Application Note:

AP-MIB-VARIO12-0108

Comparison of Flash and Glow ATP Assays with Thermo Scientific Varioskan Flash Luminometry

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Introduction

One of the most common luminescent reactions used in research is an ATP reaction catalysed by firefly luciferases. In this reaction ATP is degraded to AMP and the energy is released as visible light. ATP reaction can be used for the measurement of ATP (1-3), its degradation products ADP and AMP (4) or any enzyme activity that can be linked to ATP production or consumption (5). It can also be used for the measurement of firefly enzyme activity in applications where firefly luciferase gene is used as a reporter gene (6-9).
Light production reaction catalyzed by firefly is an oxidation reaction where a special substrate, D-luciferin, is oxidized and ATP is reduced to AMP:

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

(λ_{max} 562 nm (10-11))

When this reaction is used to measure ATP, all other substrates are kept in saturated concentrations, therefore ATP is the limiting component of the reaction and light production is directly proportional to the concentration of ATP. Firefly-ATP reaction can be controlled two different ways in common applications. Light production can be either fast flash type reaction or a stable glow type reaction, depending on the assay environment. Natural wild type firefly-ATP reaction represents this fast flash reaction and that kind of light production is obtained when just substrates and luciferase are added in the reaction mixture (1-2). However, this reaction type is not very convenient for applications and therefore the reaction is quite often chemically stabilized to produce stable long term light emission (12-13). Most of the commercial ATP assay kits use this stabilized ATP glow reaction but lately certain kits using ATP flash reaction have been introduced. This flash reaction offers improved sensitivity in ATP measurement but has a disadvantage of requiring automatic dispensers for the substrate addition. In this application note, the basic performance of Varioskan Flash microplate luminometer was tested with both glow and flash type ATP assay.

Materials and Experimental protocols

Kits and reagents

Two luminometric ATP kits were chosen for the evaluation: ATP Biomass Kit HS (BioThema AB, Sweden) representing glow type ATP reaction, and CheckLite™ HS Set (Kikkoman Corp. Japan) that is based on flash type ATP reaction. Both kits were used according to manufacturer's instructions. All tests were performed with commercial 10 umol/L ATP standard (BioThema AB, Sweden).

Instrumentation

All measurements were performed with Thermo Scientific Varioskan Flash multitechnology microplate readers that were equipped with Varioskan LumiSens high sensitivity luminometric module and automatic dispenser. Instruments were controlled using Thermo Scientific SkanIt software.

Plastics

All measurements were performed on white 96- or 384 well microplates and ATP glow reaction was measured also with white 1536-well plates. The 96-well plates used were Microlite1 96-well 12-strip plates, 384-well plates were Microlite1+ 384 square well plates (both Thermo Scientific.) and 1536-well plates were Lumitrac 600 HiBase 1536-well plates from Greiner Bio-One.

ATP Reaction Kinetics

ATP reaction kinetics of both flash and glow ATP light emission reactions were measured with white 384 well plates. A 30 ul aliquot of 0.01 µM ATP standard was added into the well and plate was placed into the microplate luminometer. An equal volume of either flash or glow ATP reagent was added with the automatic dispenser and

luminescence signal was immediately measured with 1 second integration time for 10 min (flash type ATP reaction) or with 500 ms integration time for 60 min (glow type ATP reaction). For the kinetic measurements with ATP flash chemistry, Varioskan Flash was programmed as follows:

- STEP 1: Well loop step, Execution type "by well", Well count 1.

Sets the instrument to perform the following actions well by well. All following steps are executed for one well, then next well and so on until all wells have been measured.

- STEP 2 (inside well loop): Kinetic loop step "KineticLoop1", Readings 600, Interval 0s.

This sets the instrument to perform kinetic assay with 600 kinetic points. Kinetic reading is performed as fast as possible without any interval time between the readings.

- STEP 3 (inside kinetic loop 1): Dispensing step, Dispenser 1, Dispensing volume 30 µl (for 384-well plates), Dispensing speed "High", Dispensing position "Luminometric 1", Tip priming "No", Dispense at reading 1.

Dispenses 30 µl of luciferin-luciferase reagent into the well with high speed. Dispensing is performed simultaneously with the first kinetic reading point.

- STEP 4 (inside kinetic loop 1): Measurement step, Luminometric measurement, Luminometric optics "Normal", Dynamic range "AutoRange", Measurement time 1000 ms.

