Tumorsphere culture of cancer stem cells

Application note

Our 3D Tumorsphere Medium XF has been designed to meet your requirements for the extended serial tumorsphere culture. The 3D Tumorsphere Medium XF supports the most commonly used cancer stem cell lines and primary cancer cell from tumor biopsies in tumorspheres/mammospheres culture. In contrast to the current 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. Continuous proliferation is also supported during serial passage of tumorsphere cultures (see Fig. 1). Thus, this culture system is also applicable for *in vitro* models of metastasis. Our 3D Tumorsphere Medium XF is ready-to-use and xeno-free, 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 xeno-free 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

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

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 make-up. Malignant cells that are resistant to treatment may return after all detectable 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 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, CSCs 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.

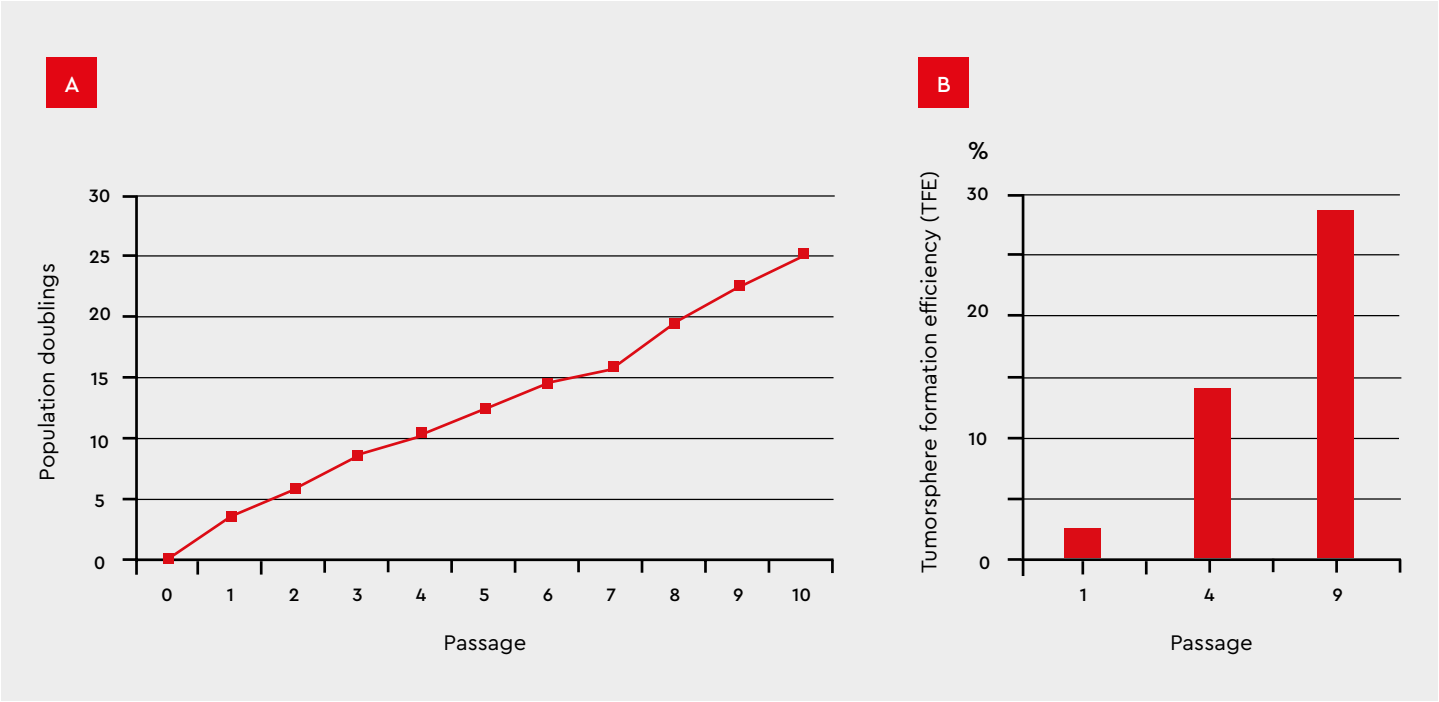

Fig. 1: Plot of cumulative population doublings of MCF-7 cells during serial passage of tumorsphere culture. A: *Forty thousand MCF-7 cells per well (10,000/ml) were plated in triplicate in the 3D Tumorsphere Medium XF using 6-well suspension culture plates. Serial passage by enzymatic dissociation according 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.*

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.

