Large Scale Expansion and Differentiation of Human Mesenchymal Stromal Cells in the Thermo Scientific Nunc Cell Factory System

Application Note

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Key Words
Cell culture, cell culture scale up, Nunc Cell Factory system, hMSC, expansion and differentiation, mesenchymal stem cell basal medium, Nunclon Delta

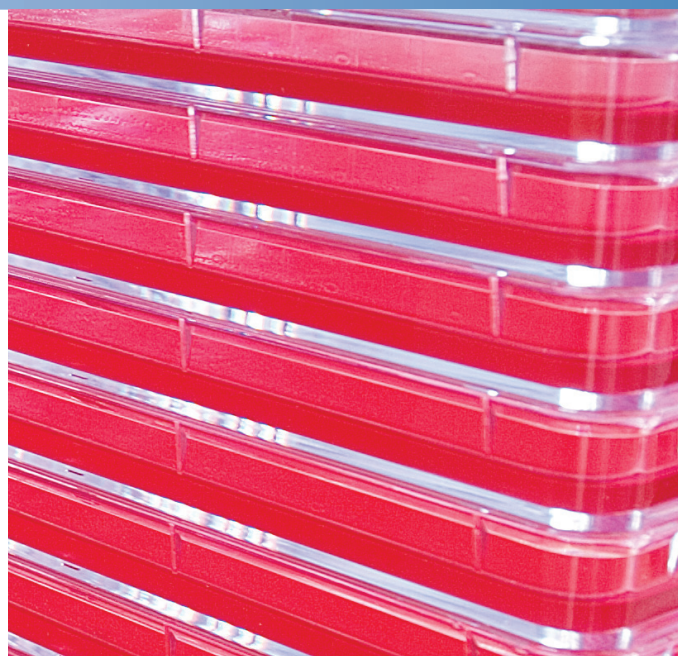
Abstract
Thermo Scientific™ Nunclon™ Delta treated Thermo Scientific™ Nunc™ Cell Factory™ systems is an effective format for the easy and rapid expansion of hMSCs. The Nunclon Delta surface is a fully synthetic chemical surface available with many options of sizes, shapes and surface area. hMSCs were removed from liquid nitrogen and plated directly onto the surfaces to be evaluated. T-25 cell culture flasks were used to determine the optimal seeding density prior to expansion of hMSC on larger formats. For expansion, 1-layer Nunc Cell Factory system, also with the Nunclon Delta surface, was seeded from a fresh cryovial. These cells were in turn used to seed a 4-layer Cell Factory systems. To ensure that the cells maintained multipotency after expansion, the expanded cells were seeded in Thermo Scientific Nunc Multidish 48-well plates and differentiated into osteoblasts or adipocytes. The seeding density chosen for expansion was 350 cells/cm² because it was the lowest seeding concentration displaying good exponential growth. The reported expansion protocol was able to increase the cell number by 136-fold, however, since Cell Factory systems are available with up to 40 layers, the expansion potential is virtually limitless. Expansion of hMSCs on the Nunclon Delta surface using mesenchymal stem cell basal medium significantly enhances the osteogenic and adipogenic potential of hMSCs and number of differentiated cells relative to α-MEM medium. Additionally, the use of for cell passage gave a slightly higher yield of adipocytes compared to cells passaged using trypsin. The methodology presented here can be applied to the large scale expansion of other cell types for use in various applications using Cell Factory systems.

