

High-Throughput Viability Assay Using an Autonomously Bioluminescent Cell Line with a Bacterial *Lux* Reporter

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Abstract

Cell viability assays are extensively used to determine cell health, evaluate growth conditions, and assess compound cytotoxicity. Most existing assays are endpoint assays, in which data are collected at one time point after termination of the experiment. The time point at which toxicity of a compound is evident, however, depends on the mechanism of that compound. An ideal cell viability assay allows the determination of compound toxicity kinetically without having to terminate the assay prematurely. We optimized and validated a reagent-addition-free cell viability assay using an autoluminescent HEK293 cell line that stably expresses bacterial luciferase and all substrates necessary for bioluminescence. This cell viability assay can be used for real-time, long-term measurement of compound cytotoxicity in live cells with a signal-to-basal ratio of 20- to 200-fold and Z-factors of ~0.6 after 24-, 48- 72-, or 96-h incubation with compound. We also found that the potencies of nine cytotoxic compounds correlated well with those measured by four other commonly used cell viability assays. The results demonstrated that this kinetic cell viability assay using the HEK293(lux) autoluminescent cell line is useful for high-throughput evaluation of compound cytotoxicity.

Keywords

bioluminescent assay, autonomously bioluminescent assay, cell viability, cytotoxicity, high-throughput screening

Introduction

Cell viability is an important parameter commonly measured in high-throughput screening (HTS) of compound libraries. Treatment of cells with a cytotoxic compound can stall cell growth and eventually lead to cell death, which can be determined by cell viability assays.^{1–3} These assays have been extensively used in lead discovery for identification of active compounds for cancer and many infectious pathogens. Cell viability assays have also been used to assess compound cytotoxicity for toxicological studies. In addition, they have been used as counterscreens in lead discovery processes to eliminate false-positive compounds.⁴ Although there are a number of cell viability assays available, few are suitable for kinetic HTS of cytotoxicity.

Luciferases, especially those derived from firefly and *Renilla*, have been extensively used as sensitive reporters for cell-based assays. In addition to its use as a reporter gene for cell-based screening assays, firefly luciferase has been used in the adenosine triphosphate (ATP) content assay for measurement of cell viability in mammalian cells and pathogens, including bacteria, fungi, and protozoa.^{5,6} Recently, a bacterial luciferase gene cassette has been transfected into mammalian cells that exhibits constitutive and continuous bioluminescence

in live cells.^{7–9} Unlike the ATP content assay using the firefly luciferase, this bacterial luciferase reporter system does not require exogenous substrate addition because it synthesizes the substrate in live cells. We report here the miniaturization and optimization of a bacterial luciferase (*Lux*) cell viability assay in 1536-well plate format for high-throughput cell viability screening. This assay was then validated by comparing its performance with other commonly used cell viability and proliferation assays, including the ATP content assay, alamar-Blue assay, CyQUANT cell proliferation assay, and MTS

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assay in 1536-well microtiter plate format. We found that the IC_{50} values of known cytotoxic compounds as tested in the Lux assay correlated very well with IC_{50} values generated by the same compounds in these other four assays. In addition, we compared the performance of the Lux assay and ATP content assay in a Library of Pharmacologically Active Compounds (LOPAC) screen. Our data demonstrate that the Lux cell viability assay is robust and sensitive for high-throughput measurement of cell viability and compound cytotoxicity in a kinetic assay format.

Materials and Methods

Reagents

Camptothecin, digitonin, 7,12 dimethylbenz(a)anthracene, etoposide, tamoxifen, terfenadine, and the LOPAC were purchased from Sigma-Aldrich (St. Louis, MO). Digoxin was purchased from Toronto Research Chemistry (North York, ON, Canada). Doxorubicin and vinblastine were purchased from AK Scientific (Palo Alto, CA). The Cell Titer-Glo Luminescent Cell Viability Assay and the Cell Titer Aqueous One Cell Proliferation (MTS) Assay kits were purchased from Promega (Milwaukee, WI). The alamar-Blue Dye and the CyQUANT Direct Cell Proliferation assay kits were purchased from Life Technologies (Grand Island, NY). All compound and assay plates were purchased from Greiner Bio-One (Monroe, NC). White, solid-bottom, tissue culture-treated plates were used for luminescence-based assays, and black, clear-bottom, tissue culture-treated plates were used for fluorescence- and absorbance-based assays.

Cell Culture

Human embryonic kidney cells (HEK293) stably transfected with the bacterial luciferase gene cassette (*luxCDA-BEfrp*) were kindly provided by 490 BioTech (Knoxville, TN) and were used for all assays. HEK293(lux) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1× sodium pyruvate, 1× non-essential amino acids, 1× antibiotic-antimycotic, and 500 µg/ml G418 (all from Life Technologies). Wild-type HEK293 cells (catalog number CRL-1573) were purchased from American Type Culture Collection. These cells were maintained in Eagle's Minimum Essential medium with 10% FBS. Cells were passaged in new flasks or seeded in assay plates upon reaching 80% confluence. For regular culture, both cell types were maintained at 37 °C, 5% CO₂, and 75% relative humidity. Both cell types were seeded in assay plates in a medium with slight modification (DMEM without phenol red and reduced FBS concentration to 1%). Assay plates

were maintained at 37 °C, 5% CO₂, and 95% relative humidity to minimize effects of evaporation.

Reagent Dispensing and Instrumentation

Cells were dispensed using a Multidrop™ Combi Reagent Dispenser (Thermo Scientific, Waltham, MA). ATP content/CellTiterGlo, alamarBlue, and MTS reagents were dispensed using the BioRAPTR FRD Microfluidic Workstation (Beckman Coulter, Brea, CA). Because the manufacturer's notes for the CyQUANT assay indicate the importance of not disrupting the cells during reagent addition, the CyQUANT reagent was dispensed using the EL406 Microplate Washer Dispenser, because it provides a lower-velocity, angled dispense (BioTek, Winooski, VT). Compound transfer between 384- and 1536-well plates was accomplished using a CyBi-Well 384-well pipettor station (Cybio, Bedford, MA). Compounds were added into assay plates using an NX-TR pin-tool workstation (Wako Automation USA, San Diego, CA). Luminescence (Lux and ATP content assays) and fluorescence (AlamarBlue) were detected using a ViewLux ultra HTS Imager (PerkinElmer, Waltham, MA). Absorbance (MTS assay) and fluorescence (CyQUANT nuclear stain) were detected using the EnVision 2104 Multilabel Plate Reader (PerkinElmer).

