APPLICATION NOTE

# ATPlite assay performance in human primary cells.


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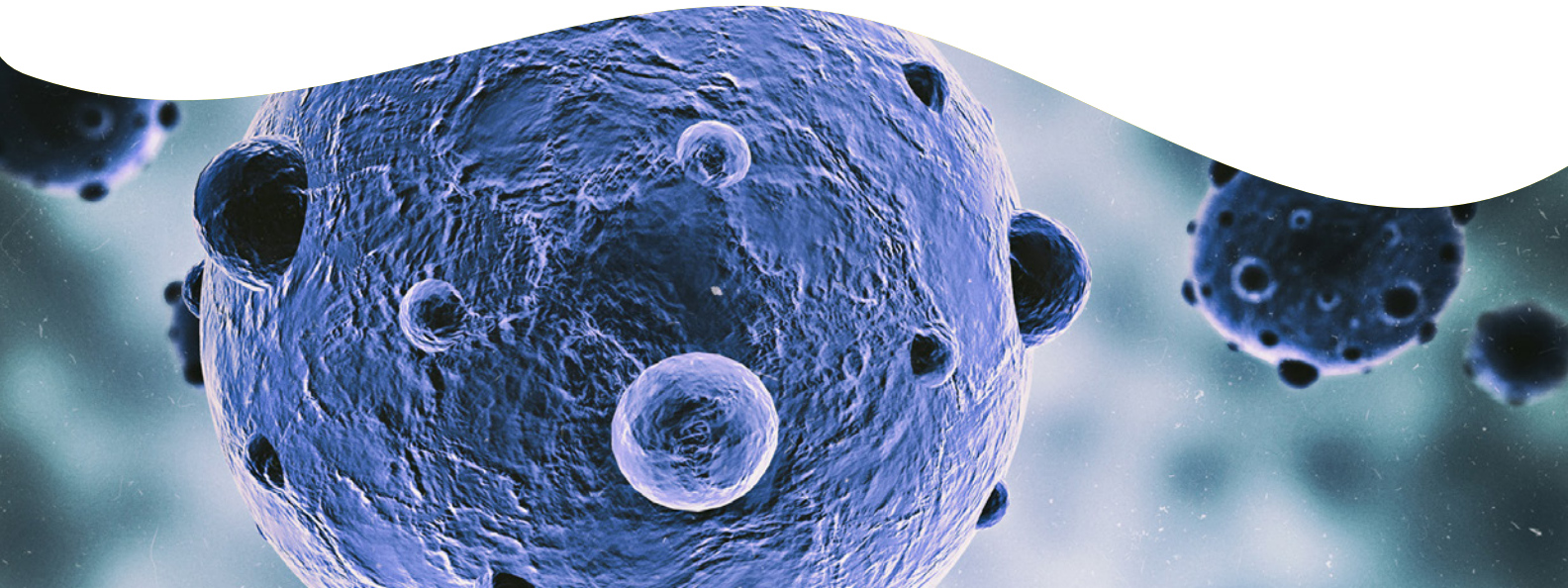
## Introduction

*In vitro* assays using primary human cells have evolved to closely mimic and predict biological responses of living organisms outside their normal biological context. In the drug development process, these assays are conducted to assess a compound's druggability. This assessment is essential to transform a compound from a lead molecule into a drug. One of the criteria for a compound to be considered druggable is the ability to cause or block a biological activity without toxic effects in an *in vitro* setting using primary human cells. As a read-out for compound-induced activity or toxicity, it is common to determine cell proliferation or cell death.

Several methods have been developed to assess proliferation and cytotoxicity. However, in drug discovery there remains a need for robust, rapid, high-throughput assays to identify and qualify novel therapies. The data presented here demonstrate the utility and benefits of Adenosine TriPhosphate (ATP)-monitoring luminescence assays for quantitative evaluation of proliferation and cytotoxicity of cultured human primary cells.

In this application note three ATP-monitoring luminescence assays were assessed and compared head-to-head: (i) ATPlite™ from Revvity, (ii) ATPlite™ 1step from Revvity and (iii) ATP assay kit from a competitor company.





## Description of ATP-detecting luminescence assays

ATP-detecting luminescence assays are an alternative to colorimetric, fluorometric and radioisotopic assays for the quantitative evaluation of proliferation and cytotoxicity of cultured mammalian cells. All three ATP-monitoring luminescence assays tested here are homogeneous, no-wash formats designed for multi-well plate use, making them ideal for automated high-throughput screening of cell proliferation and cytotoxicity.

