APPLICATION NOTE

Utilization of the ATPlite 1step Detection System for Homogeneous Automated Cytotoxicity Assays on the JANUS Cellular Workstation

Introduction

A number of strategies are currently being implemented in an effort to increase the success rate in identifying potential therapeutic lead compounds and reducing the number of late stage clinical trial failures. One approach is to perform ADME/Tox profiling at an earlier stage in the drug discovery process. The **ATPlite® 1step** Luminescence Assay System has been used to perform automated cytotoxicity assays on the **JANUS®** Cellular Workstation creating an easy-to-use, accurate integrated walk-away system for cellular assays.

ATPlite 1step assay is a homogeneous, luciferase-based luminescence detection assay system for the quantitative measurement of proliferation and cytotoxicity of cultured mammalian cells. ATP level is a general marker for determining cell viability, as it is present in all metabolically active cells and its concentration declines rapidly when cells undergo necrosis or apoptosis. Model cytotoxicity assays have been performed using both U937 suspension cells, and HepG2 adherent cells. Resulting cell viability was measured following treatment with either Staurosporine or Actinomycin D. Cell viability was determined as a function of both the time of exposure and the concentration of the test compounds.

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Figure 1. The JANUS Cellular Workstation is a fully integrated system solution providing liquid handling, detection, plate movement, capacity as well as scheduling, incubation and plate washing options.

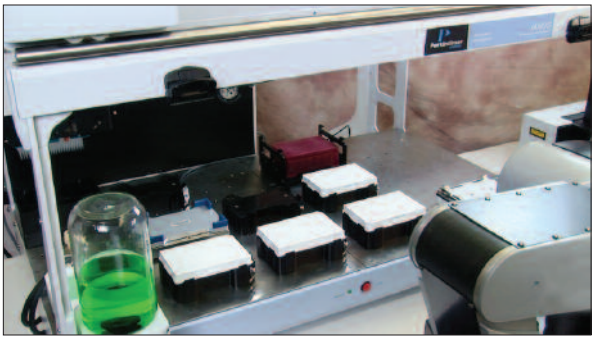


Figure 2. Typical JANUS deck layout for the ATPlite 1step assay. The reagent reservoir is highlighted here in green.

Materials and methods

The Cellular Workstation

PerkinElmer's JANUS Cellular Workstation was utilized for the ATPlite 1step assay. The Workstation was equipped with a 384-channel Modular Dispense Technology® (MDT) head (PerkinElmer, Inc.) and a Variomag Teleshake plate shaker (Quantifoil Instruments), an EnVision® 2100 Multilabel plate reader (PerkinElmer, Inc.) a BioTek® ELX405 Plate washer, a Cytomat® 2C incubator and a CRS CataLyst Express arm (Thermo-Scientific) with an integrated Shadow Delidder.

Liquid Handling

Precision liquid handling was delivered using the JANUS system. JANUS WinPREP® assay templates were developed and tested specifically for the ATPlite 1step assay application in a 384-well format. Two deck positions on the JANUS instrument are used to hold test compound plates and cell assay plates. The ATPlite 1step reagent is dispensed from a one well trough fitted with a reservoir bottle. A fourth deck position is occupied by an orbital plate shaker. The movement of the robotic arm is controlled by POLARA™ software which also schedules the sequence and timing of the steps in the assay method. Multiple plate batch runs can be optimized to efficiently utilize instruments and maintain consistent timing of liquid handling, washing, incubation, and detection steps.

Assay Automation

All steps in the assay, with the exception of cell seeding, were automated using the JANUS Cellular Workstation. Two methods and schedules were created and run for this assay. The first adds the test compounds to the assay plates containing the cells, and the second adds the ATPlite 1step reagent at the end of the final incubation. Below is the general flow of the ATPlite 1step assay automation protocol.

