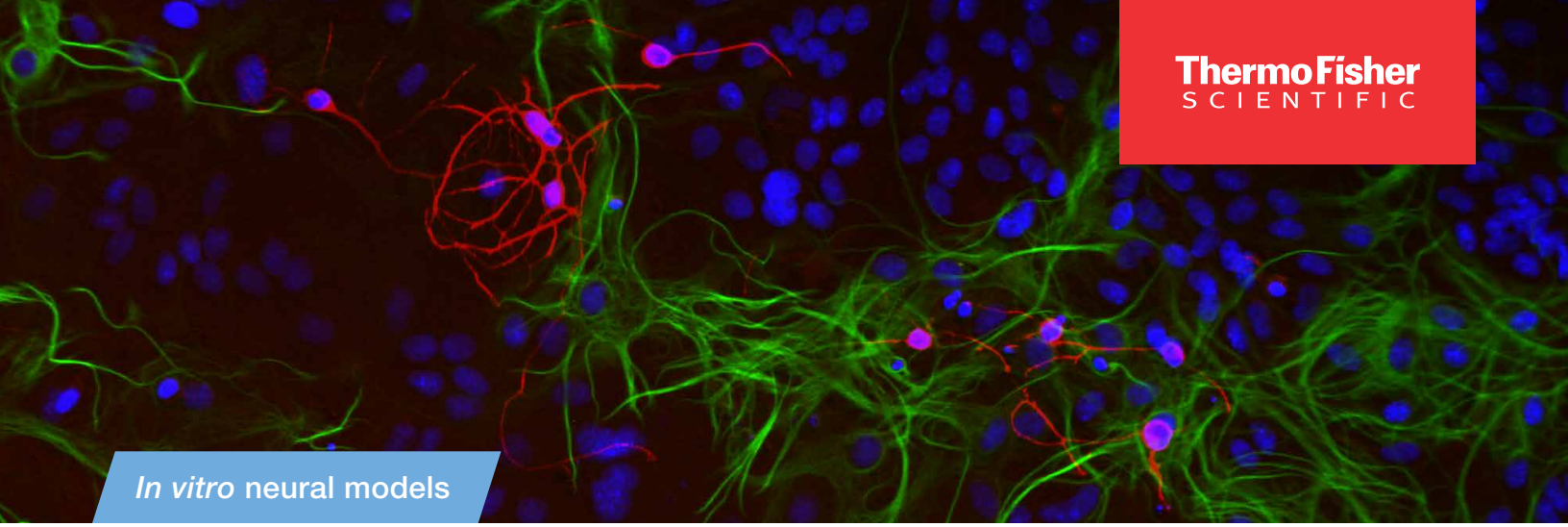

Five steps to creating a central nervous system model with inducible cell types

An inducible neuron and oligodendrocyte workflow as a platform to effectively scale differentiation of multiple cell lines for modeling the central nervous system

Introduction

Advances in stem cell biology in the 21st century have provided new opportunities to model the human central nervous system (CNS) *in vitro* and offer the potential to accurately represent phenotypes of healthy and diseased human cells. As researchers harness an increasing number of human-derived cell lines to tackle complex physiological mechanisms and disorders, the use of inducible expression of lineage-specific transcription factors to generate desired neural cell types has grown in popularity. Such approaches can provide greater ease of use, shorter culture times, better reproducibility, and greater scalability compared to directed differentiation methods. These benefits can help a greater number of scientists to employ human pluripotent stem cells (hPSCs) in their neurobiology studies.

This guide depicts the five key steps for rapidly generating specific cell types by overexpression of one or more genes known to drive a desired cell fate. From cell line generation to co-culture of multiple neural cell types, we highlight the variety of Gibco™ media and reagents from Thermo Fisher Scientific that can be used to efficiently and reproducibly obtain quality differentiated cells.

