APPLICATION NOTE

Simultaneous detection of drug efficacy and toxicity by combining HTRF, AlphaLISA, or AlphaLISA *SureFire Ultra* with ATPlite.

Key features

This application note demonstrates how compound's mechanism of action and potential cytotoxic effects can be deciphered thanks to the combination of AlphaLISA™, HTRF™ or AlphaLISA™ *SureFire® Ultra™* immunoassays with ATPlite™ 1step cell viability assay.

Introduction

The attrition of drug molecules occurs at various stages of the development process and most early-phase failures are attributed to safety and toxicity issues. In that respect, and considering the economic impact of early project termination, the biggest challenge becomes selecting the most potent and selective drug compounds while assessing their potential toxic side effects.

Revvity offers the most comprehensive panel of immunoassays to measure compound biological activities by quantifying markers of interest including secreted cytokines and intracellular phosphorylated/total proteins, thanks to the HTRF, AlphaLISA, or AlphaLISA *SureFire Ultra* proprietary technological platforms.

Additionally, ATPlite 1step luminescence assay from Revvity provides a fast and simple measurement of intracellular ATP for assessing cell viability and monitoring cytotoxic effects of drug compounds.

This application note explains how to easily combine HTRF, AlphaLISA, or AlphaLISA *SureFire Ultra* immunoassays with ATPlite 1step cell viability assay to simultaneously and in a single sample study the efficacy of drug compounds on disease pathways while identifying possible cytotoxic effects.

Preliminary recommendations

Alpha, HTRF, and ATPlite signals increase proportionally to the amount of the analyte present in the sample. When the amount of analyte becomes higher than one of the detection reagents present in the well, the maximal signal is detected and reaches a plateau. In the case of HTRF and Alpha homogeneous sandwich immunoassays, it is possible for the signal to go down if an excess of analyte oversaturates the detection reagents. This situation is known as the hook effect (Figure 1).

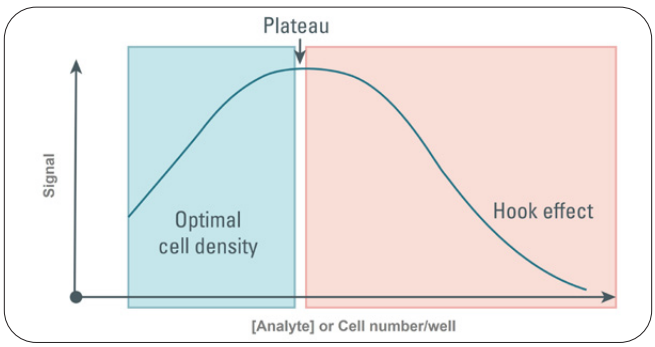

Figure 1: Detection signal intensity depending on analyte concentration and cell density.

In this application note, intracellular ATP and the marker(s) of interest, secreted or intracellular, will both be measured from the same sample. Due to this, careful optimization of cell density for each cell model, assay, lysis buffer, and experimental conditions is required to ensure that all detected analytes will be assessed at a concentration compatible with their respective assay's linear range thus avoiding hook effect. A cell density that is too high can result in one analyte being outside of its linear range and lead to its subsequent underestimation. For Alpha and HTRF assays, the sample (cell supernatant or lysate) can alternatively be pre-diluted with the cell culture medium or the lysis buffer just before detection.

1. Combining ATPlite 1step assay with the detection of secreted marker(s)

Principle

Assessing cell viability in parallel with the quantification of secreted marker(s) is of great interest to ensure that modulations of the released target(s) concentration are not caused by an effect of the tested compound(s) on cell viability, especially in the case of long-time treatment (> 2h). For example, an increase in cell secretion can be caused by a cytotoxic effect of a drug leading to cell membrane disruption. Inversely, a change in the release of the marker(s) can be indirectly triggered by a mechanism of defense of the cell facing a loss of viability.