1. Cells were dispensed off-line using the FlexDrop PLUS and the assay plates loaded into the Cytomat for a 4 h incubation at 37 °C / 5% CO_2.
2. Standards or test compounds were dispensed into 384-well plates and loaded into CataLyst Express hotels. Sets of compound and assay plates were transferred to the JANUS deck. The compounds were pipetted into the assay plates using the JANUS with the 384-channel MDT head.
3. Plates were returned to the incubator for either a 24 or 48 h incubation.
4. Plates were removed from the Cytomat incubator, delidded, and loaded onto a hotel of the CataLyst Express for 15 minutes to equilibrate to room temperature.
5. ATPlite 1step reagent was prepared in advance and loaded into the reagent reservoir of the Cellular Workstation.
6. The CataLyst Express arm then transferred the plates to the JANUS Workstation which subsequently pipetted the ATPlite 1step reagent into the assay plate.
7. Plates were transferred to the EnVision reader for luminescence detection.

Cell Culture

HepG2 and U937 cells were obtained from ATCC and cultured in an incubator at 37 °C with 5% CO_2. HepG2 cells were dissociated with 0.25 % Trypsin (Cat. No.15050-065, Invitrogen, Inc.).

HepG2 cell growth medium: MEM (Cat. No. 11095-098, Invitrogen, Inc.) with 10% FBS, 2 mM L-Glutamine, 100 mM sodium pyruvate and 10 mM HEPES.

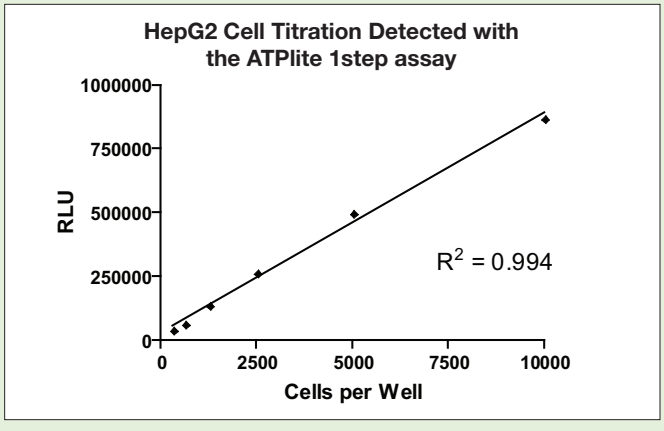


Figure 3.

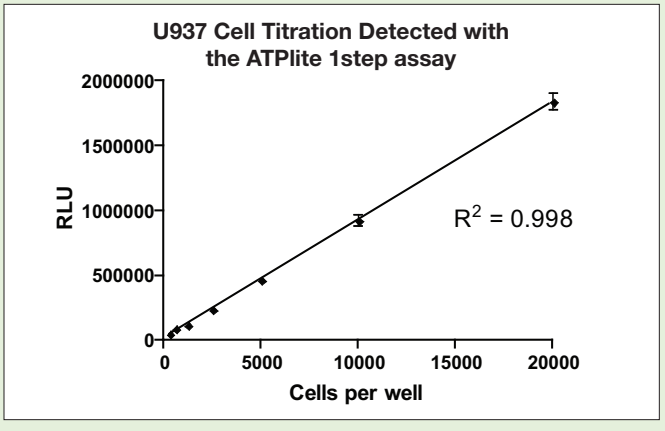


Figure 4.

U937 cell growth medium: RPMI-1640 (Cat. No.11875-135, Invitrogen, Inc.) with 10% FBS, 2 mM L-Glutamine, 100 mM sodium pyruvate and 10 mM HEPES.

Assay Reagents and Protocol

The assay was performed following the ATPlite 1step assay kit protocol (Cat. No. 6016739, PerkinElmer, Inc.). Staurosporine and Actinomycin D were obtained from Sigma, Inc.

Microplates

The assay was performed in CulturPlate™-384 white 384-well cell culture plates (Cat. No. 6007680, PerkinElmer, Inc.).

Plate Seeding

Plates were seeded prior to the start of the assay using the FlexDrop™ PLUS Precision Reagent Dispenser (PerkinElmer, Inc.).

