CELLTITER-GLO® LUMINESCENT CELL VIABILITY ASSAY: APPLICATION FOR VIABILITY STUDIES OF CELLS GROWN IN AGAROSE

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This article describes a method for using the CellTiter-Glo® Luminescent Cell Viability Assay to assess the viability of tumor cells embedded and treated in agarose.

Introduction

Analyzing human tumor cell response to treatment in situ can be difficult if the tumor sample is derived from a biopsy that includes a margin of normal tissue. Normal, adherent-dependent cell types, such as fibroblasts, often outgrow the tumor cells when disaggregated biopsy cells are expanded on plastic substrates for extended periods. This undesirable growth of normal cells can be abrogated by expanding the tumor cells in agarose—a situation that more closely mimics the tumor microenvironment. However, in order to assess tumor cell response to treatment, a sensitive cell viability assay is required that functions with cells embedded in agarose. Oncotech currently uses the EDR® Assay, an in vitro drug resistance assay that identifies, with 99% accuracy, patients who will not respond to a cancer therapeutic. The EDR® Assay excludes agents unlikely to be clinically effective, resulting in decreased side effects, improved response rates and prolonged survival of cancer patients (1,2). The EDR® Assay assesses tumor cell growth (scored by radiolabel incorporation into nascent DNA) in the presence of chemotherapeutic agents. The five-day controlled-exposure assay is performed in 24-well plates with cells embedded in agarose and scored using a proprietary algorithm. In an effort to improve assay scale and throughput and eliminate the use of radioactivity, the CellTiter-Glo® Assay protocol was modified to assess tumor cell growth.

The adapted CellTiter-Glo® Assay protocol described here reflects an improved method to evaluate tumor cell viability.

The CellTiter-Glo® Luminescent Cell Viability Assay[a,b] is a homogeneous method for determining the number of viable cells in culture. Detection is based on use of the luciferase reaction to measure the amount of ATP in viable cells. The CellTiter-Glo® Reagent does three things upon addition to cells. It lyses cell membranes to release ATP, it inhibits endogenous ATPases, and it provides the luciferin and luciferase necessary to measure ATP using a bioluminescent reaction. The assay can detect as few as 15 cells in culture medium, and although temperature equilibration of assay plates is recommended for consistency, the assay can be performed rapidly. The luminescent signal can be detected as soon as 10 minutes after adding reagent and is stable for many hours, providing excellent flexibility for batch processing of plates.

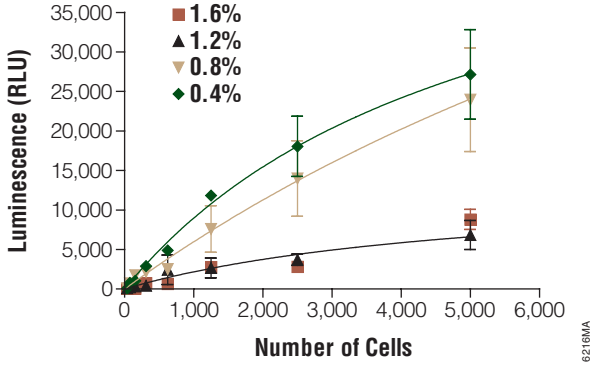

Figure 1. Cell viability analysis based on SKOV-3 cell titrations in the presence of decreasing agarose concentrations.

Here we describe a method using the CellTiter-Glo® Assay adapted to assess viability of tumor cell lines embedded and treated in agarose in a 96-well format.

Materials and Methods

Reagents and Cell Culture. Cell lines (SKOV-3, OVCAR-5 and OVCAR-8) were obtained from SAIC-Frederick, Inc. Cancer specimens were obtained from surgical specimens sent for routine Oncotech EDR® Assay testing. Viable, fresh remnant tumor cells or cryopreserved cells from cases received for the EDR® Assay were collected in accordance with an IRB-approved protocol. This protocol assures that specimens are unlinked from private patient identifiers such as name, social security number, address, and referring physician. Once unlinked, such specimens are considered exempt from 21 CFR parts 50 and 56 regarding Subject Informed Consent. RPMI-1640 cell culture medium and Fetal Bovine Serum (FBS) were obtained from Cambrex. Trypsin EDTA was obtained from Irvine Scientific, and Dulbecco's Phosphate Buffered Saline (DPBS) was purchased from Mediatech, Inc. Cells were grown in 96-well optical-bottom black plates (Nunc™) containing defined medium or medium overlaid on SeaPlaque® low-melting-temperature agarose (Cambrex).

