional supplementation with 25 µM L-glutamate up to the fourth day of culture.

Preparing Matrix

Coating culture vessels with poly-D-lysine

1. Prepare a 2 mg/mL poly-D-lysine stock solution in distilled water.
2. Dilute the poly-D-lysine stock solution 1:40 in DPBS to prepare a 50 µg/mL working solution (e.g., 125 µL of poly-D-lysine stock solution into 5 mL of DPBS).
3. Coat the surface of the culture vessel with the working solution of poly-D-lysine (150 µL/cm², e.g., 100 µL per well for a 48-well plate).
4. Incubate the culture vessel at room temperature for 1 hour.
5. Remove the poly-D-lysine solution and rinse 3 times with distilled water. Make sure to rinse the culture vessel thoroughly, because excess poly-D-lysine can be toxic to the cells.
6. Leave the coated vessels uncovered in the laminar flow hood until the wells have completely dried. You may use the dry plates immediately or store them at 4°C, wrapped tightly with Parafilm™ wrapper, for up to one week.

Isolating Neurons

1. Dissect cortex or hippocampus pairs from ten E-18 rat or mouse embryo brains. Remove all the meninges thoroughly.
2. Collect all the tissue in a conical tube containing complete Hibernate-E medium at 4°C.
3. Allow the tissue to settle to the bottom of the tube and then carefully remove excess supernatant leaving only the tissue covered by a thin layer of medium.
4. Enzymatically digest the tissue in 4 mL of Hibernate-E Medium without Ca²⁺ containing 2 mg/mL of filter-sterilized papain for 30 minutes at 30°C. Gently shake the tube every 5 minutes.
5. Add 6 mL of complete Hibernate-E medium to the tube and centrifuge for 5 minutes at 150 x *g*.
6. Remove the supernatant and resuspend the tissue in 5 mL of complete Hibernate-E medium by pipetting up and down with a fire-polished glass Pasteur pipette.
7. Let the tube stand undisturbed for 2 minutes to allow for the cell debris (if any) to settle down.
8. Transfer the cells to a new tube leaving behind all the debris.
9. Count the cells using a hemocytometer, cell counter, and trypan blue, or the Countess II Automated Cell Counter.
10. Centrifuge the tube for 4 minutes at 200 x *g*.

11. Remove the supernatant and resuspend the cell pellet in complete Neurobasal medium for culturing.

Note: Plate the cells immediately after resuspension. If you need to store the cells longer, store them in complete Hibernate-E medium at 4°C for up to 48 hours. Do not expose the neurons to ambient air at any time.

Culturing Neurons

1. Plate 0.5 x 10⁵ live cells per well in a poly-D-lysine-coated 48-well plate or an 8-chambered slide. Bring the cell suspension volume to 500 μL per well by adding complete Neurobasal medium.
2. Incubate the cells at 37°C in a humidified atmosphere of 5% CO₂ in air.
3. Feed the cells every third day by aspirating half of the medium from each well and replacing it with fresh complete Neurobasal medium.

Characterizing Neural Cells

Preparing paraformaldehyde fixing solution

20% paraformaldehyde (PFA) stock solution

1. Add PBS to 20 g of EM-grade paraformaldehyde, 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 µm 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.

4% PFA for fixing

1. Add 8 mL of 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, cool the tubes on ice.

Fixing cells

1. Remove the culture medium and gently rinse the cells without dislodging them, twice, with DPBS containing Ca²⁺ and Mg²⁺.
2. Fix the cells with 4% fresh PFA fixing solution at room temperature for 15 minutes.
3. Rinse the cells 3 times with DPBS containing Ca²⁺ and Mg²⁺.
4. Permeabilize the cells with 0.3% Triton X-100 surfactant (diluted in DPBS with Ca²⁺ and Mg²⁺) for 5 minutes at room temperature.
5. Rinse the cells 3 times with DPBS containing Ca²⁺ and Mg²⁺.

Staining cells

1. Incubate cells in 5% goat serum diluted in DPBS with Ca²⁺ and Mg²⁺ for 60 minutes at room temperature.
2. Remove the 5% goat serum solution and incubate the cells overnight with the primary antibody (mouse anti-MAP2 at 10 µg/mL and/or rabbit anti-GFAP at 4 µg/mL) diluted in 5% goat serum at 4°C. Ensure that the cell surfaces are covered uniformly with the antibody solution.
3. Wash the cells 3 times for 5 minutes with DPBS containing Ca²⁺ and Mg²⁺.
4. Incubate the cells with secondary antibody (Alexa Fluor 488 goat-anti mouse (H+L) antibody at 10 µg/mL and/or Alexa Fluor 594 goat-anti rabbit (H+L) antibody at 10 µg/mL) diluted in 5% goat serum solution for 60 minutes at room temperature.
5. Wash the cells 3 times with DPBS containing Ca²⁺ and Mg²⁺. In the last wash, counterstain the cells with DAPI solution (3 ng/mL) for 10 minutes.
6. Rinse the cells with DPBS, and if desired, mount using 3 drops of ProLong Gold Antifade Mountant per slide and seal it with a cover slip. You may store the slides in the dark at 4°C.
7. Observe the cells under the microscope using filters for FITC, Cy⁵ dye, and DAPI.

Expected Results

The thawed cortical neurons cultured in complete Neurobasal medium, i.e., Neurobasal Medium supplemented with 2% B-27 Supplement (50X), serum free and 0.5 mM GlutaMAX-I Supplement show >90% neuronal population stained with MAP2 antibody and a minimum number of astrocytes. Within 3–4 days in culture, the neurons display extensive neurite outgrowth that keeps on increasing as long as the neurons are kept healthy in culture. Note that results vary if the neurons are cultured in the presence of serum.

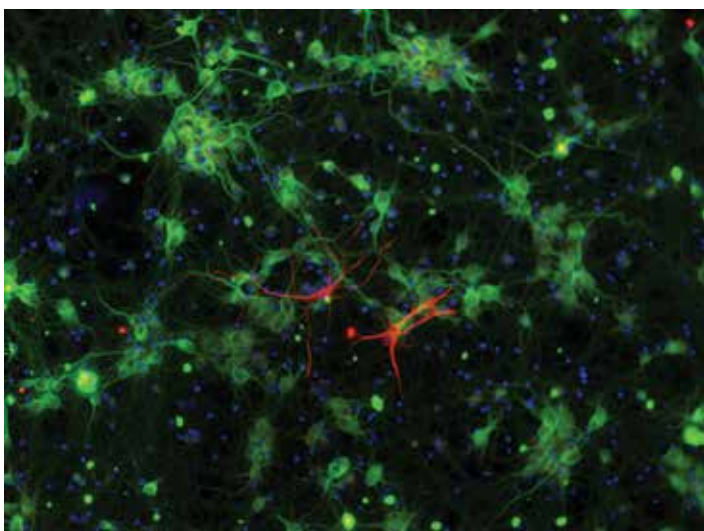


Figure 3-1. Primary rat cortical neurons. Immunofluorescence detection of primary neuronal cells stained with mouse anti-MAP2 marker (green) and presence of astrocytes as detected by rabbit anti-GFAP marker (red). Nuclei are stained with DAPI (blue).

Troubleshooting

The procedures are designed for neuronal cells grown in complete Neurobasal medium. Results may differ with culture systems grown in other complete media formulations, which can result in higher numbers of cells other than neurons (i.e., astrocytes).

Thawing and culture of cryopreserved neurons

Summary

The ability to cryopreserve primary neuronal cells is of great interest to many researchers. Such flexibility in experimental workflow allows researchers to bank cells for use in basic research, translational medicine, and cell therapy applications. In this chapter we provide detailed protocols for recovery of cells and culture in complete Neurobasal medium, which provides high post-thaw viability and recovery with superior neuronal quality.

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Required Materials

Cells

- Gibco™ Primary Rat Cortical Neurons (Cat. No. A1084001)
- Gibco™ Primary Rat Hippocampal Neurons (Cat. No. A1084101)
- Gibco™ Primary Mouse Cortical Neurons (Cat. No. A15585)
- Gibco™ Primary Mouse Hippocampal Neurons (Cat. No. A15587)

Media and reagents

- Gibco™ Neurobasal™ Medium (Cat. No. 21103049)
- Gibco™ B-27™ Supplement (50X), serum free (Cat. No. 17504044)
- Gibco™ GlutaMAX™ -I Supplement (Cat. No. 35050061)
- Gibco™ Trypan Blue Solution, 0.4% (Cat. No. 15250061)
- Poly-D-Lysine Hydrobromide (Sigma-Aldrich, Cat. No. P6407)
- 48-well plate or 8-chambered slides
- Gibco™ Distilled Water (Cat. No. 15230162)
- Gibco™ Dulbecco's Phosphate-Buffered Saline (DPBS) with Ca²⁺ and Mg²⁺ (Cat. No. 14040141)

Preparing Media

Preparing complete Neurobasal medium

The complete Neurobasal medium requires supplementation of Neurobasal Medium with B-27 Supplement (50X), serum free and GlutaMAX-I Supplement. Complete medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

To prepare 100 mL of complete Neurobasal medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally.

Component	Final conc.	Amount
Neurobasal Medium	1X	98 mL
B-27 Supplement (50X), serum free	2%	2 mL
GlutaMAX-I Supplement	0.5 mM	250 µL

For primary hippocampus neuron cultures, complete Neurobasal medium requires additional supplementation with 25 µM L-glutamate up to the fourth day of culture.

Preparing Matrix

Coating culture vessels with poly-D-lysine

1. Prepare a 2 mg/mL poly-D-lysine stock solution in distilled water.
2. Dilute the poly-D-lysine stock solution 1:40 in DPBS to prepare a 50 µg/mL working solution (e.g., 125 µL of poly-D-lysine stock solution into 5 mL of DPBS).
3. Coat the surface of the culture vessel with the working solution of poly-D-lysine (150 µL/cm², e.g., 100 µL per well for a 48-well plate).
4. Incubate the culture vessel at room temperature for 1 hour.
5. Remove the poly-D-lysine solution and rinse 3 times with distilled water. Make sure to rinse the culture vessel thoroughly, because excess poly-D-lysine can be toxic to the cells.
6. Leave the coated vessels uncovered in the laminar flow hood until the wells have completely dried. You may use the dry plates immediately or store them at 4°C, wrapped tightly with Parafilm™ wrapper, for up to one week.

Recovery of Neurons

1. Rinse a 50 mL conical culture tube with pre-warmed (37°C) complete Neurobasal medium and leave it in the laminar flow hood prior to thawing the cells.
2. If removing the vial from liquid nitrogen storage, twist cap slightly to release pressure and then retighten cap.
3. Rapidly thaw (<2 minutes) the frozen vial by gently swirling it in a 37°C water bath. Remove the vial from the water bath when only a tiny ice crystal is left (vial should still be cold to touch).
4. Transfer the vial to the laminar flow hood and disinfect it with 70% isopropyl alcohol. Tap the vial gently on the surface of the hood so that the liquid settles down to the bottom of the vial.
5. Rinse a P1000 pipette tip with complete Neurobasal medium and very gently transfer the cells to the pre-rinsed 50 mL tube (from step 1).
6. Rinse the vial with 1 mL of complete Neurobasal medium (pre-warmed to 37°C) and add to the cells in the 50 mL tube extremely slowly at the rate of one drop per second. Mix the suspension by gentle swirling after each addition.

Note: Do not add the entire amount of medium to the tube at once. This may lead to decreased cell viability due to osmotic shock.

7. Slowly add 2 mL of complete Neurobasal medium to the tube (for a total suspension volume of 4 mL). Mix the suspension very gently with the P1000 pipette without creating any air bubbles.

8. To a microcentrifuge tube containing 10 μL of Trypan Blue Solution, 0.4%, add 10 μL of the cell suspension using a pre-rinsed tip. Mix by gently tapping the tube. Determine the viable cell density using a manual (e.g., hemocytometer) counting method.

Note: Do not centrifuge the cells as they are extremely fragile upon recovery from cryopreservation.

9. It is important to rinse each pipette tip and vial with complete Neurobasal medium before using it for cell suspension to prevent the cells from sticking to the plastic.

Culturing Neurons

1. Plate $\sim 0.5 \times 10^5$ live cells per well in a poly-D-lysine-coated ($4.5 \mu\text{g}/\text{cm}^2$) 48-well plate. Dilute the cell suspension to 500 μL per well by adding complete Neurobasal medium.
2. Incubate the cells at $36\text{--}38^\circ\text{C}$ in a humidified atmosphere of 5% CO_2 in air.
3. After 4 to 24 hours of incubation, aspirate half of the medium from each well and replace with fresh complete Neurobasal medium. Return the cells to the incubator.
4. Feed the cells every third day by aspirating half of the medium from each well and replacing it with fresh medium.

Note: Do not expose neurons to ambient air at any time.

Typical Results

Thawed cortical neurons cultured in complete Neurobasal medium display extensive neurite outgrowth that continues to increase as long as they are kept healthy in culture. As opposed to non-cryopreserved neurons, post-thaw recovered neurons show dead cells due to post-thaw toxicity, and typical cultures contain dead cells that stay attached to the plate surface.

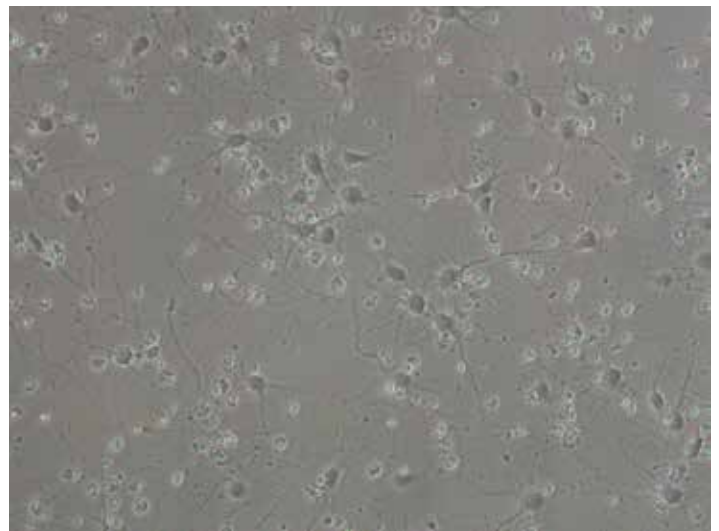


Figure 4-1. Phase-contrast image of primary rat cortical neurons recovered and cultured in complete Neurobasal medium at day 7.

Culturing human neural stem cells

Summary

Neural stem cells (NSCs) are highly valuable resources in neuroscience because of their ability to differentiate into neurons and glial cells. NSCs can be obtained by isolation from tissue or differentiation from pluripotent cells. This chapter describes methods for expanding human NSCs in cell culture and their subsequent characterization.

View this protocol online and order products at [thermofisher.com/neuroprotocol/hnsc](https://www.thermofisher.com/neuroprotocol/hnsc)

Required Materials

Cells

- Gibco™ Human Neural Stem Cells (H9-Derived) (Cat. No. N7800100)
- Gibco™ StemPro™ Neural Stem Cells (Cat. No. A15654)

Reagents

- Gibco™ StemPro™ NSC SFM (Cat. No. A1050901: This kit contains KnockOut™ DMEM/F-12 Basal Medium stored at 4°C, StemPro™ NSC SFM (Neural) Supplement stored at –20°C to –5°C in the dark, and bFGF Recombinant Human and EGF Recombinant Human stored at 4°C, desiccated.)
- Gibco™ GlutaMAX™-I Supplement (Cat. No. 35050061)
- Ascorbic Acid (Sigma-Aldrich, Cat. No. A8960)
- Heparin (Sigma-Aldrich, Cat. No. H3149)
- Gibco™ CELLstart™ CTS™ Substrate (Cat. No. A1014201)
- Gibco™ Distilled Water (Cat. No. 15230162)
- Gibco™ Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca²⁺ and Mg²⁺ (Cat. No. 14190144)
- Gibco™ Dulbecco's Phosphate-Buffered Saline (DPBS) with Ca²⁺ and Mg²⁺ (Cat. No. 14040133)

- Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent (Cat. No. A1110501)
- Gibco™ TrypLE™ Express Enzyme (1X), no phenol red (Cat. No. 12604013)

Plasticware

- Thermo Scientific™ Nunclon™ Sphera™ Flasks (Cat. No. 174951 or 174952) (for suspension culture)
- Thermo Scientific Nunclon™ Sphera™ Dishes (Cat. No. 174930, 174931, or 174932) (for suspension culture)

Preparing Media

Heparin stock preparation

1. To prepare 6,000 U/mL heparin solution (1,000X), add 1.65 mL of distilled water to 10,000 U of heparin and mix until dissolved.
2. After dissolving, filter through a 0.22-µm filter, aliquot 50–100 µL into sterile tubes, and store at –20°C.

Ascorbic acid stock preparation

1. To prepare 100 mM ascorbic acid stock solution (500X), add 17.27 mL of distilled water to 500 mg of ascorbic acid powder and mix until dissolved.
2. After dissolving, filter through a 0.22 µm filter, aliquot 100–200 µL into sterile tubes, and store at –20°C.

bFGF and EGF stock preparation

1. Reconstitute bFGF and EGF with 0.1% BSA in DPBS (Cat. No. 14040133 or 14190144) at a concentration of 20 µg/mL.
2. Aliquot 50–100 µL into sterile tubes, and store at –20°C.

Complete StemPro NSC SFM

To prepare 100 mL of complete neural stem cell culture medium, mix the components per the table below under sterile conditions. KnockOut DMEM/F-12, bFGF, EGF, and StemPro Neural Supplement are components of the StemPro NSC SFM kit. Complete medium is stable for 4 weeks when stored in the dark at 2°C to 8°C. To make larger volumes, increase the component amounts proportionally.

Component	Final conc.	Amount
KnockOut DMEM/F-12	1X	96.5 mL
bFGF (prepared as 20 µg/mL stock)	20 ng/mL	100 µL
EGF (prepared as 20 µg/mL stock)	20 ng/mL	100 µL
StemPro Neural Supplement	2%	2 mL
GlutaMAX-I Supplement	2 mM	1 mL
Heparin*	6 units/mL	100 µL
Ascorbic Acid*	200 µM	200 µL

* Optional: Can be omitted for H9 neural stem cells.

Coating culture vessels with CELLstart CTS Substrate for adherent culture

1. Dilute CELLstart CTS Substrate 1:100 in DPBS with Ca²⁺ and Mg²⁺ (e.g., 50 µL of CELLstart CTS Substrate into 5 mL of DPBS).

Note: CELLstart CTS Substrate should not be frozen, vortexed, or exposed to vigorous agitation due to potential gel formation.

2. Coat the surface of the culture vessel with the working solution of CELLstart CTS Substrate (14 mL for T-75 flask, 7 mL for T-25 flask, 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% CO₂ for 1 hour.
4. Remove the vessel from the incubator and store it until use. Remove all CELLstart CTS Substrate solution

immediately before use, and fill the vessel with complete StemPro NSC SFM.

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

Culturing Neural Stem Cells

Neural stem cell (NSC) populations can be expanded from frozen stocks and grown as adherent cultures (PSC-derived NSCs), or as suspension cultures (tissue-isolated NSCs). In either environment, change the spent culture medium every other day. When the cells in adherent culture reach >90% confluency, they are ready to be passaged. When the neurospheres in suspension culture become >3.5 mm in diameter, they are ready to be passaged. Cells cultured during expansion can be frozen down to create additional frozen stocks of higher passage number.

Thawing frozen neural stem cells

1. Prepare 10 mL of complete StemPro NSC SFM and warm to 37°C.
2. Rapidly thaw (<2 minutes) the frozen vial by gently swirling it in a 37°C water bath. Remove the vial from the water bath when only a tiny ice crystal is left (vial should be still cold to touch).
3. Transfer the vial to the laminar flow hood and disinfect it with 70% ethanol. Allow the ethanol to evaporate before opening the vial.
4. Transfer thawed cells into a 15 mL tube and add complete StemPro NSC SFM, pre-warmed to 37°C, in a dropwise manner to a total volume of 5 mL.

5. Spin down the thawed cells by centrifugation at 300 x g for 4 minutes. Aspirate and discard the supernatant.
6. Resuspend the cells in complete StemPro NSC SFM.
7. PSC-derived NSCs (H9 NSCs) can be plated on CELLstart CTS Substrate and PSCs isolated from tissue (StemPro NSCs) can be suspended in a low-attachment plate or flask.

Note: Use 1×10^5 cells/cm² seeding density for H9 NSCs and 2×10^4 to 4×10^4 cells/cm² seeding density for StemPro NSCs.

Passaging neural stem cells (adherent culture)

1. Aspirate the medium and wash with DPBS without Ca²⁺ and Mg²⁺.
 2. Add 1 mL of TrypLE Express Enzyme or StemPro Accutase reagent to the culture vessel.
- Note:** The monolayer lifts off from the culture dish within 30 seconds of application of TrypLE Express Enzyme or StemPro Accutase reagent.
3. Gently pipette to loosen monolayer into a single-cell suspension. Neutralize the treatment by adding 4 mL of medium. Do not treat the cells for longer than 3 minutes after addition of TrypLE Express Enzyme or StemPro Accutase reagent.
 4. Spin down the cells by centrifugation at 200 x g for 4 minutes. Aspirate and discard the supernatant.
 5. Resuspend the cells in complete StemPro NSC SFM.
 6. Count the cell number using a hemocytometer or Invitrogen™ Countess™ II Automated Cell Counter.

7. Plate cells in fresh complete StemPro NSC SFM on CELLstart CTS Substrate at a density of 5×10^4 cells/cm², or split the cells at a 1:4 ratio.

Passaging neural stem cells (suspension culture)

1. Transfer medium containing neurospheres into a 15 or 50 mL conical tube.
2. Leave the tube at room temperature and allow the neurospheres to settle to the bottom of tube. Alternatively, spin down the cells by centrifugation at 200 x g for 2 minutes.
3. Aspirate the supernatant carefully, and leave the neurospheres in a minimum volume of medium.
4. Wash the neurospheres with 10 mL DPBS without Ca²⁺ and Mg²⁺, aspirate the DPBS supernatant carefully, and leave the neurospheres in a minimum volume of DPBS.
5. Add 1 mL of StemPro Accutase reagent to the neurospheres and incubate the tube at 37°C for 10 minutes. Swirl the tube at 5 and 8 minutes to ensure that the cells do not aggregate or settle at the bottom of the tube.
6. Using a P1000 pipettor, break up the neurospheres by pipetting up and down 5 times. Place the tube back in the laminar flow hood for another 5–25 minutes.
7. Gently triturate neurospheres using a Pasteur pipette or P1000 pipettor to create a single-cell suspension.
8. Neutralize the treatment by adding 4 mL of complete StemPro NSC SFM.
9. Spin down the cells by centrifugation at 300 x g for 3 minutes. Aspirate and discard the supernatant, and seed cells in fresh complete StemPro NSC SFM in a suspension dish at a density of 2×10^4 to 5×10^4 cells/cm².

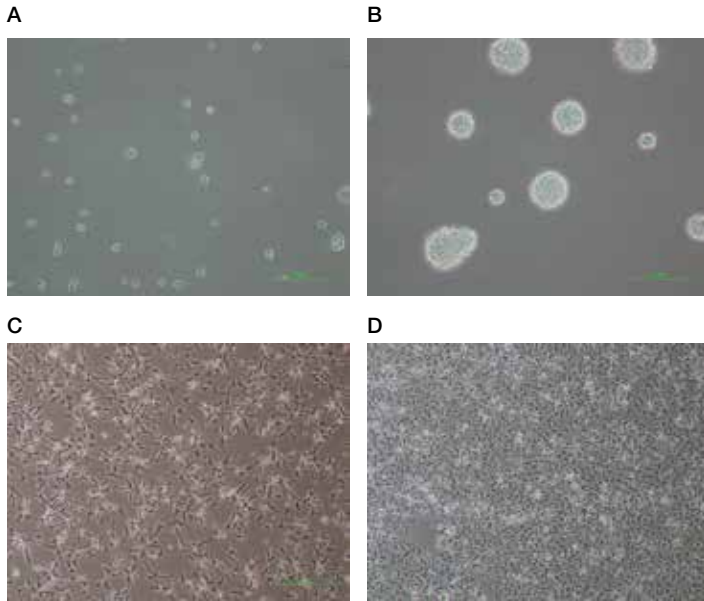


Figure 5-1. Culture of neural stem cells. Phase image of cells **(A)** 1 day after thawing of StemPro Neural Stem Cells, **(B)** 7 days after thawing of StemPro Neural Stem Cells before harvest, **(C)** 1 day after thawing of H9 neural stem cells, and **(D)** 3 days after thawing of H9 neural stem cells before harvest.

Cryopreserving neural stem cells

1. Harvest cells using method described in Passaging Neural Stem Cells (page 21).
2. Resuspend the cells in complete StemPro NSC SFM at a density of 2×10^6 cells/mL.
3. Prepare freezing medium (2X) consisting of 20% DMSO and 80% complete StemPro NSC SFM.

Note: Freezing medium (2X) can be prepared on the day of use and stored at 4°C until use.

4. Add a volume of freezing medium equal to the amount of complete StemPro NSC SFM used to resuspend the cells in a dropwise manner.
5. Prepare 1 mL aliquots (1×10^6 cells) in cryovials and place the vials in an isopropanol chamber.
6. Put the isopropanol chamber at -80°C and transfer the vials to liquid nitrogen storage the next day.

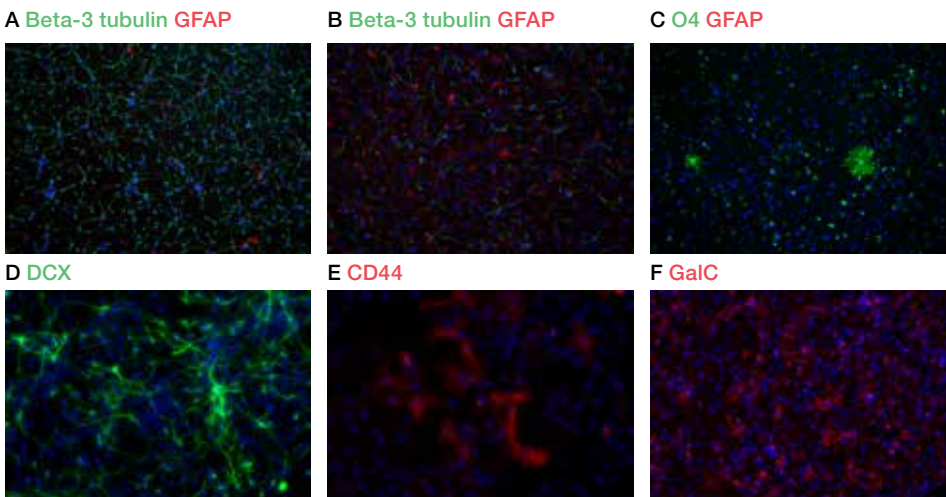


Figure 5-2. Differentiation of neural stem cells plated down on slides for differentiation. After 7 days of directed differentiation, neurons, astrocytes, and oligodendrocytes were labeled with phenotype markers of beta-3 tubulin or DCX (neurons), CD44 or GFAP (astrocytes), and O4 or GalC (oligodendrocytes). **(A-C)** StemPro Neural Stem Cells; **(D-F)** H9 neural stem cells.

Characterizing NSCs by Immunocytochemistry and PCR

Antibodies for NSC characterization

Use antibodies listed in the following table to characterize NSCs by immunocytochemistry. The Human Neural Stem Cell Immunocytochemistry Kit (Cat. No. A24354)

includes markers for neural stem cells and accompanying secondary antibodies. For details on the procedure, refer to “Immunocytochemistry” on page 80.

Category	Antigen	Type	Cat. No.	Working concentration
Neural stem cells	Nestin	Mouse IgG	A24345	1:50 dilution
	Sox1	Goat IgG	A24347	1:50 dilution
	Sox2	Rabbit IgG	A24339	1:50 dilution
	Pax6	Rabbit IgG	A24340	1:50 dilution
Proliferation	Ki67	Rabbit IgG	180191Z	1:50 dilution
	EdU	Chemical	C10086	10 μ M

Primers for NSC characterization

Use the primer sets listed in the following table to characterize NSCs by PCR. For details on the procedure, refer to “Characterizing neural cells by qPCR” on page 98.

Target	Primer	Sequence	T _m	Amplicon size	Intron size
Neural stem cells	Sox1-F	GCGGAAAGCGTTTTCTTG	53.0	406	No intron
	Sox1-R	TAATCTGACTTCTCCTCCC	50.2		
	Sox2-F	ATGCACCGCTACGACGTGA	59.3	437	No intron
	Sox2-R	CTTTTGCACCCCTCCCATT	56.0		
	Nestin-F	CAGCGTTGGAACAGAGGTTGG	58.6	389	1,142
	Nestin-R	TGGCACAGGTGTCTCAAGGGTAG	60.7		
Endogenous control	ACTB-F	ACCATGGATGATGATATCGC	58.2	281	135
	ACTB-R	TCATTGTAGAAGGTGTGGTG	54.4		

Culturing rat fetal neural stem cells

Summary

Rat neural stem cells (NSCs) serve as a well-established model for investigating mammalian brain development, disease processes, and for study of debilitating central nervous system (CNS) disorders. This protocol describes the *in vitro* expansion, passaging, and morphology of rat fetal NSCs in adherent or neurosphere suspension cultures.

View this protocol online and order products at [thermofisher.com/neuroprotocol/ratnsc](https://www.thermofisher.com/neuroprotocol/ratnsc)

Required Materials

Cells

- Gibco™ Rat Fetal Neural Stem Cells (Cat. No. N7744100) or homogeneous cell preparation from 14–18 days post-coitum rat brain tissue

Media and reagents

- Gibco™ Dulbecco's Phosphate-Buffered Saline (DPBS) with Ca²⁺ and Mg²⁺ (Cat. No. 14040141)
- Gibco™ Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca²⁺ and Mg²⁺ (Cat. No. 14190144)
- Gibco™ StemPro™ NSC SFM (Cat. No. A1050901: This kit contains KnockOut™ DMEM/F-12 Basal Medium stored at 4°C, StemPro™ NSC SFM (Neural) Supplement stored at –20°C to –5°C in the dark, and bFGF Recombinant Human and EGF Recombinant Human stored at 4°C, desiccated)
- Gibco™ StemPro Accutase™ Cell Dissociation Reagent (Cat. No. A1110501)
- Gibco™ CELLstart™ CTS™ Substrate (Cat. No. A1014201)
- Gibco™ Trypan Blue Solution, 0.4% (Cat. No. 15250061) (included with the Invitrogen™ Countess™ II Automated Cell Counter) or the Molecular Probes™ LIVE/DEAD™ Cell Vitality Assay Kit, C₁₂ Resazurin/SYTOX™ Green (Cat. No. L34951)

Special tools

- Invitrogen™ Countess™ II Automated Cell Counter (Cat. No. AMQAX1000) or hemocytometer

Preparing Media

Medium for expanding neural stem cells

Complete StemPro NSC SFM consists of KnockOut DMEM/F-12 with StemPro Neural Supplement, bFGF, EGF, and GlutaMAX-I Supplement. Complete medium is stable for 4 weeks when stored in the dark at 2°C to 8°C.

To prepare 100 mL of complete StemPro NSC SFM:

- Reconstitute bFGF and EGF with 0.1% BSA solution (in KnockOut DMEM/F-12) at a concentration of 100 µg/mL. You will need 20 µL of each per 100 mL of complete medium. Freeze unused portions in aliquots.
- Mix the following components under aseptic conditions. For larger volumes, increase the component amounts proportionally.

Component	Final concentration	Amount
KnockOut DMEM/F-12	1X	97 mL
GlutaMAX-I Supplement	2 mM	1 mL
bFGF (prepared as 100 µg/mL stock)	20 ng/mL	20 µL
EGF (prepared as 100 µg/mL stock)	20 ng/mL	20 µL
StemPro Neural Supplement	2%	2 mL

You may observe a white precipitate when thawing StemPro Neural Supplement; this precipitate will disappear when the supplement is completely thawed or dissolved.

Coating Culture Vessels with CELLstart Substrate

For adherent cultures, prepare plates with CELLstart CTS Substrate as described below.

1. Dilute CELLstart CTS Substrate 1:100 in DPBS with Ca^{2+} and Mg^{2+} (e.g., 50 μL of CELLstart CTS Substrate into 5 mL of DPBS).

Note: CELLstart CTS Substrate should not be frozen, vortexed, or exposed to vigorous agitation due to potential gel formation.

2. Coat the surface of the culture vessel with the working solution of CELLstart CTS Substrate (14 mL for a T-75 flask, 7 mL for a T-25 flask, 3.5 mL for a 60 mm dish, 2 mL for a 35 mm dish).
3. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO_2 for 1 hour.
4. Remove the vessel from the incubator and store at 4°C until use. Remove all CELLstart CTS Substrate solution immediately before use, and fill the vessel with complete StemPro NSC SFM.

