

Mouse Oligodendrocyte Differentiation Kit

For the differentiation of
mouse embryonic stem cells to oligodendrocytes

Product Number: SC15004

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PRINCIPLE OF THE ASSAY

The Mouse Oligodendrocyte Differentiation Kit is a system designed for *in vitro* neural differentiation of mouse ES cells in a serum-free environment (6). The kit contains specially formulated ITS and N-2 Plus 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 3×10^7 ES cells. This kit has been shown to generate an average of $30 \pm 5\%$ oligodendrocytes, as estimated from immunostaining various mouse ES cell lines with antibody specific to O4, an antibody that recognizes oligodendrocyte-specific glycolipid (7).

LIMITATIONS OF THE PROCEDURE

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.

The kit should not be used beyond the expiration date on the kit label.

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 of 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 Plus 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.

REAGENTS

ITS Supplement (Part 390154) - 5 mL of a 100X concentrated solution, containing bovine insulin, human transferrin and sodium selenite.

N-2 Plus Supplement (Part 390155) - 5 mL of a 100X concentrated solution, containing bovine insulin, human transferrin, sodium selenite, putrescine and progesterone.

Bovine Fibronectin Stock (Part 390213) - 1 vial containing 2 mL of a 1000X (1 mg/mL) solution of purified bovine fibronectin.

Human FGF basic (Part 390314) - 1 vial of lyophilized recombinant human FGF basic; enough to make 500 μ L of a 1000X stock.

Human EGF (Part 390315) - 1 vial of lyophilized recombinant human EGF; enough to make 100 μ L of a 1000X stock.

Human PDGF-AA (Part 390212) - 1 vial of lyophilized recombinant human PDGF-AA; enough to make 100 μ L of a 1000X stock.

STORAGE

Unopened Kit	Store at $\leq -20^{\circ}$ C in a manual defrost freezer. Do not use past kit expiration date.	
Opened Reagents	ITS Supplement	Store at 2 - 8 $^{\circ}$ C for up to 1 month or aliquot and store at $\leq -20^{\circ}$ C in a manual defrost freezer for up to 6 months.* Avoid repeated freeze-thaw cycles.
	N-2 Plus Supplement	
	Human FGF basic	
	Human EGF	
	Human PDGF-AA	
	Bovine Fibronectin	

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

OTHER SUPPLIES REQUIRED

Materials

Mouse Embryonic Stem (ES) cells (refer to reference 8 or use D3 cells from ATCC, www.atcc.org)
Mouse Embryonic Fibroblasts (MEF) feeder cells (prepared according to reference 9 or can be purchased at www.specialtymedia.com)
10 cm tissue culture dishes (Falcon Catalog # 353003 or equivalent)
10 cm bacterial culture dishes (Falcon Catalog # 351029 or equivalent)
12 mm cover slips (Assistant 1001/0012 Catalog # BA-63-3009 from www.carolina.com or equivalent)
24-well culture plates (Corning Costar Catalog # 3526 or equivalent)
15 mL centrifuge tubes (Corning Costar Catalog # 430052 or equivalent)
T-75 flasks (Corning Costar Catalog # 3375 or equivalent)
0.2 µm, 500 mL filter units (Nalgene Catalog # 161-0020 or equivalent)
0.2 µm syringe filter (PALL Gelman Laboratories Part # 4187)
10 mL syringes (BD Catalog # 309604)
Cryotubes (Nalgene Catalog # 5000-0012 or equivalent)
Serological pipettes
Pipettes and pipette tips

