

TECHNICAL BULLETIN

TUMORSPHERE CULTURE OF HUMAN BREAST CANCER CELL LINES

Sandra Dunn, PhD, Consultant • Jennifer Law, MSc, Consultant • Poh Tan, PhD, Product Manager, Epithelial & Endothelial Cell Biology

INTRODUCTION

The idea of cancer stem cells was first introduced about forty years ago, when it was shown that leukemia cells had distinct sub-populations with varying proliferation rates. Since that time, the cancer stem cell field has grown and two cancer stem cell models have emerged. One model hypothesizes that a specific subset of cells within a tumor population is tumorigenic (i.e. cancer stem cells). The other model hypothesizes that due to the heterogeneity of cells and their activities found in cancer, only some cells are inherently tumorigenic and may exhibit stem cell-like qualities.¹

Clarke *et al.* reported the existence of breast cancer stem cells which can be isolated using cell surface markers and functionally assessed *in vivo* by demonstrating *de novo* tumor formation.² In addition, it has been demonstrated that primary human breast stem/progenitor cells can be enriched in non adherent mammospheres,³ a concept adopted from the neural stem cell culture of neurospheres.⁴ Recently, it was shown that human breast cancer cell lines were also capable of generating non adherent spheres termed tumorspheres.⁵ ⁶ Of these, certain breast cancer cell lines contain a sub-population with stem cell-like properties that are also enriched in tumorspheres and,^{5, 6} because primary human breast cancer tissues are a challenge to obtain and even to culture, they are commonly used as a tool to evaluate breast cancer stem cell functionalities. This technical bulletin describes a method to culture breast cancer cell lines as tumorspheres using MammoCult® medium from STEMCELL Technologies.

PROTOCOL: TUMORSPHERE CULTURE OF BREAST CANCER CELL LINES*

Prior to mammosphere culture, breast cancer cell lines are grown as monolayers according to the supplier's recommendations on tissue culture-treated 10 cm dishes.

Ensure that cells cultured as monolayers are healthy and are 80 – 90% confluent. Over-grown cultures will have increased cell death and low quality cells.

1. On Day 1 of the mammosphere assay, remove one 80 – 90% confluent dish of cells from the incubator.
2. Aspirate culture medium from the dish. Rinse with 5 mL of warm Hank's Balanced Salt Solution (HBSS; Catalog #37250) to remove excess culture medium from the plate. Rinse twice.
3. Using a sterile cell scraper [BD Falcon, 1.8 cm blade; BD Catalog #353085], scrape cells gently from the dish. *Note: Do not trypsinize cells at this stage as this could reduce your tumorsphere forming efficiency.*
4. Resuspend scraped cells in 10 mL of complete MammoCult® medium (complete MammoCult® medium contains the MammoCult® Basal Medium (Catalog #05621), MammoCult® Proliferation Supplement, fresh Hydrocortisone (Catalog #07904) and Heparin (Catalog #07980) and transfer to a 15 mL conical tube with a pipette. *Note: Please refer to the product information sheet for details on how to prepare MammoCult® medium: <http://www.stemcell.com/technical/05620-PIS.pdf>*
5. Centrifuge the cells at 500 x g for 3 minutes at room temperature (15°C – 25°C).
6. Remove the tube from the centrifuge carefully, ensuring that the cell pellet is not disturbed. Carefully aspirate and discard the supernatant with a pipette.

*MDA-MB-231, MCF7, SKBR3, AU565, BT474, SUM149



WWW.STEMCELL.COM

29936 | VERSION 1.0.0 | AUGUST 2009

IN NORTH AMERICA

TOLL-FREE T. 1 800 667 0322 TOLL-FREE F. 1 800 567 2899

T. 1 604 877 0713 F. 1 604 877 0704 E. INFO@STEMCELL.COM

IN EUROPE

T. +33 (0)4 76 04 75 30 F. +33 (0)4 76 18 99 63 E. INFO.EU@STEMCELL.COM

IN AUSTRALIA

TOLL-FREE T. 1 800 060 350 F. +61 (03) 9338 4320 E. INFO.AUS@STEMCELL.COM

IN SINGAPORE

T. (65) 6776 7754 F. (65) 6776 7114 E. INFO.SG@STEMCELL.COM

- Resuspend the cell pellet into a single cell suspension with a 5 mL pipette in 2 mL of complete MammoCult® medium.
- Prepare a dilution of cells to enumerate cell concentration. Please refer to the cell counting protocol for detailed instructions on enumerating cells with Trypan Blue (Catalog #07050).
- Resuspend a pre-determined amount of cells in 2 mL of complete MammoCult® medium (in triplicate) in each well of a 6 well ultra-low adherent plate (Catalog #27145). The seeding density for cell lines is typically in the range of 5,000 – 20,000 cells per well of a 6 well plate.

