Human ES/iPS Neurogenesis Kit

Product Manual for the following Cat Nos.
SCR603
SCM110
SCM111

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

USA & Canada Phone: +1(800) 437-7500 Fax: +1 (951) 676-9209
www.millipore.com

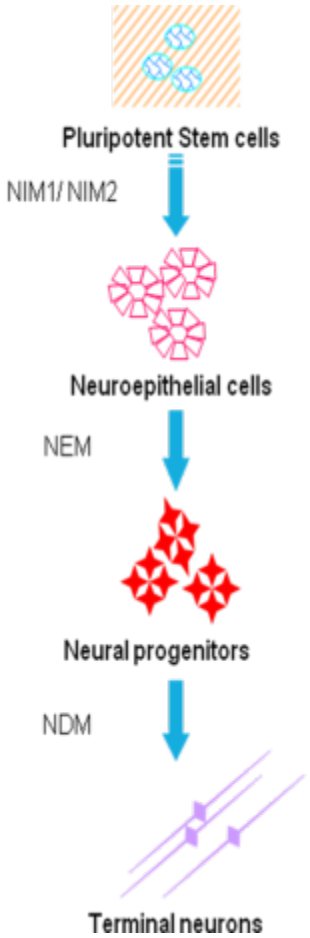
Description

EMD Millipore's Human ES/iPS Neurogenesis Kit (Cat. No. SCR603) is a robust and user-friendly kit that contains the necessary media and reagents for the rapid production of expandable neural progenitor cells and terminally differentiated neurons from pluripotent human ES and iPS cells. The media contained in this kit rely on both small molecules neural inducers and supplements to generate a highly enriched population of expandable neural progenitors and end stage neurons based on establish protocols (1,2). Neural progenitors are generated 10 days from starting cultures of traditional feeder-based and/or feeder-free cultures of undifferentiated human ES/iPS cells and can be expanded for over 3-5 passages resulting in a minimum 20-fold expansion. Typically, from 1 well of a 6-well plate containing an estimated 500,000 pluripotent human ES/iPS, approximately 5-10 million neural progenitors can be obtained after 3 passages. The resulting neural progenitor cells are multipotent and can be further differentiated to specific neural cell subtypes of interest. Included in the kit are the following:

(1) Human ES/iPS Neural Induction Medium (Cat. No. SCM110): 125 mL bottle containing optimized medium for the directed induction of human pluripotent cells to expandable neural progenitor cells. An adapation period is not required. Sufficient reagents are provided for six reactions in a 6 well plate format.

(2) ENStem-A™ Neural Expansion Medium (Cat. No. SCM004): 500 mL bottle containing optimized medium and growth factors for expanding neural progenitor cells.

(3) Human ES/iPS Neuronal Differentiation Medium (Cat. No. SCM111): 100 mL bottle containing optimized medium & reagents for the rapid differentiation of neural progenitors to terminal neurons. Sufficient reagents are provided to differentiate 24 reactions in a 24 well format for 2 weeks.



Stage 1 Neural Induction (10 days)

1. Start with undifferentiated cultures of pluripotent stem cells. Neural induction is initiated 48 hours after routine passaging of hES/iPS cells by incubation with Neural Induction Medium 1 (NIM 1). Exchange with fresh NIM 1 medium every other day for 5 days.

2. 5 days after NIM 1, harvest cells using enzyme free dissociation buffer (Cat. No. BSS-1006-B). Replate cells on Matrigel coated plates in NIM 2 medium. Exchange with fresh NIM 2 medium every other day for 5 days. Neural rosettes can be observed by day 10.

Stage 2 Neural Expansion (3 to 5 passages)

3. On day 10, dissociate the culture into single cells using Accutase and replate the cells onto Matrigel-coated plates in ENStem-A Neural Expansion Medium (NEM) (Cat. No. SCM004).

4. A high cell density expansion ratio (1:3) is recommended for the first three passages. After passage 4, cells can be expanded at a 1:5 ratio. Proliferation rates and expansion capacities may vary between cell lines and between subclones.

Stage 3 Neuronal Differentiation (10-25 days)

5. To terminally differentiate the cells, transfer neural progenitors onto poly-L-ornithine and laminin coated plates in Human ES/iPS Neuronal Differentiation Medium (NDM, Cat. No. SCM111). Dibutyryl cyclin-AMP facilitates the differentiation while ascorbic acid promotes the survival of the neuronal cells.

6. Neuronal markers such as Tuj-1 and MAP2 are expressed as early as 9 days after differentiation.

Kit Components

The Human ES/iPS Neurogenesis Kit (SCR603) conatins SCM110, SCM111 and SCM004 (Note: all components in SCR603 can be purchased seperately). All kit components should be stored at -20°C until ready to use.

1. Human ES/iPS Neural Induction Medium (Cat. No. SCM110)
    - Neural Induction Basal Medium (Part No. CS210991). One 125 mL bottle.
    - Neural Supplement 1 (50X) (Part No. CS210992). One 2.5 mL bottle.
    - Neuro2 Medium Supplement (Part No. SCM012-S). One 1.25 mL bottle.
    - GSK3 and TGFβR Inhibitor Cocktail (1000X) (Part No. CS210983-150UL). One 150 µL vial.
    - AMPK Inhibitor (1000X) (Part No. CS210995-60UL). One 60 µL vial.
2. ENStem-A™ Neural Expansion Medium (Cat. No. SCM004)
    - ENStem-A Neural Expansion Medium, 500 mL (Part No. SCM004a)
    - FGF-2, 10 µg, lyophilized (Part No. GF003-10UG)
3. Human ES/iPS Neuronal Differentiation Medium: (Cat. No. SCM111)
    - Neuronal Differentiation Basal Medium (Part No. CS211007). One 100 mL bottle.
    - Neural Supplement 1 (50X) (Part No. CS210992). One 2.5 mL bottle.
    - Adenosine 3', 5'-cyclic Monophosphate, N6, O2'-Dibutyryl-, Sodium Salt (Cat. No. 28745-25mg). One vial containing 25 mg of lyophilized powder.
    - Ascorbic Acid 2-Phosphate (Part No. 2004011). One 0.5 mL vial of 100 mM solution.

