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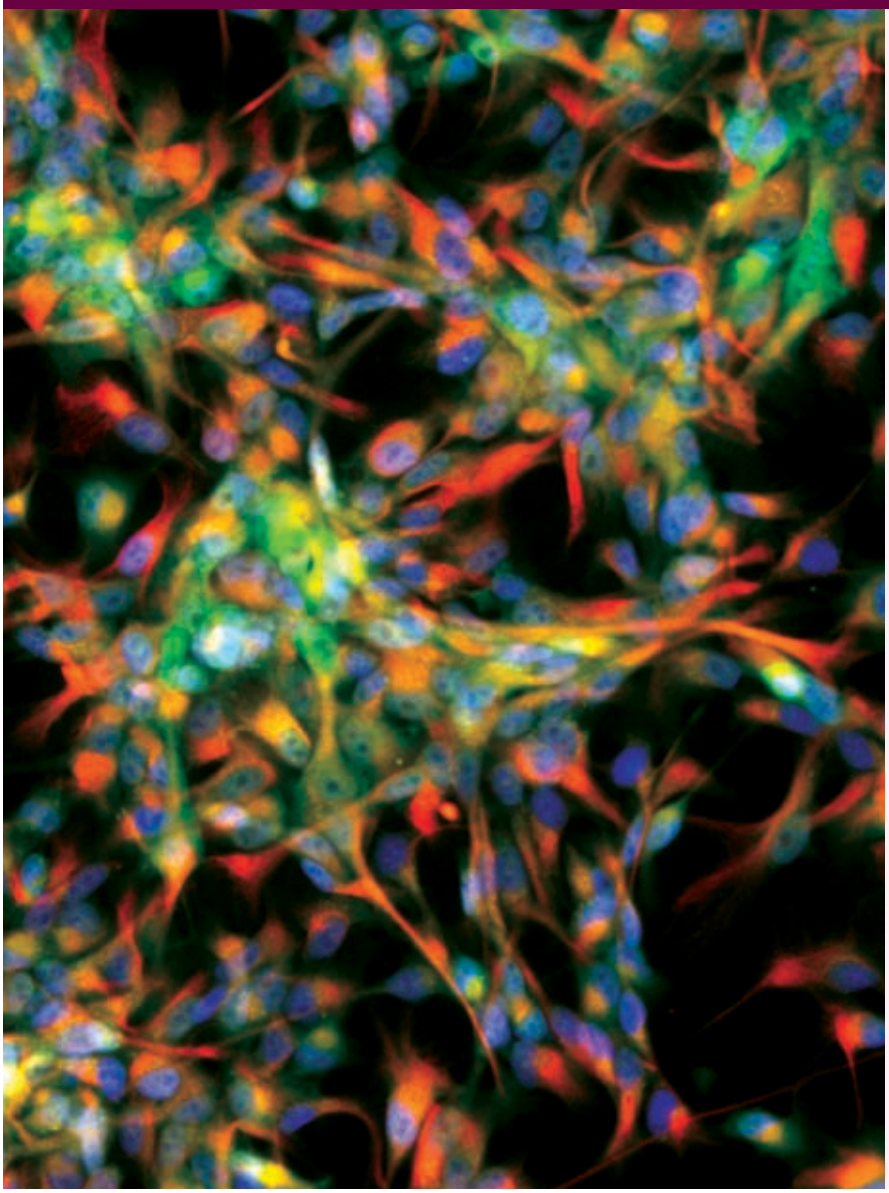

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Cover photo: MilliTrace CX Nestin GFP reporter human neural stem cells express Nestin, GFP and Sox-2 (merged image). See page 3 for complete article.

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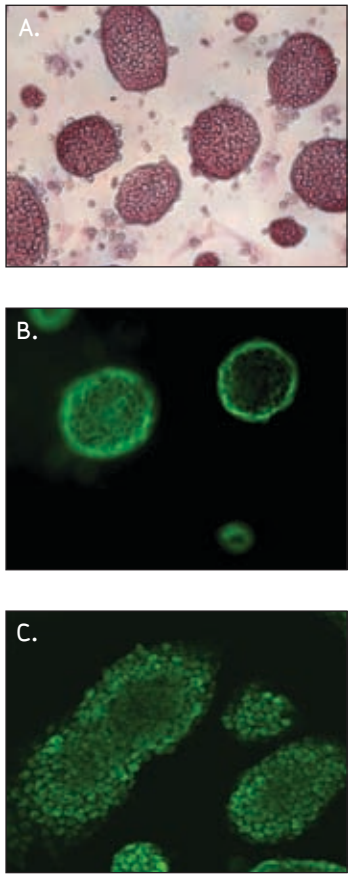

Figure 1 (right). To confirm pluripotency of ES cells after ten passages in ESGRO Complete PLUS medium supplemented with GSK3β inhibitor, cells were stained for alkaline phosphatase (A) and immunostained for SSEA-1 (B) and Oct-4 (C).

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ESGRO Complete basal Medium	500 mL	SF002-500
ESGRO Complete Derivation Kit	1 kit	SF003
ESGRO Complete Switch Kit	1 kit	SF004
ESGRO Complete Accutase Dissociation Solution	100 mL	SF006
ESGRO Complete Enzyme-Free Dissociation Solution	100 mL	SF009
ESGRO Complete Trypsin	100 mL	SF007
ESGRO Complete Freezing Medium	50 mL	SF005
ESGRO Complete Gelatin	500 mL	SF008

Characterization of MilliTrace CX Nestin GFP Reporter Human Neural Stem Cell Line

Christine Chen, Ph.D. and Vi Chu, Ph.D., Millipore Corporation, Temecula, CA

Abstract

Nestin, an intermediate filament protein, is a well-known marker that is expressed in proliferating neural progenitors and stem cells during the early stages of development in the CNS and PNS. The regulatory element of the Nestin gene is widely used as a promoter to drive expression of GFP in transgenic mice. These transgenic mice models provide an opportunity to study neural stem cell (NSC) development *in vivo* and *in vitro*. A human GFP NSC reporter cell model would be a valuable tool to examine cellular function during neurogenesis. Toward this end, a human cortical neural stem reporter cell line was established in which the expression of humanized mulleri GFP (hmGFP) was placed under the regulation of the mouse Nestin promoter. We show that the mouse Nestin promoter has been stably integrated into the cell genome and that the resulting Nestin GFP reporter human neural stem cells faithfully recapitulated the expression profile of Nestin *in vitro*, namely, GFP fluorescence remained constant during NSC propagation and steadily declined upon differentiation. The down regulation of GFP fluorescence makes it possible to monitor neuronal differentiation in live cells.

Introduction

Recently, immortalized human NSC lines derived from the cortical (CX) and ventral mesencephalon (VM) regions were created by retroviral transduction with the myc proto-onocogene[1]. Like the primary stem cells from which they are derived, ReNcell® CX and VM human NSC lines undergo continuous cell division, are karyotypically normal, and are capable of differentiating into the three major cell types of the CNS. The ReNcell CX and VM NSC lines have become useful tools for high throughput neurotoxicity screens for chemicals that are harmful to the human nervous system[2]. They are also used for proteomic analysis of proliferating and differentiating human NSCs[3], and serve as useful model systems for elucidating the role of specific genes in various CNS diseases such as Parkinson's disease[4] and stroke[5].

In this report, we describe the development and characterization of the MilliTrace CX Nestin GFP reporter human neural stem cell line. Genomic PCR on undifferentiated and differentiated cells indicated that the Nestin GFP reporter was stably integrated into the genome. During normal NSC proliferation, high levels of GFP were expressed under the regulation of the nestin promoter. Upon differentiation, GFP expression correspondingly declined, as demonstrated by microscopy observation and flow cytometry analysis. The Nestin GFP reporter human NSC line displayed a normal karyotype, proliferation rate, and multipotency similar to the non-transfected parental ReNcell CX cells. This cell line enables the stage-specific monitoring of neuronal differentiation in live cells and is thus an ideal tool for imaging, cell functional analysis, and high throughput screening.

Methods

Cell Culture: All reagents were obtained from Millipore unless otherwise indicated. Tissue cultureware was coated with 20 µg/mL of mouse laminin. The parental cell line, ReNcell CX, was maintained in ReNcell NSC maintenance medium supplemented with 20 ng/mL human recombinant FGF-2 and 20 ng/mL human EGF. Once established, the MilliTrace CX Nestin GFP reporter line was also maintained in similar growth factor conditions along with the addition of 0.25 µg/mL puromycin to maintain selection pressure. Fresh media, supplemented with growth factors and puromycin (for MilliTrace cells), was exchanged every other day. For differentiation studies, approximately $5x10^4$ cells/cm^2 were plated on a glass chamber slide or tissue cultureware in ReNcell NSC maintenance medium containing 20 ng/mL FGF-2 and 20 ng/mL EGF. Spontaneous differentiation was initiated 24 hours after the initial plating by removing the growth factors (FGF-2 and EGF) and puromycin from the medium. For optimal differentiation, puromycin was not added to the culture medium as it is an antibiotic used to select for proliferating cells containing the Nestin-GFP expression cassette. Differentiation medium was replaced every two to three days for a total of 14 days.