Abbreviations
CF1: 1-layer Nunc Cell Factory system

CF4: 4-layer Nunc Cell Factory system

CF40: 40-layer Nunc Cell Factory system

MD48: Nunc Multidish 48-well plate



Introduction
Human mesenchymal stromal cells (hMSC) are good candidates for clinical or research use because they are readily expanded in culture, have immunomodulatory potential and can differentiate into the osteogenic, chondrogenic, and adipogenic lineages. The therapeutic potential of hMSC is currently being studied as part of clinical trials to treat diseases such as graft-versus-host disease[1], osteoarthritis[2], and for the regeneration of cardiac muscle following myocardial infarcts[3]. Whether the requirements are for clinical or research use, obtaining a substantial number of cells can constitute a bottleneck for the investigator. hMSC display some plasticity in their culture conditions, but several investigators report a higher growth index and increased differentiation potential at lower seeding densities[4,5]. We present a protocol enabling the clinician or researcher to rapidly expand a population of hMSCs on the Nunclon Delta

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Surface utilizing the potential of Nunc Cell Factory system and mesenchymal stem cell basal medium. The Nunclon Delta surface is a fully synthetic chemically-modified surface that makes the otherwise very hydrophobic polystyrene surface more hydrophilic, thus facilitating cell attachment and growth. The Nunclon Delta surface is a fully synthetic chemical surface available with many options of sizes, shapes and surface area. hMSC can be expanded on a single surface from a T-25 cell culture flask up to a CF40. The combination of Nunclon Delta treated surface, mesenchymal stem cell media, and our in-house protocol allows for easier expansion and differentiation of hMSCs.

Experimental details

Methods

Cultivation of hMSC

Human mesenchymal stromal cells, hMSCs (Lonza, USA) were cultured on the Nunclon Delta surface in either α-MEM medium containing 10% FBS, 1% Penicillin/Streptomycin, and 2 mM UltraGlutamine or mesenchymal stem cell basal medium with 10% stem cell growth supplement. Cells were cultivated at 37°C with a humidified atmosphere of 5% CO_2 in a Thermo Scientific Revco™ Ultima™ II Series CO_2 Incubator.

Growth curves

To determine the optimal seeding density for the larger formats, growth curves of hMSC grown in mesenchymal stem cell and α-MEM growth media were established in T-25 flasks. hMSCs in Passage 2 (P2) were plated onto the Nunclon Delta surface. Cultures were placed in an IncuCyte™ Plus (Essen Instruments, USA) in order to measure cell proliferation. The IncuCyte Plus is located inside the incubator and eliminates the need to remove the cells from the culture environment. Cell proliferation is measured kinetically and phase-contrast images are acquired relying on the imbedded contrast based confluence algorithm to determine monolayer confluence for each image at each time point.

Expansion protocol

$5x10^5$ hMSC in P2 was thawed onto two Nunclon Delta treated CF1s with a seeding density of 350 cells/ cm^2 with either mesenchymal stem cell or α-MEM growth medium. The were cultivated for 8 days and the medium was changed on days 3 and 7. The CF1s were harvested with either trypsin or a Trypsin/EDTA alternative cell dissociation agent and were expanded onto Nunclon Delta treated CF4s with the same culture conditions used for the CF1s. The remaining cells were banked cryogenically.

Differentiation protocol

After hMSCs were expanded onto Nunclon Delta treated CF4s, cells were harvested with either trypsin or trypsin/EDTA alternative dissociation agent. Harvested cells were plated on Nunc MD48 plates with a seeding density of 5000 cells/cm^2 in mesenchymal stem cell basal medium or α-MEM media. In order to induce hMSC differentiation into adipocytes or osteoblasts, the medium was changed to either adipogenic or osteogenic differentiation medium supplemented with a stem cell growth supplement. The medium was changed every 4-5 days and the cultures were assayed on Days 3, 7, and 18 with commercial kits. The OsteoImage™ assay, which utilizes the OsteoImage PA-1501 kit (Lonza, USA), measures specific staining of the fluorescent reagent to the hydroxyapatite portion of the bone-like nodules deposited by cells. For adipogenic differentiation, the AdipoRed™ PT-7009 kit (Lonza, USA) was used. The kit utilizes Nile Red to dye the intracellular lipids formed inside the differentiating adipocytes. The accumulation of intracellular triglycerides is often used as a marker of adipocyte differentiation. Cells were counted using ChemoMetec's NucleoCounter™, an automated fluorescent microscope. The manufacturer's instructions were followed.