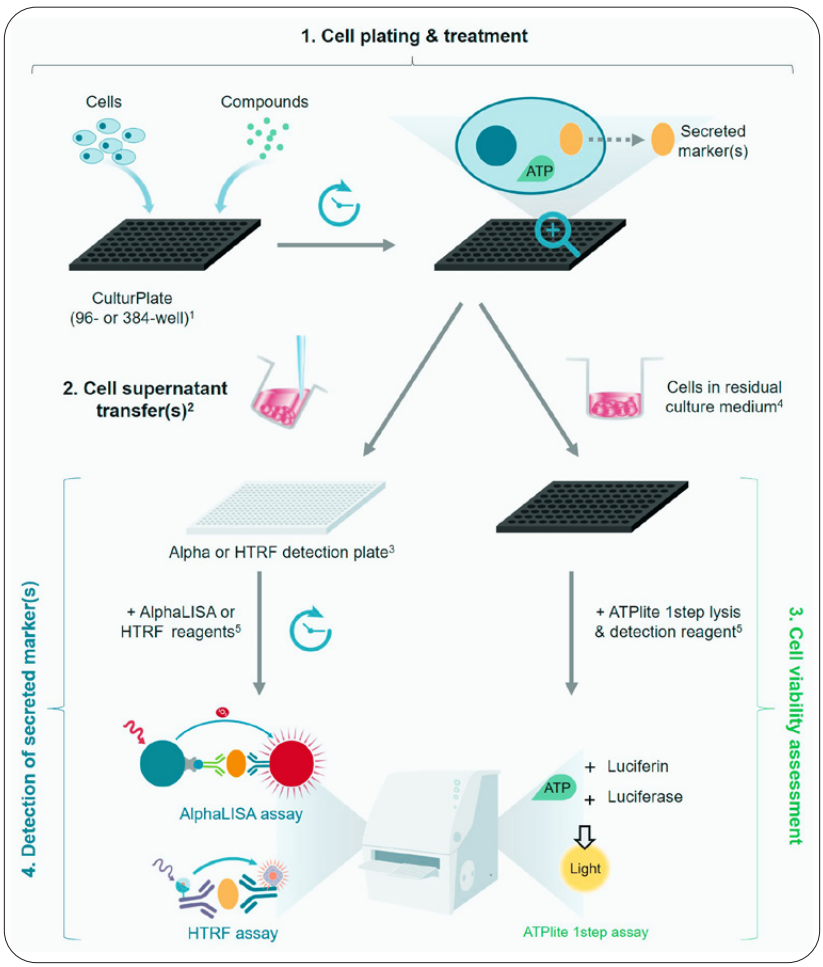

Figure 2: Workflow describing how to combine ATPlite 1step cell viability assay with the detection of secreted marker(s) using AlphaLISA and/or HTRF assays. [1]Cell treatment under 100 µL in 96-well plate, or under 25 µL in 384-well plate. [2]Sample volume mentioned in the AlphaLISA and HTRF kit manuals. Multiple transfers are required if several markers are detected. [3]Alpha or HTRF detection plate recommended in AlphaLISA and HTRF kit manuals. [4]No need to add culture medium after cell supernatant transfer. The ATPlite 1step lysis & detection reagent can be directly added on the cells, whatever the volume of residual medium (100 µL/well in 96-well plate or 25 µL/well in 384-well plate). [5]AlphaLISA, HTRF, and ATPlite 1step detection reagents are prepared, dispensed, and incubated as described in the corresponding kit manuals.

Figure 2 describes how to combine ATPlite 1step cell viability assay with the detection of secreted marker(s) using AlphaLISA and/or HTRF assay(s). Briefly, cells are plated in a tissue culture plate and treated with compound(s) for the desired time. The appropriate volume of cell supernatant is then transferred in a detection plate (as many times as needed according to the number of markers detected) for quantification of the secreted target(s) of interest by AlphaLISA or HTRF. Following cell supernatant transfer, ATPlite 1step detection reagent is directly added in the tissue culture plate containing the cells in residual culture medium to assess cell viability. After the appropriate incubation time with detection reagents, each assay is read on a compatible microplate reader.

Case study with AlphaLISA and HTRF Cytokine assays

The effect of two different compounds, LPS (Lipopolysaccharides from E. coli) and Bortezomib (proteasome inhibitor anticancer drug), was studied on the secretion of the pro-inflammatory cytokines TNF-α and IL-1β by the human monocytic leukemia cell line THP-1. Cell viability was simultaneously assessed on the same cells.

Material and methods

Material

Material and reagents used are listed in Table 1 below.

Experimental conditions

THP-1 cells were dispensed in a CulturPlate-96 (100,000 cells/well) in 80 µL complete culture medium (RPMI 1640 medium supplemented with 10% FBS and 1% Pen/Strep). After 30 minutes of incubation at 37°C - 5% CO_2, the cells were treated overnight with 20 µL of increasing concentrations of LPS or Bortezomib (both prepared in complete culture medium, supplemented with 0.1% DMSO for Bortezomib only). The day after, the plates were centrifuged (450 x g for 5 minutes at RT) to pellet the cells and the appropriate volume of cell supernatant was sequentially transferred twice into an AlphaPlate-384 or a ProxiPlate-384 Plus for measuring human TNF-α and IL-1β by AlphaLISA or HTRF, respectively. Just after cell supernatant transfer, 100 µL of ATPlite 1step lysis & detection reagent was directly added in each well of the CulturPlate-96 and the incubation/mixing/detection step was carried out as described in the ATPlite 1step kit manual. The addition of AlphaLISA and HTRF detection reagents, as well as the incubation/detection step, were also performed following the instructions detailed in the respective AlphaLISA and HTRF kit manuals. For both AlphaLISA cytokine assays and HTRF human IL-1β assay, cell supernatants were directly measured neat. For HTRF human TNF-α assay, cell supernatants were pre-diluted 10 times in complete culture medium before analysis.

Similar experiments were performed in CulturPlate-384 to combine the detection of human TNF-α (by AlphaLISA or HTRF) with ATPlite 1step cell viability assay. All experimental conditions were the same as the one described above,

Table 1: Material and reagents.