Doxorubicin HCl was obtained from Calbiochem. Cells were cultured in complete medium plus 10% FBS and maintained at 37°C under 5% CO_2. Routine passaging and preparation of cells included aspiration of medium, one equal volume wash with 1X DPBS, aspiration, trypsin-dependent release of cells (1/5 volume), dilution into medium containing 10% FBS, centrifugation and subsequent resuspension and plating. Cells were counted using standard hemacytometer techniques.

Experimental Treatment in Medium. For experiments performed in plastic plates (requiring adhesion of cells to the bottom surface of individual wells), each well of a 96-well plate was typically seeded with 3,000 cells delivered in 180µl of complete medium containing 10% FBS. Cells were allowed to attach for 24 hours prior to treatment.

Agents, such as Doxorubicin, were resuspended in DMSO to generate 20mM stocks, and aliquots were stored at –20°C. Agent titrations were performed immediately before addition to the cells. For example, Doxorubicin was diluted serially in DMSO, linearly into aqueous 1X DPBS, and finally added to the medium in each well. Twenty microliters of Doxorubicin or control (1X DPBS) was added per well, generating a total assay volume of 200µl.

Experimental Treatment in Agarose. For experiments performed in agarose, the above procedure was modified as follows. A volume of 50µl of a 1.6% agarose solution (dissolved in complete RPMI-1640 medium without FBS) was dispensed into each well and allowed to solidify at room temperature for 15 minutes. Three thousand cells diluted in 25µl medium were then thoroughly mixed with 25µl of the appropriate 2X agarose stock solution (dissolved in complete RPMI-1640 without FBS) and rapidly dispensed into each well. The solution was allowed to solidify at room temperature for 15 minutes. Complete medium (80µl) containing FBS was then added to each well, and plates were incubated for 24 hours prior to treatment as previously described.

CellTiter-Glo® Assay to Assess Cell Viability. After 24 to 48 hours of exposure to Doxorubicin (or control), the CellTiter-Glo® Luminescent Cell Viability Assay was performed as directed in Technical Bulletin #TB288. Briefly, 100µl of medium was removed from the wells of the plates containing medium, and 100µl of CellTiter-Glo® Reagent was added. From plates containing agarose, essentially all of the liquid (80µl of medium overlay plus 20µl of treatment agent) was removed by gentle multichannel pipet aspiration, and 100µl of CellTiter-Glo® Reagent was added. Plates were rocked for two minutes and incubated an additional eight minutes or longer before reading luminescent output on a BioTek Synergy™ plate reader. Sensitivity was adjusted from 175 to 250 for all reads.

Results

The CellTiter-Glo® Assay was successfully employed to assess drug-dependent cytotoxicity in tumor cells embedded in agarose. Initially we observed lower signals in agarose. This could be due to the reduced ability of the CellTiter-Glo® Assay Reagent to penetrate the agarose and lyse the cells. Or, this may also accurately reflect the restricted growth of the adherent-dependent cell types in most biopsies. Therefore we first addressed this issue by varying the final agarose concentration in the assay. We established experimentally that a "plug" of agarose below the cells was beneficial in separating cells from the plastic substrate (data not shown). We further observed that higher concentrations of agarose in the plug reduced noise in the assay (data not shown). All experiments reported here use a 1.6% agarose plug as described in the Materials and Methods section. We subsequently determined the optimal concentration of agarose in which the cells were embedded. Figure 1 depicts an initial viability analysis in which the SKOV-3 cancer cell line was titrated in 0.4%, 0.8%, 1.2% or 1.6% agarose.