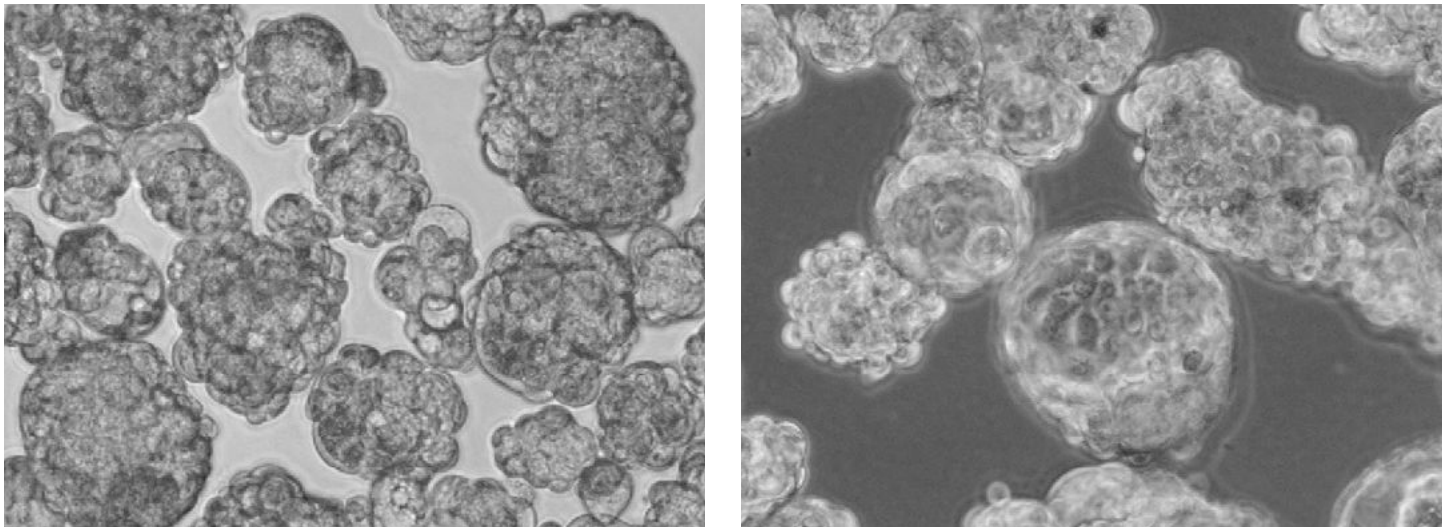

Fig. 2: Tumorsphere culture of MCF-7 mammary carcinoma cells in the 3D Tumorsphere Medium XF (C-28070) after 10 serial passages. *The tumorsphere culture was subjected to serial passage every 9 days by enzymatic dissociation according to the protocol. Robust tumorsphere formation was maintained during serial culture. See Fig. 1 for proliferation data.*