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

Expanding and Passaging of Rat NSCs

Adherent cultures

1. Resuspend the rat fetal NSCs as follows:
 - For freshly prepared rat fetal NSCs, after rinsing with DPBS with Ca^{2+} and Mg^{2+} , resuspend in warmed complete StemPro NSC SFM at a density of 1×10^7 viable cells/mL.
 - For thawed rat fetal NSCs, after determining the viable cell count, resuspend in warmed complete StemPro NSC SFM at a cell density of 1×10^7 viable cells/mL.
2. Plate rat fetal NSCs onto culture vessels coated with CELLstart CTS Substrate at a density of 5×10^4 cells/cm². See the following table for recommended seeding densities for common culture vessels.

Vessel size	Growth area	Volume of media	No. of cells
96-well plate	0.32 cm ² /well	0.1 mL	1.6×10^4
24-well plate	1.9 cm ² /well	0.5 mL	1.0×10^5
12-well plate	3.8 cm ² /well	1 mL	1.9×10^5
35 mm dish	8 cm ² /well	2 mL	4.0×10^5
6-well plate	9.6 cm ² /well	2 mL	4.8×10^5
60 mm dish	19.5 cm ²	5 mL	9.8×10^5
T-25 flask	25 cm ²	5 mL	1.3×10^6
100 mm dish	55 cm ²	10 mL	2.8×10^6
T-75 flask	75 cm ²	15 mL	3.8×10^6

3. Add the appropriate volume of cells to each culture vessel and incubate at 37°C, 5% CO_2 , and 90% humidity.
4. Re-feed the rat fetal NSC cultures every 2–3 days with fresh complete StemPro NSC SFM. The morphology of rat fetal NSCs should exhibit short stellate-like processes with uniform density (Figure 6-1).

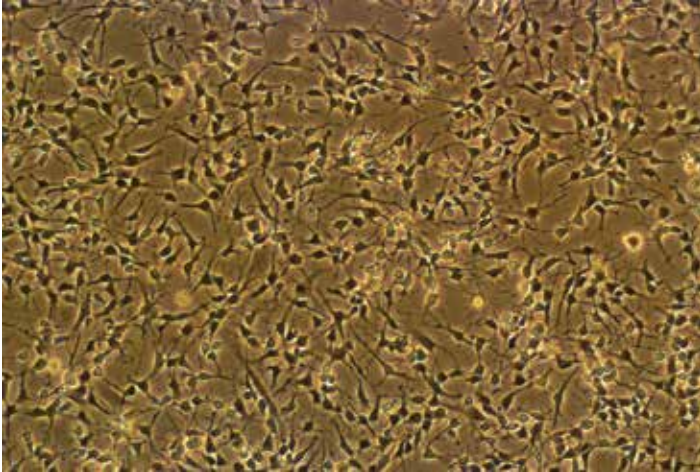


Figure 6-1. Rat fetal NSCs at passage 3 in adherent culture using complete StemPro NSC SFM.

5. When cells reach 75–90% confluency (3–4 days after seeding), the rat fetal NSC cultures are ready to be passaged.
6. Rinse the culture vessel once with DPBS without Ca^{2+} and Mg^{2+} , then remove the medium.
7. Add pre-warmed StemPro Accutase reagent and let the cells detach from the culture surface (within approximately 30 seconds).
8. After detachment, gently pipet the cells up and down to break the clumps into a uniform cell suspension and add four volumes of complete StemPro NSC SFM to the culture vessel.
9. Disperse the cells by pipetting over the culture surface several times to generate a homogeneous cell solution.
10. Transfer the cells to a sterile centrifuge tube and centrifuge at $300 \times g$ for 4 minutes at room temperature. Aspirate and discard the medium.
11. Resuspend the cell pellet in a minimal volume of pre-warmed complete StemPro NSC SFM and remove a sample for counting.
12. Determine the total number of cells and percent viability using trypan blue stain or the LIVE/DEAD Cell Vitality Assay Kit.
13. Add enough complete StemPro NSC SFM to the tube for a final cell solution of 1×10^6 viable cells/mL. Incubate at 37°C , 5% CO_2 , and 90% humidity. Rat fetal NSC cultures should not be maintained for more than 3 passages.

Important: If you are re-feeding rat fetal NSCs in a growth medium other than complete StemPro NSC SFM, ensure that the medium is supplemented with 10 ng/mL bFGF to maintain the undifferentiated state of the rat fetal NSCs.

Neurosphere suspension cultures

1. Resuspend the rat fetal NSCs as follows:
 - For freshly prepared rat fetal NSCs, after rinsing with DPBS, resuspend in warmed complete StemPro NSC SFM at a cell density of 1×10^7 viable cells/mL.
 - For thawed rat fetal NSCs, after determining the viable cell count, resuspend in warmed complete StemPro NSC SFM at a cell density of 1×10^7 viable cells/mL.
2. Plate the rat fetal NSCs onto uncoated or low-attachment culture vessels at a density of 2×10^5 viable cells/ cm^2 . See the table on the next page for recommended seeding densities.

Vessel size	Growth area	Volume of media	No. of cells
96-well plate	0.32 cm ² /well	0.1 mL	6.4 x 10 ⁴
24-well plate	1.9 cm ² /well	0.5 mL	3.8 x 10 ⁵
12-well plate	3.8 cm ² /well	1 mL	7.6 x 10 ⁵
35 mm dish	8 cm ² /well	2 mL	1.6 x 10 ⁶
6-well plate	9.6 cm ² /well	2 mL	1.9 x 10 ⁶
60 mm dish	19.5 cm ²	5 mL	3.9 x 10 ⁶
T-25 flask	25 cm ²	5 mL	5.0 x 10 ⁶
100 mm dish	55 cm ²	10 mL	1.1 x 10 ⁷
T-75 flask	75 cm ²	15 mL	3.8 x 10 ⁶

3. Add the appropriate volume of cells to each culture vessel and incubate at 37°C, 5% CO₂, and 90% humidity.
4. Carefully re-feed the neurosphere suspension of rat fetal NSCs every 2–3 days with fresh complete StemPro NSC SFM without removing any developing neurospheres. The morphology of the neurospheres should exhibit spherical and transparent multicellular complexes (Figure 6-2).

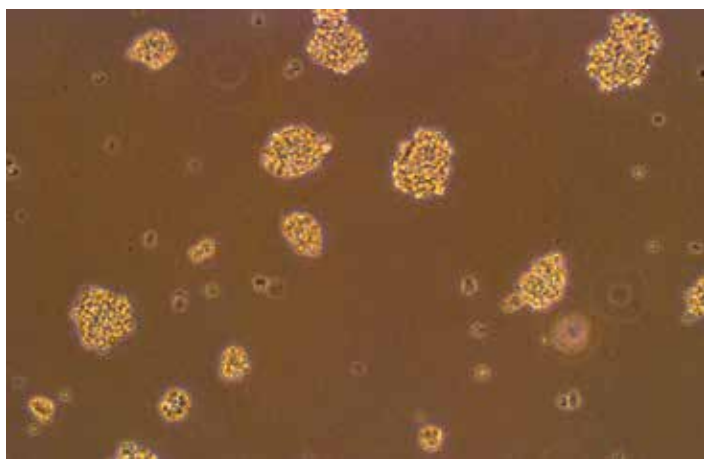


Figure 6-2. Rat fetal NSCs at passage 3 in neurosphere culture using complete StemPro NSC SFM.

5. When the neurospheres reach a diameter of 3.5 mm or larger, the rat fetal NSCs are ready to be passaged.
6. Transfer the neurosphere suspension into a sterile centrifuge tube and let the neurospheres settle by gravity or centrifuge at 200 x *g* for 2 minutes. Aspirate the supernatant carefully to leave the neurospheres in a minimal volume of medium.
7. Rinse the neurospheres once with DPBS without Ca²⁺ and Mg²⁺ and leave a minimal volume of DPBS.
8. Add 1 mL of pre-warmed StemPro Accutase reagent to the neurospheres and incubate for 10 minutes at room temperature.
9. After incubation, gently pipette the cells up and down to get a single-cell suspension and add 4 mL of complete StemPro NSC SFM to the tube.
10. Centrifuge at 300 x *g* for 4 minutes at room temperature, carefully aspirate the supernatant, resuspend in a minimal volume of pre-warmed complete StemPro NSC SFM, and remove a sample for counting on a hemocytometer or Countess II Automated Cell Counter.
11. Determine the total number of cells and percent viability.
12. Add enough complete StemPro NSC SFM to the tube for a final cell solution of 1 x 10⁷ viable cells/mL. Incubate at 37°C, 5% CO₂, and 90% humidity. Neurosphere suspension cultures should not be maintained for more than 3 passages.

Important: If you are re-feeding rat fetal NSCs in a growth medium other than complete StemPro NSC SFM, ensure that the medium is supplemented with 10 ng/mL bFGF to maintain the undifferentiated state of the rat fetal NSCs.

Differentiating glial precursor cells into astrocytes and oligodendrocytes

Summary

Glial precursor cells (GPCs), also known as glial restricted progenitors (GRP) or oligodendrocyte progenitor cells (OPCs), are cells that have the potential to differentiate into oligodendrocytes or astrocytes. The GPC population is derived from tissue or is generated from pluripotent cells by differentiation, which is induced by exogenously applied factors. Here we describe a culture system that can be adjusted to favor differentiation into either astrocytes or oligodendrocytes.

View this protocol online and order products at thermofisher.com/neuroprotocol/gpc

Required Materials

Cells

- Gibco™ Rat Glial Precursor Cells (Cat. No. N7746100)

Media and reagents

- Gibco™ Neurobasal™ Medium (Cat. No. 21103049)
- Gibco™ Astrocyte Medium (Cat. No. A1261301: This kit contains N-2 Supplement (100X) stored at -20°C ; Dulbecco's Modified Eagle Medium (DMEM) (1X) stored at 2°C to 8°C ; OneShot™ Fetal Bovine Serum, Certified stored at -20°C in the dark.)
- Gibco™ GlutaMAX™-I Supplement (Cat. No. 35050061)
- Gibco™ B-27™ Supplement (50X), serum free (Cat. No. 17504044)
- T3 (Liothyronine) (Sigma-Aldrich, Cat. No. T6397)
- Ascorbic Acid (Sigma-Aldrich, Cat. No. A8960)
- Gibco™ NT-3 Recombinant Human Protein (Cat. No. PHC7036)
- Gibco™ N-2 Supplement (100X) (Cat. No. 17502048)

- Gibco™ Fetal Bovine Serum (FBS), embryonic stem cell-qualified, US origin (Cat. No. 16141061)
- Gibco™ Geltrex™ LDEV-free Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413201)
- Gibco™ Laminin (Cat. No. 23017015)
- Poly-L-Ornithine Solution (Sigma-Aldrich, Cat. No. P4957)
- Gibco™ Distilled Water (Cat. No. 15230162)
- Gibco™ Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca^{2+} and Mg^{2+} (Cat. No. 14190144)
- Gibco™ Dulbecco's Phosphate-Buffered Saline (DPBS) with Ca^{2+} and Mg^{2+} (Cat. No. 14040133)
- Gibco™ StemPro™ NSC SFM (Cat. No. A1050901: This kit contains KnockOut™ DMEM/F-12 Basal Medium stored at 4°C , StemPro™ NSC SFM (Neural) Supplement stored at -20°C to -5°C in the dark, and bFGF Recombinant Human and EGF Recombinant Human stored at 4°C , desiccated.)

Preparing Media

T3 stock

1. To prepare 30 $\mu\text{g}/\text{mL}$ stock solution (1,000X), add 1 mL of 1 N NaOH to 1 mg of T3 powder then dilute further with 32.3 mL of DPBS with Ca^{2+} and Mg^{2+} and mix until dissolved.
2. After dissolving, filter through a 0.22 μm filter, aliquot 50–100 μL into sterile tubes, and store at -20°C .

Ascorbic acid stock

1. To prepare 100 mM ascorbic acid stock solution (500X), add 17.27 mL of distilled water to 500 mg of ascorbic acid powder and mix until dissolved.
2. After dissolving, filter through a 0.22 μm filter, aliquot 100–200 μL into sterile tubes, and store at -20°C .

NT-3 stock

1. To prepare 5 µg/mL NT-3 stock solution (1,000X), add 1 mL of DPBS with Ca²⁺ and Mg²⁺ supplemented with 0.1% BSA to 5 µg of NT-3 protein and mix until dissolved.
2. Aliquot 50–100 µL into sterile tube, and store at –20°C.

Oligodendrocyte differentiation medium

Oligodendrocyte differentiation medium uses Neurobasal Medium supplemented with B-27 Supplement (50X), serum free, GlutaMAX-I Supplement, T3, NT-3, and ascorbic acid. Complete medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

To prepare 100 mL of oligodendrocyte differentiation medium, mix the following components under aseptic conditions. For larger volumes, increase the component amounts proportionally.

Component	Final conc.	Amount
Neurobasal Medium	1X	97 mL
GlutaMAX-I Supplement	2 mM	1 mL
B-27 Supplement (50X), serum free	2%	2 mL
T3	30 ng/mL	0.1 mL
Ascorbic Acid	200 µM	0.2 mL
NT-3	5 ng/ml	0.1 mL

Astrocyte differentiation medium

Astrocyte differentiation medium uses DMEM supplemented with N-2 and FBS. Complete medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

Component	Final conc.	Amount
DMEM	1X	98 mL
N-2 Supplement	1%	1 mL
FBS	1%	1 mL

Complete StemPro NSC SFM

Complete StemPro NSC SFM consists of KnockOut DMEM/F-12 with StemPro Neural Supplement, EGF, bFGF, and GlutaMAX-I Supplement. Complete medium is stable for 4 weeks when stored in the dark at 2°C to 8°C.

To prepare 100 mL of complete medium:

1. Reconstitute bFGF and EGF with 0.1% BSA solution (in KnockOut DMEM/F-12) at a concentration of 100 µg/mL. You will need 20 µL of each per 100 mL of complete medium. Freeze unused portions in aliquots.
2. Mix the following components under aseptic conditions. For larger volumes, increase the component amounts proportionally.

Component	Final conc.	Amount
KnockOut DMEM/F-12	1X	97 mL
GlutaMAX-I Supplement	2 mM	1 mL
bFGF (prepared as 100 µg/mL stock)	20 ng/mL	20 µL
EGF (prepared as 100 µg/mL stock)	20 ng/mL	20 µL
StemPro Neural Supplement	2%	2 mL

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**Matrix for oligodendrocyte differentiation**

1. Add 2 mL of 100 µg/mL poly-L-ornithine solution to a 35 mm dish (0.5 mL for 4-well plate or slide, 0.25 mL for 8-well slide).
2. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO₂ for at least 1 hour.
3. Rinse the culture vessel 3 times with distilled water.

4. Prepare a 1:100 dilution of laminin in distilled water for a final concentration of 10 µg/mL.
5. Add 2 mL of 10 µg/mL laminin solution to a 35 mm dish (0.5 mL for 4-well plate or slide, 0.25 mL for 8-well slide).
6. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO₂ for at least 1 hour. Store it at 4°C until use.

Note: You may coat the plates in advance and store them at 2°C to 8°C, wrapped tightly with Parafilm™ wrapper, for up to 4 weeks.

Matrix for astrocyte differentiation

1. Thaw a bottle of Geltrex Basement Membrane Matrix at 2°C to 8°C.
2. Dilute Geltrex matrix 1:100 in DMEM and coat the bottom of each culture vessel by adding 2 mL of 10 µg/mL Geltrex matrix to a 35 mm dish (0.5 mL for 4-well plate or slide, 0.25 mL for 8-well slide).
3. Incubate the culture vessel at 36°C to 38°C for 1 hour.

Note: Dishes coated with Geltrex matrix can be used immediately or stored at 2°C to 8°C for up to a week, sealed with Parafilm laboratory film. Do not allow dishes to dry out. Warm stored Geltrex matrix plates to room temperature for 1 hour prior to use.

Differentiation to oligodendrocytes

1. Plate glial precursor cells on a poly-L-ornithine- and laminin-coated plate in complete StemPro NSC SFM at a density of 2.5×10^4 to 5×10^4 cells/cm².
2. On the next day, change the medium to oligodendrocyte differentiation medium.
3. Perform a half-medium change every 2–3 days thereafter. Differentiated oligodendrocytes are typically observed on day 3.

Differentiation to astrocytes

1. Plate glial precursor cells on Geltrex matrix-coated plate in complete StemPro NSC SFM at a density of 2.5×10^4 cells/cm².
2. On the next day, change the medium to astrocyte differentiation medium.
3. Change the medium every 3–4 days. Differentiated astrocytes are typically observed on days 5–7.

Xeno-free culture of neural stem cells

Summary

Neural stem cells (NSCs) derived from human embryonic stem cells (hESCs) help provide understanding for human neurogenesis and have potential for cell therapy applications related to Parkinson's disease, spinal cord injuries, and other neurological diseases. Because of this potential, standard methods of culturing NSCs raise concerns about pathogen cross-transfer from non-human sources or contamination with non-neural cells, limiting the efficiency and specificity of the differentiation protocols. These concerns have led to the development of xeno-free conditions for maintaining and expanding NSCs, which are described in this protocol. Gibco™ Cell Therapy Systems (CTS™) products help minimize the risk of contamination and variability and help meet your regulatory and quality requirements.

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Required Materials

Cells

- Neural stem cells

Media and reagents

- Gibco™ CELLstart™ CTS™ Substrate (Cat. No. A1014201)
- Gibco™ CTS™ Neurobasal™ Medium (Cat. No. A1371201)
- Gibco™ CTS™ B-27™ Supplement, XenoFree (Cat. No. A1486701)
- Gibco™ FGF-Basic Full Length CTS Recombinant Human Protein (Cat. No. CTP0261)
- Gibco™ EGF Recombinant Human Protein (Cat. No. PHG0311)
- Gibco™ CTS™ GlutaMAX™-I Supplement (Cat. No. A1286001)
- Gibco™ CTS™ TrypLE™ Select Enzyme (Cat. No. A1285901)
- Gibco™ CTS™ Dulbecco's Phosphate-Buffered Saline (DPBS) with Ca²⁺ and Mg²⁺ (Cat. No. A1285801)
- Gibco™ CTS™ Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca²⁺ or Mg²⁺ (Cat. No. A1285601)

Special tools

- 15 mL conical tube
- Tissue culture plates and flasks
- Microcentrifuge

Preparing Culture Vessels and Media

CELLstart CTS Substrate–coated vessels

Prepare culture dishes or flasks with CELLstart CTS Substrate as described below.

1. Dilute CELLstart CTS Substrate 1:100 in CTS DPBS with Ca^{2+} and Mg^{2+} (e.g., 50 μL of CELLstart CTS Substrate into 5 mL of CTS DPBS).

Note: CELLstart CTS Substrate should not be frozen, vortexed, or exposed to vigorous agitation due to potential gel formation.

2. Coat the surface of the culture vessel with the working solution of CELLstart CTS Substrate (2.5 mL for a T-25 flask or 60 mm dish, 1.5 mL for a 35 mm dish).
3. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO_2 for 1 hour.
4. Use the dish immediately after incubation. Aspirate the CELLstart CTS Substrate solution immediately before use.

Note: Prepare a freshly coated culture vessel each time before plating cells. There is no need to rinse the culture vessel before use.

Culture medium

Prepare 50 mL of culture medium as follows. The growth factors can be added immediately before use. After the culture medium has been supplemented with growth factors, aliquot the amount needed for the day and store the remaining medium at 4°C. Formulated culture medium is stable for 2 weeks if properly stored at 4°C.

Component	Amount
CTS Neurobasal Medium	49 mL
CTS GlutaMAX-I Supplement	2 mM
CTS B-27 Supplement, XenoFree	1 mL
CTS bFGF	20 ng/mL
EGF	20 ng/mL

Methods

Thawing and seeding NSCs

1. Remove a vial of cells from liquid nitrogen and quickly thaw the vial in a 37°C water bath, being careful not to immerse the vial above the level of the cap.
2. When just a small crystal of ice remains, sterilize the outside of the vial with 95% ethanol. Allow the ethanol to evaporate before opening the vial in a laminar flow hood.
3. Gently pipet the cell suspension up and down once, and place it into a 15 mL centrifuge tube.
4. Add 10 mL of warm culture medium to the tube dropwise to reduce osmotic shock.
5. Centrifuge the cell suspension at 200 x g for 5 minutes.
6. Remove the supernatant, resuspend the pellet in 5 mL of culture medium, and determine the total number of cells and percent viability.

- Seed the cells at a concentration of $>90,000$ cells/cm² onto a dish or flask that has been treated with CELLstart CTS solution. (Aspirate the CELLstart CTS solution immediately before using the dish or flask.)
- Incubate at 36°C to 38°C in a humidified atmosphere (90%) of 5% CO₂ in air.

Culture and propagation

- Twenty-four hours after seeding the cells, replace the culture medium.
- Replace the spent medium every other day with an equal volume of fresh culture medium.

Note: If the medium turns yellow, change the medium daily. Yellow medium will affect the NSC proliferation rate.

- After 3–4 days, the culture will become semi-confluent.
- To split the cell culture 1:2, aspirate the culture medium and wash the cells twice with 5 mL of CTS DPBS (without Ca²⁺ and Mg²⁺).
- Add 1 mL of CTS TrypLE Select Enzyme to dissociate the cells, and incubate for 2 minutes at 37°C.
- Add 4 mL of culture medium to neutralize the CTS TrypLE Select Enzyme activity and pipet up and down 2–3 times to get a uniform cell suspension. Check the cells under a microscope.
- Transfer the cell suspension to a 15 mL centrifuge tube.
- Centrifuge the cells at 200 x g for 5 minutes.
- Aspirate the supernatant and resuspend the cells in 10 mL of culture medium.

- Split the cell suspension into two fresh T-25 flasks that have been treated with CELLstart CTS solution. Seed each flask with 5 mL of cell suspension.
- Incubate the flasks at 37°C in a humidified atmosphere (90%) of 5% CO₂ in air.
- Grow the cells until semi-confluent, changing the culture medium once after 12 hours and every two days thereafter.
- Passage the cells when the culture reaches ~80% confluence.

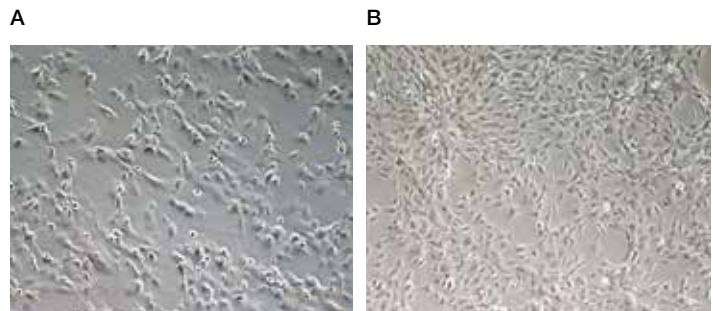


Figure 8-1. Phase-contrast microphotograph showing (A) NSCs cultured in xeno-free media 24 hours post-thaw and (B) semi-confluent NSCs cultured in xeno-free media for 3 days.

Creation of neural stem cells from human pluripotent stem cells

Summary

The derivation of neural stem cells (NSCs) is the first step in producing various neural cell types from human pluripotent stem cells (hPSCs). We have developed an efficient neural induction medium that can convert hPSCs into NSCs in one week without the need for the tedious and time-consuming process of embryoid body formation. Neural induction medium works with hPSCs cultured either in feeder-containing or feeder-free conditions.

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Required Materials

Media and reagents

- Gibco™ PSC Neural Induction Medium (Cat. No. A1647801): This kit contains 1X Neurobasal™ Medium stored at 4°C and 50X Neural Induction Supplement stored at –20°C to –5°C in the dark. After thawing of Neural Induction Supplement, aliquot 0.5 to 1 mL into sterile tubes and store at –20°C to –5°C in the dark. Avoid frequent freezing and thawing.
- Gibco™ Advanced DMEM/F12 (Cat. No. 12634010).
- Gibco™ Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413301): Thaw Geltrex matrix at 4°C overnight before using. The thawed Geltrex matrix can be kept at 2°C to 8°C for up to 2 weeks.
- Gibco™ Distilled Water (Cat. No. 15230-162)
- ROCK Inhibitor Y-27632 (Sigma-Aldrich, Cat. No. Y0503): Dissolve 1 mg of Y-27632 in 0.625 mL distilled water to make 5 mM stock solution. Sterilize the stock solution by filtering through a 0.22 µm filter. Aliquot 20–50 µL into sterile tubes and store at –20°C to –5°C in the dark for up to 1 year. The thawed Y-27632 solution can be kept at 2°C to 8°C for up to 2 weeks.

- Gibco™ Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca²⁺ and Mg²⁺ (Cat. No. 14190250)
- Gibco™ Essential 8™ Medium (Cat. No. A1517001)
- Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent (Cat. No. A1110501): Thawed Accutase reagent can be kept at 2°C to 8°C for up to 2 weeks.
- Dimethyl sulfoxide, Hybri-Max™ grade (DMSO) (Sigma-Aldrich, Cat. No. D2650)

Special tools

- Thermo Scientific™ Nunc™ Cell Scraper (Cat. No. 179693 or 179707)
- 100 µm strainer
- Thermo Scientific™ Nalgene™ General Long-Term Storage Cryogenic Tubes (Cat. No. 5000-0012 or 5000-1012)
- Thermo Scientific™ Mr. Frosty™ Freezing Container (Cat. No. 15-350-50)
- Microscope marker, such as Nikon™ microscopy object marker (Nikon™ Instruments Inc., Cat. No. MBW10020) with a Nikon microscopy C-OA 15 mm objective adapter (Nikon Instruments Inc., Cat. No. MXA20750).

Preparing Media

Neural induction medium

To prepare 100 mL of neural induction medium, mix the following components under aseptic conditions. For larger volumes, increase the component amounts proportionally.

Component	Final conc.	Amount
Neurobasal Medium	1X	98 mL
Neural Induction Supplement	1X	2 mL

The complete neural induction medium can be stored at 2°C to 8°C for up to 2 weeks. When feeding cells, warm up the appropriate amount of neural induction medium in a 37°C water bath for 5–10 minutes before feeding.

Neural expansion medium

To prepare 100 mL of neural expansion medium, mix the following components under aseptic conditions. For larger volumes, increase the component amounts proportionally.

Component	Final conc.	Amount
Neurobasal Medium	0.5X	49 mL
Advanced DMEM/F12	0.5X	49 mL
Neural Induction Supplement	1X	2 mL

The complete neural expansion medium can be stored at 2°C to 8°C for up to 2 weeks. When feeding cells, warm up the appropriate amount of neural induction medium in a 37°C water bath for 5–10 minutes before feeding.

Neural preservation medium

To prepare 10 mL of neural preservation medium, mix the following components under aseptic conditions. For larger volumes, increase the component amounts proportionally.

Component	Final conc.	Amount
Neural expansion medium	80%	8 mL
DMSO	20%	2 mL

Neural Induction

- Maintain the high quality of hPSCs including hESCs and hiPSCs by culturing in feeder-free media such as in Essential 8 Medium or in complete StemPro hESC SFM, or on mouse embryonic fibroblasts (MEFs).
- When hPSCs reach ~70–80% confluence, split hPSCs into 6-well culture plates to reach 15–25% confluence at day 1 of hPSC splitting.
- At day 1 of hPSC splitting, aspirate spent medium to remove non-attached cells and add 2.5 mL pre-warmed neural induction medium into each well of 6-well plate. Return plates to an incubator.
- The morphology of cell colonies should be uniform with a smooth edge after two days of culture in neural induction medium (Figure 9-1, panel A). Due to differentiated or partially differentiated hPSCs before neural induction, colonies of non-neural differentiation can be observed (Figure 9-1, panels B–F). Mark all colonies showing the morphology of non-neural differentiation on the bottom of the plate by using a microscope marker.

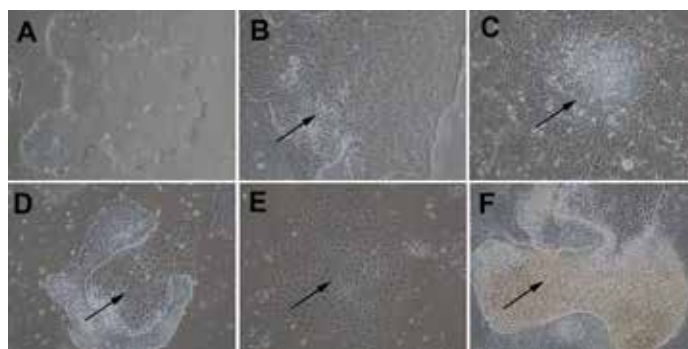


Figure 9-1. Morphology of neural and non-neural differentiated cells at day 2 of neural induction. (A) Normal morphology of cells. **(B–F)** Representative images of non-neural differentiated cells (indicated by arrows) due to the use of poor-quality starter hPSCs.

- Aspirate spent medium from each well. Remove all colonies of non-neural differentiation by pointing a Pasteur glass pipette to marked colonies to aspirate cells off. Add 2.5 mL pre-warmed neural induction medium per well and return plates to an incubator.
- At day 4 of neural induction, cells should reach confluence. Mark all colonies of non-neural differentiation by using a microscope marker.
- Aspirate spent medium from each well. Remove all colonies of non-neural differentiation by pointing a Pasteur glass pipette to marked colonies to aspirate cells off. Add 5 mL pre-warmed neural induction medium per well and return plates into an incubator.
- Feed cells every day with 5 mL neural induction medium per well until day 7 of neural induction.

NSC Expansion

1. At day 7 of neural induction, NSCs (P0) are ready to be harvested.
2. Dilute pre-thawed Geltrex matrix in Neurobasal Medium (1:100). Add appropriate amount of Geltrex solution into each culture vessel to cover the surface (e.g., 0.5–1 mL for each well of a 6-well culture plate) and incubate at least 1 hour at 37°C.
3. Aspirate the spent medium with a Pasteur glass pipette and rinse cells with DPBS once.
4. Gently add 1 mL pre-warmed StemPro Accutase Cell Dissociation Reagent to each well of 6-well plate.
5. Incubate for 5–8 minutes at 37°C until most of cells are detached from the surface of culture vessels.
6. Use a cell scraper to detach the cells off the surface of the plates.
7. Transfer cell clumps using a pipette and place cells into a 15 or 50 mL conical tube.
8. Add 1 mL DPBS to each well of 6-well plate to collect residual cells and transfer cell suspension to the conical tube.
9. Triturate cell suspension 3 times with a 5 or 10 mL pipette to break cell clumps.
10. Pass cell suspension through a 100 μm strainer and centrifuge cells at 300 x *g* for 4 minutes.
11. Aspirate supernatant, resuspend cells with appropriate amount of pre-warmed neural expansion medium (e.g., 1 mL for all cells from 1 well of a 6-well plate).
12. Determine cell concentration using preferred method.
13. Dilute cell suspension to 4×10^5 cells/mL with pre-warmed neural expansion medium.
14. Add ROCK inhibitor Y-27632 solution into cell suspension to reach final concentration of 5 μM .
15. Aspirate Geltrex solution from coated vessels and add appropriate amount of diluted cell suspension into each culture vessel to plate cells at the density of 1×10^5 cells/cm².
16. Place vessels gently in an incubator and move culture vessels in several quick back-and-forth and side-to-side motions to disperse cells across the surface of vessels.
17. Change neural expansion medium at day 1 of cell plating to eliminate Y-27632. Change neural expansion medium every other day thereafter.
18. Usually, NSCs reach confluence at day 4–5 after plating. When NSCs reach confluence, NSCs can be further expanded in neural expansion medium. For the first 3 to 4 passages, overnight treatment with 5 μM Y-27632 at the day of NSC plating is necessary to prevent cell death.

Cryopreservation of NSCs

1. After determining cell concentration at step 12 in the NSC expansion section above, dilute cell suspension to 2×10^6 to 4×10^6 cells/mL with neural expansion medium.
2. Add an equivalent volume of neural preservation medium dropwise.
3. Allocate 1 mL of cell suspension into each cryotube and freeze cells at -80°C overnight in Mr. Frosty Freezing Containers.
4. Transfer cells into liquid nitrogen tank on the next day for long-term storage.
6. Quickly remove the sticker or copy the information written on the vial in your notebook. The writing may come off the vial after spraying outside of the vial with 70% ethanol.
7. Spray outside of the vial with 70% ethanol and place in a laminar flow hood. Pipette cells gently into a sterile 15 mL conical tube using a 1 mL pipette.
8. Add 1 mL DPBS into the vial to collect residual cells.
9. Use a pipette to remove DPBS cell mixture from the vial and add it to the 15 mL conical tube dropwise. While adding, gently move the tube back and forth to mix NSCs. This reduces osmotic shock to cells.