In each of these assays, the number of viable cells in culture is determined by quantifying the ATP present. ATP is an appropriate marker for cell viability as it is present in all metabolically active cells and therefore, the amount of ATP present is directly proportional to the number of viable cells in culture. If the cell number rises due to proliferation then the amount of ATP increases; if the cell number declines due to cell death then the amount of ATP decreases.

In the assays evaluated here cellular ATP is quantified following direct lysis of the cells with suitable detergent (ATPlite 1step and Competitor ATP assay kit) or with a sodium hydroxyde-based lysis solution (ATPlite). ATP released from the lysed cells reacts in the presence of oxygen with added luciferin, luciferase, and magnesium to produce light as illustrated in the following reaction scheme.

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

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.

## Material and methods

### Cell culture and treatments

Human peripheral blood mononuclear cells (PBMCs) isolated by density gradient centrifugation from leukocyte cones were obtained from two healthy individuals donating blood components via apheresis. Cells were plated in white, tissue culture-treated 96-well CulturPlates™ (Revvity, #6005680) in RPMI-1650 medium (Sigma, #R8758) supplemented with 10% FBS (Sigma, #F9665) and 2 mM L-Glutamine (Gibso, #25030-024). Treatment of cells varied depending on what was being looked at. Each condition was set up in duplicate.

To establish the relationship between emitted light intensity and ATP concentration, a vial of lyophilized ATP standard solution from the ATPlite kit was reconstituted with water to form a stock concentration of 10 mM, and a three-fold, six-point serial dilution with a maximum concentration of 1.25 µM was performed in culture medium. In order to establish the linear range of the assay when working with cells, a two-fold, eight-point serial dilution of PBMCs with a maximum concentration of 2.5 x $10^5$ PBMCs per well was performed. Plated cells were incubated for one hour in a humidified atmosphere at 37 °C, with 5% $CO_2$ prior to running the experiment.

To look at cytotoxicity, PBMC cells were plated at a density of 1 x $10^5$ cells per well. A two-fold, seven-point serial dilution of DMSO (Sigma, #D2650) to a maximum concentration of 10% was performed in the presence of 1 µg/mL anti-CD3 (eBioscience, #14-0037-82) and 1 µg/mL anti-CD28 (eBioscience, #16-0289-85). Treated cells were incubated for 96 hours in a humidified atmosphere at 37 °C, with 5% $CO_2$ prior to running the experiment.

To look at proliferation, PBMC cells were plated at a density of 1 x $10^5$ cells per well. A two-fold, nine-point serial dilution of anti-CD3 and anti-CD28 to a maximum concentration of 4 µg/mL (per antibody) was performed. Treated cells were incubated for 96 hours in a humidified atmosphere at 37 °C, with 5% $CO_2$ prior torunning the experiment.

### ATP detection cell-based assays

Three different ATP detection assays were evaluated, ATPlite 1step (Revvity, #6016731), ATPlite (Revvity, #6016941), and a third assay from a competitor. All reagents were prepared according to the manufacturers' recommendations. The protocol used for each kit is outlined in Figure 1.

