TECHNICAL NOTE

Cell Viability Assays

Comparison of Luciferase-based Technologies for Measuring Cell Proliferation and Cytotoxicity

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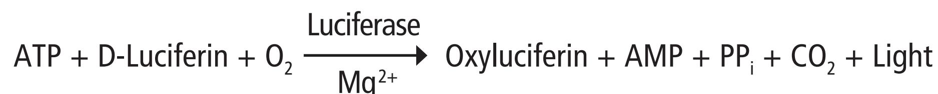
Introduction

In drug discovery, there remains a need for robust, rapid, high-throughput assays to identify and qualify novel therapies. Cytotoxicity assays are widely used in both fundamental research and drug discovery. For example, *in vitro* cytotoxicity assays can be used to identify new therapies against cancer, where the goal might be to stimulate or interfere with specific cell death mechanisms. For other therapeutic areas, cytotoxicity is an unwanted characteristic of drug candidates as cytotoxic compounds can have serious adverse effects *in vivo*.

Several methods have been developed to assess proliferation and cytotoxicity. Cell proliferation was once commonly assessed by measuring DNA synthesis via incorporation with radioactive thymidine or with labeled thymidine analogs such as bromodeoxyuridine (BrdU). A faster, simpler and more sensitive method to assess proliferation and cytotoxicity uses ATP as a marker of metabolic activity.

In this application note, four ATP-monitoring luminescence assays were assessed and compared head-to-head: ATPlite™ from PerkinElmer (referred below as "ATPlite (2steps)"), ATPlite™ 1step from PerkinElmer, and the CellTiter-Glo® (CTG) and CellTiter-Glo® 2.0 (CTG 2.0) Luminescent Cell Viability Assays from Promega.



$$\text{ATP} + \text{D-Luciferin} + O_2 \xrightarrow[\text{Mg}^{2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PP}_i + CO_2 + \text{Light}$$

Figure 1. Assay principle for ATP-based luminescence assays for measuring cell proliferation and cytotoxicity. Cellular ATP is quantified following direct lysis of the cells with suitable detergent or lysis buffer. ATP released from the lysed cells reacts with added luciferin in the presence of luciferase, oxygen and magnesium to produce light. The emitted light is then quantified with the use of a luminescence reader, whereby the measured luminescence signal is directly proportional to the amount of ATP present in the sample; and hence light intensity is representative of the number and health of cells present in the sample. The reagents have been formulated to generate a stable signal, avoiding the need to use dispensers built in the readers.

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Materials and Methods

Cell Culture

Jurkat cells (ATCC, #TIB-152) were cultured in RPMI media (ATCC, #30-2001) + 10% FBS (ThermoFisher, #26140-079). Cells were counted and serially diluted in RPMI + 10% FBS. 25 µL of each concentration of cells were added to AlphaPlate™-384 microplates (PerkinElmer, #6005350).

ATP Detection Assays

ATPlite (2steps) (PerkinElmer, #6016941), ATPlite 1step (PerkinElmer, # 6016731), CellTiter-Glo® (Promega, #G7570) and CellTiter-Glo® 2.0 (Promega, #G9241) Luminescent Cell Viability Assay kits were run according to the manuals. For each kit, the substrate solution was either reconstituted according to the manual (PerkinElmer kits) or was thawed and equilibrated at room temperature according to the manual (Promega kits). The ATP standard from the ATPlite 1step kit was serially diluted into RPMI media. The same stocks of ATP standard were used to run the standard curves for all kits for fair comparison (the Promega kits do not include an ATP standard). For each kit, 25 µL of each standard concentration was added to a 384-well AlphaPlate in replicates of 12. For ATPlite 1step, CTG and CTG 2.0, 25 µL of substrate solution was added and the plate was shaken vigorously for two minutes. For ATPlite (2steps), 12.5 µL of lysis solution was added and the plate was shaken vigorously for five minutes; then 12.5 µL of the ATPlite substrate solution was added and the plate was shaken vigorously for an additional five minutes. Each of the CTG, CTG 2.0 and ATPlite (2steps) plates were incubated at room temperature for 10 minutes prior to reading the plate. For ATPlite 1step, the plate was incubated for 5 minutes prior to reading. For the Jurkat cell titration, the same procedure was performed with 25 µL of cells (in RMPI + 10% FBS) instead of the 25 µL of ATP standard.

