Measuring cell proliferation and cytotoxicity using the ATPlite 1step system and the VICTOR Nivo multimode plate reader.

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Introduction

In cell-based assays used in basic research, viability and cytotoxicity are routinely monitored to verify the health of cells. Similar assays are also used in pre-clinical research to help characterize compound-induced cytotoxic and adverse effects. Compared to biochemical assays, cell-based assays provide more physiologically relevant data on the effect of compounds on cell proliferation, morphology and signalling pathways.

The ATPlite 1step luminescence assay system uses ATP that is present in metabolically active cells as a marker for cell viability. As cellular ATP concentration is reduced when cells undergo apoptosis and necrosis, it is a very rapid and sensitive method to monitor compound induced cytotoxic effects. The method is based on the ATP-dependent oxidation of D-Luciferin by firefly *(Photinus pyralis)* luciferase with the production of light; therefore light output is proportional to cellular ATP concentration.

For research purposes only. Not for use in diagnostic procedures.

This study demonstrates the application of the cell-based ATPlite 1step luminescence assay system on the VICTOR Nivo™ multimode plate reader to monitor compound-related cytotoxic effects on HepG2 and CHO cells in 96- and 384-well microplates.

VICTOR Nivo is a compact multimode plate reader, providing up to five detection modes: absorbance, luminescence, fluorescence intensity, time-resolved fluorescence and fluorescence polarization. The system is characterised by its small footprint and its pre-configured, but flexible, user-friendly protocols that can be easily adjusted to individual needs in a multi-user environment. The VICTOR Nivo reader offers both top and bottom detection of microplate-based assays. Bottom reading allows the plate lid to be kept closed and may enable better sensitivity in cell-based assays.

Material and methods

Human hepatocellular carcinoma cells (HepG2, DSMZ no. ACC180) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % Fetal Calf Serum (FCS), 1 % Penicillin (100 U/mL)/Streptomycin (100 µg/mL) and 2 mM glutamine. Chinese hamster ovary cells (CHO-K1, DSMZ no. ACC 110) were cultured in DMEM/F-12 medium supplemented with 10% FCS and 1 % Penicillin (100 U/mL)/Streptomycin (100 µg/mL).

For the ATPlite 1step luminescence assay, cells were washed, trypsinized, resuspended in cell culture medium and transferred to a 384-well plate (25 µl cell suspension per well) or 96-well plate (100 µl cell suspension per well) (CulturPlate-384, #6007680, CulturPlate-96, #6005680, Revvity) according to the standard protocol. Unless otherwise mentioned, after 24 h incubation, compounds (Colchicine, #C9754; 5-Fluorouridine, #F5130; Pac1, #P0115, from Sigma Aldrich) diluted in DMSO (final concentration 0.5% v/v.) were added to the cells using the Echo® Liquid Handling System 550 (Labcyte, Inc) and incubated for an additional 48 h at 37 °C with 5 % CO_2. The lyophilized substrate was dissolved in ATPlite 1step buffer and added to the cells (25 µL/well for 384-well plates, and 100 µL per well for 96-well plates). ATPlite reagent was equilibrated at room temperature before addition, as per the kit instructions. The assay plate was spun down and then placed in the VICTOR Nivo reader. The assay required an additional 60s shaking step to be added to the pre-written luminescence protocol (ATPlite Luminescence), which was easily achieved via the intuitive software interface. Data analysis was performed using GraphPad Prism® software.

Results

In order to demonstrate the suitability of the VICTOR Nivo microplate reader and the ATPlite 1step luminescence assay system to monitor compound-related cytotoxic effects, an investigation of assay stability, sensitivity and dynamic range was performed.

Dynamic range and linearity of signal

The ATP standard supplied in the assay kit was used to demonstrate the linearity and dynamic range of the ATPlite 1step luminescence assay system on the VICTOR Nivo multimode plate reader. In addition, the standard curve can be used to quantify the ATP released from viable cells. Intracellular ATP concentration is in the range of 1-10 mM depending on the cell line. The linearity was tested in the range of approx. 0.025-100 µM ATP (0.625 pmol-2.5 nmol/well (25 µL) in the 384-well format) (Figure 1). A broad dynamic range (> 5 x 10^4) and positive correlation (R^2 ~ 0.98) were detected for the full range of concentrations tested, showing that the assay is robust and has the required sensitivity for detection of changes in the ATP level of cells, and that the assay can be easily performed on the VICTOR Nivo plate reader.