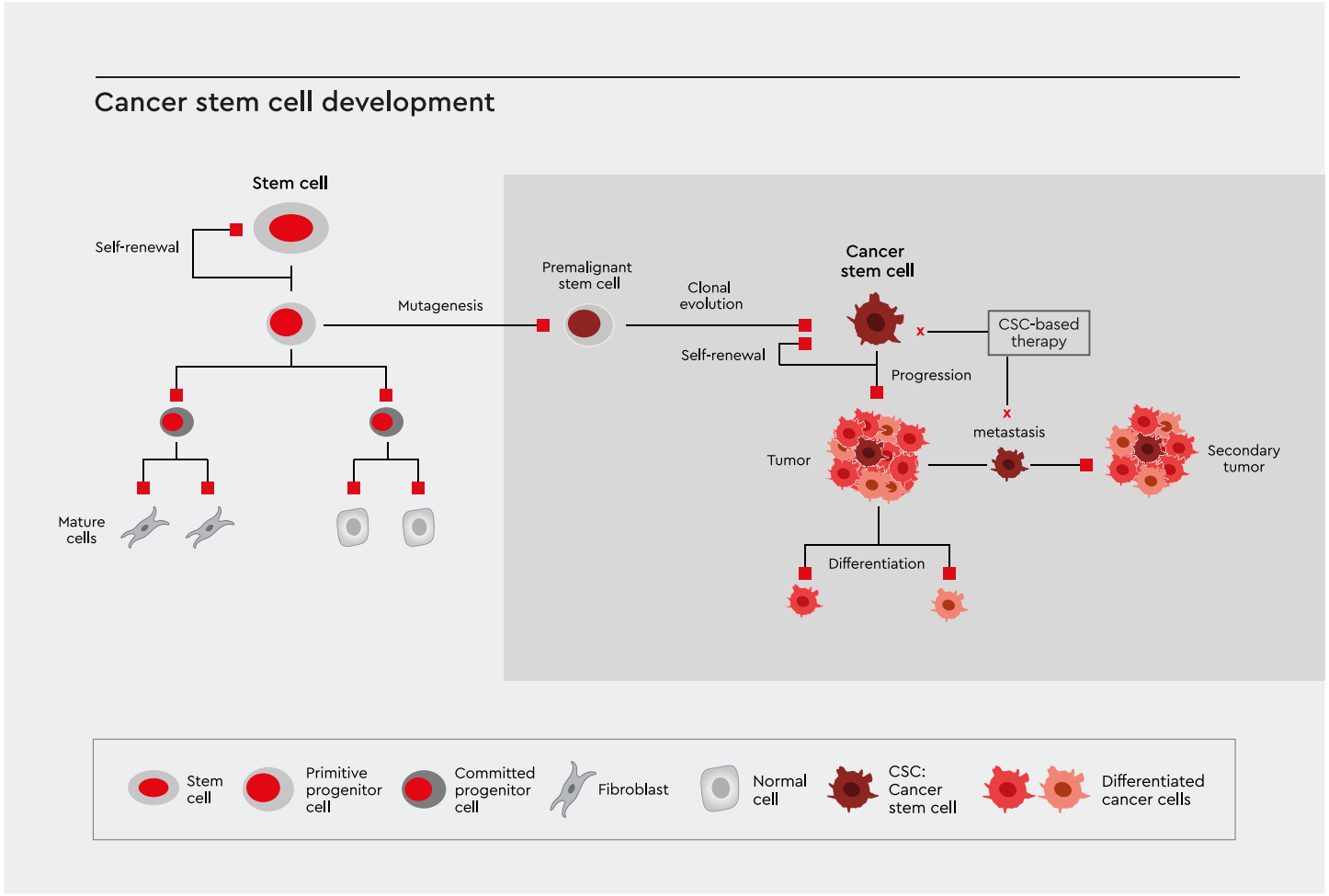

Fig. 3: Schematic overview on the origin, evolution and fate of cancer stem cells (CSC).

CSCs are self-sustaining 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.

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 CSC cell division from asymmetric to symmetric leading to elimin tion of the CSC population by blocking their self-renewal capabilities [6, 7]. For this to be achieved, detailed characterization is required.