Performs kinetic measurement with 1000 ms signal integration time, sampling speed 60 points/ minute. Total kinetic measurement time is 10 min.

Step structure of the protocol is shown in Figure 1.

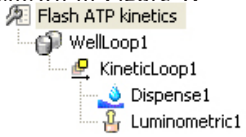

Figure 1. Protocol steps for the kinetic measurement of flash ATP reaction.

When glow type ATP chemistry was used, a different measurement protocol was used, the protocol included following actions:

- STEP 1: Kinetic loop step "KineticLoop1", Readings 60, Interval 1 min.

This sets the instrument to perform kinetic assay with 60 kinetic points with the interval time of one minute between sequential readings of the same well.

- STEP 2: (inside kinetic loop 1): Dispensing step, Dispenser 1, Dispensing volume 30 µl (for 384-well plates), Dispensing speed "High", Dispensing position "Luminometric 1", Tip priming "No", Dispense at reading 1.

Dispenses 30 µl of luciferin-luciferase reagent into all sample wells in the plate with high speed. The first kinetic measurement is taken simultaneously with the dispensing.

- STEP 3: (inside kinetic loop 1): Measurement step, Luminometric measurement, Luminometric optics "Normal", Dynamic range "AutoRange", Measurement time 500 ms.

Performs kinetic measurement with 500 ms integration time for all sample wells, then waits until defined one minute interval is filled and then repeats the measurement for 60 rounds.

Step structure of the protocol is shown in Figure 2.

ATP Sensitivity Tests

ATP sensitivity was determined for a set of Varioskan Flash units. Number of Varioskan Flash units used in each test is shown in Table I. An ATP standard dilution series was prepared between 0.1 pM to 1 µM with 10-fold dilutions. With 384-well plates 30 µl of each ATP dilution or blank solution was pipetted into the microplate wells and 30 µl of luciferin-luciferase reagent from either ATP flash or glow kit was added with the instrument's dispenser. For 96-well plates 50 µl of ATP dilution or blank solution and 50 µl of luciferin-luciferase reagent and for 1536-well plates 5 µl of both ATP dilution and reagent was used. Test included with all plate types 16 replicates of the blanks and eight replicates of the ATP dilutions. With flash type ATP reaction luminescence of each well was measured after two second delay time with four second signal integration. Measurement protocol used in this assay had following procedural steps:

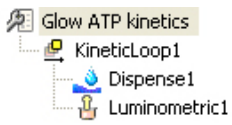

Figure 2. Protocol steps for the kinetic measurement of glow ATP reaction.

- STEP 1: Well loop step, Execution type "by well", Well count 1.

Sets the instrument to perform the following actions well by well. All following steps are executed for one well, then next well and so on until all wells have been measured.

- STEP 2: Dispensing step, Dispenser 1, Dispensing volume 30 µl (for 384-well plates), Dispensing speed "High", Dispensing position "Luminometric 1", Tip priming "No".

Dispenses 30 µl of luciferin-luciferase reagent into one sample well with high speed.

- STEP 3: Measurement step, Luminometric measurement, Luminometric optics "Normal", Dynamic range "AutoRange", Measurement time 4000 ms, Lag time 2 s.

This step first waits for 2 s delay time until the signal has been generated and then it measures the luminescent signal for 4000 ms. Resulting luminescence result is calculated and given for 1 second integration time.

Step structure of the protocol is shown in Figure 3.

When glow type ATP reaction was measured, all wells were first dispensed with the luciferin-luciferase reagent and signal was measured with one second integration time from the wells after two minute waiting period.

- STEP 1: Dispensing step, Dispenser 1, Dispensing volume 30 µl (for 384-well plates), Dispensing speed "High", Dispensing position "Luminometric 1", Tip priming "No".

Dispenses 30 µl of luciferin-luciferase reagent into all sample wells in the plate with high speed.

- STEP 2: Measurement step, Luminometric measurement, Luminometric optics "Normal", Dynamic range "AutoRange", Measurement time 1000 ms, Lag time 2 min.

Step will wait two minutes at the beginning of the step, then all wells are measured with 1000 ms integration time.
Step structure of the protocol is shown in Figure 4.

Theoretical assay sensitivity was calculated as a crossing point between the blank subtracted ATP calibration curve and 2*SD level of assay blank wells and Z-prime values were calculated according to standard formulas (14).