Recovery of Cryopreserved NSCs

1. Coat culture vessels with Geltrex solution for at least 1 hour before thawing NSCs by following the procedures of step 2 in the NSC expansion section above.
2. Wear eye protection as cryotubes stored in the liquid phase of liquid nitrogen may accidentally explode when warmed.
3. Wear ultra-low temperature cryogloves. Remove cryotubes of NSCs from the liquid nitrogen storage tank using metal forceps.
4. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.
5. When only an ice crystal remains, remove the vial from the water bath.
10. Centrifuge at $300 \times g$ for 5 minutes and aspirate the supernatant.
11. Resuspend the cell pellet in DPBS, centrifuge at $300 \times g$ for 5 minutes, and aspirate the supernatant.
12. Resuspend cell pellet in appropriate amount of pre-warmed neural expansion medium (e.g., 1 mL for all NSCs from 1 vial) and determine cell concentration using preferred method.
13. Dilute cell suspension to 4×10^5 cells/mL with pre-warmed neural expansion medium. If the passage number of the NSCs is less than 4, add ROCK inhibitor Y-27632 solution into the cell suspension to reach final concentration of $5 \mu\text{M}$.
14. Aspirate Geltrex solution from coated vessels and add an appropriate amount of diluted cell suspension into each culture vessel to plate cells at the density of 1×10^5 cells/cm².

15. Place vessels gently in an incubator and move culture vessels in several quick back-and-forth and side-to-side motions to disperse cells across the surface of vessels.
16. Change neural expansion medium every other day thereafter until NSCs reach confluence for splitting.

Differentiating neural stem cells into neurons and glial cells

Summary

The protocols in this chapter describe the steps involved in differentiating neural stem cells (NSC) to neurons, astrocytes, and oligodendrocyte lineages *in vitro*. NSCs are self-renewing multipotent stem cells that can be proliferated *in vitro* in supportive culture systems such as StemPro™ NSC SFM and can further be differentiated into downstream lineages. The protocols described herein are primarily optimized with NSCs derived from human embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC). Some optimization in terms of reagent concentration and duration of *in vitro* differentiation is expected for NSCs from other species such as rat or mouse, as well as with NSCs derived from human-specific iPSCs.

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Required Materials

Cells

- Gibco™ Human Neural Stem Cells (H9-Derived) Kit (Cat. No. N7800200: Contains hNSC (H9-Derived) stored in liquid nitrogen, KnockOut™ DMEM/F-12 Basal Medium stored at 4°C in the dark, StemPro™ Neural Supplement stored at –20°C to –5°C, and bFGF and EGF Recombinant Human proteins stored at 4°C, dessicated.)

Media and reagents

- Gibco™ Dulbecco's Modified Eagle Medium (DMEM) high glucose, pyruvate (Cat. No. 11995065)
- Gibco™ Dulbecco's Phosphate-Buffered Saline (DPBS) with Ca²⁺ and Mg²⁺ (Cat. No. 14040133)
- Gibco™ Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca²⁺ and Mg²⁺ (Cat. No. 14190144)

- Gibco™ StemPro™ NSC SFM (Cat. No. A1050901: This kit contains KnockOut™ DMEM/F-12 Basal Medium stored at 4°C, StemPro™ NSC SFM (Neural) Supplement stored at –20°C to –5°C in the dark, and bFGF Recombinant Human and EGF Recombinant Human stored at 4°C, desiccated.)
- Gibco™ N-2 Supplement (100X) (Cat. No. 17502048)
- Gibco™ B-27™ Supplement (50X), serum free (Cat. No. 17504044)
- Gibco™ Neurobasal™ Medium (Cat. No. 21103049)
- Gibco™ Antibiotic-Antimycotic (100X) solution (Cat. No. 15240096)
- Gibco™ Fetal Bovine Serum (FBS), embryonic stem cell-qualified, US origin (Cat. No. 16141061)
- Gibco™ GlutaMAX™-I Supplement (Cat. No. 35050061)
- Gibco™ CELLstart™ CTS™ Substrate (Cat. No. A1014201)
- Gibco™ Geltrex™ LDEV-Free, hESC Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413301)
- Poly-L-Ornithine (Sigma-Aldrich, Cat. No. P3655)
- Gibco™ Laminin (Cat. No. 23017015)
- Dibutyl cyclic-AMP (dBcAMP) (Sigma-Aldrich, Cat. No. D0627)
- T3 (Liothyronine) (Sigma-Aldrich, Cat. No. T6397)
- EM-grade paraformaldehyde (Electron Microscopy Services, Cat. No. 19208)
- Invitrogen™ 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Cat. No. D1306)
- Invitrogen™ ProLong™ Gold Antifade Mountant (Cat. No. P36930)
- Triton™ X-100 surfactant

Primary antibodies

Lineage marker	Antigen	Subtypes	Reactivity*	Cat. No.	Concentration
NSC	Nestin	Rabbit	Hu, Rt, Ms	MA1-110	1:100
	Sox2	Mouse IgG2a	Hu	MA1-014	1:200
Neuron	Dcx	Rabbit	Hu, Rt, Ms	48-1200	1:400
	MAP2	Mouse IgG1	Hu, Rt, Ms	13-1500	1:200
Glial	A2B5	Mouse IgM	Hu, Rt, Ms	433110	1:100
	CD44	Mouse IgG	Hu	A25528	1:50
Oligodendrocyte	GalC	Mouse IgG	Hu, Rt, Ms	Millipore MAB345	1:200
Astrocyte	GFAP	Rabbit	Hu, Rt, Ms	18-0063	1:200

* Hu = human, Rt = rat, Ms = mouse.

Secondary antibodies

Ex/Em* (color)	Alexa Fluor™ dye no.	Host	Reactivity	Cat. No.	Concentration
346/442 (blue)	350	Goat	Mouse IgM	A31552	1:1,000
		Goat	Mouse IgG	A21049	1:1,000
		Goat	Rat IgG	A21093	1:1,000
		Goat	Rabbit IgG	A21068	1:1,000
		Donkey	Goat IgG	A21081	1:1,000
495/519 (green)	488	Goat	Mouse IgM	A21042	1:1,000
		Goat	Mouse IgG	A11029	1:1,000
		Goat	Rat IgM	A21212	1:1,000
		Goat	Rat IgG	A11006	1:1,000
		Goat	Goat IgG	A11034	1:1,000
		Donkey	Mouse IgM	A11055	1:1,000
590/617 (red)	594	Goat	Mouse IgM	A21044	1:1,000
		Goat	Mouse IgG	A11029	1:1,000
		Goat	Rat IgM	A21213	1:1,000
		Goat	Rat IgG	A11007	1:1,000
		Goat	Rabbit IgG	A11037	1:1,000
		Donkey	Goat IgG	A11058	1:1,000
496, 536, 565/576 (red)	NA	Goat	Mouse IgM	M31504	1:500
		Goat	Mouse IgG	P852	1:1,000
		Goat	Rabbit IgG	P2771MP	1:1,000

* Approximate excitation and emission maxima, in nm; NA = not applicable.

Preparing Media

Complete StemPro NSC SFM

Complete StemPro NSC SFM consists of KnockOut DMEM/F-12 with StemPro Neural Supplement, EGF, bFGF, and GlutaMAX-I Supplement. Complete medium is stable for 4 weeks when stored in the dark at 2°C to 8°C.

To prepare 100 mL of complete medium:

1. Reconstitute bFGF and EGF with 0.1% BSA solution (in KnockOut DMEM/F-12) at a concentration of 100 µg/mL. You will need 20 µL of each per 100 mL of complete medium. Freeze unused portions in aliquots.
2. Mix the following components under aseptic conditions. For larger volumes, increase the component amounts proportionally. If desired, add 1 mL of Antibiotic-Antimycotic solution per 100 mL of complete medium.

Component	Final conc.	Amount
KnockOut DMEM/F-12	1X	97 mL
GlutaMAX-I Supplement	2 mM	1 mL
bFGF (prepared as 100 µg/mL stock)	20 ng/mL	20 µL
EGF (prepared as 100 µg/mL stock)	20 ng/mL	20 µL
StemPro Neural Supplement	2%	2 mL

You may observe a white precipitate when thawing StemPro Neural Supplement; this precipitate will disappear when the supplement is completely thawed or dissolved.

Neural differentiation medium

Neural differentiation medium requires supplementation of Neurobasal Medium with B-27 Supplement (50X), serum free and GlutaMAX-I Supplement. Neural differentiation medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

To prepare 100 mL of neural differentiation medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally. If desired, add 1 mL of Antibiotic-Antimycotic solution per 100 mL of medium.

Component	Final conc.	Amount
Neurobasal Medium	1X	97 mL
B-27 Supplement (50X), serum free	2%	2 mL
GlutaMAX-I Supplement	2 mM	1 mL

If faster differentiation is desired, add dibutyryl cAMP to a final concentration of 0.5 mM at day 7 for a duration of 3 days, as indicated in the differentiation protocols.

Astrocyte differentiation medium

Astrocyte differentiation medium requires supplementation of DMEM with N-2 Supplement, GlutaMAX-I Supplement, and FBS. Astrocyte differentiation medium is stable for 4 weeks when stored in the dark at 2°C to 8°C.

To prepare 100 mL of astrocyte differentiation medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally. If desired, add 1 mL of Antibiotic-Antimycotic solution per 100 mL of medium.

Component	Final conc.	Amount
DMEM	1X	97 mL
N-2 Supplement	1%	1 mL
GlutaMAX-I Supplement	2 mM	1 mL
FBS	1%	1 mL

Oligodendrocyte differentiation medium

Oligodendrocyte differentiation medium requires supplementation of Neurobasal Medium with B-27 Supplement (50X), serum free, GlutaMAX-I Supplement, and T3. Oligodendrocyte differentiation medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

To prepare 100 mL of oligodendrocyte differentiation medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally. If desired, add 1 mL of Antibiotic-Antimycotic solution per 100 mL of medium.

Component	Final conc.	Amount
Neurobasal Medium	1X	97 mL
B-27 Supplement (50X), serum free	2%	2 mL
GlutaMAX-I Supplement	2 mM	1 mL
T3*	30 ng/mL	0.1 mL

* You can prepare a 30 µg/mL T3 stock solution (1,000X) in distilled water. Filter sterilize the T3 stock solution.

Preparing Matrix

Coating culture vessels with CELLstart CTS Substrate

1. Dilute CELLstart CTS Substrate 1:100 in DPBS with Ca²⁺ and Mg²⁺ (i.e., 50 µL of CELLstart CTS Substrate into 5 mL of DPBS).
2. Coat the surface of the culture vessel with the working solution of CELLstart CTS Substrate (14 mL for T-75 flask, 7 mL for T-25 flask, 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% CO₂ in air for 1 hour.

4. Remove the vessel from the incubator and store it until use. Immediately before use, remove all CELLstart CTS Substrate 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™ wrapper, for up to 2 weeks. Do not remove CELLstart CTS Substrate solution until just prior to use. Make sure the plates do not dry out.

Coating culture vessels with Geltrex matrix

1. Thaw the Geltrex matrix bottle at 4°C overnight to prevent polymerization. The next day, dilute the Geltrex matrix 1:2 with DMEM/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 (prechilled on ice), and store the tubes at -20°C.
2. Thaw 1 tube of Geltrex matrix (0.5 mL, aliquoted as above) slowly at 4°C, and add 49.5 mL of cold DMEM/F-12 (1:100 dilution). Mix gently.
3. Cover the whole surface of each culture plate with the Geltrex matrix solution (1.5 mL for a 35 mm dish, 3 mL for 60 mm dish, 5 mL for a T-25 culture flask).
4. Seal each dish with Parafilm™ wrapper to prevent drying, and incubate 1 hour at room temperature in a laminar flow hood.
5. Immediately before use, remove all Geltrex matrix solution, wash once with DPBS with Ca²⁺ and Mg²⁺, and replace with pre-warmed complete medium.

Note: You may store the Geltrex matrix-treated dish at 4°C, wrapped tightly with Parafilm wrapper, for up to 1 month. Do not remove the Geltrex matrix solution until just prior to use.

Coating culture vessels with poly-L-ornithine and laminin

1. Dissolve poly-L-ornithine 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°C to 8°C and prepare 10 $\mu\text{g}/\text{mL}$ 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 $\mu\text{g}/\text{mL}$ 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 T-75 flask, 7 mL for T-25 flask, 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 T-75 flask, 7 mL for T-25 flask, 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 DPBS without Ca^{2+} and Mg^{2+} , and store the vessel covered with DPBS until use. Immediately before use, remove all DPBS 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 wrapper, for up to 1 week. Do not remove DPBS until just prior to use. Make sure the plates do not dry out.

Differentiating Neural Stem Cells

Neural stem cells (NSCs) will proliferate as progenitors a few times even after the complete growth medium is replaced with the appropriate differentiation medium. If the cells reach 90% confluency, it might be necessary to split the cells at a 1:2 ratio. However, do not split the cells once they reach day 9–10 of differentiation when they can get damaged during the passaging process.

Differentiation into neurons

1. Plate neural stem cells on a poly-L-ornithine- and laminin-coated culture dish in complete StemPro NSC SFM at 2.5×10^4 to 5×10^4 cells/ cm^2 .
2. After 2 days, change the medium to neural differentiation medium. Change the spent medium every 3–4 days.
3. If expedited differentiation is desired, add 0.5 mM of dibutyryl cAMP to the differentiation medium daily starting at day 7 of differentiation for 3 days.

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

Differentiation into astrocytes

1. Plate the NSCs on a Geltrex matrix–coated culture dish in complete StemPro NSC SFM at 2.5×10^4 cells/ cm^2 .
2. After 2 days, change medium to astrocyte differentiation medium. Change the spent medium every 3–4 days.

Differentiation into oligodendrocytes

1. Plate the NSCs on a poly-L-ornithine and laminin-coated culture dish in complete StemPro NSC SFM at 2.5×10^4 to 5×10^4 cells/cm².
2. After 2 days, change the medium to oligodendrocyte differentiation medium. Change the spent medium every 3–4 days.

Characterizing NSCs and Differentiated Lineages by Immunocytochemistry

Preparing paraformaldehyde fixing solution

20% paraformaldehyde (PFA) stock solution

1. Add PBS to 20 g of EM-grade paraformaldehyde, 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 µm 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.

4% PFA for fixing

1. Add 8 mL of 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, cool the tubes on ice.

Fixing cells

1. Remove culture medium and gently rinse the cells once with DPBS with Ca²⁺ and Mg²⁺, without dislodging the cells.
2. Fix the cells with 4% fresh PFA fixing solution at room temperature for 15 minutes.
3. Rinse 3 times with DPBS containing Ca²⁺ and Mg²⁺.
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 DPBS with Ca²⁺ and Mg²⁺ 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-100 surfactant in DPBS with Ca²⁺ and Mg²⁺).
- Note:** If you are using a surface antigen such as GalC, omit Triton X-100 surfactant 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 3 times for 5 minutes with DPBS containing Ca²⁺ and Mg²⁺ (if using a slide, use a staining dish with a magnetic stirrer).
 4. Incubate the cells with fluorophore-labeled secondary antibody (5% serum in DPBS with Ca²⁺ and Mg²⁺) in the dark at 37°C for 30–45 minutes.

5. Wash the cells 3 times with DPBS containing Ca^{2+} and Mg^{2+} , and in the last wash, counterstain the cells with DAPI solution (3 ng/mL) for 5–10 minutes, and rinse with DPBS.
6. If desired, mount using 3 drops of ProLong Gold Antifade Mountant per slide and seal with the cover slip. You may store the slides in the dark at 4°C.

Expected Results

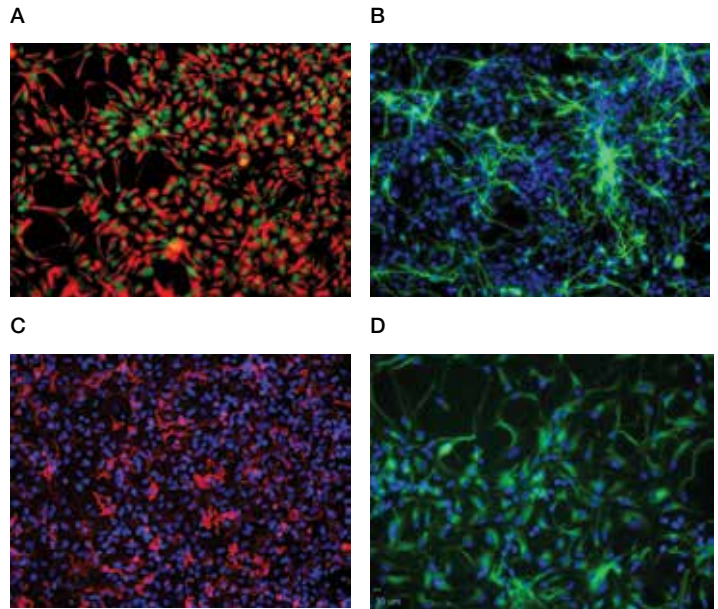


Figure 10-1. Fluorescence images (20x) of Gibco hNSCs that have been cultured in complete StemPro NSC SFM for three passages, and then allowed to differentiate into neurons, oligodendrocytes, or astrocytes. Upon directed differentiation, cells start to lose the undifferentiated NSC marker, nestin, but stain positive for the differentiated cell-type markers Dcx, GalC, and GFAP. **(A)** Cells were stained for the undifferentiated NSC markers nestin (red) and SOX2 (green) prior to directed differentiation. Cells were then differentiated into neurons and glial cells, and respectively stained **(B)** for the neuronal marker Dcx (green), **(C)** for the oligodendrocyte marker GalC (red), or **(D)** for the astrocyte marker, GFAP (green). Differentiation to neurons **(B)** and to oligodendrocytes **(C)** was observed at day 7, and to astrocytes **(D)** at day 21. The nuclei were counterstained with DAPI (blue) in panels **B–D**.

Troubleshooting

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

Possible cause	Solution
Culture medium contains bFGF	Remove bFGF from culture medium
Cell density too high and endogenous bFGF is preventing differentiation	Reduce cell density
Concentration of GlutaMAX-I Supplement is incorrect	Use GlutaMAX-I Supplement at a final concentration of 2 mM
Cells have been passaged too many times	Obtain new Gibco human neural stem cells

References

Denham M. and Dottori M (2009) Signals involved in neural differentiation of human embryonic stem cells. *Neurosignal* 17:234–241.

Elkabetz Y and Studer L (2008) Human ESC-derived neural rosettes and neural stem cell progression. *Cold Spring Harb Symp Quant Biol* 73:377–387.

Trujillo CA, Schwindt TT, Martins AH, Alves JM, Mello LE, and Ulrich H (2009) Novel perspectives of neural stem cell differentiation: From neurotransmitters to therapeutics. *Cytometry A* 75:38–53.

Differentiating PSCs to midbrain dopaminergic neurons

Summary

Midbrain dopaminergic (mDA) neurons derived from human pluripotent stem cells (hPSCs) provide an excellent alternative to primary human neurons for disease modeling of Parkinson's disease and drug screening. During brain development, mDA neurons are derived from distinct populations of cells termed midbrain floor plate (mFP) cells. In this protocol, we describe how to (1) specify hPSC to mFP cells, (2) expand and cryopreserve specified cells, and (3) revive and mature cells to mDA neurons.

View this protocol online and order products at

thermofisher.com/dopadiff

Required Materials

Cells

- Gibco™ Human Episomal iPSC Line (Cat. No. A18945) or equivalent PSC cultured in Gibco™ Essential 8™ or Essential 8™ Flex Medium (see Cat. No. information below)

Media and reagents

- Gibco PSC Dopaminergic Neuron Differentiation Kit (Cat. No. A3147701: Contains Floor Plate Specification Supplement (20X) stored at -20°C to -5°C in the dark; Floor Plate Cell Expansion Kit consisting of Floor Plate Cell Expansion Base stored at 4°C and Floor Plate Cell Expansion Supplement (50X) stored at -20°C to -5°C in the dark; and Dopaminergic Neuron Maturation Supplement (50X) stored at -20°C to -5°C in the dark.)
- Gibco Essential 8 Medium (Cat. No. A1517001), contains Basal Medium and Supplement; or Essential 8 Flex Medium Kit (Cat. No. A2858501), contains Flex Basal Medium and Flex Supplement
- Gibco™ Vitronectin (VTN-N), Recombinant Human Protein, Truncated (Cat. No. A14700)
- Gibco™ Neurobasal™ Medium (Cat. No. 21103049) (base medium for specification)

- Gibco™ DMEM/F-12, GlutaMAX™ Supplement (Cat. No. 10565018) (base medium for maturation)
- Gibco™ Laminin (Cat. No. 23017015)
- Gibco™ DPBS, without Ca^{2+} and Mg^{2+} (Cat. No. 14190144)
- Gibco™ Distilled Water (Cat. No. 15230162)
- Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent (Cat. No. A1110501)
- ROCK inhibitor Y-27632 (Sigma-Aldrich, Cat. No. Y0503)
- Dimethyl sulfoxide, Hybri-Max™ grade (DMSO) (Sigma-Aldrich, Cat. No. D2650)
- Gibco™ Human Dopaminergic Neuron Immunocytochemistry Kit (Cat. No. A29515) (for image-based analysis of intermediate floor plate progenitors and mature dopaminergic neurons)

Equipment and plasticware

- Thermo Scientific™ Nunclon™ Sphera™ Flasks (Cat. No. 174951 or 174952) (for suspension culture)
- Thermo Scientific™ Nunclon™ Sphera™ Dishes (Cat. No. 174930, 174931, or 174932) (for suspension culture)
- BioCoat™ Poly-D-Lysine-coated plates (Corning, Cat. No. 354413, 354414, or 354640) or Poly-D-Lysine (Sigma-Aldrich, Cat. No. p7280 or p9155) (to prepare poly-D-lysine and laminin double-coated culture plates)
- Thermo Scientific™ Mr. Frosty™ Freezing Container (Cat. No. 5100-0001)
- Thermo Scientific™ Nalgene™ General Long-Term Storage Cryogenic Tubes (Cat. No. 5000-0012 or 5000-1012) (for banking floor plate progenitor cells)
- 37°C humidified cell culture incubator with 5% CO_2
- Liquid nitrogen storage
- Centrifuge
- 37°C water bath
- 15 and 50 mL sterile polypropylene conical tubes
- 5, 10, 25, and 50 mL sterile pipettes

Preparing Media

Preparing specification medium

Floor Plate (FP) Specification Supplement (20X) can be thawed at 4°C or room temperature and dispensed into aliquots if desired. Complete medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

To prepare 100 mL of FP specification medium, mix the following components under sterile conditions. For larger volumes, increase the component amounts proportionally.

Component	Final conc.	Amount
Neurobasal Medium	1X	95 mL
FP Specification Supplement (20X)	1X	5 mL

Preparing expansion medium

Floor Plate (FP) Cell Expansion Supplement (20X) can be thawed at 4°C or room temperature and dispensed into aliquots if desired. Complete medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

To prepare 100 mL of FP expansion medium, mix the following components under sterile conditions. For larger volumes, increase the component amounts proportionally.

Component	Final conc.	Amount
FP Cell Expansion Base	1X	98 mL
FP Cell Expansion Supplement (50X)	1X	2 mL

Preparing maturation medium

Dopaminergic (DA) Neuron Maturation Supplement (20X) can be thawed at 4°C or room temperature and dispensed into aliquots if desired. Complete medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

To prepare 100 mL of DA neuron maturation medium, mix the following components under sterile conditions. For larger volumes, increase the component amounts proportionally.

Component	Final conc.	Amount
DMEM/F-12 medium	1X	98 mL
DA Neuron Maturation Supplement (50X)	1X	2 mL

Preparing ROCK inhibitor stock solution

To prepare 10 mM ROCK inhibitor Y-27632 solution, add 10 mg of Y-27632 to 3.125 mL of distilled water. Mix well until dissolved.

After dissolving, filter through a 0.22 µm filter, aliquot 20–50 µL into sterile tubes, and store at –20°C in the dark for up to 1 year. Thawed Y-27632 solution can be kept at 4°C for up to 4 weeks.

Preparing Matrix

Vitronectin-coated plates

1. Prepare a 1:50 dilution of vitronectin solution in DPBS without Ca^{2+} and Mg^{2+} for a final concentration of 10 $\mu\text{g}/\text{mL}$.
2. Add 1 mL of the diluted vitronectin solution to each well of a 6-well plate.
3. Incubate the coated plates at room temperature for 1 hour. The culture vessel can now be used or stored at 4°C wrapped in laboratory film for up to one week. Do not allow the vessel to dry.
4. Before use, pre-warm the culture vessel to room temperature for at least 1 hour before aspirating and discarding the vitronectin solution.

Note: It is not necessary to rinse off the culture plate after the removal of the vitronectin solution.

Laminin-coated plates

1. Thaw the required volume of 1.0 mg/mL laminin stock solution (stored at -80°C) slowly at 4°C.
2. Prepare a 1:100 dilution of laminin solution in water for a final concentration of 10 $\mu\text{g}/\text{mL}$.
3. Add 1 mL of the diluted laminin solution to each well of a 6-well plate.
4. Incubate the coated plates at 4°C overnight or at 37°C for 2 hours. The culture vessel can now be used or stored at 4°C wrapped in Parafilm™ laboratory film for up to one week. Do not allow the vessel to dry.
5. Before use, pre-warm the culture vessel to room temperature for at least 1 hour before aspirating and discarding the laminin solution.

Note: Laminin may form a gel when thawed too rapidly. Thawing the stock solution in the cold (4°C) prevents this. Thawed stock solution can be stored at 4°C for up to 1 month.

Poly-D-lysine and laminin double-coated plates

1. Prepare a 100 $\mu\text{g}/\text{mL}$ poly-D-lysine working solution in sterile, distilled water.
2. Add 1 mL of the poly-D-lysine solution to each well of a 6-well plate.
3. Incubate the coated plates at room temperature for 1–2 hours.
4. Remove the poly-D-lysine solution and rinse 3 times with distilled water.
5. Prepare a 15 $\mu\text{g}/\text{mL}$ working solution of laminin in sterile, distilled water.
6. Add 1 mL of the 15 $\mu\text{g}/\text{mL}$ laminin working solution to each well of a 6-well plate.
7. Incubate the coated plates overnight at 4°C or at 37°C for 2 hours.
8. Before use, pre-warm the culture vessel to room temperature for at least 1 hour before aspirating and discarding the laminin solution.

Note: You can use the coated culture plate immediately or store it at 4°C wrapped in laboratory film for up to one week. Do not allow the plate to dry.

Specification (PSC to Midbrain Floor Plate)

Set up hPSC culture (Day -1)

1. Prepare vitronectin-coated plate and complete Essential 8 Medium.
2. Plate a high-quality PSC culture from frozen vial or ongoing culture on vitronectin plate in Essential 8 Medium to target 20–40% confluency on the next day.
3. If plated as single cells, supplement medium with 10 μM ROCK inhibitor Y-27632 to inhibit cell death.

Note: Depending on cell line used, culture kinetics are different. Optimization is needed to find the right seeding density to get 100% confluency after 6–7 days specification. With H9 ESC lines, 30,000 cells/cm² seeding density resulted in 20–40% confluency on the next day.

Specification (Day 0–Day 10)

1. Start specification by changing medium with FP specification medium (Day 0). Aspirate the spent Essential 8 Medium containing the ROCK inhibitor and replace it with pre-warmed FP specification medium.
2. Incubate at 37°C in a humidified atmosphere of 5% CO₂ in air.
3. Replenish culture with fresh medium at day 3, 5, 7, and 9. Medium consumption will be increased over time, so use 2X volume for later points (Day 7 and 9) to compensate.

Expansion

Harvest FP progenitor (Day 10, 12, and 16)

1. Prepare laminin-coated plate and FP cell expansion medium.
2. Aspirate the spent medium from the specification culture plate and rinse the wells with DPBS with Ca²⁺ and Mg²⁺ to remove any remaining medium.
3. Aspirate the DPBS and add an appropriate volume of StemPro Accutase Cell Dissociation Reagent to fully cover the surface (1 mL per well of a 6-well plate, or 1 mL per 10 cm² of surface area).
4. Incubate the vessel at 37°C, 5% CO₂ for ~5–15 minutes, continually observing the wells for cell detachment.
5. After several minutes or when some colonies start detaching (whichever happens first), gently tap the bottom of the vessel several times. Most colonies should freely come into suspension. If all colonies do not detach, wait 1–2 minutes, and then tap the vessel again to detach the remaining colonies.
6. Transfer the cell clumps to a sterile 50 mL culture tube.
7. Rinse the wells of the specification culture plate twice with DPBS, using 4x the volume of StemPro Accutase reagent used in each well (4 mL per well of a 6-well plate). After each rinse, collect the cell clumps in the same 50 mL culture tube to ensure the recovery of all colonies.
8. Centrifuge the cell suspension at 300 x g for 3 minutes at 4°C to pellet the cells. Carefully aspirate the supernatant, leaving the cell pellet in the culture tube.

Replate FP cells (Day 10 and 12)

1. Gently flick the bottom of the tube to dislodge the cell pellet and resuspend the cells in a sufficient volume of complete expansion medium plus 5 μ M ROCK inhibitor Y-27632.
2. Use 1:2 split ratio (i.e., one plate to two plates) for FP passage 0 (FPp0) (Day 10) and use 1:4 split ratio for FP passage 1 (FPp1) (Day 16).

Note: Overnight treatment with the ROCK inhibitor is required upon passaging. The ROCK inhibitor is removed from the culture the following day when the spent medium is replaced with FP specification medium.

3. Incubate the cells overnight at 37°C in a humidified atmosphere of 5% CO₂.
4. The next day, replenish culture with fresh medium and every other day thereafter.

Cryopreserve FP passage 2 (FPp2) cells (Day 16)

1. Prepare freezing medium at 2X concentration (80% FP expansion medium + 20% DMSO) and chill at 4°C before use.
2. Calculate the volume of cells in the FPp2 cell suspension that corresponds to the number of cells you want to cryopreserve, and transfer to a sterile tube.
3. Dilute the cells to 2X the intended final frozen concentration using FP expansion medium at 4°C.
4. In a dropwise manner, add the same volume of 2X freezing medium (chilled to 4°C) as the cell suspension while gently rocking the tube back and forth.
5. Aliquot 1 mL of the cell suspension into each cryogenic vial, place the vials in a Mr. Frosty Freezing Container with isopropanol, and freeze them at -80°C overnight.

6. The next day, transfer the frozen vials to liquid nitrogen (vapor phase) for long-term storage.

Recover frozen FPp2 cells (Day 16)

1. Remove the cryogenic vial of FPp2 cells from the liquid nitrogen storage and immediately immerse it in a 37°C water bath without submerging the cap. Swirl the vial gently.
2. When only an ice crystal remains (~1–2 minutes), remove the vial from the water bath and spray the outside of it with 70% ethanol to decontaminate.
3. Pipet the cells gently into a sterile 15 mL conical tube using a 1 mL pipette.
4. Add 1 mL of DPBS without Ca²⁺ and Mg²⁺ into the vial to collect the remaining cells and transfer the cell suspension dropwise to the 15 mL conical tube. While adding, gently move the tube back and forth to mix the cells and prevent osmotic shock.
5. Add an additional 3 mL of DPBS to the cells to have a 5 mL suspension.
6. Remove a small volume of cell suspension and perform a viable cell count.
7. Centrifuge the cell suspension at 300 x g for 3 minutes to pellet the cells. Carefully aspirate the supernatant, leaving the cell pellet in the culture tube.
8. Gently flick the bottom of the tube to dislodge the cell pellet and resuspend the cells to 1.0 x 10⁶ viable cells/mL in FP expansion medium plus 5 μ M ROCK inhibitor Y-27632.

Sphere formation of FPp2 (Day 16–Day 21)

1. On day 16, harvest or thaw FPp2 cells as described, then remove a small volume of cells and perform a viable cell count.
2. Resuspend the FPp2 cells to 1.0×10^6 viable cells/mL in FP expansion medium plus 5 μ M ROCK inhibitor Y-27632.
3. Transfer cell suspension to a non-tissue culture treated vessel and adjust the volume of the cell suspension to the size of vessel.
4. Incubate the cells overnight at 37°C in a humidified atmosphere of 5% CO₂.
5. Perform a complete medium change by the centrifugation method on the next day and every other day thereafter. Transfer the spheres to a 15 mL conical tube and then centrifuge at 200 x *g* for 2 minutes. Aspirate the supernatant and discard.
6. Resuspend the spheres in fresh FP expansion medium without the ROCK inhibitor, and then transfer to original flask.
7. Pipet the sphere suspension up and down several times to prevent them from merging with each other before plating.