Results and discussion

hMSC Growth Curves

The growth of hMSCs seeded on the Nunclon Delta surface in either α-MEM medium or mesenchymal stem cell basal medium with three seeding densities was evaluated in order to determine the optimal seeding density for the expansion protocol. hMSCs were cultured for 12 days at 37°C in a humidified atmosphere of 5% CO_2. hMSC morphology and growth was monitored in the incubator using an IncuCyte Plus imager. hMSCs grown in mesenchymal stem cell basal medium displayed normal morphology on the Nunclon Delta surface after seven days of incubation (Fig. 1B).

For the first 50 hours, at 1000 and 4000 cells/cm^2, the growth of hMSCs in α-MEM medium and mesenchymal stem cell basal medium displayed a similar growth pattern (Fig. 1A), but then the cultures diverged. The growth rate of hMSC in α-MEM medium declined and reached a plateau at approximately 80% culture confluence after approximately 150 hours for cultures seeded at 4000 and 1000 cells/cm^2. The cultures grown in mesenchymal stem cell basal medium continued to grow past 80% culture confluence and were able to reach 96-99% confluence.

At the lowest seeding density of 350 cells/cm^2, the cultures seeded in α-MEM medium reached a plateau at around 75% confluence after 180 hours and cells seeded in mesenchymal stem cell basal medium reached the same level after approximately 250 hours. The purpose of our protocol is to expand a relatively low number of cells; we thus chose a seeding density of 350 cells/cm^2, which displayed good exponential growth (Fig. 1A).