Note: We recommend optimizing the seeding density for each cell line of interest.

- Recommended seeding densities for commonly used cell lines are shown below:

CELL LINE	SEEDING DENSITY (6-WELL PLATE*)
AU565	20,000 cells/well
BT474	5,000 cells/well
MDA-MB-231	5,000 cells/well
MCF7	40,000 cells/well
SKBR3	40,000 cells/well
SUM149	20,000 cells/well

*Ultra-low adherent culture plates

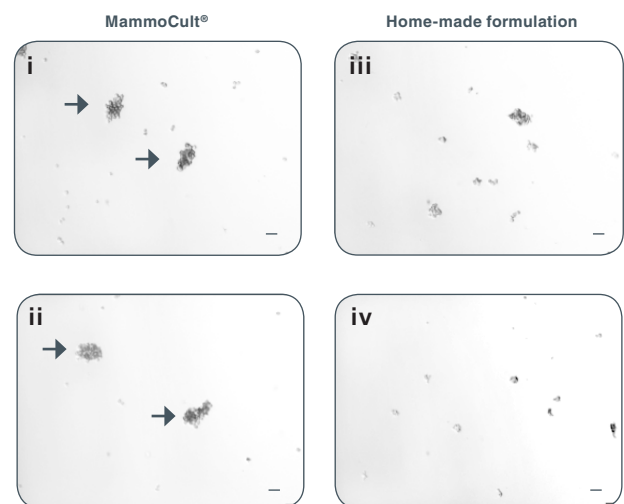
- Incubate cultures in a 5% CO₂, humidified incubator at 37°C for 7 days.
- Count the number of tumorspheres that are larger than 60µm in size. The shape and appearance of the spheres is cell line-dependent. For example, tumorspheres will appear solid and will form perfectly round spheres (SUM149, BT474, MCF7) or looser and less rounded spheres (MDA-MB-231, AU565, SKBR3). See Figure 1A and Figure 2A.

Note: To passage spheres, refer to the tumorsphere passaging protocol.

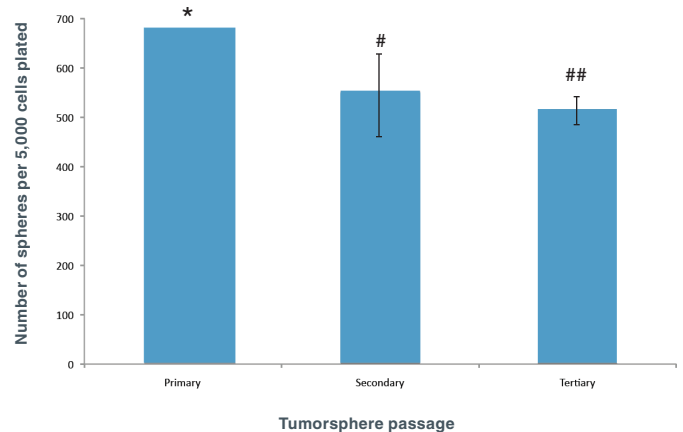
FIGURE 1: MammoCult® supports the formation of MDA-MB-231 tumorspheres in culture. Each well was seeded with 5,000 cells per well and cultured for 7 days before imaging and counting. MDA-MB-231 is an invasive breast cancer cell line and will form more loose and less rounded tumorspheres (→). A. MammoCult® supports sphere formation of MDA-MB-231 cells better (i,ii) than a widely used home-made formulation (iii,iv). B. Reproducible passages of secondary (#) and tertiary (##) spheres are cultured in MammoCult® medium.

*Spheres were too numerous to count (> 500 spheres per field of view). Scale bar = 140 µm. Tumorsphere photos and data courtesy of Dr. S. Dunn and J. Law, CFRI, Vancouver, Canada.

