# Ultrafast flash kinetic assays with Luminoskan and Fluoroskan FL microplate readers

## Introduction

Assays for measuring kinetic data are important to many research laboratories. Most kinetic assays are slow, long-lasting measurements, where the measured signals change slowly. These glow-type assays are easy to run and do not require the use of any special features from the instrumentation—reactions can be started manually using hand-held pipettes, and signal reading can be programmed to have long intervals.

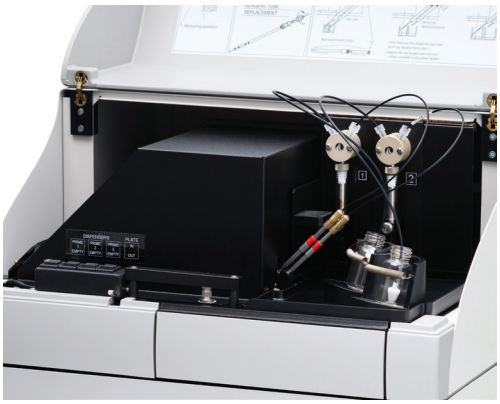
In contrast, in certain assays, kinetic response is extremely fast and the total reaction lasts for only a few seconds. Measurement of these flash-type kinetic reactions requires special features for signal reading—the reaction must be triggered using automatic addition of a reagent, the signal needs to be measured even while the triggering reagent is being added, and sample reading needs to be performed with high frequency. This note describes how these ultrafast kinetic reactions can be measured using the Thermo Scientific™ Luminoskan™ and Fluoroskan™ FL microplate readers.

Using a luminometric ATP assay with firefly luciferase and luciferin is the most common method for doing quantitative ATP measurements. There are two main types of luminometric ATP assays: glow-type assays and flash-type assays. In general, the glow-type assay is performed using a chemically stabilized glow-type luminescence reaction, where the signal is stable for an extended time. The major disadvantage of this assay is that the reaction pathway needs to be modified to create a glow-type signal, which considerably decreases the sensitivity of the assay. The flash-type ATP assays are typically 50–100 times more sensitive than the glow-type ATP assays.

When a luminometric ATP reaction is run in its natural form—a flash-type assay—the reaction reaches maximum signal intensity in less than one second. The signal intensity rapidly decreases after the maximum is reached. This flash-type assay has much higher sensitivity than the glow-type assay; however, because of the challenge presented by the reaction's unstable flash kinetics, it requires special instrument features to collect reliable data.

## Instrument configuration and assay setup

Flash kinetic assays were measured with Luminoskan and Fluoroskan FL instruments that were equipped with two reagent dispensers. These instruments have three dispensing positions, one of which is located at the measurement position. At this position, the instrument can simultaneously dispense into and take a measurement from a well, which is necessary for measuring very fast kinetic reactions. Dispensing heads are located at a 45° angle (the optics are at the top of the well), and the reagent is dispensed against the well wall, which markedly improves reagent mixing.



**The Luminoskan microplate reader can be equipped with up to 3 dispensers.**

### Assay plate preparation

The ATP standard was serially diluted 1:10 with ATP-free water to generate a concentration series between 0.1 pM and 100 nM. Then, 10 µL aliquots of the ATP standards were added into a 96-well Thermo Scientific™ Microlite™ 1+ plate. This created a final assay plate with a 7-point calibration series of increasing ATP concentrations from 1 to $10^6$ amol/well. Blank wells were not included in the assay; instead, a measurement taken before the ATP reaction triggering was used as a blank, individually for each sample.

### Assay protocol

The basic assay comprises the following steps:

1. Measure the Baseline luminescence before adding the ATP reagent to a well.

2. Add 90 µL of ATP reagent with an automatic dispenser using a high dispensing speed (to improve mixing), and simultaneously measure the flash kinetic luminescence peak in that well.

3. Repeat steps 1 and 2 for every well in the layout.

The detailed steps on steps and loops are described in the following Technical protocol. Briefly, first, the measurement baseline was followed for 5 sec. Then, the flash ATP reagent was added with an automatic dispenser to trigger the luminescence reaction, and the signal was measured for 25 sec. The longer the duration of measurement, the more effective the reaction kinetics analysis will be, but for a normal high-sensitivity ATP assay, a 1 sec baseline and 2–4 sec signal measurement would be sufficient. Both the Luminoskan and Fluoroskan FL instruments were operated by Thermo Scientific™ SkanIt™ Software. Figure 1 shows the complete protocol in the session tree.

