Generation of MCF7 spheroids in serum-free conditions

Introduction

It is widely accepted that spheroid cultures recapitulate tumor microenvironments far better than traditional monolayer cultures do [1-3]. As opposed to cells in monolayers, cells in spheroids have more physiological cell–cell and cell–matrix interactions. In addition, larger spheroids (200–500 µm in diameter) have nutritional gradients that are common in solid tumors, with a pathophysiological necrotic core forming in spheroids larger than 500 µm [1]. These attributes are important features of spheroids, as cell–cell, cell–matrix, and chemical gradients have all been implicated in driving both tumor progression and therapy resistance [1-3]. Therefore, spheroids are regarded as an important preclinical tool for assessing drug efficacy and have provided key insights into the physiology of tumor progression [1-3].

For a variety of applications, it may be better to grow cells in a phenol red–free and serum-free environment. This is especially relevant for MCF7 cells, given that they are estrogen receptor (ER)-positive and have an increase in ER signaling in response to phenol red, an estrogen mimic, and fetal bovine serum (FBS), which contains estrogen [4]. In addition to reducing the exogenous estrogen, the absence of FBS also allows for studying the effect of specific growth factors on MCF7 cells; FBS contains many types and variable amounts of growth factors which, if present, may confound the effects of specific growth factors on MCF7 cells. For these reasons, we developed a serum-free growth medium for the generation and maintenance of MCF7 spheroids. We utilized this medium to examine the impact of heat-stable basic fibroblast growth factor (HS bFGF) signaling on spheroid growth dynamics. Since native bFGF (also known as FGF2) is not stable at 37°C, media requiring bFGF supplementation are typically changed every 2–3 days. Changing medium is a well-known obstacle for spheroid culture, as spheroids may be aspirated in the process. HS bFGF offers a solution to this problem as it maintains >80% bioactivity even after 72 hr at 37°C [5].

In this application note, the materials and methods we used for the formation of spheroids as well as the spheroid growth that resulted are discussed. A step-by-step protocol can be found in Appendix A. Tips and considerations for spheroid generation, including a list of other media systems in which we have previously generated MCF7 spheroids, can be found in Appendix B.

Suggested workflow

Prior to the experiment, stocks of MCF7 cells were grown in Thermo Scientific™ Nunc™ EasYFlask™ Cell Culture Flasks in Gibco™ DMEM/F-12, HEPES, no phenol red (Cat. No. 11039047), supplemented with 10% FBS (Cat. No. 16000069), referenced below as standard culture medium.

For each experiment, two types of media were used:

- Standard culture medium: DMEM/F-12, HEPES, no phenol red, supplemented with 10% FBS
- Experimental medium: DMEM/F-12, HEPES, no phenol red (Cat. No. 11039047), with 1X Gibco™ B-27™ Plus Supplement (Cat. No. A3582801), which was supplemented with bFGF based on the desired experimental condition. Our experimental conditions were differentiated by:
  - Gibco™ HS Recombinant Human bFGF (Cat. No. PHG0360)
  - Gibco™ FGF-Basic (AA 1-155) Recombinant Human Protein (native) (Cat. No. PHG0266)
  - No bFGF (negative control)

On the day of the experiment (Figure 1) the medium was aspirated, and MCF7 cells were washed with Gibco™ DPBS (no calcium, no magnesium) (Cat. No. 14190250), then dissociated using Gibco™ TrypLE™ Express Enzyme (Cat. No. 12604021; alternatively, Cat. No. 12563029). After the cells had lifted, the standard culture medium was added to the flask and cells were counted.

Spheroids were generated using a low-adhesion U-bottom microplate, the Thermo Scientific™ Nunclon™ Sphera™ 96U-well microplate (Cat. No. 174929). Prior to cell seeding, the microplate was prepared by:

1. Adding 0.1 mL of experimental medium (containing either 20 ng/mL HS bFGF, 20 ng/mL native bFGF, or no bFGF) to all wells that were to be seeded with cells. We prepared (and later seeded) the inner 36 wells of the plate.
2. Adding 0.2 mL DPBS (no calcium, no magnesium) to all wells where there were to be no cells seeded.

