Determination of the Tumorsphere Formation Efficiency (TFE)

PromoCell®

Application note

Our 3D Tumorsphere Medium XF has been designed to meet your requirements for the extended serial 3D tumorsphere culture. The medium supports the most commonly used cancer stem cell containing cancer cell lines and primary cancer cultures. In contrast to the adherent 2D culture of cancer cells, this type of 3D culture selectively exploits inherent biologic features of cancer stem cells (CSCs), such as anoikis resistance and self-renewal (Fig. 3). Continuous proliferation is also sup-ported during serial passage of tumorsphere cultures (Fig. 2). Thus, this culture system is also applicable for *in vitro* models of metastasis.

Our 3D Tumorsphere Medium XF is ready-to-use and defined, providing a standardized culture devoid of stimuli of uncharacterized origin. This is a significant benefit in terms of CSCs which are a population of highly responsive stem cells requiring reliable and reproducible control of the self renewal/differentiation axis.

The 3D Tumorsphere Medium XF is suitable for the cost-efficient and standardized routine culture of tumorspheres/mammospheres for a wide range of cell lines (Tab. 1).

Tissue	Tested cell line	Cell line origin
Brain	U-87 MG	Grade IV glioblastoma / astrocytoma of the human brain
Breast	MCF-7	Pleural effusion of metastatic human breast adenocarcinoma
Breast	MDA-MB-231	Pleural effusion of metastatic human breast adenocarcinoma (triple-negative)
Colon	HT-29	Human colon adenocarcinoma
Colon	CT26	Mouse colon cancer cell line
Connective tissue	HT1080	Human fibrosarcoma
Liver	HepG2	Hepatocellular carcinoma of the human liver
Lung	A-549	Human lung carcinoma
Pancreas	Panc-1	Epithelioid carcinoma of the human pancreatic duct
Prostate	LNCaP	Lymph node metastasis of human prostate adenocarcinoma
Skin	A-431	Epidermoid carcinoma of the human skin

Tab. 1: *List of cell types tested for serial passage with the 3D Tumorsphere Medium XF.*

The TFE Assay

CSCs lack specific or universally applicable markers, so analysis and characterization of these unique cancer cell subpopulations remain challenging. The most widely accepted methods therefore rely on detection of specific functional cellular features. The tumorsphere formation efficiency (TFE) indicates the percentage of cells within a culture that are capable of forming a sphere from a single cell (Fig. 1). Since this property is only attributed to stemlike cells, the TFE assay remains a valuable qualitative and quantitative tool based on exclusively functional features of cells under selective culture conditions.

Originally derived by limiting dilution neurosphere assays for detecting neural (cancer) stem cells, the TFE assay in non-neuronal cancer cell cultures was adapted as an universally applicable technique. The neurosphere assay provides unique functional selectivity for cells with stemlike properties and is a useful tool for cell cultures of various other types of cancer. This TFE assay is therefore particularly useful for exploring and characterizing unidentified CSC subpopulations without the need to rely on unassured markers.