Cell number titration

To detect the effects of compounds on cell viability, it is necessary to have a positive correlation between cell number and luminescence signal. As cell lines show different activities and proliferation rates, the titration of required cell number per well should be performed for every cell line to be tested.

CHO-K1 and HepG2 cells were seeded in a 384-well CulturPlate in a 1:2 serial dilution (top concentration 2,000 cells/well) and incubated for 24-72 h. For HepG2 cells good signal linearity was observed for up to 2,000 cells/ well for 24-72 h of incubation. CHO-K1 ATP concentrations showed no correlation in cell density and proliferation for cell concentrations > 1,000 cells/well after 24 h and > 500 cells/well after 72 h of incubation (Figure 2). According to the observed signal linearity, CHO-K1 cells at a concentration of 250 cells/well and HepG2 cells at a concentration of 1,000 cells/well can be used to detect changes in cellular ATP caused by compound effects for an incubation time of up to 72 h.

To optimize the assay for 96-well microplate format, cell number titration was also performed in a CulturPlate-96. CHO-K1 and HepG2 cells were seeded in a 1:2 serial dilution starting with a highest concentration of 32,000 cells/well and incubated for 72 h. Loss of signal linearity due to contact inhibition was observed for >2000 cells/well and 4,000 cells/well, for CHO-K1 and HepG2 cells, respectively. (Figure 3). A further increase in the cell number even resulted in a slight signal decrease indicating reduction of the cell division rate and an increasing number of cells undergoing apoptosis.
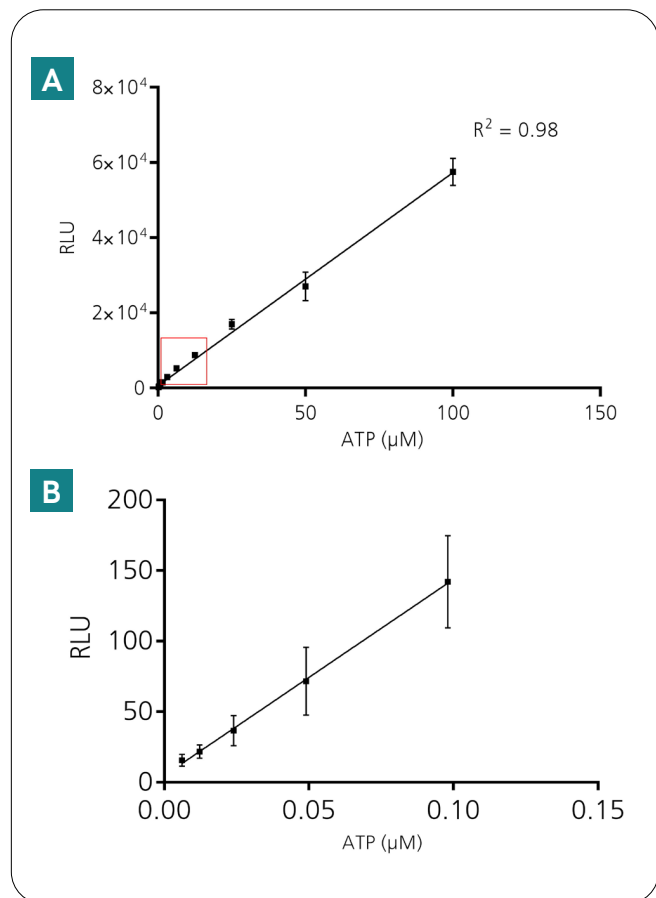

Figure 1. ATP standard dilution series for detection of assay linearity and dynamic range. The increase in luminescence signal directly correlates with the increase in ATP concentration (linear range from 0.01 - 100 μM) (A). Plot B shows the linear correlation in the range between 0.06 and 0.1 μM ATP. Values represent the mean of four replicates ± SD measured in a 384-well plate.