Cell line engineering	Characterization	Expansion	Differentiation	Co-culture
• TrueDesign Genome Editor	• Custom TaqMan probes	• Essential 8 Medium	• Laminin Mouse Protein, Natural	• Poly-D-Lysine
• TrueCut Cas9 Protein v2 and TrueGuide gRNA	• Custom TaqMan primers	• Essential 8 Flex Medium	• StemPro Accutase Cell Dissociation Reagent	• Laminin Mouse Protein, Natural
• LV-MAX Lentiviral Production System	• TaqMan hPSC Scorecard Panel	• StemFlex Medium	• StemFlex Medium	• Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix
• Neon NxT Electroporation System	• PluriTest Assay	• CultureCEPT Supplement	• Neurobasal Medium	• Neurobasal Plus Medium
• StemFlex Medium	• KaryoStat+ Assay		• B-27 Supplement	• B-27 Plus Supplement
• CultureCEPT Supplement				

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Cell line engineering

In order to induce the differentiation of a desired neural cell type directly from PSCs, a system to express the desired lineage-specific transcription factors is required. Major cell types with known transcription factors for induction are shown in Table 1. Expression of transcription factors can be done in a transient approach with a lentivirus or mRNA, or a cell line can be generated with inducible control of the desired gene engineered. A cell line approach can be advantageous because once generated, it provides a dependable source of cells with control of expression that can be used consistently. Cell line engineering begins with design of the intended construct, which must account for the gene of interest, inducible promoter, regulatory sequences, and homology arms, often making the size of the construct over 10 kb. Both lentiviral transduction and CRISPR-Cas9 technology have been demonstrated to successfully deliver a large knock-in construct to genetically modify a cell line.

After generating a cell pool with the appropriate construct, stable clones can be established to ensure long-term consistency of the system. This process has become even easier with the media systems offered by Thermo Fisher. When combining Gibco™ StemFlex™ Medium on rhLaminin-521, fluorescently sorted hPSCs can achieve up to 50% viability in 96-well plates and can be consolidated and screened in just two weeks post-sort.

mRNA approach

Synthetic mRNA can also be used to facilitate differentiation of PSCs to neural cells if a stable cell line with inducible expression is not desired. As with other approaches discussed here, it depends on the expression of lineage-specific transcription factors. Two major steps required to utilize this approach are mRNA synthesis and mRNA transfection. Synthesis is achieved using *in vitro* transcription (IVT) and an appropriate construct with the desired gene sequence. mRNA transfection can be achieved using Invitrogen™ Lipofectamine™ Stem Transfection Reagent.

Lentivirus and mRNA services

- Advanced design and production services enable you to move forward toward your next discovery. From design and synthesis to production of viral particles, Thermo Fisher offers end-to-end services to support every step in your lentivirus (LV) and mRNA production for research use. Thermo Fisher's dedicated staff of R&D scientists and project managers are ready to partner with you and your colleagues to help accelerate your research program.
- Thermo Fisher offers a complete end-to-end service for the creation of lentiviral expression constructs and virus packaging.
- Different promoters and selection markers are available for maximum flexibility for transfer plasmid construction.
- Synthesized mRNA or packaged and titered virus can be ready in as little as 4 weeks.

Table 1. Examples of cell types generated by overexpression of lineage-specific transcription factors.

Target cell	Cell to be induced	Gene to be induced	Time	Efficiency	Reference
Neuron	hPSC	*NGN2*	7 days	>90%	1
Microglia	hPSC	*SPI1*, *CEBPA*	10 days	>93%	2
OPC	hNSC	*SOX10*	7 days	>90%	3
Astrocyte	hNSC	*SOX9*	6 days	>90%	4
GABAergic neuron	hPSC	*ASCL1*, *DLX2*	5 weeks	>85%	5
DA neuron	hPSC	*ATOH1*, *NGN2*	6 weeks	>90%	6
Motor neuron	hPSC	*ISL1*, *LHX3*, *NGN2*	2 weeks	>90%	7

Tips and tricks

- The delivery and integration of large plasmids generally yields lower editing efficiencies, so antibiotic selection markers or fluorescent reporters can be used to help isolate successfully edited cells.
- Editing and cloning PSCs is stressful for cells. Gibco™ CultureCEPT™ Supplement can protect cells during the process and help ensure greater viability, increasing editing and cloning efficiencies.