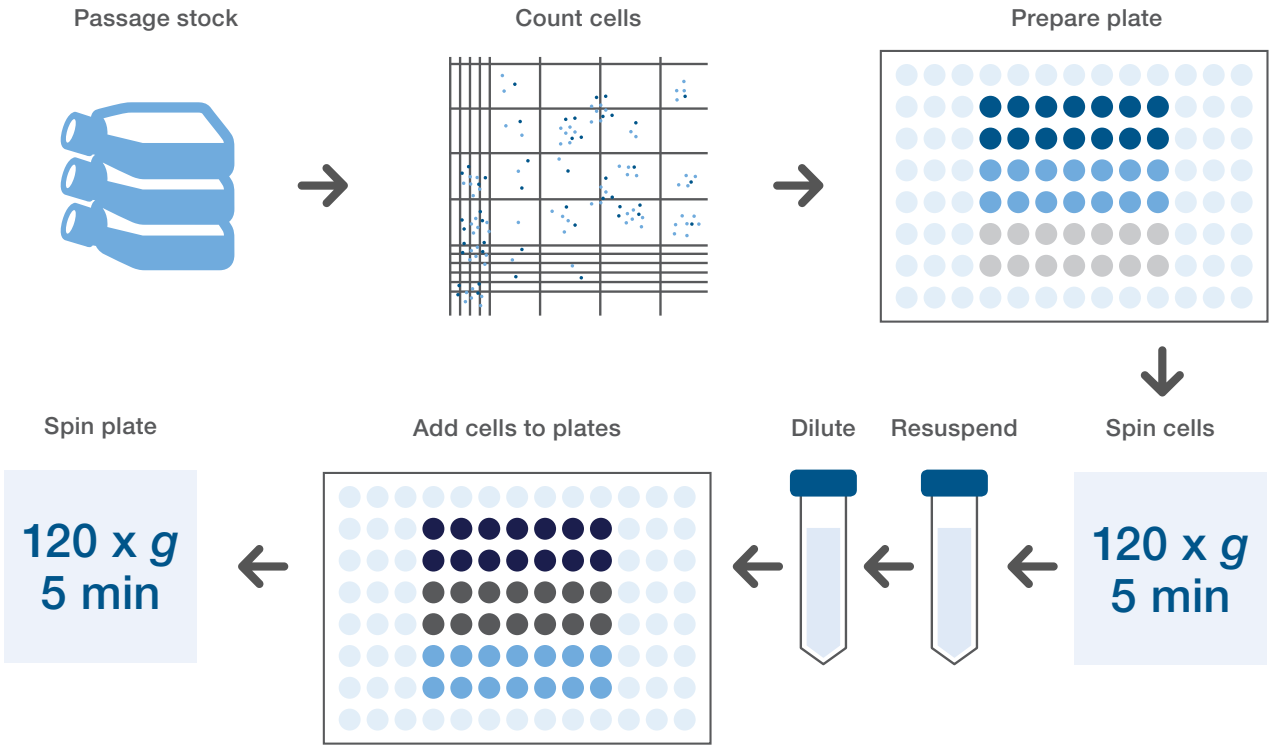

Figure 1. Suggested workflow for spheroid generation. On the day of the experiment, the MCF7 stock is passaged, counted, centrifuged, and resuspended in the serum-free experimental medium. The prepared wells of a Nunclon Sphera microplate are seeded at a density of 500 cells/well. The seeded plate is then centrifuged again to encourage spheroid development.

To prepare the cell seeding suspension, 7.2 x 10^5 cells* from the stock cell suspension were transferred to a new 15 mL conical tube. The new 15 mL conical tube was centrifuged at 120 x *g* for 5 min and the supernatant was aspirated off. The cell pellet was then resuspended in 12 mL of the experimental medium without bFGF and mixed thoroughly. From that 12 mL, 1 mL was transferred to another 15 mL conical tube containing 11 mL of the experimental medium without bFGF, for a 1:12 dilution.

This new diluted cell seeding suspension was mixed thoroughly and used to seed the prepared wells in the Nunclon Sphera microplate; 0.1 mL of cell seeding suspension was added to each well already containing 0.1 mL medium, for a total volume of 0.2 mL/well and a total of 500 cells/well.** The plate was then centrifuged at 120 x *g* for 5 min before being placed in a 37°C, 5% CO_2 incubator. The spheroids were incubated for 8 days without changing the media before analysis, but the spheroids were observed to form overnight after seeding.

