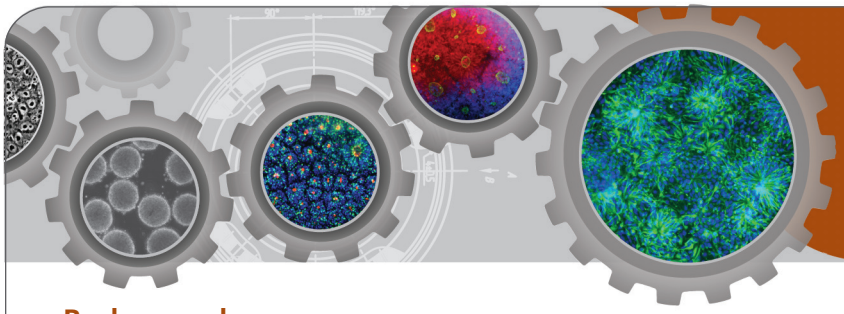

TECHNICAL BULLETIN

STEMdiff™ Neural Induction Medium:
Directed Differentiation of hPSCs
to Neural Progenitor Cells

Background

Neural progenitor cells (NPCs) are characterized by their capacity to both self-renew and generate the differentiated major tissue types of the central nervous system (CNS): neurons, astrocytes and oligodendrocytes, with the latter two cell types often referred to as glia. NPCs can be isolated from the embryonic or adult mammalian brain;[1] however in vitro propagation of primary human NPCs can be challenging due to availability in cell source and slow doubling time. In order to circumvent the challenges associated with primary human NPCs propagation, research has focused on deriving NPCs from human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs)[2] and induced pluripotent stem cells (hiPSCs).[3] Central goals of NPC research have been: 1) to optimize hPSC-derived NPC generation, 2) maintenance and expansion of NPCs, and 3) controlled differentiation of human NPCs to specific subtypes of functional end cells. hPSC-derived NPCs provide an essential tool to study NPC biology and lineage differentiation propensity, and much progress has been made in the past decade to discover factors regulating NPC self-renewal and their differentiation into specific subtypes of neurons and glia.[2]

This technical bulletin discusses the three central goals of NPC research including methods and results for NPC propagation, regionalization, and differentiation.

NPC Generation

In the last decade, many protocols have been developed for the in vitro generation of NPCs from hPSCs. While these neural differentiation protocols vary widely in their methods, a common criterion is the generation of neural "rosettes", morphologically identifiable structures containing NPCs. Validation of NPCs within neural rosettes is generally done by detection of NPC markers such as PAX6, SOX1, and Nestin by fluorescence activated cell sorting (FACS) and immunocytochemistry (ICC).[2]

Advantages of STEMdiff™ Neural Induction Medium

- Defined
- Serum-free
- Rapid and efficient neural induction
  - Neural rosettes within 6 days
  - Single cell NPC populations within 12 days

The earliest neural differentiation protocols use co-culture based methods, in which hPSCs are cultured with stromal feeder cells (e.g. MS5[4] or PA6[5] cells), which are believed to exert an undefined inducing activity to direct neural differentiation. Other neural differentiation protocols use adherent cell-based systems, in which neural fate in hPSCs is induced by the addition of supplements (such as B27 or N2A)[6,7] and pathway modulation (eg. inhibition of SMAD, TGFß, GSK3 or Notch signaling pathways).[8,9] The efficiency of these adherent cell-based methods seems to be highly dependent on cell density. Finally, embryoid body (EB)-based protocols have been described, wherein EBs are formed in poorly defined media (e.g. serum or Knockout™ serum replacement (Life Technologies).[10] The above protocols require labor-intensive optimization of feeder quality, plating densities and mechanical selection of neural rosettes in order to obtain a pure population of NPCs.

STEMCELL Technologies has developed a system for efficient neural induction using aggregate formation in AggreWell™ plates and STEMdiff™ Neural Induction Medium, a defined, serum-free medium for the rapid and efficient differentiation of hPSCs to NPCs within 12 days. AggreWell™800 plates are used to generate large aggregates of hPSCs in STEMdiff™ Neural Induction Medium, and the resulting "neural aggregates" are further cultured in the STEMdiff™ Neural Induction Medium and in AggreWell™800 plate for 5 days Afterwards, they are and then plated onto poly-L-ornithine/laminin (PLO/Lam)-coated plates, where up to 100% of the resulting colonies contain neural rosettes. Neural rosettes are then isolated enzyme-free from any neural crest-derived "flat" cells,[11] using STEMdiff™ Neural Rosette Selection Reagent. Refer to the manual "Generation of Neural Progenitor Cells from hPSCs using STEMdiff™ Neural Induction Medium" at www.stemcell.com for complete information on NPC generation and selection using STEMdiff™ Neural Induction Medium and STEMdiff™ Neural Rosette Selection Reagent.