Product highlights

- For assistance with design or execution of workflows discussed here, contact the Cell Biology Services team to help move your projects forward. Visit **here** to get started.
- **Invitrogen™ Neon™ NxT Electroporation System**—A next-generation electroporation platform with an innovative design that streamlines the mammalian cell transfection workflow.
- **Gibco™ StemFlex™ Medium**—Single-cell sorting of human induced pluripotent stem cells (hiPSCs) requires a robust medium that allows culture of single hiPSCs and extended culture of hiPSCs without the need for daily medium changes. Go to **thermofisher.com/stemflex** for protocols and additional application data.
- **Gibco™ CultureCEPT™ Supplement**—Reduces cellular stress and improves viability of stem cells, differentiated cells, and primary cells during a wide variety of handling and processing steps where cell damage and death can limit the success of a workflow.

Characterization

Proper integration of the inducible cassette should be confirmed after generation of a stable cell line. One method of confirmation is to perform qPCR using custom Applied Biosystems™ TaqMan™ probes, which will indicate transcriptional expression of the cassette. A great tool for designing and ordering custom primers and qPCR probes can be found **here**. The quality and health of the PSC line should also be assessed to help ensure that the pressures applied to cells during modification and cloning did not have any adverse effects. Thermo Fisher offers a suite of characterization services that can be used to characterize PSCs before and after genome editing and clonal isolation. The Applied Biosystems™ TaqMan™ hPSC Scorecard Panel and PluriTest™ characterization services cover the expression of markers representing the undifferentiated state and compare the transcriptional expression of the cells to other pluripotent cells. In addition, the KaryoStat+ Assay Service assesses possible genome aberrations that may have appeared in the cells. Examples of these results can be seen in Figure 1.