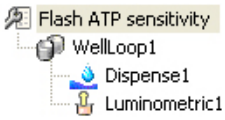

Figure 3. Protocol steps used to measure ATP sensitivity assay with flash chemistry.

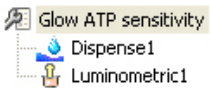

Figure 4. Protocol steps used to measure ATP sensitivity assay with glow chemistry.

Results and Discussion

ATP Reaction Kinetics

As expected, flash and glow type luminescence from the ATP reaction show totally different kinetic behaviour. Typical kinetic luminescence curves are shown in Figure 5. Flash type ATP luminescence reaction shown in Figure 5A has very fast signal generation where peak maximum is reached in about four seconds after addition of luciferin-luciferase reagent and the signal decays quite rapidly, showing little below two minutes half-life time. Therefore, this assay was measured by integrating a signal from two to six seconds, where maximal signal is emitted by the reaction. Because of this fast signal increase and decrease, this type of ATP reaction can be measured only with the instrument equipped with automatic dispenser module. If luciferin-luciferase reagent would be added into the ATP sample manually outside the instrument, the signal maximum would be totally lost before any measurements could be done. In addition, time difference between dispensing the luciferin-luciferase reagent and measuring the signal has to be constant with rather high precision for each well, otherwise incorrect results would be obtained because of signal instability.

Glow type ATP luminescence reaction in Figure 5B on the other hand has totally different reaction kinetics. Luminescence maximum is reached within the minute after ATP and luciferin-luciferase are mixed but reaction stays stable for much longer time period. After an hour, there is still more than 70% of the signal present. Therefore, when this glow reaction is used to measure ATP, it is possible to add all components manually and read the plate afterwards. This will not cause remarkable variations in the result values.

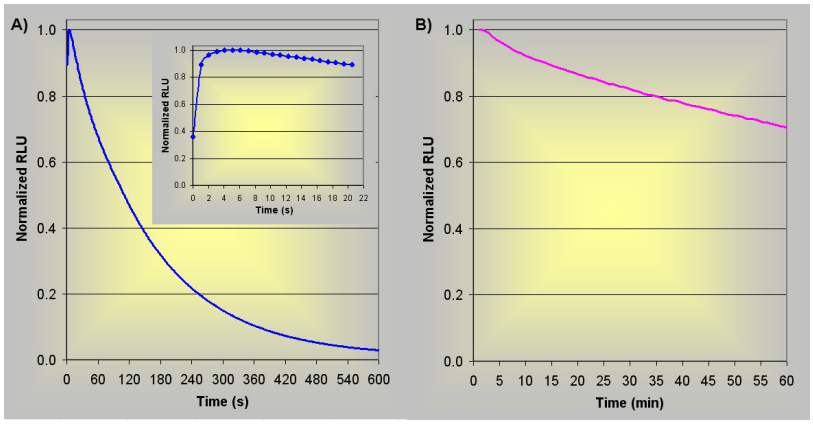

Figure 5. Kinetic curves of two luminescent ATP reactions. A) ATP flash reaction. B) ATP glow reaction.

ATP Sensitivity Tests

Sensitivity of these two ATP assays was determined with different plate formats, glow type ATP sensitivity with all three common plate formats: 96-, 384- and 1536-well plates and flash type assay only with 96- and 384-well plates.

Results of these tests are collected in Table I and examples of typical ATP standard curves are shown in Figure 6. Varioskan Flash instrument is designed to have optimal performance with 384-well plates and that is seen from these results. Both ATP assays using either flash or glow type ATP chemistry show the best sensitivity with 384-well plates. The detection limit of ATP with flash type chemistry and with this plate format is about 1.5 amol/well and with glow type chemistry about 80 amol/well. This around 50 times sensitivity difference is very understandable because of the difference between the two chemistries. With flash type ATP reaction, the light from the chemical reaction is released within the short period of time, therefore average signal over time is much higher. This is seen also from the Figure 6, the same amount of ATP in the well produces about 30 times more light in flash reaction. For example ATP sample of 300 000 amol/well gives about 5 000 000 RLU in flash reaction when glow reaction gives only 150 000 RLU with the same amount of ATP.
When the plate format is changed from 384-wells to 96-wells, assay sensitivity is decreased. This effect is not remarkable with flash type ATP assay, only about 20% decrease in the detection limit, but with glow type ATP assay the sensitivity is decreased almost 10 times. Therefore the difference between flash and glow assay sensitivity with 96-well plates is much bigger with 96-well plates than with 384-well plates, being about 400 times.