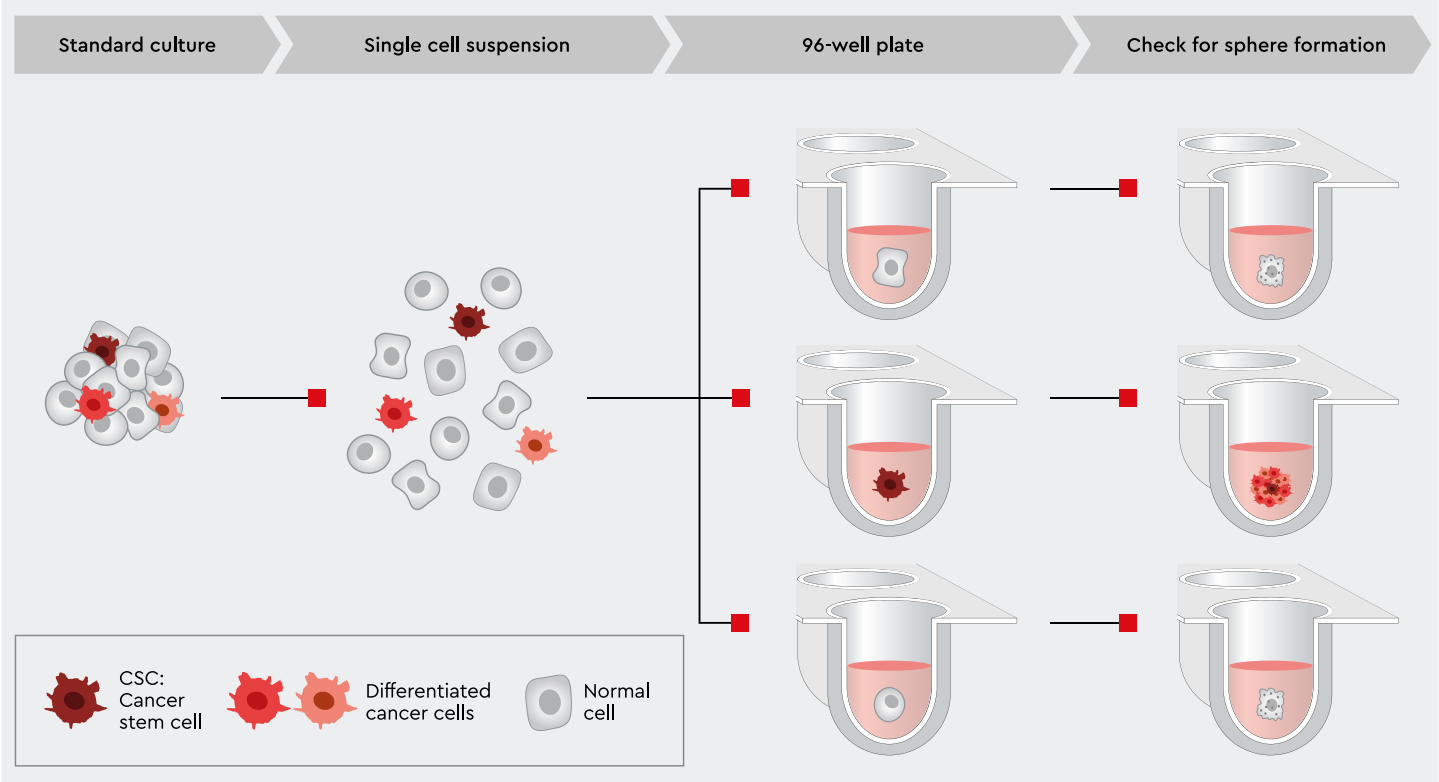

Fig. 1: Schematic flowchart of the TFE assay. *Single cancer cells obtained by enzymatic dissociation of tumorsphere cultures are plated by limiting dilution to one cell per well on 96-well plates. After an adequate incubation period, the wells originally containing the cells are analyzed for tumorsphere formation. Cells with stemlike properties are capable of forming a new tumorsphere derived from a single cell, while more restricted or differentiated tumor cells undergo anoikis. See the protocol on page 5 for details.*