	Material / Reagent	Supplier	Part number
Tissue-culture microplates	CulturPlate-96, Black opaque	Revvity	6005660/8/9
	CulturPlate-384, Black opaque	Revvity	6007660/8
Detection microplates	AlphaPlate-384, Light gray	Revvity	6005350/9
	ProxiPlate-384 Plus, White 384-shallow well	Revvity	6008280/9
AlphaLISA kits	AlphaLISA TNFα (human) Detection Kit	Revvity	AL208C/F
	AlphaLISA IL-1β (human) Detection Kit	Revvity	AL220C/F
HTRF kits	HTRF Human TNF-alpha Kit	Revvity	62HTNFAPET/G/H
	HTRF Human IL-1beta Kit	Revvity	62HIL1BPET/G/H
Cell viability kit	ATPlite 1step Luminescence Assay Kit	Revvity	6016736/1/9
Cell line	THP-1	ATCC	TIB-202
Compounds	Lipopolysaccharides (LPS)	Sigma-Aldrich	L4391
	Bortezomib	Tocris	7282

except that THP-1 cells were dispensed under 25,000 cells/well in 20 µL medium, treated with 5 µL of each compound, and 25 µL/well of ATPlite 1steplysis & detection reagent was added.

Instrumentation and data analysis

All assays were measured on the EnVision™ multimode plate reader (Revvity). The ATPlite 1step signal was measured with the standard luminescence module (infrared cut-off filter 2100-212/luminescence mirror module 2100-404). The AlphaLISA signal was detected with the standard Alpha module using the Alpha standard settings (laser excitation at 680 nm/emission at 570 nm, bandwidth 100 nm). The HTRF signal was recorded using the HTRF module "Europium cryptate donor/Red acceptor" (laser excitation at 337 nm/dual emissions at 620 nm and 665 nm).

The data were analyzed using the GraphPad Prism® software. The concentration of each cytokine was obtained by interpolation of the Alpha or HTRF signal on the corresponding standard curve. The error bars represented on histobars correspond to the standard deviations (SD) of the means of three independent wells of cells.

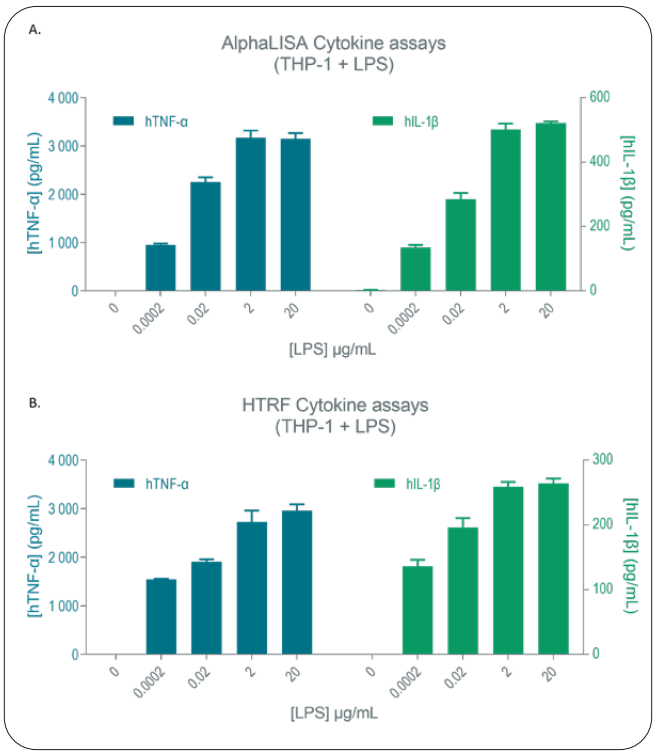

Figure 3: Quantification of human TNF-α and IL-1β secreted by THP-1 cells treated with LPS (A. AlphaLISA detection; B. HTRF detection).

Results and discussion

LPS treatment induced a dose-dependent increase in the release of the two pro-inflammatory cytokines TNF-α and IL-1β from THP-1 cells (Figure 3A & B). The secretion profiles obtained with both detection platforms AlphaLISA and HTRF were similar, with a maximal release of the two markers obtained at 2 µg/mL of LPS. As shown in Figure 4, the overnight treatment with LPS (blue bars) did not affect cell viability, even at high doses, demonstrating that the secretion of TNF-α and IL-1β is specifically due to the activation by LPS of the cell signaling pathways responsible for the release of these pro-inflammatory markers.

No secretion of TNF-α or IL-1β was detectable when cells were exposed overnight to Bortezomib, regarless of the technology used (data not represented in Figure 3). Besides, this proteasome inhibitor induced a dose-dependent decrease in cell viability, with a loss of 75% at the highest dose of 20 µM (Figure 4, red bars).