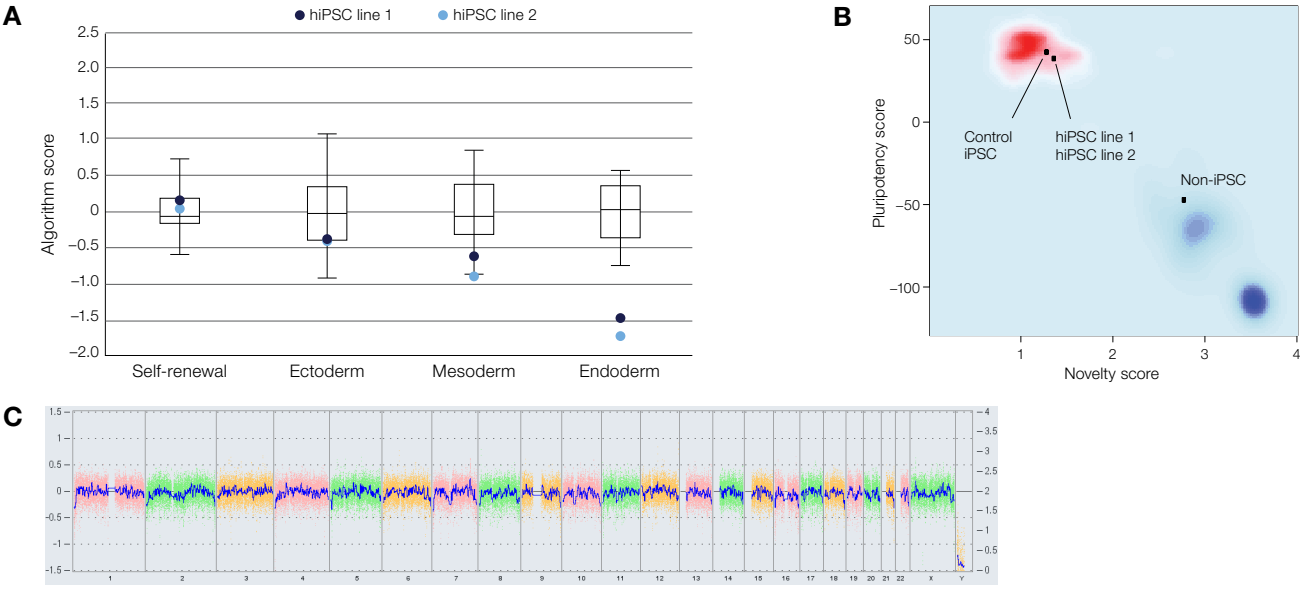

Figure 1. Stem cell characterization examples of two iPSC lines. (A) The TaqMan hPSC Scorecard Panel compares the gene expression profile of the sample to that of a reference set (colored dots and gray box plots, respectively). This assay uses over 90 genes and a static database of 13 PSCs for the comparison. **(B)** The PluriTest Assay uses microarray data to confirm pluripotency marker expression in terms of a pluripotency score (reflecting degree of pluripotency) and novelty score (reflecting degree of differentiation). This assay uses more than 36,000 transcripts and a fluid reference set of over 450 cell and tissue types for the comparison. **(C)** The KaryoStat+ Assay offers whole-genome coverage for accurate detection of copy number changes and genomic aberrations.

Tips and tricks

- Include the parental hPSC line as a control during characterization to confirm that the stable cell line characteristics are consistent and that only desired changes are obtained.
- For large knock-in genome editing, the easiest way to determine proper integration is to design custom primers across the integration junctions and screen for the presence of PCR products.
- A great tool for designing and ordering custom primers and qPCR probes can be found **here**.

Services

- **TaqMan hPSC Scorecard Panel Service**—For stem cell researchers seeking to access the characterization data that the scorecard panel provides, but who lack the resources or instrumentation to use the panel, this service offers a convenient alternative to enable obtaining the same data. The TaqMan hPSC Scorecard Panel enables verification of pluripotency and determination of lineage bias for both embryonic and induced pluripotent stem cell lines.
- **PluriTest Characterization Service**—This service leverages the Applied Biosystems™ PrimeView™ Global Gene Expression Profile Assays in combination with the PluriTest bioinformatics tool to analyze and process the transcriptome of all samples against an extensive and well-characterized reference set, allowing for global assessment of pluripotency for your submitted samples.
- **KaryoStat+ Assay Service**—Genomic stability of hiPSCs should be checked regularly, since hiPSCs are known to have an unstable genome. Traditionally, karyotypes of hiPSCs have been analyzed by G-band–based karyotyping to reveal chromosomal aberrations. Alternatively, a molecular approach can be taken where the genomic DNA of the hiPSC line is surveyed on a microarray containing DNA probes across the genome. Probe intensities are then used to obtain a detailed view of copy number and aberrations in DNA regions across the genome.

Expansion

hPSC cultures can be maintained and expanded in a wide variety of different culture systems that will allow effective differentiation, with optimal reagents dependent on the intended use of the cells. When a lean, more defined culture system is desired, Gibco™ Essential 8™ Medium provides reliable growth, maintains pluripotency, supports long-term iPSC growth without karyotypic abnormalities, and maintains the ability of iPSCs to differentiate into all three germ layers. If a weekend-free feeding schedule is desired, substituting Essential 8™ Flex Medium reduces or eliminates the need for daily feedings to allow for feeding intervals longer than 48 hours. When a more robust culture system is desired, StemFlex Medium can provide exceptional growth characteristics and greater support for more stressful growth environments such as single-cell cloning and low-density seeding applications, and also allows for a weekend-free feeding schedule similar to that of Essential 8 Flex Medium (Figure 2).

