Culturing embryonic stem cells using media filtered with Nalgene™ Rapid-Flow PES filter units.

Key words: Rapid-Flow™, PES filters, Mouse embryonic stem cells, Induced pluripotent stem cells, Leukemia inhibitory factor, Pluripotency

Abstract

Eliminating contamination is important in cell culture, and sterile filtering of media is a standard practice to reduce the possibility of introducing contaminants to the culture system. However, due to the fastidious nature of embryonic stem cells (ESC) and induced pluripotent cells (iPSC), this common precautionary measure is often curtailed for fear of removing critical media components or adding deleterious compounds during the filtration process. Therefore, many stem cell researchers forego filtering certain media components such as growth factors, leading to higher risk of contamination. The purpose of this study is to show that Thermo Fisher Scientific™ Nalgene™ Rapid-Flow™ PES filters do not remove critical media components or add harmful elements, and that ESC grown in media filtered through Rapid-Flow PES filters maintain normal growth and pluripotency.

Introduction

Maintaining pluripotent cells in culture represents a unique challenge for stem cell researchers. The culture system must be closely controlled since any changes may easily trigger spontaneous differentiation or cell death. Growth factors, such as the leukemia inhibitory factor (LIF) normally expressed in the trophoectoderm of the developing embryo, are often added to the culture media to promote long-term maintenance and prevent unwanted differentiation of pluripotent stem cells. The cost of these media supplements is often high, but they are critical in maintaining the pluripotency of cells. Therefore, it is important to ensure that the growth factors are maintained in the growth media at the proper concentrations.

Growth media for cell culture is often sterile filtered in an effort to minimize the risk of contamination. Polyethersulfone (PES) membrane filters are used for this purpose due to the low protein binding and low extractable properties of the material. Filtering media for highly sensitive stem cells, however, introduces the possibility of removing important growth factors or adding compounds from the filter that may adversely affect the culture. To avoid this, many researchers add the most critical components (e.g. LIF) to the media after filtering. While this preserves the integrity of these components, it can be a vehicle for contamination.

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Here we show that filtering stem cell growth media using Thermo Fisher Scientific™ Nalgene™ Rapid-Flow PES filter units does not remove substantial amounts of LIF or other critical components, nor does it add compounds that impair the growth of cells. In order to highlight the utility of Nalgene™ Rapid-Flow filter units in sensitive media systems, both serum-free media (2i plus LIF) and ultra-lean (Thermo Fisher Scientific™ Gibco™ Essential 8™) systems were used for stem cell culture growth. Filtering the complete stem cell growth media using Nalgene™ Rapid-Flow PES filter units does not adversely affect stem cells, allowing them to maintain their normal growth and pluripotency characteristics.

Experimental details

Mouse LIF ELISA

All reagents were prepared according to the instructions for use of the Mouse LIF ELISA Quantikine assay kit (R&D Systems). 1500 mL of ESC complete media containing LIF was prepared and divided into 500 mL aliquots. Each aliquot was filtered through ten 500 mL Rapid-Flow units of the same lot, and a sample of media was removed for testing after each filtration. Three different lots of the Rapid-Flow filters were subjected to the evaluation. New filter units were used for each filtration. Media samples were diluted 1:4 with PBS and tested according to LIF ELISA kit instructions. Plates were read on the Thermo Fisher Scientific™ Varioskan at 450 nm. A reference reading at 570 nm was subtracted from the test absorbance to eliminate background absorbance. The corrected absorbencies of the calibrator series were used to create a calibration curve with an R^2 value of 0.99. Linear regression analysis was then used to calculate the LIF concentration of the unknown samples.

Mouse Embryo Assay (MEA)

Mouse embryo assay testing was conducted using a third-party Quality Control testing laboratory. Briefly, three 50 mL batches of embryo culture media were filtered through Rapid-Flow filters from three different lots. Four 0.7 µL droplets of filtered media were placed in the wells of a Thermo Fisher Scientific™ Nunc IVF multidish, and 21 embryos were cultured in the media for each filter lot. 15 embryos were cultured separately in unfiltered media as controls. Embryos were monitored for 96 hours to determine their progression to blastocyst stage. Embryos were scored after this time, and a result greater than 70% of embryos progressing to blastocyst stage was considered a passed test.