DMSO tolerance

DMSO is widely used as a solvent for compounds therefore its tolerance by cells and the impact of increasing DMSO concentration on assay performance should be tested before compound tests are performed. CHO-K1 cells were incubated with 0.01-4 % v/v DMSO for 24 and 48 h. The results show a concentration dependent increase in % RLU signal inhibition (Figure 4) revealing DMSO tolerance of ≤ 0.5 % v/v DMSO with a maximal acceptance criteria of 20 % RLU signal inhibition.
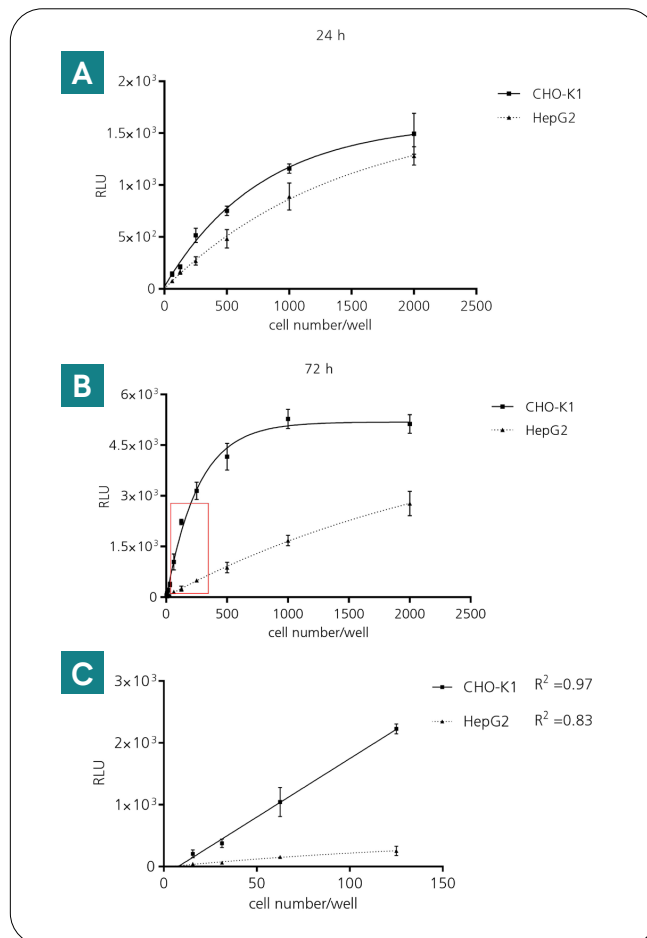

Figure 2. Correlation of cell number and luminescence signal in a 384-well plate. CHO-K1 and HepG2 cells were seeded in serial dilution, starting at 2,000 cells/well and incubated for 24h (A) to 72h (B) at 37 °C with 5% CO_2. Luminescence signal was measured after the addition of 25 μL ATPlite reagent per well and 15 min incubation at RT. Plot C shows the linear correlation in the range between 125 and 15 cells/well when cells are tested immediately after seeding. Values represent the mean of at least three replicates ± SD.
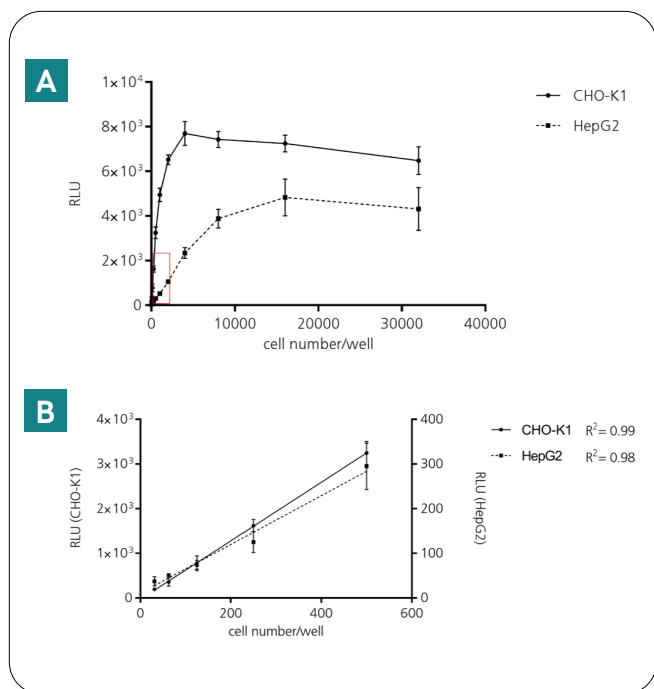
Figure 3. Correlation between cell number and detected luminescence signal measured in a 96-well plate. CHO-K1 and HepG2 cells were seeded in serial dilution, starting at 32,000 cells/well and incubated for 72 h at 37 °C with 5% CO_2 (A). Plot B shows the linear correlation in the range between 31 and 500 cells/well. Luminescence signal was measured after addition of 100 µL ATPlite reagent per well and 15 min incubation at RT. Values represent the mean of at least three replicates ± SD.
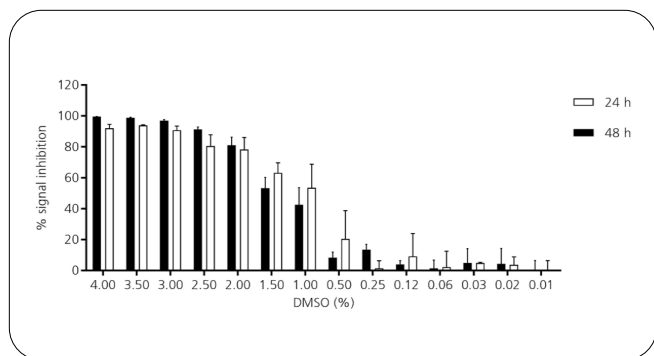