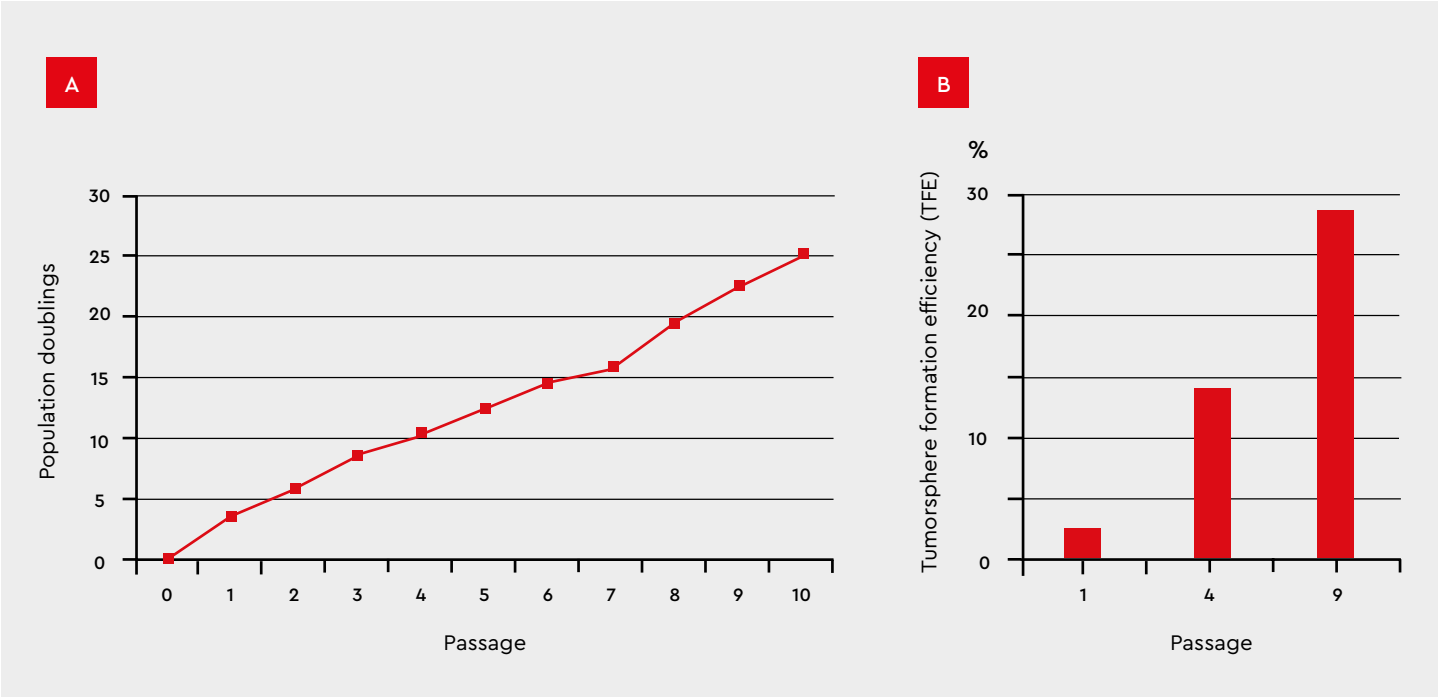

Fig. 2: Plot of cumulative population doublings of MCF-7 cells during serial passage of tumorsphere culture. A: *Forty thousand MCF-7 cells per well (10,000/ml) were plated in triplicate in the 3D Tumorsphere Medium XF using 6-well suspension culture plates. Serial passage by enzymatic dissociation according to the protocol was performed every 9 days. Tumorsphere formation and proliferation were maintained during the culture, which was discontinued after passage 10 with no sign of growth rate inhibition. The MCF-7 tumorsphere culture achieved approximately 2.5 population doublings per passage. The proliferation rate is dependent on cell line and may vary accordingly with other types of tumor cells.* ***B:*** *Serial passage of MCF-7 cells in the 3D Tumorsphere Medium XF results in significant increase of TFE from 2% in P1 to 28% in P9, respectively. See page 5 for detailed protocol.*

Where serial passage is concerned, the culture media that had been established by then did not robustly allow for sphere formation from cancer cells from other types of tumors. Indeed, this obstacle was due to the great heterogeneity and variety of metabolic re-quirements of different types of cancer cells. Our 3D Tumorsphere Medium XF, which supports established cell lines of a wide variety of cancers, fills this remaining gap. Enabling both serial passage and single cell-derived sphere formation, now all stem cell-related advantages of the sphere culture technique are available for a broad range of cancers. Depending on the culture conditions, the TFE can either remain fairly static or be distinctly dynamic. For example, in adherent serum-containing cultures of established cancer cell lines – standard culture conditions favoring differentiation – the TFE remains relatively stable at a low level. By contrast, the TFE may behave dynamically, especially after being switched from a standard culture to an environment that is more selective for stem cells. Under these conditions, sphere-forming cells may be gradually enriched (Fig. 2b). Consequently, the TFE assay is a significant, cost-effective, and reliable *in vitro* method for indirectly measuring the CSC content of cultured cancer cells at a specific point of time and/or in a certain culture state. By way of example, the repeated concomitant determination of TFE at different passages during serial culturing of cancer cells in the 3D Tumorsphere Medium XF can indirectly quantify the effects of this medium with regard to the culture's functional CSC content.

