

# Generation of cancer spheroids— tips and tricks

## Introduction

Tumor cells grown as spheroids offer an intermediate complexity between cancer cells grown in 2D monolayers and *in vivo* tumors. This potentiates their use as model systems to study tumor progression as well as to perform high-throughput screening of cytotoxic therapies, including chemotherapies and cell-based treatments.

Cancer spheroids are formed when cells are allowed to grow in suspension, as a result of which they aggregate, either on their own or with the aid of extracellular matrices. There are two factors critical in limiting variation in high-throughput assays with cancer spheroids. First, it is essential to have one spheroid per well in a multiwell plate, to reduce variability in readouts. Second, it is important that spheroids be of uniform shape and size—otherwise there can be variability between experiments. In our lab, we have tested spheroid generation conditions in nine human cell lines belonging to six cancer types. To summarize the results, we have compiled a general workflow and a few tips and tricks that would help in high-throughput generation of uniform and reproducible spheroids in Thermo Scientific™ Nunclon™ Sphera™ multiwell plates. The tips are specific to the cell type tested but can also be referred to for troubleshooting spheroid generation in other cell types.

## General workflow

1. On the day of experiment, dissociate cells using **Gibco™ TrypLE™ Express Enzyme** and then neutralize the enzyme using 4 volumes of complete medium (medium will vary depending on cell line chosen).
2. Count cells using the **Invitrogen™ Countess™ II FL Automated Cell Counter**. Cell viability should be >90%.
3. Dilute the suspension at a ratio of 1:10–1:20 in complete medium or a medium containing required additives. Seed the required number of cells in respective wells of **Nunclon Sphera 96-well plates** using **Thermo Scientific™ FinnpiPette™ F2 Multichannel Pipettes**.
4. Centrifuge plates at the required speed (250–450 x g) for 5–10 min at room temperature or 4°C, based on the use of additive (e.g., for **Gibco™ Geltrex™ matrix** addition, 4°C is necessary, and for collagen I, a temperature below 18°C is required).
5. Change the medium as necessary until spheroids are ready. Add the medium slowly along the side of the wells without touching the spheroids.

## Considerations for growing cancer spheroids

### Spheroid size

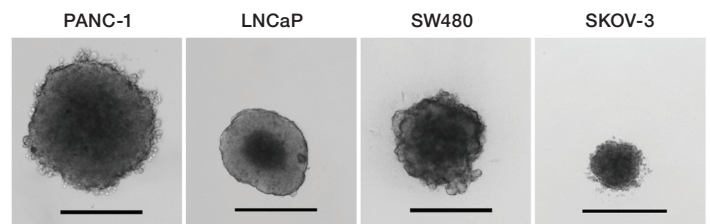
Depending on the cell line, spheroids differ in compactness. Figure 1 shows cancer spheroids generated from 5,000 cells of four different cell lines. As is evident, the seeding cell number does not correlate to spheroid size. Thus, to obtain spheroids of a specific diameter for use in a particular downstream assay, the seeding cell density for the respective cell line needs to be standardized. All brightfield images were captured using the Invitrogen™ EVOS™ M7000 Imaging System with a 4x objective with a stated otherwise.

### Time

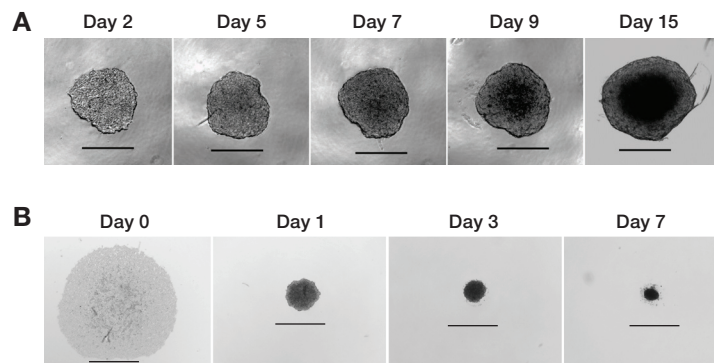
Cells have been shown to proliferate more slowly in 3D culture than in 2D culture [1]. Based on our observations, depending on the doubling time of the cells, some cancer spheroids are ready within 24 hr (for example, A549 and SKOV-3), while some might require 4–9 days (PC-3 and T47D). An ideal spheroid is translucent with a defined boundary and minimal dark core. However, certain cells, especially those that require an extracellular matrix for spheroid formation (see next section, “Extracellular matrices”), do not exhibit the ideal morphology. Figure 2 shows the morphological changes of T47D and SKOV-3 spheroids over time in culture. T47D spheroids grew in size and their cores became progressively darker over time; spheroids were ready by day 5. In contrast, SKOV-3 spheroids were ready on day 1; with increasing time in culture, the compactness increased (Figure 2B), and the cells seemed to be diverging from the spheroid.