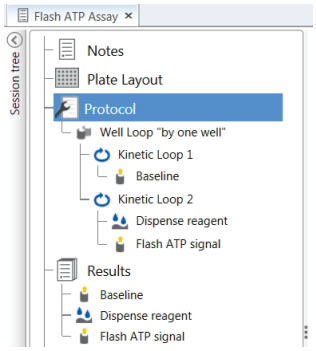



**Figure 1. Session structure for ultrafast flash kinetics reactions.**

### Technical protocol

1. **Well Loop "by one well":** This step instructs the instrument to perform the assay as described earlier in the "Assay protocol" section. One well is completed first before going to the next well.

2. **Kinetic Loop 1:** This step has a Baseline measurement as a daughter step. It governs the kinetic measurement of the baseline before the reagent is dispensed by defining the number of readings and intervals between the readings. In this assay, 50 baseline readings were obtained before triggering the reaction. The intervals between readings were set to zero, indicating that maximum sampling frequency was used.

3. **Baseline:** This daughter step of Kinetic Loop 1 is the luminometric measurement step that measures the baseline signal before the reaction begins. The Baseline measurement is repeated as many times as defined in the Kinetic Loop 1. The luminescence measurement time in this step was 100 ms. When Kinetic Loop 1 defines 50 readings, this produces an approximate total of 5 sec (50 x 0.1 sec) of baseline measurement.

4. **Kinetic Loop 2:** This step has both reagent dispensing and reading as daughter steps. Dispensing starts simultaneously with the first reading point, and measurement continues independently for the defined number of readings (multiple readings can be taken while the dispenser is adding the liquid into the well). The step was defined to measure the well 250 times.

5. **Dispense reagent:** This daughter step of Kinetic Loop 2 defines the parameters of reagent addition: volume, dispensing speed, etc. For this experiment, 90 µL of ATP reagent was dispensed at high speed. When the dispensing step is placed within the kinetic loop, dispensing is performed simultaneously with the first reading.

6. **Flash ATP signal:** This daughter step of Kinetic Loop 2 is the luminometric measurement step that measures the reaction signal. This step used the same 100 ms measurement time as the baseline reading. Therefore, with 250 readings, we get a total kinetic measurement time of about 25 sec.

Identical assay plates were tested with both the Luminoskan and Fluoroskan FL microplate readers to illustrate the performance of both instrument models in such fast, flash-type kinetic assays.

## Results and calculations

Once measurements were performed, the raw data were exported to Microsoft™ Excel™ for further calculations. First, raw kinetic data of two kinetic measurements were merged to get one kinetic data set. Then the average kinetic curves for every sample were calculated (Figure 2).

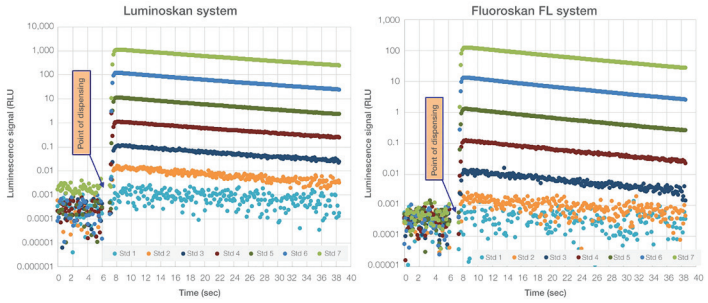



**Figure 2. Kinetic curves of the flash ATP assay with the Luminoskan and Fluoroskan FL systems.**

Next, 1.5 sec kinetic integrals of the baseline (before dispensing) and sample signals (after dispensing) were calculated for each well, as shown in Figure 3.

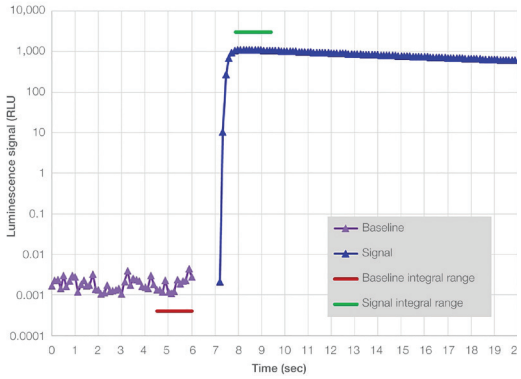



**Figure 3. Baseline and sample signal integration.** The baseline was integrated for each sample using a time range of 4.5–6 sec. The luminescence ATP reaction was triggered at 7 sec, and the real ATP signal was integrated for the time range of 8–9.5 sec.