Materials Required but Not Supplied

1. Tissue culture-wares and supplies.
2. Basement membrane protein extracts such as Matrigel (BD Cat. No. 356234)
3. DMEM medium (Cat. No. SLM-021-B)
4. Laminin (Cat. No. CC095)
5. Poly-L-ornithine (Sigma Cat. No. P4957)
6. EmbryoMax ES Cell-Qualified Ultra Pure Water, sterile H_20, 500 mL (Cat. No. TMS-006-B)
7. ENStem-A™ Neural Freezing Medium (1X) (Cat. No. SCM011).
8. EmbyroMax ES Cell-Qualified Penicillin-Streptomycin Solution, 100X (Cat. No. TMS-AB2-C) (Optional).
9. Accutase (Cat. No. SCR005)

10. EmbryoMax Dulbecco's Phosphate-Buffered Saline w/o Ca^{++} & Mg^{++}, 500 mL, (Cat. No. BSS-1006-B). Used as an enzyme free dissociation buffer.
11. Scepter 2.0 Handheld Automated Cell Counter (Cat. No. PHCC20060) or Hemacytometer
12. Fixative (e.g. 4% Paraformaldehyde in 1X PBS)
13. Millicell EZ SLIDE 8-well glass, sterile (Cat. No. PEZGS0896)
14. Blocking Solution (5% normal donkey serum, 5% BSA ± 0.1% Triton X-100 in 1X PBS)
15. Fluorescent-labeled secondary antibodies. Donkey anti-mouse IgG, Cy3-conjugated (Cat. No. AP192C) and donkey anti-rabbit IgG, Cy3-conjugated (Cat. No. AP182C)
16. 4'-6-Diamidino-2-phenylindole (DAPI) / PBS solution

Related Antibodies for Characterization

- Anti-Oct4 Antibody, clone 10H11.2 and Alexa488 and Cy3 conjugated forms (Cat. Nos. MAB4401; MAB4401A4, MAB4401C3)
- Anti-Oct4 Antibody, clone 7F9.2 and Alexa488 and Cy3 conjugated forms (Cat. Nos. MAB4419; MAB4419A4; MAB4419C3)
- Anti-Sox2 Antibody (Cat. No. AB5603)
- Anti-Sox2 Antibody, clone 10H9.1 and Alexa488 and Cy3 conjugated forms (Cat. Nos. MAB4423; MAB4423A4; MAB4423C3)
- Anti-Pax6 Antibody (Cat. No. AB2237)
- Anti-Nestin Antibody, clone 10C2 and Cy3 conjugated forms (Cat. Nos. MAB5326; MAB5326C3)
- Anti-N-Cadherin Antibody, clone 13A9 (Cat. No. 05-915)
- Anti-GFAP Antibody, clone GA5 and Cy3 conjugated forms (Cat. Nos. MAB3402; MAB3402C3)
- Anti-MAP2 Antibody, clone AP20 and Alexa488 and Cy3 conjugated forms (Cat. Nos. MAB3418; MAB3418A5; MAB3418C3)
- Anti-β III tubulin Antibody and Alexa488 conjugated forms (Cat. Nos. AB15708; AB15708A4)

Human ES/iPS Neural Induction Medium (Cat. No. SCM110)

Description	Human ES/iPS Neural Induction Medium (Cat. No. SCM110)
Kit Components	1. Neural Induction Basal Medium (Part No. CS210991). One 125 mL bottle. 2. Neural Supplement 1 (50X) (Part No. CS210992). One 2.5 mL bottle. 3. Neuro2 Medium Supplement (Part No. SCM012-S). One 1.25 mL bottle. 4. GSK3 and TGFβR Inhibitor Cocktail (1000X) (Part No. CS210983-150UL). One 150 µL vial. 5. AMPK inhibitor (1000X) (Part No. CS210995-60UL). One 60 µL vial.
Storage & Stability	Store at -20°C. Good for 3 months from date of receipt or until expiration date on bottle when reagents are handled and stored appropriately. **Please note:** The supplemented ES/iPS Neural Induction Medium should be stored at 2 to 8°C for up to 4 weeks.
Applications	Human ES/iPS Neural Induction Medium is a defined, serum-free medium that has been optimized to induce neural differentiation from human ES and iPS cells.

Quality Control

Each lot is tested for the following parameters:

Sterility testing: Negative for bacteria and fungal growth

pH: 7.2 ± 0.2

Media Preparation

All media components should be stored at -20°C until ready to be used. Thaw media components at 4°C overnight or at room temperature for 4 hours before assembling the complete media. Neural Supplement 1 (50X) and Neuro 2 Medium Supplement may be thawed at room temperature for 30 minutes before assembling the complete media.

Prepare Human ES/iPS Neural Induction Medium 1 and 2 (NIM 1 and 2)

Human ES/iPS Neural Induction Medium I (NIM 1):

49.0 mL Neural Induction Basal Medium (Part No.CS210991)
1.0 mL Neural Supplement 1 (50X) (Part No. CS210992)
0.5 mL Neuro 2 Medium Supplement (Part No. SCM012-S)
50 µL GSK3 and TGFβR Inhibitor Cocktail (1000X) (Part No. CS210983-150UL)
50 µL AMPK Inhibitor (1000X) (Part No. CS210995-60UL)

~50 mL Total volume

Mix thoroughly in a 50 mL conical tube. Store at 2 – 8°C for up to 4 weeks.

Optional: While not necessary, antibiotics may be added to the medium. If desired, add 0.5 mL 100X Penicillin-Streptomycin Solution (Cat. No. TMS-AB2-C, not included in the kit). Complete medium may be sterile filtered using a 0.22 µm filter (Cat. No. SCGP00525, not included in the kit).

Human ES/iPS Neural Induction Medium 2 (NIM 2):

49.0 mL Neural Induction Basal Medium (Part No. CS210991)
1.0 mL Neural Supplement 1 (50X) (Part No. CS210992)
0.5 mL Neuro2 Medium Supplement (Part No. SCM012-S)
50 µL GSK3 and TGFβR Inhibitor Cocktail (1000X) (Part No. CS210983-150UL)

~50 mL Total volume

Mix thoroughly in a 50 mL conical tube. Store at 2-8°C for up to 4 weeks.

Optional: While not necessary, antibiotics may be added to the medium. If desired, add 0.5 mL 100X Penicillin-Streptomycin Solution (Cat. No. TMS-AB2-C, not included in the kit). Complete medium may be sterile filtered using a 0.22 µm filter (Cat. No. SCGP00525, not included in the kit).