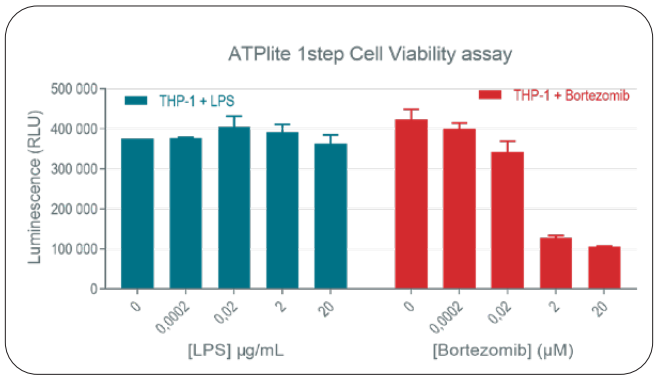

Figure 4: ATPlite 1step cell viability assay on THP-1 cells treated with LPS and Bortezomib.

It should be noted that the experiments performed in CulturPlate-384 gave similar profiles on human TNF-α secretion and cell viability (data not shown here).

2. Combining ATPlite 1step assay with the detection of intracellular marker(s)

Prerequisite

Measuring ATP and intracellular markers from the same cells and thus from the same cell lysate requires the use of a lysis buffer compatible with AlphaLISA or HTRF assay(s) and ATPlite 1step assay. The table below lists the Alpha and HTRF lysis buffers that have been validated and are compatible with ATPlite 1step assay.

Table 2: AlphaLISA and HTRF lysis buffers compatible with ATPlite 1step assay.

Lysis Buffer	Part Number
Alpha *SureFire Ultra* Lysis Buffer	ALSU-LB
HTRF Phospho-Total Protein Lysis Buffer #1*	64KL1FDF
HTRF Phospho-Total Protein Lysis Buffer #2*	64KL2FDF
HTRF Phospho-Total Protein Lysis Buffer #3*	64KL3FDF
HTRF Phospho-Total Protein Lysis Buffer #4*	64KL4FDF

*Must be supplemented with HTRF Phospho-Total Protein Blocking Reagent (64KB1AAC)

Principle

Monitoring cell viability in parallel with the detection of intracellular marker(s) is mandatory to dissociate the action of a tested compound on the modulation of the target(s) of interest from its potential effect on cell viability, especially in the case of long-time treatment (> 2h). For example, a decrease in the expression or phosphorylation level of a protein can be caused by a cytotoxic effect of a drug. In that situation, it is also recommended to include the detection of a housekeeping protein (such as GAPDH or α-Tubulin) to check that the treatment does not induce any cell detachment or decrease in global protein synthesis, and thus obtain a complete picture of the compound's mechanism of action.

Figure 5 describes how to combine ATPlite 1step cell viability assay with the detection of intracellular marker(s) using AlphaLISA *SureFire Ultra* or HTRF assay(s). Two different formats can be carried out:

Format A involves the detection of each marker of interest and ATP in separate wells/plates, which enables the multi-parametric detection of numerous analytes (e.g. phospho-protein + total protein + housekeeping protein + ATP).

Format B is based on the detection of one marker of interest and ATP in the same well, with a sequential detection of each analyte.

The first step of the protocol is shared by both formats. Briefly, cells are plated in a 96-well tissue CulturPlateTM and treated with compound(s) for the desired time. After medium

