Synthetic Laminin Peptide For Rat Neural Stem Cells

Catalog No. SCR127

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Not for use in diagnostic procedures.

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Introduction

Laminin, an extracellular matrix protein of the cell basement membrane, is involved in various biological activities including cell adhesion, proliferation, migration, invasion, and differentiation These heterotrimeric macromolecules - consisting of α, β and γ chains - are difficult to extract and purify in consistently active quantities from native tissues. While some of these laminin isoforms can be isolated from either cultured cells or placental tissues, in both cases, overall protein yields tend to be relatively low and suffer from batch to batch variations. Therefore, for many stem cell scientists, it has become necessary to test each new lot laminin to ensure that the activity level of the protein was sufficient to sustain their stem cell culture.

To overcome this time barrier, a defined synthetic laminin substrate with similar binding and functional properties of the native laminin protein is considered to be highly advantageous as it would eliminate the requirement to test and screen new lots of native protein. Millipore's Synthetic Laminin Peptide is a suitable replacement for the native laminin protein, and has been optimized to specifically support cell adhesion, proliferation and multi-lineage differentiation of rat neural stem cells (NSCs) *in vitro*. Rat neural stem cells grown on tissue culture plates coated with the synthetic laminin peptides display the characteristic neural stem cell markers, Nestin and Sox-2, and furthermore possess the capacity to preferentially differentiate down both glial and neural lineages. Millipore's Synthetic Laminin Peptide is an inexpensive, reproducible, and defined ECM substrate alternative for the *in vitro* culture of rat neural stem cells.

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Kit Components

1. Synthetic Laminin Peptide for Rat Neural Stem Cells: (Catalog No. SCR127) 15 mg total, 5 vials (3 mg per vial) of synthetic laminin peptide, lyophilized. Store at -20^{o}C.

Materials Not Supplied

1. Adult Rat Hippocampal Neural Stem Cells (Catalog No. SCR022)
2. Rat Neural Stem Cell Expansion Medium (Catalog No. SCM009)
3. Accutase™ Cell Dissociation Solution (Catalog No. SCR005)
4. Phosphate-Buffered Saline (1X PBS) (Catalog No. BSS-1005-B)
5. EmbryoMax ES Cell Qualified Ultra Pure Water, sterile H_20, 500 mL (Catalog No. TMS-006-B)
6. Rodent Neuron Differentiation Kit (Catalog No. SCR035)
7. Rodent Astrocyte Differentiation Medium (Catalog No. SCM010)
8. Tissue culture-ware
9. Fixative (e.g. 4% Paraformaldehyde in 1X PBS)
10. Blocking Solution (5% normal donkey serum, 0.3% Triton X-100 in 1X PBS)
11. Neural Stem Cell Characterization Kit (Catalog No. SCR019)
12. Fluorescent-labeled secondary antibodies. Donkey anti-mouse IgG, Cy3 conjugated (Catalog No. AP192C), donkey anti-rabbit IgG, Cy3 conjugated (Catalog No. AP182C), donkey anti-mouse IgG, FITC conjugated (Catalog No. AP192F) and donkey anti-rabbit IgG, FITC conjugated (Catalog No. AP182F) are recommended.

13. 4'-6-Diamidino-2-phenylindole (DAPI) / PBS solution
14. Nunc Lab-Tek II 8 well chamber slides (Fisher Catalog No. 12-565-8)
15. Anti-fading mounting solution
16. Hemacytometer
17. Microscope

Storage

The Synthetic Laminin Peptide (Catalog No. SCR127) is shipped lyophilized and should be stored at -20°C upon receipt. Once the peptide has been reconstituted, it should be stored at 4°C and used within 1 month.