ENStem-A Neural Expansion Medium (Cat. No. SCM004)

Description	ENStem-A Neural Expansion Medium (Cat. No. SCM004)
Kit Components	1. ENStem-A Neural Expansion Medium, 500 mL (Part No. SCM004a) 2. FGF-2, 10 µg, lyophilized (Part No. GF003-10UG)
Storage & Stability	ENStem-A Neural Expansion Medium should be stored at -20°C until ready to use. Upon thawing, fresh L-Glutamine (not provided) should be added for a final concentration of 2 mM to the expansion medium. Thawed medium should be stored at 2-8°C and given a 1-month expiration dating. Dispense into aliquots to avoid repeated heating prior to each use. **Please note:** The supplemented ENStem-A Neural Expansion Medium should be stored at 2 to 8°C for up to 1 month.
Applications	Serum free, chemically defined medium to expand and maintain neural stem cell culture.

Quality Control

Each lot is tested for the following parameters:

Sterility testing: Negative for bacterial and fungal growth

pH: 7.2 ± 0.2

Media Preparation

All media components should be stored at -20°C until ready to be used. Thaw media components at 4°C overnight or at room temperature for 4 hours before assembling the complete media.

1. FGF-2 (10 µg, lyophilized) should be reconstituted with 100 µL neutral buffer such as PBS or Tris-HCl at pH 7.6 for a stock concentration of 100 µg/mL. Dispense into aliquots to avoid repeated freeze-thaw. Store at -20°C for up to 6 months.

2. Prepare Complete ENStem-A Neural Expansion Medium:

   Complete ENStem-A Neural Expansion Medium:

   99 mL ENStem-A Neural Expansion Medium (Part No. SCM004a)
   1.0 mL 200 mM Glutamine (100X; not included in the kit)
   20 µL 100 µg/mL FGF-2 (Part No. GF003-10UG)

   ~ 100 mL Total Volume

   Mix thoroughly. Store at 2-8 for up to 1 month.

Optional: While not necessary, antibiotics may be added to the medium. If desired, add 1 mL 100X Penicillin-Streptomycin Solution (Cat. No. TMS-AB2-C, not included in the kit). Complete medium may be sterile filtered using a 0.22 µm filter (Cat. No. SCGP00525, not included in the kit).

Human ES/iPS Neuronal Differentiation Medium (Cat. No. SCM111)

Description	Human ES/iPS Neuronal Differentiation Medium (Cat. No. SCM111)
Kit Components	1. Neuronal Differentiation Basal Medium (Part No. CS211007). One 100 mL bottle. 2. Neural Supplement 1 (50X) (Part No. CS210992). One 2.5 mL bottle. 3. Adenosine 3', 5'-cyclic Monophosphate, N6, O2'-Dibutyryl-, Sodium Salt (Cat. No. 28745-25mg). One vial containing 25 mg lyophilized powder. 4. Ascorbic Acid 2-Phosphate (Part No. 2004011). One 0.5 mL vial of 100 mM solution.
Storage & Stability	Store at -20°C. Good for 3 months from date of receipt or until expiration date on bottle when reagents are handled and stored appropriately. **Please note:** The supplemented ES/iPS Neuronal Differentiation Medium should be stored at 2 to 8°C for up to 1 week.
Applications	βIII-tubulin positive neurons may be observed after 14 days of differentiation.

Quality Control

Each lot is tested for the following parameters:

Sterility testing: Negative for bacterial and fungal growth

pH: 7.2 ± 0.2

Media Preparation

All media components should be stored at -20°C until ready to be used. Thaw media components at 4°C overnight or at room temperature for 4 hours before assembling the complete media. After thawing, the Neuronal Differentiation Basal Medium, Neural Supplement 1 (50X) and Ascorbic Acid 2-Phosphate may be stored separately at 2 – 8°C for up to 1 month. For optimal results, the Complete Human ES/iPS Neuronal Differentiation Medium should always be prepared fresh.

3. Prepare a 0.5M stock of Dibutyryl cyclic AMP (1000X): Reconstitute Adenosine 3', 5'-cyclic Monophosphate, N6, O2'-Dibutyryl-, Sodium Salt (Dibutyryl cyclic-AMP) with 100 µL of sterile water. Aliquot into 25 µL per vial and store the aliquots at -20ºC for up to 3 months.

4. To make 50 mL Human ES/iPS Neuronal Differentiation Medium (NDM):

Human ES/iPS Neuronal Differentiation Medium (NDM)

49.0 mL Neuronal Differentiation Basal Medium (Part No. CS211007)
1.0 mL Neural Supplement1 (50X) (Part No. CS210992)
50 µL 0.5M Dibutyryl cyclic-AMP solution (1000X)
100 µL 100mM Ascorbic Acid 2- Phosphate solution (Part No. 2004011)

~ 50 mL Total Volume

Mix thoroughly in a 50 mL conical tube. Store at 2 – 8°C for up to 1 week.

Optional: While not necessary, antibiotics may be added to the medium. If desired, add 0.5 mL 100X Penicillin-Streptomycin Solution (Cat. No. TMS-AB2-C, not included in the kit). Complete medium may be sterile filtered using a 0.22 µm filter (Cat. No. SCGP00525, not included in the kit).

Step 1: Preparation of Coated Plates

Table 1: Volumes recommended for coating cultureware:

CultureWare	Coating Volume (mL)	Cell Culture Volume (mL)	Surface Area (cm^2)
96 well plate	0.1 mL/well	0.2 mL/well	0.34
24 well plate	0.5 mL/well	1 mL/well	2.0
6 well plate	2 mL/well	3 mL/well	9.6
T25 flask	3 mL	5 mL	25
T75 flask	8 mL	20 mL	75

MATRIGEL COATING:

Neural induction of Human ES/iPS cells and expansion of the resulting neural progenitor cells require culturewares that are coated with Matrigel. However the concentration of Matrigel differs for the two processes. Induction of human ES/iPS cells to neural progenitor cells can be performed directly on existing human ES/iPS cells that are cultured on a 1:20 diluted Matrigel coated 6-well plate whereas the expansion of neural progenitor cells require 1:50 diluted Matrigel coated culturewares. Below are general guidelines for the coating of 6- well plates and culture flasks with Matrigel.