### Extracellular matrices

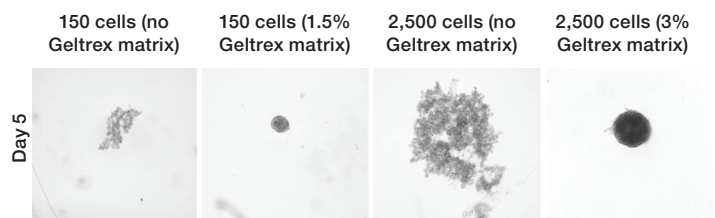
Some cell lines form spheroids on their own, while others form loose or tight cellular aggregates. Finicky cell lines require the assistance of various extracellular matrices (ECMs) to form spheroids. For example, PC-3 cells require Geltrex matrix (Figure 3). In order to optimize conditions for spheroid formation by MDA-MB-231 cells, various ECM components were tested in Nunclon Sphera 96-well plates with  $1 \times 10^4$  cells seeded per well. The day after plating, complete medium containing various ECMs was added to the spent medium, and cells were observed on day 5. As depicted in Figure 4, we found that collagen I worked best in this case to form a spheroid with a defined boundary. In all other cases, the cells formed aggregates.



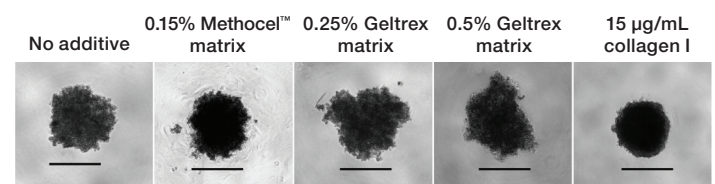
**Figure 1. Spheroids generated from cancer cell lines on a Nunclon Sphera plate.** PANC-1: pancreatic cancer; LNCaP: prostate cancer; SW480: colorectal cancer; SKOV-3: ovarian cancer. A total of 5,000 cells were seeded in each case. Scale bar: 500  $\mu$ m.



**Figure 2. Morphological change in different spheroid types.** (A) 4,000 T47D cells and (B) 10,000 SKOV-3 cells were seeded on Nunclon Sphera 96-well plates and observed on the indicated days. Scale bar: 500  $\mu$ m.



**Figure 3. PC-3 cells seeded for spheroid formation with and without Geltrex matrix.** Scale bar: 650  $\mu$ m.

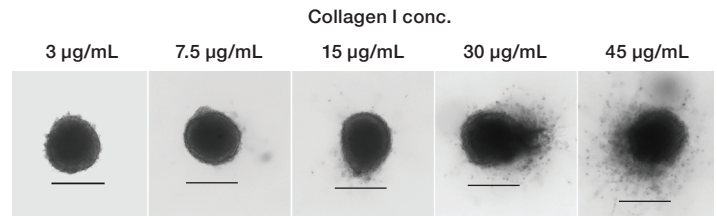


**Figure 4. Effect of ECMs on spheroid growth.** 10,000 MDA-MB-231 cells were seeded in medium supplemented with different ECMs. Scale bar: 500  $\mu$ m.

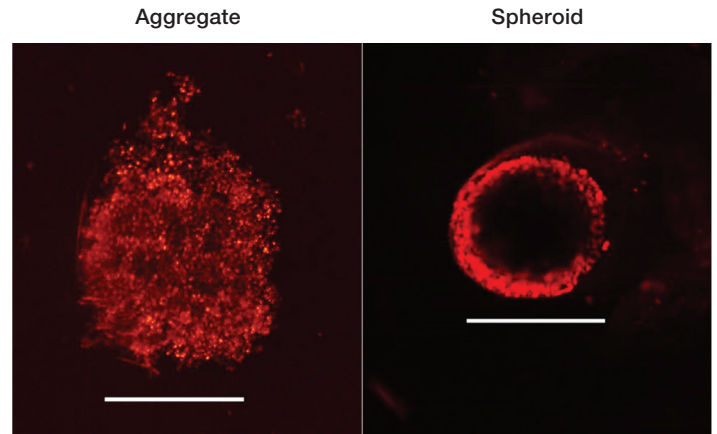
We further standardized the collagen I concentration required for spheroid formation. Subsequent testing indicated that a final concentration of 3  $\mu\text{g}/\text{mL}$  collagen I worked best, with higher concentrations leading to a disrupted spheroid morphology with cells diverging from the spheroid (Figure 5).