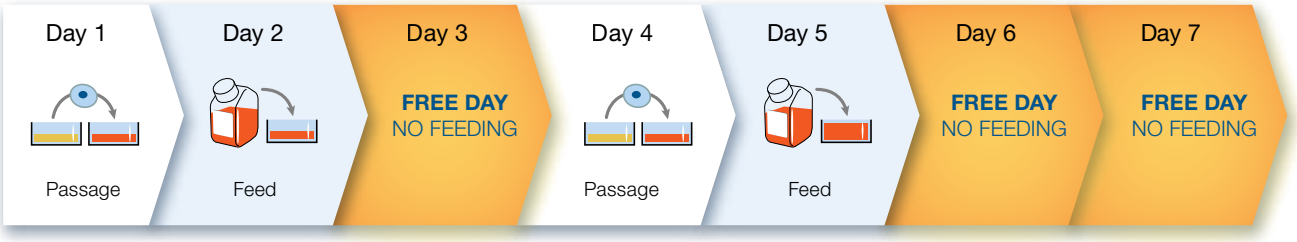

Figure 2. Weekend-free feeding schedule for Essential 8 Flex Medium and StemFlex Medium.

Tips and tricks

- Passage most iPSC lines in attachment cultures at approximately 70–80% confluency (usually at 3–5 days).
- Clump passaging protocols provide an easier method for general maintenance and expansion of cell lines, but single-cell or small-clump passaging may be required when seeding density needs to be more tightly controlled in differentiation protocols.
- Reagents developed for cell recovery, such as CultureCEPT Supplement, dramatically improve cell survival of hiPSCs after single-cell or small-clump passaging.
- Passage using a dissociation agent such as Gibco™ Versene™ Solution for clump passaging, or Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent or TrypLE™ Select Enzyme when small-clump or single-cell dissociation is required for differentiation.

Product highlights

- **Gibco™ Essential 8™ Medium**—A xeno-free and feeder-free medium specially formulated for growth and expansion of human pluripotent stem cells (hPSCs). Essential 8 Medium contains only the 8 essential components needed for stem cell culture.
- **Gibco™ Essential 8™ Flex Medium**—Provides all the benefits of Essential 8 Medium—minimized variability of components, reliable and robust cultures with a xeno-free, CGMP-produced medium—with the added benefit of flexibility.
- **Gibco™ StemFlex™ Medium**—Supports robust expansion of feeder-free PSCs. Its unique formulation offers the convenience of a flexible feeding schedule and also the ability to choose matrices and passaging reagents that best suit specific applications.
- **Gibco™ CultureCEPT™ Supplement**—Outperforms ROCK inhibitors such as Y-27632 to improve cell survival during passaging, and works with established Gibco Essential 8, StemFlex, StemScale, and Neurobasal media.

Differentiation

Once PSCs have been expanded to the desired numbers, differentiation can be rapidly achieved with induced expression of transcription factors. In this section, we describe two examples: neuron differentiation from a clonal cell line, and oligodendrocyte differentiation from a population of transduced oligodendrocyte precursor cells (OPCs) (Table 2).

Table 2. Approaches for induced differentiation of neurons and oligodendrocytes.

Scenario	*NGN2* neuron differentiation from a clonal *NGN2*-inducible PSC line	*SOX10* oligodendrocyte differentiation from PSC-derived oligodendrocyte precursor cells
Process	• PSCs to neurons without an intermediate precursor cell type • Make a stable, cryo-bankable cell line for induced differentiation of a pure neuron culture	• OPCs to oligodendrocytes • Transduce a population of precursor cells to induce a heterogeneous population of oligodendrocytes
Gene of interest	*NGN2*	*SOX10*
Benefits	• Clonal cell line • Homogeneity—neurons will express the same amount of *NGN2* and differentiate uniformly • Ideal for working with the sequence of the cell line for further gene editing (e.g., generating disease and control lines)	• Quicker—no screening of clones • Transduced cells will have varying levels of *SOX10* expression • The population of oligodendrocytes can be purified with an antibiotic to eliminate cells that were not transduced
Planning considerations	• Time is needed for screening and expansion of clones	• Start with a high-quality OPC culture • Optimize the seeding density and multiplicity of infection (MOI) for transduction

Generation of neurons

After plating singularized PSCs from an inducible cell line, *NGN2* expression is induced with addition of the transactivator doxycycline (DOX) to the medium. The overexpression of NGN2 drives differentiation of the PSCs to neurons with a few days, with the cells undergoing dramatic changes in morphology (Figure 3). In this case, a uniform culture results from differentiating a clonal inducible cell line.