Maturation

Dissociate spheres (Day 21)

1. Prepare double-coated culture plates and DA maturation medium.
2. Transfer the sphere suspension from culture vessel to a sterile 15 mL conical tube. Allow spheres to settle to the bottom of the tube (~2–5 minutes) before proceeding to the next step.
3. Carefully aspirate the spent medium, leaving the spheres at the bottom of tube in a minimal volume (~100 μ L) of the remaining medium. Resuspend the spheres in 5 mL of DPBS without Ca²⁺ and Mg²⁺.
4. Repeat steps 2 and 3, leaving the spheres at the bottom of tube in a minimal volume (~100 μ L) of DPBS.
5. Add 1 mL of StemPro Accutase Cell Dissociation Reagent to the spheres and incubate for 30 minutes at 37°C. Every 10 minutes, gently swirl the cell suspension to ensure that spheres are exposed to the StemPro Accutase reagent evenly.
6. While the spheres are incubating with the dissociation reagent, aliquot the amount of complete DA maturation medium needed for the day and warm at 37°C.
7. Gently pipet the cell suspension up and down with a P1000 pipette until all of the spheres are dispersed into a single-cell suspension.
8. Remove a small volume of cell suspension to perform a viable cell count using an automated cell counter (e.g., Invitrogen™ Countess™ II Automated Cell Counter) or a hemocytometer.

9. Centrifuge the cell suspension at 300 x *g* for 3 minutes to pellet the cells. Carefully aspirate the supernatant, leaving the cell pellet in the culture tube.
 3. Incubate the cells overnight at 37°C in a humidified atmosphere of 5% CO₂.
 4. On day 22 of differentiation (first medium change), add the same volume of fresh DA maturation medium (without the ROCK inhibitor) as the existing culture volume (e.g., 2 mL for each well of a 6-well plate).
 5. For subsequent feeds (every 2–3 days), aspirate half of the spent medium and replace it with fresh DA maturation medium.
- Plate and mature FP cells to DA neurons (Day 21–Day 35)**
1. Resuspend the cell pellet to a single-cell suspension in DA maturation medium plus 5 μM ROCK inhibitor Y-27632.
 2. Seed the double-coated culture plates with the dissociated cells at a seeding density of 1.0 x 10⁵ to 2.0 x 10⁵ cells/cm² in DA maturation medium plus 5 μM ROCK inhibitor Y-27632.

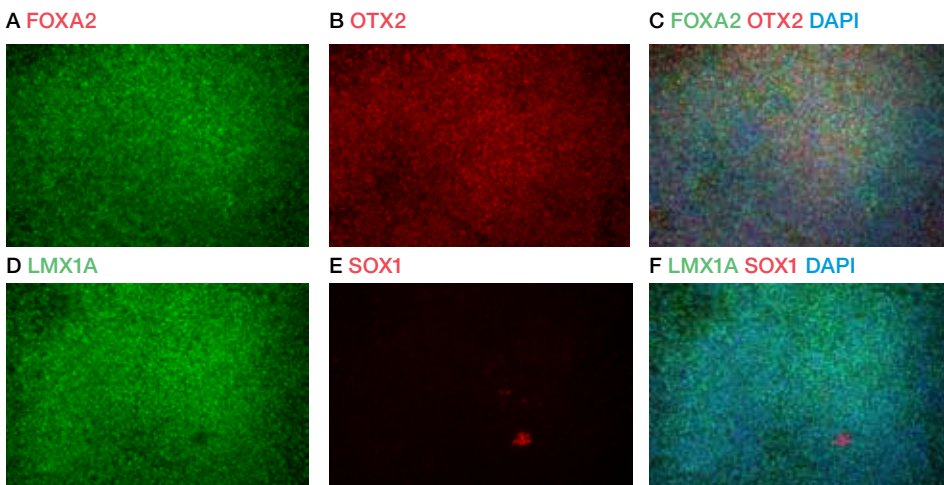


Figure 11-1. Marker expression of induced floor plate progenitor cells. hPSCs were treated with FP specification medium for 7 days and the cells were analyzed for the key phenotypic markers of the human dopaminergic neuron lineage using the Human Dopaminergic Neuron Immunocytochemistry Kit (Cat. No. A29515). **(A–C)** After floor plate specification of hPSCs, the cells express FP marker FOXA2 (green) and rostral marker OTX2 (red). **(D–E)** The specified FP cells are positive for the DA progenitor marker LMX1A (green), but negative for the neural stem cell marker SOX1 (red).

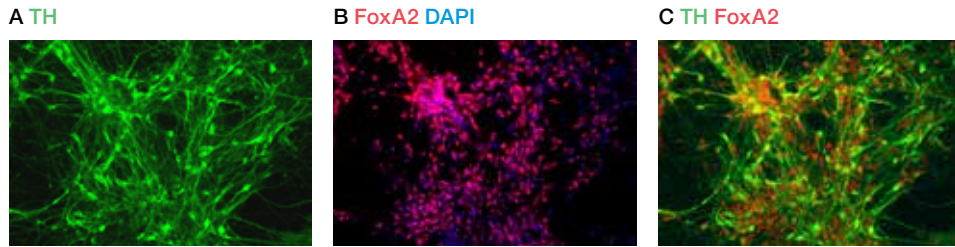


Figure 11-2. Representative images of mature dopaminergic neurons. Matured neuron after 14 days in DA maturation medium. The majority of the TH-expressing neurons also co-expressed FOXA2. **(A)** Anti-TH antibody (green); **(B)** anti-FOXA2 antibody (red) and Invitrogen™ NucBlue™ reagent (a DAPI nuclear DNA stain) (blue). **(C)** Merged image with anti-TH and anti-FOXA2 antibodies (green and red).

Derivation and culture of cortical astrocytes

Summary

Astrocytes constitute a critical mass of the central nervous system (CNS), in addition to oligodendrocytes and neurons. They are involved in adult CNS homeostasis, biochemical and nutritional support of neurons and endothelial cells that form the blood-brain barrier, perform the vast majority of synaptic glutamate uptake, and maintain extracellular potassium levels. Astroglial dysfunction has been implicated in a number of CNS pathologies. This protocol describes the preparation of primary cortical astrocytes from newborn rats or mice.

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Introduction

Astrocytes outnumber the number of neurons by approximately tenfold, and have critical roles in adult CNS homeostasis (Pekny and Nilsson, 2005). They provide biochemical and nutritional support of neurons and endothelial cells that in turn form the blood-brain barrier, perform synaptic glutamate uptake, and maintain extracellular potassium (Rothstein et al., 1996; Rothstein et al., 1994). Astroglial dysfunction has been implicated in a number of CNS pathologies including amyotrophic lateral sclerosis (ALS) and ischemic neuronal death (Maragakis and Rothstein, 2006; Takano et al., 2009). Transplant-based astrocyte replacement therapy has been shown to be a promising therapeutic strategy against neuronal death (Lepore et al., 2008) and in lessening the disease impact in ALS. Although there are few known differences between cortical and hippocampal astrocytes, it has been reported that astrocytes from different regions of the brain show different sensitivity to ischemic injury (Xu et al., 2001; Zhao and Flavin, 2000).

Sources of Rat Primary Cortical Astrocytes

Rat Primary Cortical Astrocytes are isolated from the cortices of fetal Sprague-Dawley rats at embryonic day 19 (E19) of gestation. The cells are isolated from tissue under sterile conditions, placed through one round of enzymatic dissociation and expansion in astrocyte growth medium (85% DMEM containing 4.5 g/L glucose and 15% FBS). The cells are cryopreserved at passage 1 (P1) in 90% astrocyte growth medium plus 10% DMSO. Each vial of Rat Primary Cortical Astrocytes contains 1×10^6 cells/mL that can be expanded in culture for at least one passage.

Characteristics of Rat Primary Cortical Astrocytes

- Isolated from the brain cortex of fetal Sprague-Dawley rats at E19 of gestation
- Exhibit $\geq 70\%$ viability upon thawing
- Stain $>80\%$ positive for the astrocyte specific marker, glial fibrillary acid protein (GFAP)
- Stain $\leq 10\%$ positive for neuron- and oligodendrocyte-specific markers galactocerebroside (GalC) and doublecortin (DCX)
- Exhibit a doubling time of approximately 9 days at P2
- Expandable in culture for at least one passage

Required Materials

Animals

- Newborn rats or mice at 1–2 days after birth

Media and reagents

- Ether
- 70% ethanol
- Gibco™ Distilled Water (Cat. No. 15230162)
- Acetic acid (Sigma-Aldrich, Cat. No. 34254)
- Gibco™ Collagen I Rat Protein, Tail (Cat. No. A1048301)
- Gibco™ Trypsin-EDTA (0.05%), phenol red (Cat. No. 25300054)
- Gibco™ HBSS, no calcium, no magnesium (Hanks' balanced salt solution without Ca^{2+} or Mg^{2+}) (Cat. No. 14170112)
- Gibco™ Astrocyte Medium (Cat. No. A1261301: This kit contains N-2 Supplement, 100X, stored at -20°C ; Dulbecco's Modified Eagle Medium (D-MEM) (1X), stored at 2°C to 8°C ; and OneShot™ Fetal Bovine Serum, Certified, stored at -20°C in the dark.)
- Gibco™ EGF Recombinant Human Protein (Cat. No. PHG0314)
- Gibco™ Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca^{2+} or Mg^{2+} , without phenol red (Cat. No. 14190144)
- Dibutyl cyclic-AMP (dBcAMP) (Sigma-Aldrich, Cat. No. D0627)
- Gibco™ Penicillin-Streptomycin (5,000 U/mL) (Cat. No. 15070063)

- Gibco™ Trypan Blue Solution, 0.4% (Cat. No. 15250061) (included with the Invitrogen™ Countess™ II Automated Cell Counter) or Molecular Probes™ LIVE/DEAD™ Cell Vitality Assay Kit, C_{12} Resazurin/SYTOX™ Green (Cat. No. L34951)
- Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent (Cat. No. A1110501), pre-warmed to 37°C

Equipment and plasticware

- Invitrogen™ Countess™ II Automated Cell Counter (Cat. No. AMQAX1000) or hemocytometer
- 37°C incubator with humidified atmosphere of 5% CO_2
- Uncoated, tissue-culture treated flasks, plates, or petri dishes
- Disposable, sterile 15 mL or 50 mL conical tubes, pre-rinsed with medium

Special tools

- Desiccator
- Iridectomy scissors
- 70 μm mesh cell strainer

Note: For “Expanding Rat Primary Cortical Astrocytes” on page 58, the following materials are also required:

- Culture vessels containing Rat Primary Cortical Astrocytes (100% confluent), which can be prepared using the method described in “Preparing Astrocyte-Enriched Cultures from Rat or Mouse” on page 57)
- Astrocyte growth medium (use the complete medium described in “Complete astrocyte medium” on page 57), pre-warmed to 37°C

Preparing Reagents and Media

Complete astrocyte medium

Astrocyte Medium has been specifically formulated for the growth and maintenance of human and rat astrocytes while retaining their phenotype. The medium has three components: basal medium (DMEM), N-2 Supplement, and One Shot™ Fetal Bovine Serum (FBS). Epidermal growth factor (EGF) may also be added to enhance astrocyte proliferation.

To prepare 100 mL of complete astrocyte medium, mix the following components under aseptic conditions. For larger volumes, increase the component amounts proportionally.

Component	Amount
DMEM	88 mL
N-2 Supplement	15 mL
FBS	1 mL
Penicillin-Streptomycin	1 mL
<i>Optional:</i> EGF (prepared as 100 µg/mL stock)	20 µL

Collagen

Prepare a 50 µg/mL working solution in distilled water with 0.02 M acetic acid and sterilize the solution with a 0.22 µm filter.

Dibutyryl cyclic-AMP (dBcAMP)

Prepare a 0.25 M stock solution of dBcAMP in DPBS without Ca²⁺ and Mg²⁺, aliquot into sterilized tubes, and store at -20°C.

HBSS

Chill on ice prior to use.

Preparing Astrocyte-Enriched Cultures from Rat or Mouse

1. Coat the culture vessels with collagen and let stand for 45 minutes at room temperature. Rinse with DPBS without Ca²⁺ and Mg²⁺ 2 times.
2. Anesthetize rat or mouse pups with ether in a desiccator in a laminar flow hood.
3. Remove pups from the hood and spray 70% ethanol over the animal. Decapitate the animals with scissors. Open the skull with iridectomy scissors. Remove the meninges and dissect the brain tissue from the cortices.
4. Put the cortices in a petri dish containing 5–10 mL of ice-cold HBSS. Pool the cortices from two pups in a new petri dish and wash with 5 mL of HBSS.
5. Take the petri dish to a laminar flow hood. Mince the cortices into small pieces with a scissors in a petri dish containing about 5 mL of ice-cold HBSS. Transfer the tissue to a 15 mL sterile tube. Centrifuge the tube at 200 x g for 3 minutes at 4°C and aspirate the supernatant.
6. Resuspend the tissue in 5 mL of 0.05% trypsin and incubate at 37°C for 25 minutes in a shaker bath.
7. Centrifuge the tissue suspension at 200 x g for 3 minutes, aspirate the trypsin solution with a pipette, and rinse the cells 3 times with 3 mL of HBSS.
8. Add 6 mL of complete astrocyte medium and pipet the cell suspension up and down with a 10 mL pipette to dissociate cells.

9. Filter the cell suspension through a 70 μm mesh cell strainer into a 50 mL sterile tube. Rinse the mesh with another 4 mL of complete astrocyte medium (total of 10 mL suspension).
10. Remove 10 μL of the filtrate for counting on a hemocytometer or the Countess II Automated Cell Counter.
11. Determine the total number of cells and percent viability using trypan blue stain or the LIVE/DEAD Cell Vitality Assay Kit.
12. Dilute the cell suspension to 5×10^4 viable cells/mL with complete astrocyte medium and plate the cells into culture vessels at 2.5×10^4 cells/cm².
13. Incubate the cells in a 37°C incubator with 5% CO₂ and 90% humidity.
14. Change the complete astrocyte medium the next day and then every other day until cells are confluent.
15. When confluent, feed the cells with complete astrocyte medium containing 0.25 mM dBcAMP to induce differentiation. (Dilute 0.25 M stock of dBcAMP 1:1,000 in complete astrocyte medium.)
16. Feed the cultures with dBcAMP 2 times per week and check for differentiation.
17. Astrocytes are ready for experiments 2–3 weeks after culturing.

Expanding Rat Primary Cortical Astrocytes

1. Remove the spent growth medium from the culture dish containing the cells, and store in a sterile tube to use as a washing solution.
2. Rinse the surface of the cell layer once with DPBS without Ca²⁺ and Mg²⁺ (approximately 2 mL DPBS per 10 cm² culture surface area) by adding the DPBS to the side of the vessel opposite the attached cell layer, and rocking back and forth several times.
3. Aspirate the DPBS and discard.
4. To detach the cells, add 3 mL of pre-warmed StemPro Accutase Cell Dissociation Reagent per T-75 flask; adjust volume accordingly for culture dishes of other sizes.
5. Incubate for up to 20 minutes at 37°C. Rock the cells every 5 minutes, and check for cell detachment and dissociation toward single cells under the microscope.
6. Once you observe cell detachment, gently pipette up and down to break clumps into a single-cell suspension. Stop the cell dissociation reaction by adding an equal volume of the spent medium from step 1. Disperse the medium by pipetting over the cell layer surface several times.
7. Transfer the cells to a new 15 mL or 50 mL pre-rinsed conical tube, and centrifuge at 250 x g for 5 minutes at room temperature. Aspirate and discard the supernatant.
8. Gently resuspend the cell pellet in pre-warmed complete astrocyte medium and remove a sample for counting.
9. Determine the total number of cells and percent viability using your method of choice. If necessary, add astrocyte growth medium to the cells to achieve the desired cell concentration and recount the cells.

10. Plate cells in an uncoated tissue-culture treated flask, plate, or Petri dish at a seeding density of 2×10^4 cells/cm².
11. Incubate cells at 37°C, 5% CO₂, and 90% humidity, and change growth medium every 4–5 days.
12. Astrocytes are ready for experiments 2–3 weeks after culturing.

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Cryopreserving neural stem cells

Summary

There are numerous protocols available for cryopreserving neural stem cells (NSCs) derived from human embryonic stem cells. The primary objectives of these methods are the recovery of the cells post-thaw and the retention of their multipotent properties. This chapter describes a standardized cryopreservation protocol that optimizes survival of NSCs post-thaw, while maintaining sublineage differentiation capacity of the preserved cells.

View this protocol online and order products at [thermofisher.com/neuroprotocol/cryo](https://www.thermofisher.com/neuroprotocol/cryo)

Required Materials

Cells

- Neural Stem Cells (NSCs)

Media and reagents

- Gibco™ StemPro™ NSC SFM (Cat. No. A1050901: This kit contains KnockOut™ DMEM/F-12 Basal Medium stored at 4°C, StemPro™ NSC SFM Supplement stored at –20°C to –5°C in the dark, and bFGF Recombinant Human and EGF Recombinant Human stored at 4°C, desiccated.)
- Gibco™ GlutaMAX™-I Supplement (Cat. No. 35050061)
- *Optional:* Gibco™ Antibiotic-Antimycotic (100X) (Cat. No. 15240062)
- Gibco™ TrypLE™ Select Enzyme (1X), no phenol red (Cat. No. 12563011)
- Gibco™ DPBS, without Ca²⁺ and Mg²⁺ (Cat. No. 14190144)
- 100% isopropyl alcohol
- Gibco™ Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413301)

Cryopreservation and recovery solution options:

- Gibco PSC Cryopreservation Kit (Cat. No. A2644601): Includes PSC Cryomedium and RevitaCell™ Supplement (100X), or CTS™ Synth-a-Freeze™ Medium (Cat. No. A1371301)

Tools and Equipment

- Sterile 15 mL conical tubes
- Tabletop centrifuge
- Thermo Scientific™ Nalgene™ General Long-Term Storage Cryogenic Tubes (Cat. No. 5000-1020)
- Thermo Scientific™ Mr. Frosty™ Freezing Container (Cat. No. 5100-0001)
- 37°C water bath

Preparing Media

Complete StemPro NSC SFM

Complete StemPro NSC SFM consists of KnockOut DMEM/F-12 with StemPro NSC SFM Supplement, EGF, and bFGF, combined with GlutaMAX-I Supplement. Complete medium is stable for 4 weeks when stored in the dark at 2°C to 8°C.

To prepare 50 mL of complete StemPro NSC SFM, aseptically mix the following components. For larger volumes, increase the component amounts proportionally. If desired, add 0.5 mL of Antibiotic-Antimycotic (100X) solution per 50 mL of complete medium.

Component	Final conc.	Amount
KnockOut DMEM/F-12	1X	48.5 mL
GlutaMAX-I Supplement	2 mM	0.5 mL
bFGF	20 ng/mL	1 µg
EGF	20 ng/mL	1 µg
StemPro NSC SFM Supplement*	2%	1 mL

* You may observe a white precipitate when thawing StemPro NSC SFM Supplement; this precipitate will disappear when the supplement is completely thawed or dissolved.

Freezing and recovery media

Xeno-free PSC Cryomedium (a component in Cat. No. A26444601) and animal origin-free CTS Synth-a-Freeze Medium (Cat. No. A1371301) have shown utility for cryopreservation of NSCs. Once thawed, these cryomedia are stable for up to 6 months when stored at 2°C to 8°C protected from light.

RevitaCell Supplement is provided as a 100X solution for addition to growth media (complete StemPro NSC SFM) for the first 18–24 hours post-thaw to assist in minimizing apoptosis and necrosis. RevitaCell Supplement is a chemically defined recovery supplement containing a specific ROCK inhibitor coupled with molecules that have antioxidant and free radical scavenger properties. Upon thaw, RevitaCell Supplement should be efficiently mixed by gentle inversion and dispensed into usage-size aliquots. Aliquots should be stored frozen at –20°C to –5°C protected from light. Avoid long-term (>5 days) storage at 2°C to 8°C.

Cryopreserving Neural Stem Cells

Guidelines for cryopreserving neural stem cells

- Cryopreserve NSCs when they are 80–90% confluent (2–4 days after seeding).
- Freeze NSCs at a concentration of 1×10^6 to 2.4×10^6 viable cells/mL and a volume of 1 mL/vial.
- Xeno-free PSC Cryomedium or CTS Synth-a-Freeze Medium can be used for cryopreservation of NSCs. Prechill cryomedium prior to use in cryopreservation procedures to minimize toxicity of DMSO within these formulations.
- Do not incubate the NSCs in TrypLE Select Enzyme for more than 5 minutes to avoid cell death.
- Pre-label all cryovials and Mr. Frosty Freezing Containers (filled with 250 mL of 100% isopropyl alcohol and prechilled to 4°C) prior to addition of cells.

Freezing neural stem cells

1. When NSCs are 80–90% confluent (2–4 days after seeding), aspirate the complete StemPro NSC SFM from the culture vessel.
2. Wash the cells once with DPBS without Ca^{2+} and Mg^{2+} , according to the volumes shown in Table 13-1. Aspirate the DPBS and discard.
3. Add room temperature TrypLE Select Enzyme, according to the volumes shown in Table 13-1, to the culture vessel and incubate at 37°C for 2–5 minutes.

Note: Do not incubate the NSCs in TrypLE Select Enzyme for more than 5 minutes to avoid cell death. Neutralize TrypLE Select Enzyme by adding complete StemPro NSC SFM immediately after the incubation period (according to the instructions below).

4. Detach the NSCs from the culture vessel by pipetting off the cells or by tapping the culture vessel against the heel of your hand.
5. Quickly transfer the cell suspension to a 15 mL conical tube containing the appropriate volume of complete StemPro NSC SFM (refer to Table 13-1 on the next page) to neutralize the TrypLE Select Enzyme.
6. Mix the cell suspension by gentle inversion 3 times and remove a small aliquot for assessment of cell count and viability using the Invitrogen™ Countess™ II Automated Cell Counter or traditional hemocytometer.
7. Centrifuge the NSCs at 200 x g for 5 minutes.
8. Gently aspirate the medium, being careful to avoid the cell pellet, and add prechilled (4°C) freezing medium dropwise to the cells while moving the conical tube back and forth and gently resuspend the cells to a final concentration of 1×10^6 to 2.4×10^6 viable cells/mL.
9. Transfer 1 mL of the NSC suspension in freezing medium into each pre-labeled, prechilled (4°C) cryovial.
10. Transfer the cryovials to the Mr. Frosty Freezing Container and place the container into a –80°C freezer. This procedure ensures that the cells freeze slowly at approximately –1°C/minute.
11. The next day, transfer the cells into liquid nitrogen. Note that it is important to avoid longer storage at –80°C.

Table 13-1. Reagent volumes (per well or per dish).

Culture vessel (surface area)	DPBS for wash	TrypLE Select for dissociation	StemPro NSC Medium for neutralization
6-well (10 cm ²)	2 mL	1 mL	3 mL
12-well (4 cm ²)	1 mL	0.4 mL	1.2 mL
24-well (2 cm ²)	0.5 mL	0.2 mL	0.6 mL
35 mm (10 cm ²)	2 mL	1 mL	3 mL
60 mm (20 cm ²)	4 mL	2 mL	6 mL
100 mm (60 cm ²)	12 mL	6 mL	18 mL

Guidelines for recovery of neural stem cells

- Minimize the duration of exposure of NSCs to cryomedium at 37°C by thawing until only a small ice crystal remains. Avoid longer incubation at 37°C in cryomedia solutions.
- Ensure dropwise addition of growth medium (complete StemPro NSC SFM) to the NSCs in cryomedium to avoid osmotic shock.
- When using RevitaCell Supplement, do not include additional ROCK inhibitors such as Y-27632 or Thiazovivin to the growth medium for recovery.
- Within 18–24 hours post-thaw, aspirate growth medium supplemented with RevitaCell Supplement and replenish with fresh StemPro NSC SFM in the absence of RevitaCell Supplement for the remainder of culture.

Recovery of cryopreserved neural stem cells

1. Coat the culture vessels with the appropriate substrate on which to culture your NSCs. Recommended substrates include Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413301), Gibco™ CTS CELLstart™ Substrate (Cat. No. A1014201), or Gibco™ Laminin Mouse Protein (Cat. No. 23017015). Aspirate coating matrix immediately prior to seeding of recovered NSCs.

Note: Do not allow matrices to dry out.

2. Prepare recovery medium by supplementing complete StemPro NSC SFM with RevitaCell Supplement at 1X final concentration (e.g., add 100 µL RevitaCell Supplement to 10 mL of complete StemPro NSC SFM).
3. Quickly thaw NSCs in a 37°C water bath until a small ice crystal remains.
4. Transfer the vial to the laminar flow hood and disinfect it with 70% ethanol. Allow the ethanol to evaporate prior to opening the vial.
5. Gently triturate cells and transfer to a 15 mL conical tube.
6. Add 5 mL of complete StemPro NSC SFM dropwise per 1 mL of cell suspension, while shaking the tube back and forth to avoid osmotic shock.
7. Mix the cell suspension by gentle inversion 3 times.
8. Centrifuge the NSCs at 200 x g for 5 minutes. Aspirate the supernatant and discard.

9. Resuspend the cell pellet in recovery medium (e.g., StemPro NSC SFM supplemented with 1X RevitaCell Supplement) and perform cell count using the Countess II Automated Cell Counter or traditional hemocytometer.
10. Aspirate matrix solution from precoated plates and seed NSCs at desired plating concentration for downstream assay in recovery medium (e.g., StemPro NSC SFM supplemented with 1X RevitaCell Supplement). For NSCs an initial seeding density of ~20,000–50,000 viable cells/cm² is recommended.
11. 18–24 hours post-seeding, aspirate recovery medium and replace medium with complete StemPro NSC SFM. Refresh medium every other day thereafter.

Expected Results

H9 ESC-derived NSCs cultured in complete StemPro NSC SFM were cryopreserved according to the instructions provided above and evaluated for viability. While percentage viability direct post-thaw is commonly used as a metric to assess the performance of a cryomedium solution (Figure 13-1), following cryopreservation and recovery of NSCs, additional cell death is not apparent immediately post-thaw. Additional loss of cell viability occurs over the first 24 hours post-thaw due to the processes of apoptosis and necrosis (Baust et al., 2000 and 2001) and is a direct reflection of the stress on the NSCs during the cryopreservation and recovery processes. To assess the impact of these processes, NSCs were examined under a phase-contrast microscope and assayed for viability (Figure 13-2). Cryomedia is shown to provide >70% viability direct post-thaw of cryopreserved NSCs, and RevitaCell Supplement was shown to significantly improve post-thaw recovery of NSCs cryopreserved in CTS Synth-a-Freeze Medium or PSC Cryomedium.

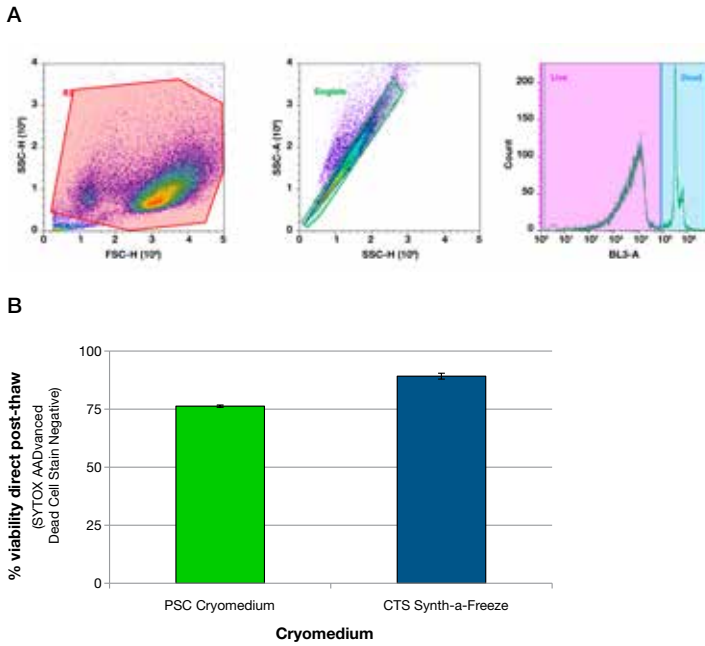


Figure 13-1. Direct post-thaw viability assessment of cryopreserved H9 ESC-derived NSCs. Post-thaw viability was assessed by SYTOX™ AADvanced™ Dead Cell Stain Kit (Cat. No. S10349) using the Attune™ NxT Flow Cytometer. Experiments for recommended cryomedia included **(A)** a gating strategy for cryopreservation and **(B)** a direct post-thaw viability percentage. Cryomedia is shown to provide >70% viability direct post-thaw of cryopreserved NSCs.

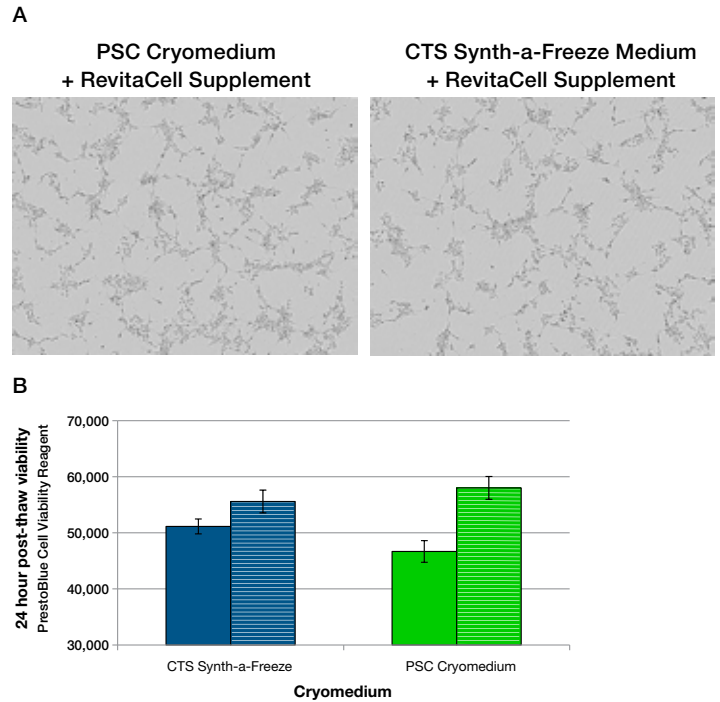


Figure 13-2. 24-hour post-thaw viability assessment of cryopreserved H9 ESC-derived NSCs. To assess 24-hour post-thaw recovery, NSCs plated on Geltrex matrix-coated plates were examined via **(A)** phase-contrast imaging and **(B)** PrestoBlue™ Cell Viability Reagent (Cat. No. A13261). RevitaCell Supplement was shown to significantly improve post-thaw recovery of NSCs cryopreserved in CTS Synth-a-Freeze Medium or PSC Cryomedium; solid bars indicate recovery in medium alone, while hatched bars indicate recovery in medium plus RevitaCell Supplement.

Troubleshooting

For troubleshooting tips regarding cryopreservation and recovery of NSCs, see below.

Problem	Possible cause	Solution
Low cell survival rate	NSCs stressed during cryopreservation	<ul style="list-style-type: none"> • Ensure that NSCs are not overly confluent prior to cryopreservation; target 80–90% confluency at time of harvest. • Ensure that TrypLE Select Enzyme is not left on the NSCs for >5 minutes. • Ensure Mr. Frosty Freezing Container contains the appropriate amount of 100% isopropyl alcohol. Be certain to refresh the container with fresh isopropyl alcohol every 5 freeze cycles to ensure proper cooling rate of about $-1^{\circ}\text{C}/\text{minute}$. • Ensure that Mr. Frosty Freezing Container, cryovials, and cryomedia solutions are prechilled to 4°C prior to use. • Ensure dropwise addition of prechilled cryomedia to the NSCs to avoid osmotic shock. • Limit the number of samples you are cryopreserving to minimize the toxicity of DMSO on your NSCs. Placing vials on ice can assist in minimizing damage during the vialing process. • Do not disturb the Mr. Frosty Freezing Container for up to 4 hours post-placement in the -80°C freezer.
	NSCs stressed during recovery from cryopreservation	<ul style="list-style-type: none"> • Ensure that cells are not left at 37°C for extended periods of time. Be certain to thaw NSCs until only a small ice crystal remains. • Ensure dropwise addition of growth medium to the NSCs to avoid osmotic shock to the cells. • Limit the number of vials thawed at one time as longer exposure of cells to cryopreservation media can have a negative impact on post-thaw viability. • Ensure addition of RevitaCell Supplement to recovery medium for the first 18–24 hours post-thaw to maximize cell survival.
Non-reproducible cryopreservation and recovery	Inconsistent cell confluency	Always harvest cells at comparable confluency.
	Inefficient mixing of cryomedia or RevitaCell Supplement	Upon thaw, ensure efficient mixing of PSC Cryomedium, CTS Synth-a-Freeze Medium, and RevitaCell Supplement by gentle inversion.