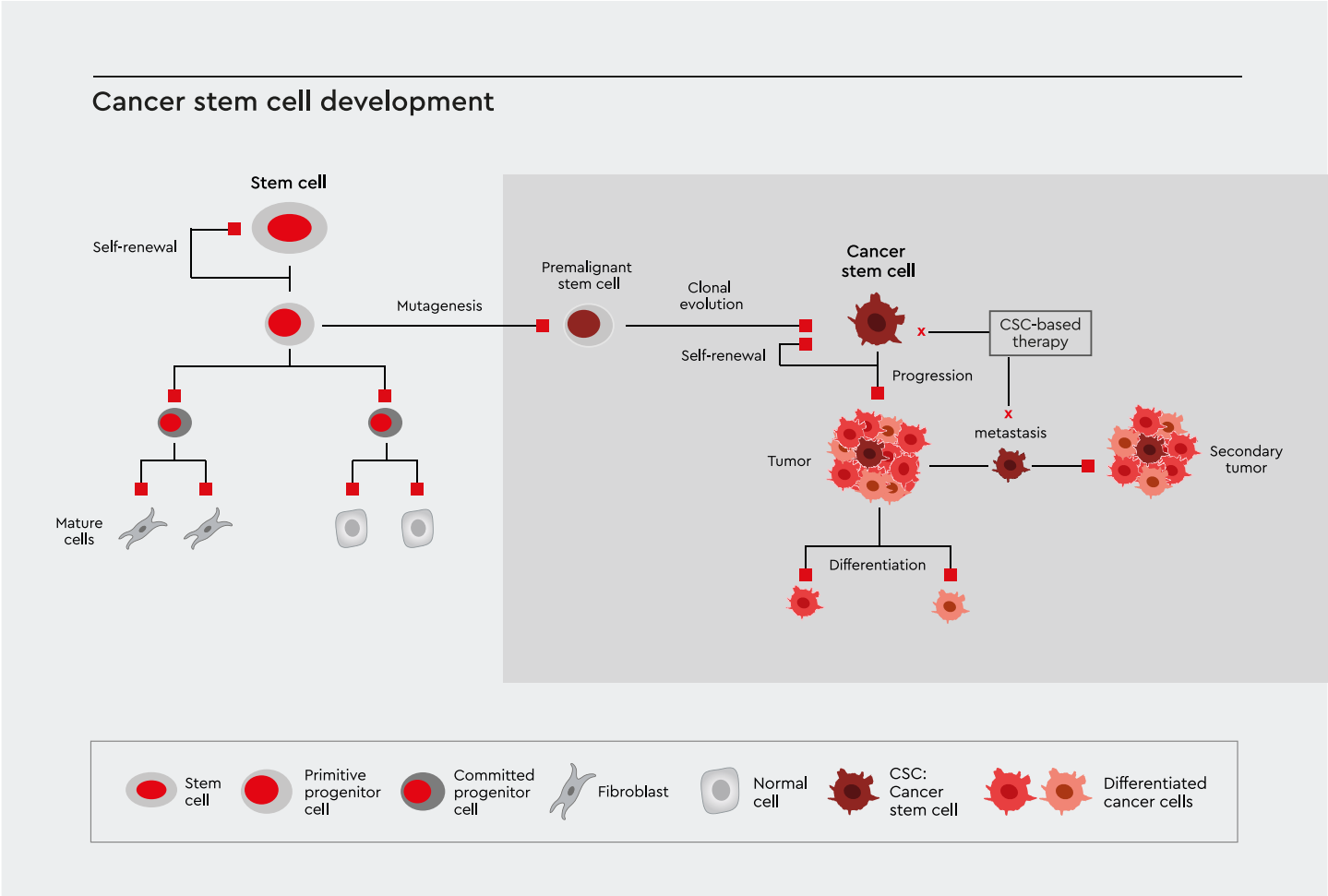

Fig. 3: *Schematic overview on the origin, evolution and fate of cancer stem cells (CSC).*