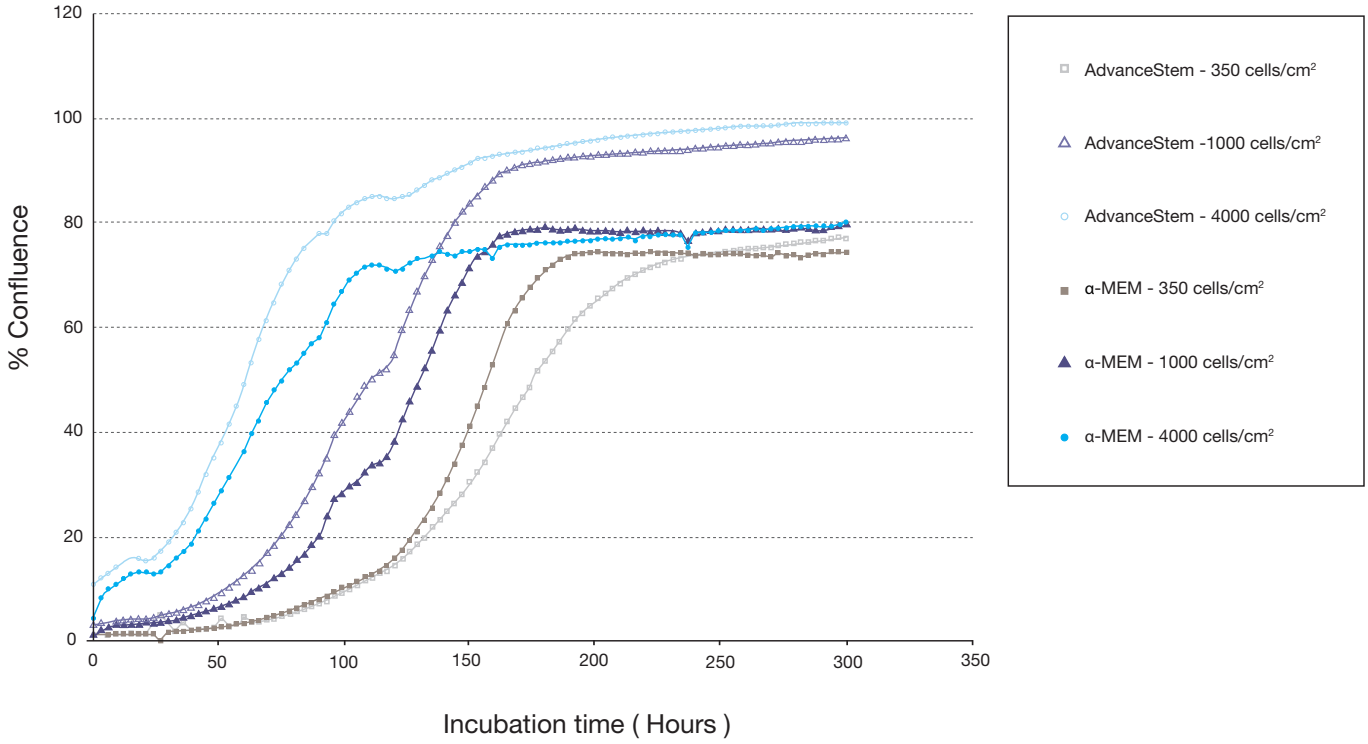


Figure 1A. Growth of hMSC in T-25 flasks with the Nunclon Delta surface in α-MEM medium or mesenchymal stem cell basal medium. Cells were seeded at three different densities: 350, 1000, or 4000 cells/cm². Cells were cultured for 12 days using standard culture conditions and the media was renewed every four days. Each data point represents the mean of 50 measurements in one flask.

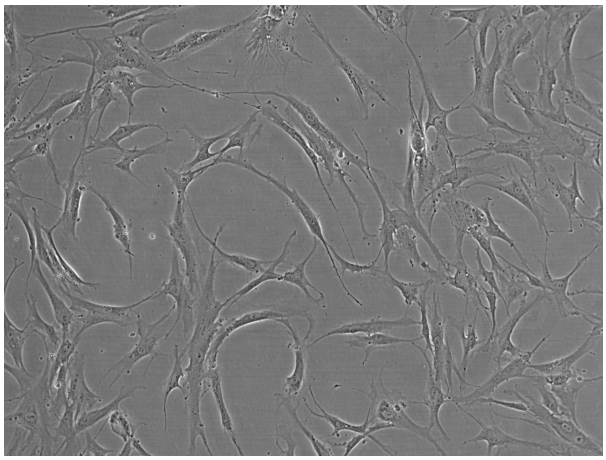
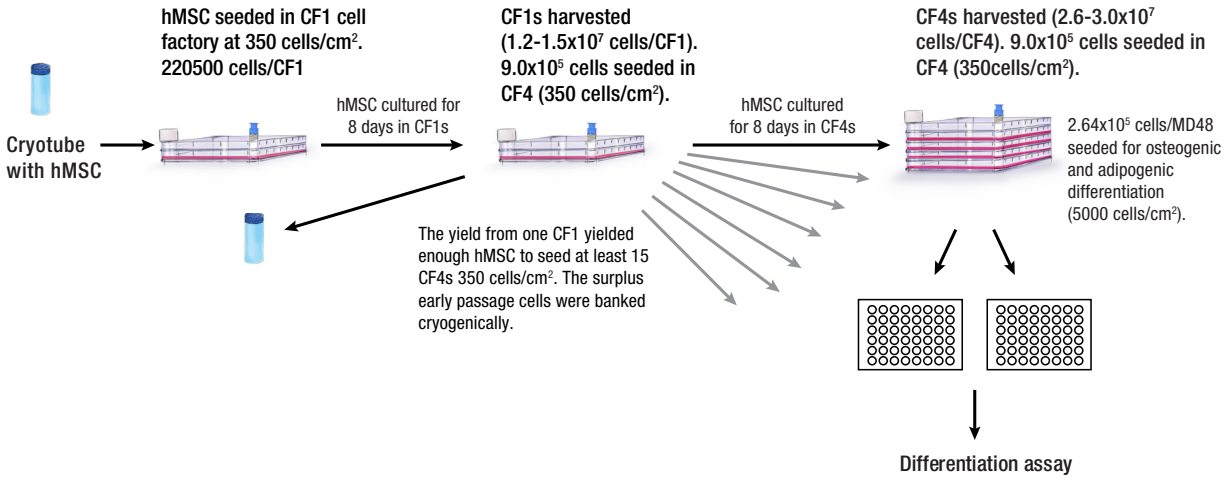
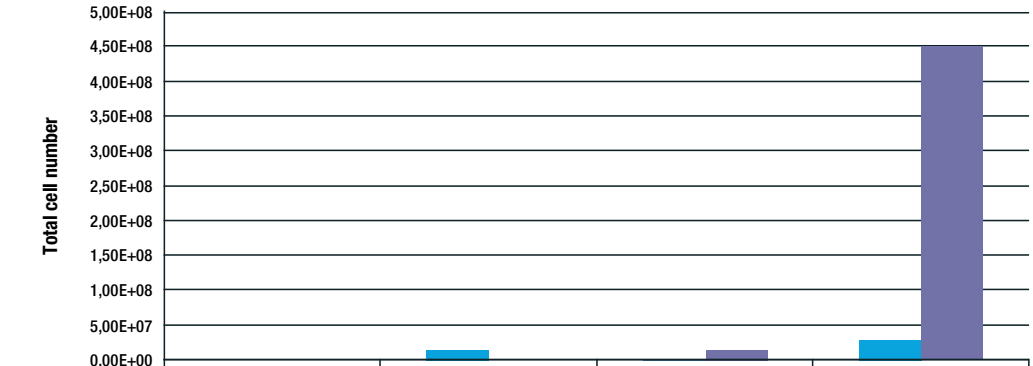