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Cryopreservation and recovery of mature differentiated neural cells

Summary

Primary neuronal cultures are indispensable in the field of neurobiology and pharmacology. Many researchers favor freshly isolated neuronal cells as they maintain their functional viability, but for convenience, an alternate route is to cryopreserve fresh cells for later use. This chapter describes the generation of cryopreserved stocks from freshly isolated neural cells, and thawing procedures for recovering the stocks.

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Required Materials

Rat brain cells

- Homogeneous cell preparation from E18 rat brain tissue (described in Isolation, culture, and characterization of cortical and hippocampal neurons (page 11)).

Media and reagents

- Gibco™ Neurobasal™ Medium (Cat. No. 21103049)
- Gibco™ B-27™ Supplement (50X), serum free (Cat. No. 17504044)
- Gibco™ GlutaMAX™-I Supplement (Cat. No. 35050061)
- Gibco™ Trypan Blue Solution, 0.4% (Cat. No. 15250061)
- Gibco™ CTS™ Synth-a-Freeze™ Medium (Cat. No. A1371301)
- Poly-D-lysine–coated plates
- *Optional:* Gibco™ RevitaCell™ Supplement (Cat. No. A2644501)

Equipment

- Thermo Scientific™ Nalgene™ General Long-Term Storage Cryogenic Tubes (Cat. No. 50001020)
- Thermo Scientific™ Mr. Frosty™ Freezing Container (Cat. No. 5100-0001)
- Freezer, –80°C
- Liquid nitrogen freezer
- Water bath set to 37°C

Cryopreservation

Guidelines for cryopreserving neural cells

- Prechill cryomedia prior to use in cryopreservation procedures to minimize toxicity of DMSO within these formulations.
- Pre-label all cryovials and Mr. Frosty Freezing Containers (filled with 250 mL of 100% isopropyl alcohol and prechilled to 4°C) prior to addition of cells.
- Pre-rinse cryovials and pipettes with complete Neurobasal medium to prevent adherence of neural cells to plastic surfaces.

Freezing neural cells

1. Isolate and prepare a suspension of rat brain cells in complete Neurobasal medium, i.e., Neurobasal Medium supplemented with 2% B-27 Supplement (50X), serum free and 0.5 mM GlutaMAX-I Supplement as described in Isolation, culture, and characterization of cortical and hippocampal neurons (page 11).
2. Count the cell number using a hemocytometer.
3. Centrifuge the cells at 200 x g for 4 minutes. Aspirate the supernatant.

4. Resuspend the cell pellet in cold CTS Synth-a-Freeze Medium at a concentration of 2.0×10^6 to 1.0×10^7 cells/mL, adding cryomedium dropwise.
5. Make 1 mL aliquots of the cells in pre-labeled, prechilled cryovials and place the vials in an isopropanol chamber at 4°C for 10 minutes. Transfer the isopropanol chamber to -80°C overnight. Note that it is important to avoid longer storage at -80°C.
6. Transfer the frozen vials to the vapor phase of liquid nitrogen storage until use is required.

Cell Recovery

Guidelines for recovery of cryopreserved neural cells

- Minimize the duration of exposure of neural cells to cryomedium at 37°C by thawing until only a small ice crystal remains. Avoid longer incubation at 37°C in cryomedia solutions.
- Ensure the addition of complete Neurobasal medium to the neural cells in cryomedium is in a dropwise manner to avoid osmotic shock.
- When using RevitaCell Supplement, do **not** include additional ROCK inhibitors such as Y-27632 or Thiazovivin to the growth medium for recovery.
- When using RevitaCell Supplement, remove half of the growth medium 18–24 hours post-thaw and replenish with fresh complete Neurobasal medium in the absence of RevitaCell Supplement for the remainder of culture.

Recovering frozen neural cells

Handle cells gently, because they are extremely fragile upon recovery from cryopreservation. It is important to rinse pipette tips and vials with complete Neurobasal medium before using them for transferring cell suspensions to avoid the cells sticking to the plastic. Do not centrifuge cells upon recovery from cryopreservation.

1. Remove one vial of frozen cells from liquid nitrogen.
2. Thaw the vial in a 37°C water bath with gentle swirling.
3. Wipe down the vial with ethanol and tap gently on a surface so that all of the medium collects at the bottom of the tube.
4. Open the vial in a laminar flow hood.
5. Rinse a pipette tip with complete Neurobasal medium and very gently transfer the cells from the vial to a pre-rinsed 15 mL tube.
6. Rinse the vial with 1 mL of pre-warmed complete Neurobasal medium, and transfer the rinse to the 15 mL tube containing the cells at a rate of one drop per second. Mix by gentle swirling after each drop.
7. Slowly add 2 mL of complete Neurobasal medium to the tube (for a total suspension volume of 4 mL).
8. Mix the suspension very gently with P1000 pipette. Avoid creating any air bubbles.
9. Add 10 μ L of cell suspension to a microcentrifuge tube containing 10 μ L of 0.4% trypan blue using a pre-rinsed tip. Mix the cells by gently tapping the tube. Determine the viable cell density using a hemocytometer.

10. Plate $\sim 1 \times 10^5$ cells per well in a poly-D-lysine-coated 48-well plate or an 8-chambered slide. Bring the cell suspension volume to 500 μL per well by adding complete Neurobasal medium.

Note: RevitaCell Supplement—a cocktail of a specific ROCK inhibitor coupled with antioxidants and free radical scavengers—may be included to improve post-thaw cell survival.

11. Incubate the cells at 37°C in a humidified atmosphere of 5% CO_2 in air.

12. Feed the cells every third day by aspirating half the medium from each well and replacing it with fresh complete Neurobasal medium.

Note: If RevitaCell Supplement is included then half of the media should be removed within 18–24 hours of cell seeding, followed by half media changes with complete Neurobasal medium alone (i.e., not supplemented with RevitaCell Supplement) every three days.

Expected Results

Cryopreserved Primary Rat Cortical Neurons (Cat. No. A10840), recovered according to the Recovering Frozen Neural Cells protocol noted above, were assessed for cell viability 24 hours post-thaw and again at 6 days post-thaw. Inclusion of RevitaCell Supplement can significantly improve the 24-hour post-thaw viability of rat cortical neurons (Figure 14-1) and provide further benefits for continued propagation of recovered neurons (Figure 14-2).

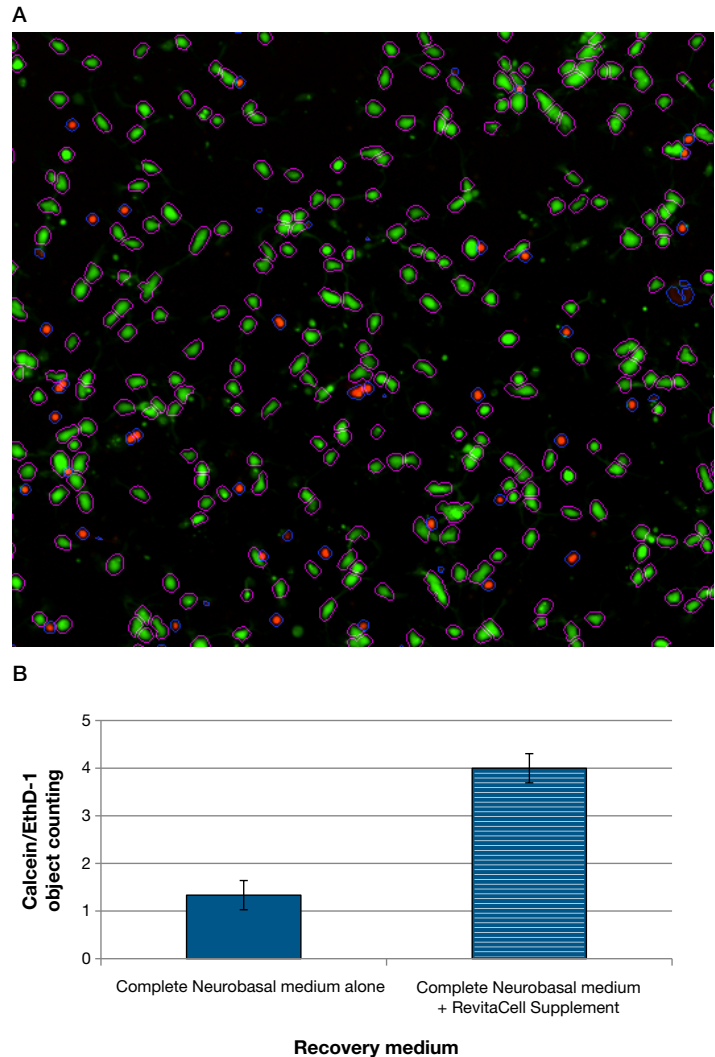
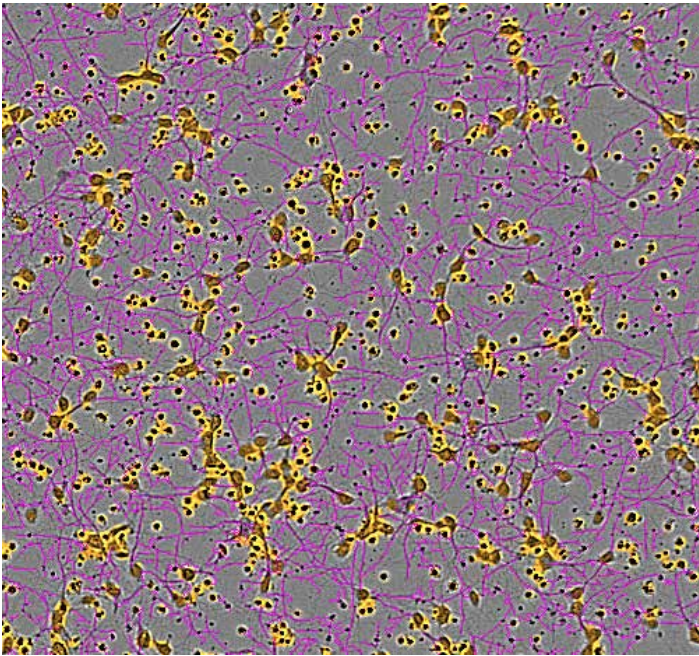


Figure 14-1. 24-hour post-thaw viability assessment of cryopreserved primary rat cortical neurons. Following 24 hours post-thaw, assessment of cell viability of cryopreserved primary rat cortical neurons was assessed using the Molecular Probes™ LIVE/DEAD™ Viability/Cytotoxicity Kit for mammalian cells (Cat. No. L3224). **(A)** Representative image following staining of rat cortical neurons with the LIVE/DEAD kit 24 hours post-thaw, taken and analyzed using the IncuCyte ZOOM™ system, shows object counting masks for live and dead objects. **(B)** Quantitation data of live (calcein AM stained) vs. dead (ethidium homodimer-1 stained) neurons indicate that RevitaCell Supplement can significantly improve post-thaw viability of rat cortical neurons.

A



B

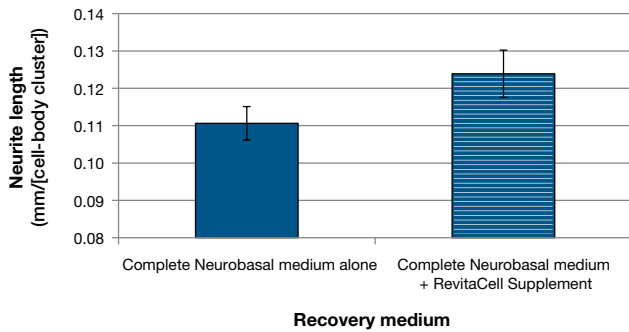


Figure 14-2. Improving cell health of cryopreserved primary rat cortical neurons using RevitaCell Supplement during recovery. Continued propagation of recovered neurons for 6 days post-thaw indicates further benefits of inclusion of RevitaCell Supplement for the first 24 hours post-thaw. **(A)** Representative image collected using IncuCyte ZOOM™ System and subsequently analyzed using NeuroTrack™ software. **(B)** Quantitation of neurite length 6 days post-thaw for cells recovered in the absence or presence of RevitaCell Supplement for the first 24 hours. RevitaCell Supplement is shown to improve cell health as indicated by longer neuronal projections per axon body.

Troubleshooting

For troubleshooting tips regarding cryopreservation and recovery of mature neurons, see below.

Problem	Possible cause	Solution
Low cell survival rate	Neurons stressed during cryopreservation	<ul style="list-style-type: none"> • Ensure Mr. Frosty Freezing Container contains the appropriate amount of 100% isopropyl alcohol. Be certain to refresh the container with fresh isopropyl alcohol every 5 freeze cycles to ensure proper cooling rate of $-1^{\circ}\text{C}/\text{minute}$. • Ensure that Mr. Frosty Freezing Container, cryovials, and cryomedia solutions are prechilled to 4°C prior to use. • Limit the number of samples you are cryopreserving to minimize the toxicity of DMSO on your neurons. Placing vials on ice can assist in minimizing damage during the vialing process. • Do not disturb the Mr. Frosty Freezing Container for up to 4 hours post-placement in the -80°C freezer. • Ensure transfer to the vapor phase of a liquid nitrogen dewar the day following cryopreservation of your neurons.
	Neurons stressed during recovery from cryopreservation	<ul style="list-style-type: none"> • Ensure that cells are not left at 37°C for extended periods of time. Be certain to thaw neurons until only a small ice crystal remains. • Ensure drop-wise addition of growth medium to the neurons to avoid osmotic shock to the cells. • Limit the number of vials thawed at one time as longer exposure of cells to cryopreservation media can have a negative impact on post-thaw viability. • Ensure addition of RevitaCell Supplement to recovery medium for the first 18–24 hours post-thaw to maximize cell survival. • 18–24 hours post-seeding, if using RevitaCell Supplement, replenish half of the medium with growth medium in the absence of RevitaCell Supplement.
Non-reproducible cryopreservation and recovery	Prewetting of plastics	Ensure prewetting of vials and plastics including conicals, pipette tips, etc. to minimize loss of neurons to the side of plastics.
	Inefficient mixing of cryomedia or RevitaCell Supplement	Upon thaw, ensure efficient mixing of CTS Synth-a-Freeze Medium and RevitaCell Supplement by gentle inversion.

Cell Analysis

Cell viability assays for neurons and neural cells

Summary

The LIVE/DEAD™ Viability/Cytotoxicity Kit provides a two-color fluorescence cell viability assay that is based on the simultaneous determination of live and dead neurons and neural stem cells (NSCs) with probes that measure two recognized parameters of cell viability: intracellular esterase activity and plasma membrane integrity.

The polyanionic dye calcein AM is well-retained within live cells, producing an intense uniform green fluorescence in live cells (excitation/emission ~495 nm/~515 nm), while ethidium homodimer-1 (EthD-1) enters cells with damaged membranes to produce a bright red fluorescence in dead cells (excitation/emission ~495 nm/~635 nm).

Protocols are provided for fluorescence microscopy or microplate analysis of adherent cells, or flow cytometry analysis of cells in suspension.

View this protocol online and order products at [thermofisher.com/neuroprotocol/cellviability](https://www.thermofisher.com/neuroprotocol/cellviability)

Required Materials

Cells

- Adherent or suspended NSCs or neurons

Media and reagents

- Molecular Probes™ LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells (Cat. No. L3224)
 - Calcein AM
 - Ethidium homodimer-1 (EthD-1)
- Gibco™ Dulbecco's Phosphate-Buffered Saline (DPBS) with Ca²⁺ and Mg²⁺ (Cat. No. 14040141)

Special tools

- Fluorescence microscope
- Attune™ NxT Flow Cytometer (if using flow cytometry)

Note: Calcein AM and EthD-1 can be viewed simultaneously with a conventional fluorescein longpass filter. The fluorescence from these dyes may also be observed separately; calcein AM can be viewed with a standard fluorescein bandpass filter and EthD-1 can be viewed with filters for propidium iodide or Texas Red™ dye.

Preparing Reagents

Prepare the reagents in the LIVE/DEAD Viability/Cytotoxicity Kit as follows:

1. Remove the stock solutions provided in the kit from the freezer and allow them to warm to room temperature.
2. Add 20 μL of the supplied 2 mM EthD-1 stock solution (Component B) to 10 mL of sterile, tissue culture–grade DPBS. Vortex to ensure thorough mixing. This prepares a $\sim 4 \mu\text{M}$ EthD-1 solution.
3. Combine the reagents by adding 5 μL of the supplied 4 mM calcein AM stock solution (Component A) to the 10 mL of EthD-1 solution in DPBS. Vortex the resulting solution to ensure thorough mixing.

Note: This reagent mixture is suitable for most neural cells. For cells with higher esterase activity, you might need to start with a lower calcein AM concentration. For further information, refer to the user manual provided with the LIVE/DEAD Viability/Cytotoxicity Kit.

The resulting working solution of $\sim 2 \mu\text{M}$ calcein AM and $\sim 4 \mu\text{M}$ EthD-1 is ready to be used. The final concentration of DMSO is $\leq 0.1\%$, a level generally innocuous to most cells.

Note: Prepare a freshly coated culture vessel each time before plating cells. There is no need to rinse the culture vessel before use.

Methods

Determining the viability of adherent cells

Adherent neurons or NSCs may be cultured on sterile glass coverslips or in a multiwell plate. A protocol for a 96-well plate is described below.

1. Aspirate the spent medium from the wells and rinse the cells gently with 200 μL of DPBS prior to the assay to remove or dilute any serum esterase activity.

Note: Serum esterases could cause some increase in extracellular fluorescence by hydrolyzing calcein AM.

2. Add 200 μL of working solution of LIVE/DEAD reagent.
3. Incubate for 30 minutes at 37°C .
4. Rinse cells with 200 μL of DPBS prior to analyzing cells either by fluorescence microscopy or by plate reader.

Determining viability of cells in suspension with flow cytometry using the Attune NxT Flow Cytometer

Allow all the reagents to come to room temperature before proceeding.

1. Make an 80-fold dilution of calcein AM (Component A) in DMSO to make a $50 \mu\text{M}$ working solution (e.g., add 2 mL of calcein AM to 158 mL DMSO).
2. Prepare a 1 mL suspension of cells with 0.1×10^6 to 5×10^6 cells/mL for each assay. Cells may be in culture medium or buffer.
3. Add 2 μL of a $50 \mu\text{M}$ calcein AM working solution and 4 μL of the 2 mM EthD-1 stock to each mL of cells. Mix the sample.

4. Incubate the cells for 15–20 minutes at room temperature, protected from light.
5. As soon as possible after the incubation period (within 1–2 hours), analyze the stained cells by flow cytometry using 488 nm excitation and measuring green fluorescence emission for calcein AM (i.e., 530/30 bandpass) and red fluorescence emission for EthD-1 (i.e., 610/20 bandpass).
6. Gate on cells to exclude debris. Using single color-stained cells, perform standard compensation. The population should separate into two groups: live cells will show green fluorescence and dead cells will show red fluorescence (Figure 15-1).

Typical Results

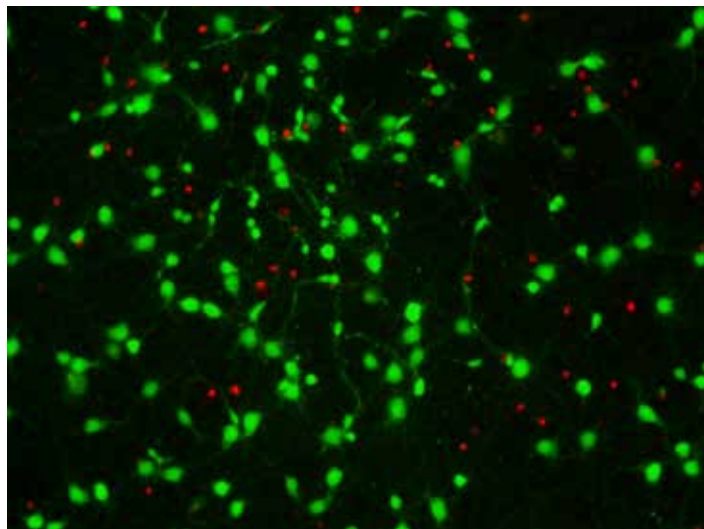


Figure 15-1. Primary rat hippocampal neurons showing live (green) and dead (red) cells using the LIVE/DEAD Viability/Cytotoxicity Kit.

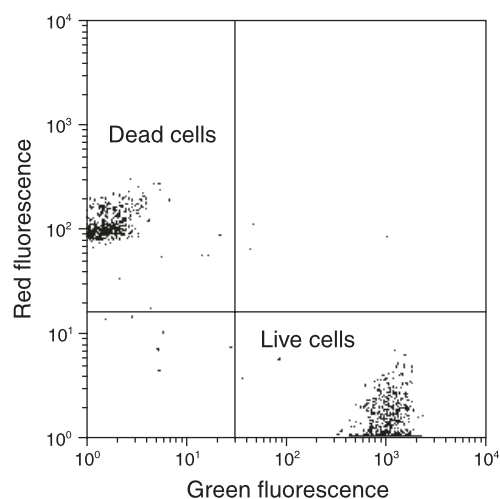


Figure 15-2. Flow cytometry viability assay using the LIVE/DEAD Viability/Cytotoxicity Kit. A 1:1 mixture of live and ethanol-fixed human B cells was stained with calcein AM and EthD-1 following the protocol provided. Flow cytometry analysis was performed with excitation at 488 nm. The resulting bivariate frequency distribution shows the clear separation of the green fluorescent (530 nm) live cell population from the red fluorescent (585 nm) dead cell population.

Markers for characterizing neural subtypes

Summary

After cells are isolated from tissue or differentiated from pluripotent precursors, the resulting population needs to be characterized to confirm whether the target population has been obtained. This chapter lists cell-type specific antibody markers commonly used for immunocytochemical (ICC) and flow cytometric analysis of neural subtypes.

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Cell-type Specific Antibodies for Characterizing Neural Subtypes

Cell type	Antigen	Antibody Cat. No.
Neural stem cells	Sox1	PA5-23351, PA5-23370
	Sox2	MA1-014, MA1-014D488, MA1-014D550, MA1-014D650, MA1-014HRP, PA1-094
	Nestin	MA1-110
	CD133	MA5-18323, PA5-38014
	Pax6	42-6600, MA1-109
Neuronal progenitors Neurons (Pan)	MAP2	MA5-12823, 13-1500, MA5-12826
	HuC/D	A21271, A21272
	Neurofilament H, M, L, Pan	711025, MA1-2012, MA1-2010, MA1-2011, 13-0500, 13-0700, 34-1000, 13-1300
	NCAM	710388, 701379, 07-5603, MA5-11563
	β III tubulin	MA1-118, 480011
	Dcx	48-1200
Dopaminergic neurons	TH	P21962, 701949, 710982
	Nurr1	MA1-195
	OTX2	701948, MA5-15854
	LMX1a	710980
Motoneurons	Isl1	MA5-15515, MA5-15516, PA5-27789
	HB9	PA5-23407
GABAergic neurons	GABA	PA5-32241
	GAD2/GAD65	39-8200, PA5-32430, PA5-22260, PA5-18369, MA5-18053
Glutamatergic neurons	vGLUT1	48-2400
	vGLUT2	42-7800
	NMDAR1	700685, 32-0500, PA3-102
	NMDAR2B	71-8600, PA3-105, A-6474, MA1-2014, 32-0700

Cell type	Antigen	Antibody Cat. No.
Cholinergic neurons	ChAT	PA1-4710, PA1-4738, PA1-9027, PA1-18313, PA5-29653
Astrocyte progenitors Astrocytes	CD44	701406, 710413, MA5-12394, MA5-13887, MA5-13890, MHCD4401, MHCD4404, RM5704
	GFAP	701406, 710413, MA5-12394, MA5-13887, MA5-13890, MHCD4401, MHCD4404, RM5704
	S100beta	701340, 710363
	Glutamine Synthetase	710963
Oligodendrocyte progenitors Oligodendrocytes	GalC	PA5-42652, PA5-27352
	NG2	37-2300, 37-2700, 41-2000
	A2B5	43-3110
	Olig2	P21954, MA5-15810
	MBP	PA5-16413
	PDGFRA	701142, 710169, PA5-16571, PA5-16742
	Olig1	PA5-21613, MA5-23954, MA5-22956
Proliferation	Ki67	701198, 710229, 08-0156, 08-1156, MA5-14520

Flow cytometry analysis

Summary

Flow cytometry is a cell analysis application that allows for single-cell multiparametric quantitative analysis of millions of cells in a short time. This provides a more comprehensive systemic analysis and understanding. Flow cytometry accomplishes this by using lasers of multiple wavelengths to excite fluorescent molecules. These fluorescent molecules can be antibody conjugates, fluorescent cellular dyes, and fluorescent proteins. The laser excites these fluorescent molecules to emit signals of varying wavelengths that are further filtered by wavelength and captured for quantitation. This ultimately allows for a more comprehensive surveying of the expression and density of particular proteins, nucleic acids, and other cell characteristics of a cell population. Flow cytometry allows for flexibility of interrogation from the simple screening of cells transfected with reporter fluorescent proteins all the way to multiparametric analysis of different cellular traits such as cell health, metabolism, DNA changes, cytokine formation, and other cell phenotypes in a large heterogeneous cell population of millions of cells.

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Required Materials

Cells

- Cells in suspension
- Adherent cells, trypsinized and in suspension buffer
- Dissociated tissue cells

Reagents and equipment

- 0.1% BSA in buffer (PBS, HBSS, etc.) or cell culture medium (Staining Buffer)
- Antibodies (primary and secondary, as needed based on experiment)
- Cell health dye reagents (e.g., Molecular Probes™ LIVE/DEAD™ reagents)
- Flow cytometer (e.g., Attune™ NxT Flow Cytometer with acoustic focusing technology)

Titration Antibodies

Determining the optimal concentration of antibody for flow cytometry

1. Dilute labeled antibodies for the appropriate antigens to be detected in staining buffer. Make dilutions of all antibodies at x1, x2, x5, x10, x20, x40, x80, and x100.
2. Prepare the cells that express the antigen to be analyzed.
3. Count the number of cells.
4. Use 1×10^6 cells for each dilution. Smaller numbers of cells ranging from 50,000 to 100,000 may work as well.
5. Centrifuge cells at $300 \times g$ for 5 minutes at 4°C and discard the supernatant.
6. Add 5 μL of antibody from each dilution into separate sample tubes containing cells.
7. Prepare negative controls of cells that have not been stained with antibody. If desired, cells labeled with an isotype control can be used.
8. Mix well and incubate cells for 25–30 minutes.

9. Wash with 3 mL of staining buffer. Discard the supernatant and resuspend the cells in 0.5 mL of staining buffer.

10. Analyze the cells by flow cytometry.

Note: Use the same cell number in every experiment. Starting with larger numbers of cells is preferred since setting up parameters during flow cytometry analysis takes time and collecting >10,000 events produces more reliable data.

Note: We recommended that you always use a dead cell stain to identify dead cells in any immunophenotyping experiment, as dead cells may nonspecifically bind antibodies and give false readings.

One-Step Staining with Fluorescently Labeled Antibodies

1. For adherent cells, trypsinize and then add staining buffer. Transfer the cells to a conical tube and centrifuge at 300 x *g* for 5 minutes. Discard the supernatant.
2. Add the appropriate amount of diluted fluorescently conjugated primary antibodies to the cell pellet.
3. Resuspend the cell pellet by gentle mixing and incubate for 25–30 minutes.
4. Wash the cells with 3 mL staining buffer. Centrifuge the cells at 300 x *g* for 5 minutes.
5. Discard the supernatant and resuspend the cells with 0.5 mL staining buffer.
6. *Optional:* Filter the cell suspension through a fine mesh filter before analysis or sorting the cells by flow cytometry.

Two-Step Staining with Biotinylated Antibodies

1. For adherent cells, trypsinize and add staining buffer. Transfer the cells to a conical tube and centrifuge at 300 x *g* for 5 minutes. Discard the supernatant.
2. Add 5 μ L of appropriately diluted biotinylated primary antibody.
3. Resuspend the cell pellet by gently mixing and incubate for 25–30 minutes.
4. Wash the cells with 3 mL staining buffer. Centrifuge the cells at 300 x *g* for 5 minutes.
5. Discard the supernatant. Add diluted streptavidin conjugated to a fluorescent tag.
6. Mix well and incubate the cells for 25–30 minutes.
7. Wash the cells with 3 mL staining buffer. Centrifuge the cells at 300 x *g*, 4°C for 5 minutes.
8. Discard the supernatant and resuspend cells with 0.5 mL of staining buffer.
9. Filter the cell suspension through a fine mesh filter before analysis or sorting the cells by flow cytometry.

Immunocytochemistry

Summary

Immunocytochemistry is a technique used to assess the presence of a specific protein or antigen in cells by use of a specific antibody that binds to it. The antibody allows visualization of the protein under a microscope. Immunocytochemistry is a valuable tool to study the presence and subcellular localization of proteins.

View this protocol online and order products at [thermofisher.com/neuroprotocol/immuno](https://www.thermofisher.com/neuroprotocol/immuno)

Required Materials

Cells

- Gibco™ Primary Rat Cortical Neurons (Cat. No. A1084001) or Gibco™ Primary Rat Hippocampal Neurons (Cat. No. A1084101)

Media and reagents

- Gibco™ Neurobasal™ Medium (Cat. No. 21103049)
- Gibco™ B-27™ Supplement (50X), serum free (Cat. No. 17504044)
- Gibco™ GlutaMAX™-I Supplement (Cat. No. 35050061)
- Gibco™ Trypan Blue Solution, 0.4% (Cat. No. 15250061)
- Gibco™ Dulbecco's Phosphate-Buffered Saline (DPBS) with Ca²⁺ and Mg²⁺ (Cat. No. 14040141)
- Gibco™ Goat Serum (Cat. No. 16210064)
- Invitrogen™ MAP2 Antibody (M13) (mouse monoclonal) (Cat. No. 13-1500)
- Invitrogen™ GFAP Antibody (rabbit, glial fibrillary acid protein) (Cat. No. PA3-16727)
- Invitrogen™ Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor™ 488 conjugate (Cat. No. A11029)
- Invitrogen™ Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor™ 594 conjugate (Cat. No. A11037)
- Invitrogen™ 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Cat. No. D1306)
- Invitrogen™ ProLong™ Gold Antifade Mountant (Cat. No. P36930)
- Paraformaldehyde (4%)
- Triton™ X-100 surfactant

Special tools

- Multi-chambered slides
- Fluorescence microscope

Methods

Treating surfaces with poly-D-lysine

Treat the multi-chambered slides used in immunocytochemistry analysis with poly-D-lysine prior to analysis, as follows:

1. Prepare a 2 mg/mL stock of poly-D-lysine in nuclease-free water. Prepare aliquots and store at -20°C .
2. Prepare a working solution of the poly-D-lysine stock from step 1 in DPBS with Ca^{2+} and Mg^{2+} at a concentration of 50 $\mu\text{g}/\text{mL}$.
3. Add 150 μL per cm^2 of poly-D-lysine in DPBS to each chamber of a multi-chambered slide (e.g., add 150 μL per chamber for an 8-chambered slide, 300 μL per chamber for a 4-chambered slide).
4. Incubate slide at room temperature for 1 hour in a laminar flow hood.
5. Aspirate the poly-D-lysine solution, and rinse 3 times with nuclease-free water.

Note: Rinse thoroughly, since extra poly-D-lysine can be toxic to the cells.

6. Leave the plates uncovered in the hood until the wells are completely dry. Plates can be used when dry or can be covered with Parafilm™ wrapper and stored at 4°C for up to two days.

Maintaining neuronal cultures

1. Thaw cryopreserved primary rat neurons according to the instructions provided with the cells.
2. Plate the cells onto a multi-chambered slide that has been treated with poly-D-lysine. Seed 1×10^5 cells per chamber in 500 μL of medium.
3. Incubate the slide at 37°C in a humidified atmosphere of 5% CO_2 in air.
4. After 24 hours of incubation, aspirate half of the medium from each well and replace with fresh medium. Return the slide to the incubator.
5. Feed the cells every third day by aspirating half of the medium from each well and replacing with fresh medium.