1. Thaw Matrigel on ice or at 2 – 8ºC overnight. Keep on ice and use pre-cooled medium and pipettes to avoid gelling of the ECM gel. **IMPORTANT: Do not thaw Matrigel at temperatures higher than 15ºC to avoid gelling.**

2. Depending upon the application, dilute the Matrigel with appropriate volumes of cold DMEM medium. Mix well.

   a. For Neural Induction of Human ES/iPS cells using NIM 1 and 2: Dilute the Matrigel 1:20 with cold DMEM medium. For example, to every 0.5 mL Matrigel, add 9.5 mL cold DMEM medium for a total volume of 10 mL. Scale accordingly to the volumes required.

   b. For Expansion of Human ES/iPS Derived Neural Progenitor Cells using ENStem-A Expansion Medium: Dilute the Matrigel 1:50 with cold DMEM medium. For example, to every 1 mL Matrigel, add 49 mL cold DMEM medium for a total volume of 50 mL. Scale accordingly to the volumes required.

3. Cover the cultureware with the recommended volumes (see Table 1). Incubate at room temperature for 1 hour or 2 – 8ºC overnight.

   Note*: If not used immediately, Matrigel coated culturewares should be sealed with parafilm to prevent evaporation and can be stored at 2 – 8ºC for up to one week or stored frozen at -20ºC for up to 3 months.*

4. Prior to seeding the cells, bring the plate back to room temperature, remove the coating solution and rinse with 1X PBS to remove residual ECMs. **IMPORTANT: Do not allow the coating to dry out.**

POLY-L-ORNITHINE AND LAMININ COATING:

For terminal differentiation, Human ES/iPS derived neural progenitor cells should be cultured on poly-L-ornithine and laminin coated culturewares before initiating terminal differentiation. The following procedures are recommended.

1. Prepare coating solution by dissolving poly-L-ornithine with distilled water to 10 µg/mL and sterilize with a 0.22 µm filter.

2. Add sufficient volumes of poly-L-ornithine solution to cover the whole surface of the tissue cultureware. Refer to Table 1 for recommended volumes. Incubate at room temperature for one hour or 2 – 8ºC overnight.

3. Thaw laminin (Cat. No. CC095), provided at 1 mg/mL on ice. Using sterile 1X PBS (Cat. No. BSS-1006-B), dilute the laminin to a final concentration of 10 µg/mL. Sterilize with a 0.22 µm filter.

4. Aspirate the poly-L-ornithine solution. Add sufficient volume of 10 µg/mL laminin solution to cover the whole surface of the tissue cultureware. Refer to Table 1 for recommended volumes. Incubate at room temperature for one hour or 2 – 8ºC overnight.

5. Coated plates and flasks can be stored in the laminin solution at -20ºC for up to 6 months. The plates should be wrapped in plastic saran wrap before storage at -20ºC.

6. Just before use, aspirate the laminin solution in the coated culturewares and wash once with 1X PBS. Add Human ES/iPS Neuronal Differentiation Medium to the tissue cultureware. **IMPORTANT: Do not allow plates or wells to dry out as it may result in uneven cell attachment.**

Step 2: Neural Induction with NIM 1 and NIM 2 Medium (SCM110)

The protocol is based on the culture of human ES/iPS cells in a 6 well plate. Volumes should be adjusted to the tissue cultureware being used. The protocol can accommodate both feeder-based and feeder-free cultures of human ES/iPS cells without the need to optimize cell density.

1. Prepare high quality human ES/iPS by passaging healthy undifferentiated colonies onto a freshly cultured mouse embryonic fibroblast feeder plate in KOSR based medium or in feeder-free conditions using Matrigel coated plates and feeder-free, serum free based medium. Manual passaging is recommended in this step to maximize the purity of undifferentiated colonies and to minimize the contamination from spontaneously differentiated cells.

2. Approximately 48 to 72 hours after passaging, remove the growth medium and change to NIM 1 medium at 0.3 mL/cm^2, (i.e. 3 mL per well of a 6 well plate). This is considered Day 0 of induction.

3. Change with fresh NIM 1 medium every other day (i.e. on day 2 and day 4) for 5 days.

4. On day 4 or 5, prepare Matrigel coated plates. Please refer to section on “Preparation of Coated Plates”. Matrigel coating should be at 1:20 dilution.

5. On day 5, carefully aspirate the NIM 1 medium. Rinse the culture with 2 mL of 1X D-PBS w/o Ca^{2+} & Mg^{2+} buffer (Cat. No. BSS-1006-B). Observe the confluency of the plate. Three-

dimensional growth of hES/iPS on the edges or in the center of the colony may be observed. Determine the passaging ratio to use. If the confluency of the plate is less than 50%, use a 1:1 split ratio. If the culture plate is 50 to 75% confluent, use a 1:2 split ratio. If the confluency is higher than 75%, use a 1:3 split ratio. The edges of colonies may lift off as the MEF culture may no longer be able to support cell attachment.

6. Carefully aspirate the 1X D-PBS w/o Ca^{2+} & Mg^{2+} buffer (Cat. No. BSS-1006-B) from each well. Add 1 mL 1X D-PBS w/o Ca^{2+} & Mg^{2+} buffer to each well and incubate at 37ºC for 5 minutes. After 5 minutes, use a p1000 pipette to dislodge the colonies from the plate and transfer the colonies to a 15 mL conical tube. Rinse well(s) with an additional 1 mL 1X D-PBS w/o Ca^{2+} & Mg^{2+} buffer to collect and add residual colonies to the cell suspension in the 15 mL conical tube.

   Note: *The use of the p1000 pipette will generate small clumps. Do not dissociate into single cell suspension as this will lead to high cell death.*

7. Centrifuge at 200 rpm for 5 minutes.

8. Remove the supernatant and resuspend the cell pellet in 2 mL NIM 2. Set aside.

9. In the meantime, remove the coating solution from a new Matrigel coated 6-well plate (1:20 Matrigel dilution) and add 2 mL NIM 2 medium to each well.

10. Depending upon the state of confluency determined from step 5, plate the cells suspension from step 8 at a 1:1 to a 1:3 split ratio (i.e., from one well of a 6 well plate to one to three wells of freshly coated Matrigel plate (1:20 Matrigel dilution).