Characterization of cancer stem cells

Various approaches have been undertaken to characterize CSCs based on marker detection. These include staining of intra- and extracellular molecules, as well as measuring the activity of certain cellular enzymes, such as aldehyde dehydrogenase (ALDH1) or small molecule transporters like the ABC transport system [8–10]. However, research is hindered by the heterogeneity of CSCs in different cancers [6, 11], as well as a lack of specificity, consistency and correlation of established markers with functional CSC features, such as tumorigenesis [6, 12]. Thus, a robust, reliable, and notably holistic, marker-based method for CSC detection and characterization has seemed a distant prospect. As a result, currently the greatest obstacle in CSC research is the isolation and purification of sufficient numbers of functional, homogenous CSC populations. At present, CSCs can only be defined experimentally by their ability to recapitulate generation of a continuously growing tumor [6].

To date, the most accepted strategies for the analysis of CSCs are generally based on the detection of their basic functional features. These include typical stem cell properties, such as self-renewal and pluripotency, as well as specific hallmarks of cancer, such as serially transferable tumorigenic potential and anoikis resistance. However, lack of a primary model system means that research is forced to rely on indirect readouts generated by alternative model systems and tests. The gold standard functional *in vivo* assay for CSCs is the serial transplantation into orthotopic sites of immunocompromised mice. However, this is laborious and results can be difficult to interpret. Established *in vitro* methods include assays for serial colony-forming units (CFU) in semi-solid media and label-retention as well as testing for the formation of serially passageable tumorspheres in a 3D suspension culture (see Figs. 2 and 4) [6]. Derived from neurosphere culture techniques of neural stem cells, the advantage of serial tumorsphere culture is the option for simultaneous determination of the sphere formation efficiency. This provides quantitative information on the temporal CSC ratio during culture on an exclusively functional basis.

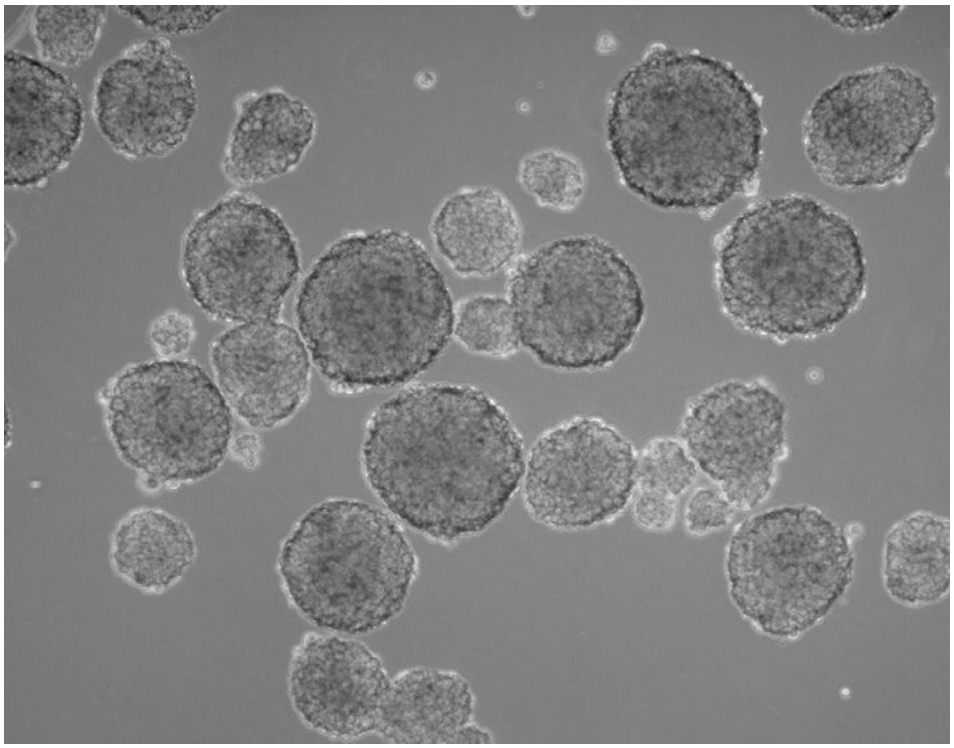

Fig. 4: Tumorsphere culture of HT1080 fibrosarcoma cells in the 3D Tumorsphere Medium XF (C-28070) after 10 serial passages. *The tumorsphere culture was subjected to serial passage every 6 days by enzymatic dissociation according to the protocol. Robust tumorsphere formation was maintained during serial culture.*

