Application Note

The Nunclon Sphera surface supports formation of three dimensional cancer spheroids in suspension

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Key Words
Nunclon Sphera, cancer spheroid, low cell binding, 3D cell culture

Abstract
While monolayer cell culture has been used extensively to study many types of cancers *in vitro*, 3 dimensional (3D) cultures of cancer spheroids better simulate the in *vivo* environment and, therefore lead to a better understanding of tumor biology. Here we introduce a new cell culture surface, Thermo Scientific™ Nunclon™ Sphera, that supports the *in vitro* formation of cancer spheroids consistently. The Nunclon Sphera surface coating inhibits cell attachment to the culture dish by blocking the adsorption of extracellular matrix (ECM) proteins (e.g. Collagen I and Fibronectin) that usually mediate cell adhesion. This was further supported by results in low cell attachment of adherent cell lines (e.g. Vero, A549, and U937) to the Nunclon Sphera surface. The functionality of the surface was demonstrated by the formation of cancer spheroids of several commonly used cancer cell lines including HeLa, MCF-7, HepG2, Panc-1, Saos-2, and A549 on the Nunclon Sphera dishes. The integrity and consistency of the Nunclon Sphera surface was showcased by spheroid culture of a cancer stem cell line, P19.CL6. When seeded at relatively low cell seeding densities, P19.CL6 spheroids were able to grow in volume over time. The performance of Nunclon Sphera in promoting cancer spheroid formation was comparable to that of a similar product by a different manufacturer, whereas use of a non-treated Petri dish resulted in significant cell attachment and failed to support cancer spheroid formation.

Introduction
The study of cell biology *in vitro* via monolayer cell culture systems is not always an accurate representation of the complex environment these cells can experience *in vivo*. Significant interaction that occurs between cells and with the extracellular matrix (ECM) is often not reflected in these simplified culture systems. Three dimensional (3D) cell culture systems better mimic complex interactions and are extremely useful in broad applications of cell biology. In human cancer biology, a 3D culture system can be used to form spheroid cell aggregates that simulate the 3D structure of tumor growth for the purpose of studying tumor cell progression and sensitivity to anticancer agents. However, variability in forming cancer spheroids has been a persistent problem that has been linked to changes in medium composition, volume, cell density, duration in culture, and most importantly the cellular interactions with the culture dish itself. More consistent results can be achieved using a high quality

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culture surface with very low binding characteristics. In this application note, we introduce a hydrophilic polymer coated surface, Thermo Scientific Nunclon Sphera, which minimizes surface variability and supports the formation of consistent cancer spheroids *in vitro*.

Materials and Methods

ECM Protein Non-specific Binding

Nunclon Sphera 96-well plates and Nunclon Delta (standard cell culture-treated) 96-well plates were coated with 100 µL/well of either 24 µg/mL FITC labeled Bovine Collagen Type I or 20 µg/mL of TAMRA labeled Fibronectin in DPBS. The plates were incubated for 24 hours at 2-8°C or 16 hours at room temperature, respectively. The solution was aspirated and plates were washed 3 times with 200 µL/well PBST (0.05% Tween 20 in PBS). The fluorescence intensity was read at Ex495/Em525 (Collagen) or Ex543/Em570 (Fibronectin) on a POWERSCAN MX (DS pharma).

Cell Adhesion

U937 cells underwent a differentiation step prior to adhesion studies. Briefly, U937 cells were cultured for 1 day in media containing phorbol 12-myristate 13-acetate (PMA) at concentration 10 ng/mL. Cells were then cultured for 2 more days in fresh media without PMA.

A similar protocol was used for cell adhesion assays for the differentiated U937 cell line and 2 other cell lines (VERO and A549). Briefly, cells were seeded onto 6-well multidishes at $1.5x10^5$ cells/cm^2 (U937) or $4x10^4$ cells/cm^2 (VERO and A549). After a 30 minute (U937) or 24 hour (VERO, A549) incubation at 37°C and 5% CO_2, culture dishes were washed and residual cells lysed with 2% Triton X-100 in phosphate buffered saline (DPBS). Dishes were incubated for another 30 minutes at 37°C before 100 µL of lysate was transferred to each well of a 96-well plate. An assay was performed using a commercial kit (Roche, 11644793001) to obtain the fluorescent intensity of the samples at 490 nm wavelength. Sample readings were compared to standards made with known quantity of cells to determine the amount of cell adhesion in the culture dishes. Results were normalized to the standard cell culture treated culture dishes.