Results

U937 and HepG2 Cell Titrations

To demonstrate a linear correlation between the cell number and luminescence signal, ten plate batches were seeded with 2-fold serial dilutions of both U937 and HepG2 cells. The plates were incubated for 4 h at 37 °C / 5% CO_2 and the ATPlite 1step assay was performed. The linear regression analysis of the response is shown in Figures 3 and 4.

The inter-plate %CV of the luminescence signal was:

- 2.7% for U937 cells
- 3.5% for HepG2 cells

Staurosporine Cytotoxicity

HepG2 and U937 cells (2,000 per well) were treated with Staurosporine, a non-selective Tyrosine kinase inhibitor, over a range from 0.2 to 25 µM and incubated for either 24 or 48 h at 37 °C / 5% CO_2. As illustrated in Figures 5-8 cell viability measured using ATPlite 1step showed that both cell lines exhibited similar time and concentration sensitivity to Staurosporine exposure.

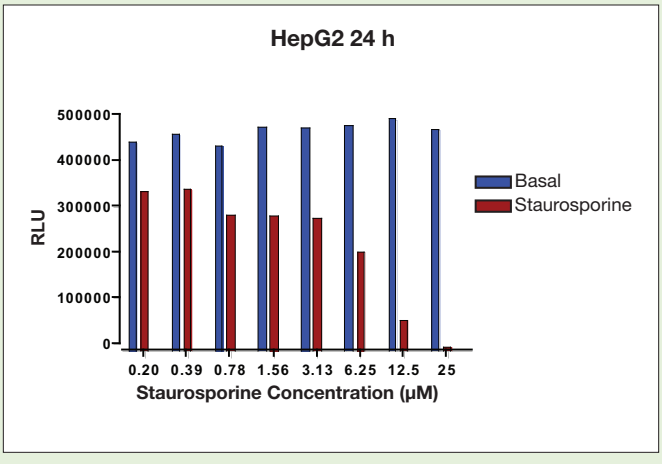
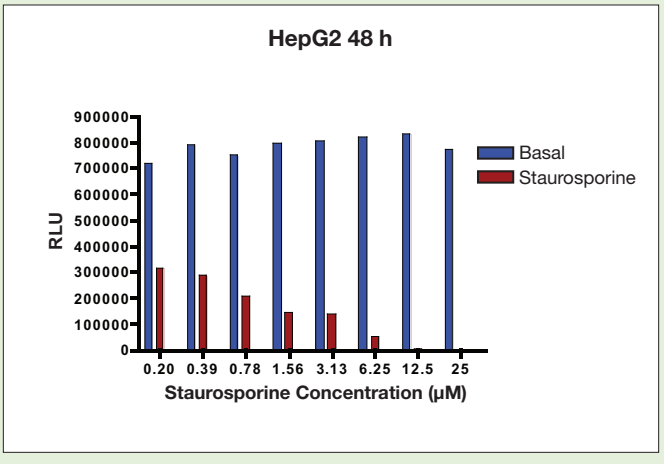


Figure 5.



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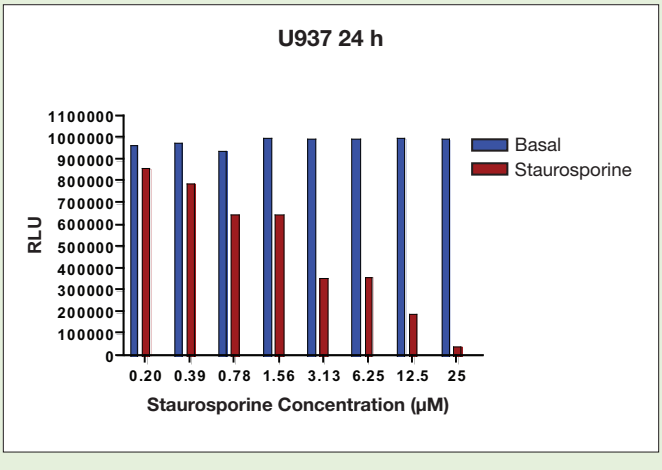