Each data set comprises at least three separate experiments. Statistical analysis was completed across all spheroids per experimental condition. Any wells in which multiple spheroids were observed to have formed were eliminated from further analysis. For statistical analyses, Student's *t*-test and analysis of variance (ANOVA) with post-hoc Tukey honestly significant difference (HSD) test were completed, as appropriate for the data set.

Results and discussion

We found that MCF7 spheroids formed in all experimental conditions after overnight incubation. There was no significant difference in the size of the spheroids one day after seeding. In all conditions, a seeding density of 500 cells/well resulted in ~200 µm diameter spheroids (Figure 2).

However, after 8 days in culture, there were significant differences in the size and morphology of spheroids (Figure 3). Endpoint live/dead staining and subsequent confocal imaging confirmed that all spheroids were viable (Figure 3A). Spheroids exposed to bFGF expanded significantly more over the course of 8 days than those that were not exposed to any bFGF (Figure 3B). In addition, Invitrogen™ PrestoBlue™ viability reagent revealed that the spheroids grown in HS bFGF were not only larger in area but also contained a higher number of viable cells than the spheroids grown in native bFGF (Figure 3C).

Qualitatively, there were notable morphological differences between the spheroids exposed to bFGF and those that were not (Figure 3). Even though only wells with a single spheroid grown after one day of incubation were included for further analysis (Figure 2), small and self-contained spheroids (separated from the original bulk of the spheroid) were observed to have formed in all conditions after 8 days in culture (Figure 3A). These smaller spheroids were more numerous and widespread in the bFGF-treated conditions (white arrows, Figure 3A), contributing to what may be described as a different bulk morphology for spheroids in those conditions.

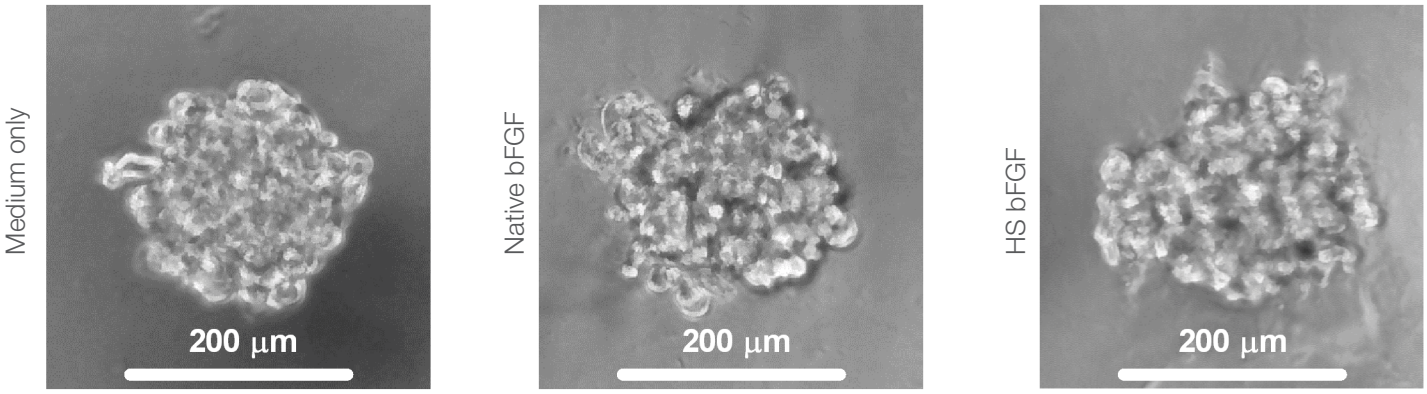

Figure 2. MCF7 cells form ~200 µm diameter spheroids after one day of incubation in all experimental conditions. Regardless of the bFGF supplementation condition, the 500 cells/well seeding density resulted in spheroids that were ~200 µm in diameter after only one day post-seeding.

* This will need to be adjusted depending on the number of wells you would like to seed. This determination is also based on the desired final seeding density, which was 500 cells/well for this study.

** A density of 500 cells/well was found to be optimal for our purposes, but spheroids also formed at 1,000 cells/well in our experience. See Appendix B for more information on how to choose seeding density.