Background

"The term 'malignancy' refers to cancerous cells that have the ability to spread to other sites in the body (metastasize) or to invade nearby (locally) and destroy tissues. Malignant cells tend to have fast, uncontrolled growth and do not die normally due to changes in their genetic makeup. Malignant cells that are resistant to treatment may return after all detect- able traces of them have been removed or destroyed." (Medline Encyclopedia)

History of cancer

Paleopathologic findings indicate that cancer has been recognized for millennia. The first written documentation of human cancer dates from 3000 BC, with the first description of metastasis going back to the year 50 AD [1]. More than 5000 years later, many types of cancer still remain incurable and prove fatal, particularly in cases of recurrence after seemingly successful primary treatment. In 1863, almost 160 years ago, the pathologist Rudolf Virchow was the first to propose the model of cancer stem cells stating that 'immature cells' represent the origin of cancer [2]. In 1959 Pierce introduced the term 'cancer stem cell' [3], while in 1997 Bonnet described a specific subpopulation of CSCs as 'cancer driver cells' in myeloid leukemia [4]. Recently, CSC have been identified in various cancers including hematopoietic malignancies and a range of solid tumors [5].

Research and therapy for malignancy

Cancer research is one of today´s major research fields producing thousands of publications every year. The most frequently studied malignancies are cancers of the breast, lung, liver, colon, skin, pancreas and nervous system. Traditional therapeutic approaches aim to eliminate as much of the tumor mass as possible by means of surgery, irradiation, chemotherapy and biologics. However, accumulating evidence suggests these measures target the more harmless, rapidly dividing cell mass of the tumor and do not eradicate the putative root of the disease – CSCs. In the cancer stem cell model of tumors, CSCs are defined as a small subset of malignant cells with the exclusive ability to self-renew and maintain the tumor. They can differentiate into a heterogeneous mass of non-tumorigenic cancer cell types, which usually constitute the majority of the tumor [6]. It is clear in this context that CSCs, despite their malignant phenotype, share common hallmarks of normal stem cells (Fig. 3), assigning extraordinary biologic potential to these cells. These combined capabilities account for one of the greatest risks in the treatment of malignancies: metastasis. CSCs are self-renewing and largely resistant to anoikis, the term for apoptosis induced by the detachment of adherently growing cells from the extracellular matrix. Therefore, CSCs can separate from the primary tumor, travel and spread through the body where they may form secondary tumors (metastases) in distant organs. Metastases may develop quickly or over several years after seemingly successful treatment of the primary tumor. It is believed that relapse is caused by quiescent CSCs that are able to evade current therapeutic regimens by using protective mechanisms mediated by their stem cell properties. It is clear that cancer research needs to be realigned, especially when exploring new clinical strategies to treat malignancies. CSCs are now considered to be a new therapeutic target and it is believed that their elimination could lead to permanent remission or even cure. This might be achieved by direct eradication of the CSCs or by specific adaptation of CSCs cell division from asymmetric to symmetric leading to elimination of the CSC population by blocking their self-renewal capabilities [6, 7]. For this to be achieved, detailed characterization is required.