Tumorsphere culture protocol

I. Initiation of the tumorsphere culture

Materials

- 3D Tumorsphere Medium XF (C-28070 or C-28075)
- Phosphate Buffered Saline without Ca^{++}/Mg^{++} (PBS, C-40232)
- Detach-Kit (C-41210)
- 6-well Suspension Culture Plates (e.g., Greiner Bio One, No. 657 185)
- Adherently growing human cancer cells (for initial tumorsphere culture set-up)

Use aseptic techniques and a laminar flow bench.

1

Harvest the adherent cells

Detach the cells of a human CSC-containing adherently growing cancer cell line using your standard procedures. The cells should be 80–90% confluent and in good condition. Centrifuge the cell suspension for 5 minutes at 300 x g and aspirate the supernatant. Resuspend the cells in a small volume, e.g., 3–5 ml, of the PromoCell 3D Tumorsphere Medium XF.

2

Count the cells

Count the cells using your routine method and adjust the volume with 3D Tumorsphere Medium XF to obtain a concentration of 1 million cells/ml.

3

Set up the tumorsphere culture

Seed the cells in appropriate suspension culture vessels at 10,000 cells/ml, e.g., 40,000 cells in 4 ml of fresh 3D Tumorsphere Medium XF in each well of a 6-well suspension culture plate.

4

Allow the tumorspheres to grow

Incubate the culture for 4–10 days, depending on the cell type used. Add one-half of the culture volume of fresh 3D Tumorsphere Medium XF every 3–4 days. Do not change the medium.

5

Passage of the tumorsphere culture

The tumorspheres should be passaged (section B below) before they start to develop a dark center. Depending on the cell type used optimal passage should occur after 4–10 days.

Note: If a defined size range of tumorspheres is required, e.g., 100–200 µm, filters may be used for fast and easy sorting of tumorspheres according to size. Use a 200 µm cell strainer to filter the tumorspheres and collect the flow-through. Then, filter the flow-through again using a 70–100 µm cell strainer and flush back and collect the tumorspheres retained in the filter. The remaining fraction of tumorspheres will have a defined range of diameters from approx. 100 to 200 µm.

II. Serial passage of tumorsphere cultures

6

Collect the tumorspheres

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

7

Gravity sedimentation of the tumorspheres

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.

8

Wash the tumorspheres

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

9

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 B5 (below) must be determined empirically 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 according to the supplier`s instructions.

10

Break down remaining cell aggregates

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 as normal but tilt the pipet tip slightly at the bottom of the tube when expelling the cells. The shear forces generated facilitate the break up 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 as cell viability will be compromised. If in doubt, monitor the dissociation process microscopically. Non-dissociated cell aggregates may be removed by passing the cell suspension through a 40 µm cell strainer. When using recombinant trypsin use fresh 3D Tumorsphere Medium XF for inactivation instead of TNS.

11

Determine the cell number and viability

Make up to 5 ml with fresh 3D Tumorsphere Medium XF and determine the cell number and viability. Centrifuge the cells for 5 minutes at 300 x g. Discard the supernatant and resuspend the cells in fresh 3D Tumorsphere Medium XF at 1 million cells/ml.

Note: Alternatively, the cells may be resuspended in buffer, e.g., PBS without Ca^{++}/Mg^{++} plus 0.5% albumin plus 2 mM EDTA, and used for further experiments and/or analytical procedures.

12

Plate the Cells

Reseed the cells at 10,000 cells/ml in new suspension culture vessels. Typically, 6-well plates with 40,000 cells in 4 ml of medium per well are used.

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
Primary Cancer Culture System	250 ml	C-28081
Cancer Cell Line Medium XF	250 ml	C-28077
Dulbecco's PBS, without Ca^{++}/Mg^{++}	500 ml	C-40232
DetachKit	3 × 125 ml	C-41210

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