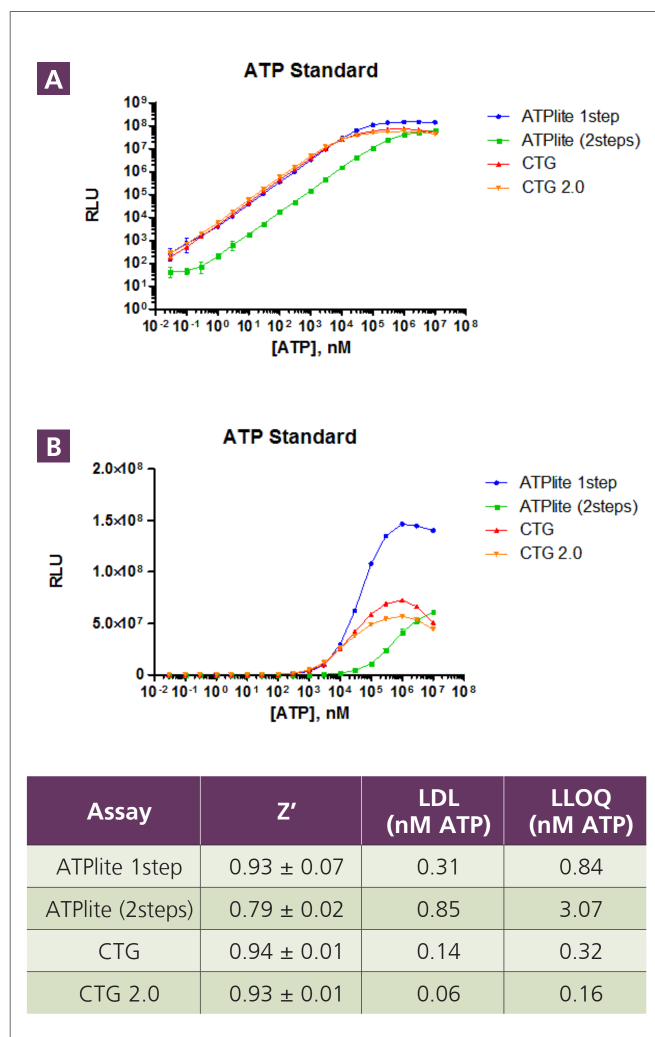
Instrument and Data Analysis

All assays were read with an EnVision® 2105 multimode plate reader using ultra-sensitive luminescence settings and a 0.1s measurement time per well. All data were analyzed using GraphPad Prism®. The standard curves and cell titration curves were analyzed using a nonlinear regression with a four-parameter logistic equation (sigmoidal dose-response curve with variable slope). The Lowest Detection Limit (LDL) was calculated using the interpolation of the average of the background + 3X its standard deviation (SD) on the curve fit using only the points in the range of signal intensity. The Lowest Limit of Quantification (LLOQ) was calculated as the interpolation of the average of the background + 10X its SD of the background on the curve fit using only the points in the range of signal intensity . Z' was calculated using the following equation: Z'= 1- (3* (SD (high value) + SD (low value))/ (Average (high value) - Average (low value))). Z' values reported are the average ± SD of Z' for 1 µM to 1 mM ATP each value compared with background.

Results

ATP Standard Curve

Assay performance was first assessed by running ATP standard curves using detection reagents from each kit. Both ATPlite (2steps) and ATPlite 1step appear to have a wider dynamic range compared to each of the CTG or CTG 2.0 kits (Figure 2). A wider dynamic range is advantageous, as it makes it less likely samples will require dilution to fall within the sensitive range of the assay. ATPlite 1step also showed a sensitivity (measured by LDL and LLOQ) comparable to the CTG and CTG 2.0 kits, with a similar signal window. The ATPlite (2steps) assay emitted less light and was more variable and slightly less sensitive than the other assays (although largely covering the physiological range of ATP concentrations, see detailed explanations in conclusion). The reproducibility of each assay was assessed by Z' calculations. All four assays had robust Z' values greater than 0.75, indicating all four assays are amenable to higher throughput assays such as cytotoxicity screening.



Assay	Z'	LDL (nM ATP)	LLOQ (nM ATP)
ATPlite 1step	0.93 ± 0.07	0.31	0.84
ATPlite (2steps)	0.79 ± 0.02	0.85	3.07
CTG	0.94 ± 0.01	0.14	0.32
CTG 2.0	0.93 ± 0.01	0.06	0.16

Figure 2. ATP standard curves. The graph shows the raw luminescence signal for the standard curve, for each assay, with the light emission (Y axis) presented as a logarithmic scale to visualize the full dynamic range (A), or as linear scale to better visualize the differences between kits at the highest ATP concentrations (B). Reproducibility (Z') and sensitivity (LDL and LLOQ) are indicated in the table below the graphs.