Stem Cell Culture

Mouse embryonic stem cells (mESC) were obtained from an established culture of 129/SVEV mESC (Millipore). One batch of 2i plus LIF growth medium[1,2] was prepared using Gibco™ media components and divided in half, with one half filtered once using a Rapid-Flow filter unit and the other half kept as a control. mESC were seeded on Nunc 6-well Multidishes, coated with Gibco™ Attachment Factor (AF) for at least 5 minutes prior to seeding, using separate wells for filtered media and control. Cultures were maintained on 6-well dishes coated with AF through five passages. Cells were incubated for two to three days before passaging and media was changed on each day between passages. All feeding and passaging was performed with the proper medium batch corresponding to the test condition. On the fifth passage, cells were seeded into an additional Nunc 12-well plate for flow cytometry analysis, and a 48-well plate for immunostaining of pluripotency markers. On day 2 of passage 5, mESC were harvested for flow cytometry analysis. 3 wells were harvested, immunostained for SSEA-1 (a cell surface pluripotency marker), and analyzed separetly using a Thermo Fisher Scientific™ Attune NxT. On day 3 of passage 5, bright field photographs were taken, cells were harvested and counted to determine proliferation, and unharvested cells were fixed for immunocytochemistry.

Cells harvested in the 5th passage were then subjected to an embryoid body differentiation protocol[3]. Harvested cells were cultured in differentiation media in hanging-drop format, 600 cells per drop. After two days, embryoid bodies were washed with fresh differentiation media into an untreated dish and cultured in suspension for an additional 3 days. Individual embryoid bodies were then transferred into Nunc 48-well cell culture plates with fresh differentiation media for an additional 5 days (10 days total differentiation). On day 10, embryoid bodies were fixed and prepared for immunostaining.

Human Gibco™ Episomal iPSC were obtained from an established culture of iPSC. One batch of Gibco™ Essential 8™ media was prepared and divided in half, with one half

filtered once using a Rapid-Flow filter unit and the other half kept as control media. Nunc 6-well multidishes were coated for one hour with Gibco™ recombinant human vitronectin. Cells were seeded using separate wells for filtered media and control. Cultures were maintained for 5 passages, passaging every three to four days with daily media changes between passages. On the 5th passage, cells were seeded into a Nunc 48-well multidish for immunostaining as well as a Nunc 6-well multidish to determine proliferation.

Immunocytochemistry

Immunocytochemistry was performed in Nunc 48-well plates. For mESC, anti-Oct-4 and anti-SOX-2 (Thermo Fisher Scientific™ Molecular Probes™) were used as primaries. For mouse embryoid bodies, anti-mouse AFP (R&D Systems), anti-SMA and anti-TUJ1 (Molecular Probes™) were used as primaries. For human iPSC, anti-Oct-4, anti-SOX-2, anti-SSEA-4, and anti-TRA-1-160 were used as primaries (Molecular Probes™). Cells were fixed, permeablized, and blocked prior to adding primaries. Primary antibodies were diluted appropriately in blocking solution and were added for an overnight incubation at 2-8°C. The cells were rinsed with Dulbecco's phosphate-buffered saline (DPBS) three times and the appropriate secondary antibodies diluted in blocking solution were added and incubated for one hour at room temperature. Cells were rinsed three times with DPBS and NucBlue was incubated for 5 minutes at room temperature. Cells were rinsed two additional times with DPBS. The cells were stored in the second DPBS wash in the dark at 2-8°C until image analysis was completed the appropriate secondary antibodies diluted in blocking solution were added and incubated for one hour at room temperature. Cells were rinsed three times with DPBS and NucBlue was incubated for 5 minutes at room temperature. Cells were rinsed two additional times with DPBS. The cells were stored in the second DPBS wash in the dark at 2-8°C until image analysis was completed.