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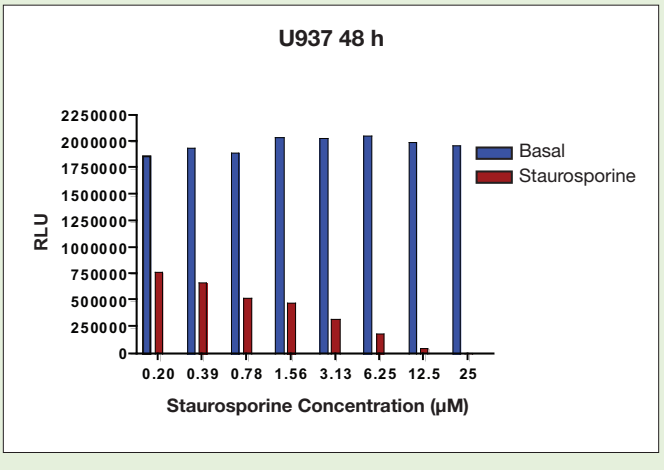


Figure 8.

Actinomycin D Cytotoxicity

Actinomycin D binds to DNA and blocks the transcription of mRNA by RNA polymerase. The cytotoxicity of Actinomycin D to U937 cells was tested under the same conditions as described above for Staurosporine. Actinomycin D demonstrated approximately 10-fold higher cytotoxic potency compared to Staurosporine treated cells, see Figures 9 and 10.

Z' Analysis with U937 Cells

A Z' analysis of Staurosporine or Actinomycin D cytotoxicity was performed using U937 cells at 2,000 cells per well. Thirty-two wells of cells treated with either 25 µM Staurosporine or 5 µM Actinomycin D were compared to 32 wells of basal cells following either 24 or 48 h of incubation. Representative data for the two time points is shown in Table 1. Z' values for 24 h exposure with

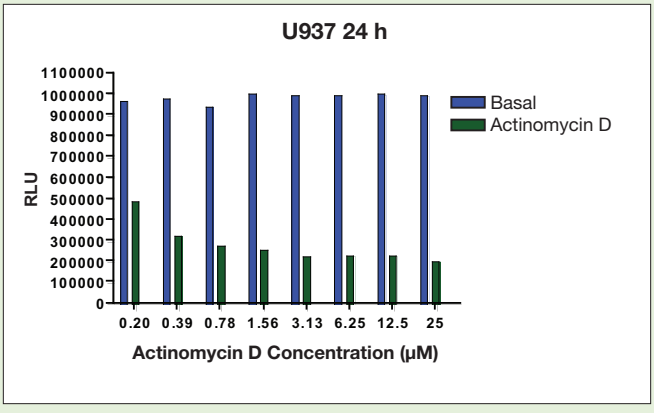
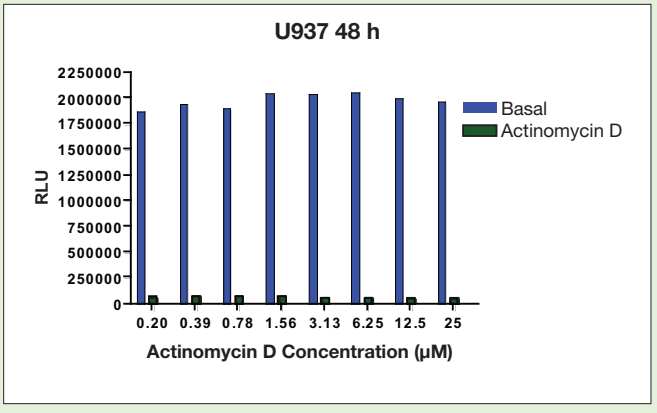


Figure 9.



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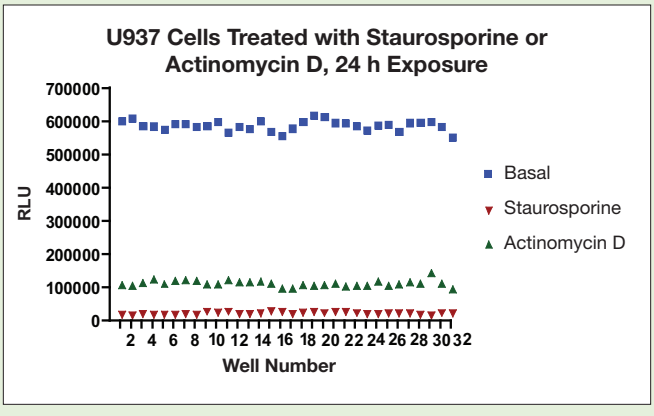


Figure 11.