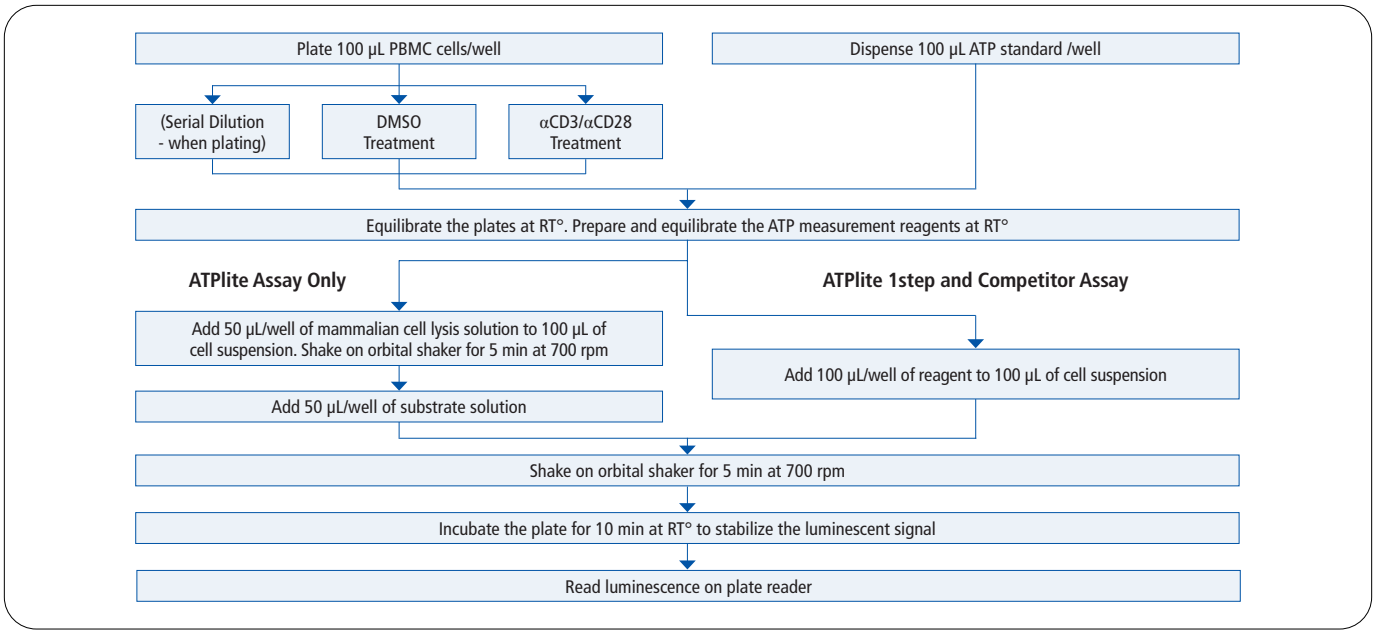



Figure 1: ATP detection assay workflow.

### Instrumentation and data analysis

All assays were measured on the PHERAstar® FS multimode plate reader (BMG Labtech) using Top optic Lum plus with a gain of 3600 and a focal height of 12.5 mM. The time setting used was 0.1 sec and the measurement interval was 1.0 sec. The data were analyzed using the GraphPad Prism® software. The ATP or cells serial dilution curves were generated using a linear regression. The DMSO toxicity and the proliferation activity curves were generated using a four-parameter logistics equation with variable slope.

## Results and discussion

### ATP standard curve

Assay sensitivity to ATP was determined by examining serial dilutions of ATP (three-fold, 1.25 µM max.). Figure 2 shows that for all three kits, ATP detection was linear over three magnitudes ranging from ~1 nM to 1 µM. Over this dynamic range the detection ratios between kits remained constant. The ATPlite 1step kit showed the largest signal-to-background (S/B) ratio compared to the competitor kit and the ATPlite kit. This superior assay window indicates a high sensitivity to ATP that allows reliable detection of small changes in ATP concentration.
The lower signal intensity obtained with the ATPlite assay was expected, as it is a consequence of the stabilization over time of the signal emitted by this kit, providing a half-life of five hours, while the other assays should be read within the first 30 minutes after reagent addition.

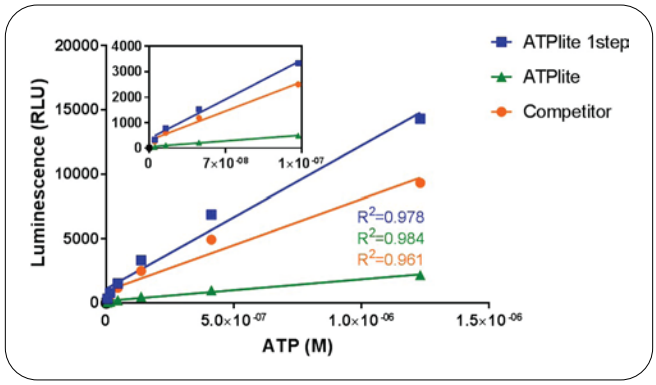



Figure 2: ATP standard detection curve. Insert shows linearity at low ATP concentrations. For each data point, mean ±SD are graphed, however, error bars are not visible as they are within the range of the dimension of the symbols.