Compound Plate Preparation

Stock DMSO solutions for each compound were serially diluted 1:3 in a 384-well polypropylene plate (Greiner Bio-One). For serial dilution, 20 µl of the compound solution was transferred into 40 µl DMSO. After mixing three times, 20 µl was removed and added to a subsequent well containing 40 µl DMSO. This process gave three wells with highest concentration of the compounds and a 14-point titration. Then, the compound solutions were transferred from 384- to 1536-well polypropylene plates with the CyBi pipettor station. The resulting 1536-well compound plates, containing 5 µl/well, were sealed and stored in a desiccator cabinet in the dark.

The compound library was prepared and handled as previously described.⁵ Briefly, the LOPAC is composed of 1280 unique chemical entities that are pharmacologically active. This library has been extensively used in HTS assay development to evaluate assay performance. All compounds were dissolved in DMSO to yield a 10 mM stock solution. This 10 mM stock was serially diluted (1:5) in 384-well plates in a seven-point titration and transferred to 1536-well plates using the CyBi pipettor station.

The following controls were used for all assays: assay media without cells, DMSO (basal signal), digitonin titration (top concentration ~300–375 µM, assay-dependent), and tamoxifen titration (top concentration ~250–300 µM, assay-dependent).

Table 1. Protocol for the Lux Cell Viability Assay in the 1536-Well Plate Format.

Step	Parameter	Value	Description
1	Seed cells	5 μ l/well	500 HEK293(lux) cells/well; white, solid-bottom plates
2	Centrifuge	1 min, 1000 rpm	To settle cell suspension in well
3	Incubate	1 h	37 °C, 5% CO ₂ , 95% relative humidity
4	Pin compound	23 nl	Compound in DMSO
5	Incubate	24, 48, and 72 h	37 °C, 5% CO ₂ , 95% relative humidity
6	Incubate	10 min	Room temperature
7	Read	200 s exposure, 2 x binning	Luminescence mode, ViewLux plate reader

Cell Viability Assays

Cell density and incubation temperature were first examined and optimized for robust signal-to-basal (S/B) ratios and Z-factors [% coefficients of variation (CV) < 12%; Z-factor > 0.5] for up to 72 h using the Lux autoluminescence assay. Once the optimal conditions were chosen, the Lux assay was performed in parallel with the other assays to validate the ability of the Lux assay to evaluate the cytotoxicity of nine known toxic compounds in a 1536-well plate format. The Lux assay protocol is summarized in **Table 1**. Briefly, 5 μ l/well of HEK293(lux) cell suspension was seeded in white, 1536-well assay plates using the Multi-Drop dispenser. The assay plates were centrifuged at 1000 rpm for 1 min to settle cells in the wells, followed by 1 h incubation. Compound was then transferred into the assay plates at 23 nl/well via pin tool. Assay plates were then incubated for 72 h at 37 °C with luminescence detected every 24 h.

Because the cells produce light autonomously (i.e., no substrate or other additional reagents are necessary), the Lux assay plates could be read directly out of the incubator. It was found, however, that the signal increased as plates equilibrated to room temperature (RT). Because well-to-well variability in signal also increased with increased time at RT, the assay plates were kept at RT for 10 min after the 37 °C incubation before luminescence detection using the ViewLux (200 s exposure, 2 \times binning). This was found to achieve a maximal S/B ratio and reduced variability.

The ATP content assay was performed as recommended by the vendor. Briefly, after seeding the plates with HEK293(lux) cells and incubating with compound as previously described, the plates were removed from the incubator and allowed to equilibrate to RT (**Suppl. Table S1**). Cell-Titer Glo (CTG) reagent (Promega) was dispensed at 4 μ l/well using the BioRAPTR FRD Microfluidic Workstation. The plates were incubated in the dark at RT for 10 min prior to detection by the ViewLux plate reader (1 s exposure, 1 \times binning). To assess if stable expression of *lux* potentially affected cell health in HEK293(lux) cells, the ATP content assay was also performed in a comparison with the wild-type HEK293 cells.

The alamarBlue, CyQUANT, and MTS assay protocols were similar to the Lux and ATP content assays, but with a few differences (**Suppl. Tables S2–S4**). Rather than white, solid-bottom plates, black, clear-bottom plates were used for these three assays. The alamarBlue assay was conducted as described previously^{10,11} (**Suppl. Table S2**). The total assay medium per well in this protocol was 4 μ l rather than 5 μ l, which slightly changed the concentration range of compounds tested in this assay. The CyQUANT assay was performed per the manufacturer's instructions (**Suppl. Table S3**). The Cell Titer Aqueous One Cell Proliferation (MTS) assay using the protocol from Promega was miniaturized to a 1536-well format (**Suppl. Table S4**). As per the manufacturer's recommendation, we corrected for background absorbance by preparing a control plate, which consisted of 5 μ l assay medium, pinned compound, and 1 μ l MTS reagent. The absorbance values obtained from this control plate were subtracted as background for the results obtained from the assay plates.

Data Analysis

The primary screening data and curve fitting were analyzed using software developed at the National Institutes of Health Chemical Genomics Center.¹² The dose–response curves, correlation curves, and scatterplots were calculated and plotted using GraphPad Prism software (GraphPad Software, San Diego, CA). S/B ratios were calculated by dividing the luminescence signals from wells with HEK293lux cells pinned with DMSO by those from wells without cells or with the highest concentration of cytotoxic compound (digitonin/tamoxifen). Z-factors were calculated using the following equation: $Z = 1 - (3(SD_{total} + SD_{basal}) / (\text{mean}_{total} - \text{mean}_{basal}))$, where SD_{total} is the standard deviation of DMSO-treated wells, SD_{basal} is the standard deviation of wells treated with 300 μ M digitonin (or no cell control wells), mean_{total} is the mean of DMSO-treated wells, mean_{basal} is the mean of 300 μ M digitonin-treated wells (or no cell control wells),¹³ and % CV = $(SD/\text{mean}) \times 100$, which is expressed as a percentage. Unless otherwise noted, all values are expressed as the mean \pm SD ($n = 3$).