Immunocytochemistry analysis

1. Before proceeding, prepare a solution of 5% goat serum in DPBS with Ca^{2+} and Mg^{2+} . This solution will be used to coat the cells before antibody detection and to dilute the antibody. Prepare enough solution to completely coat the cells twice.
2. When you are ready to perform the immunocytochemistry procedure, aspirate the supernatant from each chamber and rinse the cells twice with DPBS with Ca^{2+} and Mg^{2+} .
3. Treat the cells with 4% paraformaldehyde for 20 minutes to fix them.
4. Rinse the cells 3 times with DPBS with Ca^{2+} and Mg^{2+} .
5. Permeabilize the cells with 0.3% Triton X-100 surfactant (diluted in DPBS with Ca^{2+} and Mg^{2+}) for 5 minutes at room temperature.
6. Rinse the cells 3 times with DPBS with Ca^{2+} and Mg^{2+} .
7. Add enough 5% goat serum solution from step 1 to the cells to coat them, and incubate for 60 minutes at room temperature.
8. Remove the solution from the wells and coat the cells with primary antibody (mouse anti-MAP2, 10 $\mu\text{g}/\text{mL}$, and/or rabbit anti-GFAP, 4 $\mu\text{g}/\text{mL}$) diluted in 5% goat serum solution.
9. Incubate the coated cells at 2°C to 8°C overnight.
10. Rinse the cells 3 times with DPBS with Ca^{2+} and Mg^{2+} .
11. Treat the cells with a secondary antibody (Alexa Fluor™ 488 goat anti-mouse IgG (H+L), 10 $\mu\text{g}/\text{mL}$, and/or Alexa Fluor 594 goat anti-rabbit IgG (H+L), 10 $\mu\text{g}/\text{mL}$) diluted in 5% goat serum solution.
12. Incubate for 60 minutes at room temperature.
13. Rinse the cells 3 times with DPBS with Ca^{2+} and Mg^{2+} .
14. Stain the cells with a DAPI solution (3 ng/mL) for 10 minutes.
15. Mount the cells with ProLong Gold Antifade Mountant and observe them under the microscope using filters for FITC, Cy⁵ dye, and DAPI.

Typical Results

Thawed cortical neurons cultured in complete Neurobasal medium, i.e., Neurobasal Medium supplemented with B-27 Supplement (50X), serum free and GlutaMAX-1 Supplement, show a >90% neuron population with a minimum number of astrocytes when stained with MAP2 antibody. Within 3–4 days in culture, the neurons display extensive neurite outgrowth that continues to increase as long as they are kept healthy in culture. Results vary if neurons are cultured in the presence of serum.

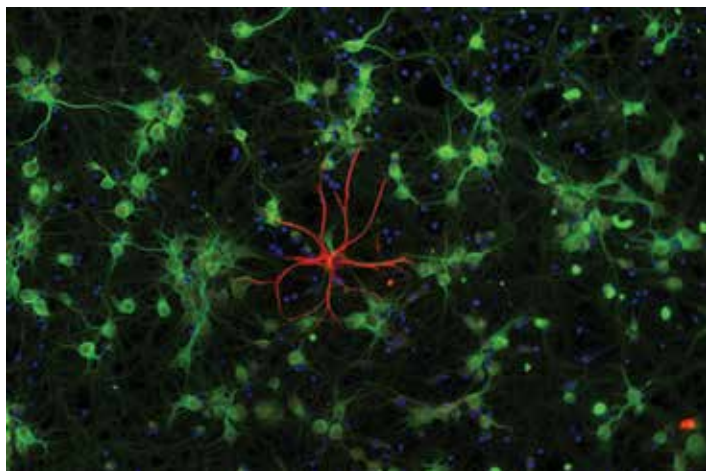


Figure 18-1. Primary rat hippocampal neurons. Immunofluorescence detection of primary neuronal cells stained with mouse anti-MAP2 antibody (green) and astrocytes stained with rabbit anti-GFAP antibody (red). Nuclei are stained with DAPI (blue).

Quantitative image analysis

Summary

Cellular imaging technology has long contributed to significant advances in cell biology research. In fact, the ability to visualize individual cells was a founding moment in the field of cell biology. As technology has continued to develop, cellular imaging progressed from a useful qualitative method to a powerful and essential quantitative analysis tool for cell-based assays. Two technological developments that have synergistically assisted the emergence of quantitative image analysis are automated fluorescence microscopes and powerful image analysis software. The automation of microscopes has enabled accurate and consistent exposure times and precise image acquisition, which are two critical factors for intensity-based comparisons of cellular markers. The development of user-friendly image analysis software with an array of dynamic image analysis algorithms and informatics tools enables scientists to make hundreds of parametric measurements and are adaptable to a broad range of cell types and applications. Quantitative image analysis is now an accurate and efficient method for cellular analysis.

This convergence of technological advancements has resulted in a new area of quantitative image analysis called **High-Content Analysis (HCA)** or High-Content Screening (HCS). HCA can be described as a set of analytical methods using automated microscopy, multi-parameter image processing, and visualization tools, to extract quantitative data from cell populations. The high-throughput and data-rich nature of HCA makes it a valuable method for biological research, cell characterization, and drug discovery studies. This chapter will present examples of implementing HCA for cell type characterization and measurement of cell type purity along a neuronal differentiation workflow. Examples of HCA data visualization will also be illustrated.

HCS Studio™ Cell Analysis Software

The **Target Activation BioApplication** is a general-purpose assay for intensity-based measurements of molecular localization with broad applicability across multiple disciplines. This assay calculates measurements of fluorescent indicators of choice on a cell-by-cell basis in up to six channels, where a channel represents a fluorophore or a specific exposure condition. An object mask is generated for channel 1 primary objects (nuclei, cell bodies, organelles, etc.), and is used to define the measurement area for fluorescent intensity in downstream channels. The software allows you to visualize object-intensity histograms for each image and enables you to set object intensity-based thresholds for each channel. Population and subpopulation analysis features allow you to quantify the percentage of positively stained cells for each channel as well as subpopulations of co-stained cells.

The **Neuronal Profiling BioApplication** enables quantification of morphological changes in neurons, allowing control over selecting neurites based on morphological as well as intensity differences. Selection of neurons is possible through the use of nucleus, cell body, and neurite object identification parameters and identifying subpopulations of neurons through quantification of multiple biological characteristics.

Implementation of HCA for Measuring Neuronal Differentiation Efficiency

The **Gibco™ PSC Dopaminergic Neuron Differentiation Kit** (Cat. No. A3147701) enables the differentiation of pluripotent stem cells (PSCs) to midbrain dopaminergic neurons. The workflow for this kit is a three-step process (Figure 19-1). hPSCs are first induced in Floor Plate Specification Medium into midbrain-specified floor plate progenitor (FP) cells. Next, FP cells are expanded as adherent cultures in Floor Plate Cell Expansion Medium and then cultured in suspension to form spheres. Finally, the spheres are differentiated into mature dopaminergic neurons in Dopaminergic Neuron Maturation Medium.

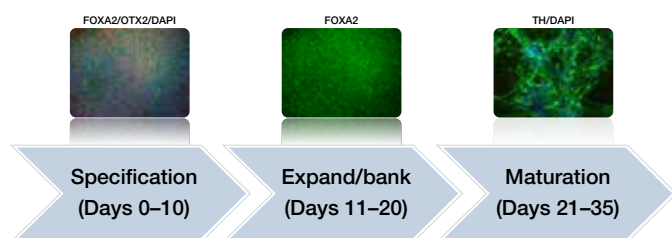


Figure 19-1. Simplified workflow diagram from midbrain-specified FP cells through expansion/banking to differentiated, mature dopaminergic neurons

To assess the efficiency of differentiation at key points along this protocol, the **Gibco Human Dopaminergic Neuron Immunocytochemistry Kit** (Cat. No. A29515) was used for optimal image-based analysis of three key markers of neural differentiation: OTX2 and FOXA2 for characterization and quantification of FP progenitor cells (Figure 19-2), and tyrosine hydroxylase (TH) for identification of matured dopaminergic neurons (Figure 19-3).

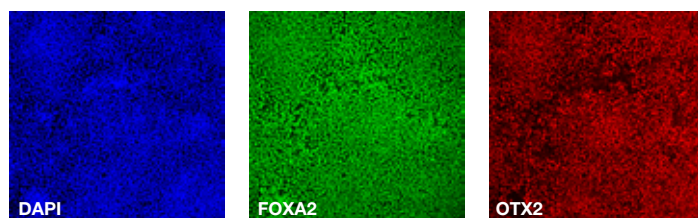


Figure 19-2. Floor plate progenitor cell characterization.

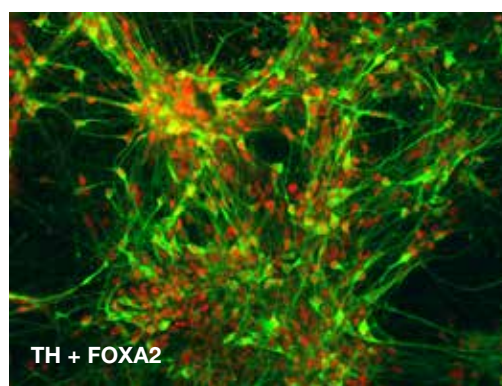


Figure 19-3. Midbrain dopaminergic neurons.

The image acquisition and analysis presented in this chapter was performed using the Thermo Scientific™ **CellInsight™ CX5 High-Content Screening (HCS) Platform** ([thermofisher.com/hcs](https://www.thermofisher.com/hcs)). The CX5 platform includes the HCS Studio Cell Analysis Software that contains over 20 pre-established and highly customizable assays.

Quantification of Percent FP Cells using HCA

The efficiency of the specification step can be measured by quantifying the percentage of FP progenitor cells using the Target Activation image analysis assay. For this example, the objective of the Target Activation assay is to measure the percentage of cells that are expressing both FOXA2 and OTX2. Channel 1 DAPI-stained nuclei are used to generate a primary object mask (Figure 19-4). Channels 2 and 3 are, respectively, the FOXA2 and OTX2 stains where intensity measurements will be performed. The primary object mask is duplicated for channels 2 and 3 where average intensity measurements are made for each object. Intensity-based thresholds are then set for each of channels 2 and 3. Once the thresholds are set, the software can calculate the percentage of co-stained cells. For the image set in Figure 19-4, the Target Activation assay calculated that 92.3% of the cells are co-stained for FOXA2 and OTX2. Figure 19-5 illustrates the data visualization features of the HCS Studio software. Figure 19-5, panel A demonstrates setting thresholds for each individual channel. Figure 19-5, panel B demonstrates the data visualization of the co-stained subpopulation of cells.

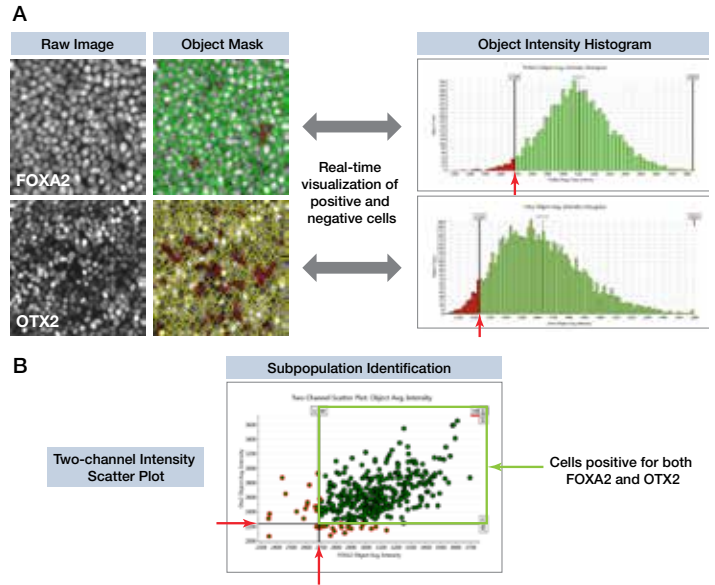


Figure 19-5. Object intensity threshold setting and subpopulation data visualization.

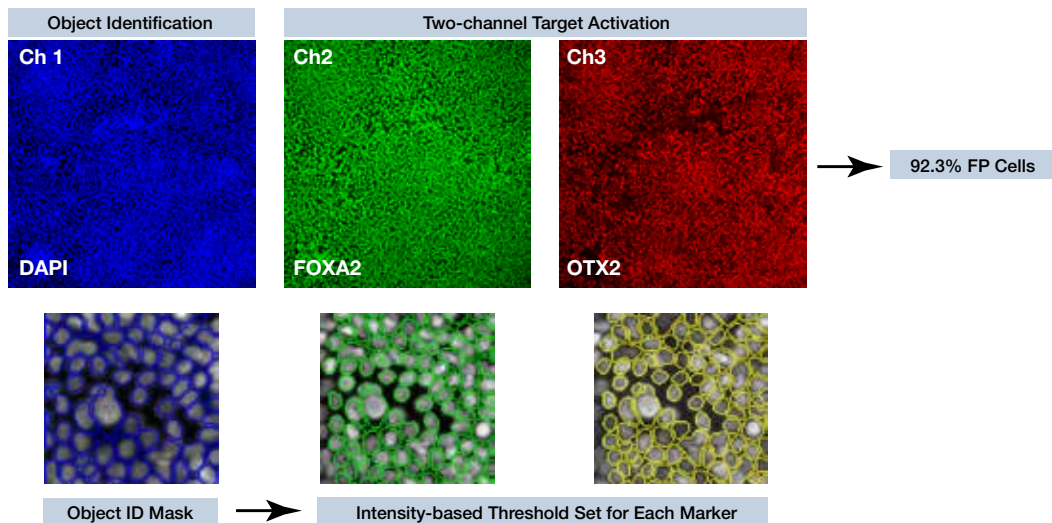


Figure 19-4. Quantification of percent FP cells.

Quantification of Percent Dopaminergic Neurons using HCA

Upon completion of the maturation step, the percentage of dopaminergic neurons can be quantified using the Neuronal Profiling assay (Figure 19-6). In channel 1, a mask is generated to identify and count DAPI-stained nuclei. For channel 2, a mask is generated to identify and count TH-positive cell bodies. The software can then calculate the percentage of TH-positive cells for each image set. For the image set in Figure 19-6, the neuronal profiling assay calculated 31.3% dopaminergic neurons.

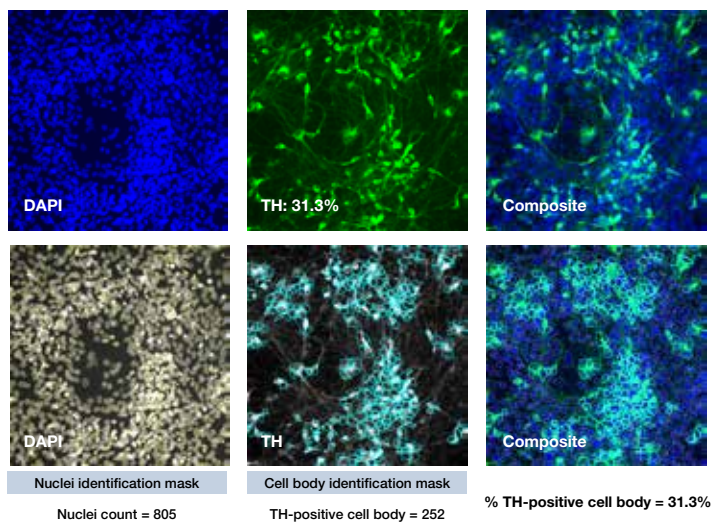


Figure 19-6. Quantification of dopaminergic neurons using the Neuronal Profiling assay.

Intracellular calcium assay using Fluo-4

Summary

The following protocol describes how to perform Fluo-4 dye-based measurements of cytosolic calcium changes in neural stem cells in response to neurotransmitter applications.

View this protocol online and order products at [thermofisher.com/neuroprotocol/fluo4](https://www.thermofisher.com/neuroprotocol/fluo4)

Required Materials

Cells

- Neural stem cells, cultured on poly-D-lysine-coated 96-well plate or other culture vessel

Reagents

- Gibco™ HBSS, with Ca²⁺ and Mg²⁺, no phenol red (Hanks' Balanced Salt Solution) (Cat. No. 14025134)
- Molecular Probes™ Fluo-4, AM, cell permeant (Cat. No. F14201)
- Molecular Probes™ Pluronic™ F-127 (20% Solution in DMSO) (Cat. No. P3000MP)
- DMSO, Anhydrous (Dimethyl sulfoxide) (Cat. No. D12345)
- Neurotransmitters or ligands (e.g., acetylcholine, glutamate)

Tools and equipment

- Fluorescence inverted microscope
- Invitrogen™ EVOS™ FL Imaging System or EVOS FL Auto Imaging System ([thermofisher.com/evos](https://www.thermofisher.com/evos))
- Invitrogen™ EVOS™ Light Cube, GFP (Cat. No. AMEP4651)

Preparing Reagents

Fluo-4 AM loading solution

Fluo-4 AM loading solution consists of 3 μM Fluo-4 AM (reconstituted in DMSO) and 0.1% Pluronic F-127 in HBSS. Use the Fluo-4 AM loading solution as soon as possible after preparation to avoid decomposition with subsequent loss of cell loading capacity.

1. To reconstitute Fluo-4 AM, add 44 μL of DMSO to one vial of Fluo-4 AM (50 μg) and vortex thoroughly. Store the Fluo-4 AM reconstituted in DMSO protected from light, frozen, and desiccated for up to one week.
2. Add 9 μL of Pluronic F-127 to the reconstituted Fluo-4 AM and vortex thoroughly.

Note: Because Fluo-4 AM is relatively insoluble in aqueous solutions, addition of the low-toxicity dispersing agent Pluronic F-127 facilitates cell loading. However, Pluronic F-127 may decrease the stability of AM esters, so it should only be added to working stocks (e.g., the loading solution).

3. Add 50 μL of the ~86 μM Fluo-4 AM/Pluronic F-127 solution to 14.3 mL of HBSS.

Loading NSCs with Fluo-4 AM loading solution

1. Wash the NSCs with 100 μL of HBSS.
2. Load the NSCs with 100 μL of Fluo-4 AM loading solution per well of a 96-well plate. Adjust the volume as appropriate for other culture vessels.
3. Incubate the NSCs in the dark at room temperature for ~60 minutes.
4. Wash the Fluo-4-loaded NSCs with 100 μL of HBSS and maintain at room temperature in the dark until data acquisition.

Data Acquisition

1. Place the 96-well plate containing the Fluo-4-loaded NSCs in an inverted microscope (e.g., EVOS FL Imaging System or EVOS FL Auto Imaging System) for visual inspection and fluorescent imaging.
2. To acquire and analyze data, define regions of interest around a random series of cells using your software of choice (e.g., MetaFluor™ software, MDS Analytical Technologies).

Note: The NSCs should display a typical neuronal morphology with dendritic and axonal processes clearly recognizable by cellular polarity and proportionate size.

3. Identify 50–100 neurons for data acquisition and analysis in each well examined.
4. Excite the NSCs with 488 nm light and collect images from 520 nm emitted light (FITC/GFP setting or EVOS GFP Light Cube) with a CCD or digital camera.
5. Challenge the cells in one well with a neurotransmitter or other ligand. For example, add 20 μL of 3 mM acetylcholine to achieve a final concentration of 500 μM acetylcholine in the well.
6. Collect the data using the appropriate software (e.g., MetaFluor software, MDS Analytical Technologies).
7. Repeat the procedure for each neurotransmitter or ligand of interest in separate wells. Use the following final concentrations for each well: 500 μM glutamate, 500 μM dopamine (add 500 μM ascorbic acid with dopamine to prevent dopamine oxidation), 500 μM γ -aminobutyric acid, and 500 μM ATP.

Data Analysis

1. Integrate the acquired Fluo-4 520 nm emission signal for each region of interest, normalize to the first ten data points (F/F_0), and then plot against time.
2. Set the response criteria. For example, an NSC might be considered responsive to a given neurotransmitter or ligand if the resulting normalized signal rises more than 10% within 60 seconds following neurotransmitter addition compared to the baseline signal. The number of NSCs that exhibit clear changes in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) depends on the neurotransmitter and differentiation state of the NSCs.

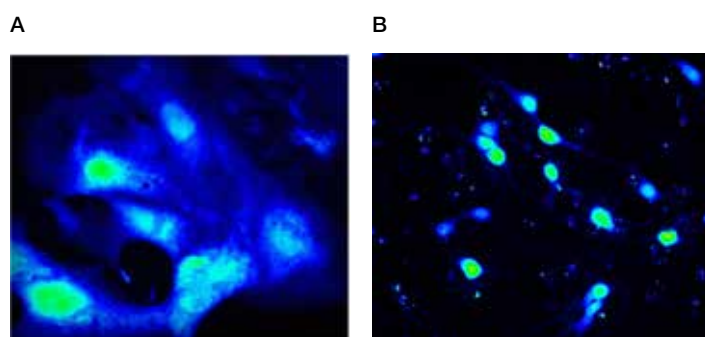


Figure 20-1. Detection of intracellular calcium using Fluo-4, AM. (A) Calcium waves in beating cardiomyocytes. (B) Calcium spikes in firing neurons.

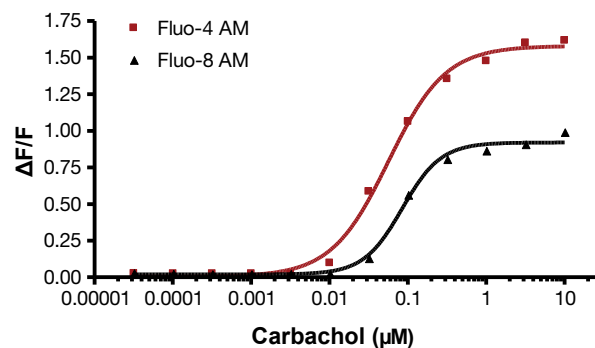


Figure 20-2. Comparison of Fluo-4 versus Fluo-8. Similar EC_{50} values are observed with Fluo-4 and Fluo-8 AM dyes for a dose response of carbachol on M1 CHO-K1 cells, but the $\Delta F/F$ (signal to background) is higher for Fluo-4, AM.

Measuring membrane potential using the FluoVolt Kit

Summary

The FluoVolt™ membrane potential dye represents the next generation in voltage-sensitive probes and brings together the best characteristics of the fast- and slow-response membrane potential probes: it has a sub-millisecond response time to changes in membrane potential and displays a high magnitude of response. The following protocol describes how to perform FluoVolt™ dye-based measurements of changes in membrane potential.

View this protocol online and order products at [thermofisher.com/order/catalog/product/F10488](https://www.thermofisher.com/order/catalog/product/F10488)

Required Materials

Cells

- Neural stem cells, cultured on poly-D-lysine-coated 96-well plate or other culture vessel

Media and reagents

- Molecular Probes™ FluoVolt™ Membrane Potential Kit (Cat. No. F10488)
 - FluoVolt Dye (1000X concentrate)
 - PowerLoad™ Concentrate (100X)
 - Neuro Background Suppressor (10X concentrate)
- Molecular Probes™ Live Cell Imaging Solution (LCIS) (Cat. No. A14291DJ)
- Gibco™ HBSS, with Ca²⁺ and Mg²⁺, no phenol red (Hanks' Balanced Salt Solution) (Cat. No. 14025134)
- Invitrogen™ EVOS™ FL Imaging System or EVOS™ FL Auto Imaging System ([thermofisher.com/evos](https://www.thermofisher.com/evos))
- Molecular Probes™ Valinomycin (Cat. No. V1644)
- *Optional:* Glucose (for preparing 20 mM Glucose Stock in LCIS)
- *Optional:* Molecular Probes™ Probenecid, Water Soluble (Cat. No. P36400)

Preparing Reagents

Preparing FluoVolt loading solution

The protocol below provides instructions for performing the membrane potential assay using cells grown in a 35 mm dish with 2 mL of culture medium.

To a 15 mL tube, add the following reagents in the order listed below to prepare fresh FluoVolt loading solution:

Component	Amount
100X PowerLoad Concentrate	100 µL
FluoVolt dye, 1,000X	10 µL
Vortex to mix.	
Physiological buffer of choice or 20 mM Glucose Stock in LCIS	10 mL
Invert the tube to mix.	
<i>Optional:</i> 100X Probenecid stock solution*	100 µL

* Add 100 µL of 100X Probenecid stock solution to prevent extrusion of cytosolic dye by anion pumps, which can decrease loading efficiency on some cell types.

Preparing 20 mM glucose stock in LCIS

Dilute a 2 M glucose stock solution 1:100 into LCIS for a final glucose concentration of 20 mM. Keep this solution clean and free of contaminants to prevent bacterial, fungal, or yeast growth once glucose has been added.

Loading NSCs with FluoVolt Membrane Potential Dye

1. Remove medium from adherent cells and wash cells twice in physiological buffer of choice or LCIS.
2. Add 2 mL of FluoVolt loading solution (page 90) to cells, and incubate cells at room temperature for 15–30 minutes.
3. Remove FluoVolt loading solution, and wash cells twice in physiological buffer of choice or LCIS.
4. Add 2 mL of physiological buffer of choice or 20 mM glucose stock in LCIS. Cells are now ready for live-cell imaging.

Optional: To suppress background fluorescence, add 1:10 diluted Neuro Background Suppressor solution.

Image Cells Loaded with FluoVolt Dye

Standard FITC settings can be used to visualize the membrane staining of FluoVolt dye. Short exposures (10 milliseconds or less) are possible with pixel 2 x 2 binning or greater, but will depend on hardware configurations to measure rapid or successive depolarizations. To confirm positive responses from the dye, treat cells with 10 μ M Valinomycin (a potassium ionophore) for 30 minutes, and then add an equal volume of isotonic potassium chloride (KCl) solution to depolarize the cells.

Note: Isotonic KCl is composed of 140 mM KCl, 5 mM NaCl, 1.8 mM CaCl_2 , 1.0 mM MgCl_2 , 20 mM HEPES, 20 mM Glucose and adjusted to pH 7.4 with NaOH.

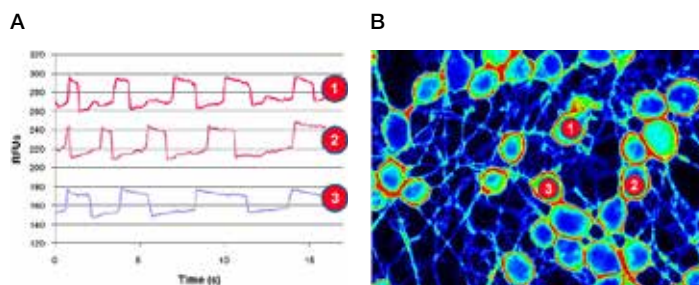


Figure 21-1. Differentiated NG-108 cells were loaded with FluoVolt membrane potential dye. Cells were imaged with 10 millisecond illumination pulses and images acquired with 2X binning. The three selected traces (A) show fluorogenic responses from the dye as selected cells (B) spontaneously depolarize and repolarize in culture.

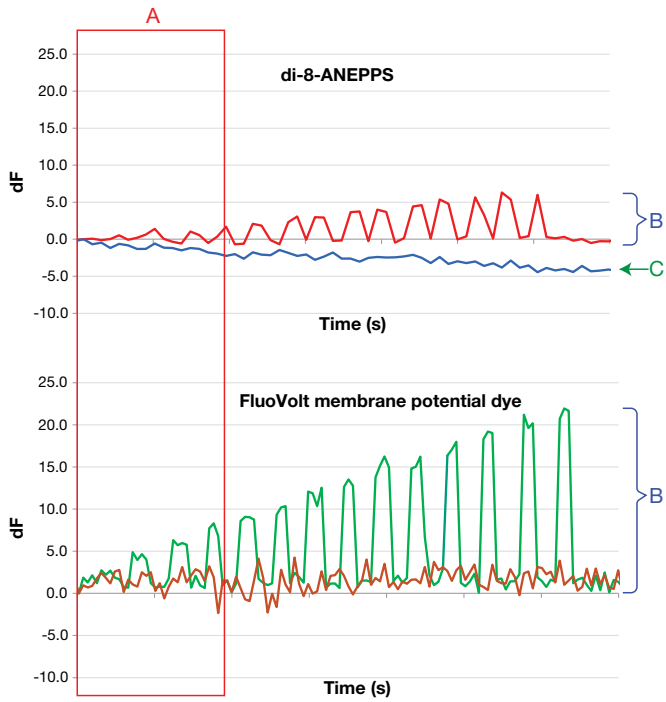


Figure 21-2. FluoVolt membrane potential dye versus di-8-ANEPPS.
(A) Weaker signals, detected earlier by the FluoVolt membrane potential dye. A series of 10 mV steps in voltage clamp from -100 to $+30$ mV. Changes as small as 10 mV can be detected with the FluoVolt membrane potential dye. **(B)** Greater magnitude of response displayed with the FluoVolt membrane potential dye. **(C)** Unclamped control cell loaded with di-8-ANEPPS shows photobleaching.

Molecular Characterization

PCR primers for molecular characterization of neural subtypes

Summary

After cells are isolated from tissue or differentiated from pluripotent precursors, the resulting populations should be characterized to confirm the presence of the target population of cells. Further analysis can help define molecular mechanisms underlying biological differences between different groups of cells (donors, treatments, etc). The table below lists PCR primers that can be used in quantitative polymerase chain reactions (qPCR) to measure the expression levels of specific genes for characterizing neural stem cells (NSCs), mature neurons, and neural subtypes as well as the neuronal supporting cell types,

including oligodendrocytes and astrocytes. Custom primers for qPCR expression analysis using Applied Biosystems™ TaqMan™ probe-based assays and SYBR™ Green dye-based master mixes can be designed and purchased on our website ([thermofisher.com/primers](https://www.thermofisher.com/primers)). Alternatively, preconfigured primers and arrays can be searched by disease, pathways, or biological process.

View this protocol online and order products at [thermofisher.com/pcr](https://www.thermofisher.com/pcr)

Target	Primer	Sequence	T _m (°C)	Amplicon size (bp)	Intron size (bp)
Neural stem cells	SOX1-F	GCGGAAAGCGTTTTCTTG	53.0	406	No intron
	SOX1-R	TAATCTGACTTCTCCTCCC	50.2		
	SOX2-F	ATGCACCGCTACGACGTGA	59.3	437	No intron
	SOX2-R	CTTTTGCACCCCTCCCATTT	56.0		
	NESTIN-F	CAGCGTTGGAACAGAGGTTGG	58.6	389	1,142
	NESTIN-R	TGGCACAGGTGTCTCAAGGGTAG	60.7		
Oligodendrocytes	MAG-F	TCTGGATTATGATTCAGCC	49.7	366	159
	MAG-R	GCTCTGAGAAGGTGTAAGTGG	54.7		
	OSP-F	ACTGCTGCTGACTGTTCTTC	55.1	283	5,714
	OSP-R	GTAGAAACGGTTTTACCAA	50.8		
Astrocytes	ALDH1L1-F	TCACAGAAGTCTAACCTGCC	55.5	398	21,837
	ALDH1L1-R	AGTGACGGGTGATAGATGAT	54.4		
	GFAP-F	GTACCAGGACCTGCTCAAT	55.0	321	2,989
	GFAP-R	CAACTATCCTGCTTCTGCTC	55.3		
Neurons	MAP2-F	CCACCTGAGATTAAGGATCA	55.1	482	11,798
	MAP-R	GGCTTACTTTGCTTCTCTGA	55.0		
	ChAT-F	ACTGGGTGTCTGAGTACTGG	55.0	451	7,692
	ChAT-R	TTGGAAGCCATTTGACTAT	54.9		
Endogenous control	ACTB-F	ACCATGGATGATGATATCGC	58.2	281	135
	ACTB-R	TCATTGTAGAAGGTGTGGTG	54.4		

Target	Primer	Sequence	T _m (°C)	Amplicon size (bp)	Intron size (bp)
GABAergic/ glutamatergic neurons	GAD1-F	GTCGAGGACTCTGGACAGTA	55.3	357	12,277
	GAD1-R	GGAAGCAGATCTCTAGCAAA	54.9		
Serotonergic neurons	SLC6A4-F	GCCTTTTACATTGCTTCCTA	54.8	447	2,251
	SLC6A4-R	CCAATTGGGTTTCAAGTAGA	55.2		
Cholinergic neurons	ChAT-F	ACTGGGTGTCTGAGTACTGG	55.0	451	7,692
	ChAT-R	TTGGAAGCCATTTGACTAT	54.9		
Dopaminergic neurons	TH-F	TCATCACCTGGTCACCAAGTT	56.0	126	656
	TH-R	GGTCGCCGTGCCTGTACT	60.0		

RNA isolation and cDNA preparation from neural cells

Summary

A rapid method of analysis for determining the identity of neural stem cells (NSCs), mature neurons (and specific subtypes), and glial cells involves the early detection of differentiation and lineage-specific markers tracked at the RNA level. This protocol follows methodologies described in the PureLink™ RNA Mini Kit manual for isolating total RNA from neuronal cell types, followed by cDNA synthesis using SuperScript™ IV Reverse Transcriptase. The following protocol gives you a step-by-step procedure for template preparation required for RT-PCR or RT-qPCR.

