MILLIPORE

Mouse Embryonic Stem Cell Neurogenesis Kit

10 Reactions

Catalogue No. SCR101

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

USA & Canada
Phone: +1(800) 437-7500 • Fax: +1 (951) 676-9209 • Europe +44 (0) 23 8026 2233
Australia +61 3 9839 2000 • Germany +49-6192-207300 • ISO Registered worldwide
www.Millipore.com

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Introduction

Numerous neurological disorders, including Parkinson Disease, Alzheimer Disease, Amyotrophic Lateral Sclerosis (ALS) and spin cord injuries, remain inoperable disorders that are caused by an absence of functional neurons within the central nervous system. The expansion of functional neurons for transplantation and biomedical research remains inefficient.

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass (ICM) of preimplantation embryos and are capable of unlimited, undifferentiated proliferation *in vitro* under appropriate cell culture conditions. These cells have the unique ability to differentiate into cells comprising all three embryonic germ layers (ectoderm, mesoderm and endoderm). Because of their pluripotent and robust growth characteristics, embryonic stem cells have been used to model neurogenesis and to generate large quantities of functional neurons for both *in vitro* and *in vivo* studies.

Product Description

Millipore's Mouse Embryonic Stem Cell Neurogenesis Kit (Catalogue No. SCR101) provides a system designed for the neural differentiation of mouse ES cells. The kit contains all the reagents necessary to fully differentiate mouse ES cells into βIII-tubulin positive neurons *in vitro* following a modified 4-/4+ differentiation protocol developed by Gottlieb et. al (1). The kit provides enough reagents for ten separate differentiation reactions. Included in the kit are the following:

(1) One vial each of poly-L-ornithine and laminin, two key substrates that provide proper support for cell attachment and spreading.

(2) One 100 mL bottle of Accutase, an enzymatic reagent for the detachment of cells.

(3) Five 100 mL bottles of a specially formulated medium that has been optimized and qualified to support the formation of embryoid bodies.

(4) One vial of Neural Inducer A Solution.

(5) One vial each of SSEA-1 and βIII-tubulin monoclonal antibodies to characterize the starting undifferentiated mouse ES cell populations and to validate the resulting neurons produced from the differentiation protocol provided, respectively.

Kit Components

Sufficient reagents are provided in the kit for 10 separate differentiation reactions.

1. Embryoid Body (EB) Formation Medium: (Catalogue No. SCM018) Five (5) 100 mL bottles. Store at -20°C.
2. Accutase: (Catalogue No. SCR005) One (1) 100 mL bottle. Store at -20°C.
3. Neural Inducer A Solution: (Catalogue No. 2005820) One 200 µL vial of 500 µM retinoic acid. Store at -20°C.
4. Mouse Laminin, 200UG: (Catalogue No. CC095-200UG) One vial of 200 µg mouse laminin. Store at -20°C.
5. Poly-L-ornithine Solution: (Catalogue No. CS201485) One (1) vial of 2 mg poly-L-ornithine (10 mg/mL). Store at -20°C.
6. Mouse anti-SSEA-1, 50UG: (Catalogue No. MAB4301-50UG) One (1) vial of 50 µg IgM monoclonal. Store at -20°C.
7. Mouse anti-βIII tubulin, 50UL: (Catalogue No. MAB1637-50UL) One (1) vial of 50 µL IgG monoclonal. Store at -20°C.

Stability and Storage

When stored at the recommended storage conditions (refer to Kit Components), components are stable up to the expiration date listed on the bottle. Do not expose to elevated temperatures. Discard any remaining reagents after the expiration date.

Embryoid Body (EB) Formation Medium: Medium should be stored at -20°C until ready to use. At -20°C the medium is stable for up to six months. Medium is provided in convenient 100 mL bottles. Prior to initial use, thaw frozen media at 2 to 8°C overnight or until it has become completely equilibrated. Upon thawing, medium should be stored at 2 to 8°C and given a 1-month expiration dating.

Accutase: Stable when stored at -20°C. Refer to lot expiration date. Recommended storage upon receipt is -20°C. After thawing, Accutase may be stored for up to 2 months at 2 to 8°C. DO NOT STORE AT ROOM TEMP.