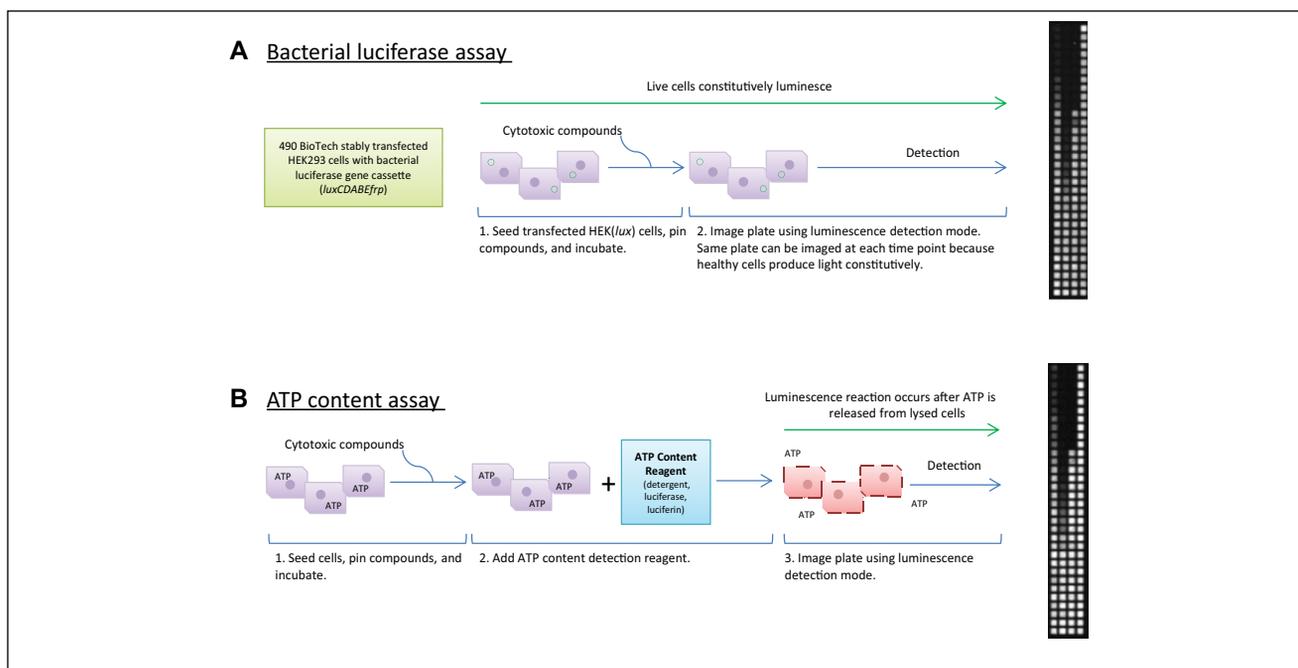


Figure 1. Schematic description of the Lux assay (bacterial luciferase) in comparison with the classical adenosine triphosphate (ATP) content assay. **(A)** The Lux cell viability assay: Cells stably transfected with a mammalian optimized bacterial luciferase gene cassette (*lux*) are able to luminesce without a need of reagent addition. This constitutive luminescence property enables kinetic monitoring of cell viability. **(B)** The ATP content cell viability assay: The detection reagent mixture, which lyses cells to release intracellular ATP, is added after cells are treated with compound. The ATP, which is the limiting reagent, interacts with the luciferase and luciferin in the reaction mixture to produce luminescence signal. It is an endpoint cell viability measurement.

Results

Bacterial Luciferase Assay Principle

The bacterial gene cassette (*lux*) encodes six components that are necessary for transfected cells to luminesce. The heterodimeric bacterial luciferase is encoded by *luxA* and *luxB* genes. In addition, a reductase, a transferase, and a synthase are encoded by *luxC*, *luxD*, and *luxE* genes, respectively. These components form a complex that converts endogenous aliphatic compounds, originally destined for lipid biogenesis, to an aldehyde substrate for the bacterial luciferase. Flavin reductase, the gene product encoded by *frp*, shifts the FMN–FMNH₂ balance toward a more reduced state.^{8,14} FMNH₂ is a cofactor of the enzyme reaction that facilitates the light production by bacterial luciferase. The unique ability to generate a bacterial luciferase and its substrate in live cells enables healthy cells to luminesce constitutively without a need for exogenous reagents.^{7–9} For assessing cell viability and compound cytotoxicity, these cells can be incubated with compounds throughout a period of time, and the cytotoxicity of compounds can be determined kinetically by the loss of luminescence signal (**Fig. 1A**).

The commonly used ATP content assay, which is also a luminescence-based assay, relies on reagent addition to lyse

cells and deliver an exogenous enzyme and substrate for luminescence generation (**Fig. 1B**). Because the cells must be lysed to release ATP, which interacts with luciferase and luciferin in the reagent to produce light, it is only possible to record a snapshot of cell health at a given time point. This ATP content assay has been extensively used for high-throughput screening of cell viability and compound cytotoxicity. Because it is well characterized and popularly used, it was selected as a control assay to evaluate this new Lux cell viability assay.