Laminin Peptide Coating Protocol

1. Resuspend the Synthetic Laminin Peptide (3 mg/vial) with 1 mL sterile DMSO in a sterile environment.
2. Vortex for a few seconds and let sit until fully dissolved.
3. Dilute peptide to a final concentration of 150 μg/mL by adding 19 mL of sterile filtered water to the 1 mL reconstituted Synthetic Laminin Peptide (from step 1 above).
4. Sterile filter using a 0.2 μm filter.
5. Add sufficient volume of the sterile filtered Synthetic Laminin Peptide solution (150 μg/mL) to cover the entire plate or flask. The following volumes are recommended.

Type of plate/flask	Volume of laminin peptide solution (150 μg/mL) required
24-well plate	0.25 mL per well
12 well plate	0.5 mL per well
6 well plate	1 mL per well
T25 flask, or 6 cm plate	3 mL
T75 flask or 10-cm plate	5 mL

6. Incubate overnight at room temperature in a laminar flow hood.
7. Just before use, aspirate the laminin peptide solution in the coated plates.

Thawing of Cells

1. Do not thaw the cells until the recommended medium and appropriately coated plasticware and/or glassware are on hand.
2. Remove the vial of adult rat hippocampal neural stem cells from liquid nitrogen and incubate in a 37°C water bath. Closely monitor the cells until they are completely thawed. Maximum cell viability is dependent on the rapid and complete thawing of frozen cells. **IMPORTANT: Do not vortex the cells.**

3. As soon as the cells are completely thawed, disinfect the outside of the vial with 70% ethanol. Proceed immediately to the next step.
4. In a laminar flow hood, use a 1 or 2 mL pipette to transfer the cells to a sterile 15 mL conical tube. Take extra care to avoid introducing any bubbles during the transfer process.
5. Using a 10 mL pipette, slowly add dropwise 9 mL Neural Stem Cell Basal Medium (Catalog No. SCM003) (pre-warmed to 37°C) to the 15 mL conical tube. **IMPORTANT: Do not add the whole volume of media to the cells all at once. This may result in decreased cell viability due to osmotic shock.**
6. Gently mix the cell suspension by slowly pipeting up and down twice. Take extra care to avoid introducing any bubbles. **IMPORTANT: Do not vortex the cells.**
7. Centrifuge the tube at 300 x g for 2-3 minutes to pellet the cells.
8. Decant as much of the supernatant as possible. *NOTE: Steps 4-8 are necessary to remove residual cryopreservative (DMSO).*
9. Resuspend the cells in a total volume of 10 mL Neural Stem Cell Basal Medium (Catalog No. SCM003) (pre-warmed to 37°C) containing freshly added 20 ng/mL FGF-2.

   Note: *FGF-2 should always be added fresh to the Neural Stem Cell Basal Medium.*
10. Plate the cell mixture onto a Synthetic Laminin Peptide coated 10-cm tissue culture plate.
11. Incubate the cells at 37°C in a 5% CO_2 humidified incubator.
12. The next day, exchange the medium with fresh Neural Stem Cell Basal Medium (pre-warmed to 37°C) containing 20 ng/mL FGF-2. Exchange with fresh medium containing FGF-2 every other day thereafter.
13. When the cells are approximately 80% confluent, they can be dissociated with Accutase™ Cell Dissociation Solution and passaged or alternatively frozen for later use.

Subculturing

1. Carefully remove the medium from the Synthetic Laminin coated 10-cm tissue culture plate containing the confluent layer of adult rat neural stem cells.
2. Apply 3-5 mL of Accutase and incubate in a 37°C incubator for 3 minutes.
3. Inspect the plate and ensure the complete detachment of cells by gently tapping the side of the plate with the palm of your hand.
4. Apply 5 mL Neural Stem Cell Basal Medium (pre-warmed to 37°C) to the plate.
5. Transfer the dissociated cells to a 15 mL conical tube.
6. Centrifuge the tube at 300 x g for 2-3 minutes to pellet the cells.
7. Discard the supernatant.
8. Apply 2 mL of Neural Stem Cell Basal Medium containing 20 ng/mL FGF-2 to the conical tube and resuspend the cells thoroughly.
9. Count the number of cells using a hemacytometer.
10. Plate the cells to the desired density into the appropriate Synthetic Laminin Peptide coated flasks, plates or wells in Neural Stem Cell Basal Medium containing 20 ng/mL FGF-2. (NOTE: We typically plate approximately ~2 million cells on the Synthetic Laminin Peptide coated 10-cm plates or T75 flasks).