Generating Nestin GFP reporter cell line: The MilliTrace CX Nestin GFP reporter cell line was generated by electroporation of the ReNcell CX line with a proprietary bicistronic plasmid that contained hmGFP under the control of the mouse Nestin promoter. The electroporation

procedure was optimized to allow high transfection efficiency with minimal cell death. After electroporation, cells were maintained in ReNcell NSC maintenance medium supplemented with 20 ng/mL FGF and 20 ng/mL EGF for 48 hours prior to selection with 0.25 µg/mL puromycin. After five weeks of puromycin selection, four stable cell lines (named B1 – 4) were expanded and banked for further characterization.

Immunofluorescent staining: Cells were rinsed once with 1X PBS and fixed with 4% paraformaldehyde at room temperature for 15 minutes. After fixation, cells were washed four times and then permeabilized with PBS containing 0.1% Triton X-100 and 1% BSA at room temperature for 1 hour. Primary antibodies generated in mouse and rabbit were used at the following concentrations: mouse anti-Nestin, human specific (1:500), rabbit anti-Sox-2 (1:1000), mouse anti-βIII tubulin (1:1000) and rabbit anti-GFAP (1:250). Secondary antibodies, Alexa Fluor® 555-conjugated goat anti-mouse and Cy5-conjugated goat anti-rabbit, were used at 1:500 dilutions. Nuclei were stained with DAPI prior to mounting the cells. Image acquisition was performed on a Leica DMI 6000 microscope and a Hamamatsu CCD camera.

Flow cytometry analysis: Two independent cell lines (B1 and B2) were chosen to be further characterized by flow cytometry. For both cell lines, samples were taken from proliferating and differentiating cultures. The B1 line was differentiated for 15 days and the B2 line was differentiated for 17 days. Cells were harvested with Accutase™ dissociation solution, washed once with 1X PBS, and then fixed with 4% paraformaldehyde at room temperature for 15 minutes. After fixation, cells were washed four times and then resuspended in 1X PBS at 10^5 cells/mL. GFP levels in fixed cells were analyzed by flow cytometry.

PCR and Western blot analysis: Genomic DNA was extracted from 10^5 cells using an alkaline lysis procedure. 100 ng of genomic DNA was used to amplify a 350 bp fragment containing the Nestin regulatory element and the 5′ end of hmGFP. Protein samples were prepared by lysing the cell pellet with 50 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM EDTA, and 1% Triton X-100 containing protease inhibitors from Sigma. Protein concentration was determined by a Bradford assay obtained from BioRad. Total proteins (2 µg) were subjected to SDS-PAGE and then Western blot analysis.

Results

To confirm stable integration of the Nestin-GFP reporter cassette in the cells, genomic DNA was isolated and analyzed using PCR (Figure 1B). In both undifferentiated (lane 2) and differentiated samples (lane 3), a 350 bp PCR fragment corresponding to the size of the amplified reporter cassette was obtained.

Nestin is known to be preferentially expressed in proliferating NSC and is downregulated upon differentiation. To demonstrate the faithful expression of the Nestin gene, Western blot analysis was performed on undifferentiated and differentiated cell samples of the MilliTrace CX Nestin GFP reporter line (Figure 1D). A 220-240 kDa band corresponding to the nestin protein was observed only in the undifferentiated sample (Lane 1) and was not detected in the differentiated cells (Lane 2)(Figure 1D).

Visualization of the cells with a FITC filter on a fluorescent microscope indicated a dramatic downregulation of GFP expression in differentiated cells as compared to the undifferentiated cell population (Figure 1C). Overall, the data indicated that the Nestin-GFP reporter cassette was stably integrated into the cell genome (Figure 1B) and that the expression of GFP accurately reflected the differentiation status of the cell (Figure 1C, D).

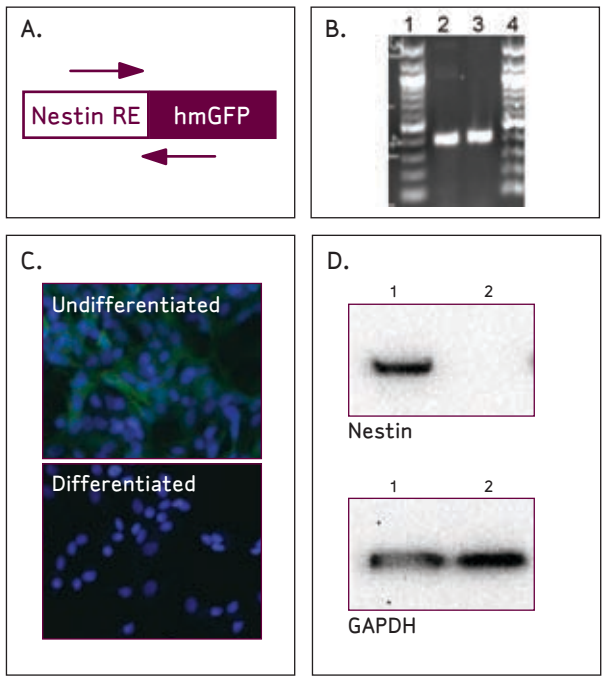

Figure 1. (A) Schematic diagram of PCR primers designed to amplify the Nestin-hmGFP region of the expression cassette. A 350 bp PCR fragment corresponding in size to the amplified Nestin-hmGFP region is observed in genomic DNA isolated from undifferentiated (B, lane 2) and differentiated cells (B, lane 3). Lanes 1 and lane 4 are molecular weight markers with 100bp interval DNA ladders. (C) GFP expression of undifferentiated and differentiated cells as viewed on a fluorescent microscope using the FITC filter. Cell nuclei were visualized with DAPI (blue). (D) Western blot analysis of Nestin protein expression in undifferentiated (lane 1) and differentiated (lane 2) cell lysates. House-keeping gene, GAPDH was used as a loading control. Nestin was detected with anti-Nestin, clone 10C2 (Catalogue No. MAB5326) and the loading control GAPDH was detected with anti-GAPDH, clone 6C5 (Catalogue No. MAB374).

To quantify the change in GFP expression upon differentiation, flow cytometry was performed on two independent puromycin-resistant cell lines, B1 and B2 (Figure 2). Both analyses were performed at the same time using similar acquisition and threshold parameters. In proliferating cultures of the B1 and B2 cell lines, a significant majority of the NSCs expressed high levels of GFP (98.9% for B1 and 98.3% for B2). However, after 15-17 days of differentiation, GFP expression was significantly downregulated in both cell lines (3.7% for B1 and 6.6% for B2). For both cell lines, differentiation resulted in significant downregulation of GFP expression.

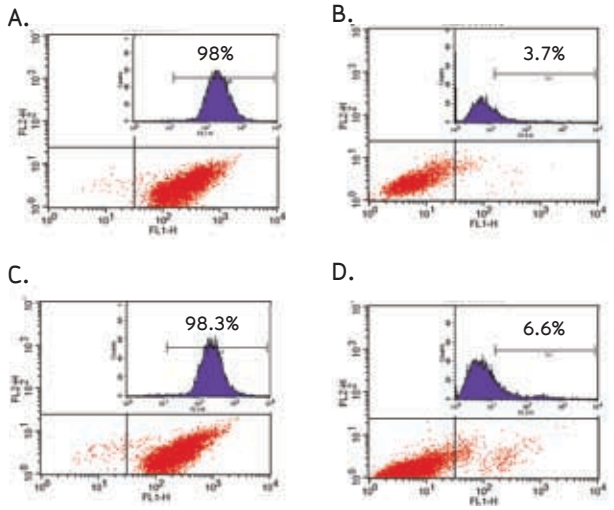

Figure 2. Flow cytometry analysis on the number of GFP-expressing cells in undifferentiated (A, C) and after 15-17 days of spontaneous differentiation (B, D) of cell lines B1 and B2. In total, 10,000 events were analyzed from each sample. The number of GFP expressed cells significantly decreased from 98.9% to 3.7% in B1 cell line and 98.3% to 6.6% in B2 cell line after 15 and 17 days of differentiation, respectively.

To examine the genomic integrity of the cells after transformation, cytogenetic analysis was performed on the four stable cell lines. All four cell lines demonstrated an apparently normal male karyotype (46, XY). Figure 3 shows a representative karyotype from one of the lines. All four stable cell lines also exhibited proliferation rates similar to the parental untransfected ReNcell CX line (data not show).

Figure 3. MilliTrace CX Nestin GFP reporter human NSC line is karyotypically normal. Cytogenetic analysis was performed by Cell Line Genetics on twenty G-banded metaphase cells. All twenty cells demonstrated an apparently normal male karyotype (46, XY). No abnormal cells were detected.