Neural Inducer A Solution: Solution is light sensitive and is readily oxidized upon exposure to air. Neural Inducer A Solution should be stored in working aliquots (10-20 μL) at -20°C for up to 1 year after date of receipt. Prolonged exposure to air and light will result in significant loss of activity.

Laminin Solution: Solution is stable for up to six months at -20°C.

Poly-L-ornithine Solution: Solution is stable for up to 2 years at -20°C.

Mouse anti-SSEA-1 and Mouse anti-βIII tubulin antibodies should be maintained at -20°C in undiluted aliquots for up to 12 months after date of receipt. Avoid repeated freeze/thaw cycles.

Materials Required But Not Provided

1. Cryopreserved Mouse Embryonic Stem Cells (Cat. Nos. SCR011, SCR012, SCC013, CMTI-1, and CMTI-2)
2. EmbryoMax® Complete ES Cell Media w/15% FBS and LIF; 500 mL (Cat. No. ES-101-B)
3. ESGRO® mLIF Medium Supplement (Cat. No. ESG1106, ESG1107)
4. EmbryoMax ES Cell Qualified 0.1% Gelatin Solution, 500 mL (Catalogue No. ES-006-B)
5. Petri Dishes (BD Cat. No. 351008)
6. Phosphate-Buffered Saline (1X PBS) (Cat. No. BSS-1005-B)
7. Fixative (e.g. 4% Paraformaldehyde in 1X PBS)
8. Blocking Solution (5% normal donkey serum, 0.3% Triton X-100 in 1X PBS)
9. Non-Permeable Blocking Solution (5% normal donkey serum in 1X PBS)
10. Fluorescent-labeled secondary antibodies. Donkey anti-mouse IgG Cy3 conjugated (Catalogue No. AP192C) and donkey anti-mouse IgM, Cy3 conjugated (Jackson Laboratories Cat. No. 715-165-140) are recommended
11. Isotype controls (e.g. mouse IgG (Cat. No. PP54) and mouse IgM (Catalogue No. PP50)
12. 4'-6-Diamidino-2-phenylindole (DAPI) / PBS solution
13. Anti-fading mounting solution (DABCO/PVA)
14. Hemacytometer

15. Trypan Blue
16. Microscope with appropriate fluorescent filters
17. Nunc Lab-Tek II 8 well chamber slides (Fisher Cat. No. 12-565-8)

Removal of Feeders from Mouse ES Cell Culture

If mouse embryonic stem cells are grown on a mouse embryonic fibroblast feeder (MEF) layer, it is important that the feeder layer is removed before mouse ES cells are differentiated. The following protocol is recommended.

1. Carefully remove the medium used to culture mouse ES cells from the 10-cm tissue culture plate and wash the plate twice with 1X PBS.
2. Apply 5 mL Accutase and incubate in a 37°C incubator for 3-5 minutes.
3. Inspect the plate and ensure the complete detachment of the cells (both ES and MEFs) by gently tapping the side of the plate with the palm of your hand.
4. Apply 5 mL Complete ES Cell Media w/ 15% Serum and LIF (Cat. No. ES-101-B) (pre-warmed to 37°C) to the plate and use this medium to collect the cells onto a 15 mL conical tube.

   Note: *For users who would like to make their own ES growth medium, the final media composition should be DMEM-High Glucose, 15% FBS, 1X Glutamine, 1X Non-Essential Amino Acids, 1X β-mercaptoethanol, and 1X PSF).*

5. Centrifuge the tube at 300 xg for 2-3 minutes to pellet the cells.
6. Discard the supernatant.
7. Apply 10 mL Complete ES Cell Media w/ 15% Serum and LIF (pre-warmed to 37°C) to the conical tube and resuspend the cells thoroughly. **IMPORTANT: Do not vortex.**
8. Transfer the cell suspension to a fresh sterile 10-cm plate that has been pre-coated with 0.1% gelatin solution.
9. Incubate in 37°C incubator for 30 minutes.
10. After 30 minutes, remove the medium and any non-attached cells and transfer this cell suspension onto another 0.1% gelatin coated 10-cm plate.