### Sensitivity to Endogenous ATP produced by human PBMCs

Sensitivity to ATP produced by human PBMCs was determined by examining serial dilutions of PBMC suspensions (two-fold, 2.5 x $10^5$ PBMCs max). For all three kits, ATP detection was linear over two orders of magnitude ranging from 1.9 x $10^3$ to 2.5 x $10^5$ PBMCs, as shown in Figure 3. Over this dynamic range the detection ratios between kits remained constant. ATP from PBMCs was detected with the highest sensitivity by the ATPlite 1step kit followed by the competitor kit and the ATPlite kit, as expected (see comments relative to Figure 1). Using the standard curves from Figure 2, luminescence can be translated to ATP concentrations where 2.5 x $10^5$ PBMCs corresponded to ~2.5 x $10^{-7}$M ATP (donor A) and ~4 x $10^{-7}$M ATP (donor B) for all three kits. The slope of regression fit is shallower for donor A compared to donor B, indicating

donor-specific differences in which donor B's PBMCs are metabolically more active than donor A's PBMCs. These results demonstrate that PBMC numbers are reflected in the amount of ATP detected by the three ATP-detecting luminescence assays tested.

### Detection of PBMC cytotoxicity

DMSO was used as an exemplary compound to induce cytotoxicity in human PBMCs. Figure 4 shows that increasing concentrations of DMSO, incubated for 96 hours with the PBMCs, resulted in a decrease of luminescence signal reflecting the compromised viability of cells in presence of DMSO. The rank order of $IC_{50}$ values was comparable between all three kits tested; but once again, the largest assay window was obtained with the ATPlite 1step. Interestingly, donor A's PBMCs responded more heterogeneously to DMSO than donor B's PBMCs, as indicated by the shallower slope of donor A's curve fit. These results demonstrate that induced PBMC cell death results in a decrease of ATP and can be detected by all three ATP-detecting luminescence kits, and that all three kits were able to detect similarly the donor-specific differences.