Generation of oligodendrocytes

Oligodendrocytes can be rapidly obtained by forced *SOX10* expression in oligodendrocyte precursor cells (OPCs) derived from PSCs. In this case, after 4 days of initial directed differentiation from PSCs to OPCs, cells are replated, transduced with a lentivirus that constitutively expresses *SOX10*, and allowed to mature in culture for 10 days. Mature oligodendrocytes staining positively for GalC are obtained within 15 days (Figure 4). Alternatively, OPCs can be efficiently differentiated to mature oligodendrocytes by adding a transactivator to the induction medium if using an inducible cell line designed for *SOX10*.

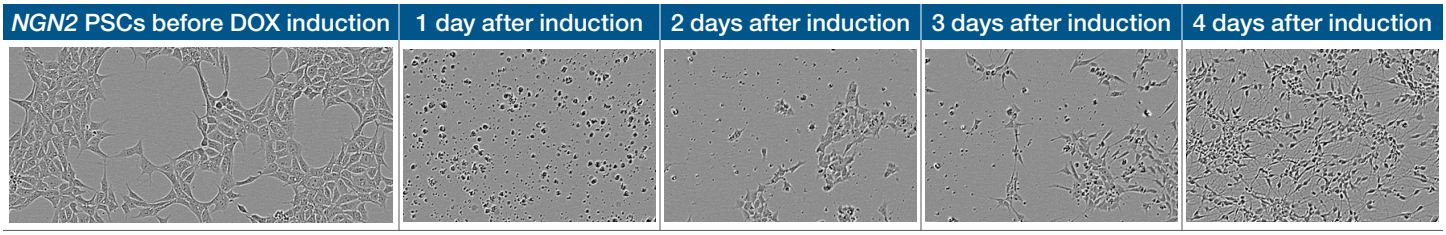

Figure 3. Bright-field images of a clonal *NGN2* PSC line (seeded at 60,000 cell/cm^2) before and after DOX induction for neuron differentation. Uniform neuronal morphology is observed throughout the culture by day 4.

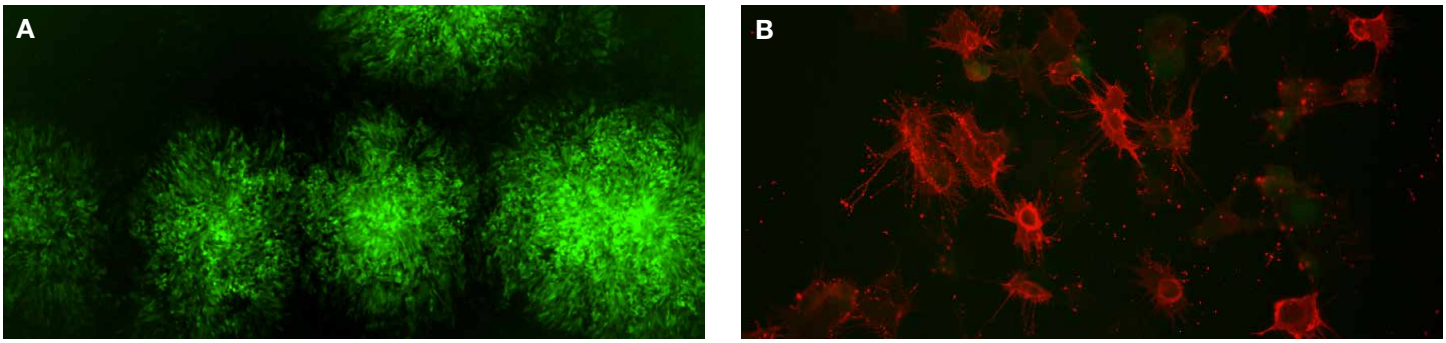

Figure 4. Induced oligodendrocytes generated by lentiviral transduction of OPCs to overexpress *SOX10*. (A) 4 days of directed differentiation of PSCs established an *OLIG2*-expressing OPC population (green: OLIG2-GFP reporter protein). **(B)** OPCs were transduced on day 5 to overexpress *SOX10*, allowed to mature in culture for 10 days, and fixed on day 15 for staining (green: OLIG2-GFP reporter protein; red: GALC ICC staining).