    Note: *Mouse embryonic fibroblasts (MEFs) adhere more readily to the tissue-culture plate than undifferentiated mouse ES cells and thus a significant number of MEFs can be removed by this differential adhesion step.*

11. Repeat step 9 and 10 two to three more times.
12. Repeat the whole procedure (steps 1 through 11) for at least three passages to ensure that any residual MEFs are removed from the mouse ES cell culture.

Formation of Embryoid Bodies (4- Stage)

1. Carefully remove the medium used to culture feeder-free mouse ES cells from the 10-cm tissue culture plate and wash the plate twice with 1X PBS.
2. Apply 5 mL Accutase and incubate in a 37°C incubator for 3-5 minutes.
3. Inspect the plate and ensure the complete detachment of the cells (both ES and feeders) by gently tapping the side of the plate with the palm of your hand.
4. Apply 5 mL Embryoid Body (EB) Formation medium (pre-warmed to 37°C) to the plate and use this medium to collect the detached cells and transfer the cell suspension to a 15 mL conical tube.
5. Centrifuge the tube at 300 xg for 2-3 minutes to pellet cells.
6. Discard the supernatant.
7. Apply 2 mL EB Formation Medium to the conical tube and resuspend the cells thoroughly. **IMPORTANT: Do not vortex.**
8. Count the number of cells using a hemacytometer.
9. Aliquot 2-3 x 10^6 cells in 10 mL EB Formation Medium and place in a sterile 10-cm bacterial Petri dish or ultra low attachment Petri dish.
10. Incubate the cells in 37°C, 9-10% CO_2 incubator for two days. After two days, there should be numerous floating embyroid bodies (EBs) formed (refer to Figure 3).
11. On the second day, transfer the floating EBs and the entire medium in the 10-cm Petri dish to a 50 mL conical tube.
12. Wash the 10-cm Petri dish twice with 10 mL EB Formation Medium and transfer each wash to the same 50-mL conical tube.
13. Leave the conical tube at room temperature for 15 minutes to allow the EBs to settle to the bottom of the tube.

14. Using a 10 mL or larger pipette, carefully remove the supernatant.

    Note: *We do not recommend aspirating with a vacuum.*

15. Resuspend the EBs in 10 mL EB Formation Medium (pre-warmed to 37°C).

16. Transfer the entire cell suspension to a sterile 10-cm Petri dish. Incubate the EBs in 37°C, 9-10% CO_2 incubator for an additional two days.

Induction of Neuronal Differentiation (4+ Stage)

1. After a total of 4 days in suspension culture, collect the EBs to a 50 mL conical tube.

2. Wash the 10-cm Petri dish twice with 10 mL EB Formation Medium and transfer each wash to the same 50 mL conical tube.

3. Leave the conical tube at room temperature for 15 minutes to allow the EBs to settle to the bottom of the tube. Set aside.

4. Prepare the Neural Induction medium by adding 10 μL Neural Inducer A Solution to 10 mL EB Formation Medium.

5. To the tube containing the EBs, carefully remove and discard the supernatant with a 10 mL or larger pipette.

   Note: *We do not recommend aspirating the supernatant with a vacuum.*

6. Resuspend the EBs in 10 mL Neural Induction Medium (from step 4) and transfer the suspension to a fresh Petri dish.

7. Incubate the dish of cells in 37°C, 9-10% CO_2 incubator for two days.

8. After two days, repeat steps 1 through 7 for a total of 4 days in the Neural Induction Medium.

Preparation of Coated Glass Slides (for 8-well chamber slides)

1. Dilute poly-L-ornithine stock solution (10 mg/mL) with water to yield 50 μg/mL solution.

2. Add enough of the poly-L-ornithine solution (50 μg/mL) to cover the whole surface of the each well. Use 0.5 mL volume for each well of an 8-well chamber slide. Incubate overnight at room temperature.

3. The next day, rinse each well with sterile water. Aspirate after each rinse.

4. Using sterile 1X PBS, dilute the laminin to a final concentration of 5 µg/mL.

5. Add enough laminin (5 µg/mL) solution to each well to cover the surface. Use 0.5 mL volume for each well of an 8-well chamber slide. Incubate for 4 hours at 37°C.

6. Coated slides can be stored in the laminin solution at -20°C for 6-8 months. The tray of slides should be wrapped in plastic saran wrap before storage at -20°C.