Cancer Cell Line Cultivation

Cancer cell lines (HeLa, HepG2, A549, MCF-7, P19.CL6, Panc-1, and Saos-2) were maintained on the Nunclon Delta surface in the appropriate HyClone media containing 10-15% FBS (HyClone, SH3007103) at 37°C and 5% CO_2 before they were subjected to cancer spheroid formation in various low cell binding dishes.

Spheroid Formation

Cell lines HeLa, HepG2, A549, and MCF-7 were seeded into Nunclon Sphera and Brand C 6-well multidishes at a density of $2x10^4$ cells/well (HeLa, HepG2, A549) or $4x10^4$ cells/well (MCF-7) in the appropriate HyClone media containing 10% FBS (Hyclone, SH3007103). Untreated and cell culture-treated dishes were also seeded as controls. Cells were incubated at 37°C and 5% CO_2, and fresh media was added after 2-3 days. After 7 days cells were imaged, the size of spheres was measured, and the number of spheres and total cell count per well were determined.

To monitor the effect of the Nunclon Sphera coating on cancer spheroid growth, the P19.CL6 cancer stem cells were plated at density of 100, 500, 1,000, 2,500 and 5,000 cells/well in Nunclon Sphera 96 well U bottom plates. Cells were incubated for 5 days and spheroid size was measured by microscopic examination on days 1, 3, and 5.

Results and Discussion

The adsorption of ECM to the Nunclon Sphera surface is extremely low

Common ECM proteins are known to mediate cell attachment to culture surfaces. In order for adherent cells to form spheroids in suspension, the culture vessel must encourage the aggregation of cells through cell-cell binding by preventing ECM binding to the plastic surface. In this study, both Collagen I and Fibronectin binding was minimal on the Nunclon Sphera surface. This is demonstrated through the extremely low fluorescence intensity following overnight incubation of solution with the ECM proteins (Figure 1). This suggests that unlike the standard cell culture-treated surface, the Nunclon Sphera surface has minimal binding interactions with the ECM, consequently discouraging the cells from attaching to the surface.

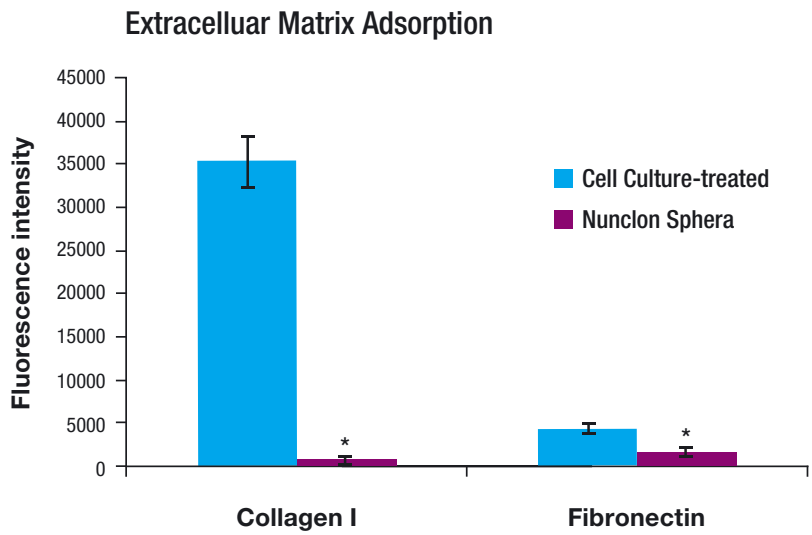


Figure 1. The adsorption of Collagen I and Fibronectin to the Nunclon Sphera surface is extremely low compared to the standard cell culture-treated surface (*, Student's T test, $p<0.01$).