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Maintenance and Expansion of NPCs

Mouse PSC-derived NPCs have been shown to selectively survive and grow in serum-free media.[12,13] This finding has since been translated into culture of hPSC-derived NPCs, which are traditionally cultured in a base medium such as DMEM/F12.[14,15] Supplements such as N2A[15] and/or B27[6,7] are required for survival of NPCs, and growth factors such as basic fibroblast growth factor (bFGF) or epidermal growth factor (EGF) enhance the expansion rate of NPCs.[1,2,17]

Currently the effect of cytokine addition is a point of debate in the literature and a number of factors are being investigated for their influence on NPC properties. Recently, concerns have been raised about neural rosette-derived NPCs which were expanded with bFGF and EGF, in regards to losing their capacity to be regionalized to specific neuronal subtypes[2,16] or to transition to a more glial-restricted progenitor cell.[9] Conversely, it has also been reported that NPCs can be maintained with the addition of bFGF and EGF in culture for over 100 passages while retaining their developmental responsiveness to regionalizing cytokines.[17]

NPC plating density and its effect on NPC propagation and expansion has not been systematically approached in literature; in fact, the reported densities for plating single cell NPCs after harvest from neural rosette structures vary from 1 x 10^5 cells per cm^2 [2; (15)] to 1 x 10^6 cells per cm^2.[2; (18)] For differentiation, NPCs are usually plated at lower densities,[14] indicating that cell density affects the self-renewing and differentiation capacity of NPCs.

Controlled Differentiation of NPCs

A major focus of NPC research is maintaining their potential to be regionalized by developmental cues in vitro, which mimic the spatio-temporal patterning of the developing brain during embryogenesis. It has been described, that NPCs obtained from hPSC lines display a certain specification towards "anterior regions" of the developing embryonic brain.[3,20] In order to be able to differentiate NPCs in vitro towards diverse neuronal subtypes, NPCs need to be regionalized at an early timepoint by inductive signals. The next section describes how NPCs derived in STEMdiff™ Neural Induction Medium can be specified into region-specific NPCs representative of the ventral or dorsal forebrain.

The propensity of NPCs to aquire any cell fate along the dorso-ventral and anterio-posterior axes of the developing brain is an important attribute of NPCs as it enables the generation of various terminally differentiated neuronal cells in vitro.[9,14,19-23,26]

To give an example of such a terminal differentiation protocol, the following describes a method to differentiate NPCs into dopaminergic (DA) neurons of the ventral midbrain, demonstrating that regionalized NPCs derived in STEMdiff™ Neural Induction Medium can differentiate into terminal neuronal subtypes.

hPSC Differentiation to NPCs Using STEMdiff™ Neural Induction Medium

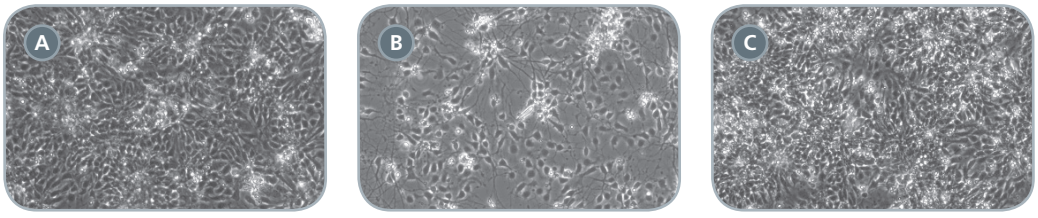
Propagation of NPCs as Single Cells

NPC-containing neural rosettes can be readily obtained using the reagents and protocol described in the manual "Generation of Neural Progenitor Cells from hPSCs using STEMdiff™ Neural Induction Medium" at www.stemcell.com. The following example demonstrates how NPCs obtained from that system can be further propagated as single cells.