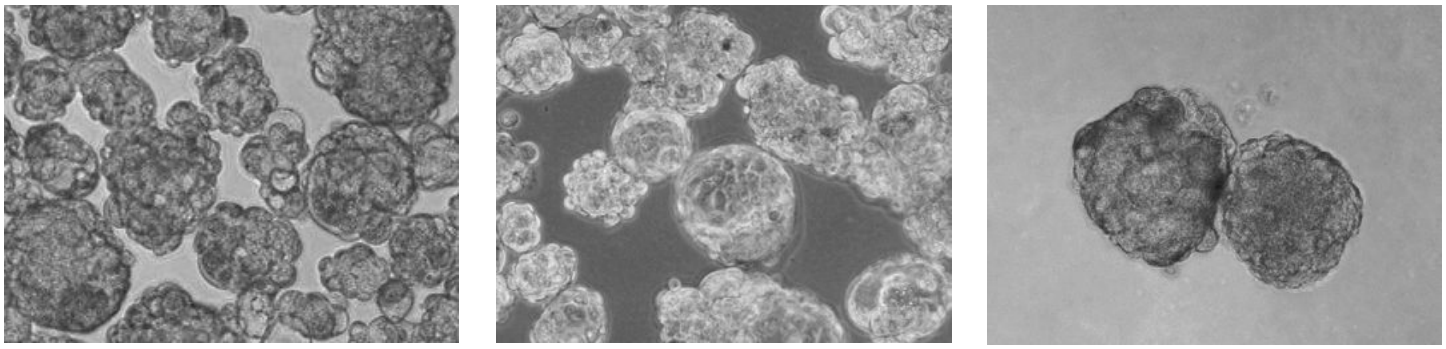

Fig. 4: Tumorsphere culture of MCF-7 mammary carcinoma cells in our 3D Tumorsphere Medium XF (C-28070) after 10 serial passages. *The tumorsphere culture was subjected to serial passage every 9 days by enzymatic dissociation according to the protocol. Robust tumorsphere formation was maintained during serial culture. See Fig. 2 for proliferation data.*

TFE assay protocol

This procedure largely corresponds (steps 1–5) to the subculture protocol of tumorsphere cultures, which routinely requires generation of a single-cell suspension.

I. Generation of a single-cell suspension

Materials

- Tumorspheres (see application note "Tumorsphere Culture of Cancer Stem Cells (CSCs)")
- 3D Tumorsphere Medium XF (C-28070 and C-28075)
- Phosphate buffered saline without Ca^{++}/Mg^{++} (PBS, C-40232)
- DetachKit (C-41210)
- 96-well u-bottom suspension plates (e.g., Greiner Bio One, No. 650 185)
- 40 µm cell strainer

Use aseptic techniques and a laminar flow bench.

1

Collect the tumorspheres (day 0)

Transfer the 3D Tumorsphere Medium XF containing the tumorspheres into 15 ml conical tubes using a serological pipet.

2

Gravity sedimentation of the tumorspheres (day 0)

Allow the spheres to settle by gravity sedimentation for 10 minutes at room temperature. Aspirate the supernatant but leave approximately 200 µl in the conical tube. Do not aspirate the tumorspheres.

3

Wash the tumorspheres (day 0)

Repeat the sedimentation (step 2 on the previous page) with an equal volume of PBS. Gently aspirate the PBS leaving approximately 200 µl in the conical tube.

4

Enzymatic digestion of the tumorspheres

Add 1 ml of trypsin-EDTA to the tumorspheres and incubate for 2–4 minutes at room temperature. Keep the spheres resuspended in the trypsin solution by pipetting up and down once every 30 seconds. Avoid sedimentation of the spheres.

Note: The optimal incubation time required to achieve complete dissociation in step A5 (below) must be empirically determined by the user for each cell type. While 2–3 minutes will be optimal in most cases, tumorspheres of some cell types, e.g. MCF-7, may need longer incubation, especially in higher passages. If a completely defined dissociation process is preferred, a recombinant trypsin solution may be used as an alternative dissociation reagent while following the supplier's instructions.

5

Break down remaining cell aggregates (day 0)

Pipet the spheres up and down 10–20 times using a 1000 µl pipet tip to generate a single-cell suspension. Aspirate the cell suspension in the normal way but slightly tilt the pipet tip at the bottom of the tube when expelling the cells. The generated shear forces facilitate the breakup of any residual cell aggregates. Perform a visual check to confirm that no large cell aggregates remain. Immediately after trituration, add twice the volume of Trypsin Neutralization Solution (TNS).

Note: Do not over-triturate since this would compromise cell viability. If in doubt, monitor the dissociation process microscopically. When using recombinant trypsin, use fresh 3D Tumorsphere Medium XF for inactivation instead of TNS.

6

Determine the cell number and viability (day 0)

Make up to 5 ml with fresh 3D Tumorsphere Medium XF and centrifuge the cells for 5 minutes at 300 x g. Discard the supernatant and resuspend the cells in 5 ml of the fresh medium. Pass the cell suspension through a 40 µm cell strainer to obtain a single-cell suspension. Then determine the cell count and viability.