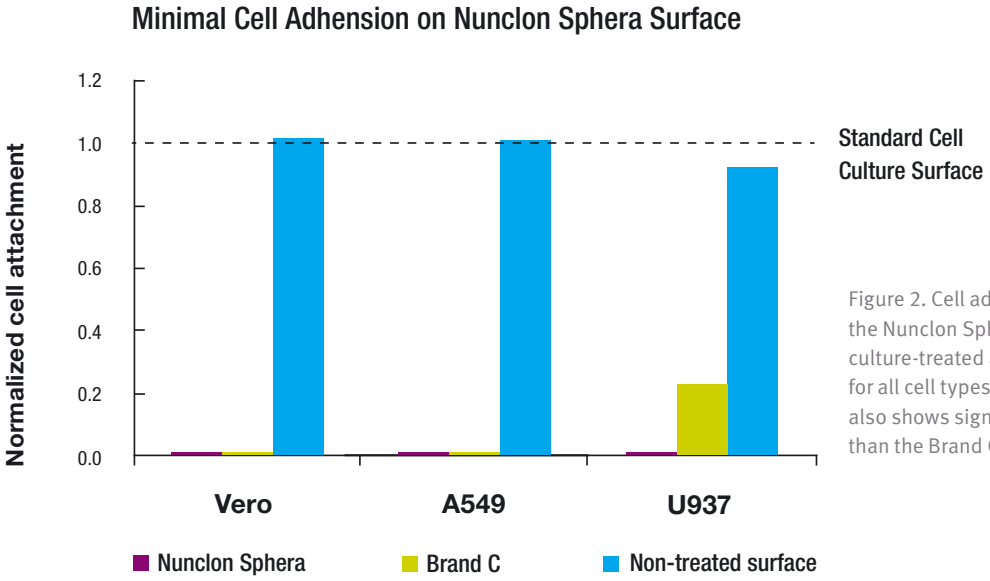


Figure 2. Cell adhesion is much lower in the Nunclon Sphera surface than in cell culture-treated and non-treated controls for all cell types tested. Nunclon Sphera also shows significantly lower adhesion than the Brand C surface with U937 cells.

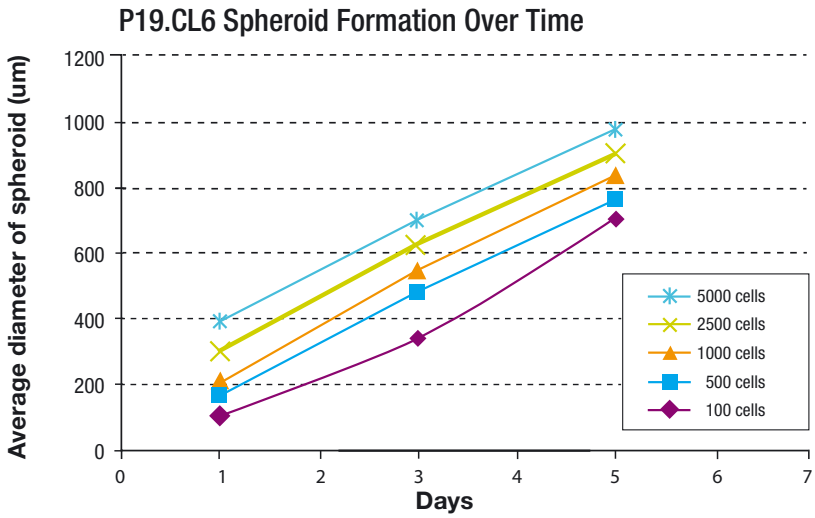


Figure 3. Cancer stem cell spheroids grow in size over time (average of 4 separate measurements) when cultured on the Nunclon Sphera surface at a rate associated with the initial seeding density.

Surface adhesion of cells is extremely low on Nunclon Sphera

In order to verify that adherent cells show little attachment to the Nunclon Sphera surface, the quantity of adhered cells was assayed in cell culture dishes with either the Nunclon Sphera surface, a low cell binding surface by a different manufacturer (Brand C), or the non-treated polystyrene surface commonly used for non-adherent cell culture. A standard cell culture-treated surface was used as a positive control for cell adhesion. The Nunclon Sphera surface demonstrated virtually no cell adhesion for all cell lines tested whereas the Brand C attracted some differentiated U937 cells to the surface (Figure 2). The non-treated polystyrene surface showed significant cell adhesion, comparable to the cell culture-treated surface, presumably due to the presence of the serum and abundant ECM proteins in the culture system (Figure 2). While the non-treated polystyrene surface is unsuitable for spheroid culture, the Nunclon Sphera surface with the low adhesion properties demonstrates its feasibility in applications where preventing cells from binding to the culture dish are desired.