In additional experiments, lower concentrations of agarose did not improve the overall assay signal. In fact, signal-to-noise ratios decreased at agarose concentrations below 0.4% as demonstrated in Figure 2. Thus, 0.4% was determined to be the optimal agarose concentration in which to embed cells. We then examined additional cell lines in the CellTiter-Glo® Assay on plastic and in 0.4% agarose to establish the reproducibility of Doxorubicin-dependent growth inhibition.

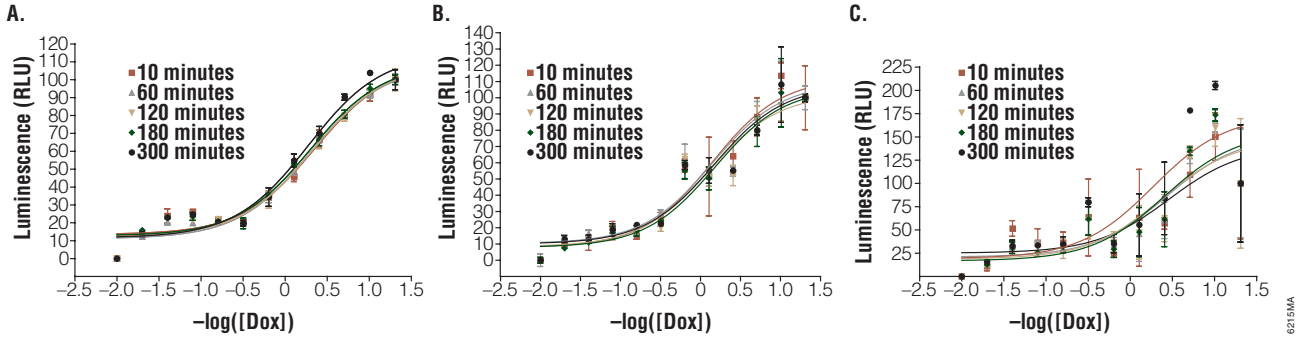

Figure 2. Doxorubicin dose responses in OVCAR-8 cells grown on plastic substrate (Panel A) and in 0.4% (Panel B) and 0.2% (Panel C) agarose.

Luminescent Viability Assay for Cells Grown in Agarose

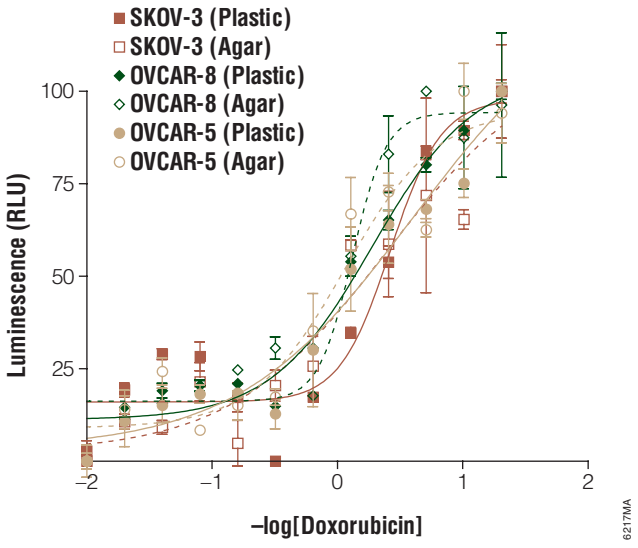

Figure 3. Doxorubicin dose responses in ovarian cancer cell lines grown on plastic substrate versus agarose. The CellTiter-Glo® Assay was performed on three ovarian cancer cell lines plated on plastic substrate or in 0.4% agarose and exposed to varying concentrations of Doxorubicin as described in Materials and Methods.