Figure 4. DMSO tolerance of CHO-K1 cells. CHO-K1 cells were seeded at a concentration of 250 cells/well and incubated for 24 h at 37 °C with 5% CO_2. At the end of this incubation time DMSO was added in increasing concentrations and cells were incubated for additional 24 h or 48 h. Luminescence signal was measured after addition of 25 µL ATPlite reagent per well and 15 min incubation at RT. Values represent the mean of at least three replicates ± SD measured in a 384-well plate.

Cytotoxicity Test

Three reference compounds, colchicine, 5-fluorouridine and 6-mercaptopurine, were selected for testing of their cytotoxic effects on CHO-K1 cells. Colchicine is a widely used substance for disruption of mitosis through binding to

tubulin and thus inhibition of its polymerisation. Inhibition of cell growth can be observed when cells are incubated with colchicine. 5-fluorouridine is a pyrimidine nucleoside analog and shows anti-cancer and cytotoxic activity. 6-mercaptopurine is an inhibitor of nucleic acid synthesis by interference with purine metabolism that can result in an immunosuppressive effect. To evaluate the impact of these compounds on cell viability, CHO-K1 cells were incubated for 48 h with increasing concentrations of colchicine, 5-fluorouridine or 6-mercaptopurine (1:2 serial dilution) at 37 °C and 5 % CO_2.

The results revealed that cell viability, as measured with the ATPlite 1step luminescence assay, was significantly reduced when cells were incubated with colchicine and 5-fluorouridine. Both compounds showed similar cytotoxic potency with IC_{50} values of 28 and 54 nM respectively (Figure 5). Although IC_{50} values for cytotoxicity are highly dependent on assay and cell line, a similar range for the colchicine IC_{50} was shown by Thomopoulou et al[1] for THP-1 and Jurkat cells of 20.4 nM and 13.5 nM respectively. In contrast, 6-mercaptopurine showed only a weak impact on the ATP level in CHO-K1 cells with a maximum inhibition effect of ~ 50 % at 40 µM. Therefore colchicine and 5-fluorouridine can be used as good positive controls for detection of cytotoxicity in CHO-K1 cells.