H9 hESCs were differentiated into neural aggregates and then plated onto PLO/Lam-coated dishes, where neural rosettes abundantly appeared and were readily selected using STEMdiff™ Neural Rosette Selection Reagent. The selected neural rosettes were then re-plated onto fresh PLO/Lam-coated dishes, and once those cultures reached 80-90% confluence, single NPCs were extracted using ACCUTASE™ for 5 minutes at 37°C. After inactivating the ACCUTASE™ with DMEM/F12, the cells were collected, spun at 350 x *g* and then resuspended in 0.5 - 1.0 mL of culture medium. Three types of culture media were tested for propagation of NPCs: 1) DMEM/F12 with 1X N2A; 2) DMEM/F12 with 1X B27; and 3) DMEM/F12 with 1X N2A and 1X B27. Cells were plated at a density of 8 x 10^4 cells/cm^2 in each of the three test media. Five days after plating, the morphology of NPCs was evaluated revealing morphological differences in the different media: NPCs cultured with N2A (Medium 1) grew to approximately 90-95% confluence and displayed few processes, which would be indicative of the presence of further differentiating cells such as neurons (Figure 1A). NPCs maintained in DMEM/F12 with 1X B27 (Medium 2) appeared less confluent and more prominent processes were visible, indicating the presence of differentiating NPCs (Figure 1B). A differentiation promoting effect of B27 has been described before.[2,17] In Medium 3, containing a combination of N2A and B27, the NPCs showed a phenotype comparable to Medium 1.

Using this ACCUTASE™-based method of single cell dissociation, NPCs were propagated for 2-3 passages in all three media. Split ratios between passages in the media tested were typically between 1:2 and 1:3. To obtain higher expansion of NPCs, culture conditions (including addition of supplements, growth factors such as bFGF and EGF, and cell plating densities) may need to be further optimized by individual researchers for individual downstream applications, with the caveat that there is some evidence for an effect of cytokines on downstream NPC differentiation potential.

FIGURE 1. Propagation of NPCs derived in STEMdiff™ Neural Induction Medium



NPCs are shown 5 days after plating at a density of 8 x 10^4 cells/cm^2 onto PLO/Lam-coated dishes, in 1 of 3 test media. A) NPCs cultured in Medium 1 (DMEM/F12 + 1X N2A). B) NPCs cultured in Medium 2 (DMEM/F12 + 1X B27) and C) NPCs cultured in Medium 3 (DMEM/F12 + 1X N2A + 1X B27).

Dorso-Ventral Regionalization of NPCs

As discussed above, the demonstration of NPC-responsiveness to instructive neural patterning cues in vitro is a central topic in stem cell research. The following section shows that NPCs generated and isolated with STEMdiff™ Neural Induction Medium and STEMdiff™ Neural Rosette Selection Reagent have the propensity to be regionalized according to in vivo spatio-temporal developmental cues.

Dorso-ventral patterning of the developing embryonic CNS, often referred to as neural tube, is accomplished mainly by the opposing actions of members of the Bone Morphogenetic Protein (BMP) family and sonic hedgehog (Shh). BMPs are expressed in the dorsal portions of the neural tube, and specify dorsal fates throughout the neural tube (i.e. in the spinal cord[19]).

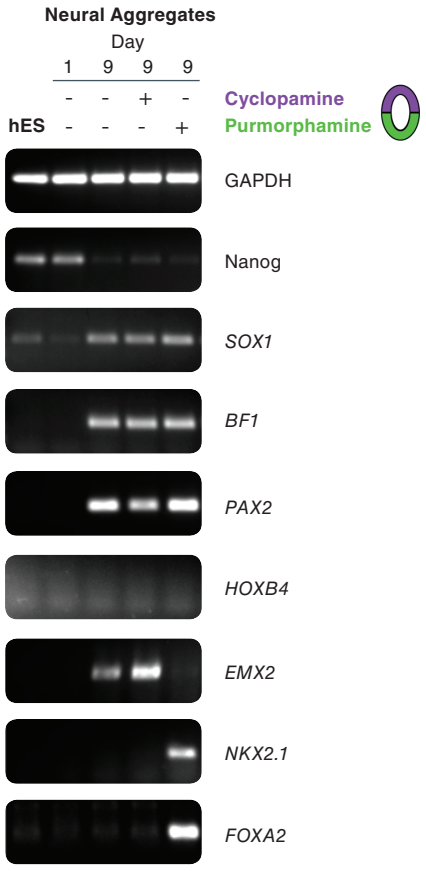
Shh is secreted from the notochord, underlying the ventral part of the developing neural tube and therefore specifies ventral fates such as GABAergic neurons of the ventral forebrain,[20] dopaminergic (DA) neurons of the ventral midbrain[21] or motoneurons of the spinal cord.[23] It has been demonstrated that activators and inhibitors of Shh can specify NPC towards ventral or dorsal fates, respectively: Purmorphamine is a commonly used Shh activator, which induces a ventral fate in NPCs[23] and cyclopamine is a commonly used Shh inhibitor,[24] which induces dorsal neural fates.