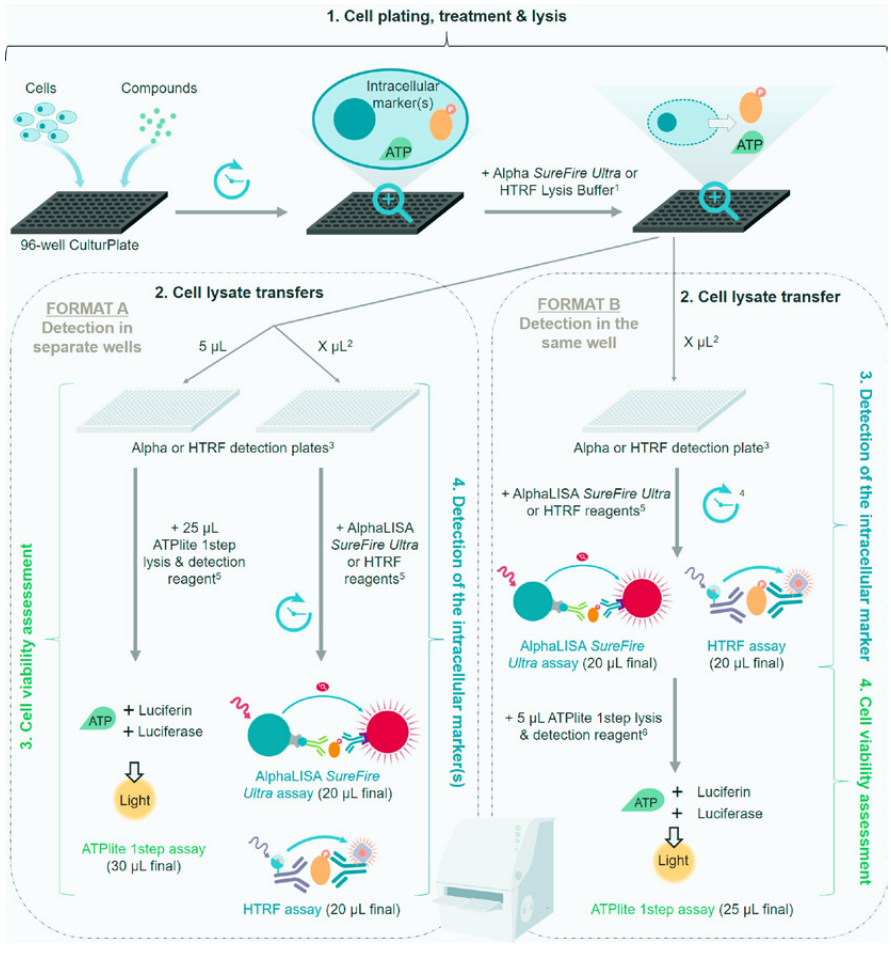

Figure 5: Workflow describing how to combine ATPlite 1step cell viability assay with the detection of intracellular marker(s) using AlphaLISA *SureFire Ultra* or HTRF assays. [1]The lysis step (volume of lysis buffer/ incubation time/shaking) must be carried out as described in the corresponding AlphaLISA *SureFire Ultra* or HTRF kit manuals. Refer to Table 2 for compatible Alpha and HTRF lysis buffers. [2]Sample volume mentioned in the AlphaLISA *SureFire Ultra* and HTRF kit manuals. Multiple transfers are required if several markers are detected. [3]AlphaPlate-384, Light gray or HTRF 96-well low volume white plate. [4]The total incubation time of the AlphaLISA *SureFire Ultra* or HTRF detection step must not exceed 4h to avoid ATP degradation. [5]AlphaLISA *SureFire Ultra*, HTRF and ATPlite 1step detection reagents are prepared, dispensed, and incubated as described in the corresponding kit manuals. [6]The ATPlite 1step lysis & detection reagent must be prepared 2.5 times more concentrated than what is mentioned in the kit manual (reconstitute the lyophilized substrate with a volume 2.5 times lower than the one mentioned).

removal, cells are lysed with the appropriate volume of the Alpha or HTRF lysis buffer provided in the kit(s) used to detect the intracellular marker(s) of interest (cf. Table 2 for lysis buffers compatible with ATPlite 1step assay).

Format A then requires the transfer of 5 µL of cell lysate into a first detection plate and the addition of 25 µL of ATPlite 1step lysis & detection reagent to measure cell viability. Into a second detection plate, the appropriate volume of the same cell lysate is sequentially dispensed (as many times as needed according to the number of markers of interest) and the corresponding AlphaLISA *SureFire Ultra* or HTRF detection reagents are added to quantify the level of each marker. After the appropriate incubation time with detection reagents, each assay is read on a compatible microplate reader.

Format B involves the transfer of the appropriate volume of cell lysate into a detection plate and the addition of the AlphaLISA *SureFire Ultra* or HTRF detection reagents used to quantify the level of the marker of interest. After the incubation time recommended in the corresponding kit manual (total incubation time must not exceed 4h to avoid ATP degradation), the Alpha or HTRF signal is recorded on a compatible microplate reader. Following plate reading, 5 µL of ATPlite 1step lysis & detection reagent (prepared 2.5 times more concentrated than what is mentioned in the kit manual) is added to measure ATP cellular content. After the proper mixing/incubation of the plate, the luminescence assay is read on a compatible microplate reader.

Case study with AlphaLISA *SureFire Ultra* and HTRF Phospho-/Total protein assays

The effect of the MEK inhibitor PD98059 was studied on the phosphorylation of the MAP kinase ERK1/2. Independent experiments were performed, on the human epidermoid carcinoma cell line A-431 using AlphaLISA *SureFire Ultra* assays, and on the human breast cancer cell line MCF7 using HTRF assays. Format A was carried out to measure the levels of phospho-ERK1/2 (Thr202/Tyr204), total ERK, GAPDH (housekeeping protein), and ATP (cell viability) from the same cell lysate. Format B was also performed with the sequential detection in the same well of phospho-ERK1/2 (Thr202/Tyr204) and ATP (cell viability).

Material and methods

Material

Material and reagents used are listed in Table 3 below.

Table 3: Material and reagents.

	Material / Reagent	Supplier	Part number
Tissue-culture microplates	CulturPlate-96, Black opaque	Revvity	6005660/8/9
Detection microplates	AlphaPlate-384, Light gray	Revvity	6005350/9
	HTRF 96-well low volume white plate	Revvity	66PL96005/25/100
AlphaLISA *SureFire Ultra* kits	AlphaLISA *SureFire Ultra* p-ERK1/2 (Thr202/Tyr204) Assay Kit	Revvity	ALSU-PERK-A500/10K/50K
	AlphaLISA *SureFire Ultra* Total ERK1/2 Assay Kit	Revvity	ALSU-TERK-A500/10K/50K
	AlphaLISA *SureFire Ultra* Total GAPDH (Human/Mouse) Assay Kit	Revvity	ALSU-TGAPD-B500/10K/50K
HTRF kits	HTRF Advanced phospho-ERK (Thr202/Tyr204) Cellular Kit	Revvity	64AERPET/G/H
	HTRF Total ERK Cellular Kit	Revvity	64NRKPET/G/H
	HTRF GAPDH Housekeeping Cellular Kit	Revvity	64GAPDHPEG/H
Cell viability kit	ATPlite 1step Luminescence Assay Kit	Revvity	6016736/1/9
Cell lines	A-431	ATCC	CRL-1555
	MCF7	ATCC	HTB-22
Compound	PD98059 (MEK inhibitor)	Selleckchem	S1177