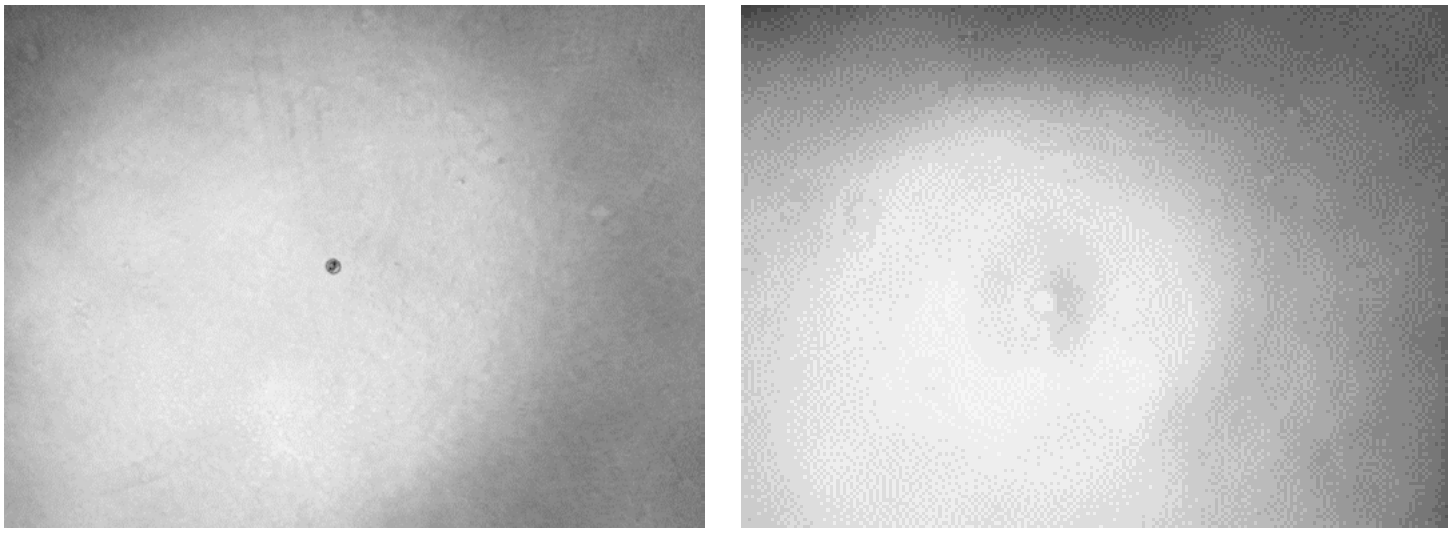

Fig. 5: Setup for the Tumorsphere Formation Efficiency (TFE) detection assay. Left: *A single cell seeded in an individual well of a 96-well u-bottom plate.* ***Right:*** *An empty well without a cell, to be excluded from analysis.*

II. Tumorsphere Formation Efficiency (TFE) Assay

7

Dilute the single cell suspension (day 0)

Dilute an aliquot of the single-cell suspension obtained in I. with an appropriate amount of 3D Tumorsphere Medium XF in order to obtain a concentration of 10 viable cells per ml of this medium. Prepare at least 50 ml of this diluted cell suspension.

8

Plate the single cells (day 0)

Distribute the diluted cell suspension at 100 µl per well in 96-well u-bottom suspension culture plates. Seed 3 to 5 entire plates. On the same day, check the single wells for the presence of a single cell using a microscope (Fig. 5). Mark wells without a cell.

9

Let the tumorspheres grow (day 0–4+)

Incubate the plates in the incubator at 37°C and 5% CO_2.

10

Add fresh medium (day 4–6)

On day 4 to 6, add 100 µl of fresh 3D Tumorsphere Medium XF to each well.

Note: Do not change the medium, only add the fresh medium.

11

Evaluate the results (day 7–12+)

Check the wells of the seeded 96-well plates for sphere formation (Fig. 6). Omit wells marked as "without cell" from analysis. Divide the number of spheres counted by the number of total cell-containing seeded wells and multiply by 100. The result is the TFE percentage (% TFE).

