Differentiation of mouse pluripotent stem cells to motor neurons

Pub. No. MAN0016688 **Rev.** A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Introduction

Biological cell models are powerful tools that allow researchers to study and investigate a variety of topics from developmental biology to translational medicine. Mouse pluripotent stem cells (mPSCs) are particularly useful because they enable genetic modification at the cellular level as well as the creation of whole animal models.

Over the last years, several reports have introduced methods for the *in vitro* generation of specific neuronal subtypes from mPSCs. This protocol based on literature describes how to differentiate motor neurons from mPSCs maintained in either KnockOut™ Serum Replacement–Multi-Species medium on MEFs or in feeder-free medium.

Required materials

Item	Source
Mouse pluripotent stem cells	—
C57BL/6 Mouse Embryonic Fibroblasts, Irradiated	MTI-GlobalStem, GSC-6202G
Attachment Factor Protein (1X)	S006100
DMEM/F-12, GlutaMAX™ Supplement	10565
Neurobasal™ Medium	21103
KnockOut™ Serum Replacement–Multi-Species	A31815
2-Mercaptoethanol	21985
DPBS, no calcium, no magnesium	14190
StemPro™ Accutase™ Cell Dissociation Reagent	A11105
Retinoic Acid	Sigma-Aldrich, R2625
Sonic Hedgehog C25II Recombinant Mouse Protein	PMC8034
Nunclon™ Sphera™ Dishes	174930, 174931, or 174932 (for suspension)
Mouse Laminin Protein, Natural	23017
Poly-D-Lysine	Sigma-Aldrich, P7280, P9155
Human Neural Stem Cell Immunocytochemistry Kit	A24354

Prepare media and plates

Prepare retinoic acid stock

1. To prepare 50 mM stock solution (100,000X), add 3.33 mL of DMSO solution to 50 mg Retinoic Acid (RA) and mix until dissolved.
2. Dilute 50 mM stock solution further to 5 mM (10,000X) with DMSO solution.
3. Aliquot 50–500 µL into sterile tubes, and store at –20°C.

Prepare Sonic hedgehog stock

1. Reconstitute Sonic hedgehog (Shh) with 0.1% BSA PBS solution at a concentration of 200 µg/mL.
2. Aliquot 50–100 µL into sterile tubes, and store at –20°C.

Prepare differentiation medium

Aseptically mix the following components:

Component	Final concentration	Amount[1]
DMEM/F-12, GlutaMAX™ Supplement	0.5X	45 mL
Neurobasal™ Medium	0.5X	45 mL
KnockOut™ Serum Replacement–Multi-Species	10%	10 mL
2-Mercaptoethanol	55 µM	100 µL

[1] To make larger volumes, increase the component amounts proportionally.

Prepare mPSC Culture Medium (500 mL of complete medium)

Aseptically mix the following components:

Reagent	Volume
DMEM/F-12, GlutaMAX™ Supplement	395 mL
KnockOut™ Serum Replacement–Multi-Species	100 mL
MEM Non-Essential Amino Acids Solution	5 mL
2-Mercaptoethanol	500 µL
LIF Recombinant Mouse Protein[1], 10 µg/mL	500 µL

[1] Alternatively, for best results, add LIF fresh to an aliquot of medium before use. Final concentration should be 10 ng/mL.

Complete mPSC Culture Medium can be stored at 2–8°C for up to 3 weeks.

Coat culture vessels with Attachment Factor

Attachment Factor (AF) is a sterile solution (1X) containing 0.1% gelatin.

1. Cover the whole surface of each new culture vessel with AF solution and incubate the vessels for 30 minutes at 37°C or for 2 hours at room temperature.
2. Use sterile technique in a laminar flow culture hood, and completely remove the AF solution from the culture vessel by aspiration just prior to use.

   It is not necessary to wash the culture surface before adding cells or medium. Coated vessels may be used immediately or stored at room temperature for up to 24 hours.

Prepare Poly-D-Lysine and laminin double-coated plates

1. Prepare a 100 µg/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 to 2 hours.
4. Remove the Poly-D-Lysine solution and rinse 3 times with distilled water.
5. Prepare a 15 µg/mL working solution of laminin in sterile in distilled water.
6. Add 1 mL of the 15 µg/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.

Prepare mPSCs before differentiation

Cells co-cultured with mouse embryonic fibroblasts need to be depleted of fibroblasts prior to differentiation. To deplete fibroblasts, passage cells on a AF- or gelatin-coated plate without feeder cells, as described in "Passage mouse pluripotent stem cells". After two passages without feeder cells, cultures may be considered depleted of fibroblasts and may be used for downstream differentiation.

