Supplemental Table 1

Cost comparison for spheroid assays.

Dye/Kit	Manufacturer (catalog #)	# of assays/kit	Price/kit in US $	US $/1000 assays	Recommended use/well	Ref.
Acridine Orange	Thermo Fisher Scientific (A3568)	1×10^7	70	**0.007**	0.01 µg	This study
AQUEOUS MTS Reagent	Promega (G1111)	2.5×10^4	922	**36.88**	20 µl MTS (3.9 mM)	[1]
LIVE/DEAD viability/cyto-toxicity kit	Thermo Fisher Scientific (L3224)	8000 – 10,000	400	**40 – 50**	5 µl Calcein AM (8 µM) and 5 µl EthD-1 (16 µM)	[2]
WST-1 reagent	Roche (11644807001)	2500	468	**187.2**	10 µl	This study
CellTiter-Glo Luminescent Cell viability assay	Promega (G9683)	1000	418	**418**	100 µl	[3]
Cultrex 3D Spheroid Fluorometric Assay	Trevigen (3510-096-K)	250	295	**1180**	10 µl	

1. **Hoffmann, O.I., C. Ilmberger, S. Magosch, M. Joka, K.W. Jauch, and B. Mayer.** 2015. Impact of the spheroid model complexity on drug response. J Biotechnol *205*:14-23.
2. **Hsiao, A.Y., Y.C. Tung, X. Qu, L.R. Patel, K.J. Pienta, and S. Takayama.** 2012. 384 hanging drop arrays give excellent Z-factors and allow versatile formation of co-culture spheroids. Biotechnol Bioeng *109*:1293-1304.
3. **Vinci, M., S. Gowan, F. Boxall, L. Patterson, M. Zimmermann, W. Court, C. Lomas, M. Mendiola, et al.** 2012. Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. BMC Biol *10*:29.

Preparation and Analysis of Acridine Orange Stained Neurospheres

PROTOCOL FOR:

A Simple, Low-Cost Staining Method for Rapid-Throughput Analysis of Tumor Spheroids

Frank Eckerdt[1, *], Angel Alvarez[2], Jonathan Bell[1], Constadina Arvanitis[3], Asneha Iqbal[1, 4], Ahmet D. Arslan[1], Bo Hu[2], Shi-Yuan Cheng[2], Stewart Goldman[1, 4], and Leonidas C. Platanias[1, 5]

[1]*Robert H. Lurie Comprehensive Cancer Center and Division of Hematology- Oncology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA*

[2]*Department of Neurology, Robert H. Lurie Comprehensive Cancer Center, Northwestern Brain Tumor Institute, Northwestern University Feinberg School of Medicine, Chicago, IL USA*

[3]*Center for Advanced Microscopy and Department of Pathology, Northwestern University Feinberg School of Medicine, Chicago, IL USA*

[4]*Division of Hematology and Oncology, Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, IL USA*

[5]*Department of Medicine, Jesse Brown VA Medical Center, Chicago, IL, USA*

[*]*Address correspondence to: Frank Eckerdt, Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, 303 East Superior Street, Lurie 3-220, Chicago, IL 60611, Email: frank.eckerdt@northwestern.edu*

BioTechniques Vol#:pp-pp (Month Year)

LEGEND

REAGENTS

DMEM/F12 (Thermo Fisher Scientific, Grand Islands, NY catalog # 10565-018)
B27 Supplement (Thermo Fisher Scientific, Grand Islands, NY catalog # 17504-044)
Heparin (Sigma-Aldrich, St. Louis, MD catalog # H3149)
EGF (20 ng/ml, Peprotech, Rocky Hill, NJ catalog # AF-100-15)
basic FGF (20 ng/ml, Peprotech, Rocky Hill, NJ catalog # 100-18B)
TrypLE Express (Thermo Fisher Scientific, Grand Island, NY catalog # 12604-013)
Acridine Orange (Thermo Fisher Scientific, Grand Island, NY catalog #A3568)

PROCEDURE

GENERATION AND SEEDING OF NEUROSPHERE CELLS:

1. Trypsinize adherent growing U87 cells, collect in PBS and centrifuge at 1000 rpm for 5 min.
2. Resuspend in PBS and count cells.
3. Seed 4,000,000 cells into 75 cm^2 low adhesion flask, (Greiner bio-one, cat. # 658195) in 12 ml cancer stem cell medium (CSC medium).
4. Allow neurosphere formation for > 4 days, replace medium every other day. Resulting neurospheres can be cultured for up to eight passages.
5. Prepare round bottom 96-well plates (96 Well Suspension Culture Plate from Greiner bio-one, cat #650185) with 50 μl CSC medium per well.
6. Dissociate U87 neurospheres with TrypLE Express into single cells, centrifuge at 1000 rpm for 5 min and resuspend in 1 ml PBS.
7. Subject cells to flow cytometry using a BD FACSAria2 Special Order Research Product (SORP) instrument (San Jose, CA) in a biosafety cabinet and sort cells by forward-scattered light (FSC) vs. side-scattered light (SSC). Seed 1000 cells/well into the round bottom 96-well plates prepared in step 5.
8. Add 50 μl of CSC medium with or without 2X inhibitors, resulting in a total volume of 100 μl CSC medium per well.
9. Incubate at 37˚C in a CO_2 incubator for 14 days.

*This protocol is not limited to U87 cells and can be used for most adherent growing cell lines (composition of CSC medium might need to be adjusted for non-neuronal cell lines). In case of highly “sticky” cells, Ultra Low Attachment 96-well round bottom plates (Corning, cat. #7007) can be used to facilitate sphere formation. Also, other Cell Sorting Instruments can be used for sorting cells.

ACRIDINE ORANGE STAINING OF NEUROSPHERES:

10. To each well add 1 μl of Acridine Orange (10 μg/ml) for 1 hour at 37˚C in a CO_2 incubator.

Cells can remain with acridine orange overnight at 37˚C in a CO_2 incubator for analysis on the next day.

IMAGING AND ANALYSIS OF NEUROSPHERES

11. If using NIS-Elements software and a 10x air PlanApo objective, Image Acquisition is set to "Capture Large Images Setting" using the 3 x 3 tile function (resulting image size is 2282.35 x 2282.35 μm).
12. General Analysis Parameters include:
    Threshold: ≥ 300. (Threshold should be adjusted according to the fluorescence intensity of the cell line.) Size: ≥ 100, Circularity: ≥ 0.12
13. Using these settings, image capture and data collection will require 5 minutes, 18 seconds.

RECIPES

Cancer Stem Cell (CSC) medium (511.3 ml)

Component	Vulume (ml)	[final]
DMEM/F12	500 ml	1 x
B27 Supplement (50x)	10 ml	1 x
Heparin (25 mg/ml)	100 μl	5 μg/ml
EGF (0.1 mg/ml)	100 μl	20 ng/ml
bFGF (0.1 mg/ml)	100 μl	20 ng/ml
Gentamicin (50 mg/ml)	1 ml	0.1 mg/ml

* Stock solution for EGF is prepared in ddH_2O with 1 % BSA, for bFGF in 5 mM Tris pH 7.6, 0.1%BSA. Instead of Gentamicin, Pen Strep (1%, Thermo Fisher Scientific, Grand Islands, NY) can be used.