A. MDA-MB-231 Tumorspheres



B. Reproducible MDA-MB-231 Tumorspheres in MammoCult®

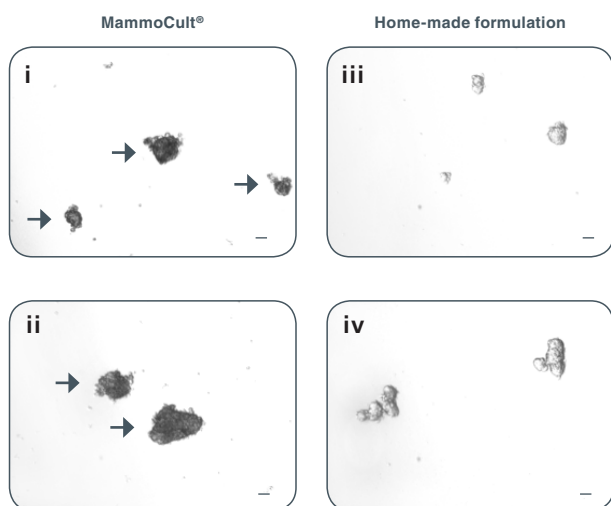


TECHNICAL BULLETIN

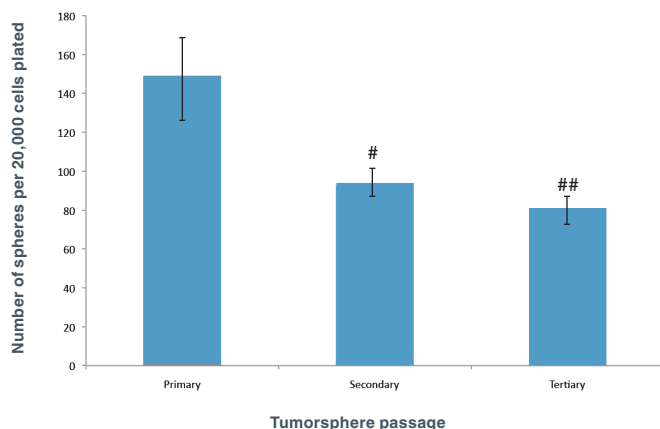
TUMORSPHERE CULTURE OF HUMAN BREAST CANCER CELL LINES

FIGURE 2: Better quality of SUM149-derived tumorspheres cultured in MammoCult® compared to a widely used home-made formulation. Each well was seeded with 20,000 cells per well and cultured for 7 days before imaging and counting. A. SUM149 cells form tight mammospheres more efficiently in MammoCult® (→) (i,ii) than in the widely used home-made formulation (iii,iv). B. Reproducible passages of secondary (#) and tertiary (##) spheres are cultured in MammoCult® medium. Scale bar = 140 µm. Tumorsphere photos and data courtesy of Dr. S. Dunn and J. Law, CFRI, Vancouver, Canada.

A. SUM149 Tumorspheres



B. Reproducible SUM149 Tumorspheres in MammoCult®



PROTOCOL: PASSAGING TUMORSPHERES

1. Harvest tumorspheres after 7 days in culture. Collect the entire culture into a 50 mL conical tube and centrifuge at 350 x g for 5 minutes.

Note: Tumorspheres should be passaged when they are ~60 µm in diameter and before they develop a dark center. Depending on the number of spheres present in each well, it may be necessary to pool multiple wells to obtain maximum number of tumorspheres for subsequent passages.

2. Aspirate as much supernatant as possible without disturbing the pellet. *Note: The pellet may be very loose.*
3. Add 0.5 – 1 mL of pre-warmed Trypsin-EDTA (Catalog #07901). Use a P1000 pipette with a sterile plastic tip and set the volume to slightly less than the approximate volume of the remaining medium (for example: if volume of remaining medium is 800 µL, set the volume of the pipettor to 700 µL to avoid creating bubbles). Pre-wet the tip with complete MammoCult® medium to reduce cells sticking inside the tip.
4. Triturate tumorspheres by slightly tilting the tip and pressing it against the bottom or side of the tube to generate resistance in order to break up the tumorspheres. Rinse the side of the tube during trituration to remove the remaining spheres that are attached to the side of the tube.

If some tumorspheres are not dissociated after 1.5 minutes (this usually occurs at later passages), trituration can be extended to a maximum of 2 minutes.

