StemXVivo™

Mouse Oligodendrocyte Differentiation Kit

Catalog Number SC004

For the differentiation of mouse pluripotent stem cells to oligodendrocytes.

This package insert must be read in its entirety before using this product.
For laboratory research use only. Not for diagnostic use.
The safety and efficacy of this product in diagnostic or
other clinical uses has not been established.

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INTRODUCTION

Oligodendrocytes are myelinating cells in the central nervous system (CNS) that form the myelin sheath of axons to support rapid nerve conduction. In CNS disorders, such as stroke, multiple sclerosis and spinal cord injury, demyelination of axons contributes to functional deficit. Studies have demonstrated that enhanced remyelination of damaged CNS axons through transplantation can restore functions lost as a consequence of demyelination (1, 2). However, the approach of neural transplantation therapy is limited by the availability of a rich, effective source of oligodendrocyte precursors for myelin regeneration.

Pluripotent stem cells (PSCs) can give rise to a collection of differentiated cells and tissues, including glia cells and neurons (4). They may serve as a potential source of replacement oligodendrocytes in CNS demyelination disorders. Several groups have reported methods for generating oligodendrocytes from PSCs and have identified conditions required for functional oligodendrocyte differentiation (5-8). Experimental models established from these studies might facilitate the *in vitro* examination of the underlying mechanisms controlling cell lineage and fate commitment. Moreover, these systems may provide a tool for developing more effective treatments for CNS disorders resulting from axon demyelination (5, 6).

PRINCIPLE OF THE ASSAY

The StemXVivo™ Mouse Oligodendrocyte Differentiation Kit is a system designed for *in vitro* neural differentiation of mouse PSCs in a serum-free environment (6). The kit contains specially formulated ITS and N-2 MAX Supplements, which are used to select and enrich neural precursor populations that are characterized by Nestin and A2B5 staining. Bovine Fibronectin is included to provide proper support for cell attachment and spreading. A growth factor panel, consisting of Human Fibroblast Growth Factor basic (FGF basic), Human Epidermal Growth Factor (EGF) and Human Platelet-derived Growth Factor AA (PDGF-AA), is included for effective oligodendrocyte differentiation. The quantity of each component provided in the kit is estimated to be sufficient for the induction of 3 x 10^7 PSCs. This kit has been shown to generate an average of 30 ± 5% oligodendrocytes, as estimated from immunostaining various mouse pluripotent stem cell lines with an antibody specific to O4, which recognizes an oligodendrocyte-specific glycolipid (9).

LIMITATIONS OF THE PROCEDURE

- FOR LABORATORY RESEARCH USE ONLY. NOT FOR DIAGNOSTIC USE.
- Do not mix or substitute reagents with those from other lots or sources.
- This kit should not be used beyond the expiration date on the kit label.
- The safety and efficacy of this product in diagnostic or other clinical uses has not been established.
- The quality of the embryonic stem cells and any variation in this procedure can cause variation in the efficiency of oligodendrocyte generation.

PRECAUTIONS

The acute and chronic effects of overexposure to reagents in this kit are unknown. Safe laboratory procedures should be followed, and protective clothing should be worn when handling kit reagents.

The ITS and N-2 MAX Supplements contain human transferrin. This transferrin was tested at the donor level using an FDA licensed method and found to be non-reactive for anti-HIV-1/2 and Hepatitis B surface antigen. As no testing can offer complete assurance of freedom from infectious agents, these reagents should be handled as if capable of transmitting infection.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store unopened kit at ≤ -20 °C in a manual defrost freezer. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
ITS Supplement	390154	5 mL of a 100X concentrated solution containing bovine insulin, human transferrin and sodium selenite.	Store at 2-8 °C for up to 1 month or aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 6 months.* Avoid repeated freeze-thaw cycles.
N-2 MAX Supplement	390524	5 mL of a 100X concentrated solution containing recombinant human insulin, human transferrin, sodium selenite, putrescine and progesterone.	
Bovine Fibronectin Stock	390213	1 vial containing 2 mL of a 1000X (1 mg/mL) solution of purified bovine fibronectin.	
Human FGF basic	390314	1 vial of lyophilized recombinant human FGF basic; enough to make 500 µL of a 1000X stock.	
Human EGF	390315	1 vial of lyophilized recombinant human EGF; enough to make 100 µL of a 1000X stock.	
Human PDGF-AA	390212	1 vial of lyophilized recombinant human PDGF-AA; enough to make 100 µL of a 1000X stock.	