The baseline values were then subtracted from the sample signals, as shown in Table 1.

**Table 1. Summary of results for the flash ATP assay.**

| | Std 1 | Std 2 | Std 3 | Std 4 | Std 5 | Std 6 | Std 7 |
|---|---|---|---|---|---|---|---|
| ATP (amol/well) | 1 | 10 | 100 | 1,000 | 10,000 | 100,000 | 1,000,000 |
| **Luminoskan system** | | | | | | | |
| Baseline | 0.0044 | 0.0005 | 0.0051 | 0.0045 | 0.0057 | 0.0047 | 0.0193 |
| Signal | 0.0197 | 0.1843 | 1.5146 | 14.9067 | 152.5524 | 1,631.2200 | 15,174.4050 |
| Baseline-subtracted signal | 0.0153 | 0.1788 | 1.5095 | 14.9022 | 152.5467 | 1,631.2153 | 15,174.3857 |
| **Fluoroskan FL system** | | | | | | | |
| Baseline | 0.0027 | 0.0030 | 0.0019 | 0.0038 | 0.0037 | 0.0014 | 0.0054 |
| Signal | 0.0047 | 0.0210 | 0.1475 | 1.4759 | 15.9021 | 165.0994 | 1,512.4150 |
| Baseline-subtracted signal | 0.0020 | 0.0180 | 0.1456 | 1.4721 | 15.8984 | 165.0980 | 1,512.4096 |

Baseline-subtracted signals were plotted against the ATP concentration to get the linear calibration curves shown in Figure 4. Detection limit values were calculated using the common principle: 3 times standard deviation for limit of detection (LOD) and 10 times standard deviation for limit of quantitation (LOQ). The dynamic range was calculated based on the known theoretical maximum relative light unit (RLU) values that the instruments can detect and the detection limit. LOD, LOQ, and dynamic range values are shown in Table 2.

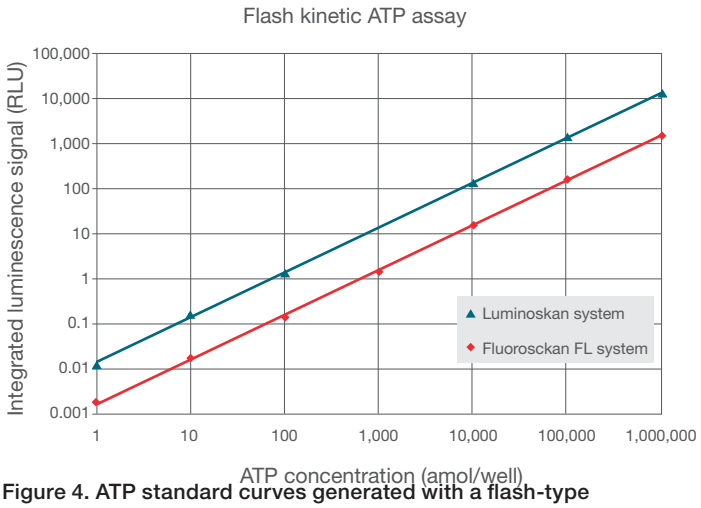



**Figure 4. ATP standard curves generated with a flash-type luminescence ATP assay.**

**Table 2. Key characteristics of the performance of the flash ATP assay.**

| Instrument | LOD (amol/well) | LOQ (amol/well) | Dynamic range (decades) |
|---|---|---|---|
| Luminoskan luminometer | 0.14 | 0.47 | 7.5 |
| Fluoroskan FL fluorometer and luminometer | 0.51 | 1.71 | 7.9 |

## Discussion and summary

This flash-type kinetic assay is quite easy to do when the instrument is capable of dispensing and reading without delay. When a reaction is so fast that the signal reaches its maximum within about one second after dispensing has started, both dispensing and reading must be performed simultaneously at exactly the same location inside the instrument.

The Luminoskan and Fluoroskan FL instruments both perform this assay with sub-attomolar detection limits—supporting the high sensitivity of flash-type kinetic assays. In flash-type assays, energy from all chemical reactions is released within a few seconds; in contrast, glow-type reactions release the same amout of energy over a long period of time. This improves the signal-to-background and signal-to-noise ratios remarkably; and therefore, flash-type assays clearly offer better sensitivity than glow-type assays. The most sensitive glow-type ATP assays have a detection limit in the range of 100–200 amol of ATP. When this is compared to the <1 amol detection limit we obtained with a flash-type kinetic assay, the difference is significant.

Find out more at **thermofisher.com/luminoskan**

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