To further corroborate the Lux reporter system for use in cell viability assays, we compared the HEK293(*lux*) cells' autoluminescence performance to three other highly regarded and widely used cell viability and proliferation assays. Both the alamarBlue and Cell Titer 96 Aqueous One Cell Proliferation (MTS) assays rely on functional enzymes in live cells to give an estimate of viable cells. In the alamarBlue assay, resazurin, a pro-fluorescence compound, can permeate cell membranes and is reduced to resofurin, which emits red fluorescence. The MTS assay uses a tetrazolium reagent that originally has yellow and is reduced to formazan in live cells with absorbance at 490 nm. Unlike the MTT assay, the MTS dye is soluble in assay medium, and cell wash steps are not needed for this MTS assay. The third cell viability assay was the CyQUANT Direct Cell Proliferation assay, which has two components to assess cell membrane integrity and DNA content in live cells:

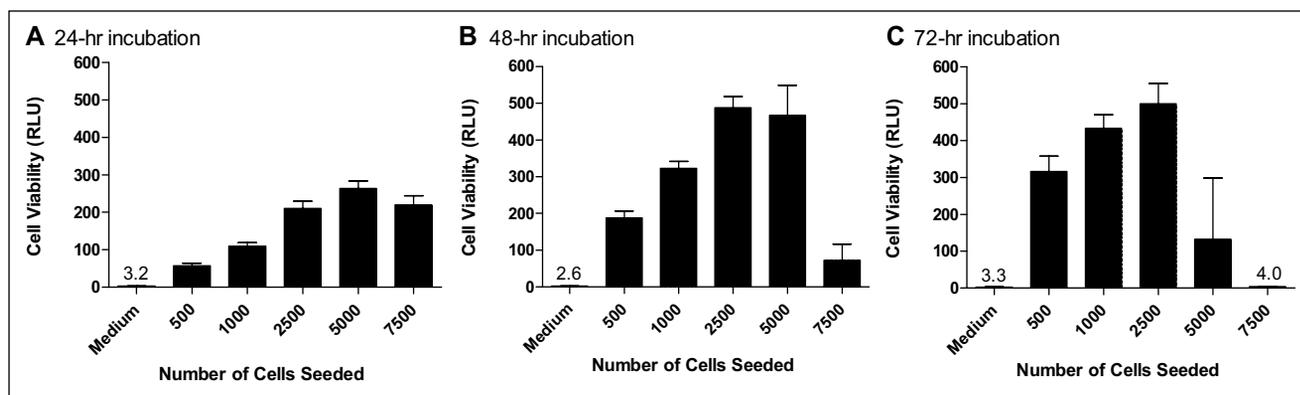


Figure 2. Optimization of cell density and incubation time. Cells were seeded at 500, 1000, 2500, 5000, and 7500 cells/well in one 1536-well assay plate. Because viable HEK293(lux) cells produce light continuously without a need of reagent addition, the luminescence signals in the same plate were detected at (A) 24, (B) 48, and (C) 72 h.

Table 2. Results of the Lux Assay with Regard to Cell Density and Cell Culture Time in 1536-Well Plate Format.

Parameter	Number of Cells/Well	Compound Incubation Time (h)			
		24	48	72	96
S/B	500	18	72	104	160
	1000	34	123	143	153
	2500	65	187	165	134
	5000	82	179	44	1.4
	7500	68	26	1	1.0
% CV	500	12.4	10.2	13.1	14.2
	1000	8.3	6	8.6	12
	2500	9.1	6.3	10.9	49
	5000	7.5	17.6	124.5	117
	7500	11.1	54.9	22.6	16.6
Z-factor	500	0.58	0.68	0.6	0.57
	1000	0.73	0.82	0.74	0.63
	2500	0.72	0.81	0.67	-0.48
	5000	0.77	0.47	-2.84	-12.0
	7500	0.66	-0.73	-3.9	8.1

Signal corresponds to wells seeded with cells, whereas background corresponds to wells containing assay medium only. CV, coefficient of variation; S/B, signal-to-basal ratio.

a membrane-permeable green fluorescent DNA-binding dye and a membrane impermeable quencher dye that suppresses fluorescence in extracellular medium, so cell wash steps are eliminated from this assay.

Assay Optimization for the Bacterial Luciferase Assay

Cell density per well of HEK293(lux) cells was first optimized for the appropriate S/B ratio during an incubation time of 96 h. The cells were seeded at 500, 1000, 2500, 5000, and 7500 cells/well in white 1536-well plates at a volume of 5 μ L/well in medium. The wells without cells (cell culture medium only) were used as a negative control for the basal signal. Because the HEK293(lux) cells

constitutively luminesce, cell viability can be determined kinetically with multiple reads from the same assay plate. We measured the luminescence signals once every 24 h during a 96-h time course. Generally, the S/B ratios increased with increasing cell density; we found that at the two highest cell densities, the luminescence signals decreased due to overcrowding of cells and possible partial cell death (Fig. 2 and Table 2). Although 1000 and 2500 cells/well yielded higher S/B ratios, 500 cells/well was selected as an optimized condition because it yielded high S/B ratios of 18 (24 h), 72 (48 h), 104 (72 h), and 160 (96 h) -fold with good % CVs and Z-factor values (Table 2). In addition, a lower cell density of 500 cells/well in 5 μ L/well of assay medium may minimize the chance of depleting medium nutrients during a 96-h assay in 1536-well plates.