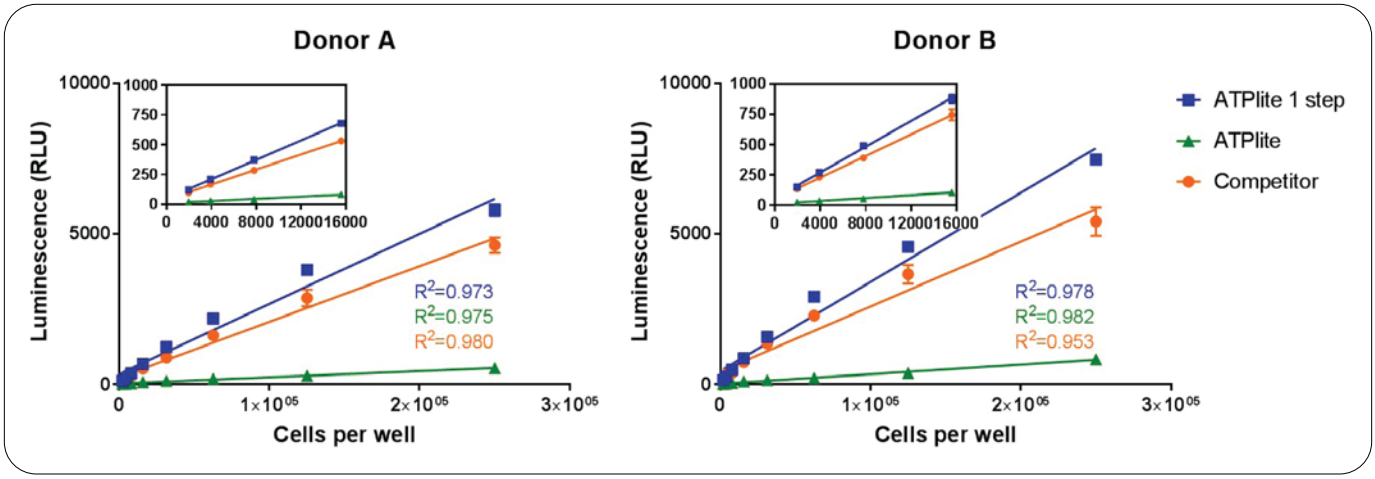
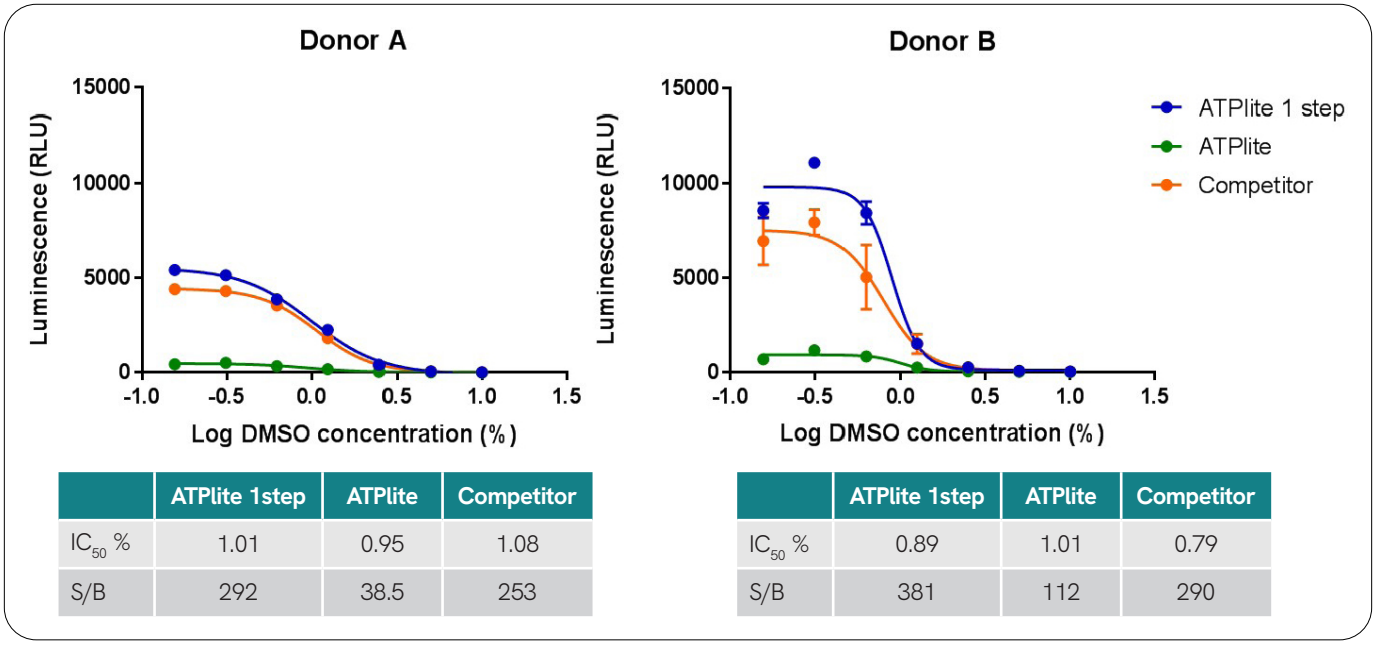



Figure 3: Detection of endogenous ATP. Serial dilution of human primary PBMCs from two donors. Insert shows linearity at low cell numbers per well. For each data point, mean ±SD are graphed.