11. Change with fresh NIM 2 medium every other day for an additional 5 days. Total induction = 10 days.

12. On day 10, a high percentage of cells should have a neural rosette structure (See Figure 1). Manually remove non-rosette like structures from the culture plate and aspirate the medium. Rinse the cells with 1 mL/well Accutase (Cat. No. SCR005). Aspirate and add 1 mL/well Accutase and incubate at 37ºC for 5 minutes to dissociate the cells.

13. Transfer the single cell suspension to a 15 mL conical tube with a p1000 pipette and rinse the plate with 1 mL NIM 2 medium to collect any residual cells. Combine both cell suspensions and centrifuge at 1000 rpm for 5 minutes.

14. Remove the supernatant. The cell pellet may be resuspended in 5 mL ENStem-A Expansion Medium (Cat. No.SCM004) and plated on a T25 flask coated with 1:50 dilution of Matrigel. For expansion, proceed immediately to Step 3 (see below). Alternatively, the cell pellet may be cryopreserved for later experiments. In the event of cryopreservation, use ENStem-A Freezing Medium (Cat. No. SCM011). It is recommended to bank the cells at a density higher than $1x10^6$ cells per vial.

Step 3: Neural Progenitor Expansion with ENStem-A Medium (SCM004)

The protocol is based on the expansion of neural progenitors starting in T25 flasks. The volume should be adjusted according to the tissue culture vessel used.

1. Prepare tissue cultureware according to the section on "**Preparation of Coated Plates**". We recommend starting with T25 flasks coated with a 1:50 diluted Matrigel Solution.

2. Prepare Complete ENStem-A Expansion Medium according to the section on "**Media Preparation**" on pg. 6. Briefly, after thawing, supplement the medium with 1X Glutamine and 20 ng/mL FGF-2 prior to use.

3. Early passage neural progenitors should be maintained at a high cell density (0.5 – 1 x 10^5 cells/cm^2). Hence we recommend transitioning Day 10 neural induced cell pellets (from Step 2 section, number 14) from 1 – 3 wells of a 6-well plate to a T25 flask coated with a 1:50 diluted Matrigel. Plate the cell suspension in 5 mL Complete ENStem-A Expansion Medium containing 1X Glutamine and 20 ng/mL FGF in a T25 Matrigel coated (1:50 dilution) flask.

4. Replace with fresh Complete ENStem-A Expansion Medium containing 1X Glutamine and 20 ng/mL FGF every other day until the flask is 80-90% confluent (approximately 3-4 days).

5. When cells are approximately 80 – 90% confluent, they can be dissociated with Accutase™ and passaged or alternatively frozen for later use. At this point, cells are designated p0 Neural Progenitors.

6. To passage, rinse the plate or flask once with 1X PBS and aspirate. Add sufficient amounts of Accutase to cover the surface of the flask (use 3-5 mL Accutase for T25 flasks). Incubate at 37ºC for 3 to 5 minutes.

7. Inspect the plate and ensure the complete detachment of cells by gently tapping the side of the plate with the palm of your hand.

8. Transfer the dissociated cells to a 15 mL conical tube. Add an equal amount (3 – 5 mL) of ENStem-A Expansion Medium to collect any residual cells.

9. Centrifuge at 1000 rpm for 5 minutes. Discard the supernatant and resuspend the cell pellet in 2 mL Complete ENStem-A Expansion Medium containing 1X Glutamine and 20 ng/mL FGF. Count the number of cells using a Scepter or hemacytometer. Determine cell viability using Tryphan blue exclusion.

10. Plate cells at 0.5 – 1 x 10^5 cells/cm^2 or 1:3 ratio for the first three passages. Split cells every three days. After the third passage, the plating density can be lowered to 1 – 2 x 10^4 cells/cm^2. For differentiation studies, it is recommended to use cells before passage 5.

11. The following plating density and flasks are recommended for the first 3 passages:

    a. P0 Neural Progenitors: Transition Day 10 neural induced cells from 6-well plates to T25 flask (i.e. p0 Neural Progenitors). Plate approximately 1 x10^6 cells per T25 flask.

    b. From p1 to p3 Neural Progenitors: Count and plate 3.75 x 10^6 cells per T75 flask at each passage up to passage 3.

    c. From p3 to p5 Neural Progenitors: Count and plate 1.5 x 10^6 cells per T75 flask at each passage from p3 to p5.

12. For cryopreservation, harvest cells as described above and resuspend cells with ENStem-A Neural Freezing Medium (Cat. No. SCM011). It is recommended to bank the cells at a cell density higher than 1 x 10^6 cells per vial.

Step 4: Neuronal differentiation with Human ES/iPS Neuronal Differentiation Medium (SCM111)

The protocol is based on the terminal differentiation of Human ES/iPS derived NPCs in 24 well plates.

1. Coat 24 well plates with poly-L-ornithine and laminin. Please refer to the section "**Preparation of Coated Plates**".
2. Prepare the Human ES/iPS Neuronal Differentiation Medium. Please refer to the section "**Media Preparation**" on pg. 7.
3. Before using, remove the poly-L-ornithine and laminin coating solution and add 0.5 mL Complete ENStem-A Expansion Medium containing 1X Glutamine and 20 ng/mL FGF-2 to each well.
4. Plate human ES/iPS derived neural progenitor cells at 1 – 2 x10^4 cells/cm^2 or 20,000 to 40,000 cells per well of a 24 well plate in Complete ENStem-A Expansion Medium containing 1X glutamine and 20 ng/mL FGF-2. Total volume per well = 1 mL. At this plating density, cells should be 50 – 70% confluent by the next day. Incubate overnight at 37ºC.
5. The next day, carefully remove approximately 80% of the medium (i.e. this corresponds to 0.8 mL volume) and replace with 0.8 mL per well of fresh Complete Human ES/iPS Neuronal Differentiation Medium (Cat. No. SCM111). Total volume per well = 1 mL.

   Note: *Subsequent media changes should always be done at 80% volume (i.e. 0.8 mL) to avoid perturbation to cell attachment during media changes*.
6. Change with fresh Complete Human ES/iPS Neuronal Differentiation Medium every other day for 10 to 14 days. Significant neurite outgrowth should be observed after 9 days of differentiation.
7. Cells can be fixed and stored at 2 to 8ºC in 1X PBS with 0.1% sodium azide for up to 3 months.

Step 5: Immunostaining Protocol for 24 well plate (Optional)

Human NPCs and terminally differentiated neurons can be characterized using the Human Neural Stem Cell Characterization Kit (Cat. No. SCR060, not provided, available separately).