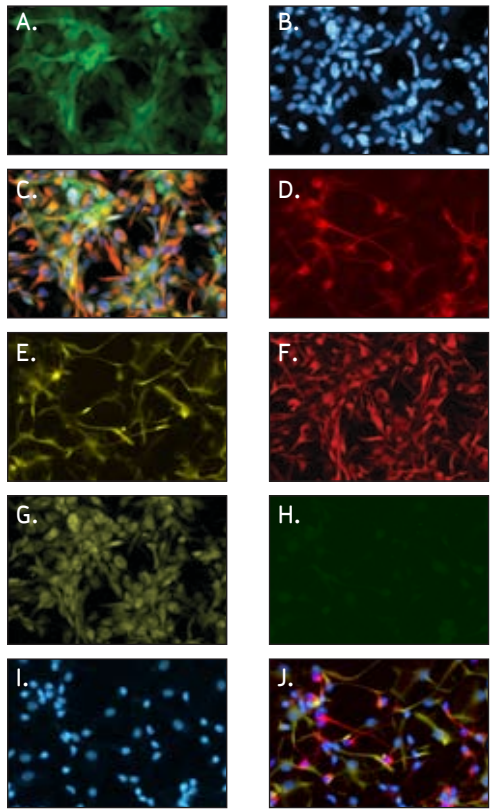

Figure 4. MilliTrace CX Nestin GFP reporter human neural stem cells express GFP (A) along with the appropriate NSC markers, Nestin (B, red) and Sox-2 (D, yellow) in proliferating cultures of undifferentiated neural stem cells. After two weeks of differentiation, GFP expression is down regulated (F) and lineage-specific markers, βIII-tubulin (G, red) and GFAP (I, yellow) are expressed indicating the presence of neurons and astrocytes, respectively. Under identical exposure time, GFP intensity decreased approximately 10-fold after 14 days of spontaneous differentiation (compare A to F). Nestin was detected by monoclonal MAB5326 (1: 500 dilution); Sox-2 was detected by polyclonal AB5603 (1:1000 dilution), βIII-tubulin was detected by monoclonal MAB1637 (1:1000 dilution); and GFAP was detected by polyclonal AB5804 (1/250 dilution).

MilliTrace CX Nestin GFP reporter human NSCs displayed the immunochemical staining characteristics of NSCs; under proliferative culture conditions, the majority were positively stained for Nestin (Figure 4B) and Sox-2 (4D) and negatively stained for the neuronal and astrocyte restricted lineage markers, βIII-tubulin and GFAP, respectively (data not shown). MilliTrace CX Nestin GFP reporter human NSCs are moreover multipotent; they can readily differentiate into βIII-tubulin positive neurons (Figure 4G) and GFAP positive astrocytes (Figure 4I) following a 14-17 day spontaneous differentiation protocol. Collectively, these results demonstrate the capacity of human NSCs to stably express Nestin-hmGFP, while still retaining the self-renewal and multipotentiality characteristics of neural stem cells.

Conclusions

MilliTrace CX Nestin GFP reporter human neural stem cells can be an important tool for researchers interested in live monitoring of the differentiation status of their human neural stem cells. The cells have been fully characterized to appropriately express GFP under the regulation of the mouse Nestin promoter and can be differentiated into neuronal and astrocytic lineages.

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Related Products

Description	Catalogue No.
MilliTrace CX Nestin GFP Reporter Human Neural Stem Cell Kit	SCR096
MilliTrace ReNcell Neural Stem Cell Maintenance Media Kit	SCM043
MilliTrace CX Constitutive GFP Reporter Human Neural Stem Cell Kit	SCR095
ReNcell CX Human Neural Progenitor Cells & Media Kit	SCC007
ReNcell NSC Maintenance Medium	SCM005
ReNcell NSC Freezing Medium	SCM007
Mouse Laminin	CC095
Accutase Cell Dissociation Solution	SCR005
Human Neural Stem Cell Characterization Kit	SCR060
Mouse Anti-Nestin, human specific	MAB5326
Rabbit Anti-Sox-2	AB5603
Mouse Anti-βIII Tubulin	MAB1637
Rabbit Anti-GFAP	AB5804
Human Recombinant FGF-2 (Basic FGF)	GF003
Human Recombinant EGF	GF001

Differentiation of Rat Mesenchymal Stem Cells Under Shear Stress

Carolyn Conant, Ph.D., Fluxion Biosciences, San Francisco, CA

Abstract

Stem cell research has the potential to produce novel treatments for previously incurable diseases and injuries. For studies of stem cell differentiation, scientists have traditionally used biochemical stimulation. However, shear stress can also be used. Here we demonstrate the differentiation of rat mesenchymal stem cells into von Willebrand factor (vWF)-expressing endothelial cells as a result of shear stress.

Introduction

In the lab, stem cell differentiation is induced using one of two ways: biochemical stimulation, the more traditional method; or mechanical stimulation, such as shear stress. Each method instigates a slightly different signaling pathway. Shear stress activates SRC family kinases and FLK-1 (with Cbl) to activate JNK and ERK. VEGF, commonly used in biochemical stimulation, elicits FLK-1/Nckβ association to later activate JNK and ERK[1].

Shear stress is used as a stimulus for differentiation especially for cell types that naturally respond to physiological shear, such as endothelial cells[2,3,4,5]. Differentiation of cells into specific cell types and subsequent production of biomaterials is also facilitated by mechanical forces such as shear. This is the case with chondrocytes used to produce cartilage[6].

Mesenchymal stem cells may be differentiated under different conditions into several types of specialized cells. Application of shear stress to mesenchymal stem cells or addition of VEGF may influence their differentiation to endothelial cells, which exclusively express and secrete vonWillebrand factor (vWF)[7,8]. We compared the differentiation of mesenchymal stem cells into vWF-expressing endothelial cells due to shear stress or biochemical stimulation.

Methods

Cryopreserved adult Fisher 344 rat mesenchymal stem cells (Millipore, Catalogue No. SCR027) were propagated in their accompanying mesenchymal stem cell medium as directed by the manufacturer. For shear stress experiments, the BioFlux system from Fluxion Bioscience was used. Channels were coated with poly-l-lysine at room temperature for one hour and then washed with spent medium from the culture of the stem cells. Cells were seeded into the channels at 5 x 10^6 cells/mL and grown overnight by gravity flow in mesenchymal stem cell expansion medium (Millipore, Catalogue No. SCM015). The next day, media was changed to CO_2-independent medium (Invitrogen®) supplemented with either 10% FBS, 2% FBS, or 2% FBS with 50 ng/mL recombinant rat vascular endothelial growth factor (VEGF) added. An alternative to using CO_2-independent media would be to use the optional gas hookup to apply a 5% CO_2 gas mixture as the pressure source. The BioFlux plate was placed on the heater plate; media were either perfused by gravity flow or at 1 dyn/cm^2 for 48-100 hours. Additional media was added as needed. Following perfusion, cells were fixed with 1% paraformaldehyde for 30 minutes, blocked, and stained with a primary antibody (rabbit) against the endothelial marker vWF (Abcam) as well as the actin marker phalloidin, conjugated with Alexa Fluor 594 (Invitrogen). Cells were washed with 0.5% BSA in PBS for 10 minutes at 1 dyn/cm^2 and the secondary antibody was added (anti-rabbit, conjugated with Alexa Fluor 488). Cells were washed in PBS for 10 minutes at 1 dyn/cm^2 and Hoechst 33342 was added in PBS by gravity flow. Images were captured using a Nikon TS100 microscope and a CCD camera (QICAM).

Results

The mesenchymal stem cells differentiated into endothelial cells under both mechanical and biochemical stimulation conditions. We found that most cells began to express vWF in the cytosol after 48 hours of shear stress even without addition of VEGF. The most evident differentiation (marked by expression of cytosolic vWF) occurred without the addition of VEGF in rich media under shear (Figure 1, 10% FBS, shear stress). This sample also differentiated into a tight colony – a type of cell morphology that was not observed in any of the other differentiation conditions. Differentiation also occurred in the VEGF/shear stress-treated cells (Figure 1, VEGF/2% FBS, shear stress).

Discussion & Conclusion

Mechanotransduction is a very important factor in certain types of stem cell differentiation and remains an area of active research. Depending on the scenario, it can offer

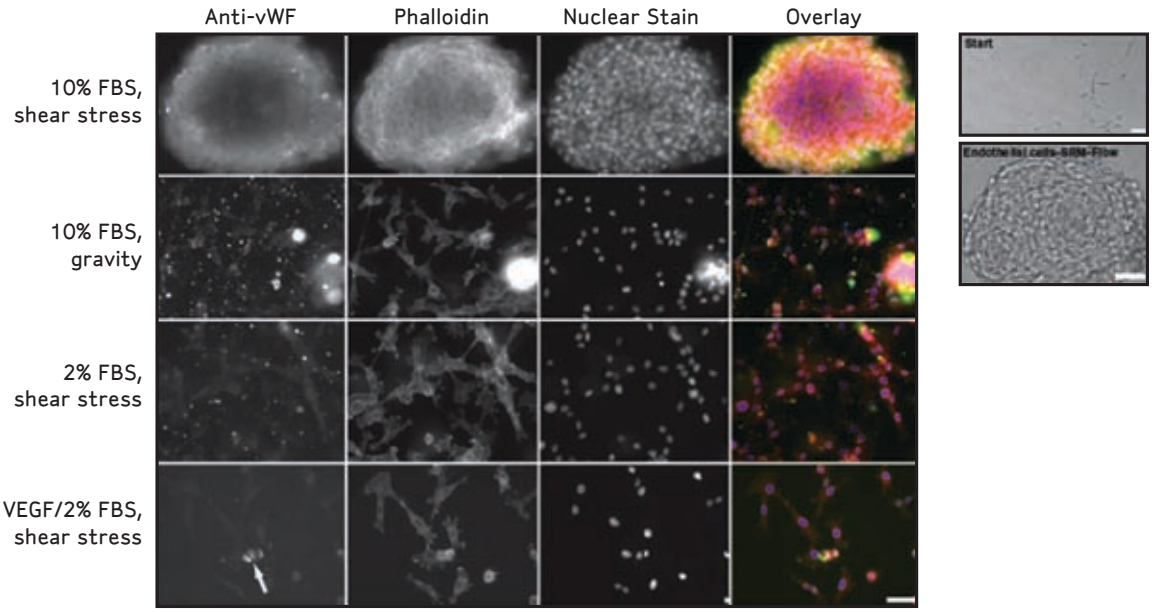

Figure 1. Micrographs of mesenchymal stem cells before (start) and after flow. Scale bars = 50 µm. Arrow indicates vWF expressing cells in the VEGF condition. All fluorescence micrographs are after 48 hours of continuous flow or no flow as indicated.