Figure 1B. Normal hMSC morphology in mesenchymal stem cell basal medium after 7 days of incubation on the Nunclon Delta surface. Seeding density: 350 cells/cm².

Expansion of hMSC on Nunclon Delta treated Cell Factory systems

hMSC cultured in α-MEM medium and mesenchymal stem cell basal medium on Nunclon Delta treated Cell Factory systems was effective in generating a large population of hMSCs for either differentiation or cryogenic storage. Cells were removed from liquid nitrogen and were plated on two CF1s with a seeding density of 350 cells/cm². The yield from one CF1 yielded enough hMSC to seed at least fifteen CF4s. Here, the cells were harvested from the CF1 with either trypsin or a Trypsin/EDTA alternative cell dissociation agent and were used to seed one CF4. The remaining cells were banked cryogenically. Figure 2 displays the actual number of cells seeded and the actual yield obtained together with the potential yield of 4.50 x 10^8 cells if all cells had been used for scale up.





	CF1 seeding population	CF1 harvest population	CF4 seeding population	CF4 harvest population
In our experiment	220500	1,50x10⁷	9,60x10⁵	3,00x10⁷
Potential			1,44x10⁷	4,50x10⁸

Table displaying the actual number of cells seeded and the actual yield together with the potential yield if all cells had been used.

Figure 2. Schematic of our in-house hMSC expansion protocol using Nunclon Delta treated Cell Factory systems

hMSCs maintain their multipotency after large scale expansion in Nunclon Delta treated Cell Factory systems

In order to verify that the cells had maintained their ability to differentiate after expansion in both types of growth medium on Nunclon Delta treated Cell Factory systems with both dissociation agents, the cells were differentiated into osteoblasts or adipocytes on Nunc MD48s with a seeding density of 5000 cells/cm². Differentiation was induced using either osteogenic or adipogenic differentiation media and was monitored on Days 3, 7, and 18 using commercial kits.