Tips and tricks

- Successful induction depends on optimal passaging methods, seeding density, seeding medium, and differentiation medium.
  - Single-cell passaging using StemPro Accutase Cell Dissociation Reagent and seeding densities in the range of 6 x 10^4 to 8 x 10^4 cells/cm^2 will help ensure evenly distributed cells with appropriate cell-to-cell communication for uniform differentiation.
  - Seed PSCs into the same PSC medium and matrix (StemFlex Medium and laminin) used for expansion, before starting DOX induction. Once the cells attach to the matrix, the medium can be changed from PSC medium to induction medium (Gibco™ Neurobasal™ Medium with Gibco™ B-27™ Supplement).
  - DOX can also be added to the maturation medium of an *NGN2* differentiation to facilitate conversion of any uninduced progenitor cells; however, DOX must be removed following differentiation if the neurons are to be co-cultured with another cell type that isn't engineered to be resistant to DOX.

Product highlights

- **Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent**—A cell detachment solution of proteolytic and collagenolytic enzymes that performs exceptionally well in detaching primary and stem cells while maintaining high cell viability compared to animal-origin enzymes such as trypsin.
- **Gibco™ Neurobasal™ Medium**—A classic basal medium formulation used by neuroscientists for over 30 years. This neuronal cell culture medium supports long-term maintenance and maturation of pure prenatal and embryonic neuronal cell populations.
- **Gibco™ B-27™ Supplement**—A defined yet complex mixture of antioxidant enzymes, proteins, vitamins, and fatty acids that are combined in optimized ratios to support neuronal survival in culture.

Co-culture

Maintain a representative co-culture system to model the central nervous system

Co-culture models that accurately represent the complexity of the brain are critical for successful downstream studies. Therefore, a culture system including a variety of neural cell types, such as the three neural lineages (astrocytes, oligodendrocytes, and neurons), is often desired.

Populations of different cell types can be derived separately using induced differentiation methods described previously or directed differentiation techniques, and then combined into the same culture. The individual populations can be combined by replating nascent differentiated cells and culturing in a maturation medium. Inclusion of CultureCEPT Supplement improves cell survival during replating steps. For robust and enduring neural co-cultures, we recommend utilizing the Gibco™ B-27™ Plus Neuronal Culture System. The medium is specifically designed to maintain cell viability of neural cultures over extended culture periods, enabling reliable results in downstream applications.