5. Add 5 mL of cold HBSS (Catalog #37250) + 2% FBS (Catalog #06100) and centrifuge the cell suspension at 350 x g for 5 minutes.
6. Aspirate the supernatant and resuspend the pellet in 0.5 mL of complete MammoCult® medium (complete MammoCult® medium contains the MammoCult® Basal Medium (Catalog #05621), MammoCult® Proliferation Supplement (Catalog #05622), fresh Hydrocortisone (Catalog #07904) and Heparin (Catalog #07904)) and perform a viable cell count using Trypan Blue (Catalog #07050). To perform a viable cell count refer to the cell counting protocol.

Complete MammoCult® medium allows for the generation of subsequent tumorspheres. See Figure 1B and 2B.

TECHNICAL BULLETIN

TUMORSPHERE CULTURE OF HUMAN BREAST CANCER CELL LINES

PROTOCOL: CELL COUNTING

Use a hemacytometer that has a 0.100 mm depth chamber (eg. Neubauer hemacytometer).

TRYPAN BLUE EXCLUSION

Trypan Blue stains dead cells but does not stain live cells. The dye enters cells with compromised membranes to stain them blue. Thus, live and dead cells can be distinguished by their ability to exclude the blue dye.

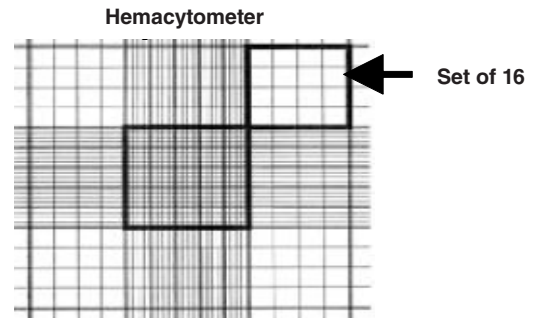
Ensure that the cell suspension to be counted is completely re-suspended. Before the cells settle, place a suitable volume of a cell suspension (20-200 μ L) in a centrifuge tube.

1. Add an equal volume of 0.4% Trypan Blue (Catalog #07050) and gently mix. Incubate the mixture for 5 minutes at room temperature (15°C – 25°C). Resuspend the cell mixture after incubation.
2. Prepare a hemacytometer by first cleaning the chamber surface with 70% ethanol. Wipe dry. Position the cover-slip over the chambers.
3. Using a P20 pipette, place 10 μ L of stained cells into the hemacytometer chamber.
4. Using a hand tally counter, count the number of viable (unstained) cells in an area of 16 squares. When counting, always count only live (bright) cells that look healthy.
5. Move the hemacytometer to another set of 16 squares and carry on counting until all 4 sets of 16 corner squares are counted. See Figure 3.
6. Total the cell count from all 4 sets of 16 corner squares. Divide this number by 4 to find the average number. Multiply the average number by 2 (to adjust for the 1:2 dilution factor with trypan blue). Then, multiply by 1×10^4 to obtain the concentration of cells per mL.

Example: If the total cell count from all 4 sets of squares is 350 and the dilution factor is 2, then the concentration of cells is:

$$350/4 \times 2 \times (1 \times 10^4) = 1.75 \times 10^6 \text{ cells per mL.}$$

FIGURE 3: A sketch of the grid seen on a hemacytometer. Cells are counted in the four sets of 16 squares at each corner of the grid.



REFERENCES

1. Charafe-Jauffret E, *et al.* Pathobiology. 75: 75 – 84, 2008
2. Al-Hajj M, *et al.* PNAS. 100: 3983 – 3988, 2003
3. Dontu G, *et al.* Genes and Dev. 17: 1253 – 1270, 2003
4. Reynolds BA, *et al.* Science. 255: 1707 – 1710, 1992
5. Fillmore CM, *et al.* Breast. Can Res. 2:R25. Epub 2008
6. Charafe-Jauffret E, *et al.* Cancer Res. 69: 1302 – 1313, 2009

PRODUCT INFORMATION

PRODUCT	CATALOG #	QUANTITY
MammoCult® Medium	05620	500 mL
Ultra-Low Adherent Dishes (6-well plates)	27145	5 plates
Heparin	07980	2 mL
Hydrocortisone	07904	100 mg
Trypan Blue	07050	100 mL
HBSS	37250	500 mL