Z' and Inter-plate Variability Determination

In order to evaluate assay quality and robustness, Z' analysis[2] and plate to plate variation were tested. Colchicine (1 µM) was used as a positive control for inhibition of cell viability and DMSO (0.5% v/v) was added to wells representing the negative control. For each condition 16 wells were analyzed. The calculated Z' of 0.71 (Figure 6) reveals a robust assay suitable for multiple compound tests. The variation of the signal in the DMSO control may be caused by the long incubation time of cells in the microplate (72 h total incubation time), which can result in evaporation effects, DMSO-related cytotoxic effects and increased depletion of nutrients in cell culture medium. To determine inter-plate variability, five cell plates were seeded and incubated with 1 µM colchicine or 0.5% DMSO in parallel. Z' analysis was performed as described above. Plates showed Z' values > 0.8 and CV of ~ 12% in the positive control and mostly < 10% in the negative control, therefore revealing good assay reproducibility (Table 1).
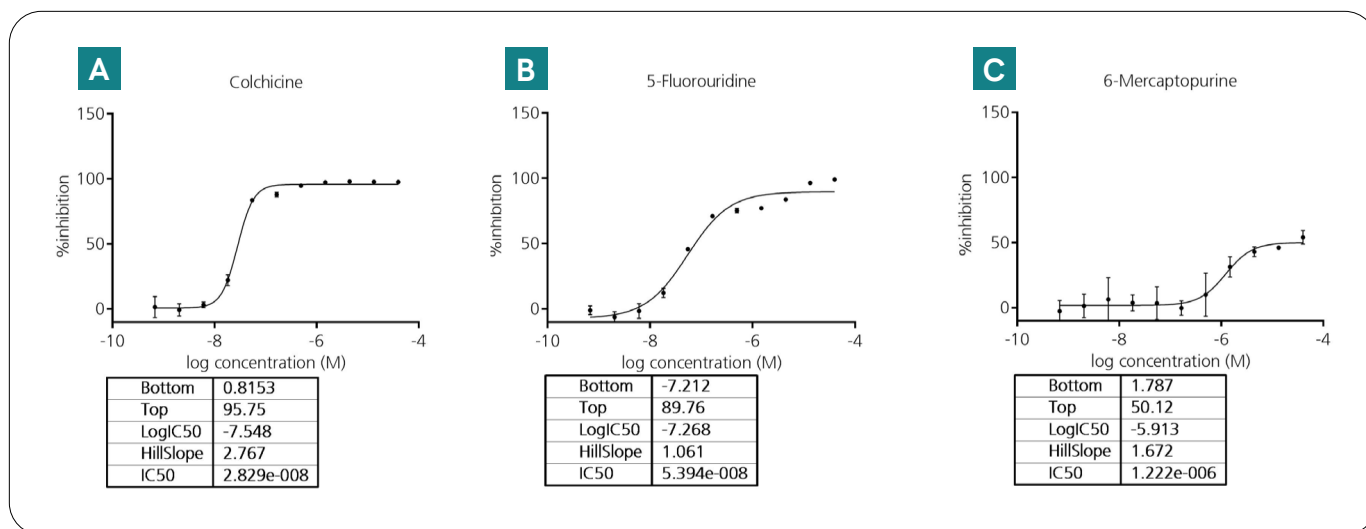
Figure 5. Evaluation of cytotoxic effect of colchicine (A), 5-fluorouridine (B) or 6-mercaptopurine (C) in CHO-K1 cells. CHO-K1 cells were seeded at a concentration of 250 cells/well and incubated for 24 h at 37 °C and 5% CO_2. By the end of the incubation time compounds or DMSO (0.5 % v/v) were added in increasing concentrations and cells were incubated for an additional 48 h. Luminescence signal was measured after addition of 25 µL ATPlite reagent per well and 15 min incubation at RT. Values represent the mean of at least three replicates ± SD measured in a 384-well plate.