1. After two weeks of differentiation, fix the cells by incubation in freshly prepared 4% paraformaldehyde in 1X PBS for 10 minutes at room temperature.
2. Carefully aspirate the fixative and rinse four times (5 – 10 minutes each) with 1X PBS.
3. Prepare the blocking solution (e.g. 5% BSA, 5% normal donkey serum in 1X PBS). For intracellular staining, add 0.1% TX-100 to the blocking solution to permeate the cells.
4. Carefully aspirate the 1X PBS wash and add the blocking solution. Incubate at room temperature for 2 hours or overnight at 4ºC. **IMPORTANT: Do not shake the cells.**
5. Dilute the primary antibodies to working concentrations in the appropriate blocking solutions.
6. In a separate control well, depending upon the specific antibody used, add equivalent concentrations of mouse IgG (1 mg/mL) or rabbit IgG (1 mg/mL) to 0.5 mL of the appropriate blocking solution.

7. Carefully remove the blocking solution from each well and add the appropriate diluted primary antibodies to each well. Incubate at room temperature for 4 hours, or 2 to 8°C overnight. **IMPORTANT: Do not shake.**

8. Remove the primary antibody solution. Wash the cells four times with blocking solution (5 minutes each wash).

9. Dilute secondary antibodies to 1 to 5 μg/mL concentration (1:200 to 1:1000 dilution) with the blocking solution.

10. Remove the last wash and add the appropriate diluted secondary antibody to each well. Incubate at room temperature for 1 hour. Cover the plate with tinfoil to protect from the light.

11. Remove the secondary antibody solution. Wash 4 times (5 minutes each) with 1X PBS.

12. Prepare DAPI dye: dilute the DAPI with 1X PBS to 1 μg/mL (1:10,000 dilution).

13. Remove the last wash; add DAPI staining solution and incubate at room temperature for 15 minutes.

14. Remove the DAPI solution; wash twice with 1X PBS.

15. Visualize the cell staining with a fluorescent microscope. ***Note:*** *Be sure to use the correct filter to visualize fluorescent-labeled cells.*

Results:

The following are representative results obtained using the Human ES/iPS Neural Induction Medium (Cat. No. SCM110) and Human ES/iPS Neuronal Differentiation Medium (Cat. No. SCM111) on human ES and iPS cells.

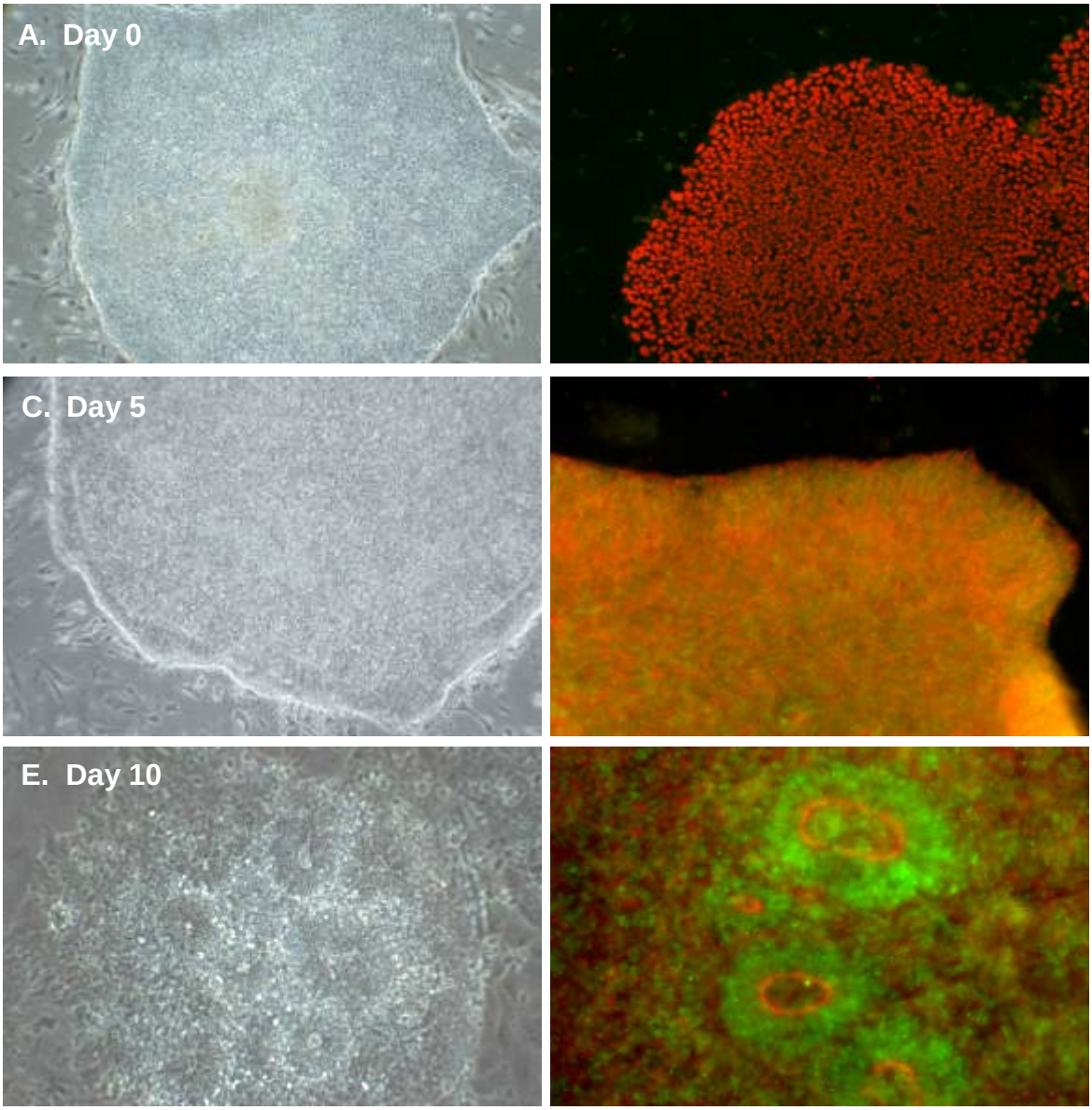


Figure 1. Neural Induction of human pluripotent iPS cells. Human iPS cells (passage 5 to 7) generated using the Human STEMCCA Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit (Cat. No. SCR545) were cultured on MEFs with 20% KOSR medium prior to differentiation **(A, B)**. Neural differentiation was initiated by replacing KOSR medium with Neural Induction Medium 1 (NIM 1) for 5 days **(C, D)**. On day 5, significant 3D growth can be observed around the edges of the colonies. After 5 days, cells were harvested from the plate with non enzymatic dissociation buffer and plated onto Matrigel™ coated plates with Neural Induction Medium 2 (NIM 2) for an additional 5 days. After 10 days of induction, >80% cells are Pax6 positive and polarized neural rosettes can be visualized by N-cadherin (N-Cad) staining **(E, F)**.