7. Just before use, aspirate the laminin solution in the coated wells and wash the wells once with 1X PBS. Aspirate the 1X PBS before plating the cells.

Expansion of Neuronal Cells from Embryoid Bodies (8- Stage)

1. From step 8 of section titled "Induction of Neuronal Differentiation," gently swirl the 10-cm Petri dish containing the neural induced EBs counterclockwise for several circular rotations.

   Note: *Use caution to avoid sloshing the media over the side of the plate as this may increase the risk of cell contamination. The swirling process helps localize all of the EBs to the center of the 10-cm Petri dish.*

2. Immediately place the 10-cm Petri dish under a microscope and count the number of EBs that have localized to the center of the 10-cm Petri dish. This is your total number of EBs in the culture. Typical yields are 50 to 100 EBs per 10-cm Petri dish.

3. Using a 10 mL pipette, collect and transfer the EB suspension to a 50 mL conical tube.

4. Wash the 10-cm Petri dish twice with 10 mL EB Formation Medium and transfer each wash to the same 50 mL conical tube.

5. Leave the conical tube at room temperature for 15 minutes to allow the EBs to settle to the bottom of the tube.

6. Using a 10 mL or larger pipette, remove and discard the supernatant.

7. Resuspend the EBs in 2 mL EB Formation Medium (pre-warmed to 37°C).

8. Plate approximately 10 to 20 EBs to each well of a poly-L-ornithine and laminin coated 8-well chamber slide (please refer to the section on Preparation of Coated Glass Slides).

9. Exchange the medium in each well with 0.5 mL fresh EB Formation Medium every two days for a total of 8 days. Neuronal like cells and their

processes should extend and migrate from the attached EBs in as little as four days (Figure 5).

***Note**: Use extreme care when exchanging the medium as the cells are loosely adherent. Always leave behind a small volume of medium to ensure that the cells do not dry out.*

Staining Protocol (for 8-well chamber slides)

1. Carefully aspirate the medium and wash the wells three times with 1X PBS (5 minutes per wash).

   ***Note:** Use extreme care when exchanging media as the cells are loosely adherent. Always leave behind a small volume of medium to ensure that the cells do not dry out.*

2. Fix the cells by incubation in 4% paraformaldehyde for 30-40 minutes at room temperature.

3. Carefully aspirate the fixative and rinse three times (5-10 minutes each) with 1X PBS. Aspirate after each rinse.

4. Wash the cells with 0.5 mL Non-Permeable Blocking Solution (5% normal donkey serum in 1X PBS) three times (5 minutes each). Aspirate after each wash.

5. Apply 0.25 mL fresh Blocking Solution for at least 2 hours at room temperature or overnight at 4°C. **IMPORTANT: Do not shake the cells**.

   ***Note:** For optimal results, use the Blocking Solution (e.g. 5% normal donkey serum, 0.3% Triton X-100 in 1X PBS) with the antibody directed against the neuronal cell marker, βIII-tubulin. Use the Non-Permeable Blocking Solution (5% normal donkey serum in 1X PBS) with the antibody directed against the mouse ES cell marker, SSEA-1.*

6. Dilute the primary antibodies included in this kit to working concentrations in the appropriate blocking solutions. For optimal results, the following antibody dilutions are recommended for immunocytochemistry (see images):

   Mouse anti-SSEA-1, IgM monoclonal: 1/500 dilution of 1.7 mg/mL, final 3.4 μg/mL

   Mouse anti-βIII-tubulin, IgG monoclonal: 1/500 to 1/1000 dilution of monoclonal antibody

7. In a separate control well, depending upon the specific antibody used, add equivalent concentrations of mouse IgG or mouse IgM to 0.25 mL of the appropriate blocking solution.

8. Incubate the cells in primary antibody overnight at 4°C. **IMPORTANT: Do not shake.**

9. The next day, wash the cells three times with 1X PBS (5-10 minutes each wash) and three times with the appropriate blocking solution.

10. At the completion of the last wash, leave the cells in the appropriate blocking solution for at least 30 minutes.

11. Dilute secondary antibodies in the appropriate blocking solution just before use. The following secondary antibody can be used, donkey anti-mouse IgG Cy3 conjugated (Catalogue No. AP192C) and donkey anti-mouse IgM Cy3 conjugated (Jacksons Laboratories) at a 1:500 dilution.