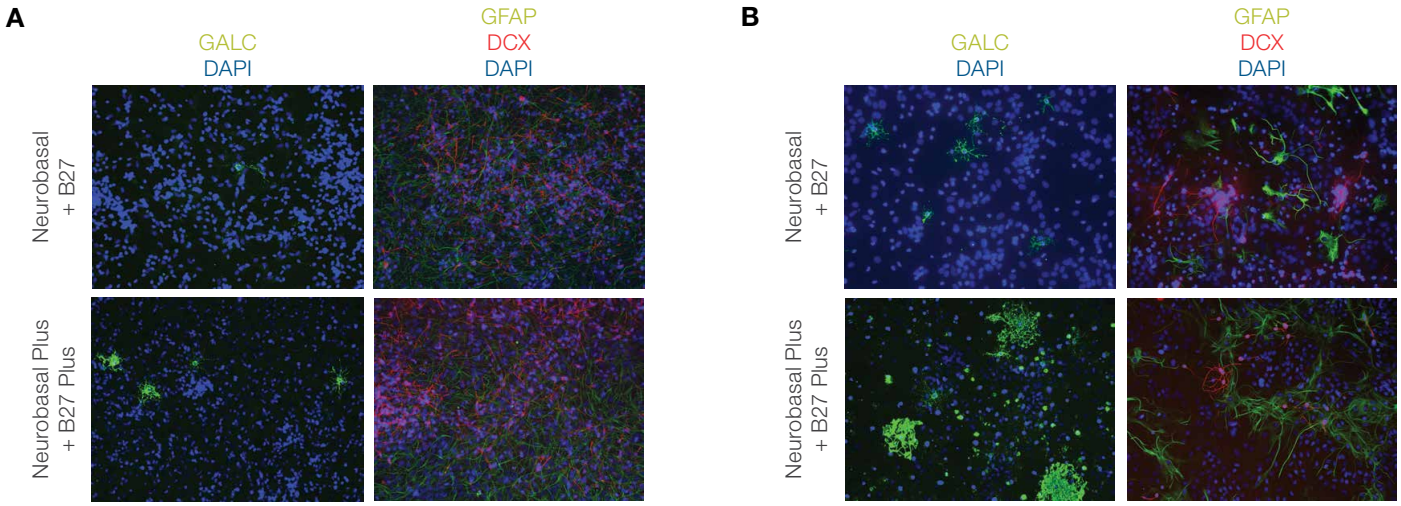

Figure 5. Immunocytochemistry of neural lineage markers in co-cultures derived from neural stem cells and maintained in the Neurobasal or the Neurobasal Plus media systems. (A) Human neural stem cells following 7 days of differentiation in maturation medium, and 14 days of maintenance in Neurobasal or Neurobasal Plus medium. Co-cultures were stained for GALC (oligodendrocyte marker), DCX (neuronal marker), and GFAP (astrocyte marker). **(B)** Rat neural stem cells following 7 days of differentiation in maturation medium, and 21 days of maintenance in Neurobasal or Neurobasal Plus medium. Co-cultures were stained for GALC (oligodendrocyte marker), DCX (neuronal marker), and GFAP (astrocyte marker).

Tips and tricks

- To ensure differentiation of neural stem cells into the three neural lineages, immunocytochemistry can be used to confirm the presence of markers of the three neural cell subtypes (Figure 5).
- Co-cultures are best plated onto cell culture dishes that are double-coated. Use a first layer of Gibco™ poly-D-lysine followed by a second coating of Gibco™ Geltrex™ matrix or Gibco™ laminin mouse protein.
- When combining different cell types that have been derived separately, consider initially plating one cell type and then, after several days, plating the next cell type on top.
- Inclusion of CultureCEPT Supplement improves cell survival during replating steps.

Product highlights

- **Gibco™ Poly-D-Lysine**—A ready-to-use product, consisting of a chemically synthesized extracellular matrix that facilitates cell adhesion to tissue culture–treated plastic and glass for neuronal culture applications.
- **Gibco™ Neurobasal™ Plus Medium**—The next generation of one of the most cited neuronal cell culture products, Neurobasal Medium. Optimization of key amino acids and buffering components provides the highest rates of neuronal cell survival when combined with B-27 Plus Supplement.
- **Gibco™ B-27™ Plus Supplement**—An optimized serum-free supplement that provides the highest rate of *in vitro* survival of primary rodent and human stem cell–derived neurons when used with Neurobasal Plus Medium. B-27 Plus Supplement also improves electrophysiological activity and enhances functional maturity, versus other neuronal cell culture media systems.

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Ordering information

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TrueGuide gRNA	Selection tool
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StemFlex Medium	A3349401
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Custom TaqMan primers	Custom
TaqMan hPSC Scorecard Panel	A15876
PluriTest Assay Service	A38154
KaryoStat+ Assay Service	A52849
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StemFlex Medium	A3349401
CultureCEPT Supplement	A56799
Differentiation	
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StemPro Accutase Cell Dissociation Reagent	A1110501
StemFlex Medium	A3349401
Neurobasal Medium	21103049
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