certain advantages as an *in vitro* model when compared to the traditional biochemical stimulation methods. For example, during development of an organism, growth factors and other compounds are present to instigate differentiation, but mechanical stimulation may be the primary mediator in a situation like wound healing in an adult organism with an injury to a blood vessel. Both routes may also interact synergistically. Shear stress also appears to be a faster method, at least in mesenchymal stem cells. In these tests, vWF was expressed in the shear stress samples after only 48 hours, while commonly published times for biochemical stimulation are around seven days – more than double that of the shear stress differentiation.

This same experimental setup – using the same readily-available cryopreserved mesenchymal stem cells and the BioFlux system – could be used to evaluate the role of other influences in the differentiation process, such as temperature, gas composition, and shear stress pressures/times.

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Related Products

Description	Catalogue No.
Cryopreserved Rat Mesenchymal Stem Cells, 1 x 10^6 cells	SCR027
Mesenchymal Stem Cell Expansion Medium, 1X, 500 mL	SCM015

Western Blotting: Passive Diffusion vs. Vacuum-Driven Immunodetection

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Abstract

Western blotting is a popular technique among biological researchers in many different disciplines. Although highly versatile, it involves a lengthy immunodetection step that has not changed much since the technique was first introduced. Here we show three independent evaluations of a new vacuum-based immunodetection system for Western blotting. This new system reduces the immunodetection step to under half an hour, while providing results equivalent to those of the traditional method.

Introduction

The Western blot is a powerful tool used extensively in protein research to detect and compare the relative levels of proteins without the need for their prior purification. Its widespread appeal is based on its overall simplicity, coupled with the high resolution of proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) prior to membrane transfer. This versatile technique continues to develop with approaches that now enable more rapid electrophoresis, faster protein transfer, increased miniaturization, and higher specificity. Additionally, recent enhancements in chemiluminescent detection have significantly increased both the sensitivity and flexibility of Western blots. While much attention has focused on improving these aspects of Western blotting, the immunodetection step has remained much the same—a lengthy process typically requiring 4-30 hours or more of elapsed time to complete.

Millipore has developed the SNAP i.d.® protein detection system as a faster alternative. This new system uses a vacuum rather than passive diffusion during blocking, washing, and antibody incubations to reduce the time required for immunodetection. It is compatible with multiple membrane types and detection methods, and follows the same sequence of steps as traditional immunodetection; however, unlike traditional methods, the SNAP i.d. system can complete the immunodetection step in less than half an hour.

To compare the different methods, we asked three scientists from the academic or private sector to run their samples in parallel using both the traditional technique and the SNAP i.d. system. The results from their experiments are briefly reviewed.

Methods

Three different samples were tested in parallel with both the traditional Western blotting method and the SNAP i.d. system.

FLAG®-TAGGED PROTEIN

The first sample was a FLAG-tagged protein. Traditional Western blot detection used a mouse anti-FLAG primary antibody at a 1:1000 dilution in 10 mL, incubated at room temperature (RT) for one hour. This was followed by an anti-mouse secondary antibody conjugated with HRP at a 1:20000 dilution in 10 mL, incubated at RT for half an hour. The blocking step used 3% BSA in PBS with Tween® detergent, incubated at RT for two hours. The total immunodetection time was four hours and 10 minutes.

The SNAP i.d. detection used the same antibodies at different dilutions. The primary antibody was diluted 1:300 in 1.5 mL and incubated at RT for 10 minutes. The secondary antibody was diluted 1:20000 in 2 mL and incubated at RT for 10 minutes. The blocking step used 1% BSA in PBS with Tween detergent, incubated at RT for 10 seconds. The total immunodetection time was 22 minutes.

WT PROLACTIN IN MCF-7 CELLS

The second sample type tested was MCF-7 cells, stimulated and non-stimulated with prolactin. The traditional Western blot detection used a mouse anti-PRLR-ECD antibody to detect the wild-type prolactin receptor. 10 mL of this primary antibody was used at a concentration of 0.5 µg/mL and was incubated overnight at 4 °C. This was followed by 10 mL of an anti-mouse secondary antibody conjugated with HRP, incubated for one hour at RT. The blocking step used 3% BSA in 0.1% tris-buffered saline with Tween-20 detergent, incubated at RT for one hour. The total immunodetection time was 16.5 hours.

The SNAP i.d. detection system used the same antibodies at different dilutions. 1.5 mL of the primary

antibody was used at a concentration of 3.3 µg/mL and incubated at RT for 10 minutes. The secondary antibody used 1.5 mL and was incubated at RT for 10 minutes. The blocking step used 0.5% milk in 0.1% tris-buffered saline with Tween detergent, incubated at RT for 10 seconds. The total immunodetection time was 22 minutes.

MCM3 IN A HCT116 PROTEIN EXTRACT

The third sample type was a HCT116 protein extract. 5 mL of the primary anti-MCM3 antibody at a concentration of 0.4 µg/mL was incubated overnight at 4 °C. This was followed by 5 mL of an AffiniPure™ anti-igG secondary conjugated with HRP at a concentration of 0.2 µg/mL in 5 mL, incubated at RT for 45 minutes. The blocking step used 5% milk in 0.1% PBS with Tween-20 detergent, incubated at RT for one hour. The total immunodetection time was four hours and 10 minutes.

The SNAP i.d. detection used the same antibodies at different dilutions. The primary antibody was diluted to 1.3 µg/mL in 1.5 mL and incubated at RT for 10 minutes. The secondary antibody was diluted to 1.3 µg/mL in 1.5 mL and incubated at RT for 10 minutes. The blocking step used 0.5% milk in 0.1% tris-buffered saline with Tween detergent, incubated at RT for 10 seconds. The total immunodetection time was 22 minutes.

Results

In each test, the immunodetection performed with the SNAP i.d. system was completed in less than half an hour, as compared to between four and 16 hours for the traditional method. Likewise, the SNAP i.d. blots tended to have lower background and more intense signals, providing higher quality results than the traditional method. The SNAP i.d. system also generally used less antibody (higher concentrations but much smaller volumes) than the traditional method.

For the first test with the FLAG-tagged protein (Figure 1), the SNAP i.d. system (B) was able to perform the immunodetection steps in 22 minutes—this is 91% faster than the standard Western blotting method (A). The system also used three times less primary antibody, five times less secondary antibody, three times less bovine serum albumin (BSA), and the obtained signal was more intense.

For the second experiment testing for the prolactin receptor in MCF-7 cells (Figure 2), the SNAP i.d. system (B) was fifty times faster than the standard Western blotting method (A). In this experiment, approximately the same quantity of primary antibody was used. However, the SNAP i.d. system used six times less secondary antibody and blocking was instantaneous, performed using milk instead of BSA. The background for the SNAP i.d. detection was noticeably lower than that of the traditional method for this test.

For the third and final experiment testing for MCM3 in a HCT116 protein extract (Figure 3), the SNAP i.d. system (B) was fifty times faster than the standard Western blotting method (A).

While this experiment used approximately the same quantity of the primary antibody for both methods, the SNAP i.d. system used five times less secondary antibody. Blocking was also instantaneous for the system.