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

Materials

- Mouse pluripotent stem cells (PSCs) (refer to reference 10 or use D3 cells from ATCC, www.atcc.org)
- Irradiated Mouse Embryonic Fibroblast (iMEF) (R&D Systems, Catalog # PSC001)
- 10 cm tissue culture plates
- 10 cm bacterial culture plates
- 12 mm coverslips
- 24-well culture plates
- 15 mL centrifuge tubes
- 0.2 µm, 500 mL filter units
- 0.2 µm syringe filter
- 10 mL syringes
- Cryotubes
- Serological pipettes
- Pipettes and pipette tips

Reagents

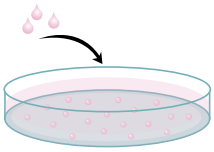
- Dulbecco's Modified Eagle Medium (DMEM)
- DMEM/F-12, no HEPES
- Fetal Bovine Serum, ES Cell Qualified
- Phosphate Buffered Saline (PBS)
- 0.05% Trypsin/EDTA
- Gelatin
- ESGRO® (recombinant mouse LIF) (Millipore, Catalog # ESG1106 or equivalent)
- Knock-out DMEM
- MEM Non-essential AA Solution
- Penicillin-Streptomycin-Glutamine, 100X
- Penicillin-Streptomycin, 100X
- 2-Mercaptoethanol, 1000X
- Glucose
- L-Glutamine
- Sodium Bicarbonate, $NaHCO_3$
- Poly-L-ornithine
- T3 (3,3',5-Triiodo-L-thyronine, sodium salt)
- Sterile, deionized water
- BSA, very low endotoxin (Millipore, Catalog # 81-068-3 or equivalent)
- Acetic Acid

Equipment

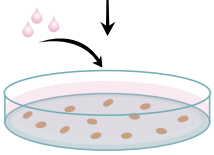
- 37 °C and 5% CO_2 incubator
- 37 °C water bath
- 60 °C hot plate
- Centrifuge
- Hemocytometer
- Microscope

PROCEDURE OUTLINE

Selection of Nestin-positive Cells (10-12 Days)

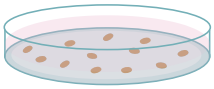


Generate embryoid bodies (EB) from pluripotent stem cells.
Transfer EB to a 10 cm culture plate containing KO-ES Media.
Culture the cells for 24 h at 37 °C and 5% CO_2.

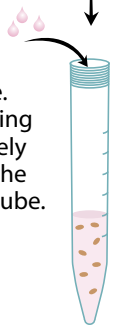


Replace the KO-ES Media with ITS/Fibronectin Media.
Culture the cells for 6-8 days at 37 °C and 5% CO_2.
Replace the media every 2 days.
Verify successful differentiation by staining cells for Nestin.

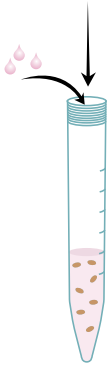
Induction of A285-Positive Cells (12 Days)



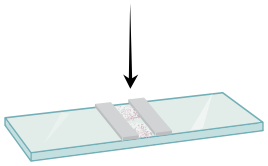
Wash the cells twice with sterile PBS.
Dissociate the cells with 0.05% Trypsin/EDTA.
Add 5 mL of KO-ES Media to neutralize the trypsin.



Transfer the cells to a 15 mL tube.
Remove the cell clumps by allowing the tube to stand for approximately 5 minutes and then transferring the suspended cells to a new 15 mL tube.



Centrifuge the samples at 220 x g for 5 minutes.
Resuspend the cell pellet in N-2 MAX/FGF Media.



Perform a cell count.

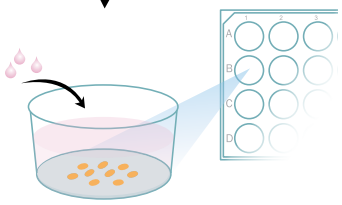
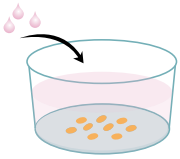


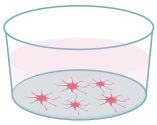
Plate the cells at 1 x 10^5 cells/well in 500 µL of N-2 MAX/FGF media on Poly-L-ornithine/Fibronectin-coated plates.
Replace the media daily:
- N-2 MAX/FGF media daily for 4 days.
- N-2 MAX/FGF/EGF media for the next 4 days.
- N-2 MAX/FGF/PDGF-AA media daily for the final 4 days.