Results and discussion

Nalgene™ Rapid-Flow PES filters does not diminish LIF content in the mESC growth media

The amount of LIF in mESC complete media was measured following successive filtration. ELISA results indicate that one time filtration did not appreciably affect the LIF concentration (Figure 1). Even over the course of ten filtrations, no more than 5% of LIF in the growth media is lost to filtration (Figure 1).

Nalgene™ Rapid-Flow PES filters does not add deleterious compounds to the filtered media

The highly sensitive Mouse Embryo Assay was conducted to assess potential harmful additions to the culture media from the filtration process. For two out of three tested filter lots, 95% of mouse embryos tested grew to blastocyst stage by 96 hours. For the remaining filter lot, 100% of the embryos progressed to blastocyst stage after 96 hours. These results indicate that filtering of media using Nalgene™ Rapid Flow PES filters does not add any deleterious compounds to the media that will affect cell growth.

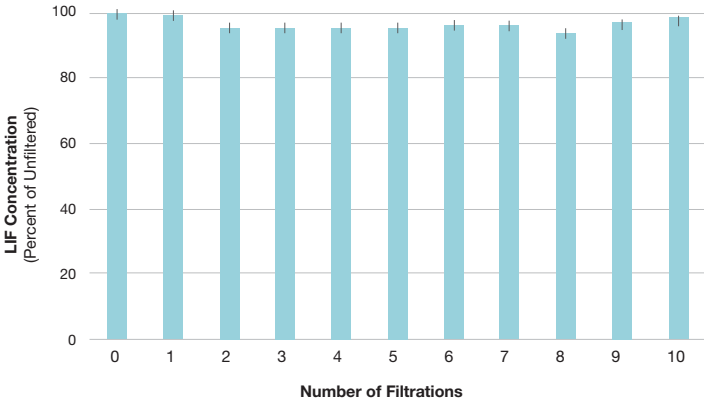

Figure 1. The retention of LIF in the complete mESC growth media following multiple filtrations using Nalgene™ Rapid-Flow PES filters.

Media filtered with Nalgene™ Rapid-Flow PES filters support Stem Cell growth and maintain their pluripotency

Stem cells (mESC and iPSC) were cultured through five passages using filtered medium. The mESC proliferated well throughout the span of the culture (Figure 2)

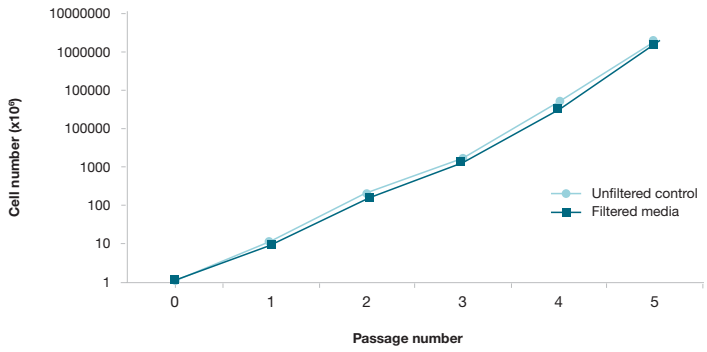

Figure 2. Proliferation of mESC during 5 passages in culture.

and displayed normal mESC morphology (Figure 3, brightfield). The mESC pluripotency was evaluated by immunofluorescence staining of Oct-4 and SOX-2 markers (Figure 3). In both brightfield and immunostained conditions, mESC cultured in filtered media yield comparable results as that of the control. Flow cytometry of SSEA-1 expressing cells shows no significant difference (Student's T-test, $p > 0.05$) between filtered media and unfiltered control (Figure 4). These results indicate that mESC pluripotency was maintained throughout the time in culture with filtered medium containing LIF.