Marked differences between hMSCs expanded in mesenchymal stem cell basal medium and hMSCs expanded in α-MEM medium was observed during differentiation. hMSCs expanded in mesenchymal stem cell basal medium displayed a 59% higher signal using the OsteoImage assay compared to cells expanded in α-MEM medium (Fig. 3). Regarding adipogenic differentiation, hMSCs expanded in mesenchymal stem cell basal medium produced significantly more adipocytes than cells expanded in α-MEM medium. Additionally, cells passaged with a typsin/EDTA alternative dissociation agent displayed a slightly higher signal using the AdipoRed assay when compared to cells passaged with trypsin. This can be interpreted as an increased yield of hMSCs that successfully differentiated into adipocytes resulting from the use of a trypsin/EDTA alternative as the dissociation agent (Fig. 4).

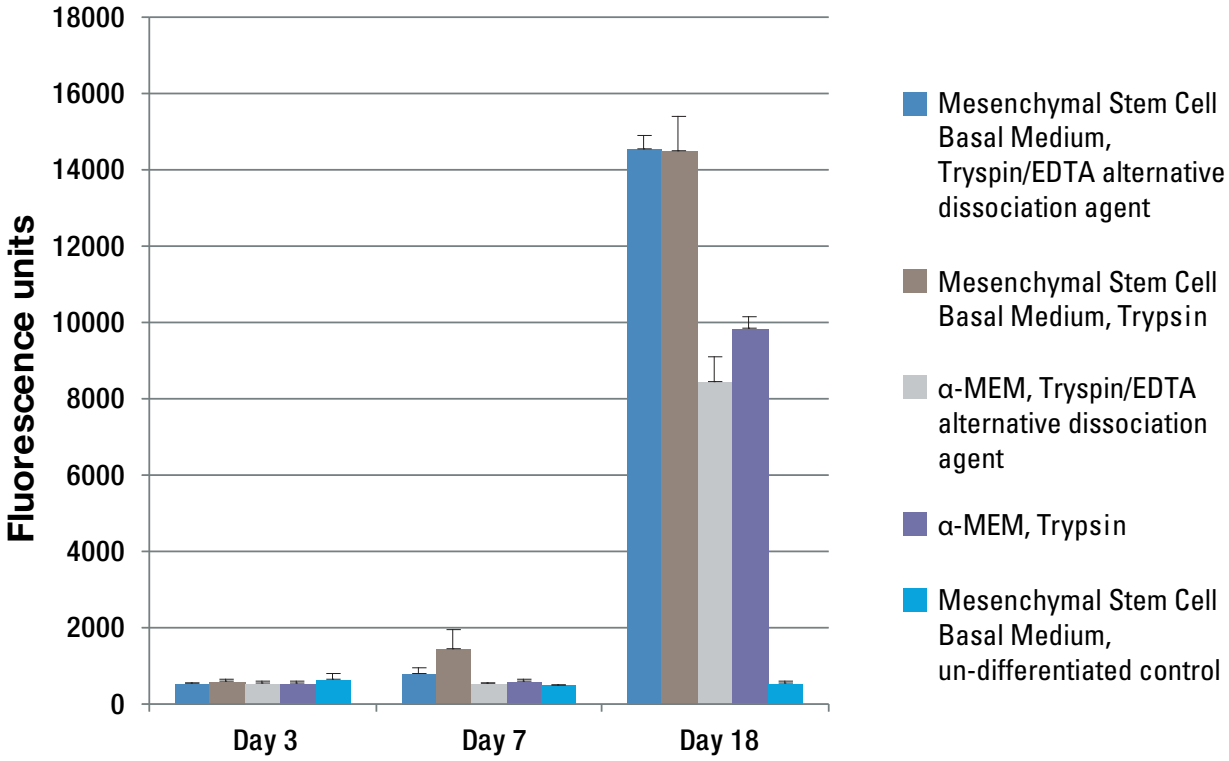


Figure 3. hMSCs differentiated to osteoblasts on Nunc MD48 plates with osteogenic differentiation media containing stem cell growth supplement were assayed for differentiation with the OsteoImage PA-1501 kit on days 3, 7, and 18. Data is displayed as relative fluorescence units.