Verify successful induction by staining cells for A2B5.

Differentiation of A2B5 Cells to Oligodendrocytes (6-8 Days)



Replace the media on A2B5 cells with N-2 MAX/T3 Media.
Replace the media every 2 days for 6-8 days.



Verify successful differentiation by staining cells for expression of Oligodendrocyte Marker O4.

PREPARATION OF REAGENTS FOR PLURIPOTENT STEM CELL PLATING & EXPANSION

Use serological pipettes to transfer and remove solutions.

Note: *The procedures described here are for mouse pluripotent stem cells. If using a different starting population, these procedures may need to be modified.*

0.1% Gelatin Solution - Add 0.25 g gelatin to 250 mL deionized water to make a 0.1% solution. Dissolve by heating to 60 °C. Water may evaporate during the heating process. Additional water should be added to bring the final volume up to 250 mL. Sterile filter using a 500 mL, 0.2 µm filter unit and store at room temperature for up to 6 months.

MEF Media - Mix the following sterile ingredients to make 50.5 mL of media. Store at 2-8 °C for up to 1 month.

Item	Amount	Final Concentration
DMEM	45 mL	90%
Fetal Bovine Serum	5 mL	10%
100X Penicillin-Streptomycin-Glutamine	0.5 mL	100 U/mL Penicillin, 100 µg/mL Streptomycin, 2 mM L-Glutamine

KO-ES Media - Mix the following sterile ingredients to make 510.5 mL of media. Store at 2-8 °C for up to 1 month.

Item	Amount	Final Concentration
Knock-out DMEM	425 mL	85%
Fetal Bovine Serum	75 mL	15%
MEM Non-Essential AA Solution	5 mL	100 µM
100X Penicillin-Streptomycin-Glutamine	5 mL	100 U/mL Penicillin, 100 µg/mL Streptomycin, 2 mM L-Glutamine
2-Mercaptoethanol	0.5 mL	55 µM

KO-ES/ESGRO Media - Dilute the ESGRO in KO-ES Media to make a 1400 U/mL solution. Prepare fresh as needed.

PREPARATION OF MOUSE PLURIPOTENT STEM CELLS

Use serological pipettes to transfer and remove solutions.

THAWING AND PLATING OF THE iMEF FEEDER CELLS

1. Gelatin coat one 100 mm tissue culture plate by covering the surface of the plate with 0.1% sterile gelatin for 15 minutes. One vial of 6 x 10^6 iMEF is enough for two 100 mm plates.
2. Warm the MEF Media to 37 °C.
3. Thaw the vial of iMEF cells by quickly warming the cryotube in a 37 °C water bath until the cells are just thawed and then immediately transfer the contents of one vial to a 15 mL conical tube containing at least 5 mL of pre-warmed MEF Media. Rinse the vial with an additional 1 mL of media to ensure the removal of all the cells.
4. Centrifuge at 200 x g in a clinical centrifuge for 5 minutes.
5. Remove the supernatant and flick the pellet.
6. Aspirate the 0.1% gelatin from the plate(s).
7. Resuspend the iMEF cells from step 5 (above) in 10 mL of MEF Media and transfer to the gelatin-coated plate at a density of approximately 3 x 10^6 cells/100 mm plate. Incubate for 24 hours at 37 °C and 5% CO_2.

PLURIPOTENT STEM CELL CULTURE

1. Twenty-four hours after the iMEF feeder cells are plated, warm the KO-ES/ESGRO Media in a 37 °C water bath for 20 minutes. In a 15 mL tube, resuspend 3 x 10^6 PSCs from a frozen stock vial with 10 mL of warmed KO-ES/ESGRO Media.
2. Remove the MEF Media from the feeder cells and wash once with 5 mL of KO-ES Media.
3. Add the PSC suspension on top of the feeder cells and incubate the culture at 37 °C and 5% CO_2. Twenty-four hours after the PSCs are plated, the cells should become attached to the feeder cell layer.
4. Feed the cells daily by replacing with fresh KO-ES/ESGRO Media. PSCs should start proliferating as indicated by the increasing size of colonies on top of the feeder cell layer.
5. Incubate the cells for 2 days (or until just before individual colonies contact each other) at 37 °C and 5% CO_2. Harvest the cells as follows:
    a. Remove the media. Wash the feeder cells 3 times with 10 mL of sterile PBS.
    b. Add 1 mL of 0.05% Trypsin/EDTA. Incubate for 5 minutes at 37 °C and 5% CO_2.
    c. Tap the plate gently to dislodge the cells. Add 5 mL of KO-ES Media to neutralize the Trypsin.
    d. Transfer the cells to a 15 mL tube by gentle pipetting and centrifuge for 5 minutes at 220 x g.
    e. Remove the supernatant and gently resuspend the PSCs with 5 mL of KO-ES/ESGRO Media. Count the cells. At this point, cells can be used in Stage I of this protocol.