These results are significant in several ways. First, while bFGF is not necessary for MCF7 spheroid generation and growth, it does contribute to greater proliferation. This effect is supported not only by the spheroids' size differences between the bFGF-free and bFGF-containing conditions, but also by the increased cell number observed with the use of stabilized bFGF vs. native bFGF. This is significant given that the effect of bFGF on MCF7 proliferation has been debated in the literature [6].

Second, the morphology observed in the bFGF-containing conditions may have important implications. A different bulk morphology, as opposed to the mass-like morphology that is typical of MCF7 cells, is associated with an invasive phenotype and enhanced ability of cells to break away from the primary tumor *in vivo* [7,8]. Morphological changes may be indicative of bFGF inducing a more invasive phenotype in the MCF7 cells, as has been demonstrated for the ductal carcinoma line T-47D when exposed to exogenous bFGF [9]; however, more work needs to be completed in order to confirm this hypothesis.

It must be noted that these observations would have been impossible without the use of HS bFGF. HS bFGF directly enabled the evidence of induction of MCF7 proliferation with stable bFGF signaling. In addition, if only native bFGF was used, frequent media changes would have disturbed the spheroids and likely confounded any attempts to make observations on the effect of bFGF on MCF7 spheroid morphology. Therefore, we highly recommend HS bFGF to be used in any media formulation that calls for bFGF.

Conclusions

In lieu of a phenol red– and serum-containing media system in which estrogen signaling in MCF7 cells may occur, we demonstrate that DMEM/F-12 (HEPES, no phenol red) with 1X B-27 Plus Supplement may be used in Nunclon Sphera 96U-well microplates to generate MCF7 spheroids. A seeding density of 500 cells/well generated ~200 µm diameter spheroids after one day of incubation. The utility of this serum-free system was demonstrated in an effort to understand the effect of bFGF on MCF7 spheroid growth. However, this method of MCF7 spheroid generation may be applicable more broadly for any study in which the inclusion of FBS may be confounding (e.g., hormone and growth factor studies).