View this protocol online and order products at [thermofisher.com/neuroprotocol/rna](https://www.thermofisher.com/neuroprotocol/rna)

Required Materials

Cells

- Neuronal cell types of interest

Reagents and equipment

- Invitrogen™ PureLink™ RNA Mini Kit (Cat. No. 12183018A)
 - RNA Lysis Buffer
 - Wash Buffer I
 - Wash Buffer II
 - RNase-free water
 - RNA spin cartridges
 - Collection tubes
 - RNA recovery tubes
- Gibco™ 2-Mercaptoethanol (Cat. No. 21985023)

- Invitrogen™ SuperScript™ IV First-Strand Synthesis System (Cat. No. 18091050)
 - SuperScript IV Reverse Transcriptase
 - 5X SSIV Buffer Mix
 - 10 mM dNTP Mix
 - 0.1 M DTT
 - 50 μM oligo(dT)₂₀
 - Random hexamers (50 ng/mL)
 - Ribonuclease inhibitor
 - Ribonuclease H (RNase H)
- 70% ethanol
- Gibco™ TrypLE™ Express Enzyme (1X), no phenol red (Cat. No. 12604013)
- Gibco™ Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca²⁺ and Mg²⁺ (Cat. No. 14190144)
- Invitrogen™ Ribonuclease H (RNase H) (Cat. No. 18021071)
- Invitrogen™ BlueJuice™ Gel Loading Buffer (10X) (Cat. No. 10816015)
- Tabletop centrifuge
- DEPC-treated water

RNA Isolation

Isolating RNA

Important: Perform all steps on ice unless noted otherwise. For all incubations, heat the thermocyclers in advance. Prechill all reagents and thaw all frozen reagents and cells immediately prior to use. To prevent RNase contamination, wear disposable gloves while handling all materials and use of sterile disposable plasticware and RNase-free pipette tips with aerosol barriers is recommended. Always wear appropriate personal protective equipment (PPE) when working in a laboratory environment.

1. Prepare RNA Lysis Solution by adding 10 μL 2-mercaptoethanol per mL of RNA Lysis Solution.

- Remove media from T-25 flasks, rinse once with Dulbecco's phosphate-buffered saline (DPBS) without Ca^{2+} and Mg^{2+} , and treat cells with 1 mL of pre-warmed TrypLE Express Enzyme for 10 minutes at 37°C.
- Harvest the cells and place them into 15 mL centrifuge tubes. Take 100 μL of the sample and obtain a viable cell count.
- Centrifuge the cells in a tabletop centrifuge for 7 minutes at 100 x *g*. Discard the supernatant.
- Freeze the cells overnight in a -80°C freezer; frozen cell pellets can be stored at least a month if desired. Alternatively, freshly isolated cell pellets can be processed directly by going to step 6.
- Allow the cell pellet to thaw. Add 0.5 mL of RNA Lysis Solution for each T-25 flask harvested for the pellet (0.5 mL per 2×10^6 to 5×10^6 cells). Pipet the cells ~20 times until the pellet is disrupted.
- Transfer 0.5 mL of cell lysis solution to 1.5 mL RNase-free microcentrifuge tubes and centrifuge at room temperature for 2 minutes at 12,000 x *g* (12,000 rpm).
- Add 0.5 mL (1 volume) of 70% ethanol to each tube, and vortex the suspension 5–10 times.
- Apply a 600 μL aliquot of sample to the RNA Spin Cartridge. Centrifuge at room temperature for 15–30 seconds at 12,000 x *g*, then discard the flow-through. Continue applying 600 μL aliquots of the same RNA sample to the spin cartridge until the entire sample has been processed.
- Add 700 μL Wash Buffer I to the spin cartridge and centrifuge at room temperature for 15–30 seconds at 12,000 x *g*. Discard the flow-through and the tube. Place the spin cartridge into a clean 2 mL RNA Wash Tube.
- Add 500 μL Wash Buffer II (containing ethanol) to the spin cartridge and centrifuge at room temperature for 15–30 seconds at 12,000 x *g*. Discard the flow-through.
- Repeat step 11.
- Centrifuge for 1 minute to dry the cartridge.
- Place the cartridge into a clean RNA Recovery Tube. Add 40 μL of RNase-free water to the cartridge, and let it stand for 1 minute. Centrifuge the cartridge at room temperature for 2 minutes at 12,000 x *g*. Add an additional 40 μL of RNase-free water to the cartridge and repeat the step. Yield should be about 60–300 μg total RNA.

Note: Always allow time for the RNase-free water to percolate into the cartridge bed. Do not spin the cartridge immediately because it may result in partial recovery and alter the yield of RNA.

Determining RNA quality

- Measure ratio of absorbance at 260 nm and 280 nm by analyzing 1 μL of the RNA sample using a Thermo Scientific™ NanoDrop™ spectrophotometer. Conduct readings 3 times, and use the average as the final value. Wipe down the analysis stage with a lab tissue wetted with DEPC-treated water before and after measuring each RNA sample. The A_{260}/A_{280} of pure RNA is ~2.

Note: The yield and quality of the isolated RNA depends on the type and age of the starting material, in addition to how the material was collected and preserved.

2. Prepare the RNA samples for RNA gel analysis as follows:

Component	Amount
RNA sample	1 μ L
BlueJuice Gel Loading Buffer (10X)	1 μ L
DEPC-treated water	8 μ L

3. Mix the components and load the samples onto individual wells of an agarose gel. Use 10 μ L of 0.1 kb and 1 kb molecular weight markers to estimate the molecular weight size of ribosomal RNA bands. Use 10 μ L DEPC-treated water for empty wells. Run samples for 30 minutes, visualize the bands on an UV light box, capture the gel image, and perform band intensity measurements.

RNA storage

Store RNA samples at -80°C or process them further for cDNA synthesis.

cDNA Preparation

First-Strand cDNA synthesis

This protocol follows the methodologies described in the instructions for SuperScript IV First-Strand Synthesis System.

- Mix and briefly centrifuge each component before use. Pre-heat the thermocycler to 65°C . Note that oligo (dT)₂₀ may be substituted for random hexamers primer solution.
- Combine the following components on ice in a 0.2 mL thin-walled PCR tube. Use a volume containing up to 1 μ g of total RNA for the reaction.

Component	Amount
10 mM dNTP Mix	1 μ L
Random hexamers (50 ng/ μ L)	1 μ L
RNA (1 μ g)	x μ L
DEPC-treated water	to 13 μ L

- Incubate the reaction in the thermocycler at 65°C for 5 minutes, and then immediately place on ice for at least 1 minute. Collect the contents of the tube by brief centrifugation.
- Vortex and briefly centrifuge the 5X SSIV Buffer Mix.
- Prepare the reverse transcriptase reaction mix in a separate tube using the components listed below:

Component	Amount
5X SSIV Buffer Mix	4 μ L
100 mM DTT	1 μ L
Ribonuclease Inhibitor	1 μ L
SuperScript IV Reverse Transcriptase	1 μ L

- Cap the tube, mix, and then briefly centrifuge the contents.
- Add the reverse transcriptase reaction mix to the annealed RNA.
- Incubate the tube at 50°C for 10 minutes.
- Terminate reaction by incubating at 80°C for 10 minutes, then chill the tube on ice.
- Add 1 μ L of RNase H to the sample, and incubate at 37°C for 20 minutes.
- Store the cDNA samples at -20°C or proceed to PCR amplification.

Characterizing neural cells by qPCR

Summary

Quantitative polymerase chain reaction (qPCR) is one of the most accurate and sensitive methods for studying gene regulation, and can be used to measure the expression levels of specific genes in a wide variety of neuronal cell models. Understanding gene patterns can provide significant insight into diverse biological processes including the regulation of differentiation and maturation, the impact of different cell-to-cell interactions, and mechanisms of disease and aging.

Here we provide guidelines and a general protocol for performing qPCR using the Applied Biosystems™ 7300 Real-Time PCR System and Platinum™ SYBR™ Green qPCR SuperMix-UDG with ROX™ Reference Dye.

View this protocol online and order products at thermofisher.com/neuroprotocol/qpcr

Required Materials

Starting material

- cDNA generated from total RNA isolated from neural cells (see “RNA isolation and cDNA preparation from neural cells” on page 95).

Media and reagents

- Invitrogen™ Platinum SYBR Green qPCR SuperMix-UDG (Cat. No. 11733038)
- Custom primers (thermofisher.com/oligos)
- DEPC-treated water

Special tools

- Applied Biosystems™ 7300 Real-Time PCR System or similar instrument
- 0.2 mL microcentrifuge tubes or 96-well or 384-well PCR plates
- Vortex mixer
- Microcentrifuge

Methods

Template preparation

For qPCR, prepare a 1:10 dilution series of cDNA generated from 10 pg to 1 µg of total RNA using the protocol described in RNA Isolation and cDNA preparation from neural cells (page 95).

Real-time PCR instruments

Platinum SYBR Green qPCR SuperMix-UDG can be used with a variety of real-time PCR instruments, including but not limited to the following Applied Biosystems™ instruments: StepOne™, ViiA™ 7, StepOnePlus™, 7300, and 7500 Real-Time PCR Systems; ABI PRISM™ 7000, 7700, and 7900HT systems; and GeneAmp™ 5700 system. Optimal cycling conditions will vary with different instruments.

Primer design

Primer design is one of the most important parameters when using a SYBR Green qPCR detection system. We strongly recommend using a primer design program such as the OligoPerfect™ Designer (thermofisher.com/oligos) or Vector NTI Advance™ software (thermofisher.com/vectorntiadvance). When designing primers, the amplicon length should be approximately 80–250 bp and should not exceed 500 bp. Optimal results may require a titration of primer concentrations between 100 nM and 500 nM. A final concentration of 200 nM per primer is effective for most reactions.

ROX Reference Dye

ROX Reference Dye is recommended to normalize the fluorescent reporter signal for instruments that are compatible with that option. ROX dye is supplied as a separate tube in Platinum SYBR Green qPCR SuperMix-UDG at a 25 µM concentration. Use the following table to determine the amount of ROX to use with a particular instrument.

Instrument	Amount of ROX dye per 50 µL reaction	Final ROX dye conc.
Applied Biosystems 7300, 7000, 7700, 7900HT, and 7900HT Fast	1.0 µL	500 nM
Applied Biosystems 7500	0.1 µL	50 nM

Protocol for qPCR

In this section we provide a step-by-step protocol for qPCR on the Applied Biosystems 7300 Real-Time PCR System in 20 µL assays. For protocols for specific instruments, visit thermofisher.com/qpcr

1. Program your real-time instrument as shown below. Optimal temperatures and incubation times may vary.
 - 50°C for 2 minutes (UDG incubation)
 - 95°C for 2 minutes
 - 40–50 cycles of:
 - 95°C, 15 seconds
 - 60°C, 30 seconds

Melting curve analysis: Program the instrument for melting curve analysis to identify the presence of primer dimers and analyze the specificity of the reaction. A typical melting curve program is listed below (see your instrument documentation for details):

- 95°C for ~30 seconds
- 45°C for ~30 seconds
- 99°C for ~30 seconds

Use a 2% ramp rate with data collection from 45°C to 99°C.

Note: For the following steps, do not touch the bottom of each tube, and be sure to use powder-free gloves to handle all reagents and plasticware.

- For each reaction, add the following components to a 0.2 mL microcentrifuge tube or each well of a PCR plate. Volumes for a single 20 μ L reaction are listed. For multiple reactions, prepare a master mix of common components, add the appropriate volume to each tube or plate well, and then add the unique reaction components (e.g., template). For no-template controls, add an equivalent volume of water in lieu of template.

Component	Amount
Platinum SYBR Green qPCR SuperMix-UDG	10 μ L
ROX Reference Dye (amount specified for 7300 system)	0.4 μ L
Forward primer, 10 μ L	0.4 μ L
Reverse primer, 10 μ L	0.4 μ L
Template cDNA (1:10 dilution series from 10 pg to 1 μ g total RNA)	1–2 μ L
DEPC-treated water	to 20 μ L

- Cap or seal the reaction tube/PCR plate, and gently mix. Make sure that all components are at the bottom of the tube/plate; centrifuge briefly if needed.
- Place the reactions in a preheated real-time instrument programmed as described in step 1. Collect the data and analyze the results using the instrument software.

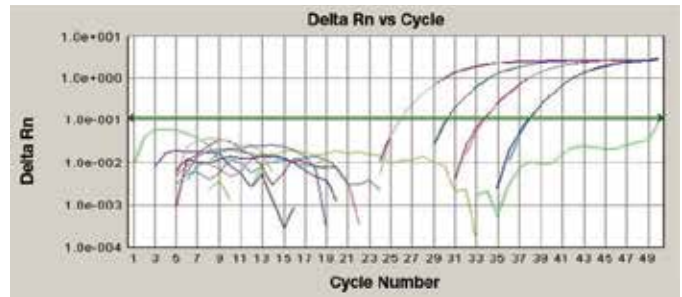


Figure 24-1. qPCR detection of nestin transcripts in human embryonic stem cell-derived NSCs.

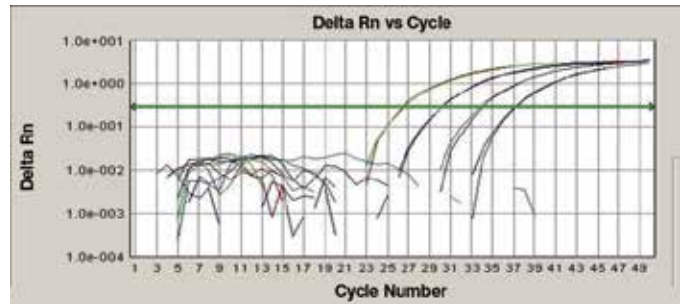


Figure 24-2. qPCR detection of Sox1 transcripts in human embryonic stem cell-derived NSCs.

Transfection

Lipid-mediated transfection of neuronal cells

Culture Conditions

The following table summarizes the culture conditions for various neural cell lines, including neural stem cells. For detailed instructions on culturing and passaging these

cells, refer to the to the instructions supplied with the specific cell line you are using.

Cell type	Media	Culture conditions
Human Neural Stem Cells	Complete Gibco™ StemPro™ NSC SFM ¹	<ul style="list-style-type: none"> Adherent culture on Gibco™ CELLstart™ CTS™ Substrate-, fibronectin-, or poly-L-ornithine-coated culture vessels 37°C, humidified atmosphere of 5% CO₂ in air Exchange spent medium every other day
Human Astrocytes	Complete Gibco™ Astrocyte Medium ¹	<ul style="list-style-type: none"> Adherent culture on Gibco™ Geltrex™ matrix-coated tissue culture vessels 37°C, humidified atmosphere of 5% CO₂ in air Exchange spent medium every 3–4 days
Rat Fetal Neural Stem Cells	Complete StemPro NSC SFM ¹	<ul style="list-style-type: none"> Adherent culture on CELLstart CTS Substrate-, fibronectin-, or poly-L-ornithine-coated culture vessels 37°C, humidified atmosphere of 5% CO₂ in air Exchange spent medium every 3–4 days
Rat Primary Cortical Astrocytes	Complete Astrocyte Medium ^{1,2}	<ul style="list-style-type: none"> Adherent culture on standard culture vessels 37°C, humidified atmosphere of 5% CO₂ in air Exchange spent medium every 2–3 days
Rat Glial Precursor Cells	Complete StemPro NSC SFM ¹ , supplemented with 10 ng/mL PDGF-AA	<ul style="list-style-type: none"> Adherent culture on CELLstart CTS Substrate- or poly-L-ornithine-coated culture vessels 37°C, humidified atmosphere of 5% CO₂ in air Exchange spent medium every other day

¹ See Media Preparation on page 111 for instructions on preparing complete StemPro NSC SFM and complete Astrocyte Medium.

² For increased proliferation of rat astrocytes, you can supplement complete Astrocyte Medium (DMEM with 1X N-2 Supplement and 10% One Shot™ FBS) with 20 ng/mL EGF. Adding EGF to human astrocyte cultures can increase proliferation, but may result in morphological or phenotypic changes.

Summary

The following protocols provide instructions for lipid-mediated transfection of plasmid DNA, siRNA, or mRNA into various neural cells using Invitrogen™ Lipofectamine™ 3000, Lipofectamine 2000, Lipofectamine RNAiMAX™, and Lipofectamine MessengerMAX™ Transfection Reagents.

- Lipofectamine 3000 Reagent is a next-generation, broad-spectrum reagent for the delivery of plasmid DNA into immortalized neural cells with low cytotoxicity.
- Lipofectamine 2000 Reagent is a broad spectrum, animal origin-free formulation recommended for the delivery of plasmid DNA into primary neurons with low cytotoxicity.
- Lipofectamine RNAiMAX is a leading RNAi transfection reagent optimized for the transfection of siRNA and Invitrogen™ Stealth RNAi™ duplexes into all neuronal-based cell types, immortalized and primary.
- Lipofectamine MessengerMAX reagent is an mRNA transfection reagent that offers an alternative strategy to transfect astrocytes, neural stem cells, neurons, and other primary cells. If you are not satisfied with the results obtained transfecting plasmid DNA, we highly recommend this as an alternative for superior transfection results. Visit [thermofisher.com/messengermax](https://www.thermofisher.com/messengermax) to learn more about the benefits of mRNA transfection.

View this protocol online and order products at [thermofisher.com/transfection](https://www.thermofisher.com/transfection)

Best Practices

The following guidelines are meant to improve your transfection workflow and its subsequent results.

- While our standard protocol has been significantly simplified to reduce the number of optimization parameters, we recommend that you use best practices to further optimize your specific experimental protocol.
- While not always necessary, if you use antibiotics during transfection, test your conditions thoroughly. Note that adding antibiotics to media during transfection may result in cell death.
- Maintain the same seeding conditions between experiments. When using immortalized cell lines, try to use post-thaw cells between passages 4 and 25; ensure the cells are healthy, and are between 70–90% confluent before transfection. In some instances, certain cells achieve better results at lower confluency. In addition, be sure to maintain consistent confluency across each well.
- Use Gibco™ Opti-MEM™ I Reduced Serum Medium to dilute the payload (DNA, siRNA, or mRNA) and the appropriate transfection reagent. Transfections can be performed both in the presence or absence of serum. If serum-free medium is required, be sure to test it for compatibility with the transfection reagent.

Optimization: A How-to Example

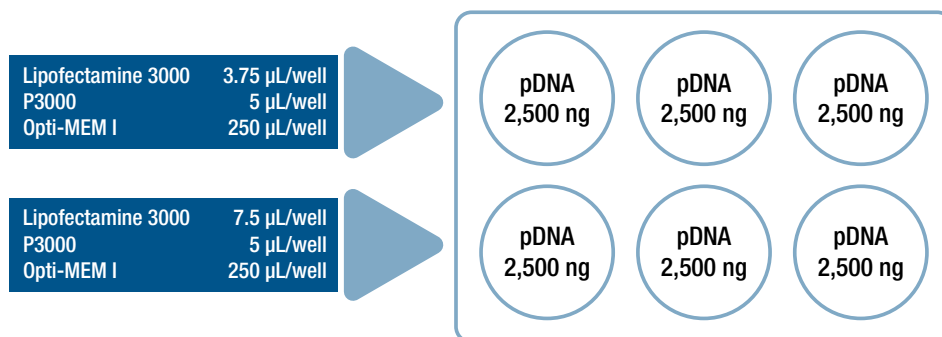
Optimizing your transfection experiment is one of the most important steps in achieving the best results. The final results can be heavily influenced by the number of cells, cell confluency, payload quality, incubation time, culturing medium, and other factors.

The example below helps you plan initial optimization experiments using the standard transfection protocols as the starting points. It varies reagent doses and incubation times, but allows for adjustment of other parameters, including payload amounts, as needed.

Example

Plate format	6-well	
Cells	SH-SY5Y: 70% confluent	
Payload	Plasmid DNA: 2,500 ng	Constant
Recommended reagent doses*	Lipofectamine 3000 reagent: 3.75 μ L/well and 7.5 μ L/well	Variable
Reagent 2	P3000™ reagent: 5 μ L/well	Constant
Medium	Opti-MEM I Reduced Serum Medium: 250 μ L/well	Constant
Incubation time	2 days, 3 days, or 4 days at 37°C	Variable

Day 2–4 incubation at 37°C



* The recommended doses for Lipofectamine 3000 reagent were identified through extensive testing. It was determined that these two doses were the most effective across dozens of cell types.

Transfecting Plasmid DNA (Immortalized Neuronal Cells and Neural Stem Cells)—Protocol

Materials needed

- Plasmid DNA of interest (0.5–5 µg/µL)
- Invitrogen™ Lipofectamine™ 3000 Reagent (Cat. No. L3000008)
 - Lipofectamine 3000 reagent
 - P3000 reagent
- Gibco™ Opti-MEM™ I Reduced Serum Medium (Cat. No. 31985062)
- Eppendorf tubes

Note: The following protocol has two reagent doses (dose 1 and dose 2) that are needed for the initial optimization only.

Step	Description	Component	6-well format*	
			Dose 1/well	Dose 2/well
1	Seed cells to be 70–90% confluent at transfection	Adherent cells	0.25–1 x 10 ⁶	0.25–1 x 10 ⁶
2	Prepare reagent mix: Dilute Lipofectamine 3000 reagent in Opti-MEM I Reduced Serum Medium	Opti-MEM I medium	125 µL	125 µL
		Lipofectamine 3000 reagent	3.75 µL	7.5 µL
2a	Vortex reagent mix		2–3 seconds	
3	Dilute DNA: Prepare master mix of DNA by diluting DNA in Opti-MEM I medium, then add P3000 reagent	Opti-MEM I	125 µL	125 µL
		DNA	2.5 µg (2,500 ng)	2.5 µg (2,500 ng)
		P3000 reagent (2 µL/µg DNA)	5 µL	5 µL
4	Add diluted DNA (step 3) to each tube of reagent mix (step 2) in a 1:1 ratio	Reagent mix	125 µL	125 µL
		Diluted DNA	125 µL	125 µL
4a	Incubate DNA-reagent complex		10–15 minutes at room temperature	
5	Add DNA-reagent complex to cells			
6	Incubate transfected cells		Incubate cells for 2–4 days at 37°C	
7	Analyze the transfected cells			

* See the experimental scaling table below to adjust for other plate formats.

Experiment scaling

Culture vessel	Confluency	Reagent mix 1 (step 2)			Diluted DNA (step 3)		
		Dose 1	Dose 2	Opti-MEM I	P3000	DNA	Opti-MEM I
6 well	0.25–1 x 10 ⁶	3.75 µL	7.5 µL	125 µL	5 µL	2.5 µg (2,500 ng)	125 µL
24 well	0.5–2 x 10 ⁵	0.75 µL	1.5 µL	25 µL	1 µL	0.5 µg (500 ng)	25 µL
96 well	1–4 x 10 ⁴	0.15 µL	0.3 µL	5 µL	0.2 µL	0.1 µg (100 ng)	5 µL

Transfecting Plasmid DNA (Primary Neuronal Cells)–Protocol

Materials needed

- Plasmid DNA of interest (100 ng/μL or higher)
- Invitrogen™ Lipofectamine™ 2000 Transfection Reagent (Cat. No. 11668030)
- Gibco™ Opti-MEM™ I Reduced Serum Medium (Cat. No. 31985062)
- Eppendorf tubes

Note: The following protocol has four reagent doses (dose 1–4) that are needed for the initial optimization only.

Step	Description	Component	6-well format*			
			Dose 1/well	Dose 2/well	Dose 3/well	Dose 4/well
1	Seed cells to be 70–90% confluent at transfection	Adherent cells	0.25–1 x 10 ⁶	0.25–1 x 10 ⁶	0.25–1 x 10 ⁶	0.25–1 x 10 ⁶
2	Prepare reagent mix: Dilute Lipofectamine 2000 reagent in Opti-MEM I Reduced Serum Medium and transfer to an Eppendorf tube	Opti-MEM I medium	150 μL	150 μL	150 μL	150 μL
		Lipofectamine 2000 reagent	6 μL	9 μL	12 μL	15 μL
2a	Vortex reagent mix		2–3 seconds			
3	Dilute DNA in Opti-MEM I Reduced Serum Medium and transfer to an Eppendorf tube	Opti-MEM I medium	150 μL	150 μL	150 μL	150 μL
		DNA	2.5 μg (2,500 ng)	2.5 μg (2,500 ng)	2.5 μg (2,500 ng)	2.5 μg (2,500 ng)
4	Add diluted DNA (step 3) to each tube of reagent mix (step 2) in a 1:1 ratio	Reagent mix	150 μL	150 μL	150 μL	150 μL
		Dilute DNA	150 μL	150 μL	150 μL	150 μL
4a	Incubate DNA-reagent complex		5 minutes at room temperature			
5	Add DNA-reagent complex to cells					
6	Incubate transfected cells		Incubate cells for 1–3 days at 37°C			
7	Analyze the transfected cells					

* See the experimental scaling table below to adjust for other plate formats.

Experiment scaling

Culture vessel	Confluency	Reagent mix (step 2)				Diluted DNA (step 3)	
		Dose 1	Dose 2	Dose 3	Dose 4	DNA	Opti-MEM I
6 well	0.25–1 x 10 ⁶	6 μL	9 μL	12 μL	15 μL	2.5 μg (2,500 ng)	150 μL
24 well	0.5–2 x 10 ⁵	2 μL	3 μL	4 μL	5 μL	0.5 μg (500 ng)	50 μL
96 well	1–4 x 10 ⁴	1 μL	1.5 μL	2 μL	2.5 μL	0.1 μg (100 ng)	25 μL

Transfection

Expected Results

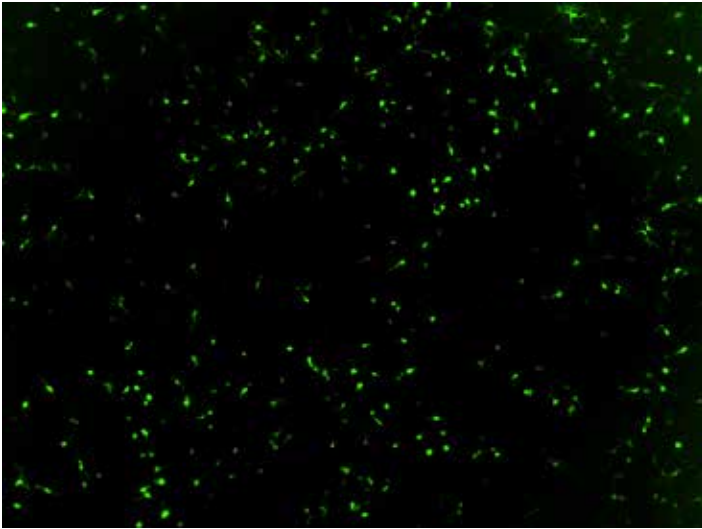


Figure 25-1. Plasmid DNA delivered by Lipofectamine 2000 reagent in primary cortical neurons (freshly isolated from E16 mouse; 5 days *in vitro*). GFP expression, analyzed 24 hours posttransfection, was 13%.

Transfecting siRNA (Immortalized Neuronal Cells and Primary Neuronal Cells)–Protocol

Materials needed

- Invitrogen™ *Silencer*™ Select siRNAs (thermofisher.com/sirna)
- Gibco™ Opti-MEM™ I Reduced Serum Medium (Cat. No. 31985062)
- Invitrogen™ Lipofectamine™ RNAiMAX Transfection Reagent (Cat. No. 13778075)
- Eppendorf tubes

Step	Description	Component	6-well format* Dose/well
1	Seed cells to be 60–80% confluent at transfection	Adherent cells	0.25–1 × 10 ⁶
2	Prepare reagent mix: Dilute Lipofectamine RNAiMAX reagent in Opti-MEM I Reduced Serum Medium in one Eppendorf tube	Opti-MEM I medium	150 µL
		Lipofectamine RNAiMAX reagent	7.5 µL
2a	Vortex reagent mix		2–3 seconds
3	Dilute <i>Silencer</i> Select siRNA in Opti-MEM I medium in one Eppendorf tube	Opti-MEM I medium	150 µL
		siRNA (10 µM)	30 pmol
4	Add diluted siRNA (step 3) to each tube of reagent mix (step 2) in a 1:1 ratio	Reagent mix	150 µL
		Diluted siRNA	150 µL
4a	Incubate siRNA-reagent complex		5 minutes at room temperature
5	Add siRNA-reagent complex to cells		
6	Incubate transfected cells		Incubate cells for 1–3 days at 37°C
7	Analyze the transfected cells		

* See the experimental scaling table below to adjust for other plate formats.

Experiment scaling

Culture vessel	Confluency	Reagent mix (step 2)		Diluted siRNA (step 3)	
		Dose	Opti-MEM I	siRNA	Opti-MEM I
6 well	0.25–1 × 10 ⁶	7.5 µL	150 µL	30 pmol	150 µL
24 well	0.5–2 × 10 ⁵	1.5 µL	50 µL	5 pmol	50 µL
96 well	1–4 × 10 ⁴	0.5 µL	25 µL	1 pmol	25 µL

Transfecting mRNA (Immortalized and Primary Neuronal Cells)–Protocol

Materials needed

- mRNA of interest (0.5–5 µg/µL)
 - Invitrogen™ mMESSAGE mMACHINE™ T7 ULTRA Transcription Kit (Cat. No. AM1345)
 - Invitrogen™ Lipofectamine™ MessengerMAX Transfection Reagent (Cat. No. LMRNA008)
 - Gibco™ Opti-MEM™ I Reduced Serum Medium (Cat. No. 31985062)
 - Eppendorf tubes
- Note:** The following protocol has two reagent doses (Dose 1 and Dose 2) that are needed for the initial optimization only.

Step	Description	Component	6-well format*	
			Dose 1/well	Dose 2/well
1	Seed cells to be 70–90% confluent at transfection	Adherent cells	0.25–1 x 10 ⁶	0.25–1 x 10 ⁶
2	Prepare reagent mix: Dilute MessengerMAX reagent in Opti-MEM I Reduced Serum Medium	Opti-MEM I medium	125 µL	125 µL
		MessengerMAX reagent	3.75 µL	7.5 µL
2a	Vortex reagent mix		2–3 seconds	
2b	Incubate reagent mix		10 minutes at room temperature	
3	Dilute mRNA in Opti-MEM I medium	Opti-MEM I medium	125 µL	125 µL
		mRNA	2.5 µg (2,500 ng)	2.5 µg (2,500 ng)
4	Add diluted mRNA (step 3) to each tube of reagent mix (step 2) in a 1:1 ratio	Reagent mix	125 µL	125 µL
		Diluted mRNA	125 µL	125 µL
4a	Incubate mRNA-reagent complex		5 minutes at room temperature	
5	Add mRNA-reagent complex to cells			
6	Incubate transfected cells		Incubate cells for 2–4 days at 37°C	
7	Analyze the transfected cells			

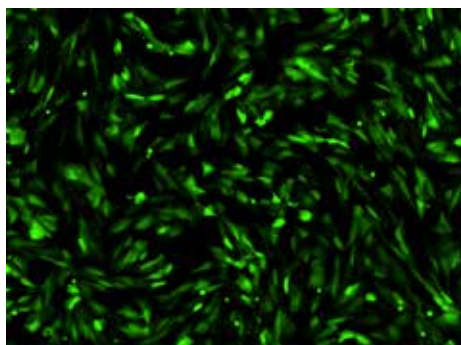
* See the Experimental Scaling table below to adjust for other plate formats.

Experiment scaling

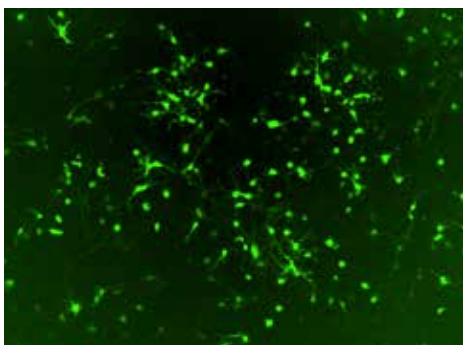
Culture vessel	Confluency	Reagent mix 1 (step 2)			Dilute mRNA (step 3)	
		Dose 1	Dose 2	Opti-MEM I	mRNA	Opti-MEM I
6 well	0.25–1 x 10 ⁶	3.75 µL	7.5 µL	125 µL	2.5 µg (2,500 ng)	125 µL
24 well	0.5–2 x 10 ⁵	0.75 µL	1.5 µL	25 µL	0.5 µg (500 ng)	25 µL
96 well	1–4 x 10 ⁴	0.15 µL	0.3 µL	5 µL	0.1 µg (100 ng)	5 µL

Expected Results

A GFP expression 62%



B GFP expression 76%



C GFP expression 92%

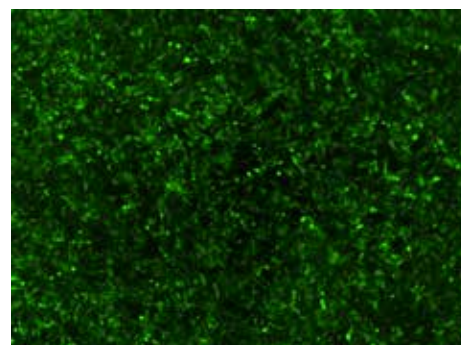


Figure 26-1. Lipofectamine MessengerMAX reagent was used to deliver mRNA encoding GFP (500 ng/well). Cells used included **(A)** primary cortical neurons (freshly isolated from E16 mouse; 5 days *in vitro*); **(B)** SK-N-SH cells (neuroblastoma) in a 24-well format, and **(C)** Gibco™ Human Neural Stem Cells (H9-Derived, Cat. No. N7800100) (hNSCs; 250 ng/well of mRNA was used in a 48-well format). GFP expression (noted above) was analyzed 24 hours posttransfection.