STAGE I: EXPANSION OF UNDIFFERENTIATED PLURIPOTENT STEM CELLS (3-4 DAYS)

1. Warm the KO-ES/ESGRO Media in a 37 °C water bath for 20 minutes.
2. Seed 3 x 10^6 PSCs in 10 mL of warmed KO-ES/ESGRO Media on a gelatin-coated plate.

   Note: *Some MEF cells may be carried over to this stage. However, they will adhere to the plate at Stage II and will be separated from the floating cells after the embryoid bodies are formed.*
3. Culture the cells for 3-4 days at 37 °C and 5% CO_2, or until just before the cell colonies contact each other. Replace the KO-ES/ESGRO Media **daily.**

STAGE II: FORMATION OF EMBRYOID BODIES (4 DAYS)

1. Discard the media. Wash the cells from Stage I twice with 10 mL of sterile PBS.
2. Add 1 mL of 0.05% Trypsin/EDTA solution. Incubate for 5 minutes at 37 °C and 5% CO_2.
3. Gently tap the plate to dislodge the cells. Add 5 mL of KO-ES Media to neutralize the Trypsin.
4. Transfer the cells to a 15 mL tube by gentle pipetting. Centrifuge for 5 minutes at 220 x g.
5. Remove the supernatant and resuspend the cell pellet in 5 mL of KO-ES Media. Count the cells. Approximately 1.5 x 10^7 cells/plate can be expected from the harvest.
6. Seed 2 x 10^6 cells on a 10 cm bacterial culture plate containing 10 mL of KO-ES Media.
7. Culture the cells for 4 days at 37 °C and 5% CO_2. Change the media on day 2 in the following manner:
   a. Transfer the embryoid bodies (EB) to a 15 mL tube by gently pipetting. **Do not centrifuge.**
   b. Allow the tube to stand until the EB settle to the bottom (about 5 minutes).
   c. Remove the media. Add 10 mL of fresh KO-ES Media.
   d. Transfer the EB back into the original dish by gently pipetting.
8. On day 4, proceed to Stage III.

STAGE III: SELECTION OF NESTIN-POSITIVE CELLS (6-8 DAYS)

REAGENT PREPARATION FOR THE SELECTION OF NESTIN-POSITIVE CELLS

ITS Media - Mix the following ingredients with deionized water to make 500 mL of media. Adjust the pH to 7.5 ± 0.2. Sterile filter the solution using a 500 mL, 0.2 µm filter unit and add 5 mL of 100X Penicillin-Streptomycin. Store at 2-8 °C for up to 2 weeks.

Item	Amount
DMEM/F-12*	6 g (495 mL*)
Glucose	775 mg
L-Glutamine	36.5 mg
$NaHCO_3$	1.2 g
ITS Supplement (100X)	5 mL

*Liquid DMEM/F-12 (no HEPES) can be used as an alternative to the powder form.

Bovine Fibronectin Stock - Allow the Bovine Fibronectin Stock to stand for 30 minutes at room temperature **without agitation.** Swirl very gently. Aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

ITS/Fibronectin Media - Dilute the Bovine Fibronectin Stock 200-fold in ITS Media to make a 5 µg/mL solution (e.g. 50 µL of Bovine Fibronectin Stock in 10 mL of ITS Media). Mix by gently swirling without vortexing. Prepare fresh as needed.