Discussion & Conclusion

These three tests demonstrated the ability of the SNAP i.d. system to generate results that are equivalent to or better than the standard Western blotting method, while requiring less time and less material. The innovative use of the vacuum to drive reagents through the membrane rather than allowing them to slowly diffuse into it greatly reduces the time required for immunodetection, and offers other

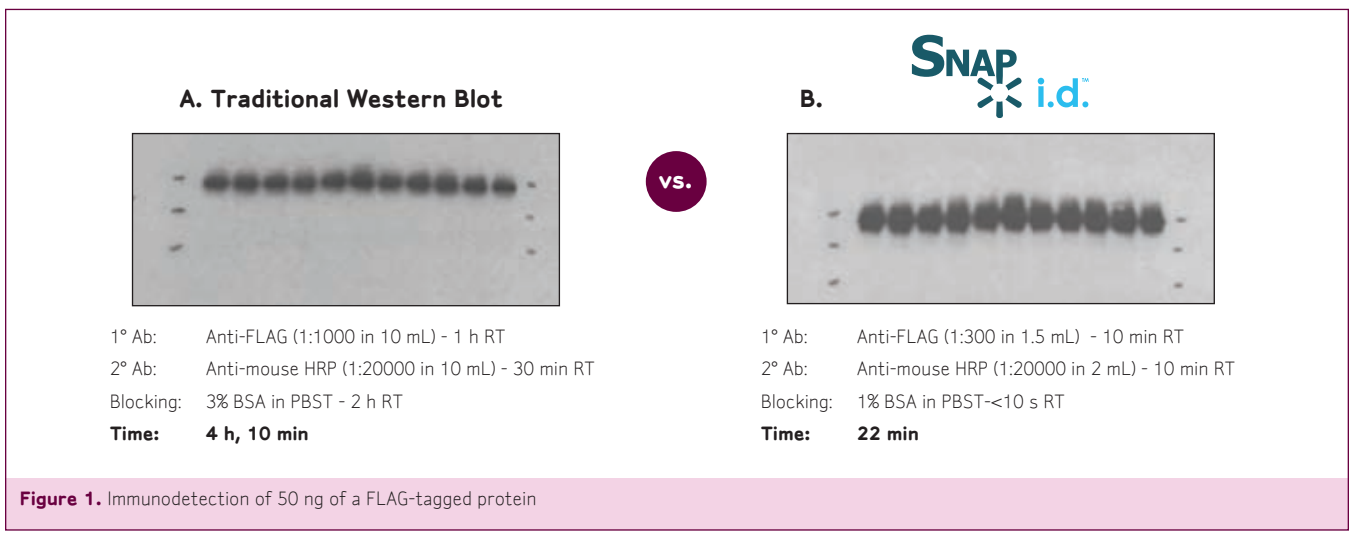
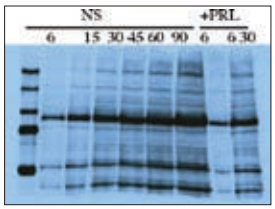


Figure 1. Immunodetection of 50 ng of a FLAG-tagged protein

benefits as well. There were instances where the system yielded blots with either a reduced background or an improved signal relative to the traditional blot. The application of a vacuum dramatically increases the efficiency of blocking, such that it can be achieved nearly instantaneously even with low concentrations of blocking buffers. Furthermore, the use of higher antibody concentrations in a proportionally smaller volume allows users to decrease incubation times and reduce the amount of antibody required for each experiment.

As with any new technology, optimization is required for maximal performance. For the SNAP i.d. system, optimization of both antibody concentrations (primary and secondary antibodies) and blocking buffers is essential. Because of the speed of immunodetection with this system, though, optimization can be accomplished much more quickly than before. Once the SNAP i.d. system is optimized, users can expect results similar to those obtained with standard immunodetection but in a fraction of the processing time.

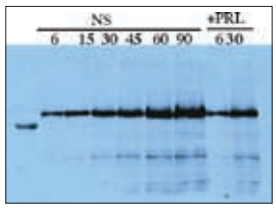
A. Traditional Western Blot



1° Ab: Mouse anti-PRLR-ECD (0.5 µg/mL in 10 mL) - O/N 4 °C
2° Ab: Anti-mouse HRP (10 mL) - 1 h RT
Blocking: TBST (0.1%), 3 % BSA - 1 h RT
Time: 16 h, 30 min

vs.

B. SNAP i.d.™



1° Ab: Mouse anti-PRLR-ECD (3.3 µg/mL in 1.5 mL) - 10 min RT
2° Ab: Anti-mouse HRP (1.5 mL) - 10 min RT
Blocking: TBST (0.1%), 0.5% milk <10 s RT
Time: 22 min

Figure 2. Expression of WT prolactin receptor, +/- prolactin stimulated, in MCF-7 cells

A. Traditional Western Blot

1° Ab: Anti-MCM3 (0.4 µg/mL in 5 mL) - O/N 4° C
2° Ab: Peroxidase-conjugated AffiniPure IgG (0.2 µg/mL in 5 mL)- 45 min RT
Blocking: PBST (0.1%), 5% milk - 1 h RT
Time: 16 h, 30 min

vs.

B. SNAP i.d.™

1° Ab: Anti-MCM3 (1.3 µg/mL in 1.5 mL) - 10 min RT
2° Ab: Peroxidase-conjugated AffiniPure IgG (1.3 µg/mL in 1.5 mL)- 10 min RT
Blocking: PBST (0.1%), 0.5 % Milk <10 s RT
Time: 22 min

Figure 3. Immunodetection of MCM3 from a HCT116 protein extract

Related Products

Description	Catalogue No.
SNAP i.d. Protein Detection System	WBAVDBASE
SNAP i.d. Single Blot Holder	WBAVDBH01
SNAP i.d. Double Blot Holder	WBAVDBH02
SNAP i.d. Triple Blot Holder	WBAVDBH03
SNAP i.d. Antibody Collection Tray	WBAVDABTR
SNAP i.d. Blot Roller	WBAVDROLL
Immobilon Western Chemiluminescent HRP Substrate, 100 mL	WBKLS00100

Stem Cell Testing of Millicell 24-Well Plates

Sónia Gil and Amy Botica, Millipore Corporation, Temecula, CA

Abstract

Stem cell research has revolutionized cell biology by allowing a better understanding of cellular differentiation and providing a potential cell source for cell therapy and tissue engineering. Typically, embryonic stem (ES) cells are maintained on solid bottom, tissue-culture-treated plates, but microporous membrane-based culture offers many unique advantages over the traditional solid bottom plates, especially for co-culture experiments. Here we show that Millicell 24-well cell culture plates can be used for stem cell research without harming the cells or affecting pluripotency.

Introduction

Microporous membrane-based cell culture offers many advantages over traditional solid bottom plates. Cells grown on membranes are able to access media from both their apical and basolateral sides. This three-dimensional environment enhances stem cell morphology, creating a better model of *in vivo* growth and differentiation. The cells are also separated from the contents of the lower well, which can be very useful for co-culture research.

As with any component in the stem cell culturing process, it is crucial to ensure that the cultureware, whether solid-bottom or membrane-based, does not harm the cells or leach compounds to the media. To meet this need, Millipore has instigated a stem cell testing program for many of our sterile filtration and cultureware products.

The first products to be given the stem cell tested label were the Stericup® and Steritop® filters with the Millipore Express® PLUS membrane. These sterile filtration devices can be used for filtering stem cell culture media without removing significant amounts of crucial growth factors or adding deleterious components to the media. Cells cultured in media filtered through Stericup devices retained their pluripotency for at least five passages.

For this new study, three lots of Millipore's Millicell 24-well plates were tested for use in stem cell culture. After culturing murine embryonic stem cells in the plates for five passages, the colonies were evaluated for pluripotency based on cell morphology, alkaline phosphatase staining, and expression of the stem cell markers Oct-4 and NANOG.

Methods

STEM CELL CULTURE

Cells were initially cultured in T75 flasks on mitotically inactive murine embryonic fibroblast (MEF) feeder layers until ready for passaging. Cells were then lifted from the plates and separated from the MEF layer. Millicell 24-well culture plates were coated with 0.1% gelatin in DPBS and seeded with 1500 cells per well in 400 µL murine embryonic stem cell media (DMEM, bovine serum, glutamax-1, non-essential amino acids, 2-mercaptoethanol, and ESGRO® murine leukemia inhibitory factor). Cells were passaged and re-seeded onto Millicell plates five times before analysis.

ALKALINE PHOSPHATASE STAINING

Cells cultured in the Millicell plates were fixed with 3.7% formaldehyde in PBS for 1-2 minutes, rinsed, and stained with Millipore's alkaline phosphatase detection kit according to the manufacturer's protocol.

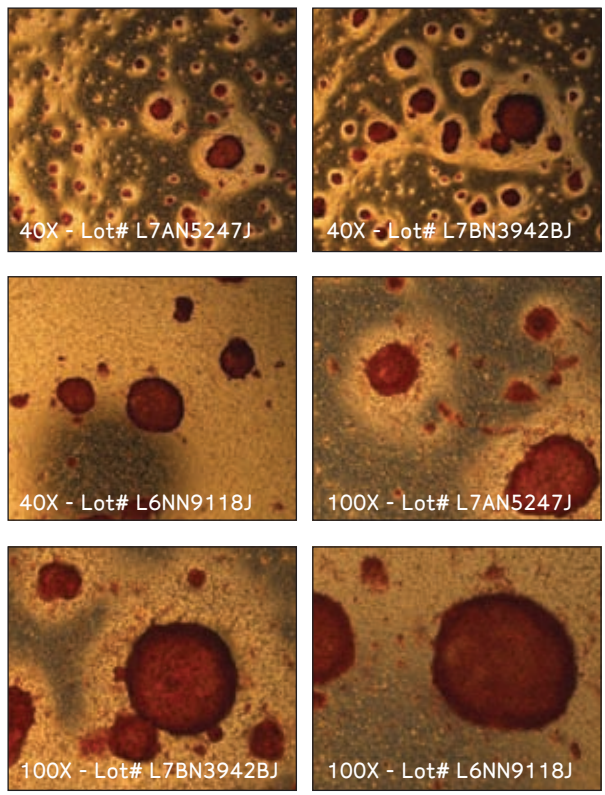


Figure 1. Alkaline phosphatase staining of mES colonies.