Transfecting neural cells using the Neon Transfection System

Summary

The Invitrogen™ Neon™ Transfection System is a benchtop electroporation device that uses the pipette tip as an electroporation chamber to efficiently transfect mammalian cells including primary cells and stem cells.

Instructions for using the Neon Transfection System for transfecting neural cells are described below. For detailed instructions, including several cell specific protocols, refer to [thermofisher.com/neon](https://www.thermofisher.com/neon)

For detailed information on culture conditions for various neural cell lines, refer to the instructions supplied with the specific cell line you are using.

View this protocol online and order products at [thermofisher.com/neuroprotocol/neon](https://www.thermofisher.com/neuroprotocol/neon)

Required Materials

Cells

- Neural cell line of interest

Media and reagents

- Growth medium and growth factors appropriate for your neural cell line
- Plasmid DNA of interest (1–5 µg/mL in deionized water or TE buffer)
- Gibco™ Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca²⁺ and Mg²⁺ (Cat. No. 14190144)
- Invitrogen Neon™ Transfection System (Cat. No. MPK5000)
- Invitrogen™ Neon™ Transfection System 10 µL Kit (Cat. No. MPK1096) or Neon™ Transfection System 100 µL Kit (Cat. No. MPK10096)
- Appropriate tissue culture plates and supplies
- Gibco™ StemPro™ NSC SFM (Cat. No. A1050901: This kit contains KnockOut™ DMEM/F-12 Basal Medium stored at 4°C, StemPro™ NSC SFM Supplement stored at –20 to –5°C in the dark, and bFGF Recombinant Human and EGF Recombinant Human stored at 4°C, desiccated.)
- Gibco™ Astrocyte Medium (Cat. No. A1261301: This kit contains N-2 Supplement (100X) stored at –20°C; Dulbecco's Modified Eagle Medium (DMEM) (1X) stores at 2°C to 8°C; OneShot™ Fetal Bovine Serum, Certified stored at –20°C in the dark.)

Preparing Media

Use complete StemPro NSC SFM for human neural stem cells or rat fetal neural stem cells and complete astrocyte medium for human astrocytes, rat primary cortical astrocytes, or rat glial precursor cells. Also refer to Culture Conditions on page 101 of the previous chapter for additional information.

Complete StemPro NSC SFM

To prepare 100 mL of complete StemPro NSC SFM, aseptically mix the components listed in the table below. Complete medium is stable for up to 4 weeks when stored in the dark at 4°C.

Component	Final conc.	Amount
KnockOut™ DMEM/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

Complete astrocyte medium

Use for human astrocytes, rat primary cortical astrocytes, or rat glial precursor cells

To prepare 100 mL of complete astrocyte medium, aseptically mix the components listed in the table below. Complete medium is stable for up to 2 weeks when stored in the dark at 4°C.

Component	Final conc.	Amount
DMEM	1X	89 mL
N-2 Supplement	1X	1 mL
FBS	10%	10 mL
EGF	20 ng/mL	2 µg

Note: Adding EGF at a final concentration of 20 ng/mL can increase proliferation, but may result in morphological and phenotypic changes in human astrocytes.

Transfection Protocol

Use this procedure to transfect plasmid DNA into hNSCs in a 24-well format using the 10-µL Neon kit. All amounts and volumes are given on a per-well basis.

1. Cultivate the required number of cells in the appropriate growth medium (see table on the next page) such that the cells are 70–90% confluent on the day of the experiment.
2. On the day of the experiment, harvest and wash cells in DPBS without Ca²⁺ and Mg²⁺.
3. Resuspend the cell pellet in Resuspension Buffer R (included with Neon kits) at the appropriate final density (see the table on the next page).
4. Prepare 24-well plates by filling the wells with 0.5 mL of the appropriate growth medium without antibiotics and pre-incubate plates at 37°C in a humidified 5% CO₂ incubator. If using other plate formats, adjust the volume accordingly.
5. Turn on the Neon unit and enter the following electroporation parameters in the **Input** window. Alternatively, press the **Database** button and select the appropriate transfection protocol (if you have already added the electroporation parameters for your cell type). For detailed instructions, refer to the manual supplied with the Neon unit.

Cell type	Cell density	Pulse voltage (V)	Pulse width (ms)	Pulse number	Neon Tip
Human Neural Stem Cells	1 x 10 ⁷ cells/mL	1,400	20	2	10 µL
		1,600	20	1	
		1,700	20	1	
Human Astrocytes	1 x 10 ⁷ cells/mL	1,100	30	1	10 µL
		1,200	40	1	
Rat Fetal Neural Stem Cells	1 x 10 ⁷ cells/mL	1,300	20	2	10 µL
		1,500	10	3	
		1,600	10	3	
Rat Primary Cortical Astrocytes	0.5 x 10 ⁷ cells/mL	1,400	20	2	10 µL
		1,400	30	1	
		1,700	20	1	
Rat Glial Precursor Cells	1 x 10 ⁷ cells/mL	1,300	10	3	10 µL
		1,500	20	1	

- Fill the Neon Tube with 3 mL of Buffer E. (Use Buffer E2 if you are using the 100 µL Neon Tip.)
- Insert the Neon Tube into the Neon Pipette Station until you hear a click, indicating that the tube has locked in position.
- Transfer 0.5 µg of plasmid DNA into a sterile, 1.5 mL microcentrifuge tube.

Note: The quality and concentration of DNA used for electroporation plays an important role for the transfection efficiency. We strongly recommend using high-quality plasmid purification kits such as Invitrogen™ PureLink™ HiPure Plasmid DNA Purification kits to prepare DNA.
- Add 1 mL of cells (resuspended in step 3) to the tube containing the plasmid DNA and gently mix.
- Insert a 10 µL Neon Tip into the Neon Pipette.
- Press the push-button on the Neon Pipette to the first stop and immerse the Neon Tip into the cell-DNA mixture. Slowly release the push-button on the pipette to aspirate the cell-DNA mixture into the Neon Tip.
- Insert the Neon Pipette with the sample vertically into the Neon Tube placed in the Neon Pipette Station until you hear a click, indicating that the pipette has locked in position.
- Ensure that you have entered the appropriate electroporation parameters and press **Start** on the Neon touchscreen.

The Neon device delivers the electric pulse according to the parameters entered in step 5, and the touchscreen displays **Complete** to indicate that electroporation is complete.

Remove the Neon Pipette from the Neon Pipette Station and immediately transfer the samples from the Neon Tip into the prepared culture plate containing the appropriate pre-warmed complete growth medium without antibiotics.

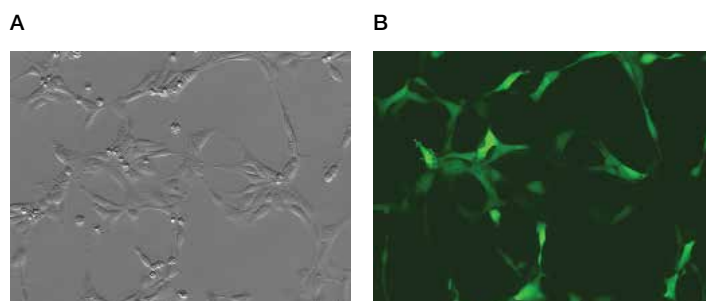
Discard the Neon Tip into an appropriate biological hazardous waste container.

14. Repeat steps 10–13 for the remaining samples.
15. Gently rock the plate to assure even distribution of the cells. Incubate the plate at 37°C in a humidified 5% CO₂ incubator.
16. Assay the samples to determine the transfection efficiency (e.g., fluorescence microscopy or functional assay).

Expected Results

Human Neural Stem Cells

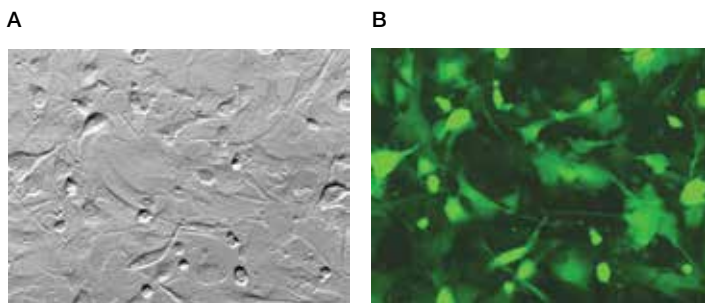
Gibco™ Human Neural Stem Cells (H9-Derived) (Cat. No. N7800100), cultured in complete StemPro NSC SFM, were transfected with 0.5 µg of a plasmid encoding the Emerald Green Fluorescent Protein (EmGFP) using the Neon Transfection System with the parameters listed in the following table. 48 hours posttransfection, the cells were analyzed by light **(A)** and fluorescence microscopy **(B)**.



Cell density (cells/mL)	Pulse voltage (V)	Pulse width (ms)	Pulse number	Transfection efficiency	Viability	Neon Tip
1 × 10 ⁷	1,400	20	2	82%	95%	10 µL
	1,600	20	1	84%	95%	
	1,700	20	1	87%	96%	

Human Astrocytes

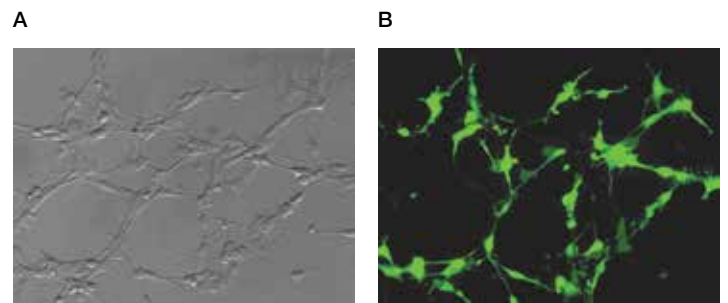
Gibco™ Human Astrocytes (Cat. No. N7805100) were transfected using the Neon Transfection System and 0.5 µg of a plasmid encoding the Emerald Green Fluorescent Protein (EmGFP); 24 hours post-electroporation, the cells were analyzed by light (A) and fluorescence microscopy (B).



Cell density (cells/mL)	Pulse voltage (V)	Pulse width (ms)	Pulse number	Transfection efficiency	Viability	Neon Tip
1 × 10 ⁷	1,100	30	1	92%	97%	10 µL
	1,200	40	1	93%	97%	

Rat Fetal Neural Stem Cells

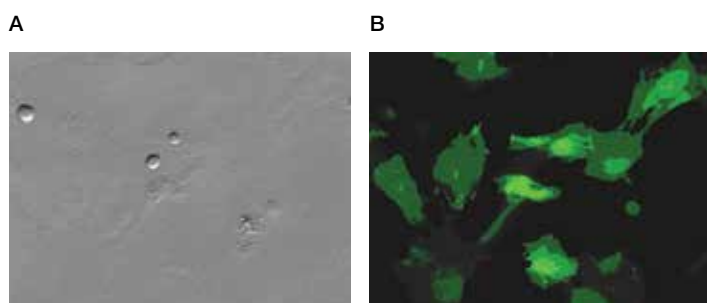
Gibco™ Rat Fetal Neural Stem Cells (Cat. No. N7744100) were transfected using the Neon Transfection System and 0.5 µg of a plasmid encoding the Emerald Green Fluorescent Protein (EmGFP); 24 hours post-electroporation, the cells were analyzed by light (A) and fluorescence microscopy (B).



Cell density (cells/mL)	Pulse voltage (V)	Pulse width (ms)	Pulse number	Transfection efficiency	Viability	Neon Tip
1 × 10 ⁷	1,100	30	1	92%	97%	10 µL
	1,200	40	1	93%	97%	

Rat Primary Cortical Astrocytes

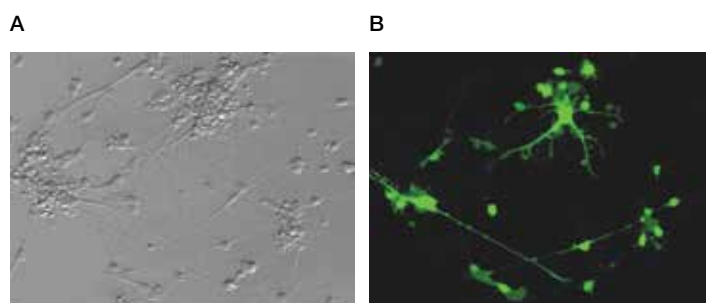
Gibco™ Rat Primary Cortical Astrocytes (Cat. No. N7745100) were transfected using the Neon Transfection System and 0.5 µg of a plasmid encoding the Emerald Green Fluorescent Protein (EmGFP); 24 hours post-electroporation, the cells were analyzed by light (A) and fluorescence microscopy (B).



Cell density (cells/mL)	Pulse voltage (V)	Pulse width (ms)	Pulse number	Transfection efficiency	Viability	Neon Tip
0.5 x 10 ⁷	1,400	20	2	69%	87%	10 µL
	1,400	30	1	71%	89%	
	1,700	20	1	71%	90%	

Rat Glial Precursor Cells

Gibco™ Rat Glial Precursor Cells (Cat. No. N7746100) were transfected using the Neon Transfection System and 0.5 µg of a plasmid encoding the Emerald Green Fluorescent Protein (EmGFP); 24 hours post-electroporation, the cells were analyzed by light (A) and fluorescence microscopy (B).



Cell density (cells/mL)	Pulse voltage (V)	Pulse width (ms)	Pulse number	Transfection efficiency	Viability	Neon tip
1 x 10 ⁷	1,300	10	3	49%	78%	10 µL
	1,500	20	1	44%	64%	

Troubleshooting

For troubleshooting tips regarding the Neon Transfection System, see the table below. For troubleshooting tips regarding the culture and passaging of your cells, refer to the manual provided with the cells.

Problem	Possible cause	Solution
Connection failure	No Neon Tip is inserted or the Neon Tip is inserted incorrectly	Make sure that the Neon Tip is inserted into Neon Pipette correctly as described. There should be no gap between the tip and the top head of the pipette.
Arcing (sparks)	Air bubbles in the Neon Tip	Avoid any air bubbles in the Neon Tip while aspirating the sample.
	High voltage or pulse length settings	Reduce the voltage or pulse length settings.
Low cell survival rate	Poor DNA quality	<ul style="list-style-type: none"> • Use high-quality plasmid DNA for transfection (use high-quality plasmid purification kits such as PureLink HiPure Plasmid DNA Purification kits) to prepare DNA. • Resuspend the purified DNA in deionized water or TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at a concentration between 0.5–5 µg/µL. • Check the purity of the purified DNA preparation by measurement of the A_{260}/A_{280} ratio. The ratio should be at least 1.8 for electroporation. • Do not precipitate DNA with ethanol to concentrate DNA. Concentrated DNA by ethanol precipitation shows poor transfection efficiency and cell viability due to salt contamination.
	Cells are stressed or damaged	<ul style="list-style-type: none"> • Avoid severe conditions during cell harvesting, especially high-speed centrifugation, and pipette cells gently. • Avoid using over-confluent cells or cells at high densities as this may affect the cell survival after electroporation. • After electroporation, immediately plate the cells into pre-warmed culture medium without antibiotics.
	Multiple use of the same Neon Tip	Do not use the same Neon Tip for electroporation more than 2 times because the repeated application of electric pulses reduces the tip quality and impairs its physical integrity.

Problem	Possible cause	Solution
Low transfection efficiency	Poor plasmid DNA quality or the plasmid DNA is low	<ul style="list-style-type: none"> • Use high-quality plasmid DNA for transfection. • Start with 0.5 µg plasmid DNA per sample.
	Incorrect cell density	Use the recommended cell densities of 1×10^5 cells per 10 µL per sample (i.e., 1×10^7 cells/mL).
	Incorrect electroporation parameters	Use the recommended voltage, pulse width, and pulse number. We recommend optimizing the electroporation parameters using the preprogrammed 24-well optimization protocol available on the Neon unit.
	Mycoplasma-contaminated cells	Test cells for Mycoplasma contamination. Start a new culture from a fresh stock.
Nonreproducible transfection efficiency	Inconsistent cell confluency or passage number	Always use cells with low passage number and harvest cells with comparable confluency levels.
	Multiple use of the same Neon Tip or the same Neon Tube	<ul style="list-style-type: none"> • Do not use the same Neon Tip for more than 2 times because the repeated application of electric pulses reduces the tip quality and impairs its physical integrity. • Do not use the same Neon Tube for more than 10 times. • Always use a new Neon Tip and Neon Tube for different plasmid DNA samples to avoid any cross-contamination.

Appendix

Thermo Fisher Scientific Products

Overview

Thermo Fisher Scientific provides you with all of your neural cell culture needs through its Gibco™ Cell Culture Media and offers products including reagents, media, sera, and growth factors to support the growth of a range of neural

cell lines. All cell culture media products available from Thermo Fisher Scientific are tested for contamination to help ensure their quality, safety, consistency, and regulatory compliance.

Ordering information

Cells

Product	Quantity	Cat. No.
Human		
Human Astrocytes	1 mL	N7805100
Human Episomal iPSC line	1 x 10 ⁶ cells/vial	A18945
Human Neural Stem Cells (H9-Derived)	1 mL	N7800100
Human Neural Stem Cells (H9-Derived) Kit	1 kit	N7800200
StemPro Neural Stem Cells	1 x 10 ⁶ cells	A15654
	5 x 10 ⁶ cells	A15655
Mouse		
Primary Mouse Cortical Neurons	1 x 10 ⁶ cells	A15585
	4 x 10 ⁶ cells	A15586
Primary Mouse Hippocampal Neurons	1 x 10 ⁶ cells	A15587
Rat		
Primary Rat Cortical Neurons	1 x 10 ⁶ cells	A1084001
	4 x 10 ⁶ cells	A1084002
Primary Rat Hippocampal Neurons	1 mL	A1084101
Rat Fetal Neural Stem Cells	1 mL	N7744100
Rat Glial Precursor Cells	1 mL	N7746100

Media and Supplements

Product	Quantity	Cat. No.
Advanced DMEM/F12	500 mL	12634010
	10 x 500 mL	12634028
Astrocyte Medium	500 mL	A1261301
CTS (Cell Therapy Systems) DPBS, without calcium chloride, without magnesium chloride	1 L	A1285601
B-27 Supplement (50X), serum free	10 mL	17504044
	100 mL	17504001
CTS B-27 Supplement, XenoFree	10 mL	A1486701
CTS DPBS, calcium, magnesium	1,000 mL	A1285801
CTS GlutaMAX-I Supplement	100 mL	A1286001
CTS Neurobasal Medium	500 mL	A1371201
Dulbecco's Modified Eagle Medium (DMEM, high glucose, pyruvate) [†]	500 mL	11995065
	500 mL	10565018
DMEM/F-12, GlutaMAX supplement	10 x 500 mL	10565042
	500 mL	A1517001
Essential 8 Medium	500 mL	A1517001
Essential 8 Flex Medium Kit	1 kit	A2858501
Fetal Bovine Serum, embryonic stem cell-qualified, US origin (FBS) [†]	100 mL	16141061
	500 mL	16141079
GlutaMAX-I Supplement	100 mL	35050061
	20 x 100 mL	35050079
Goat Serum, New Zealand origin [†]	100 mL	16210064
	500 mL	16210072
Hibernate-E Medium	500 mL	A1247601
N-2 Supplement (100X)	5 mL	17502048
	50 mL	17502001
Neurobasal Medium	500 mL	21103049

Media and Supplements

Product	Quantity	Cat. No.
Opti-MEM I Reduced Serum Medium	100 mL	31985062
	500 mL	31985070
	10 x 500 mL	31985088
PSC Dopaminergic Neuron Differentiation Kit	1 kit	A3147701
PSC Neural Induction Medium	500 mL	A1647801
RevitaCell Supplement (100X)	5 mL	A2644501
StemPro NSC SFM	1 kit	A1050901

Substrates, Matrices, and Bioscaffolds

Product	Quantity	Cat. No.
CELLstart CTS Substrate	2 mL	A1014201
Collagen I Rat Protein, Tail	20 mL	A1048301
Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	1 mL	A1413301
	5 mL	A1413302
Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix	1 mL	A1413201
	5 mL	A1413202
Laminin Mouse Protein, Natural	1 mg	23017015
Vitronectin (VTN-N) Recombinant Human Protein, Truncated	1 mL	A14700
	10 mL	A31804

Reagents

Product	Quantity	Cat. No.
2-Mercaptoethanol (55 mM) (β -Mercaptoethanol)	50 mL	21985023
	20 mL	15240096
Antibiotic-Antimycotic (100X)	100 mL	15240062
	20 x 100 mL	15240112
CTS Synth-a-Freeze Medium	50 mL	A1371301

Reagents

Product	Quantity	Cat. No.
CTS TrypLE Select Enzyme	100 mL	A1285901
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	10 mg	D1306
DMSO, Anhydrous (dimethylsulfoxide)	10 x 3 mL	D12345
FluoVolt Membrane Potential Kit	1 kit	F10488
	0.1 mL	L3000001
	0.75 mL	L3000008
Lipofectamine 3000 Transfection Reagent	1.5 mL	L3000015
	5 x 1.5 mL	L3000075
	15 mL	L3000150
	0.3 mL	11668030
	0.75 mL	11668027
Lipofectamine 2000 Transfection Reagent	1.5 mL	11668019
	15 mL	11668500
	0.1 mL	LMRNA001
	0.3 mL	LMRNA003
Lipofectamine MessengerMAX Transfection Reagent	0.75 mL	LMRNA008
	1.5 mL	LMRNA015
	15 mL	LMRNA150
	0.1 mL	13778100
	0.3 mL	13778030
Lipofectamine RNAiMAX Transfection Reagent	0.75 mL	13778075
	1.5 mL	13778150
	15 mL	13778500
Live Cell Imaging Solution (LCIS)	500 mL	A14291DJ
Penicillin-Streptomycin (5,000 U/mL)	100 mL	15070063
Pluronic F-127 (20% Solution in DMSO)	1 mL	P3000MP
Probenecid, Water Soluble (Cat. No. P36400)	10 x 77 mg	P36400

Reagents

Product	Quantity	Cat. No.
ProLong Gold Antifade Mountant	2 mL	P10144
	10 mL	P36930
	5 x 2 mL	P36934
PSC Cryopreservation Kit	50 mL	A2644601
StemPro Accutase Cell Dissociation Reagent	100 mL	A1110501
Trypan Blue Solution, 0.4%	100 mL	15250061
	100 mL	12604013
	500 mL	12604021
	20 x 100 mL	12604039
	5 L	12604054
TrypLE Express Enzyme (1X), no phenol red	100 mL	12563011
	500 mL	12563029
Trypsin-EDTA (0.05%), phenol red	100 mL	25300054
	500 mL	25300062
	20 x 100 mL	25300120
Valinomycin	25 mg	V1644

Growth Factors, Purified Proteins, and Antibodies*

Product	Quantity	Cat. No.
EGF Recombinant Human Protein	5 µg	10605HNAE5
	10 µg	PHG0314
	25 µg	PHG0315
	5 x 5 µg	10605HNAE25
	50 µg	10605HNAE50
	100 µg	PHG0311
	5 x 50 µg	10605HNAE250
	1 mg	PHG0313

Growth Factors, Purified Proteins, and Antibodies*

Product	Quantity	Cat. No.
FGF-Basic Full Length CTS Recombinant Human Protein	100 µg	CTP0261
	1 mg	CTP0263
GFAP Antibody	50 µL	PA3-16727
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 488 conjugate	500 µL	A-11029
Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 594 conjugate	500 µL	A-11037
MAP2 Antibody (M13)	100 µg	13-1500
NT-3 Recombinant Human Protein	10 µg	PHC7036

* For additional antibodies, see pages 40 and 76.

Buffers and Balanced Salt Solutions

Product	Quantity	Cat. No.
CTS (Cell Therapy Systems) DPBS, calcium, magnesium (CTS Dulbecco's Phosphate-Buffered Saline (DPBS))	1,000 mL	A1285801
CTS DPBS, without calcium chloride, without magnesium chloride (CTS Dulbecco's Phosphate-Buffered Saline without Ca ²⁺ or Mg ²⁺)	1 L	A1285601
	100 mL	14040141
	500 mL	14040133
DPBS, calcium, magnesium (Dulbecco's Phosphate-Buffered Saline (DPBS) with Ca ²⁺ and Mg ²⁺)	1,000 mL	14040117
	10 x 500 mL	14040182
	6 x 1,000 mL	14040216
	500 mL	14190144
DPBS, no calcium, no magnesium (Dulbecco's Phosphate-Buffered Saline without Ca ²⁺ or Mg ²⁺)	1,000 mL	14190136
	5 L	14190342
	10 x 500 mL	14190250
	6 x 1,000 mL	14190235
	10 L	14190359
	20 L	14190367

Buffers and Balanced Salt Solutions

Product	Quantity	Cat. No.
HBSS, calcium, magnesium, no phenol red [†] (Hanks' Balanced Salt Solution with Ca ²⁺ and Mg ²⁺ and without phenol red)	500 mL	14025092
	1,000 mL	14025076
	10 x 500 mL	14025134
	6 x 1,000 mL	14025126
HBSS, no calcium, no magnesium [†] (Hanks' Balanced Salt Solution without Ca ²⁺ or Mg ²⁺)	100 mL	14170120
	500 mL	14170112
	10 x 500 mL	14170161

Assay Kits

Product	Quantity	Cat. No.
Fluo-4, AM, cell permeant	10 x 50 µg	F14201
Human Dopaminergic Neuron Immunocytochemistry Kit	40 reactions	A29515
LIVE/DEAD Cell Vitality Assay Kit, C ₁₂ Resazurin/SYTOX Green	1 kit	L34951
LIVE/DEAD Viability/Cytotoxicity Kit, for mammalian cells	1 kit	L3224
mMESSAGE mMACHINE T7 ULTRA Transcription Kit	10 reactions	AM1345
	25 x 2 reactions	MPK1025
Neon Transfection System 10 µL Kit	96 x 2 reactions	MPK1096
	25 x 2 reactions	MPK10025
Neon Transfection System 100 µL Kit	96 x 2 reactions	MPK10096
	100 reactions	11733038
Platinum SYBR Green qPCR SuperMix-UDG	500 reactions	11733046
	10 preps	12183020
PureLink RNA Mini Kit	50 preps	12183018A
	250 preps	12183025
SuperScript III First-Strand Synthesis SuperMix	50 reactions	18080400

Plasticware

Product	Quantity	Cat. No.
Nalgene General Long-Term Storage Cryogenic Tubes (1.0 mL capacity)	Case of 500	5000-1012
Nalgene General Long-Term Storage Cryogenic Tubes (1.2 mL capacity)	Case of 500	5000-0012
Nalgene General Long-Term Storage Cryogenic Tubes (1.5 mL capacity)	Case of 500	5000-1020
Nunc Cell Scrapers (23 cm handle for use with 25 to 80 cm ² flasks)	Case of 250	179693
Nunc Cell Scrapers (32 cm handle, for use with 75 to 175 cm ² flasks)	Case of 250	179707
Nunclon Sphera Flasks (T25 Cell Culture Flask)	6 units/bag, 18 units/case	174951
Nunclon Sphera Flasks (T75 Cell Culture Flask)	4 units/bag, 24 units/case	174952
Nunclon Sphera Dishes (Multidish 24-Well)	1 unit/pack, 7 units/case	174930
Nunclon Sphera Dishes (Multidish 12-Well)	1 unit/pack, 7 units/case	174931
Nunclon Sphera Dishes (Multidish 6-Well)	1 unit/pack, 7 units/case	174932
Nunclon Sphera Dishes (Dish 35mm)	5 units/pack, 20 units/case	174943
Nunclon Sphera Dishes (Dish 60mm)	5 units/pack, 20 units/case	174944
Nunclon Sphera Dishes (Dish 90mm)	5 units/pack, 20 units/case	174945

Instruments and Software

Product	Quantity	Cat. No.
Applied Biosystems Real-Time PCR Systems		thermofisher.com/qpcr
Attune NxT Flow Cytometer		thermofisher.com/attune
CellInsight CX5 High-Content Screening (HCS) Platform		thermofisher.com/hcs
Countess II Automated Cell Counter		thermofisher.com/countess
EVOS FL Auto Imaging System		thermofisher.com/evosflauto
EVOS FL Imaging System		thermofisher.com/evosfl
HCS Studio 2.0 Cell Analysis Software, client installation	1 each	SX000041A
Neon Transfection System	1 each	MPK5000

Accessory Products

Product	Quantity	Cat. No.
BlueJuice Gel Loading Buffer (10X)	3 x 1 mL	10816-015
Custom primers	thermofisher.com/oligos	
Distilled Water	100 mL	15230170
	500 mL	15230162
	1,000 mL	15230147
Mr. Frosty Freezing Container (1.0 to 2.0 mL tube capacity)	1 each	5100-0001
Ribonuclease H (RNase H)	30 units	18021014
	120 units	18021071
<i>Silencer</i> Select siRNAs	thermofisher.com/sirna	
SuperScript IV First-Strand Synthesis System	50 reactions	18091050
	200 reactions	18091200

Resources for more information

Books

- **Neural Stem Cell Assays**, edited by Navjot Kaur and Mohan Vemuri, Wiley Press, 2015.
- **Developmental Biology, 9th edition**, edited by Scott F. Gilbert Sinauer Associates, 2010.
- **Neural Stem Cells, 2nd edition**, edited by Leslie P. Weiner, Humana Press, 2008.
- **Neural Development and Stem Cells, 2nd edition**, edited by Mahendra S. Rao, Humana Press, 2006.
- **Protocols for Neural Cell Culture, 4th edition**, edited by Laurie C. Doering, Humana Press, 2010.
- **Protocols for Neural Cell Culture, 3rd edition**, edited by Sergey Fedoroff and Arleen Richardson, Humana Press, 2001.

Journals

- *Neuron*, www.cell.com/neuron
- *Development*, <http://dev.biologists.org>
- *The Journal of Neuroscience*, www.jneurosci.org
- *European Journal of Neuroscience*, www.onlinelibrary.wiley.com/journal/10.1111/%28ISSN%291460-9568
- *Developmental Neurobiology*, www.onlinelibrary.wiley.com/journal/10.1002/%28ISSN%291097-4695
- *Nature Reviews Neuroscience*, www.nature.com/nrn/index.html
- *Cell Stem Cell*, <http://www.cell.com/cell-stem-cell/home>

Organizations

- American Academy of Neurology, www.aan.com
- Society for Neuroscience, www.sfn.org
- European Neuroscience and Society Network, www.lse.ac.uk/collections/ENSN
- Federation of European Neuroscience Societies, www.fens.mdc-berlin.de
- The Japanese Neuroscience Society, www.jnss.org/en/
- International Society for Stem Cell Research, www.isscr.org

Government Sites

- National Institute of Neurological Disorders and Stroke (NINDS), www.ninds.nih.gov
- National Institute of Mental Health (NIMH), www.nimh.nih.gov
- Food and Drug Administration (FDA), www.fda.gov
- National Institute of Child Health and Human Development (NICHD), www.nichd.nih.gov
- NCBI PubMed, www.ncbi.nlm.nih.gov/pubmed
- National Institutes of Health Entrez Databases, www.ncbi.nlm.nih.gov/Database/index.html
- National Library of Medicine's MEDLINEplus, www.ncbi.nlm.nih.gov/Database/index.html

Websites

- The Dana Foundation, www.dana.org
- Neuroanatomy and Neuropathology on the Internet, www.neuropat.dote.hu
- Neuromuscular Disease Center at Washington University School of Medicine, St. Louis, www.neuromuscular.wustl.edu
- Neuroscience Information Framework, www.neuinfo.org

Technical Support

Web Resources

Visit the [thermofisher.com](https://www.thermofisher.com) website for:

- Technical resources, including manuals, vector maps and sequences, application notes, Safety Data Sheets (SDSs), FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Thermo Fisher Online Catalog
- Additional product information and special offers
- Warranty information

Contact Us

For more information or technical assistance please consult our website at [thermofisher.com/contactus](https://www.thermofisher.com/contactus)

SDS

Safety Data Sheets (SDSs) are available at [thermofisher.com/sds](https://www.thermofisher.com/sds)

Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to [thermofisher.com/support](https://www.thermofisher.com/support) and search for the Certificate of Analysis by product lot number, which is printed on the product packaging (tube, pouch, or box).

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Find out more at thermofisher.com/neuroprotocols

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