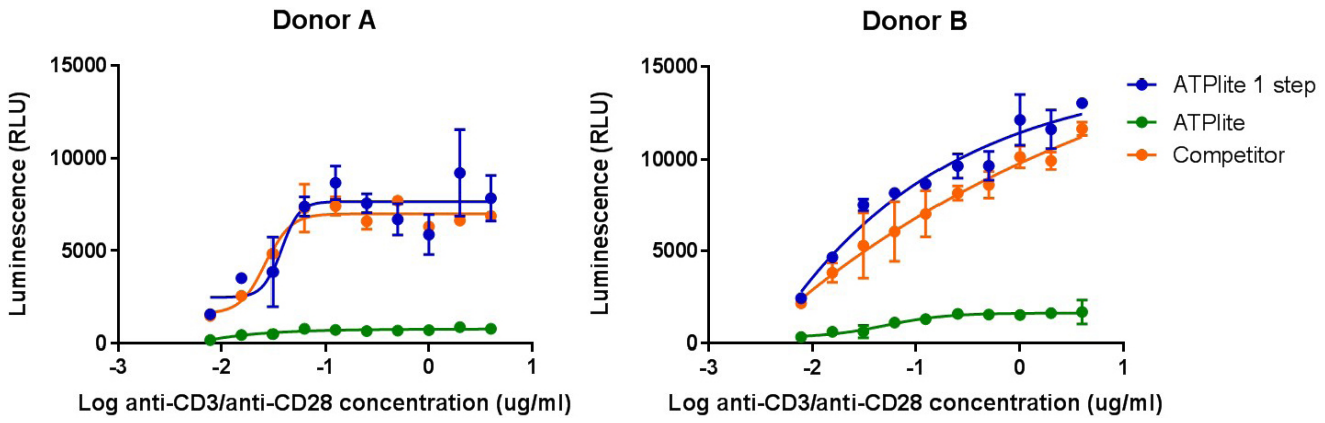

| | ATPlite 1step | ATPlite | Competitor |
|---|---|---|---|
| $IC_{50}$ % | 1.01 | 0.95 | 1.08 |
| S/B | 292 | 38.5 | 253 |

| | ATPlite 1step | ATPlite | Competitor |
|---|---|---|---|
| $IC_{50}$ % | 0.89 | 1.01 | 0.79 |
| S/B | 381 | 112 | 290 |

Figure 4: Detection of cytotoxicity shown via the serial dilution of DMSO in human primary PBMCs cultures from two donors. For each data point, mean ±SD are graphed. S/B were calculated as the luminescent signal obtained the lowest DMSO concentration (i.e. 0.15%) divided by the luminescent signal obtained at the highest DMSO concentration (i.e. 10%).

### Detection of PBMC proliferation

The antibody combination anti-CD3/anti-CD28 was used as an exemplary reagent to induce cell proliferation in PBMCs. Indeed, antibodies specific for the TCR-CD3 complex provide an initial activation signal, but proliferation is dependent on a costimulatory signal, usually provided via the CD28 molecule.[1] The results in Figure 5 show that increasing concentrations of anti-CD3/anti-CD28 lead to an increase of luminescence signal, reflecting induced cell proliferation. As expected, the rank order of $EC_{50}$ values from donor A was comparable between the ATPlite 1step kit and the competitor kit. Interestingly, while donor A's PBMCs reach their maximum proliferative capacity at 0.625 µg/mL anti-CD3/anti-CD28, donor B's PBMCs still responded with increased proliferation to 4 µg/mL anti-CD3/ anti-CD28 without reaching a plateau when using ATPlite 1step and the competitor product. These results indicate donor-to- donor variability and illustrate the advantage of ATPlite over ATPlite 1step and the competitor product for PBMCs from donor B: while curve fitting failed for the proliferative PBMC response of donor B with ATPlite 1step and the competitor product due to an incomplete concentration response curve; an $EC_{50}$ could be determined with ATPlite. This could be due to the differences in cell lysis efficiency as ATPlite cell lysis is more aggressive than ATPlite and the competitor product. These results demonstrate that induced PBMC proliferation results in an increase of ATP and can be detected by ATP-detecting luminescence kits with high degree of sensitivity.




| | ATPlite 1step | ATPlite | Competitor |
|---|---|---|---|
| $EC_{50}$ (µg/mL) | 0.04 | 0.03 | 0.03 |
| S/B | 5.0 | 4.2 | 4.5 |

| | ATPlite 1step | ATPlite | Competitor |
|---|---|---|---|
| $EC_{50}$ (µg/mL) | No curve fit | 0.05 | No curve fit |
| S/B | 5.8 | 5.6 | 5.4 |

Figure 5: Serial dilution of anti-CD3 and anti-CD28 in human primary PBMCs cultures from two donors. For each data point, mean ±SD are graphed.

## Conclusion

The results indicate that the ATPlite 1step and the competitor ATP detection assays provide comparable results. However, the ATPlite 1step kit outperforms the competitor kit in terms of S/B ratio. The ATPlite may be useful for detecting larger changes in ATP at higher ATP concentrations of a homogenous cell population; while it generates less light intensity (as stated by the manufacturer), it has the advantage of providing a signal that is more stable over time (half-life > 4 hours), which provides more flexibility in the workflow. It is important to note that light intensity and assay sensitivity are not the same thing. Whether the ATPlite assay is less sensitive than ATPlite 1step assay would require further investigation (i.e. Lowest Detection Limit calculation, by engaging more replicates of each condition).

The high S/B ratio and easy assay format makes ATPlite 1step an excellent assay technology for detecting cell proliferation as well as cell death in primary human PBMCs.

## References

1. Frauwirth, K.A., Thompson, C.B., (2002) Activation and inhibition of lymphocytes by costimulation. Journal of Clinical Investigation 109:295- 299.

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