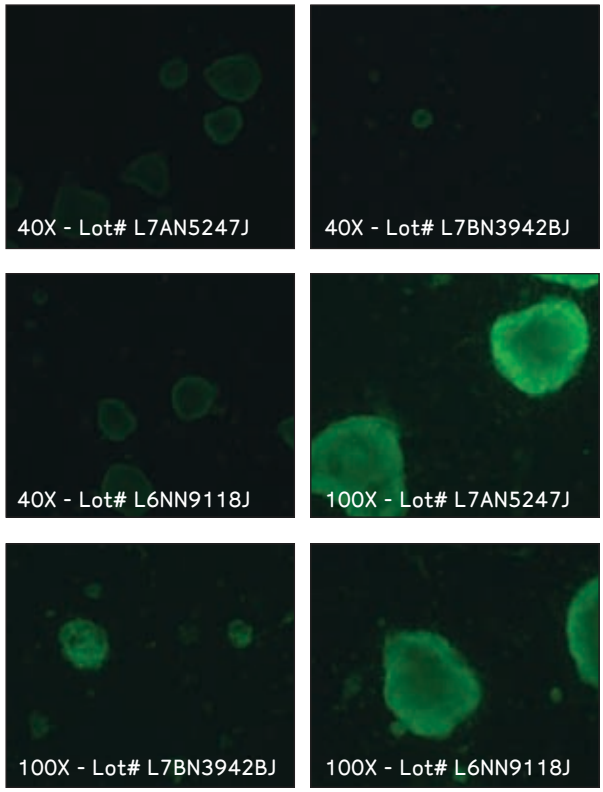


Figure 2. NANOG staining of mES colonies.

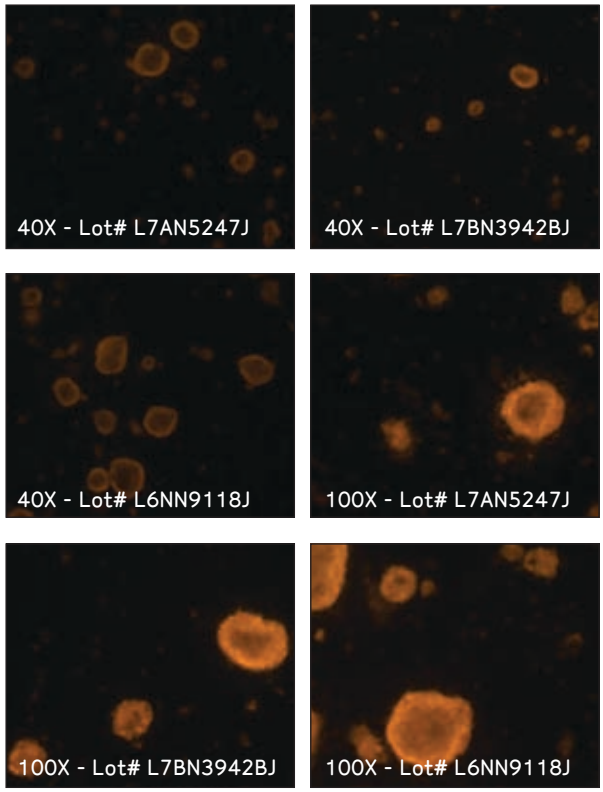


Figure 3. Nanog staining of mES colonies.

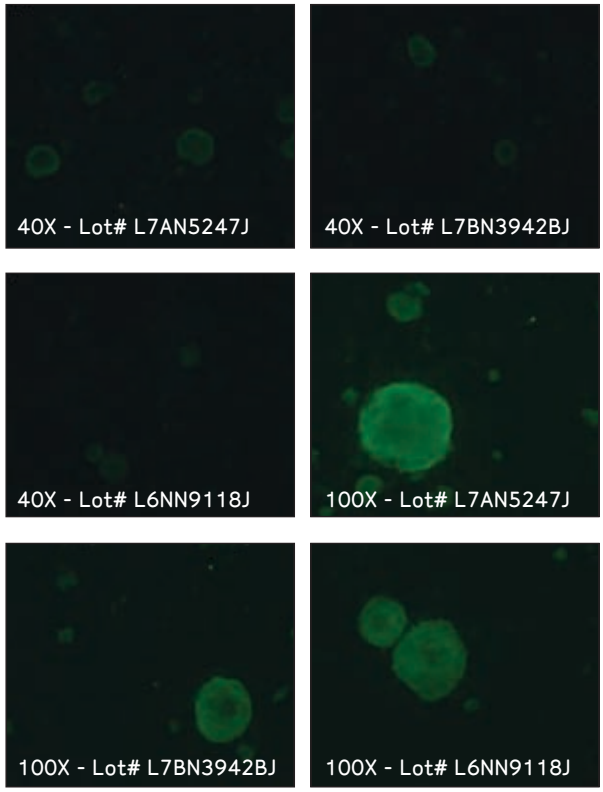


Figure 4. Oct-4 staining of mES colonies.

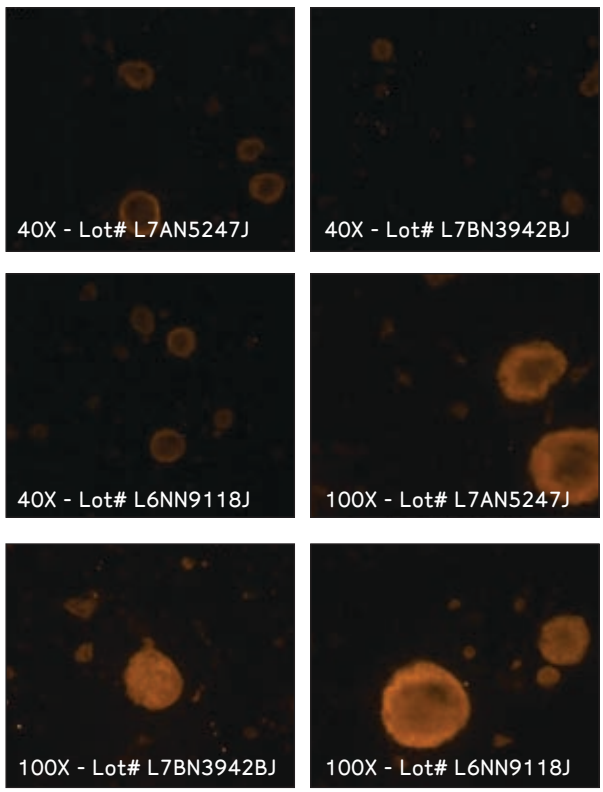


Figure 5. Oct-4 staining of mES colonies.

OCT-4 AND NANOG STAINING

Cells cultured in the Millicell plates were fixed with 3.7% formaldehyde in PBS for five minutes. Cells were then rinsed and incubated for one hour with the primary antibody in a solution of 1% FBS, 0.1% saponin, and DPBS. Antibody dilutions were 1:200 for Oct-4 and 1:400 for NANOG. The wells were then washed and incubated for 30 minutes with the secondary antibody in DPBS. Secondary antibody dilutions were 1:100 for Oct-4 and 1:200 for NANOG.

Results

Three lots of Millicell plates were evaluated. The results from all three were roughly equivalent, eliminating possibility of lot-to-lot variations in the plates affecting cell culture. After five passages, the embryonic stem cell colonies were distinct with sharp borders; morphology similar to that of cells cultured in standard solid-bottom plates (data not shown). They demonstrated high levels of alkaline phosphatase staining (Fig. 1). The colonies also evenly expressed the stem cell markers Oct-4 (Fig. 4&5) and NANOG (Fig. 2&3).

Discussion & Conclusion

The data clearly demonstrates that the murine embryonic stem cells tested in all three Millicell lots maintained their pluripotency after five passages. As a result, the Millicell plates have been given the Stem Cell Tested label, joining the previously-validated Stericup and Steritop filtration devices.

Now that these plates have been used successfully in stem cell culture, scientists have more options in designing future assays. Since membrane-based cell culture keeps cells separated from the contents of the lower well, it is useful for co-culture studies, which cover a broad range of stem cell research. Feeder cells can be placed below the membrane and thus separated from the stem cell colonies, so the feeder cells do not need to be mitotically inactivated as is necessary for traditional culture methods. Likewise, the separation makes for easier passaging and isolation, as the feeder cells are already out of direct contact with the stem cells. This can be useful for down stream applications such as differentiation, embryoid body formation, or molecular analysis. In addition, other cell types can be placed in the lower well to study indirect cell-cell interactions that influence differentiation or interfere with cell development. Or, as in our tests, the cells can be cultured entirely without feeders, while benefiting from the improved morphology that results from accessing media from both sides. Many more applications are possible.