The following data is courtesy of Dr. Kim and Dr. Ghosh from the University of California, San Diego, USA. Neural aggregates were formed from HUES9 hESCs in AggreWell™800 and STEMdiff™ Neural Induction Medium for 5 days, and then plated onto PLO/Lam-coated dishes in STEMdiff™ Neural Induction Medium. After plating the neural aggregates in the same medium, purmorphamine or cyclopamine were added (at a concentration of 2 µM) for 3 days, to induce ventral or dorsal neural tube fates, respectively. RT-PCR assay was performed on the starting hESCs, cells harvested 24 hours after aggregate formation (Day 1), and the neural aggregates cultured in STEMdiff™ Neural Induction, alone or supplemented with purmorphamine or cyclopamine for a 3-day regionalization induction (Day 9).

RT-PCR results (Figure 2) show that the pluripotency marker Nanog was highly expressed in pluripotent cells and immediately after aggregate formation (Day 1), but was down-regulated in neural aggregates cultures (Day 9), independent of regionalization. Neural aggregates cultured in STEMdiff™ Neural Induction Medium without the addition of regionalization induction factors expressed the forebrain marker *BF1* and the midbrain marker *PAX2*, but did not express the hindbrain marker *HOXB4*. This code of transcription factor expression is indicative of NPCs within neural aggregates having adopted a forebrain/midbrain identity. Furthermore, as shown in Figure 2, the dorsal forebrain marker *EMX2* is expressed in "untreated" neural aggregates. These results confirm previous reports on the "default" developmental fate of hESC-derived NPCs, which was identified as being a forebrain fate.[3,20]

Cyclopamine treatment induced only slightly higher expression of the dorsal marker *EMX2* compared to the untreated control and did not alter expression of the ventral markers *NKX2.1* or *FOXA2*. These results are to be expected as NPCs obtained from HUES9 cells in this protocol already have a dorsal forebrain identity. Conversely, purmorphamine was able to induce expression of the ventral markers *NKX2.1* and *FOXA2*, and to repress expression of the dorsal marker *EMX2*. For both substances, no effect was observed on anterio-posterior patterning, as shown by constant expression levels of B *BF1* and *PAX2* and absence of *HOXB4* in treated neural aggregates. These results demonstrate that the "default" fate of HUES9-derived neural aggregates in this system can be changed from "dorsal forebrain" to "ventral forebrain" and that neural aggregates obtained with STEMdiff™ Neural Induction Medium are responsive to developmental cues.

FIGURE 2. RT-PCR data on dorso-ventral patterning of neural aggregates derived in STEMdiff™ Neural Induction Medium



GAPDH expression shows equal amounts of PCR product in all samples, serving as a loading-control. Transcripts for Nanog, a pluripotency gene, can be found in hESCs (Nanog: lane 1) and neural aggregates at Day 1 (lane 2). By Day 9, very faint expression is detected. Expression of the neural marker *SOX1* can be detected in Day 1 and Day 9 neural aggregates. *BF1* expression and *PAX2* expression in Day 9 neural aggregates indicate forebrain and midbrain specification of untreated aggregates (both: lane 3). *HOXB4*, a hindbrain marker is absent throughout. (Positive PCR control not shown).

After addition of the dorsalizing agent cyclopamine, *EMX2* expression is slightly induced (*EMX2*: lane 4) compared to "untreated" control levels (*EMX2*: lane 3). The ventralizing purmorphamine, conversely, induces ventral marker of the developing brain: *NKX2.1* and *FOXA2* (both: lane 5). At the same time, expression of the dorsal marker *EMX2* (expressed in untreated neural aggregates) is strongly repressed. Note that forebrain and midbrain marker *BF1* and *PAX2* are not changed by treatment with either cyclopamine or purmorphamine.

Data is courtesy of Dr. Kim and Dr. Ghosh UCSD, San Diego, USA.