To verify spheroid formation, we stained the cellular entities with Invitrogen™ Dil, a lipophilic membrane stain. All cells in the aggregate were easily accessible to the dye, whereas the compactness of the spheroid prevented the dye from entering the core (Figure 6).

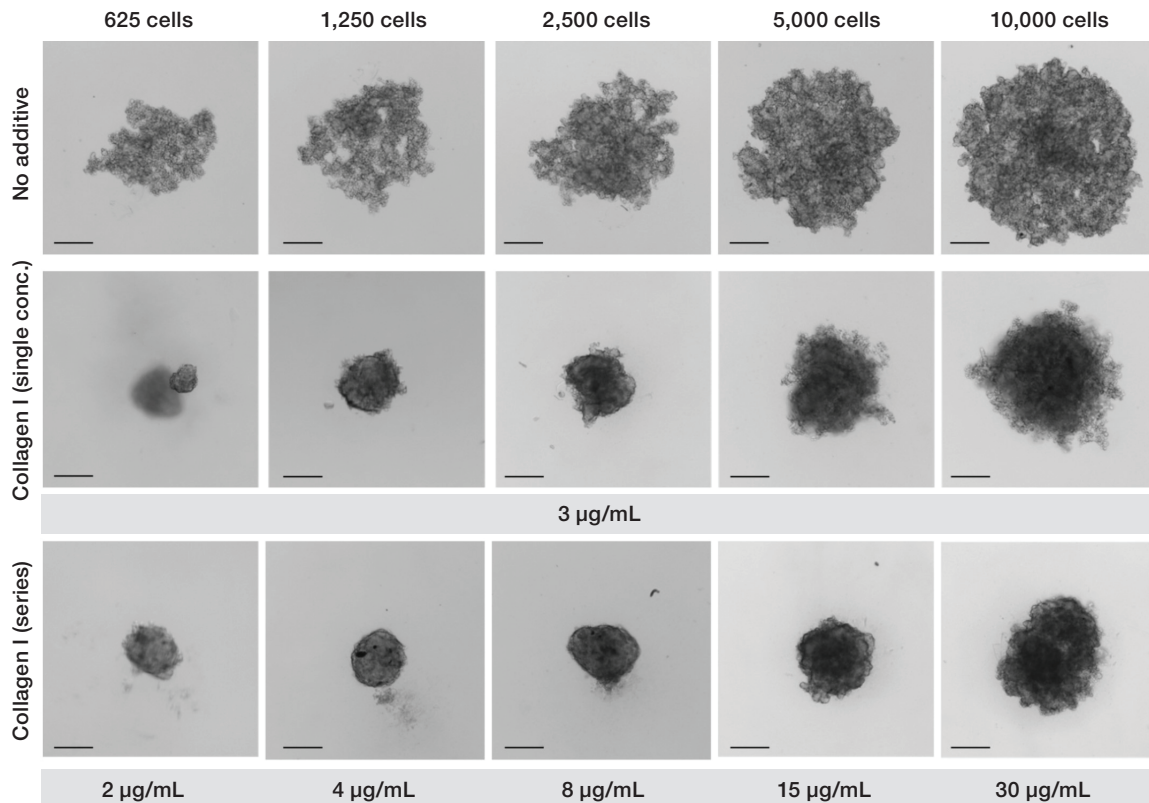
While a single concentration of ECM worked for MDA-MB-231 cells, some cell lines such as SW480 required different ECM concentrations based on the seeding density. As seen in Figure 7, a concentration of 3  $\mu\text{g}/\text{mL}$  of collagen I worked for 625–2,500 cells only. However, increasing the collagen I concentration for higher cell densities formed better spheroids than those formed using a single lower concentration of collagen I for those cell densities.



**Figure 5. Standardizing collagen I concentration.** 10,000 MDA-MB-231 cells were seeded in medium supplemented with different concentrations of collagen I. Scale bar: 500  $\mu\text{m}$ .



**Figure 6. Verification of spheroid formation.** 5,000 MDA-MB-231 cells in medium with or without collagen I were seeded onto Nunclon Sphera plates and stained with Dil 4 days later. Images were captured using the Thermo Scientific™ CellInsight™ CX7 High Content Analysis Platform with a 4x objective. Scale bar: 400  $\mu\text{m}$ .