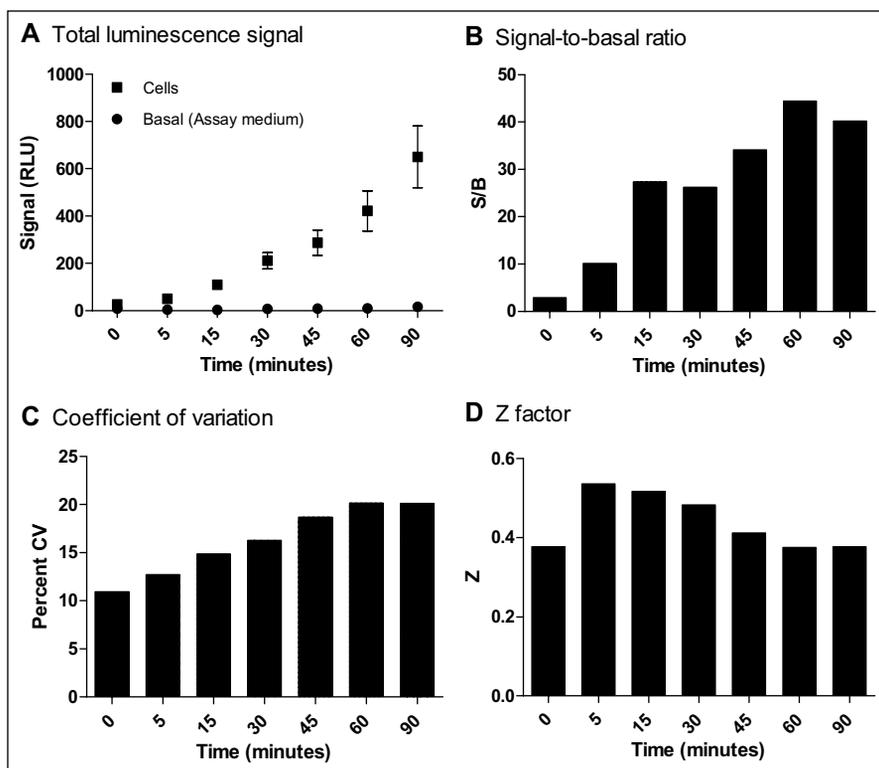


Figure 3. Optimization of the Lux assay window with the time at room temperature. Cells were seeded at 500 cells/well and incubated for 24 h. The assay plate was removed from the 37 °C incubator and placed at room temperature before detection. The luminescence signals were detected at 0, 15, 30, 45, 60, and 90 min after the assay plate was removed from the 37 °C incubator. Total luminescence signal increased with time when the assay plate waited at room temperature before detection (**A**). Whereas the signal-to-basal (signal = wells + cells; basal = assay medium only) ratio (**B**) increased throughout time, (**C**) the variability increased, and (**D**) the Z-factor decreased.

We noticed that luminescence from the cells in 1536-well plates increased throughout the time with the assay plates waited at RT to be read after being removed from the 37 °C incubator. We then sought to determine the optimal equilibration time at RT to achieve the maximal signal from the cells with the lowest well-to-well variability. Following 24 h incubation at 37 °C, the assay plate was removed from the incubator and placed into the plate reader for luminescence detection. The plate remained at RT and was subsequently read 5, 15, 30, 45, 60, and 90 min after the initial detection. We found that the total luminescence signal increased steadily during the 90 min (**Fig. 3A**). The S/B ratios show some variation in comparison to the total luminescence mainly due to differences in the basal signal (**Fig. 3B**). Although S/B ratios increased with increasing time at RT, we found that well-to-well variability also increased throughout time (**Fig. 3D**), ultimately resulting in a decreased Z-factor (**Fig. 3C**). Thus, a 10-min incubation at RT was selected for further experiments because it yielded a S/B ratio of 20-fold with acceptable % CV and Z values.

Comparison of Compound Cytotoxicity with Wild-Type HEK293 Cell Line

To assess if stable expression of *lux* affects cell viability and response to treatment with cytotoxic compounds, cytotoxicity assays were performed in parallel using HEK293(*lux*) and wild-type (WT) cell lines. Because the ATP content

reagent mixture lyses cells to bring ATP in contact with luciferase and luciferin, separate plates were seeded for each time point. Plates were read at 24, 48, and 72 h after the addition of ATP content reagent mixture in three assay plates (**Suppl. Table S1**). Although the IC_{50} correlation comparing HEK293(*lux*) and WT-HEK293 was relatively weak at 24 h ($R^2 = 0.4841$), it was stronger at 48 ($R^2 = 0.8468$) and 72 h ($R^2 = 0.6335$) (**Suppl. Fig. S4**). Digitonin, digoxin, tamoxifen, and terfenadine gave IC_{50} ratios (WT/Lux cells) between 1 and 2 for all three time points (i.e., these compounds were similarly toxic in both cell types; **Suppl. Fig. S8**). Camptothecin, doxorubicin, and etoposide were more potent in the HEK293(*lux*) cells after 24-h incubation, but more potent in WT-HEK293 at 48 and 72 h (IC_{50} ratios < 1). Vinblastine was also more potent in WT-HEK293 cells at 48 and 72 h.

Assay Validation with Known Cytotoxic Compounds

To evaluate the Lux cell viability assay for determination of compound cytotoxicity, we selected nine known cytotoxic compounds and tested them in the Lux assay in parallel with the ATP content assay. We found that the IC_{50} values of most of these compounds, after incubations of 24, 48, and 72 h with the HEK293(*lux*) cells, were similar to those determined in the ATP content assay (**Table 3**). The

Table 3. IC₅₀ Values of 9 Known Compounds Determined from Lux Assay and ATP Content Assay.

Compound	IC ₅₀ (μM)					
	24 h		48 h		72 h	
	CTG	Lux	CTG	Lux	CTG	Lux
Camptothecin	0.040	0.067	0.019	0.042	0.024	0.032
Digitonin	2.34	6.50	2.86	3.24	3.01	3.49
Digoxin	0.063	0.09	0.065	0.125	0.067	0.138
Doxorubicin	0.159	0.71	0.113	0.577	0.143	0.82
7,12 dimethylbenz(a)anthracene	36.1	11.0	49.8	98.0	71.3	65.1
Etoposide	1.93	10.5	1.37	5.550	1.75	5.16
Tamoxifen	12.1	14.2	11.1	11.2	8.57	10.0
Terfenadine	7.75	7.37	5.46	6.70	4.92	6.29
Vinblastine	0.031	0.008	0.024	0.06	0.026	0.044

ATP, adenosine triphosphate; CTG, Cell-Titer Glo reagent.