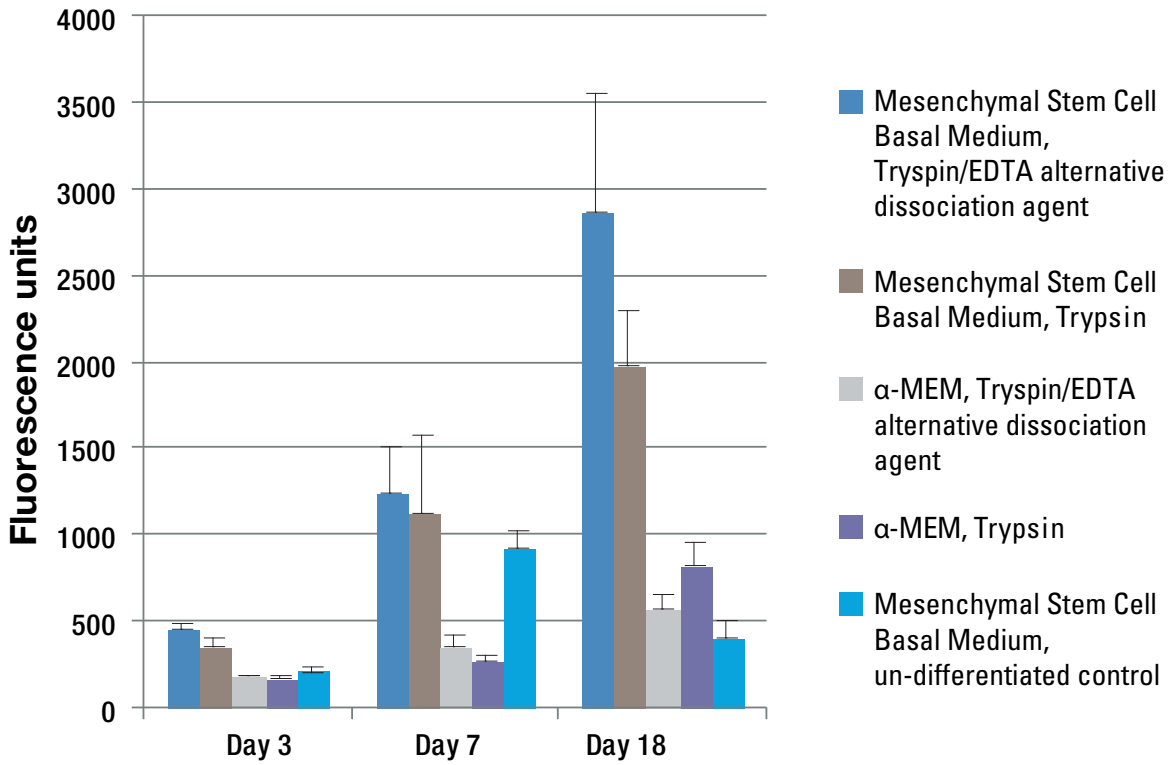


Figure 4. hMSCs differentiated to adipocytes on Nunc MD48 plates with adipogenic differentiation media containing a stem cell growth supplement were assayed for differentiation with the AdipoRed PT-7009 kit on Days 3, 7, and 18. Data is displayed as relative fluorescence units.

Conclusions

- Nunclon Delta treated Nunc Cell Factory systems are an effective format for the easy and rapid expansion of hMSCs.
- Expansion of hMSCs on the Nunclon Delta surface using mesenchymal stem cell basal medium significantly enhances the osteogenic and adipogenic potential of hMSCs and number of differentiated cells relative to α-MEM medium.
- Harvesting cells with a trypsin/EDTA alternative dissociation agent increases the yield of adipocytes compared to trypsin.
- The methodology presented here can be applied to the large scale expansion of other cell types for the use in various applications using Nunc Cell Factory systems.

References

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