The Nunclon Sphera surface has no deleterious effects on cell growth

While cells do not adhere to the Nunclon Sphera, it is also critical to verify that the surface coating does not hamper the normal growth of the culture. To demonstrate cell growth, P19.CL6 mouse cancer stem cells were plated at 5 different densities on the Nunclon Sphera surface and spheroids were able to grow in size for 5 days. There was an approximate 6.5 fold increase in spheroid size at the lowest seeding density while there was only a 2.5 fold increase at the highest seeding density (Figure 3). These results suggest that the seeding density affects the growth rate of spheroids. Additionally, the spheroids of all initial seeding densities were able to grow in volume, indicating that the Nunclon Sphera polymer coating has no adverse effect on cell survival and proliferation.

The Nunclon Sphera surface supports spheroid formation and growth of multiple cancer cell lines

The key element for any low cell binding surface is its performance in forming and growing 3D spheroids in culture. To test this, several cancer cell lines were grown in dishes of various low cell binding surfaces, including the Nunclon Sphera surface, the Brand C surface by a different manufacturer, and the non-treated Petri dish. The standard cell culture-treated Nunclon Delta dish was used as a negative control for spheroid formation. The formation and growth of spheroids were assessed after a week of incubation. Microscopic examination demonstrated that the Nunclon Sphera surface consistently supported spheroid formation of all cancer cells, and the spheroids maintained healthy and normal morphology in culture (Figure 4). Brand C performed comparably to the Nunclon Sphera surface with the spheroids slightly less uniformed in size (Figure 4). Interestingly, the non-treated Petri dish, which is made of hydrophobic polystyrene and is routinely used for suspension cell culture, failed to support cancer spheroid growth resulting in significant cell attachment of all cancer cell lines tested (Figure 4). In addition to the HeLa, MCF-7, and HepG2 cells shown, similar results were obtained with Panc-1, Saos-2, and A549 cells (data not shown).