Differentiation of NPCs into Dopaminergic Neurons

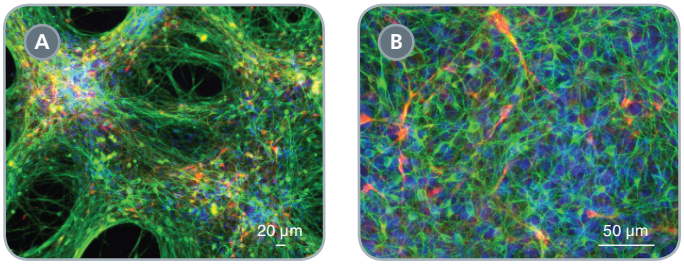
Protocols for the differentiation of dopaminergic (DA) neurons have been described using any of the three classes of neural differentiation protocols described in the above sections.[9,14,15,18] DA neurons develop in the anterior ventral midbrain region of the developing brain and thus, their differentiation from NPCs requires ventralizing factors such as Shh, which has been shown to act synergistically with FGF-8 to induce the ventral anterior midbrain fate.[21] The addition of FGF-8 in this context can be seen as a "posteriorizing" factor, given that hPSCs acquire a telecephalic fate by default. In the developing embryonic brain, NPCs which develop in the ventral anterior midbrain will be instructed by both secreted factors to differentiate into DA neurons at a later stage. It has recently been shown that timing of factor addition is crucial for the induction of midbrain DA neuronal cell fate.[18,26]

The following data is courtesy of Dr. Sonntag from Harvard Medical School, McLean Hospital, Belmont, USA. The protocol of Yan et al.[14] was adapted for the induction of DA neurons. Aggregates were obtained using mechanical dissection from H7 hESCs grown on MS-5 feeder cells, and were cultured in suspension in Knockout™ DMEM (Life Technologies) supplemented with 15% Knockout™ Serum Replacement (Life Technologies) for 2 days to maximize cell survival, before switching the aggregates to STEMdiff™ Neural Induction Medium for a further 2 days of non-adherent aggregate culture. On Day 4, aggregates were plated in STEMdiff™ Neural Induction Medium with 200 ng/mL Shh and 100 ng/mL FGF-8 (e.g. isoform FGF8-b). Neural rosettes appeared within 10 days, were mechanically selected and re-plated in STEMdiff™ Neural Induction Medium, as before with the addition of both Shh and FGF-8 for another 7 days. Subsequently, NPCs were differentiated to DA neurons using a modified procedure by Yan et al. (Sonntag et al., unpublished data).

ICC was performed using standard protocols using antibodies against tyrosine hydroxylase (TH), a marker for DA neurons and neuron-specific class III beta-tubulin (TUJ-1), a neuronal marker. Figure 3 shows clusters of neurons, stained in green for TUJ-1 and prominent interspersed expression of TH.

This protocol shows that NPCs derived in STEMdiff™ Neural Induction Medium can be directed using the appropriate morphogens to dopaminergic neurons.

FIGURE 3. TH positive dopaminergic neurons are induced from NPCs derived in STEMdiff™ Neural Induction Medium



A) Clusters of neurons differentiated from NPCs according to a modified protocol by Yan et al. stained with TUJ-1 (green) with interspersed TH positive (red) DA neurons. B) Higher magnification of a group of DA neurons (red). DAPI staining indicates all cell bodies. (Dr. Sonntag, McLean Hospital, Belmont, USA).

Summary

By using the STEMdiff™ Neural Induction System (STEMdiff™ Neural Induction Medium and STEMdiff™ Neural Rosette Selection Reagent) in combination with high quality pluripotent stem cells, >95% NPC purity can be obtained. These NPCs are capable of responding to dorsalizing and ventralizing cues and have the ability to differentiate to dopamingeric neurons. For more information, visit **www.stemcell.com** or contact **techsupport@stemcell.com**.

PRODUCT	QUANTITY	CATALOG #
STEMdiff™ Neural Induction Medium	100 mL	05831
STEMdiff™ Neural Rosette Selection Reagent	100 mL	05832

Support Products

PRODUCT	CATALOG #
mTeSR™1 Defined Maintenance Medium	05850 / 05870 / 05875 / 05857
mFreSR™ Defined Cryopreservation Medium	05855 / 05854
ACCUTASE™	07920
DMEM/F12	36254
Dispase (1 mg/mL)	07923
Y-27632	07171 / 07172
AggreWell™800 plates	27865 / 27965
37 µm Reversible Cell Strainer, Small/Large	27215 / 27250
N2 Supplement-A	07152
Human Basic Fibroblast Growth Factor (rh bFGF)	02634/02654
Human Epidermal Growth Factor (EGF)	02633/02653

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