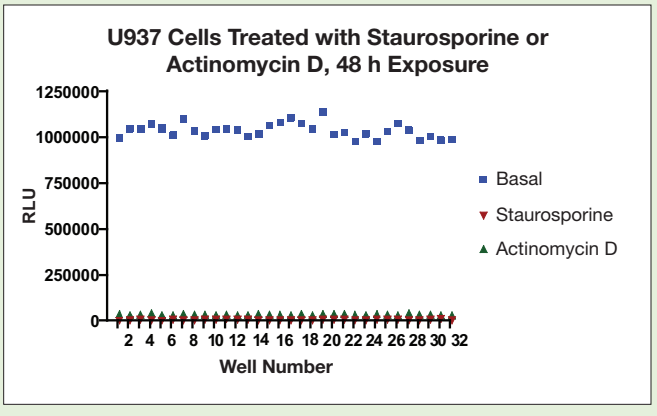


Figure 12.

Table 1.

Plate:	1	2	3	4	5	6	7	8	9	10
Z' 48 h Exposure of U937 Cells to Staurosporine	0.85	0.83	0.81	0.82	0.81	0.78	0.79	0.79	0.88	0.85
Z' 48 h Exposure of U937 Cells to Actinomycin	0.85	0.83	0.80	0.83	0.82	0.82	0.83	0.89	0.88	0.87
Z' 48 h exposure of HepG2 Cells to Staurosporine	0.69	0.76	0.73	0.77	0.74	0.76	0.74	0.68	0.64	0.77

either Staurosporine or Actinomycin D were better than 0.73 and 0.71 respectively (data not shown). Figures 11 and 12 show Z' analysis data for U937 Cells exposed to Actinomycin D for 24 h and 48 h respectively.

Z' Analysis with HepG2 Cells

A Z' analysis of Staurosporine cytotoxicity was performed using HepG2 cells. A 10 plate batch was seeded at 2,000 cells per well and treated with 25 µM Staurosporine for 48 h. As illustrated in Figure 13 the Z' was calculated comparing the response of 48 wells of basal versus 48 wells of treated cells. Table 1 shows the average Z' for the 10 plates was 0.72.

Inter-Plate Variability of a Ten Plate Batch

To test the inter-plate variability of a multiple plate batch run, a ten plate batch of 2,000 U937 cells per well was treated with 5 µM Actinomycin D, or 25 µM Staurosporine. The plates were incubated for either 24 or 48 h at 37 °C / 5% CO_2 and an ATPlite 1step assay was performed using the JANUS Cellular Workstation. The average signal, standard deviation and %CV for the various test conditions at the two time points is shown in Figures 14 and 15.

Assay throughput

The ATPlite 1step assay was efficiently automated providing more than 30,000 data points within an 8 h workday. This represents approximately an 8-fold increase in productivity over manual methods. Additionally, we often ran methods overnight increasing our productivity to a 16 h workday with over 60,000 data points. The stability of the ATPlite reagents combined with the true walk-away automation capabilities of the JANUS Cellular Workstation produced consistent and reliable data and results.