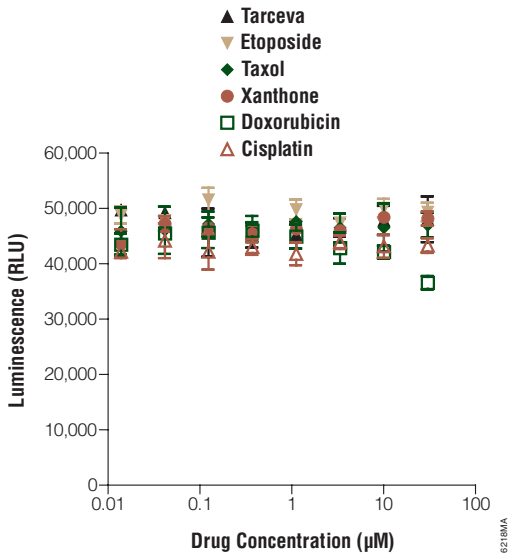

Figure 4. Doxorubicin has no significant inhibitory effect on luciferase activity. OVCAR-8 cells were used to provide cellular ATP. The drugs were diluted as described in Materials and Methods, and 80µl of each aqueous dilution were delivered to a 96-well plate containing 3,000 OVCAR-8 cells in a volume of 20µl. One hundred microliters of CellTiter-Glo® Reagent were added immediately, and the assay was performed as described in Technical Bulletin #TB288.

The luminescent signal obtained in the presence of agarose is considerably lower than that on plastic (Figure 3). Increasing the incubation period after addition of the CellTiter-Glo® Assay Reagent did not significantly alter signal intensity or background. Interestingly, EC_{50} values (the extrapolated effective concentration at which growth of 50% of the cells is inhibited) for Doxorubicin in 0.4% agarose were consistently lower compared to Doxorubicin in 0.2% agarose and plastic (see Table 1). To assess potential drug-dependent direct inhibition of luciferase activity, each chemotherapeutic agent was titrated against CellTiter-Glo® Reagent (Figure 4). Only Doxorubicin exhibited an inhibitory effect in the assay, demonstrating approximately 10% inhibition at the highest concentration (30µM). None of the other chemotherapeutic agents showed significant inhibitory effects on luciferase. Thus the CellTiter-Glo® Assay is compatible with many chemotherapeutic agents even at considerably high concentrations.

Because the assay was developed to test disaggregated cells derived from tumor biopsies, the CellTiter-Glo® Assay was used in conjunction with the optimized conditions described above to assess tumor cell drug response in three tumors with three drugs (Doxorubicin, Etoposide and CiSplatin) in triplicate. In order to correlate the results of this experiment with those of the previously determined in-house EDR® Assay, cells were embedded and incubated in agarose for 48 hours prior to exposure to the drugs. The CellTiter-Glo® Assay was

Table 1. EC_{50} Values for Doxorubicin for Cells Grown in Agarose and on Plastic.

Cell Line and Condition	10 minutes	60 minutes	120 minutes	180 minutes	300 minutes	Mean	S.D.
OVCAR-5 on plastic	2.14	2.12	1.89	1.87	1.59	1.89	0.22
OVCAR-5 in 0.4% agarose	0.28	0.53	0.55	0.48	0.53	0.53	0.11
OVCAR-5 in 0.2% agarose	3.36	1.39	0.72	0.61	1.73	1.39	1.11
OVCAR-8 on plastic	2.25	2.02	2.12	2.00	1.88	2.02	0.14
OVCAR-8 in 0.4% agarose	1.39	1.41	1.29	1.50	1.48	1.41	0.08
OVCAR-8 in 0.2% agarose	2.86	2.73	2.34	2.56	1.87	2.56	0.39
SKOV-3 on plastic	4.39	4.52	3.68	4.28	4.64	4.39	0.37
SKOV-3 in 0.4% agarose	1.39	1.41	1.29	1.50	1.48	1.41	0.08
SKOV-3 in 0.2% agarose	N.A.	0.68	0.64	0.41	0.34	0.53	0.17