The very high density 1536-well plate was tested only with glow type ATP chemistry and it was shown to produce about the same ATP sensitivity than 96-well plate, having the detection limit value of 200 amol/well ATP, both slightly lower than with 384-well plate.

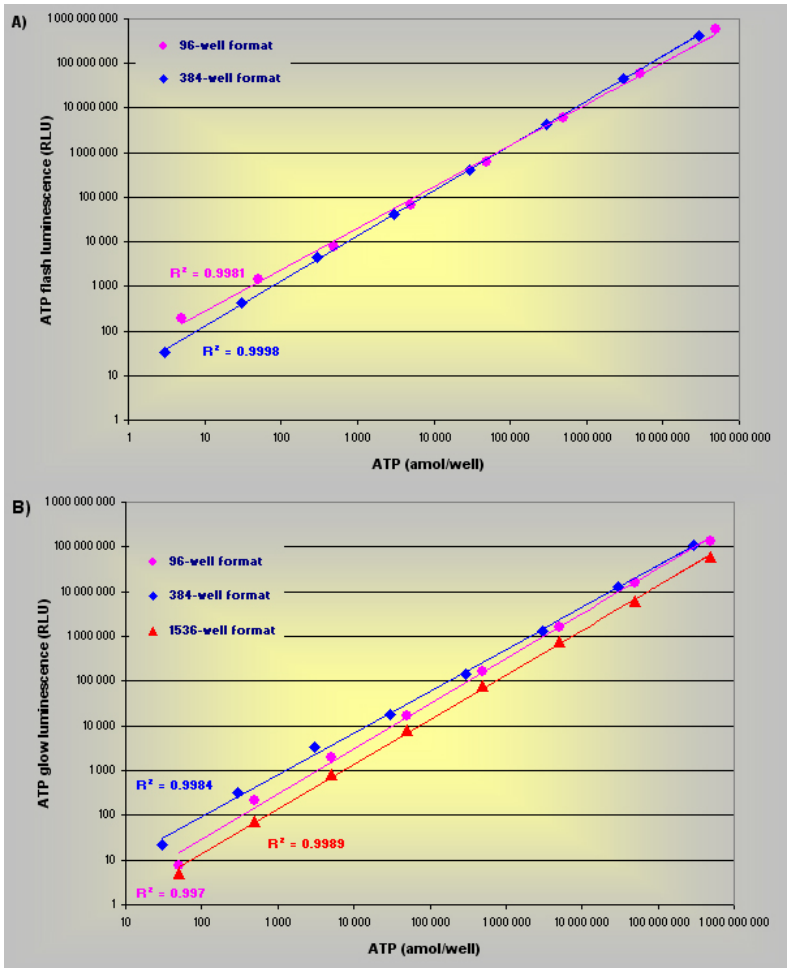

Figure 6. Typical ATP calibration curves with different plate formats. A) Flash type ATP assay. B) Glow type ATP assay.

Table I. Sensitivity test results of flash and glow ATP assays with Varioskan Flash.

Plate format	ATP Flash chemistry			ATP Glow Chemistry			Glow/ Flash ratio
	Average detection limit (amol/well))	Range, (Min-Max)	Number of Varioskan Flash units	Average detection limit (amol/well))	Range, (Min-Max)	Number of Varioskan Flash units	
96	1.8	0.8 – 2.6	7	130	600 - 900	4	409
384	1.5	0.5 – 2.9	30	80	60 - 130	6	52
1536	--	--	--	200	180 - 210	2	--

All sensitivity tests were performed with several Varioskan Flash units. As expected, there are differences between the performances of the individual instruments in these ATP assays. Every instrument is always individual in its performance because there are always some differences in electronics, noise levels etc. The total performance range in the detection limits between the instruments is anyhow amazingly narrow. When the average ATP detection limit with the flash chemistry and 384-well plates is for example 1.8 amol/well, all tested 30 instrument units had their detection limit below 3 amol/well ATP. Figure 7. shows the cumulative distribution of these detection limit values of the individual Varioskan Flash instruments. This distribution is clearly following expected cumulative Gaussian distribution.