PROCEDURE FOR THE SELECTION OF NESTIN-POSITIVE CELLS

1. At the end of Stage II, transfer the EB to a 15 mL tube by gently pipetting.
2. Allow the tube to stand until the EB settle to the bottom. Remove half of the media.
3. Rinse the original plate with 5 mL of fresh KO-ES Media and add to the 15 mL tube by gently pipetting.
4. Transfer the EB to a 10 cm tissue culture plate by gentle pipetting. Culture for 24 hours at 37 °C and 5% CO_2. At this time, the EB should become attached.
5. Change the media from KO-ES Media to 10 mL of ITS/Fibronectin Media.
6. Culture the cells for 6-8 days at 37 °C and 5% CO_2. Change the ITS/Fibronectin Media every 2 days. During this period, a monolayer will grow from the attached EB.

STAGE IV: INDUCTION OF A2B5-POSITIVE CELLS (12 DAYS)

REAGENT PREPARATION FOR THE INDUCTION OF A2B5-POSITIVE CELLS

N-2 MAX Media - Mix the following ingredients with deionized water to make 500 mL of media. Adjust the pH to 7.2 ± 0.2. Sterile filter the solution using a 500 mL, 0.2 µm filter unit and add 5 mL of 100X Penicillin-Streptomycin. Store **in the dark** at 2-8 °C for up to 2 weeks.

Item	Amount
DMEM/F-12*	6 g (495 mL)*
Glucose	775 mg
L-Glutamine	36.5 mg
$NaHCO_3$	845 mg
N-2 MAX Media Supplement (100X)	5 mL

*Liquid DMEM/F-12 (no HEPES) can be used as an alternative to the powder form.

0.1% BSA in PBS - Dissolve 10 mg of BSA in 10 mL of PBS. Sterile filter the solution using a 0.2 µm syringe filter and store at 2-8 °C for up to 3 months.

10 mM Acetic Acid - Add 10 µL of concentrated Acetic Acid (17.4 M) into 17.4 mL of sterile deionized water.

0.1% BSA in 10 mM Acetic Acid - Dissolve 10 mg of BSA in 10 mL of 10 mM Acetic Acid. Sterile filter the solution using a 0.2 µm syringe filter and store at 2-8 °C for up to 3 months.

FGF basic Stock (1000X) - Add 500 µL of sterile 0.1% BSA in PBS to the Human FGF basic vial. Aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

EGF Stock (1000X) - Add 100 µL of sterile 0.1% BSA in 10 mM acetic acid to the Human EGF vial. Aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

PDGF-AA Stock (1000X) - Add 100 µL of sterile 0.1% BSA in PBS to the vial of Human PDGF-AA. Aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

N-2 MAX/FGF Media - Dilute the FGF basic Stock 1000-fold in N-2 MAX Media. Prepare fresh as needed.

N-2 MAX/FGF/EGF Media - Dilute the FGF basic and EGF Stocks 1000-fold in N-2 MAX Media. Prepare fresh as needed**.**

N-2 MAX/FGF/PDGF-AA Media - Dilute the FGF basic and PDGF-AA Stocks 1000-fold in N-2 MAX Media. Prepare fresh as needed.

Poly-L-ornithine Stock (1000X) - Dissolve the Poly-L-ornithine in sterile PBS to make a 15 mg/mL stock. Aliquot and store at ≤-20 °C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

Poly-L-ornithine Solution (1X) - Dilute the Poly-L-ornithine Stock 1000-fold in sterile PBS to make a 15 µg/mL solution. Prepare fresh as needed.

Fibronectin Solution (1X) - Dilute the Fibronectin Stock 1000-fold in sterile PBS to make a 1 µg/mL solution. Mix by gently swirling, without vortexing. Prepare fresh as needed.

POLY-L-oRNITHINE/FIBRONECTIN-COATED PLATES

1. Insert a sterile cover slip (sterilized with 95% EtOH and flamed) into each well of a 24-well plate.
2. Add 0.5 mL of Poly-L-ornithine Solution (1X) to each well. Incubate overnight at 37° C.
3. Discard the Poly-L-ornithine Solution. Wash each well 3 times with 1 mL of sterile PBS.
4. Add 0.5 mL of sterile PBS to each well. Incubate overnight at 37 °C.
5. Discard the PBS. Wash each well once with 1 mL of sterile PBS.
6. Add 0.5 mL of Fibronectin Solution (1X) to each well. Incubate at 37 °C for 3 to 30 hours.
7. Discard the Fibronectin Solution. Wash each well once with 1 mL of sterile PBS before use.