Experimental conditions

A-431 cells were plated in a CulturPlate-96 under 5,000 cells/well in complete culture medium (DMEM with GlutaMAX™ supplemented with 10% FBS, 1% Pen/Strep, 1% Non-Essential Amino Acids, and 2 mM HEPES). MCF7 cells were plated in a CulturPlate-96 at 25,000 cells/well in complete culture medium (MEM alpha medium supplemented with 10% FBS, 1% Pen/Strep, 1% Non-Essential Amino Acids, and 2 mM HEPES). After an overnight incubation at 37°C - 5% CO_2, the cells were treated with increasing doses of PD98059 (prepared in complete culture medium supplemented with 0.1% DMSO) for an additional overnight incubation at 37°C - 5% CO_2. After medium removal, A-431 cells were lysed with the Alpha *SureFire Ultra* Lysis Buffer (1X), and MCF7 cells were lysed with the HTRF Phospho-Total Protein Lysis Buffer #1 supplemented with the HTRF Phospho-Total Protein Blocking Reagent (1X) (for more details about cell plating, treatment, and lysis, please refer to the corresponding AlphaLISA *SureFire Ultra* and HTRF kit manuals).

The detection step based on format A was carried out as mentioned in the previous section for the multi-parametric analysis of the levels of phospho-ERK1/2 (Thr202/Tyr204), total ERK1/2, GAPDH, and ATP. All AlphaLISA *SureFire Ultra* assays were read after a total incubation time of 2.5h (1h incubation after acceptor mix addition and 1.5h incubation after donor mix addition). All HTRF assays were read after an overnight incubation.

The detection step based on format B was also performed as previously described for the combined detection in the same well of phospho-ERK1/2 (Thr202/Tyr204) and ATP levels. The AlphaLISA *SureFire Ultra* phospho-ERK1/2 assay was read after a total incubation time of 2.5h (1h incubation after acceptor mix addition and 1.5h incubation after donor mix addition). The HTRF phospho-ERK1/2 assay was read after a 4h incubation.

Cell lysate transfer and preparation/addition of AlphaLISA *SureFire Ultra* or HTRF detection reagents, as well as the incubation/detection steps, were carried out following the instructions detailed in the respective AlphaLISA and HTRF kit manuals.

For all AlphaLISA *SureFire Ultra* and HTRF assays, cell lysates were measured neat and diluted to ensure working in the linear range of each assay (the data presented are the ones on neat lysates).

Instrumentation and data analysis

All assays were measured on the EnVision multimode plate reader (Revvity). The ATPlite 1step signal was measured with the standard luminescence module (infrared cut-off filter 2100-212/luminescence mirror module 2100-404). The AlphaLISA signal was detected with the standard Alpha module using the Alpha standard settings (laser excitation at 680 nm/emission at 570 nm, bandwidth 100 nm). The HTRF signal was recorded using the HTRF module "Europium cryptate donor/Red acceptor" (laser excitation at 337 nm/dual emissions at 620 nm and 665 nm).

The data were analyzed using the GraphPad Prism® software. Sigmoid dose-response curves and associated IC_{50} values were obtained using a four-parameter logistic model with variable slope. The error bars represented on the points correspond to the SD of the means of three independent wells of cells.

Results and discussion

AlphaLISA *SureFire Ultra* assays combined with ATPlite 1step assay on A-431 cells

The results obtained with format A are presented in Figure 6A & B. Cell treatment with PD98059 induced a dose-dependent decrease in the phosphorylation level of ERK1/2 at Thr202/Tyr204 (blue curve), with an IC_{50} value close to 2 µM which is in accordance with the literature. The level of Total ERK1/2 remained stable, except at the highest dose of compound for which there is a signal decrease of approximately 30% (red points). Similarly, the ATPlite 1step signal (orange points) followed the same profile with a 45% signal loss at 10 µM of PD98059, demonstrating a cytotoxic effect in this condition. However, the level of the housekeeping protein GAPDH was unchanged whatever the doses of compound (green points), meaning that the decrease in Total ERK1/2 and cell viability was not due to cell detachment.

The data obtained with format B (Figure 6C & D) show similar results as the one obtained with format A on Phospho-ERK1/2 and cell viability. It therefore demonstrates that AlphaLISA *SureFire Ultra* and ATPlite 1step assays can be sequentially detected in the same well without any interference.