Another very important assay quality parameter is Z-prime value. It is a numerical calculation that reflects the assay capability to separate positive results from the negative ones (14). Theoretical maximum of the Z-prime value is 1.0 that suggests that reliability of the separation of two signals is 100.0%. This is of course never possible to be met in real life because there is always some standard deviation in the signal values. If the Z-prime value is over 0.5, that assay can be normally considered good and reliable (14) but sometimes values around 0.6 or higher might be required. Calculated Z-prime values against the assay blanks over the whole ATP calibration series are shown for both flash and glow ATP chemistries and all plate formats in Figure 8.

Figure 8. clearly shows the excellent reliability of these ATP assays. With the flash type ATP assay, that 3 amol/well ATP sample that is very close to the theoretical detection limit shows already almost sufficient Z-prime values. Theoretical detection limit is calculated based on blank SD only when Z-prime calculation formula includes both blank and standard SD values. Therefore, calculated theoretical detection limit is always below the concentration range where good Z-prime values are obtained. With the ATP sample of 30 amol/well Z-prime values are clearly over required 0.5 level, with both 96- and 384-well formats.

When the Z-prime values of glow type ATP assay are analyzed, it is immediately noticed that higher ATP amounts than with flash chemistry are required for the good z-prime level. About 300 amol/well ATP is required before calculated Z-prime values are constantly over required level.

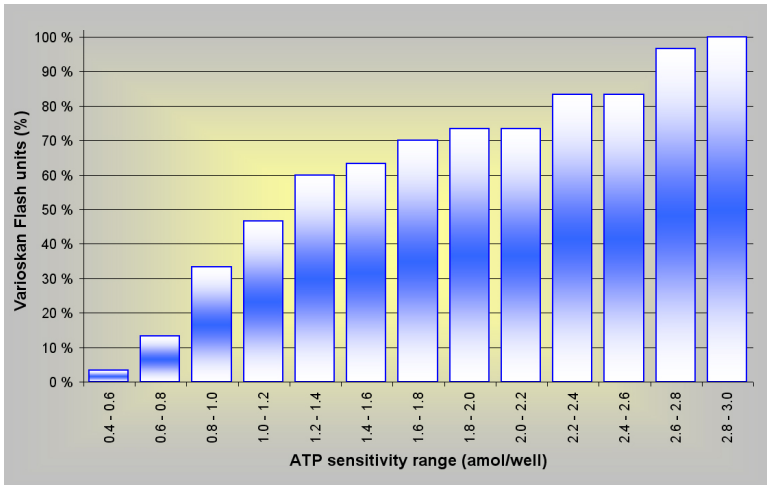

Figure 7. Cumulative distribution of the Varioskan Flash detection limits in flash type ATP assay with 384-well plates.

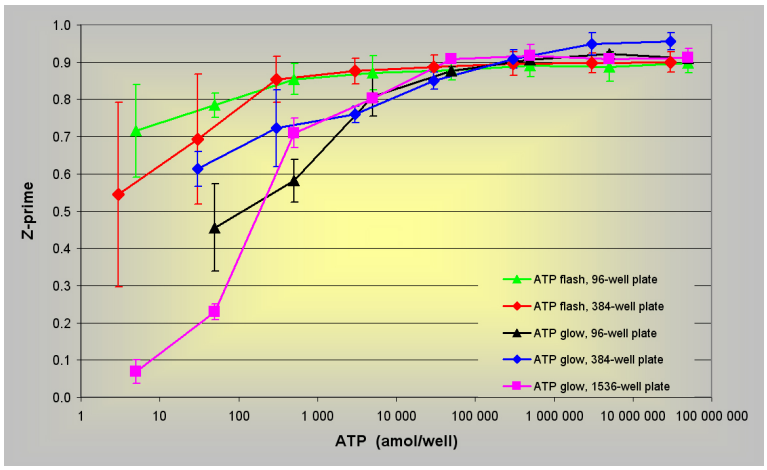

Figure 8. The Z-prime values of both flash and glow type ATP assays measured with 96-, 384- and 1536-well plate formats.

Conclusions

These results clearly show the high sensitivity and reliability of ATP assays with Varioskan Flash luminometry. ATP assay using flash type chemistry gives better assay sensitivity than assays using glow type chemistry and 384-well plate format gives the best sensitivity with both types of chemistries. For the statistically reliable results, around 10-30 amol of ATP is required in the sample with flash type ATP assay and about 300 -500 amol/well is required for the glow type assay.

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