Reagents

Dulbecco's Modified Eagle Medium (DMEM) (GIBCO Catalog # 11965-092 or equivalent)
DMEM/F-12, no HEPES (GIBCO Catalog # 12500-062 or equivalent)
Fetal Bovine Serum, ES Cell Qualified (GIBCO Catalog # 16141-061 or equivalent)
Mitomycin C (Sigma Catalog # M0503 or equivalent)
Phosphate Buffered Saline (PBS) (GIBCO Catalog # 10010-023 or equivalent)
0.05% Trypsin/EDTA (GIBCO Catalog # 25300-054 or equivalent)
Gelatin (Sigma Catalog # G2500 or equivalent)
ESGRO[®] (recombinant mouse LIF) (Chemicon Catalog # ESG1106 or equivalent)
Knock-out DMEM (GIBCO Catalog # 10829-018 or equivalent)
MEM Non-essential AA Solution (GIBCO Catalog # 11140-050 or equivalent)
Penicillin-Streptomycin-Glutamine, 100X (GIBCO Catalog # 10378-016 or equivalent)
Penicillin-Streptomycin, 100X (GIBCO Catalog # 15140-148 or equivalent)
2-Mercaptoethanol, 1000X (GIBCO Catalog # 21985-023 or equivalent)
Glucose (Sigma Catalog # G6152 or equivalent)
L-Glutamine (Sigma Catalog # G5763 or equivalent)
Sodium Bicarbonate, NaHCO₃ (Sigma Catalog # S5761 or equivalent)
Poly-L-ornithine (Sigma Catalog # P3655 or equivalent)
T3 (3,3',5-Triiodo-L-thyronine sodium salt) (Sigma Catalog # T5516 or equivalent)
Sterile, deionized water
BSA, very low endotoxin (Serologicals Proteins Inc. Catalog # 81-068-3 or equivalent)
Acetic acid (J.T. Baker Catalog # 9508-03 or equivalent)

Equipment

37° C and 5% CO₂ incubator
37°C water bath
60° C hot plate
Centrifuge
Hemocytometer
Microscope

PROCEDURE OUTLINE

Stage I: Expansion of Undifferentiated
ES Cells (3 - 4 days)



Stage II: Formation of Embryoid
Bodies (4 days)



Stage III: Selection of Nestin-positive
Cells (6 - 8 days)



Stage IV: Induction of A2B5-positive
Cells (12 days)



Stage V: Differentiation to
Oligodendrocytes
(6 - 8 days)

Total: 31 - 36 days

REAGENT AND MATERIAL PREPARATION

Use serological pipettes to transfer and remove solutions.

Preparation of ES Cells

Mitomycin C Stock (100X) - Add 2 mL of sterile, deionized water to a 2 mg vial of Mitomycin C to make a 100X concentrate (final concentration of 1 mg/mL). Protect from light. Store at 2 - 8° C for up to 6 weeks.

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.

Gelatin-coated Dishes - Prepare immediately before use.

1. Add 10 mL of 0.1% sterile Gelatin Solution to a 10 cm tissue culture dish (0.127 mL/cm²).
2. Incubate at room temperature for 15 minutes.
3. Remove the 0.1% Gelatin Solution.
4. Air dry for 30 minutes by leaving the lid half-open in a hood at room temperature.

MEF Medium - Mix the following sterile ingredients to make 505 mL of medium. Store at 2 - 8° C for up to 1 month.

Item	Amount	Final Concentration
DMEM	450 mL	90%
Fetal Bovine Serum	50 mL	10%
100X Penicillin-Streptomycin-Glutamine	5 mL	100 U/mL Penicillin 100 µg/mL Streptomycin 2 mM L-Glutamine

KO-ES Medium - Mix the following sterile ingredients to make 510.5 mL of medium. 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

Stage I: Expansion of Undifferentiated ES Cells

0.1% Gelatin Solution - See previous recipe.

Gelatin-coated Dishes - See previous recipe.

KO-ES Medium - See previous recipe.

Stage II: Formation of Embryoid Bodies

KO-ES Medium - See previous recipe.

Stage III: Selection of Nestin-positive Cells

ITS Medium - Mix the following ingredients with deionized water to make 500 mL of medium. Adjust the pH to 7.5 ± 0.2 . Sterile filter the solution using a 500 mL 0.2 μ m 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
Glucose	775 mg
L-Glutamine	36.5 mg
NaHCO ₃	1.2 g
ITS Supplement (100X)	5 mL

Fibronectin Stock (1000X) - Allow the stock to stand for 30 minutes at room temperature, without agitation. Swirl very gently. Aliquot and store at $\leq -20^{\circ}$ C in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

ITS/Fibronectin Medium - Dilute Fibronectin Stock (1000X) 200-fold in ITS Medium to make a 5 μ g/mL solution (e.g. 50 μ L of Fibronectin Stock in 10 mL of ITS Medium). Prepare fresh as needed.