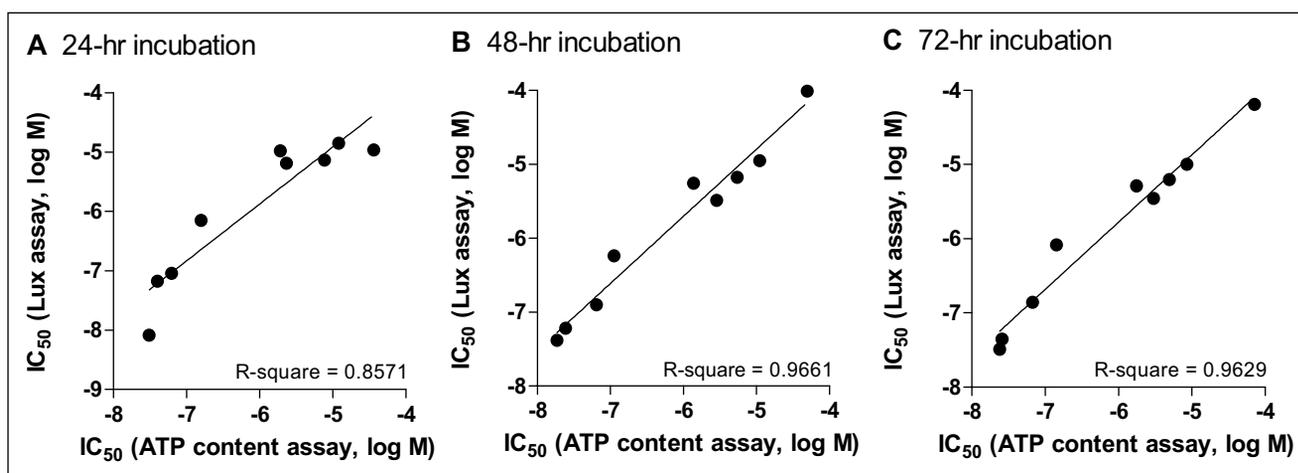


Figure 4. Correlation of compound cytotoxicity (IC₅₀s) measured by the Lux and ATP content cell viability assays. Direct correlations were observed when the nine known compounds were incubated with the cells for 24 ($R^2 = 0.86$), 48 ($R^2 = 0.97$), and 72 h ($R^2 = 0.96$).

cell-killing effects of these nine compounds correlated well in two assays with an R^2 value of 0.86 for 24-h incubation and ~0.96 for both 48- and 72-h incubations (Fig. 4). These results validate the sensitivity and reliability of the Lux assay in measuring compound cytotoxicity.

To further validate the Lux assay, we tested the nine cytotoxic compounds in three other commonly used assays: the alamarBlue, CyQUANT, and MTS assays. For these experiments, cytotoxicity of compounds was measured after 24- and 48-h compound incubations with cells. A summary of the statistics generated from the experiments is included in the Supplementary Material (Suppl. Table S5). The IC₅₀s of the compounds in the alamarBlue and MTT assays correlated strongly at both incubation times with those obtained from both the Lux and the ATP content assays (R -squared > 0.9; Suppl. Figs. S1 & S3 and Table

S6). The 24- and 48-h compound incubation times for the CyQUANT assay (Suppl. Fig. S2) gave reasonable IC₅₀ correlations with the Lux and ATP content assays (24-h: Lux vs. CyQUANT, R^2 value = 0.86; 24-h: ATP assay vs. CyQUANT, R^2 value = 0.73; 48-h: Lux vs. CyQUANT, R^2 value = 0.89; and 48-h: ATP assay vs. CyQUANT, R^2 value = 0.97). The IC₅₀ values for these three assays align with the Lux and ATP content assays (Suppl. Tables S6 and S7).

Cell Viability Screen Using the LOPAC Compound Library

To assess the performance of the Lux cell viability assay in a 1536-well plate format, the S/B ratio, % CV, and the Z-factor were examined using a DMSO plate. DMSO is the

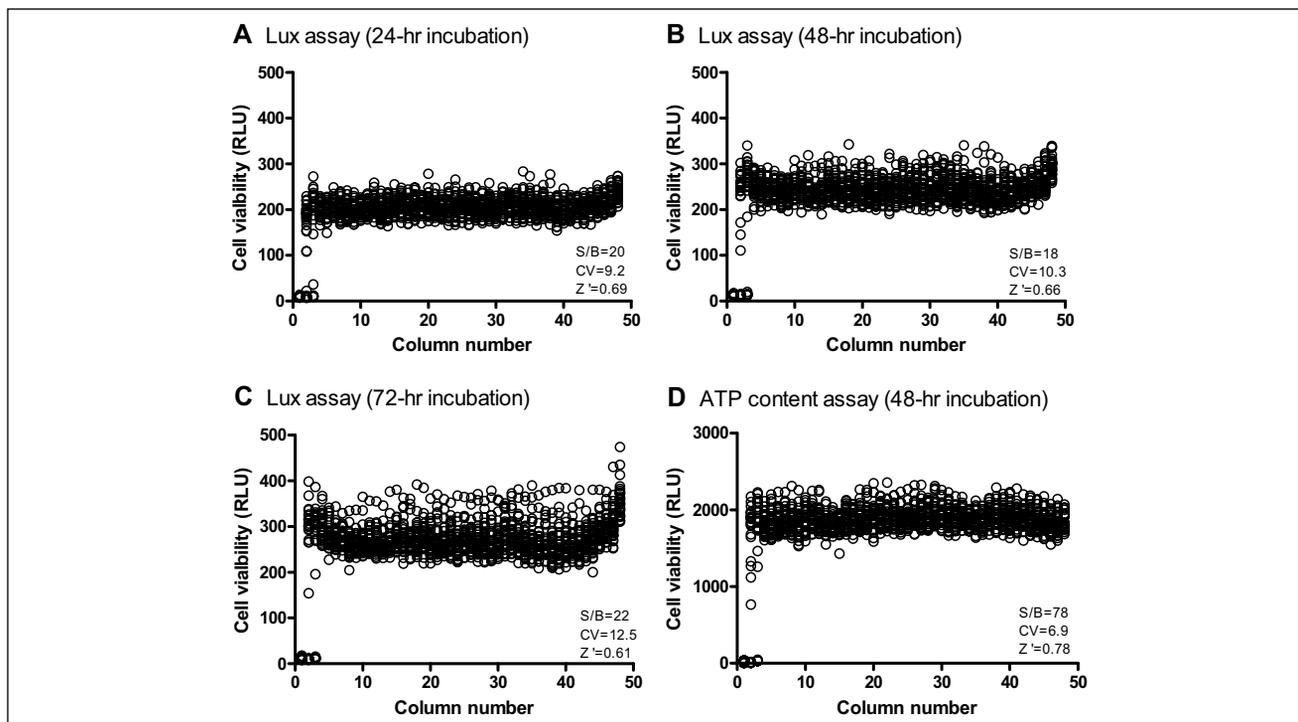


Figure 5. Scatterplots of DMSO plate screening in 1536-well plates. Luminescence was detected for 500 cells/well after (A) 24, (B) 48, and (C) 72 h of incubation with DMSO (final concentration of 0.5%). (D) The adenosine triphosphate content assay using 500 cells/well with 48-h incubation with compound was used for comparison (signal = cells + DMSO only; basal = cells + [digitonin]_{highest}).