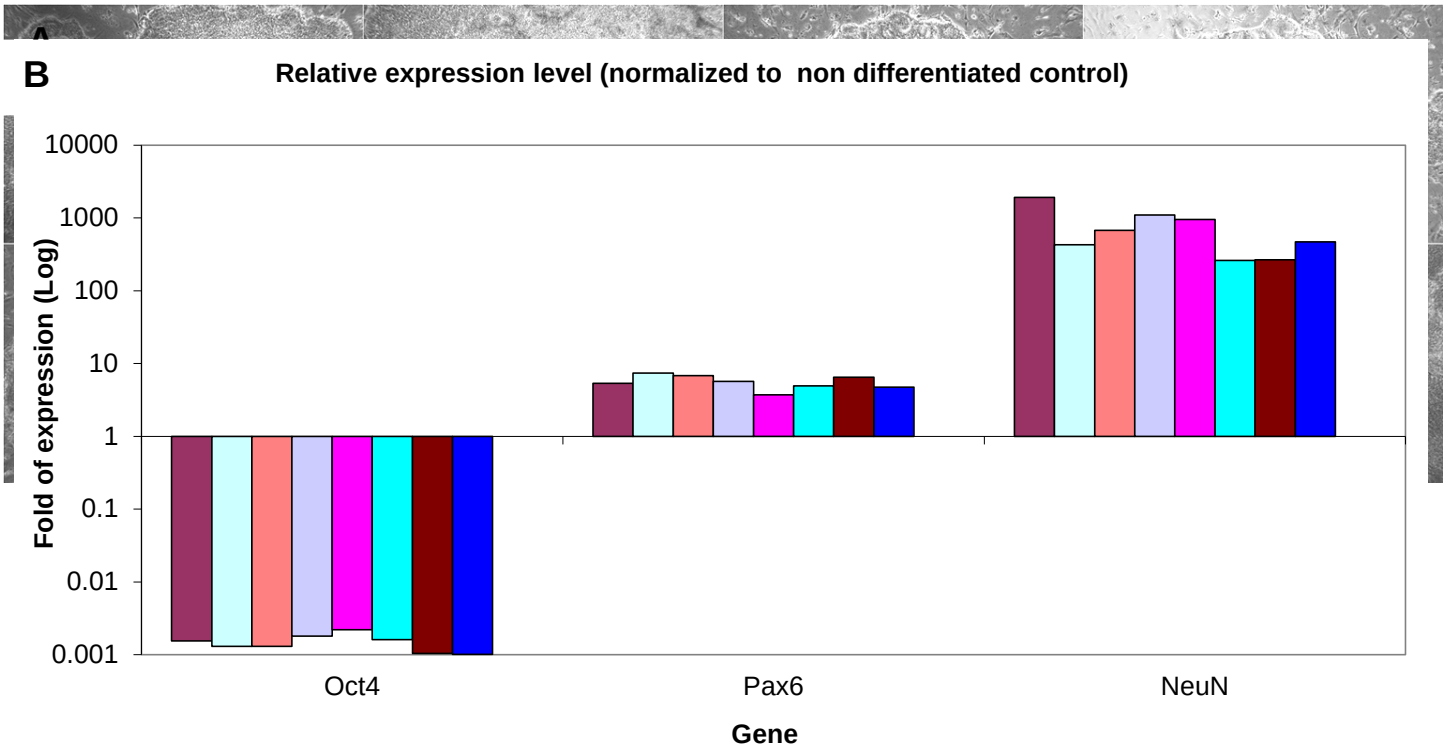


Figure 2. Lot-to-lot consistency of Neural Induction Medium (Cat. No. SCM110). Eight separate lots of neural induction medium were manufactured and QC tested for their ability to induce neural differentiation of H9 human ES cell line (passage 40 – 50) cultured on MEFs in 20% KOSR medium. (**A**) Distinctive neural rosettes can be observed in all eight lots of Day 5 induced cultures. (**B**) Quantitative RT-PCR was performed on Day 10 induced cells from each of the 8 test lots. Relative expression level was normalized to non-differentiated pluripotent controls. All eight lots resulted in significant downregulation of the pluripotent Oct-4 gene expression and concomitant upregulation of Pax 6 and NeuN neural gene expressions.