Passage mouse pluripotent stem cells

1. Aspirate spent medium and feed mPSCs with pre-warmed mPSC culture medium 2–4 hours before passaging.
2. Aspirate the spent medium from the dish and rinse the dish twice with DPBS, no calcium, no magnesium.
3. Aspirate DPBS and add StemPro™ Accutase™ Cell Dissociation Reagent to the dish containing mPSCs.
4. Incubate the dish for 3–5 minutes in a 37°C, 5% CO_2 incubator until individual single cells start to round up.
5. Gently pipet StemPro™ Accutase™ Cell Dissociation Reagent up and down to get a single-cell suspension using a 1-mL pipette.

   Make sure to pipet gently to minimize the formation of bubbles.
6. Add 2X volume of mPSC Culture Medium to the dish to stop the dissociation reaction.
7. Transfer the mPSC suspension into a 15-mL conical tube and centrifuge the tube at 200 × g for 5 minutes to pellet the cells.
8. Carefully aspirate the supernatant from the mPSC pellet.
9. Resuspend the pellet with an appropriate amount of mPSC Culture Medium.
10. Plate cells on a new AF- or gelatin-coated dish at 30,000 ~50,000 cells/cm^2 and return the dish to the incubator. See "Coat culture vessels with Attachment Factor" on page 2

    Note: Incubate for about 2 days before passaging again or before using for differentiation.

Differentiation workflow

Day	Procedure
0	EB formation in differentiation medium
1	Change medium
2	Split EBs 1:4 in differentiation medium containg RA and Shh
4	Plate spheres on coated plate
7	Characterize motor neurons

Differentiate and culture embryoid bodies (EBs)

1. Harvest cells as described in "Passage mouse pluripotent stem cells".
2. Plate the mPSCs into Petri dishes or Nunclon™ Sphera™ Dishes in differentiation medium at 2 × 10^5 cells/mL (i.e. 2 × 10^6 cells per 100-mm dish).
3. Incubate the cells in a 37°C, 5% CO_2 incubator to allow them to form EBs.
4. On the next day, feed EBs with fresh differentiation medium by transferring EBs into a 15-ml conical tube and spinning the tube at 200 x g for 1 minute.
5. Resuspend EBs in fresh mPSC differentiation medium and replate them in a new Petri dish or Sphera™ dish.

6. On the next day, divide the EBs into 4 new dishes with fresh differentiation medium supplemented with 0.5 µM RA and 200 ng/mL Shh protein.

   Note: After two days, cells should be enriched with Sox1 positive progenitor cells. Sox1 and other neural progenitor markers can be analyzed using the Human Neural Stem Cell Immunocytochemistry Kit (Cat. No. A24354). See Figure 1 A and B.

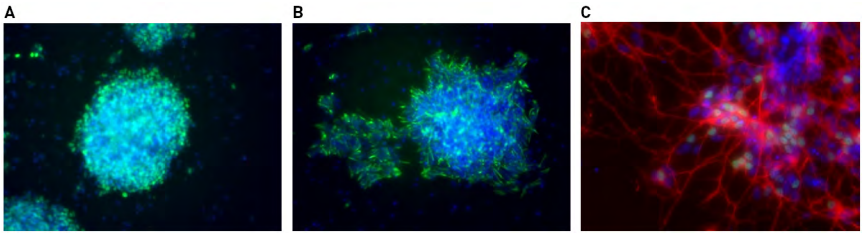

Figure 1 Phenotypic markers for neural progenitors and motor neurons

After 5 days of differentiation, cells express neural progenitor markers Sox1 (A) and Nestin (B). C: After 7 days of differentiation, cells express motor neuron marker HB9 (green) and neuronal marker Dcx (red). NucBlue™ reagent (a DAPI nuclear DNA stain) (blue) was used to label all cells.

Plate progenitors for motor neuron differentiation

1. Prepare a double-coated plate using Poly-D-Lysine (15–100 µg/mL) and Laminin (10–100 µg/mL). See "Prepare Poly-D-Lysine and laminin double-coated plates" on page 2.

2. Resuspend spheres in fresh differentiation medium containing RA and Shh. Transfer spheres and medium into the double-coated well(s) at a 1:1 to 1:2 transfer ratio.

   Note: Do not let coated plate dry out as this will prevent attachment of cells later. Minimize the time between removing the laminin solution and adding the cell suspension.

   Note: After 3 days, cells should be enriched with HB9 positive motor neurons. See Figure 1C.

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