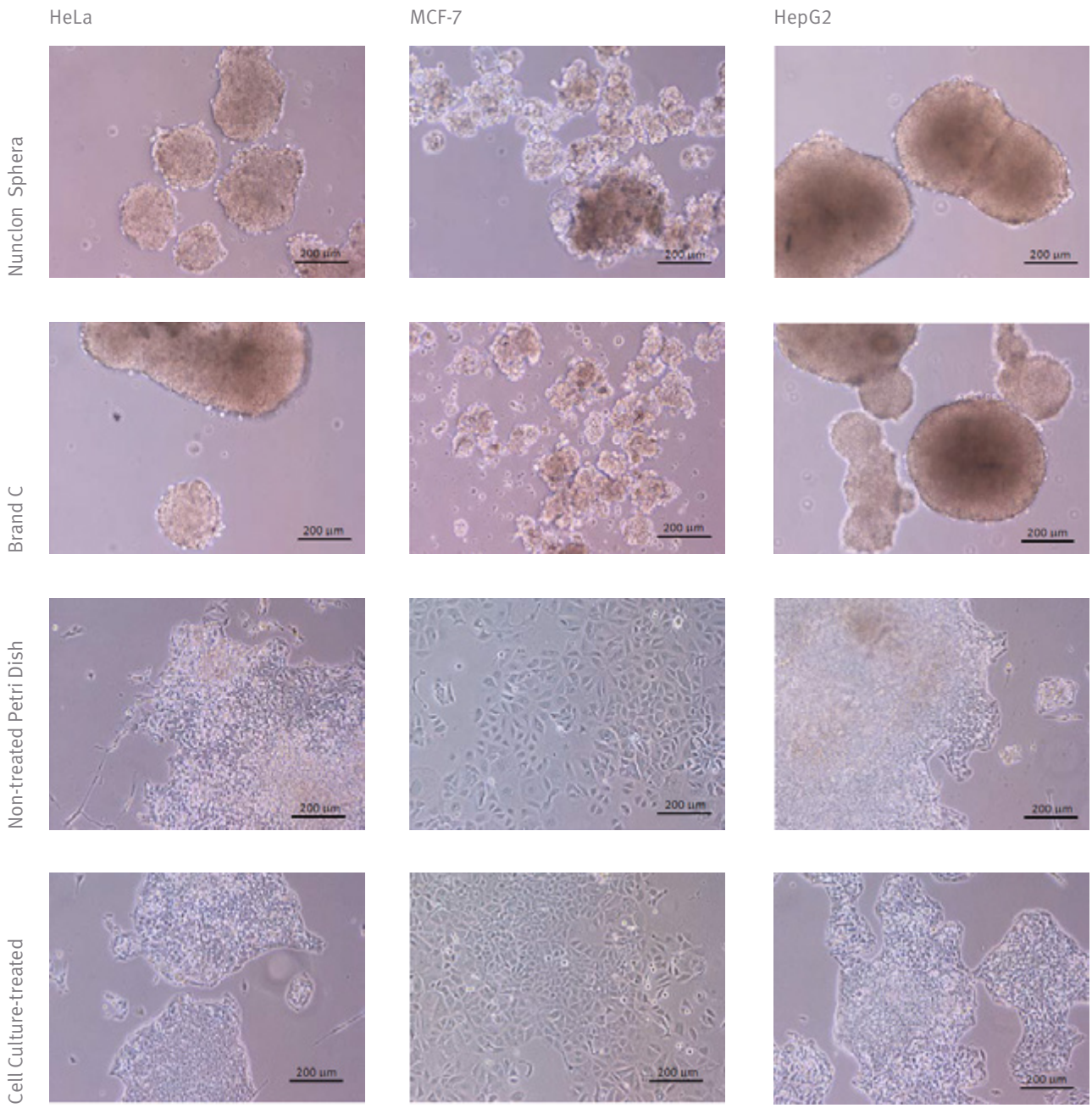
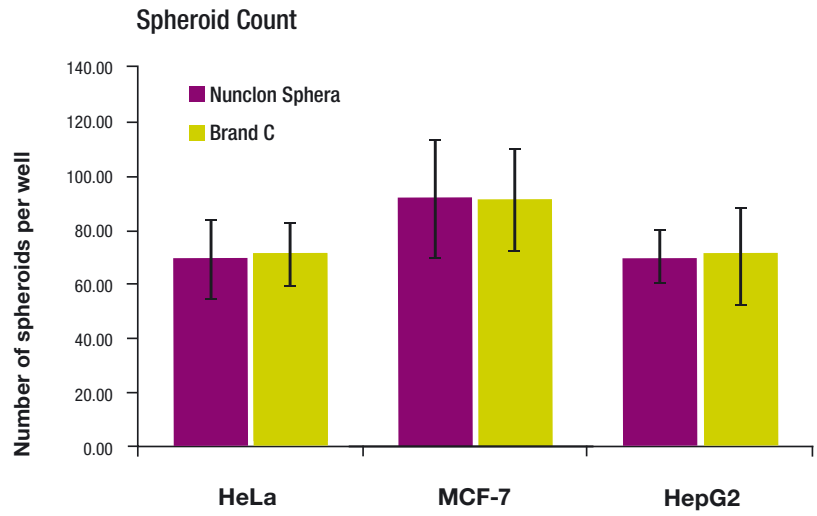
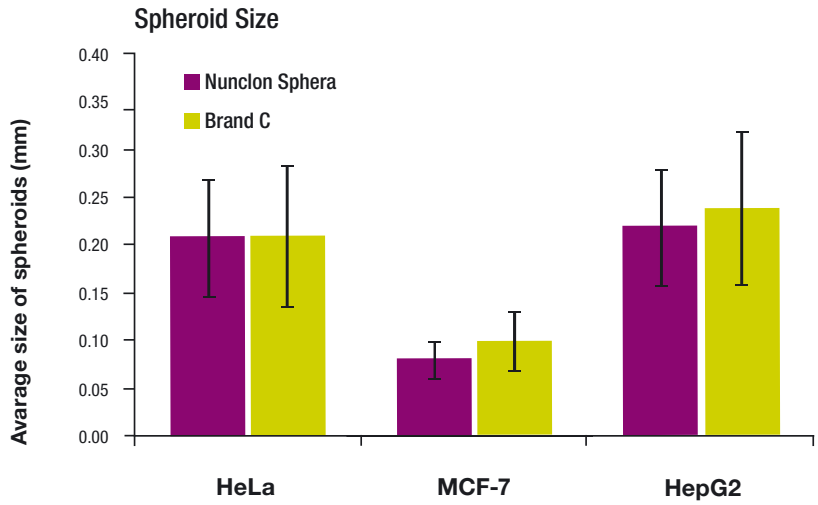


Figure 4. Nunclon Sphera effectively supports the formation of sizable cancer spheroids in culture. HeLa, MCF-7, and HepG2 cells were grown in Nunclon Sphera, Brand C, non-treated Petri, and standard cell culture-treated dishes.