Staining Protocol (for 24-well tissue culture plates)

1. The 24-well tissue culture plates should be coated with 150 µg/mL Synthetic Laminin Peptide (please refer to the section on Preparation of Coated Plates).
2. Plate out 50,000-100,000 cells per well into an appropriately coated 24-well tissue culture plate in Neural Stem Cell Basal Medium containing 20 ng/mL FGF-2. Total volume per well = 0.75 mL. At this density, the cells should be ~50%-70% confluent by the next day.
3. The next day, carefully aspirate the medium and wash the wells two times with 1X PBS. Be careful to not aspirate the cells.

   Note: *Use extreme care when exchanging media as the NSC are loosely adherent. Always leave behind a small volume of medium to ensure that the cells do not dry out.*
4. Fix the cells by incubation in 4% paraformaldehyde for 30-40 minutes at room temperature.
5. Carefully aspirate the fixative and rinse three times (5-10 minutes each) with 1X PBS.
6. Apply a blocking solution (e.g. 5% normal donkey serum, 0.3% Triton X-100 in 1X PBS) for at least 2 hours at room temperature or overnight at 4^{o}C. **IMPORTANT: Do not shake the cells.**
7. 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:

   - Mouse anti-Nestin: 1:200 dilution based on 1 mg/mL, final 5 ng/µL
   - Rabbit anti-Sox 2: 1:1000 dilution based on 1 mg/mL, final 1 ng/µL
   - Mouse anti-MAP-2: 1:200 dilution based on 1 mg/mL, final 5 ng/µL
   - Rabbit anti-GFAP: 1:250 dilution of rabbit serum
8. 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. For example, to obtain a 1/200 dilution of mouse anti-Nestin (1 mg/mL), 2.5 µL of the antibody is added to 0.5 mL volume of the blocking solution. In an adjacent control well, add 2.5 µL mouse IgG (1 mg/mL) control antibody to 0.5 mL of the blocking solution.
9. Incubate the cells in primary antibodies overnight at 4°C. **IMPORTANT: Do not shake.**
10. The next day, wash the cells twice with 1X PBS (5-10 minutes each wash) and twice with the blocking solution.
11. At the completion of the last wash, leave the cells in blocking solution for at least 30 minutes.
12. Dilute secondary antibodies in the blocking solution just before use. The following secondary antibodies can be used: donkey anti-mouse IgG Cy3 conjugated (Cat. No. AP192C), donkey anti-mouse IgG FITC conjugated (Cat. No. AP192F), donkey anti-rabbit IgG Cy3 conjugated (Cat. No. AP182C), and donkey anti-rabbit IgG FITC conjugated (Cat. No. AP182F) antibodies at a 1:250 or 1:500 dilution.
13. Overlay the cells with the appropriate donkey anti-mouse and anti-rabbit secondary antibodies that are conjugated to fluorescent molecules for 2 hours at room temperature.
14. Wash 3-5 times (5-10 minutes each) with 1X PBS.
15. Counterstain the cell nuclei with DAPI / 1X PBS solution.
16. Visualize the cell staining with a fluorescent microscope.

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

Results

Characterization of rat neural stem cells cultured on Synthetic Laminin peptide ECM matrices.