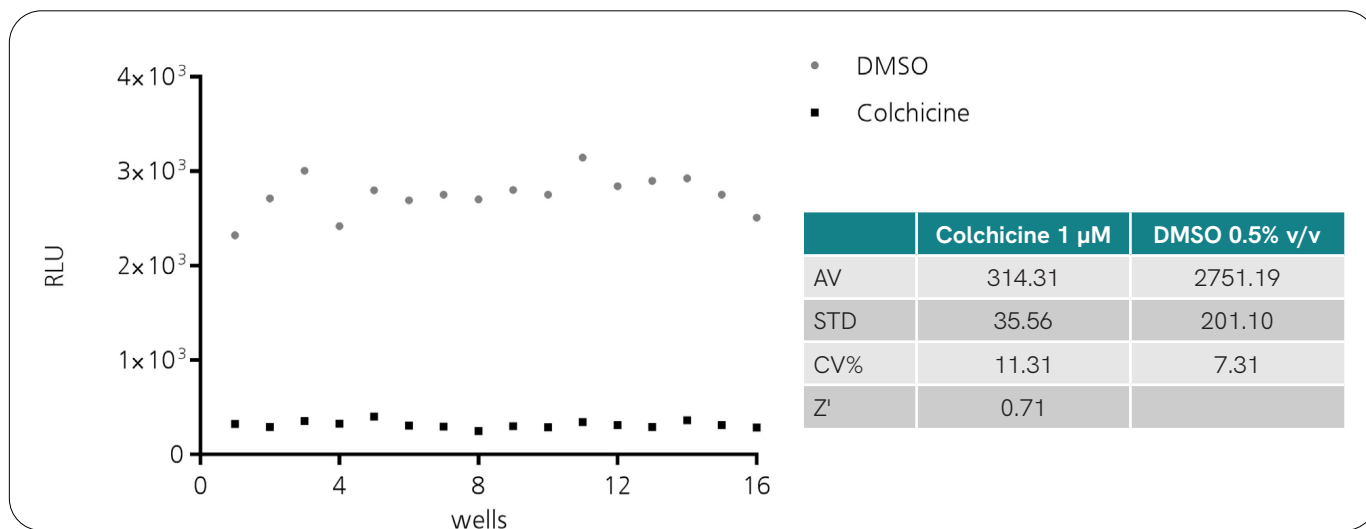|	Colchicine 1 µM	DMSO 0.5% v/v
AV	314.31	2751.19
STD	35.56	201.10
CV%	11.31	7.31
Z'	0.71	

Figure 6. Calculation of assay robustness using Z'. CHO-K1 cells were seeded in a 384-well plate at a concentration of 250 cells/well and incubated for 24 h at 37 °C and 5 % CO_2. At the end of the incubation time, colchicine (1 µM) or DMSO (0.5 % v/v) was added to 16 wells each and cells were incubated for additional 48 h. Luminescence signal was measured after addition of 25 µL ATPlite reagent per well and 15 min incubation at RT.

Conclusions

This study shows the application of the ATPlite 1step Luminescence assay system and the VICTOR Nivo multimode plate reader to monitor compound-related cytotoxic effects. The pre-installed luminescence protocol provided by the software of the VICTOR Nivo reader was ideally-suited to the requirements of the assay and was easily adapted when necessary. In addition, the bottom reading capability of the reader can maximize signal detection in a cell-based assay such as that used here (not tested in this study). The simplicity and stability of the ATPlite 1step luminescence assay system allowed fast optimization of assay conditions for the selected cell lines. The dynamic range and assay linearity were in agreement with the assay manual. The high assay quality, shown by high Z' values and low inter-plate variation, demonstrates its suitability for the testing of multiple compounds.

References

1. Thomopoulou, P., Sachs, J. Teusch, N., Mariappan, A., Gopalakrishnan, J. and Schmalz, H.-G. New Colchicine-Derived Triazoles and Their Influence on Cytotoxicity and Microtubule Morphology. ACS Med. Chem.Lett. 2016, 7, 188 – 191.

2. Zhang, JH., Chung, TD. and Oldenburg, KR. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen. 1999;4 (2):67-73.

Table 1. Inter-plate variability (CV) and Z' calculation of the ATPlite 1step assay in CHO-K1 cells incubated with colchicine and DMSO as positive and negative control for cytotoxicity, respectively.

	Plate 1		Plate 2		Plate 3		Plate 4		Plate 5	
	Colchicine 1 µM	DMSO	Colchicine 1 µM	DMSO	Colchicine 1 µM	DMSO	Colchicine 1 µM	DMSO	Colchicine 1 µM	DMSO
AV [RLU]	283.8	2539.0	314.3	2751.2	374.8	2831.4	352.5	2792.6	331.4	2757.3
STD [RLU]	34.6	329.1	35.6	201.1	51.8	253.9	44.0	254.0	55.3	256.9
CV [%]	12.2	12.9	11.3	7.31	13.8	8.9	12.49	9.10	16.7	9.3
Z'	0.52		0.71		0.63		0.63		0.61	