Millipore Products

Description	Catalogue No.
Millicell 24-Well Cell Culture Plates	PSRP010R5
Stericup-GP, 0.22 µm, radio-sterilized	SCGPU10RE
PluriStem 129/S6 Murine ES cells	SCR012
EmbryoMax® Primary Mouse Embryo Fibroblasts, strain CF1, mytomycin C treated, passage 3	PMEF-CF
ESGRO mLIF Supplement	ESG1106
Anti-Oct-4, clone 10H11.2	MAB4401
Anti-Nanog, N-terminus	AB5731
Alkaline Phosphatase Detection Kit	SCR004
Goat Anti-Rabbit IgG, Cy2 conjugated	AP132J
Goat Anti-Mouse IgG, Cy2 conjugated	AP124J
Goat Anti-Rabbit IgG, Cy3 conjugated	AP132C
Goat Anti-Mouse IgG & IgM, Cy2 conjugated	AP130C

CELLnTEC™ Products for Gingival Research

Jim Johnson, CELLnTEC Advanced Cell Systems

With humans living longer, periodontal disease and gingivial aging has become an increasingly relevant area of medical research. Aside from the obvious quality of life issues resulting from these diseases, damaged gingivial boundaries can also be a gateway for other health issues. In order to tackle theses problems, it is crucial to understanding the mechanisms of normal gum development and replenishment.

To provide an *in vivo*-like cell model for periodontal research, CELLnTEC has created optimized gingival keratinocyte culture media and primary gingival keratinocyte cells isolated in specialized, progenitor cell targeted (PCT) medium. PCT media use a signaling-based approach to medium formulation, resulting in cell culture media that specifically target progenitor cells. These media encourage cells to remain undifferentiated, enabling them to maintain the proliferative phenotype for longer periods than cells grown in traditional, older media formulations.

Progenitor Cell Targeted (PCT) Oral Culture Media

By mimicking the microenvironment for the adult stem cell, CELLnTEC's PCT media enhance the growth of progenitor cells, resulting in higher isolation efficiencies, and longer *in vitro* lifespan. Non-PCT formulations are also available for use during and after differentiation. Unlike self-made or commercial media, which typically expire within a month after supplements are added, CELLnTEC media are stable for three months when stored at 4 ºC. This extended stability is an added benefit of CELLnTEC's state-of-the-art formulations compared to the older formulations.

Primary Gingival Cells

Many researchers do not have access to a source of tissue to isolate gingival keratinocytes. Commercial sources of gingival keratinocytes, having been isolated in traditional media formulations, quickly reach senescence and stop dividing, thereby limiting the amount and types of experiments that can be performed. As a result of their excellent modeling and undifferentiated phenotype, CELLnTEC primary gingival cells are ideal starting material for a number of different experiments including establishing 3D *in vitro* models.

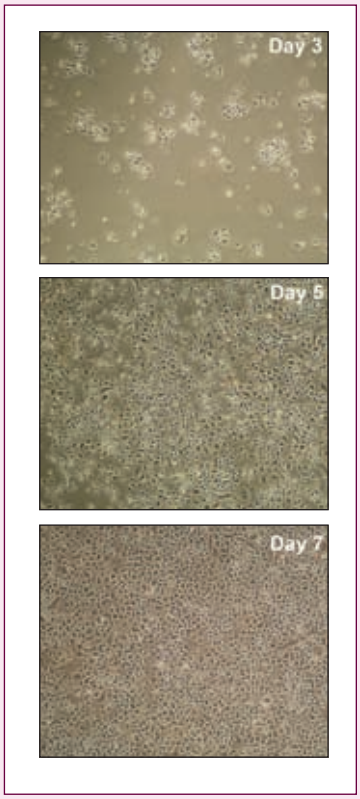


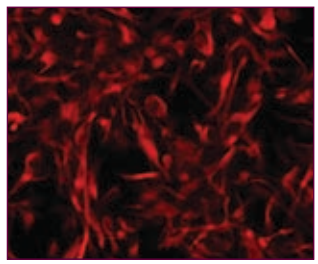
Figure 1 (right). Primary human gingival epithelium progenitor cells isolated in CnT-24 medium. Frozen cells were thawed and plated at 4 x 10^3 cells/cm^2 in CnT-24 medium.

Description	Qty/pk	Catalogue No.
Gingival Cell Culture Media Kits (basal medium with supplements)		
PCT Oral Epithelium Medium, defined	500 mL kit	CnT-24
Oral Epithelium Medium, defined	500 mL kit	CnT-32
Primary Human Gingival Keratinocyte Cell Kit (cells plus medium)		
Gingival Epithelium Progenitors, pooled	>5 x 10^5 cells	HGEPp.05
Gingival Epithelium Progenitors, pooled	>1.5 x 10^6 cells	HGEPp.15
Gingival Epithelium Progenitors, single donor	>5 x 10^5 cells	HGEPs.05
Related Products		
Antibiotic/Antimycotic Solution (100X)	100 mL	CnT-ABM
Antibiotic/Antimycotic Solution (200X, single aliquots)	10 x 2.5 mL	CnT-ABM10
Antibiotic/Antimycotic Solution (200X, single aliquots)	20 x 2.5 mL	CnT-ABM20

Exciting New Products for Cell Biology Research

STEM CELL RESEARCH

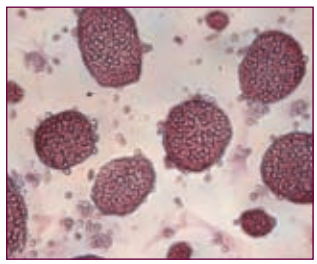
MilliTrace CX Nestin GFP Reporter Human Neural Stem Cell Kit



This kit provides a quick, convenient method to study the role of nestin and other factors in neural stem cell differentiation, maintenance and self-renewal. MilliTrace CX Nestin GFP reporter human neural stem cells are labeled with the humanized mulleri green fluorescent protein (hmGFP) under the regulation of the Nestin promoter. Upon differentiation, Nestin is down-regulated and GFP expression is switched off. In addition to the cells, the kit contains expansion medium to help maintain expression of the transgene. Also available from Millipore are human neural stem cells that constitutively express hmGFP.

Description	Qty/Pk	Cat. No.
MilliTrace CX Nestin GFP Reporter Human Neural Stem Cell Kit	10^6 viable cells, plus 500 mL expansion medium	SCR096
MilliTrace VM Constitutive GFP Reporter Human Neural Stem Cell Kit	10^6 viable cells, plus 500 mL expansion medium	SCR092
MilliTrace CX Constitutive GFP Reporter Human Neural Stem Cell Kit	10^6 viable cells, plus 500 mL expansion medium	SCR095

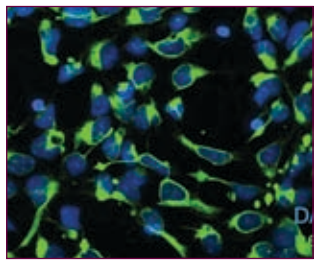
ESGRO Complete PLUS Medium for Mouse ES Cell Culture



For improved serum-free and feeder-free mouse embryonic stem (ES) cell culture, Millipore has developed ESGRO Complete PLUS, a two-component system containing the original ESGRO Complete clonal grade medium, plus an optimized GSK3β inhibitor supplement to help maintain pluripotency. The new formulation eliminates the drawbacks associated with the use of FBS and feeder layers, while enhancing the growth and maintenance of undifferentiated mouse ES cells in controlled conditions.

Description	Qty/Pk	Cat. No.
ESGRO Complete PLUS Clonal Grade Medium	100 mL	SF001-100P
ESGRO Complete PLUS Clonal Grade Medium	500 mL	SF001-500P

Animal-Free Recombinant Human Basic Fibroblast Growth Factor



Millipore's recombinant human basic fibroblast growth factor (FGF) has been developed without animal-derived ingredients and supports human ES cell and induced pluripotent stem cell (iPSC) maintenance and expansion. With our newly introduced 100 µg pack size, we have several options for you to choose from. Don't see what you need here? Custom packaging is available; please inquire for details.

Description	Qty/Pk	Cat. No.
Basic FGF, animal-free, recombinant human	50 µg	GF003
Basic FGF, animal-free, recombinant human	100 µg NEW SIZE!	GF003AF-100UG
Basic FGF, animal-free, recombinant human	1 mg	GF003AF-1MG

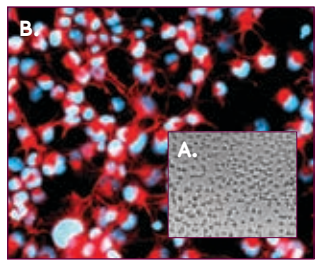
PluriStem® Mouse Embryonic Stem Cell Lines

The PluriStem range of murine ES cells are derived from well characterized, common inbred mouse strains. These new mouse ES cell lines are provided at passage 9, have an apparent normal male karyotype (40, XY), and have been genotyped by SNP analysis.