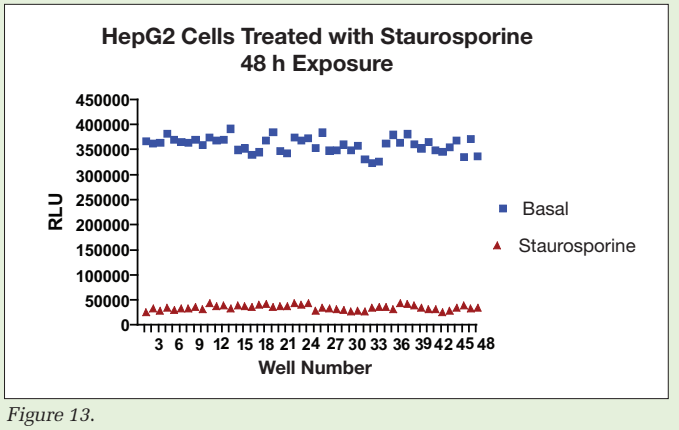
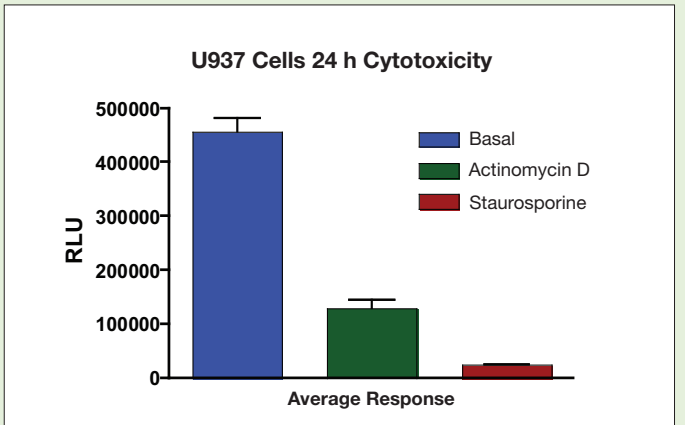
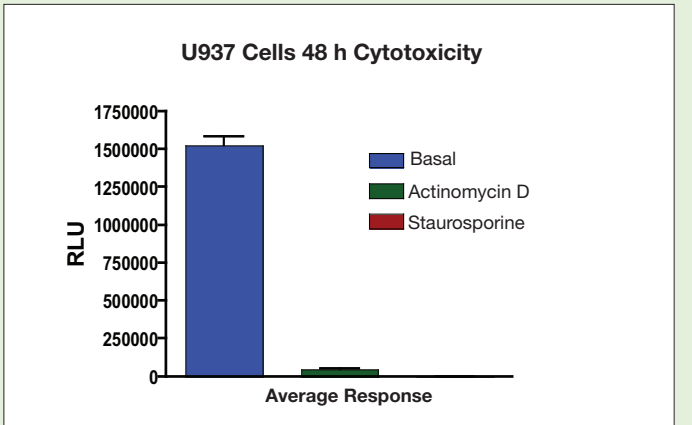


Figure 13.



24 h:	Basal	Actinomycin D	Staurosporine
Average	457,411	130,263	25,283
Std Dev	25,595	14,944	1,890
%CV	5.6	11.5	3.6

Figure 14.



48 h:	Basal	Actinomycin D	Staurosporine
Average	1,529,771	51,365	3,002
Std Dev	58,868	3,903	272
%CV	3.9	7.6	9.1

Figure 15.

Conclusions

The JANUS Cellular Workstation has been used to automate cell-based cytotoxicity assays using ATPlite 1step technology to measure cellular ATP levels as a marker for cell viability. Assay throughput can be easily scaled up 8 fold or greater as compared to manual methods. The combination of PerkinElmer's high quality luminescence assay reagent with the precision and speed of the automation and detection technologies results in superior Z' values with low inter-plate variability, a must for all medium and high throughput applications.

ATPlite 1step luminescence assay system is an ideal assay platform for automated screening applications.

- Provides the simplicity of single reagent addition in a homogeneous format.
- Long term stability of the reconstituted assay reagent permits continuous processing of plates over a single day.
- Easily scalable between 96-, 384-, and 1536-well formats.

JANUS Cellular Workstation is a fully integrated and automated platform ideal for functional and cellular screening applications.

- Precise JANUS liquid handling plus the superior speed and detection sensitivity of the EnVision plate reader increases productivity while generating reliable results.
- Effectively integrates and schedules assay method steps, including liquid handling, plate washing, incubation and detection, maintaining plate-to-plate consistency and data integrity for true walk-away automation.
- Pre-tested application templates and application guides for ATPlite, britelite®, steadylite and LANCE® cAMP assays provide quick and easy application set up and results.

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