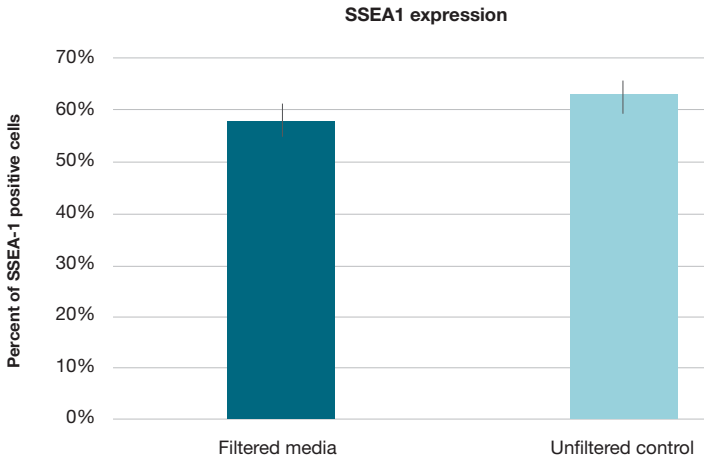

Figure 4. Flow cytometry results showing the percentage of cells expressing pluripotency marker SSEA-1 in filtered media compared to unfiltered controls.

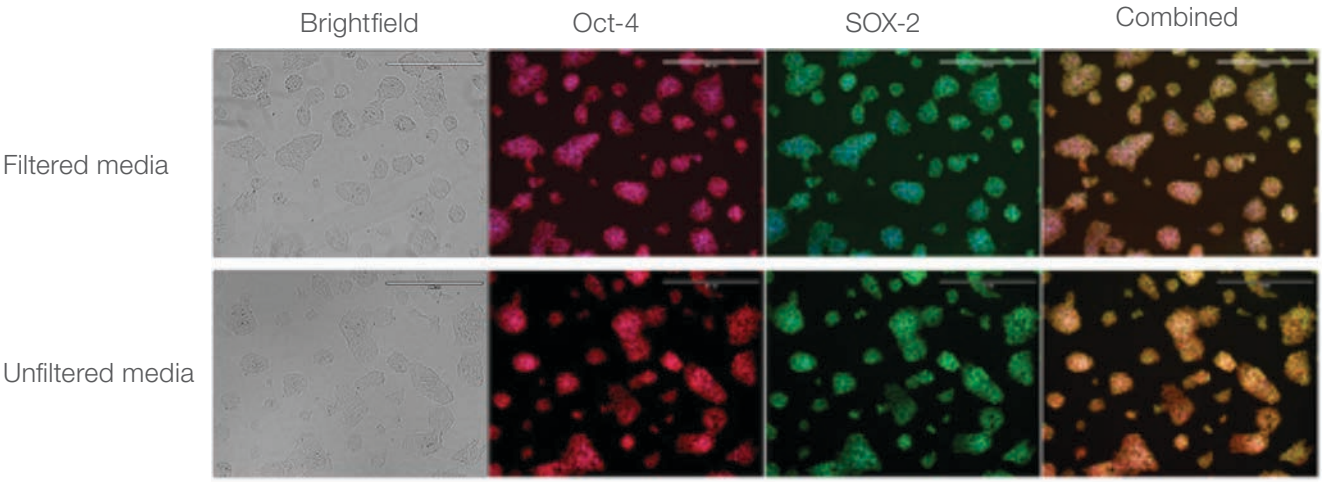

Figure 3. Immunofluorescence staining of pluripotent markers on mESC after being cultured for 5 passages in filtered media. Cells were counterstained with Thermo Fisher Scientific™ Invitrogen™ NucBlue™ nuclear stain.

Gibco™ iPSC culture also proliferated well throughout the culture, with a 14.7-fold increase in cell number during the 5th passage over seeding density in filtered media compared to a 7.4-fold increase in cell number in unfiltered controls. The iPSC pluripotency was evaluated by immunofluorescence staining of Oct-4, SOX-2, SSEA-4, and TRA-1-60 markers (Figure 5). In both brightfield and immunostained conditions, iPSC cultured in filtered media yield comparable results as that of the control. These results indicate that iPSC pluripotency was maintained throughout the time in culture with filtered medium.