Luminescent Viability Assay for Cells Grown in Agarose

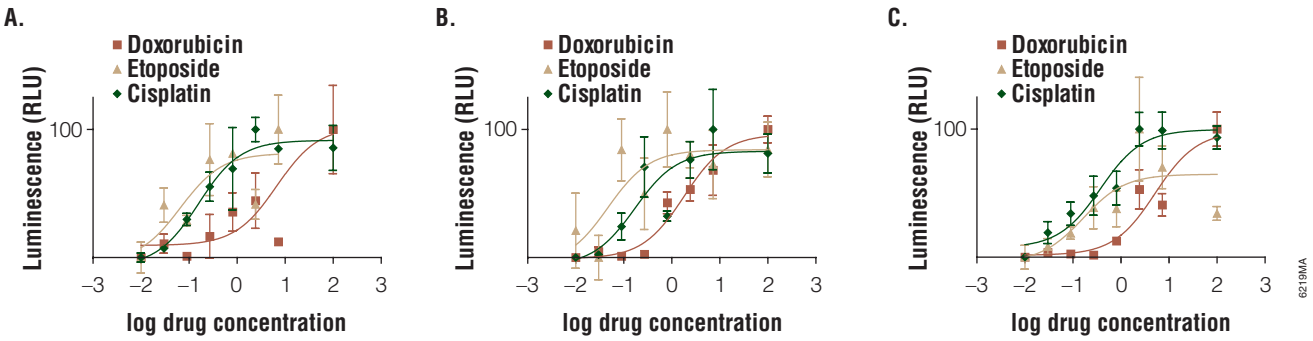

Figure 5. Doxorubicin, Etoposide and CiSplatin dose responses in disaggregated human ovarian tumor cells grown in 0.4% agarose and exposed to varying concentrations of the drugs. Panel A. OVA 001 cells. **Panel B**. OVA 002 cells. **Panel C**. OVA 003 cells. See Materials and Methods for experimental details.

Table 2. Dose Response of Ovarian Tumor Cells Obtained with the CellTiter-Glo® Assay and the EDR® Assay.

	Doxorubicin (DOXIL)			Etoposide (VPI6)			Cisplatin (CPLAT)		
	CellTiter-Glo® Assay	EDR® Assay		CellTiter-Glo® Assay	EDR® Assay		CellTiter-Glo® Assay	EDR® Assay	
Sample	EC_{50}	Status	Percent Inhibition @ 0.17µM	EC_{50}	Status	Percent Inhibition @ 0.17µM	EC_{50}	Status	Percent Inhibition @ 0.17µM
OVA 001	6.66	LDR	78.8	0.067	LDR	90.40	0.14	LDR	95.1
OVA 002	1.80	LDR	91	0.047	LDR	90.30	0.08	LDR	80.1
OVA 003	5.18	LDR	87.1	0.163	LDR	93.50	0.39	LDR	79.1

performed after five days of exposure to the drugs. The data were plotted as dose response curves (Figure 5).

The ovarian tumor specimens behaved similarly in the assay exhibiting similar drug-dependent dose response curves. Each of these tumors was reported as LDR (Low Drug Resistant) in the EDR® Assay for this set of three drugs (Table 2).

Summary

The results for both ovarian tumor cell lines and disaggregated tumor biopsies embedded and treated in 0.4% agarose conclusively demonstrate that the CellTiter-Glo® Assay readily and rapidly reports cytotoxicity in such cultures in a reproducible and reliable manner. The adapted CellTiter-Glo® Assay protocol described here reflects an improved method to evaluate tumor cell viability in an environment that more closely resembles that of the native tumor and that selectively excludes growth of adherent dependent cell types present in most biopsies. Furthermore, preliminary comparisons suggest good correlation between the EDR® and the CellTiter-Glo® assays. Additional experiments to supplement and reinforce this correlation are currently under way.

References

1. Kern, D.H. and Weisenthal, L.M. (1990) *J. Natl. Cancer Inst.* **82**, 582–8.
2. Mehta, R.S. *et al.* (2001) *Breast Cancer Res. Treat.* **66**, 225–37.

Protocol

CellTiter-Glo® Luminescent Cell Viability Assay Technical Bulletin #TB288
(www.promega.com/tbs/tb288/tb288.html)

Ordering Information

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay	10ml	G7570
	10 × 10ml	G7571
	100ml	G7572
	10 × 100ml	G7573

For Laboratory Use.

[a]U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other patents and patents pending.

[b]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.