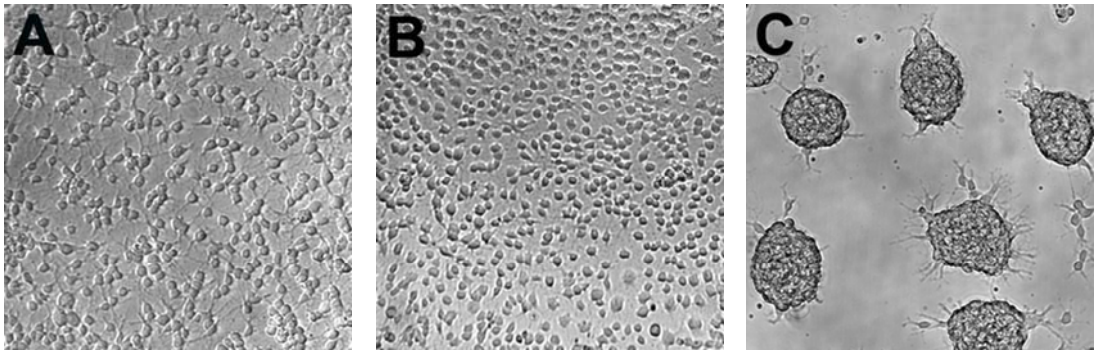

Figure 1. Phase bright images of rat hippocampal neural stem cells grown on Synthetic Laminin Peptide coated tissue culture flasks. Rat hippocampal neural stem cells were grown in monolayers for 10 passages on (**A**) poly-L-ornithine and laminin coated (positive control) or on (**B**) Synthetic Laminin Peptide coated (150 μg/mL final) T25 flasks in Neural Stem Cell Basal Media containing 20 ng/mL FGF-2 (Catalog No. SCM009). Without the laminin protein or Synthetic Laminin Peptides as an ECM matrices, rat NSCs clump up as neurospheres after two days in culture (**C**).

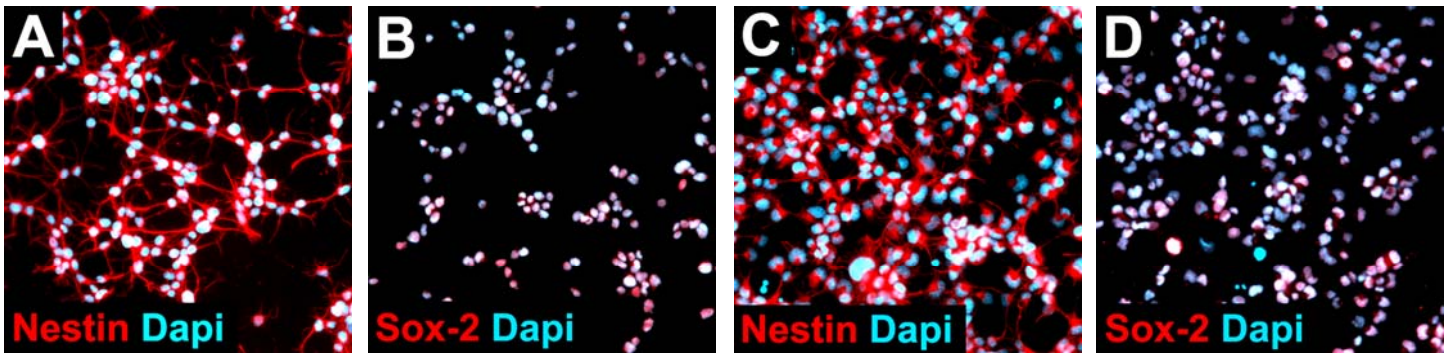

Figure 2. Rat NSCs cultured on Synthetic Laminin Peptide coated tissue cultureware express multipotent NSC markers, Nestin (C) and Sox-2 (D). Cells were cultured for over 10 passages on either poly-L-ornthine and laminin coated T-25 flasks (**A, B**) or on Synthetic Laminin Peptide coated T-25 flasks (**C, D**). For immunocytochemical characterization, cells were cultured in Neural Stem Cell Basal Medium containing 20 ng/mL FGF at 50,000 cells / well in Synthetic Laminin Peptide (150 μg/mL, final concentration) coated 24 well tissue culture plate overnight before fixing and staining.