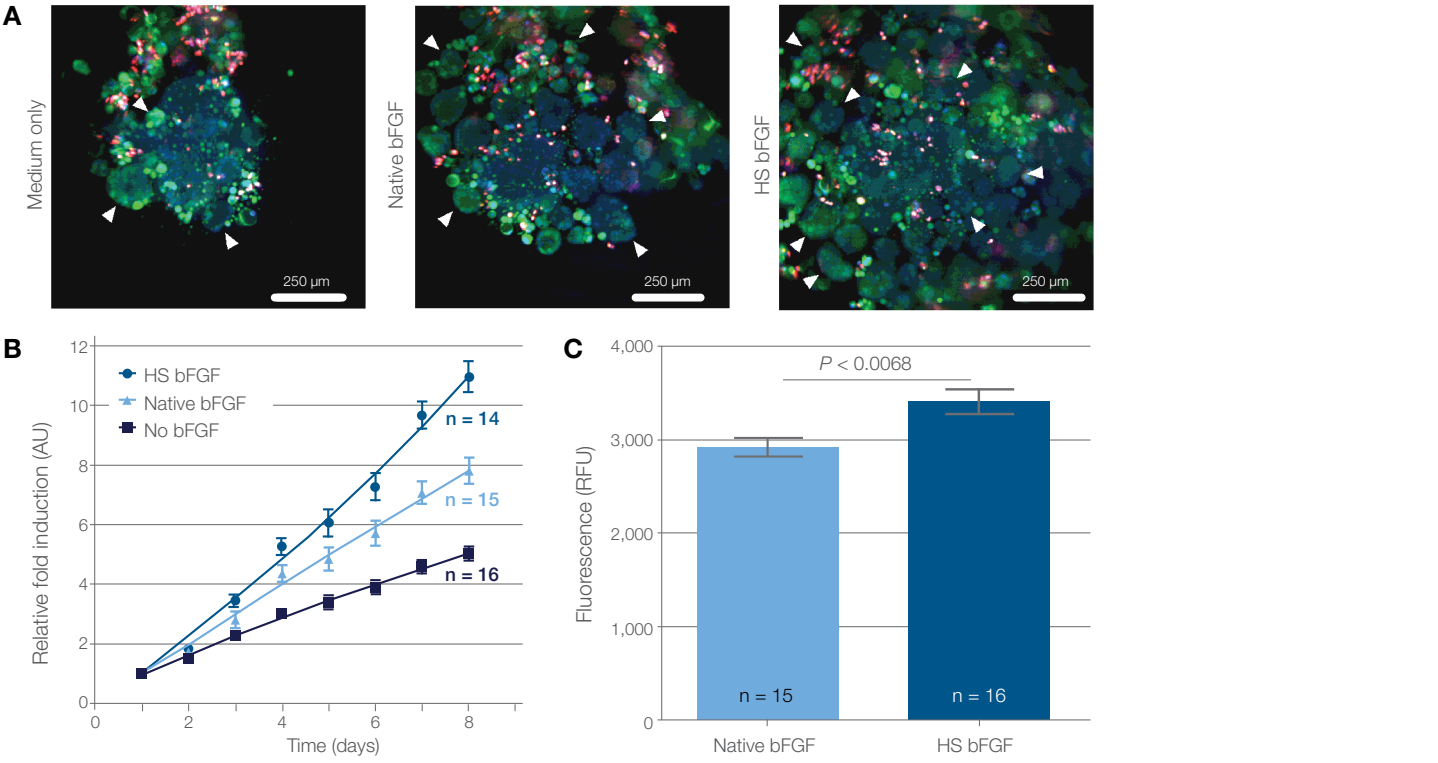

Figure 3. bFGF induced larger spheroids and an altered spheroid morphology. (A) Eight days post-seeding, spheroids in all conditions were viable, as shown by live/dead staining (live = green, dead = red, cell nuclei = blue). By visual inspection, it was evident that spheroids exposed to bFGF were larger and had a different bulk morphology than the spheroids grown in the non-bFGF condition. **(B)** Analysis of relative growth (change in spheroid size, normalized to the initial size) over the course of 8 days revealed that HS bFGF supported the largest increase in size, and spheroids grown in both bFGF conditions maintained faster growth than spheroids in the non-bFGF condition; $P < 0.0001$ at day 8. **(C)** The PrestoBlue assay on spheroids 8 days post-seeding showed a higher number of viable cells in spheroids in the HS bFGF condition vs. those in the native bFGF condition.

Appendix A

Spheroid generation protocol

Prior to the experiment, stocks of MCF7 cells were grown in Nunc EasYFlask Cell Culture Flasks in DMEM/F-12, HEPES, no phenol red (Cat. No. 11039047), supplemented with 10% FBS (Cat. No. 16000069), referenced below as the standard culture medium.

For each experiment, two types of media were used:

- Standard culture medium: DMEM/F-12, HEPES, no phenol red (Cat. No. 11039047), supplemented with 10% FBS (Cat. No. 16000069)
- Experimental medium: DMEM/F-12, HEPES, no phenol red (Cat. No. 11039047), and 1X B-27 Plus Supplement (Cat. No. A3582801), to which HS bFGF (Cat. No. PHG0360) or native bFGF (Cat. No. PHG0266) was added as needed for the experiment. Based on our results, a final concentration of 10 ng/mL HS bFGF is recommended for long-term (~1 week) growth of a spheroid without medium change.

1. Cells in the flask were washed twice with DPBS (no calcium, no magnesium) (Cat. No. 14190250), then dissociated using TrypLE enzyme (Cat. No. 12604021; alternatively, Cat. No. 12563029) for no more than 5 min.
2. After the cells had lifted, the standard culture medium was added to the flask. The cell suspension was transferred to a 15 mL conical tube, and the viable cell density was determined.
3. A Nunclon Sphera 96U-well plate was prepared:
   - To all wells that were to be seeded with cells, 0.1 mL of experimental medium (containing either 20 ng/mL HS bFGF, 20 ng/mL native bFGF, or no bFGF) was added.
   - To all other wells of the plate, 0.2 mL DPBS (no calcium, no magnesium) was added.
4. To prepare the seeding suspension:
   - From the stock cell suspension, 7.2 x 10^5 cells* were transferred to a new 15 mL conical tube.
   - The new 15 mL conical tube was centrifuged at 120 x *g* for 5 min, the supernatant was aspirated off, and the cell pellet was resuspended in 12 mL of experimental medium and mixed thoroughly (7.2 x 10^5 cells/12 mL = 6 x 10^4 cells/mL).
   - From that 12 mL, 1 mL was transferred to another 15 mL conical tube containing 11 mL of experimental medium (1:12 dilution); this new cell seeding suspension was then mixed thoroughly (6 x 10^4 cells/12 mL = 5 x 10^3 cells/mL) before it was used to seed the plate.
   - The cell seeding suspension (5 x 10^3 cells/mL) was used to seed the prepared wells in the Nunclon Sphera 96U-well plate; 0.1 mL of the seeding suspension was added to each well already containing 0.1 mL medium, for a total volume of 0.2 mL/well and a total of 500 cells/well.**
5. The plate was then centrifuged at 120 x *g* for 5 min before being placed in a 37°C, 5% CO_2 incubator.
6. The spheroids were incubated for 8 days without changing the media before analysis, but the spheroids were observed to form overnight after seeding.