To quantify the performance of the surfaces, assessments were made for the number and size of the cancer spheroids, as well as the total number of cells in the well after a week of incubation. The Nunclon Sphera surface achieved similar results as the Brand C surface in all categories, with no significant differences in the number of spheroids, the size of the spheroids, or the total number of cells (Figure 5).





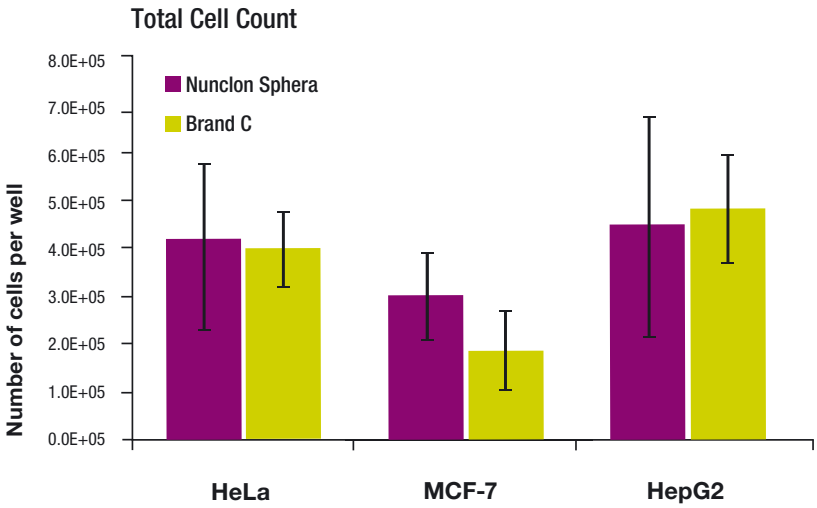


Figure 5. Nunclon Sphera performs as well as the Brand C in the number of spheroids formed, the size of spheroids grown, and the total number of cells grown per well.

Conclusions

The Nunclon Sphera surface consistently demonstrates minimal ECM protein binding, extremely low cell attachment, and supports good formation and proliferation of spheroids across several commonly used cancer cell lines. These results indicate that the Nunclon Sphera surface is an excellent choice for 3D cancer spheroid culture.

- Minimal adsorption of ECM proteins and extremely low cell adherence highlight the low binding properties of the Nunclon Sphera surface.
- The Nunclon Sphera polymer coating has no adverse effects on cell survival and proliferation as demonstrated by cancer spheroids with low seeding density grown both in number and size over time.
- The Nunclon Sphera surface effectively supports the formation of spheroids by many different cancer cell lines, providing a consistent culture system for spheroid growth and aiding in 3D cancer cell modeling *in vitro*.

Ordering Information

Thermo Scientific Cat. No.	Description	Units per bag	Units per case
174925	Microwell 96U-Well Plate, Round Bottom, Well Volume 300 µL	1	8
174927	Microwell 96F-Well Plate, Flat Bottom, Well Volume 400 µL	1	8
174929	Microwell 96U-Well Plate, Round Bottom Bulk Pack	1	8
174930	Multidish 24-Well, Culture Area 1.9 cm^2	1	7
174931	Multidish 12-Well, Culture Area 3.8 cm^2	1	7
174932	Multidish 6-Well, Culture Area 9.6 cm^2	1	7
174943	Dish 35 MM, Culture Area 8.8 cm^2	5	20
174944	Dish 60 MM, Culture Area 21.5 cm^2	5	20
174945	Dish 90 MM, Culture Area 56.7 cm^2	5	20
174951	T25 Cell Culture Flask, Culture Area 25 cm^2	6	18
174952	T75 Cell Culture Flask, Culture Area 75 cm^2	4	24

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