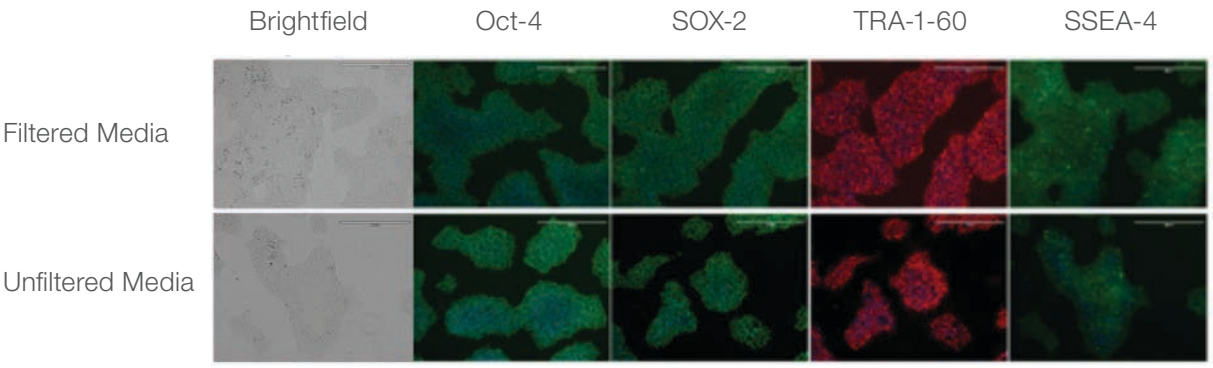

Figure 5. Immunofluorescence staining of pluripotent markers on human iPSC after being cultured for 5 passages in filtered media. Cells were counterstained with Invitrogen™ NucBlue™ nuclear stain.

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Mouse embryoid bodies were generated following the 5th passage in filtered media to demonstrate the cells' maintained capability to generate all three germ layers. Positive immunofluorescence staining of AFP-expressing cells (endodermal lineage), SMA-expressing cells (mesodermal lineage), and TUJ1 (ectodermal lineage), were confirmed in mouse EBs after fixing and staining (Figure 6).

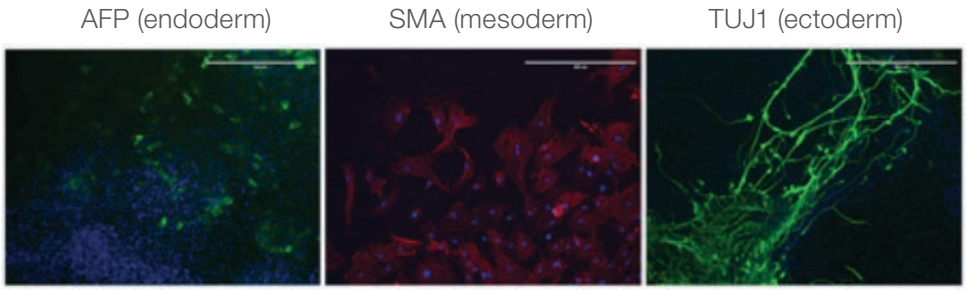

Figure 6. Immunofluorescence stains of mouse EBs showing all 3 germ layer lineages present in EBs generated from mESC maintained in filtered media for 5 passages.

Discussion

For the evaluation of LIF concentration, multiple media filtrations were conducted during this study in order to magnify any possible effect the filtration may have. For each successive filtration a new filter unit was used so that if any LIF that had been removed by filtration, the drop in concentration would be detectable during successive filtrations. Such stringent conditions are unlikely under normal application usage, however such a study ensures that any minimal effect that filtering may have becomes apparent during experimentation.

In experiments evaluating maintenance of stem cell cultures in filtered media, despite filtering what is already very lean media, stem cells continued to grow well and maintain pluripotency throughout the passages. Pluripotency was also confirmed through spontaneous differentiation of all three germ layers in embryoid bodies derived from mESC maintained on filtered media. These results indicate Nalgene™ Rapid-Flow™ PES filters are a safe solution for maintaining sterility in stem cell cultures.

Conclusion

- Nalgene™ Rapid-Flow filter units do not retain nor add components to the filtered media that impact the cell culture.
- Sterile filtration of complete ESC growth media using Nalgene™ Rapid-Flow filter units does not adversely affect growth or pluripotency of the stem cells.
- Nalgene™ Rapid-Flow PES filter units are a safe solution for maintaining sterility in stem cell cultures.

References

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