* This will need to be adjusted depending on the number of wells you would like to seed. This determination is also based on the desired final seeding density, which was 500 cells/well for this study.

** A density of 500 cells/well was found to be optimal for our purposes, but spheroids were also formed at a density of 1,000 cells/well in our experience. See Appendix B for more information on how to choose seeding density.

Appendix B

Tips and considerations for MCF7 spheroid generation using Nunclon Sphera plates

- If you are using bFGF in your serum-free formulation, the HS Recombinant Human bFGF is highly recommended. Native bFGF is not stable in standard culture conditions (i.e., 37°C); we have demonstrated a loss of ~80% bFGF bioactivity after 72 hours at 37°C [5]. Although changing the medium on your spheroids is not impossible, care should be taken to minimize the risk of unintended spheroid aspiration. HS bFGF maintains >80% bFGF bioactivity after 72 hours at 37°C [5] and thus eliminates the need to change the medium.

- Final spheroid size may be optimized by varying the seeding density (number of cells per well):

  – For this study, we tested seeding densities of both 500 cells/well and 1,000 cells/well, and continued with 500 cells/well due to the relatively long length of our study.

  – The seeding density will be dependent on the application, especially with regard to drug testing. Although spheroids ≤200 µm in diameter are more representative of physiological cell–cell and cell–matrix interactions than 2D culture, they will likely not represent the hypoxia gradient and necrotic core associated with many solid tumors; spheroids of 200–500 µm, or larger, are needed in order to have these pathophysiological attributes [1].

- We have found that centrifugation of the Nunclon Sphera microplate post-seeding, in combination with the U-bottom configuration of the plate, best encourages spheroid development.

- In our experience, MCF7 spheroids were formed overnight in all of the following media conditions:

  – DMEM/F-12, GlutaMAX Supplement (Cat. No. 10565018) and 10% FBS (Cat. No. 16000069)

  – DMEM/F-12, HEPES, no phenol red (Cat. No. 11039047) and 1X B-27 Plus Supplement (Cat. No. A3582801)

  – DMEM/F-12, HEPES, no phenol red (Cat. No. 11039047), 1X B-27 Plus Supplement (Cat. No. A3582801), and 10 ng/mL HS bFGF (Cat. No. PHG0360)

  – DMEM/F-12, HEPES, no phenol red (Cat. No. 11039047), 1X B-27 Plus Supplement (Cat. No. A3582801), and 10 ng/mL bFGF (Cat. No. PHG0266)

  – DMEM/F-12, GlutaMAX Supplement (Cat. No. 10565018) and 1X B-27 Plus Supplement (Cat. No. A3582801)

  – DMEM/F-12, GlutaMAX Supplement (Cat. No. 10565018), 1X B-27 Plus Supplement (Cat. No. A3582801), and 10 ng/mL HS bFGF (Cat. No. PHG0360)

  – DMEM/F-12, GlutaMAX Supplement (Cat. No. 10565018), 1X B-27 Plus Supplement (Cat. No. A3582801), and 10 ng/mL bFGF (Cat. No. PHG0266)

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Ordering information

Product	Cat. No.
DMEM/F-12, HEPES, no phenol red	11039047
FBS, certified, US origin	16000
B-27 Plus Supplement	A3582801
DPBS, no calcium, no magnesium	14190
Heat Stable Recombinant Human bFGF Protein	PHG0360
TrypLE Express Enzyme (1X)	12604
Nunc 15 mL Conical Sterile Polypropylene Centrifuge Tubes	339651
Nunc EasYFlask Cell Culture Flasks	156499
Nunclon Sphera 96U-Well Microplate	174929
CellInsight CX7 High Content Analysis Platform	CX7A1110

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