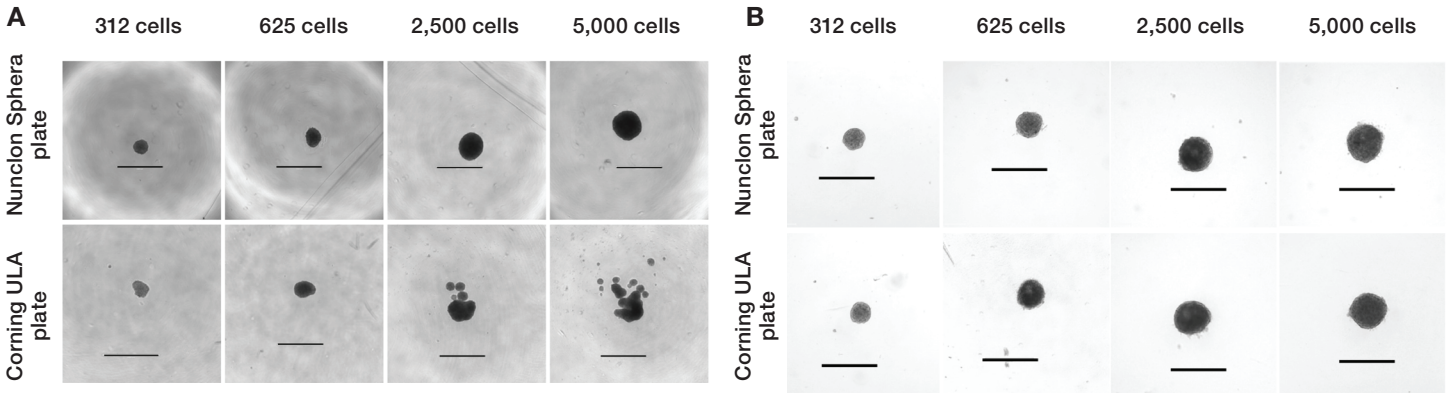


**Figure 7. Higher seeding densities may require more concentrated ECM.** SW480 cells were seeded in medium supplemented with either a single concentration or a concentration series of collagen I with increasing cell seeding density. Scale bar: 650  $\mu\text{m}$ .

## Plastic surface

The primary requirement for spheroid formation is a non-adherent surface. The Nunclon Sphera plates help to repel the cells from settling at the bottom and facilitate uniform spheroid formation upon centrifugation. We compared this surface to Corning™ ULA plates for spheroid formation. In our observations, out of nine cell lines tested,

approximately 50% of cell lines formed satellite colonies around the spheroids on the Corning ULA surface. This was more evident at higher cell densities, as in the case of HepG2 (Figure 8A, lower panel). On the other hand, Nunclon Sphera plates helped in consistent formation of a single spheroid per well for all cell lines tested.



**Figure 8. Effect of surface on spheroid formation in different cell lines.** (A) HepG2 cells were seeded for spheroid formation on a Nunclon Sphera plate (top panel) and a Corning ULA plate (bottom panel). (B) PC-3 cells were seeded in medium supplemented with Geltrex matrix on a Nunclon Sphera plate (top panel) and a Corning ULA plate (bottom panel) for spheroid formation. Scale bar: 1,000 μm.

## Conclusion

By using the right plastic surface, medium, and extracellular matrix, and following the tips and tricks, uniform and reproducible cancer spheroids can be generated easily. In our observations, cells that have a circular morphology and cells that grow in clusters can form spheroids on their own. However, cells that

don't grow in clusters require ECM support. Cells with elongated morphology vary in their requirement for ECM, so spheroid-generating conditions need to be optimized for each cell line. Our portfolio supports robust generation, characterization, and high-throughput applications and analyses of 3D cancer spheroids.

## Ordering information

Product	Cat. No.
<b>Plastics</b>	
Nunc EasYFlask Cell Culture Flasks	156499
Nunclon Sphera 96-well plate	174925
Matrix Reagent Reservoirs	8094
Nunc 15 mL and 50 mL Conical Sterile Polypropylene Centrifuge Tubes	339650, 339652
<b>Instruments</b>	
EVOS M7000 Imaging System	AMF7000
CellInsight CX7 High Content Analysis Platform	CX7A1110
Countess II Automated Cell Counter	AMQAX1000
Finnpipette F2 Multichannel Pipettes	4662030, 4662060
<b>Reagents</b>	
Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	A1413301
Collagen I, Rat Tail	A1048301
Dil Stain	D3911

## Reference

1. Anna C et al. (2013) Impact of the 3D microenvironment on phenotype, gene expression, and EGFR inhibition of colorectal cancer cell lines. *PLoS One* 8(3):e59689.



For additional resources for spheroid protocols and applications, go to [thermofisher.com/sphera](https://thermofisher.com/sphera)

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