PROCEDURE FOR THE INDUCTION OF A2B5-POSITIVE CELLS

1. Remove the ITS/Fibronectin Media from the cell culture in Stage III. Wash the attached cells twice with 10 mL of sterile PBS. Remove the PBS.
2. Add 1 mL of 0.05% Trypsin/EDTA solution. Incubate at 37 °C and 5% CO_2 for 5 minutes. Gently tap the plate to dislodge the cells. Add 5 mL of KO-ES Media to neutralize the Trypsin.
3. Transfer the cells to a 15 mL tube by gently pipetting. Remove the cell clumps (remnants of EB) by allowing the tube to stand just long enough to allow the cell clumps to settle to the bottom (about 5 minutes).
4. Transfer the suspended cells to a new 15 mL tube by gently pipetting.
5. Centrifuge the suspension for 5 minutes at 220 x g to pellet the cells.
6. Discard the supernatant and resuspend the cell pellet **in N-2 MAX/FGF Media.**
7. Count the cells and seed on a Poly-L-ornithine/Fibronectin-coated 24-well plate.
   Note: *Seeding density: 1 x 10^5 cells/well in 500 µL of N-2 MAX/FGF Media.*
8. Feed the cells with **N-2 MAX/FGF Media** every day for 4 days, followed by **N-2 MAX/FGF/EGF Media** for an additional 4 days, and lastly, **N-2 MAX/FGF/PDGF-AA Media** for the final 4 days.
   Note: *The cell number is expected to increase 2 to 3-fold during this period.*
9. **Optional:** Remove and fix one coverslip for staining with Anti-A2B5 Antibody.

STAGE V: DIFFERENTIATION TO OLIGODENDROCYTES (6-8 DAYS)

REAGENT PREPARATION FOR DIFFERENTIATION TO OLIGODENDROCYTES

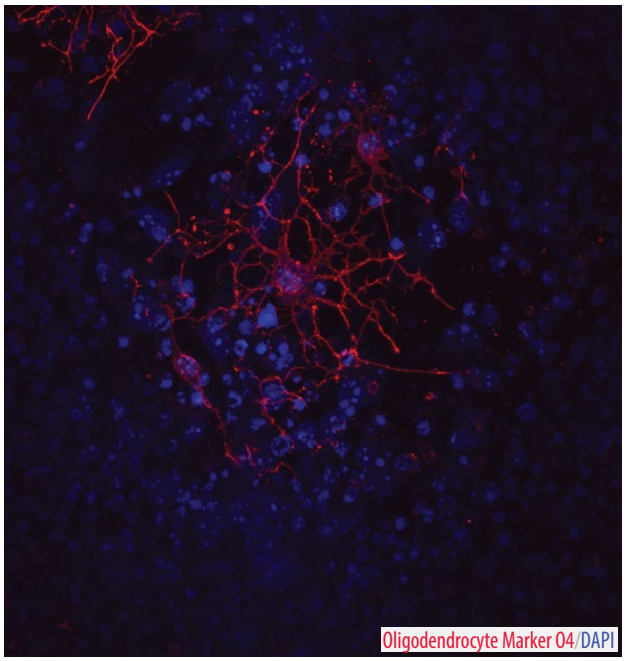
T3 Stock - Prepare a 20 µg/mL stock solution according to the manufacturer's instructions.

N-2 MAX/T3 Media - Dilute the T3 Stock 666-fold in N-2 MAX Media to make a 30 ng/mL solution. Prepare fresh as needed.

PROCEDURE FOR DIFFERENTIATION TO OLIGODENDROCYTES

1. Induce differentiation of expanded A2B5-positive cells from Stage IV by culturing the cells in N-2 MAX/T3 Media for 6-8 days. Change the N-2 MAX/T3 Media every 2 days.
2. After 6-8 days, oligodendrocytes can be identified by staining with O4 antibody (refer to references 6 and 9).

DATA EXAMPLE



Mouse Oligodendrocytes Generated Using the Mouse Oligodendrocyte Differentiation Kit. D3 mouse embryonic stem cells were expanded in KO-ES Media and differentiated into oligodendrocytes using the StemXVivo Mouse Oligodendrocyte Differentiation Kit. Oligodendrocytes were detected using Mouse Anti-Human/Mouse/Rat/Chicken Oligodendrocyte Marker O4 Monoclonal Antibody (R&D Systems, Catalog # MAB1326). The cells were stained with NorthernLights™-557 Affinity Purified Goat Anti-Mouse Secondary Antibody (R&D Systems, Catalog #NL019). The nuclei were counterstained with DAPI.

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NOTES