12. Overlay the cells with the secondary antibody blocking solution for 2 hours at room temperature in the dark.

13. Wash 3-5 times (5-10 minutes each) with 1X PBS.

14. Counterstain the cell nuclei with DAPI / 1X PBS solution.

15. Mount a glass coverslip over the chamber slides using antifading mounting solution (e.g. DABCO/PVA)

16. Visualize the cell staining with a fluorescent microscope.

    Note: *Be sure to use the correct filter to visualize fluorescent-labeled cells.*

Results

Overview: 16-day protocol for mouse ES cell differentiation to neurons

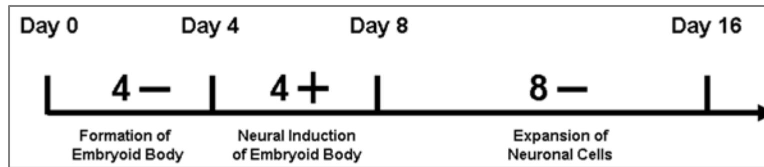

Figure 1. Mouse ES cells are cultured in EB Formation Medium in a non-adhesive 10-cm Petri dish for 4 days in the absence of LIF (**4- Condition**) to help form tight clusters of cells called embryoid body (EB). EB formation is the critical first step in the spontaneous differentiation of ES cells. More directed differentiation of ES cells to the neuronal lineage occurs upon the addition of retinoic acid (Neural Inducer A Solution) to the culture for an additional 4 days (**4+ Condition**). Neural induced EBs are subsequently transferred to poly-L-ornithine and laminin coated slides and cultured in EB Formation Medium for an additional 8 days (**8- Condition**). Neurons emanate, migrate and extend long processes from attached EBs in as little as 4 days after the transfer to the coated slides.

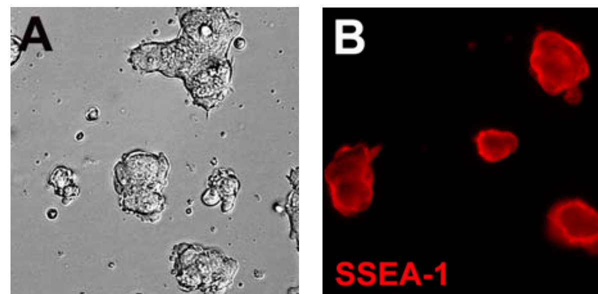

Figure 2. Undifferentiated mouse ES cells (Catalogue No. SCR012) display the characteristic tight round colonies with a high nuclear to cytoplasmic ratio, (**A**, 40X magnification) and stain positively for mouse ES cell marker SSEA-1 (**B,** 40X magnification).

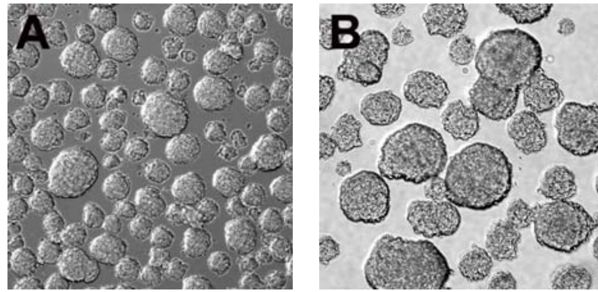

Figure 3. Formation of embryoid bodies (EB) after the culture of dissociated mouse ES cells in EB Formation Medium for 2 days (**A**) and 4 days (**B**) on a non-adhesive Petri dish. 10X magnification.

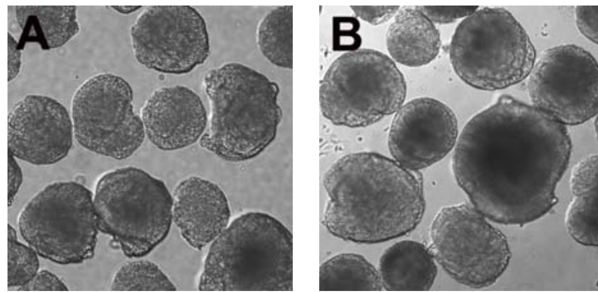

Figure 4. Morphology of EBs after treatment with Neural Inducer A Solution for 2 days (**A**) and 4 days (**B**). 10X magnification.