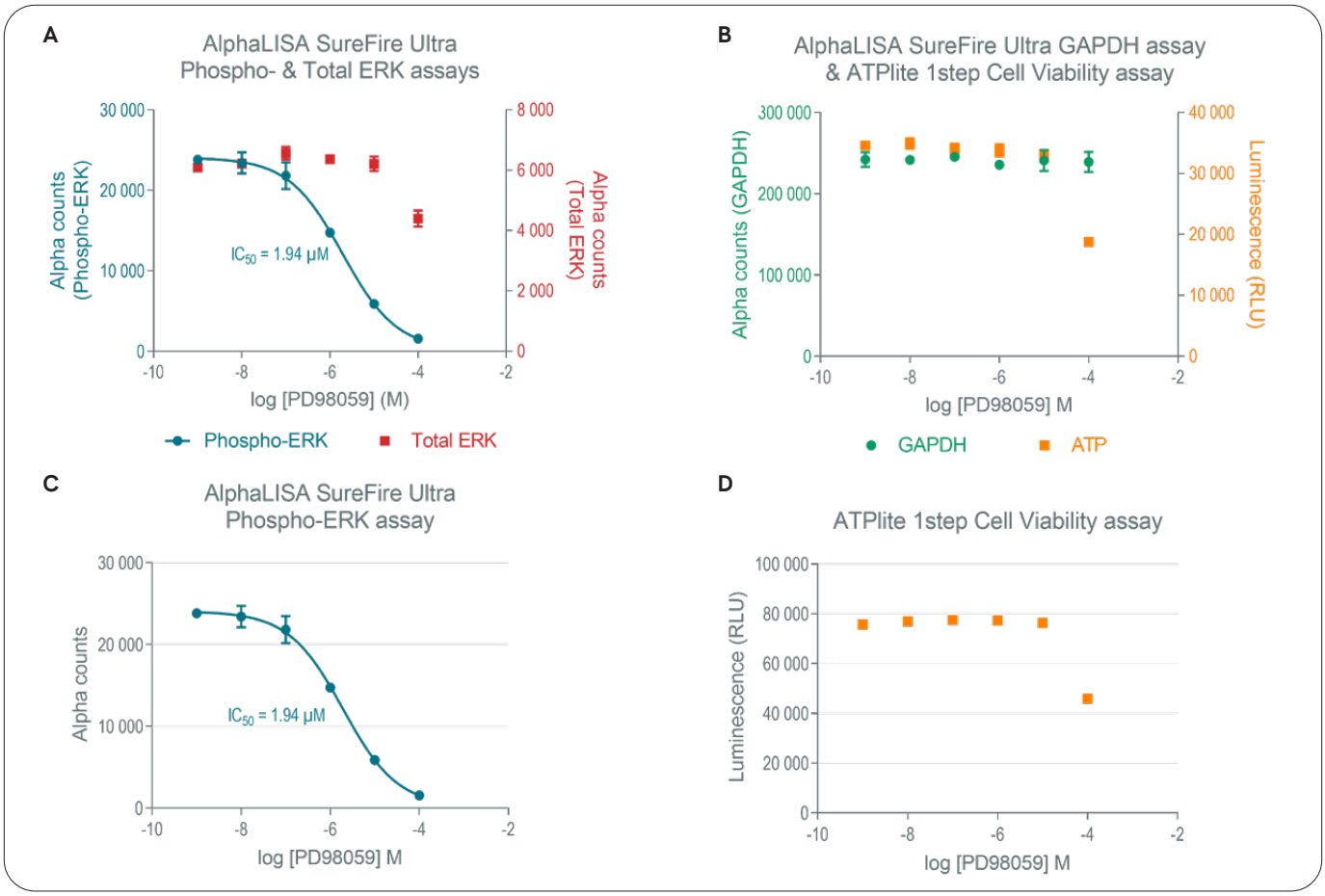

Figure 6: Combination of AlphaLISA *SureFire Ultra* assays with ATPlite 1step assay on A-431 cells treated with PD98059 (A. AlphaLISA *SureFire Ultra* Phospho-ERK1/2 & Total ERK1/2 assays using format A; B. AlphaLISA *SureFire Ultra* GAPDH assay and ATPlite 1step assay using format A; C. AlphaLISA *SureFire Ultra* Phospho-ERK1/2 assay using format B; D. ATPlite 1step assay using format B).

HTRF assays combined with ATPlite 1step assay on MCF7 cells

The results obtained with format A are presented in Figure 7A & B. As seen on A-431 cells with AlphaLISA *SureFire Ultra* ERK1/2 assays, MCF7 cells treated with PD98059 displayed a dose-dependent decrease in the phosphorylation level of ERK1/2 at Thr202/Tyr204 (blue curve), with an IC_{50} value close to 1 µM. The level of Total ERK1/2 was stable with a signal loss of about 30% observed only at the highest concentration of compound (red points). Nevertheless, both the ATP content (orange points) and GAPDH level (green points) showed a significant decrease at the two highest doses of PD98059, with a maximal decrease of 55% at 10 µM. These data demonstrate that MCF7 cells are more affected by the treatment than A-431 cells. Besides inducing a higher loss of cell viability, the compound also induced a cell detachment of this cell line as highlighted by the GAPDH signal decrease.