solvent for all compounds used in this study. Digitonin and tamoxifen were used as positive control compounds and were examined in a titration in each assay plate. For the bacterial luciferase, the luminescence signals were determined kinetically after 24-, 48- and 72-h incubation of DMSO or compounds with cells. We found that the S/B ratio remained relatively constant after the 24-, 48- and 72-h compound incubation (S/B ratio of 20-, 18-, and 22-fold, respectively). The values of % CVs and Z-factors were similar for these three time points (Fig. 5A–C), indicating a robust cell viability assay for HTS. We also did a parallel DMSO plate test for the ATP content assay using HEK293(lux) cells. Only the result of 48-h incubation was obtained for this ATP content assay because it is an endpoint assay. The S/B ratio, % CV, and Z-factor were 78-fold, 6.9, and 0.78, respectively (Fig. 5D). The % CV and Z-factor are comparable with those in the bacterial luciferase assay, but the S/B ratio is greater than that in the bacterial luciferase assay.

We then screened the LOPAC compound library with this Lux assay with a titration of compound concentrations ranging from 0.003 to 46 μM . The compounds were incubated with the cells for 48 h. A total of 76 compounds with IC_{50} values less than 5 μM were selected as cytotoxic compounds from this screen. We also did a parallel cytotoxicity screen with the LOPAC library using the ATP content assay. We found that the cytotoxic potencies of compounds from the

LOPAC library correlated well in the two assays (Fig. 6C). Together, the results demonstrate that the Lux cell viability assay is validated for high-throughput screening of compound cytotoxicity.

Discussion

Cell viability assays are frequently used in HTS to determine the cytotoxicity of compounds. These assays have been used to assess compound toxicity against diverse cell types, including mammalian, bacterial, and protozoan cells, during lead discovery.^{5,6} In addition, they can be used as counterscreens in parallel with other assays to eliminate false positives that arise in HTS campaigns.⁴ A common assay to determine cell viability in HTS is the ATP content assay, which relies on cellular ATP in live cells as the limiting reagent in a luciferase enzyme reaction. Wells in assay plates containing healthy cells contain higher concentrations of ATP and therefore produce higher luminescence signals. In contrast, wells in assay plates with nonviable cells contain little to no ATP that produce weak to undetectable luminescence signals. The ATP content assay is amenable to HTS for a number of reasons: it has been optimized to an “add-mix-detect” format that is convenient for screening large compound libraries, it provides robust S/B ratios, and it is sensitive for use in a miniaturized assay format. It is only capable of capturing cell viability at a given time point, however, because the cells must be lysed to determine

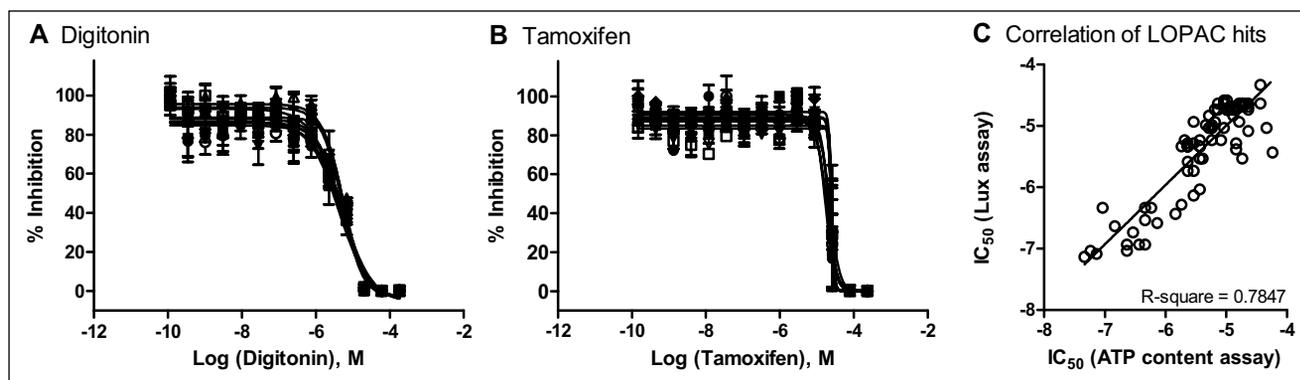


Figure 6. Results of the Library of Pharmacologically Active Compounds (LOPAC) library screen using the Lux cell viability assay. HEK293(lux) cells at 500 cells/well were incubated with compounds for 48 h before the detection of compound cytotoxicity. Digitonin and tamoxifen were used as positive control compounds whose cytotoxic activities were determined in all nine assay plates. Concentration–response curves of both (A) digitonin and (B) tamoxifen were similar among the assay plates, which is indicative of the consistency and reproducibility of the Lux cell viability assay in different plates. (C) A strong correlation of IC_{50} values of cytotoxic compounds in the LOPAC library was determined for the LUX and adenosine triphosphate content cell viability assays.

the amount of intracellular ATP. To determine cytotoxicity at various time points, separate plates have to be prepared for each endpoint of interest. Thus, a kinetic experiment in this assay format greatly increases the consumption of reagents and time.