To look more closely at the dynamic range of each assay, the raw luminescence signal for each assay was normalized to the ATPlite 1step raw luminescence signal (Figure 3). Compared to ATPlite 1step, the luminescence signal for the CTG and CTG 2.0 assays drops significantly around 1 µM ATP. This suggests that the ATPlite 1step assay will better discriminate differences in ATP above this concentration, compared to the CTG and CTG 2.0 assays. This may be particularly important in cancer studies, where rapidly proliferating tumor cells demand high levels of ATP, where ATP production is demonstrated to be significantly higher with respect to invasive vs. non-invasive cancer cells, and where intratumoral extracellular ATP concentrations have been shown to be 1,000X higher than in non-tumoral tissues.[1-3]

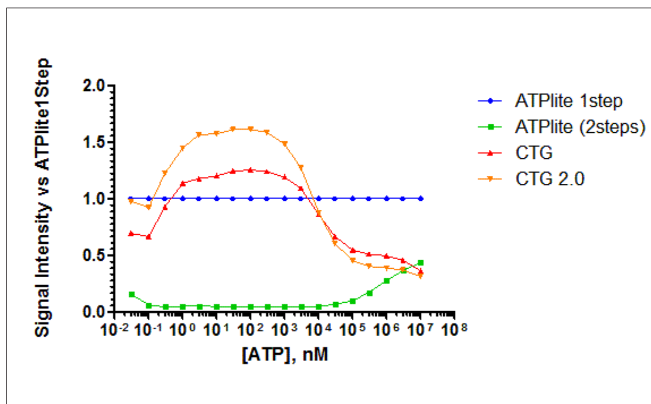
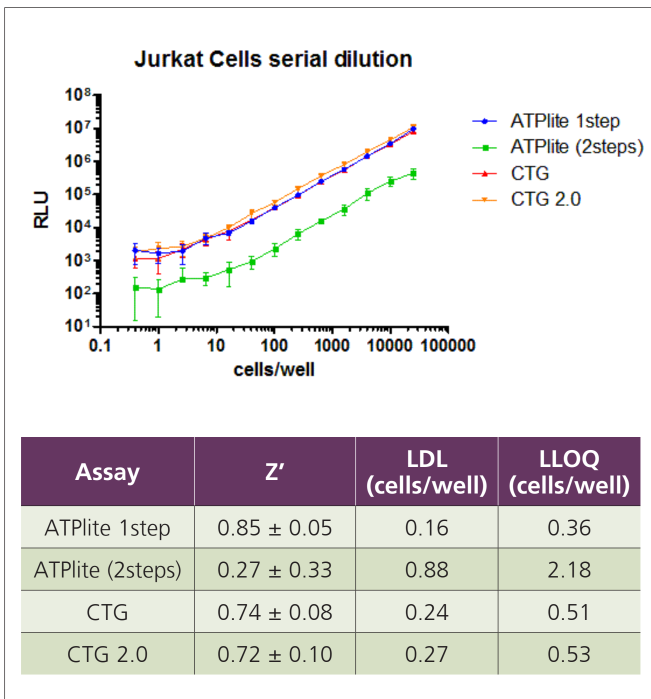


Figure 3. Signal for ATP standard curves, normalized to signal for the ATPlite 1step assay.

Cellular assays

Next, the performance of each assay was compared using serial dilutions of Jurkat cells to mimic differences in proliferation. Again, the ATPlite 1step, CTG and CTG 2.0 kits had comparable performance with respect to reproducibility (Z' value) and sensitivity (LDL and LLOQ; see Figure 4). It is interesting to note that all four kits exhibit sensitivities beyond what would be required for most cell proliferation or cytotoxicity assays: much less than 100 cells/well could be precisely detected by all four assays.



Assay	Z'	LDL (cells/well)	LLOQ (cells/well)
ATPlite 1step	0.85 ± 0.05	0.16	0.36
ATPlite (2steps)	0.27 ± 0.33	0.88	2.18
CTG	0.74 ± 0.08	0.24	0.51
CTG 2.0	0.72 ± 0.10	0.27	0.53

Figure 4. Serial dilution of Jurkat cells. Raw signal for each assay is shown in the graph. Reproducibility (as measured by Z') and sensitivity (as measured by LDL and LLOQ) are indicated in the table below the graph.

Conclusions

We compared four luciferase-based assays that utilize ATP as a metabolic marker of cytotoxicity and cell proliferation. ATPlite 1step showed the same high level of performance as the CTG and CTG 2.0 assays in terms of assay sensitivity and reproducibility. Additionally, data demonstrated that the ATPlite 1step and ATPlite (2steps) assays have a wider range than the CTG and CTG 2.0 assays, eliminating the potential need for sample dilution. The ATPlite (2steps) assay, though it requires two addition steps, has the advantage that it also permits a pause in the workflow, where cell lysates can be prepared on different days for different types or durations of cell treatment (for example) and stored at -20°C until all samples are thawed and the second reagent added for testing all the samples on the same day. ATPlite assays provide a fast and simple luminescent measurement for quantitation of cell numbers, cell proliferation, and cytotoxic effects for drug discovery and basic research.

References

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