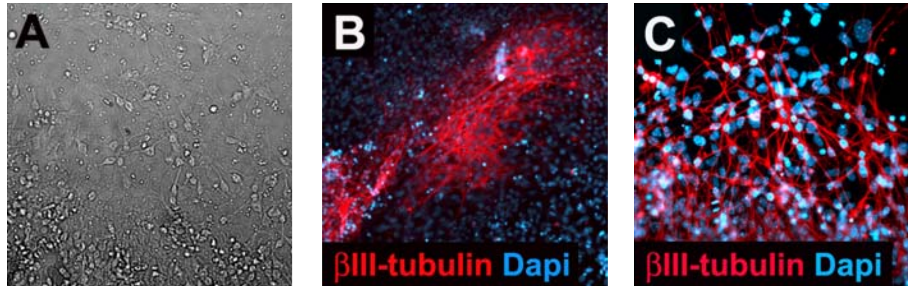

Figure 5. Differentiation of mouse ES cells to βIII-tubulin positive neurons (**B**, 20X magnification; **C,** 40X magnification). Bright-field image (**A**, 40X magnification).

*For color images, please go to www.millipore.com.

References

1. Bain, G., Kitchens, D., Yao, M., Huettner, J. E., and Gottlieb, D. (1995). Embryonic stem cells express neuronal properties *in vitro*. *Dev Biol.* **168 (2)**: 342-357.
2. Du, Z. W., and Zhang, S. C. (2004). Neural differentiation from embryonic stem cells: which way? *Stem Cells Dev,* **13(4)**: 372-381.
3. Ying, Q. L. Stavridis, M. Griffiths, D., Li, M. and Smith, A. (2003). Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat Biotechnol.* **21(2)**: 183-186.

Related Products

The following stem cell products are available from MILLIPORE as separate items:

1. Cryopreserved Mouse Embryonic Stem Cells: (Catalogue Nos. SCR011, SCR012, SCC013, CMTI-1, and CMTI-2)
2. Alkaline Phosphatase Detection Kit: (Catalogue No. SCR004)
3. Quantitative Alkaline Phosphatase ES Cell Characterization Kit: (Catalogue No. SCR066)
4. ES Cell Characterization Kit: (Catalogue No. SCR001)
5. Neuron-Glial Marker Sampler Kit: (Catalogue No. NS130)
6. Embryonic Stem Cell Derived Neuron Integration and Characterization Kit: (Catalogue No. NS140).
7. Dopaminergic Neuron Integration and Characterization Kit: (Catalogue No. NS145)
8. Cryopreserved Mouse Cortical Neural Stem Cells: (Catalogue No. SCR029)
9. Mouse Cortical Neural Stem Cell Expansion Kit: (Catalogue No. SCR032)
10. Cryopreserved Mouse Spinal Cord Neural Stem Cells: (Catalogue No. SCR031)
11. Mouse Spinal Cord Neural Stem Cell Expansion Kit: (Catalogue No. SCR033)
12. Adult Rat Hippocampal Neural Stem Cell Expansion Kit: (Catalogue No. SCR034)
13. Cryopreserved Adult Rat Hippocampal Neural Stem Cells: (Catalogue No. SCR022)
14. Neural Stem Cell Expansion Medium: (Catalogue No. SCM003)
15. Rat Hippocampal Neuron Kit: (Catalogue No. SCR009)
16. Cryopreserved Rat Hippocampal Neurons: (Catalogue No. SCR010)
17. Mouse anti-SSEA-1, 100 µg: (Catalogue No. MAB4301)
18. Mouse anti-Nestin, 100 µg: (Catalogue No. MAB353)
19. Mouse anti-βIII-tubulin, 100 µL: (Catalogue No. MAB1637)
20. Mouse Embryonic Stem Cell Adipogenesis Kit: (Catalogue No. SCR100)
21. Mouse IgM, purified 1 mg: (Catalogue No. PP50)
22. Mouse IgG, purified 10 mg: (Catalogue No. PP54)

Warranty

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