Several other cell viability assays with an endpoint measurement are available for high-throughput screening, and we also assessed their performance in HTS. The results of these assays have been used to further validate the data from the bacterial luciferase assay. Like the ATP content assay, these other viability assays also follow an “add-mix-detect” model; however, they use other aspects of cellular metabolism and viability to estimate cell health. The MTS assays use a chromogenic dye as an indicator, which is oxidized in mitochondria of healthy cells to produce a colored formazan product.¹⁵ We were able to generate a MTS assay protocol that performed very well in a 1536-well format (Suppl. Table S4). In addition, the IC_{50} s of the cytotoxic compounds that we tested were similar to that found in the bacterial luciferase assay (Suppl. Table S6). We also optimized a protocol for the alamarBlue assay in 1536-well format. This is a pro-fluorescence dye assay that requires metabolism by cells to yield fluorescence.^{16,17} Again, it is less commonly used in HTS because it can yield high background. As with the MTS assay, however, we were able to optimize a protocol that gave very good performance in 1536-well plates, and generate IC_{50} s comparable to the other assays tested. The third assay that we optimized for a 1536-well format to compare with the bacterial luciferase assay was the CyQUANT Cell Proliferation Assay that measures cell number and viability based on membrane integrity and intercalation of a DNA dye. This assay did not perform as well under certain assay conditions as the other assays we tested, but it still gave decent assay performance (Suppl. Tables S5 and S6).

Enzyme release assays measure the intracellular enzymes in extracellular medium released through damaged cell membranes. Intracellular enzymes, such as lactate dehydrogenase^{18,19} and proteases,^{20,21} are released after membrane integrity is disrupted by cytotoxic compounds. The activity of released enzymes can be determined by addition fluorogenic or chromogenic substrate. The assay windows of these enzyme release assays are usually high because the basal signal is usually low. This is because almost no enzymes are present in the medium of healthy cells. Enzyme release assays are time sensitive, however, because the enzymes are quickly degraded (within minutes or a few hours) after they are released from damaged cells.

The Lux cell viability assay uses cell lines transfected with a cassette of six genes. The transfected cells have the ability to constitutively luminesce without the addition of exogenous reagents. This provides several advantages over established cell viability assays. First, it can be used to assess the cell viability and compound cytotoxicity kinetically over an extended time period in one 1536-well assay plate. Second, the ability to read the plate at multiple time points reduces reagents and supplies consumed. Third, no reagent addition step simplifies the assay procedure, which is ideal for automated, robotic screens. Finally, compound cytotoxicity data can be obtained in parallel with the pharmacological data during the interrogation of compound libraries if the HEK293(lux) cells are co-transfected with a specific reporter gene for other cellular functions (i.e., a GPCR, ion channel, or enzyme).

In the Lux assay, the luminescence signal from HEK293(lux) cells was weaker than the signal from the ATP content assay; a 200-s exposure time was required for the detection in the Lux assay compared to a 1-s exposure time for the ATP content assay. This is not surprising, considering that the luminescence signal for the Lux assay is

limited by the intracellular expression levels of the components of the luminescence reaction. We find that the S/B ratio for the Lux assay increases with increasing cell number per well, up to a certain cell density, at which point the S/B ratio decreases due to overcrowding of cells (**Table 2**). Our studies showed that lower cell densities (500 and 1000 cells/well) yielded a wide S/B window (S/B ratios of 160 and 153, respectively) with low % CV and a Z-factor greater than 0.5 (**Table 2**). Although the S/B ratio in the Lux assay was smaller than that of the ATP content assay, its assay performance still correlated well with the ATP content assay and other cell viability and proliferation assays tested.

In addition, we noticed a few factors that affected luminescence output. One was temperature for assay plate incubation. After removing assay plates from the incubator, the signal increased at each time point during the course of 90 min after the incubation at RT. Although the signal increased throughout time, the well-to-well variability also increased. For this reason, we chose a 10-min incubation at RT because it provided an S/B ratio of 20-fold with low variability between wells (% CV = 10%; Z-factor = 0.7). Another factor that affected luminescence output was media volume in the well, presumably due to interference in luminescent light transmission through the media. Wells with lower volumes appeared to produce brighter luminescence signals. This was especially noticeable at time points of 48-h and longer. Although the assay performed well even after a 96-h incubation with compound (at lower cell densities; see **Table 2**), maintaining any cell type for this long time in a 1536-well format is challenging due to the potential evaporation of media from the plate, overcrowding at higher cell densities, and depletion of medium nutrients. Overcrowding and depletion of medium are likely the reasons why the higher cell densities tested—2500, 5000, and 7500 cells per well—performed poorly at longer incubation timepoints.

Constitutive expression of certain genes can affect cell health in engineered transgenic cell lines. Xu and colleagues⁹ performed a comparison using WT and HEK293(lux) cells in which both cell types were treated with serially diluted Zeocin and reduction in cell viability was determined using the MTT assay. They found that both cell lines responded similarly to Zeocin. To further evaluate the HEK293(lux) cells, we performed cytotoxicity assays in parallel using both HEK293(lux) and WT-HEK293 cells using cytotoxic compounds. Our results indicate that although the HEK293(lux) cells are more sensitive for a few compounds after 24-h compound treatment, this was not the trend for all of the compounds tested (**Suppl. Fig. S4**). In addition, we found that the IC₅₀s of cytotoxic compounds were more similar at 48- and 72-h postcompound addition (R^2 values of 0.8468 and 0.633 for compound incubation at 48 and 72 h, respectively). Our data thus indicate that the HEK293(lux) cell line has similar sensitivity to cytotoxic

compounds compared to the untransfected cell line; however, compound screening with a larger collection of compounds may be needed for an overall evaluation of the sensitivity difference.

In conclusion, we have optimized a Lux cell viability assay in a 1536-well plate format that does not require addition of substrate or detection reagents. The assay is robust with a high S/B ratio and low well-to-well variability throughout a 72-h duration and can be used for the kinetic measurement of compound cytotoxicity in one assay plate. In addition, the data from nine known cytotoxic compounds showed a strong correlation of IC₅₀ values when comparing results from the Lux assay with results obtained in the ATP content assay, alamarBlue assay, CyQUANT cell proliferation assay, and MTS assay. These results thus validate the Lux assay as a reliable cell viability assay. The ability of the Lux assay to evaluate cytotoxic compounds has also been validated using the LOPAC library, which identified the same set of cytotoxic compounds as those found in the ATP content assay. Therefore, our results demonstrate that the Lux assay is a robust cell viability assay for kinetic determination of compound cytotoxicity. In addition, because the Lux assay does not require any additional reagents, it has the potential to be multiplexed with other cellular functional assays for HTS.

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