The data obtained with format B (Figure 7C & D) are similar to the ones obtained with format A on Phospho-ERK1/2 and cell viability, showing that HTRF and ATPlite 1step assays can also be sequentially detected in the same well without any interference.

Conclusion

This application note demonstrates that combining HTRF, AlphaLISA or AlphaLISA *SureFire Ultra* immunoassays with ATPlite 1step detection gives a complete picture of the compound's mechanism of action and related potential side effects.

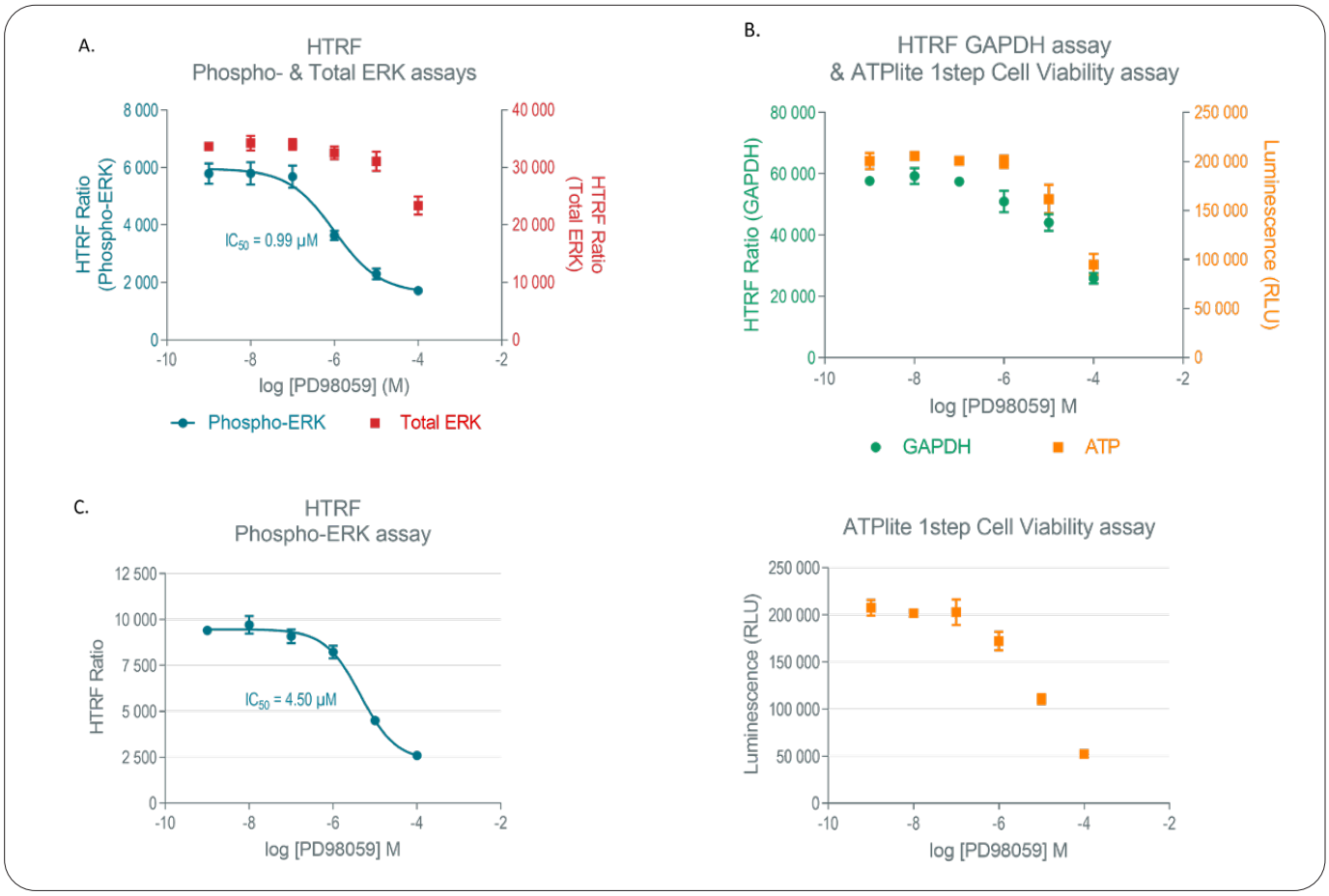

Figure 7: Combination of HTRF assays with ATPlite 1step assay on MCF7 cells treated with PD98059 (A. HTRF Phospho-ERK1/2 & Total ERK1/2 assays using format A; B. HTRF GAPDH assay and ATPlite 1step assay using format A; C. HTRF Phospho-ERK1/2 assay using format B; D. ATPlite 1step assay using format B).

The case studies presented here clearly highlight that ATPlite 1step luminescence assay system is complementary to the HTRF/AlphaLISA cytokine assays and HTRF/AlphaLISA *SureFire Ultra* intracellular phospho/total protein assays and ideally suited to determine the pattern of drug compounds from efficacy to safety evaluation.

The lysis buffer compatibility and low sample volume for detection offer the advantage that both assays can be performed from a unique well and on the same lysates for intracellular phospho-/total protein detection.

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