Stage IV: Induction of A2B5-positive Cells

N-2 Plus Medium - Mix the following ingredients with deionized water to make 500 mL of medium. 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
Glucose	775 mg
L-Glutamine	36.5 mg
NaHCO ₃	845 mg
N-2 Plus Supplement (100X)	5 mL

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 $\leq -20^\circ\text{C}$ 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 $< -20^\circ\text{C}$ 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 human PDGF-AA vial. Aliquot and store at $\leq -20^\circ\text{C}$ in a manual defrost freezer for up to 6 months. Avoid repeated freeze-thaw cycles.

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

N-2 Plus/FGF/EGF Medium - Dilute the FGF basic and EGF Stocks 1000-fold in N-2 Plus Medium. Prepare fresh as needed.

N-2 Plus/FGF/PDGF-AA Medium - Dilute the FGF basic and PDGF-AA Stocks 1000-fold in N-2 Plus Medium. 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 $\leq -20^\circ\text{C}$ 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 1X solution (15 $\mu\text{g/mL}$). Prepare fresh as needed.

Fibronectin Solution (1X) - Dilute the Fibronectin Stock 1000-fold in sterile PBS to make a 1X solution (1 $\mu\text{g/mL}$). Mix by gentle 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.

Stage V: Differentiation to Oligodendrocytes

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

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

PREPARATION OF ES CELLS

Use serological pipettes to transfer and remove solutions.

MEF Feeder Cells

1. Plate the MEF feeder cells from the frozen stock ($1 - 2 \times 10^6$ cells) into a T-75 flask containing 25 mL of MEF Medium. Incubate at 37° C and 5% CO₂. Cells should become adherent after 4 hours.
2. Feed the cell culture daily by replacing with fresh medium until subconfluent.
3. Remove the medium. Add 10 mL of MEF Medium supplemented with 0.1 mL of Mitomycin C Stock (final concentration of 10µg/mL). Incubate for 3 hours at 37° C and 5% CO₂.
4. Remove the MEF Medium containing Mitomycin C. Wash the feeder cells 3 times with 10 mL of sterile PBS.
5. Add 2 mL of 0.05% Trypsin/EDTA. Incubate for 5 minutes at 37° C and 5% CO₂.
6. Tap the flask to dislodge the feeder cells. Add 10 mL of MEF Medium to neutralize the trypsin.
7. Transfer the cells to a 15 mL tube by gentle pipetting. Centrifuge for 5 minutes at 220 x g.
8. Remove the supernate and resuspend the feeder cells with 10 mL of MEF Medium. Count the cells. At this point, extra cells can be frozen down for future use, according to the procedure described in reference 9.
9. Seed 3×10^6 Mitomycin C-treated feeder cells in 10 mL of MEF Medium in a Gelatin-coated Dish. Incubate for 24 hours at 37° C and 5% CO₂.

ES Cell Culture

1. Twenty-four hours after the Mitomycin C-treated feeder cells are plated, warm the KO-ES Medium containing ESGRO (1400 U/mL) in a 37° C water bath for 20 minutes. In a 15 mL tube, resuspend 3×10^6 ES cells from a frozen stock vial with 10 mL of warmed KO-ES Medium containing ESGRO.
2. Remove the MEF Medium from the feeder cells and wash once with 5 mL of KO-ES Medium.
3. Add the ES cell suspension on top of the feeder cells and incubate the culture at 37° C and 5% CO₂. Twenty-four hours after the ES cells are plated, the ES cells should become attached to the feeder cell layer.
4. Feed the cells daily by replacing with fresh KO-ES Medium containing ESGRO. ES cells should start proliferating as indicated by increasing size of colonies on top of the feeder cell layer. Refer to reference 9 for images of the cell colonies.
5. Incubate the cells for 2 days (or before individual colonies contact each other) at 37° C and 5% CO₂. Harvest the cells as follows:
 - a. Remove the medium. 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% CO₂.
 - c. Tap the dish gently to dislodge the cells. Add 5 mL of KO-ES Medium to neutralize the trypsin.
 - d. Transfer the cells to a 15 mL tube by gentle pipetting. Centrifuge for 5 minutes at 220 x g.
 - e. Remove the supernate and gently resuspend the ES cells with 5 mL of KO-ES Medium containing ESGRO. Count the cells. At this point, cells can be either frozen down by following the protocol described in ***Disaggregation, expansion and feeding of transfected ES clones*** (<http://medicine.wustl.edu/~escore/>) or used in Stage I of this protocol.