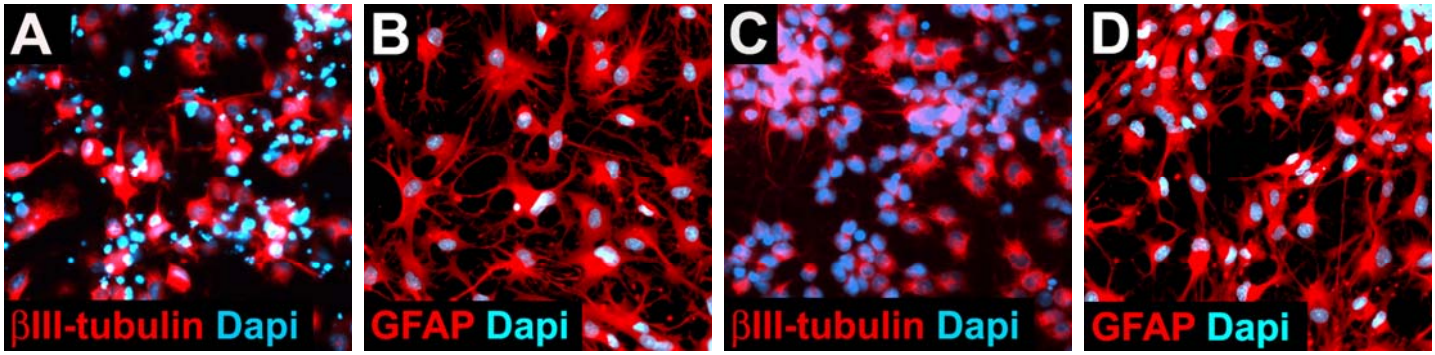

Figure 3. Rat NSCs cultured long term on laminin peptide coated flasks can be preferentially differentiated into βIII-tubulin positive neurons (C) and GFAP positive astrocytes (D). Rat NSCs were preferentially differentiated to neurons following 4 days treatment with retinoic acid and forskolin (Catalog No. SCR035). Rat NSCs were differentiated into GFAP positive astrocytes following 7 days treatment with BMP-4, FBS and LIF (Catalog No. SCM010). Cells display similar multipotentiality as those cultured on poly-L-ornthine and laminin coated positive control (**A, B**). Cells were differentiated in the specified conditions on a Synthetic Laminin Peptide coated (150 μg/mL, final concentration) 24-well tissue culture dish.

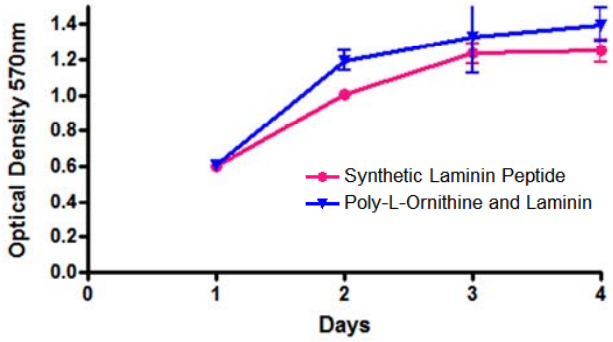

Figure 4. Rat NSCs cultured on laminin peptide coated plates display similar cell proliferation profiles as cells cultured on poly-L-ornithine and laminin positive control. Cell proliferation in T-25 flasks was monitored on days 1, 2, 3, and 4 following Millipore's MTT Cell Proliferation Assay (Catalog No. CT02), respectively. Rat NSCs had been cultured for at least 5 passages in the respective Synthetic Laminin Peptide or poly-L-ornithine and laminin positive control coated tissue culture flasks before analysis was initiated.

References

Asbrock, N., Kendall, S., Hozumi, K., Nomizu, M., Kovelman, R., and Chu, V.T. (2008). Identification of synthetic laminin peptides that support the maintenance, proliferation and multi-lineage differentiation of rat neural stem cells. 2008 ASCB conference, poster.

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