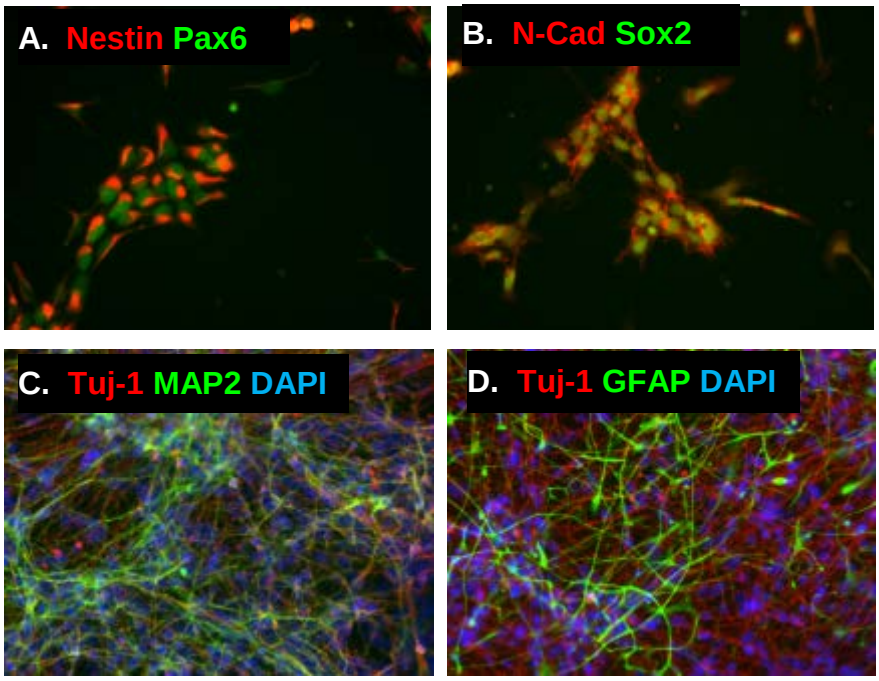


Figure 3. Terminal differentiation of human iPS-derived neural progenitor cells using Human ES/iPS Neuronal Differentiation Medium (Cat. No. SCM111). Neural progenitor cells derived from human iPS were passaged as single cells from passage 2. At passage 3, cells were fixed for progenitor marker expressions: **(A)** Pax6 and Nestin **(B)** Sox2 and N-Cad. Approximately 30K cells per well were plated on poly-L-ornithine and laminin coated 24 well plates before initiating terminal differentiation. Cells formed extensive neurite networks as soon as 9 days after differentiation. A high percentage of cells expressed neuronal markers, Tuj1 and MAP2 **(C)** with a small number of cells expressing GFAP **(D)**.

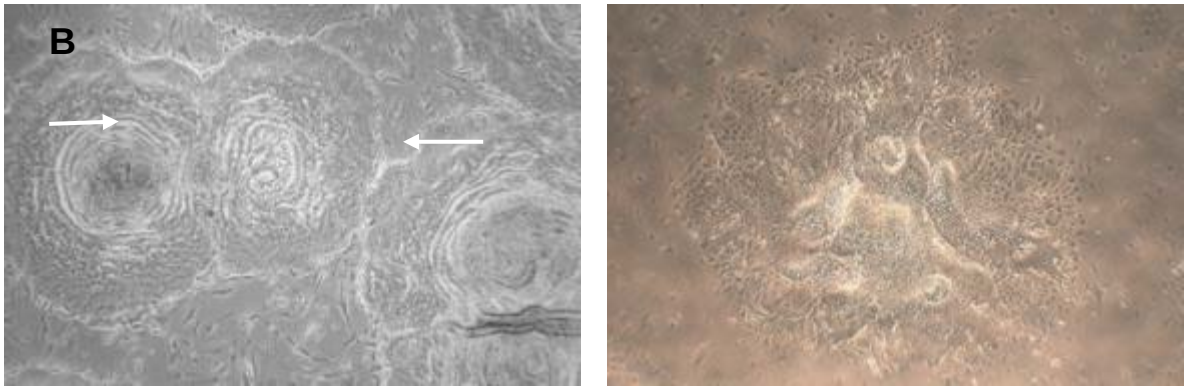


Figure 4. Examples of neural (**A**) and non-neural (**B**) progenies that may arise in the differentiated culture. Neural tubular-like colonies are observed in A whereas more flattened cells along the edges of (white arrow) are suggestive of non-neural progenies.

Troubleshooting

Problem	Possible Cause	Solution
High cell death during the neural induction process	• Low quality iPS cell line with incomplete transformation or high expression of transforming factors	• Remove transgenes from human iPS cells (i.e. using Cre-Lox)
	• Clonal variations	• Select alternative iPS clones
	• Suboptimal reagents	• Check shelf life and storage conditions of all the reagents.
Mixed cell population after the 10 day neural induction period.	• The starting human ES and iPS cultures were not of good quality.	• The neural induction procedure is dependent upon the cell density. Therefore, carefully select and manually passage undifferentiated human ES/iPS colonies.
	• High proportion of differentiated cells in starting human ES/iPS culture.	• Remove the differentiated sections from undifferentiated colonies.
		• Remove differentiated colonies that contain non neural progenies (Fig 4B) before proceeding to NIM 2.
Slow expansion of neural progenitors	• Clonal variations and cell line variations.	• Some lines expand slower than others. Typically doubling time is approximately 48 hour. Establish neural progenitor from two to three independent lines and choose the best one to continue.
	• Aggressive expansion with a high split passage ratio.	• Neural progenitors should be passaged at a high cell density for the first 3 passages. Cells should be plate at 0.5 – 1 x 10^5 cells/cm^2 which typically correspond to ~1 x 10^6 cells for T25 flask and 3.75 x 10^6 cells for T75 flask.

References

1. Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., and Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat. Biotechnol. 27,275–280.3.

2. Li, W., Sun, W. S., Zhang, Y., Wei, W., Ambasuhan, R., Xia, P., Talantova, M., Lin, T., Kim, J., Wang, X., Kim, W. R., Lipton, S. A., Zhang, K., and Ding, S. (2011). Rapid induction and long-term sel-renewal of primitive neural precursors from human embryonic stem cells by small molecule inhibitors. Proc. Natl. Acad. Sci. USA 108, 8299-8304.

Warranty

Millipore Corporation ("Millipore") warrants its products will meet their applicable published specifications when used in accordance with their applicable instructions for a period of one year from shipment of the products. **MILLIPORE MAKES NO OTHER WARRANTY, EXPRESSED OR IMPLIED. THERE IS NO WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE.** The warranty provided herein and the data, specifications and descriptions of Millipore products appearing in Millipore's published catalogues and product literature may not be altered except by express written agreement signed by an officer of Millipore. Representations, oral or written, which are inconsistent with this warranty or such publications are not authorized and if given, should not be relied upon.

In the event of a breach of the foregoing warranty, Millipore's sole obligation shall be to repair or replace, at its option, the applicable product or part thereof, provided the customer notifies Millipore promptly of any such breach. If after exercising reasonable efforts, Millipore is unable to repair or replace the product or part, then Millipore shall refund to the Company all monies paid for such applicable Product. **MILLIPORE SHALL NOT BE LIABLE FOR CONSEQUENTIAL, INCIDENTAL, SPECIAL OR ANY OTHER DAMAGES RESULTING FROM ECONOMIC LOSS OR PROPERTY DAMAGE SUSTAINED BY ANY COMPANY CUSTOMER FROM THE USE OF ITS PRODUCTS.**

Unless otherwise stated in our catalog or other company documentation accompanying the product(s), our products are intended for research use only and are not to be used for any other purpose, which includes but is not limited to, unauthorized commercial uses, in vitro diagnostic uses, ex vivo or in vivo therapeutic uses or any type of consumption or application to humans or animals.