PROCEDURE

Stage I: Expansion of Undifferentiated ES Cells

1. Warm the KO-ES Medium containing ESGRO in a 37°C water bath for 20 minutes.
2. Seed 3×10^6 ES cells in 10 mL of warmed KO-ES Medium containing ESGRO on a Gelatin-coated Dish.

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 they form Embryoid bodies.

3. Culture the cells for 3 - 4 days according to the procedure outline at 37°C and 5% CO₂ or before cell colonies contact each other. Replace the KO-ES Medium containing ESGRO daily.

Stage II: Formation of Embryoid Bodies

1. Discard the medium. 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% CO₂.
3. Gently tap the dish to dislodge the cells. Add 5 mL of KO-ES Medium 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 supernate and resuspend the cell pellet in 5 mL of KO-ES Medium. Obtain a cell count. Approximately 15×10^6 cells/dish can be expected from the harvest.
6. Seed 2×10^6 cells on a 10 cm bacterial culture dish containing 10 mL of KO-ES Medium.
7. Culture the cells for 4 days. Change the medium on day 2 in the following manner:
 - a. Transfer the embryoid bodies (EB) to a 15 mL tube by gentle pipetting. Do not centrifuge.
 - b. Allow the tube to stand until the EB settle to the bottom (about 5 minutes).
 - c. Remove the medium. Add 10 mL of fresh KO-ES Medium.
 - d. Transfer the EB back into the original dish.
8. On day 4, proceed to Stage III.

Stage III: Selection of Nestin-positive Cells

1. At the end of Stage II, transfer the EB to a 15 mL tube by gentle pipetting.
2. Allow the tube to stand until the EB settle to the bottom. Remove half of the medium.
3. Rinse the original dish with 5 mL of fresh KO-ES Medium and add to the 15 mL tube.
4. Transfer the EB to a 10 cm tissue culture dish by gentle pipetting. Culture for 24 hours at 37° C and 5% CO₂. At this time, the EBs should become attached.
5. Change the medium from KO-ES Medium to 10 mL ITS/Fibronectin Medium.
6. Culture the cells for 6 - 8 days. Change the ITS/Fibronectin Medium every 2 days. During this period, a monolayer will grow from the attached EBs.

Stage IV: Induction of A2B5-positive cells

1. Remove the ITS/Fibronectin Medium 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 for 5 minutes. Gently tap the dish to dislodge the cells. Add 5 mL of KO-ES Medium to neutralize the trypsin.
3. Transfer the cells to a 15 mL tube by gentle pipetting. Remove the cell clumps (remnants of Ebs) 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 15 mL tube by gentle pipetting.
5. Centrifuge the suspension for 5 minutes at 220 x g to pellet the cells.
6. Discard the supernate and resuspend the cell pellet in N-2 Plus/FGF Medium.
7. Count the cells and seed on a Poly-L-ornithine/Fibronectin Coated Plate.

Note: Seeding Density: 1×10^5 cells/well in 500 μ L of N-2 Plus/FGF Medium. .

8. Feed the cells with N-2 Plus/FGF Medium every day for 4 days, followed by N-2 Plus/FGF/EGF Medium for an additional 4 days, and lastly, N-2 Plus/FGF/PDGF-AA Medium for the final 4 days.

Note: During the period, cell number is expected to increase 2 to 3-fold.

Stage V: Differentiation to Oligodendrocytes

1. Induce differentiation of expanded A2B5-positive cells from Stage IV by feeding the cells with N-2 Plus/T3 Medium for 6 - 8 days. Change the N-2 Plus/T3 Medium every 2 days.
2. After 6 - 8 days, oligodendrocytes can be identified by staining with O4 antibody (refer to references 6 and 7).

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NOTES