Description	Qty/Pk	Cat. No.
PluriStem C57BL/6N Mouse ES Cell Line	2 vials, 2.5 x 10^6 cells ea.	SCC050
PluriStem BALB/c Mouse ES Cell Line	2 vials, 2.5 x 10^6 cells ea.	SCC052
PluriStem FVB/N Mouse ES Cell Line	2 vials, 2.5 x 10^6 cells ea.	SCC053
PluriStem DBA/2 Mouse ES Cell Line	2 vials, 2.5 x 10^6 cells ea.	SCC054
PluriStem C3H Mouse ES Cell Line	2 vials, 2.5 x 10^6 cells ea.	SCC055

Extracellular Matrix (ECM) Proteins

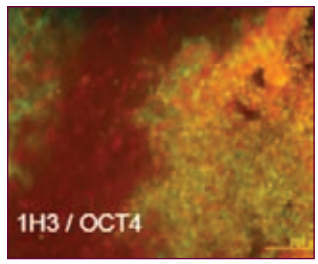
Millipore's synthetic laminin peptide is a defined ECM substrate that has been specifically optimized to support the cell adhesion, proliferation, and multi-lineage differentiation of rat neural stem cells (NSCs) *in vitro*. Rat neural stem cells grown on tissue culture plates coated with this synthetic laminin peptide (A) display the characteristic neural stem cell markers, Nestin (B) and Sox-2, and furthermore possess the capacity to preferentially differentiate down both glial and neural lineages.



Description	Qty/Pk	Cat. No.
Synthetic Laminin Peptide for Rat Neural Stem Cells	5 x 3 mg	SCR127

Stem Cell Antibodies

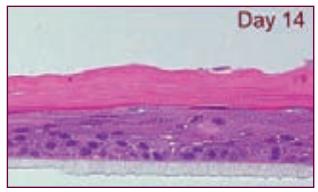
hPlurES-1, clone 1H3 (green), is a novel monoclonal antibody that identifies a 65 kDa antigen that is expressed on the cell surface of human embryonic stem cells. This unique antibody recognizes a protein epitope that appears to be expressed within a subset of Oct-4-positive (red) human embryonic stem cell populations, as indicated by double-staining analysis using flow cytometry and immunocytochemistry.



Description	Qty/Pk	Cat. No.
hPlurES-1 Antibody, clone 1H3	100 µg	MAB4395

PRIMARY CELLS

Epidermal Keratinocyte 3D Prime Medium for Generating 3D Skin Models



This novel 3D cell culture medium enables the easy and reliable generation of 3D epidermal models in your own laboratory, on your own schedule, when used with CELLnTEC human epidermal keratinocytes and Millipore's Millicell membrane inserts. The media can also be used with human keratinocytes you have isolated and grown in CELLnTEC's CnT-07 or CnT-57 media. Human epidermal keratinocytes cultured in this medium can generate a 3D model of human epidermis, with all layers (stratum corneum, granulosum, spinosum, basale), within 14-18 days. The 3D medium is fully defined, serum-free, BPE-free, and optimized for 3D epidermal growth. Full, detailed protocols for establishment of 3D keratinocyte models and histological sectioning and staining are available.

Description	Qty/Pk	Cat. No.
Epidermal Keratinocyte 3D Prime Medium, defined	100 mL	CnT-02-3DP1
Epidermal Keratinocyte 3D Prime Medium, defined	500 mL	CnT-02-3DP5

Related Products for 3D culture

Description	Qty/Pk	Cat. No.
PCT Epidermal Keratinocyte Medium, Low BPE (for 2D isolation and growth)	500 mL	CnT-57
PCT Epidermal Keratinocyte Medium, defined (for 2D isolation and growth)	500 mL	CnT-07
Human Epidermal Keratinocyte Progenitors, pooled donor	5 x 10^5 cells	HPEKP.05
Human Epidermal Keratinocyte Progenitors, single donor	5 x 10^5 cells	HPEKS.05
Millicell Single Well Inserts, PCF, 0.4 µm pore size, 30 mm	50	PIHP03050
Millicell Single Well Inserts, PCF, 0.4 µm pore size, 12 mm	50	PIHP01250

CELL CULTUREWARE

Elispot Strips

Millipore is now offering 8-well Elispot strips to give you greater flexibility in your research. MultiScreen®$_{HTS}$ filter plates and strips provide high protein binding capacity with low background staining and reliable sensitivity from lot to lot. Both the plates and strips are designed with a flat membrane for enhanced imaging on a range of systems including Zeiss and AID devices. Plates are available with either a removable underdrain or no underdrain, and the strips are provided without an underdrain for easy imaging and with a convenient tray for washing. The Elispot strips are a great way to optimize your assay before moving to the higher throughput 96-well plate.

Description	Qty/Pk	Cat. No.
Multiscreen 8-well Strip Support, sterile	10	M8IPFRAME
Multiscreen Plate, hydrophobic PVDF, sterile, 0.45 µm pore size, 8-well strips	10 plates	M8IPS4510

Introducing EndoGRO™ Media and Cells

Reliable, Viable, Endothelial Cell Culture

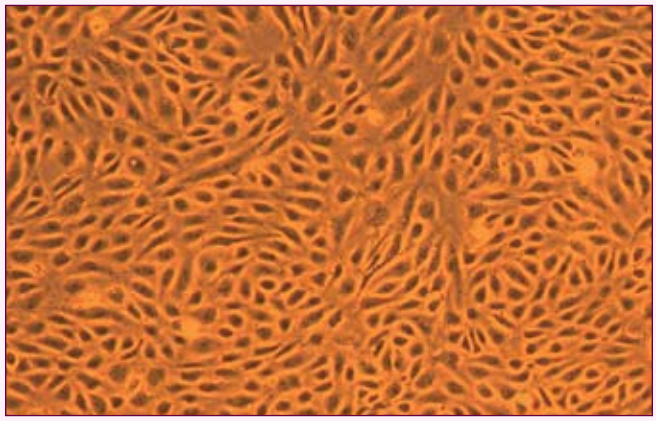
The new product line includes four uniquely optimized media formulations for large vessel and microvascular endothelial cells, as well as low passage human umbilical vein endothelial cells (HUVEC). Used together, these tools provide an ideal model for vascular biology research.

EndoGRO Media:

- Lower serum concentrations than standard endothelial cell culture media
- Dramatically improves proliferation rates while maintaining excellent cell morphology
- No phenol red or antimicrobials, which can cause cell stress and masking effects
- Extensive QC and exacting standards to ensure lot-to-lot consistency
- Special UV packaging helps prevent light damage
- Built-in temperature gauges assist in contamination-free media warming

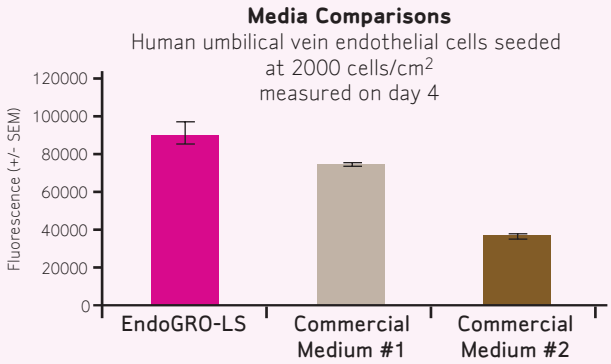
EndoGRO HUVEC:

- Provided at passage one for highest viability
- Extensive culture life – greater than 15 population doublings
- Extensive QC testing for sterility, viability, and morphology
- Ideal model system when used with EndoGRO media



EndoGRO HUVEC, P2, 6 days after innoculation at 100x.

Media Type	Description
EndoGRO-LS	Low serum culture media for human endothelial cells, HUVEC, aortic endothelial cells, and other large vessel endothelial cells
EndoGRO-VEGF	Low serum, VEGF-supplemented media for the rapid proliferation of human endothelial cells, HUVEC, aortic endothelial cells, and other large vessel endothelial cells
EndoGRO-MV	Low serum culture media for human microvascular endothelial cells
EndoGRO-MV-VEGF	Low serum, VEGF-supplemented media for the rapid proliferation of human microvascular endothelial cells



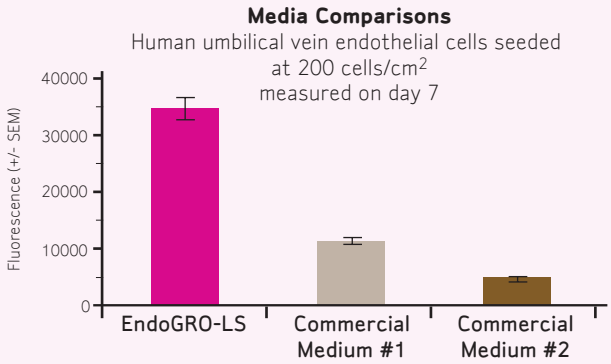


Figure 1. A comparison of proliferation rates of HUVEC cultured in EndoGRO media and other commercially available media at two different seeding densities. EndoGRO media shows superior proliferation at both seeding densities.

Description	Qty/Pk	Catalogue No.
EndoGRO-LS Complete Media Kit*	500 mL	SCME001
EndoGRO-VEGF Complete Media Kit	500 mL	SCME002
EndoGRO-MV-VEGF Complete Media Kit	500 mL	SCME003
EndoGRO-MV Complete Media Kit*	500 mL	SCME004
EndoGRO HUVEC	5 x 10^5 cells	SCCE001

*Not currently available in Europe.

Check out our new
Stem Cell Microsite by visiting
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