Note: To ensure reproducible results, it is important to establish a specific definition for "positive sphere formation". It is recommended to use the mean diameter of the cell aggregate as a readout parameter, which can be easily measured using the measure/ruler function of the microscope image capture software or an optical grid. Ideally, the median sphere diameter of the specific cell line will already be known from preceding 3D cultures. Intact MCF-7 spheres, for example, exhibit a mean diameter of >150 µm after 10–12 days. Smaller aggregates are therefore not counted as positive. If the corresponding sphere diameter is unknown, set your own cutoff value by measuring the size of some "nice spheres" within the assay. Aggregates <80 µm should never be considered as spheres. While most cell lines will form tumorspheres from a single cell within 7–12 days, it may be necessary to adjust the duration of the growth phase for very fast-growing or very slowly proliferating cell lines in order to obtain unambiguously evaluable results.

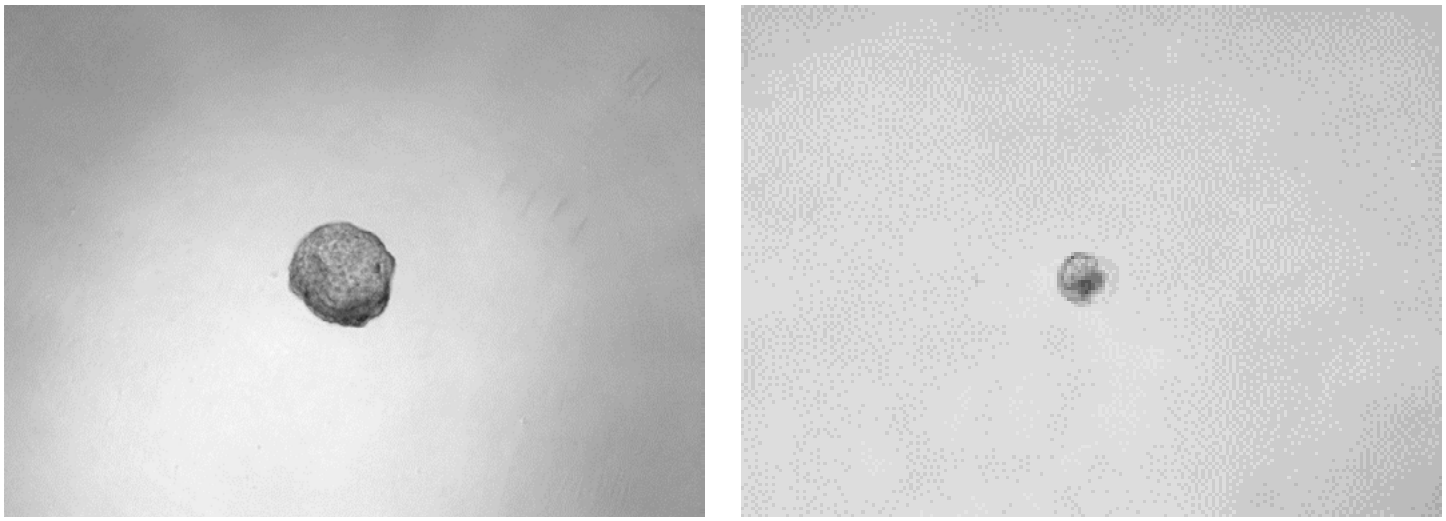

Fig. 6: Expected results of the TFE Assay. Left: *Tumorsphere derived from a single MCF-7 cell larger than the cutoff size of ≥150 µm: "positive for tumorsphere formation".* ***Right:*** *Cell aggregate smaller than the cutoff size rated as "negative for tumorsphere formation". The dark center indicates the onset of degeneration.*

Products

Media	Size	Catalog number
3D Tumorsphere Medium XF	250 ml	C-28070
3D Tumorsphere Medium XF, phenol red-free	250 ml	C-28075

Related Products

Media	Size	Catalog number
Dulbecco's PBS, without Ca^{++}/Mg^{++}	500 ml	C-40232
DetachKit	3 × 125 ml	C-41210

References

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