Application Note:

AP-MIB-VARIO14-0708

Optimization of Instrument Settings and Measurement Parameters for Kinetic Fluorometric and Luminometric Assays

Reija-Riitta Harinen, Jorma Lampinen, Marika Raitio. ThermoFisher Scientific Oy, Vantaa, Finland.

Abstract

The speed of reaction kinetics of fluorometric and luminometric assays sets certain important requirements for the detection instrument and measurement parameters. Fluorometric peroxide / peroxidase assay, luminometric ATP assay and luminescence producing reaction between Aequorin and calcium were used as examples for optimizing measurements of different types of kinetic reactions in microplate format. The results present many possible ways of selecting optimal instrument settings according to the reaction speed.

Introduction

When fluorometric and luminometric assays are performed in microplate format, a high number of samples can be analyzed at a time. The speed of the reaction kinetics of the assay determines the requirements for the detection instrument and measurement parameters for achieving the best possible performance of the assay. If the reaction is started by adding the starting reagent to microplate wells, the speed of the reaction kinetics determines if it's possible to add the reagent manually or if an automatic instrument dispenser must be used. If the signal peak is reached quickly after reagent addition and the fluorescence or luminescence decays rapidly after that, manual reagent addition may be out of the question, as the delay between reagent addition and signal detection may weaken the assay sensitivity considerably. Therefore fast reactions must be performed with an instrument equipped with automatic dispensers. If the reaction is extremely fast and light detection must be started simultaneously with reagent addition, the dispensing position inside the instrument must be in the measurement position and the dispenser head must point directly to the well to be measured. That way the progress of the reaction can be followed from the beginning.

In some kinetic measurements the baseline signal is first measured for a certain period of time before starting the reaction with reagent addition. For that kind of assays the user must be able to program the kinetic measurement protocol so that it's possible to select from the software settings at which kinetic reading the reagent is dispensed.

It must also be possible to choose in which order the kinetic readings of different wells are measured after reagent addition. If the reaction is really fast, all the kinetic readings of the first well must be measured instantly after reagent addition before proceeding to dispense and measure the next well. Otherwise the peak of the signal may be lost while the other wells are measured, or the kinetic curve of the beginning of the reaction can form incorrectly. If the interval of the kinetic readings can be long enough, the reagent can first be dispensed to all the wells before starting to measure the kinetic readings by plate. So the consecutive kinetic readings following the dispensing can either be measured by one well, the whole plate or by certain group of wells according to the rate of the kinetic reaction.

The selection of dispensing speed should also be included in the software settings. The more viscose the dispensed solution is, the faster the dispensing speed should be. The optimal dispensing speed is also important for reagent mixing in the well. If the speed is too low, the reagents may not get mixed well and fast enough. And if the speed is set too high, the reagents may splash outside the well.

Some assays include several reagent addition steps, therefore instrument with more than one dispenser may be required. For example a very commonly used luminometric reporter gene assay, DLR™ (Dual Luciferase® Reporter Assay, Promega, USA) includes two reagent addition steps for which the instrument dispensers are essential.

Materials and methods

All the fluorometric and luminometric measurements were performed with Thermo Scientific Varioskan® Flash, a spectral scanning multitechnology microplate reader. The instrument was controlled via Thermo Scientific SkanIt® Software.

Amplex® Red hydrogen peroxide / peroxidase assay kit (Invitrogen, USA) was used for kinetic fluorometric measurements. In the presence of peroxidase the Amplex Red reagent reacts with H2O2 to produce resorufin, which can be detected fluorometrically. Amplex Red reagent and HRP (horseradish peroxidase) were mixed, to concentrations 100 µM and 2 mU/ml respectively, and 50 µl of that solution was added to microplate wells. The fluorometric reaction was started by adding 50 µl of 10 µM H2O2 to the wells. The same assay was performed by either dispensing the H2O2 solution at the first or at the fifth kinetic reading. The fluorescence was measured with excitation at 550 nm and emission at 590 nm. The assays were performed on black Microfluor 1 96 well plates (Thermo Scientific).

Two ATP detection kits were used for measurements of luminescence kinetics. One was the CheckLiteTM HS Set (Kikkoman Corp., Japan) producing fast flash type luminescence and the other was the ATP Biomass HS kit (BioThema AB, Sweden) producing more stable glow type luminescence. The ATP detection of both of the kits is based on the light producing reaction between D-luciferin and ATP catalyzed by firefly luciferase enzyme. The difference in luminescence stability of flash and glow reactions is caused by the chemical stabilization of the glow type reaction. The kinetics of flash and glow reactions was measured by adding 30 µl of 0.01 µM ATP to the wells. The reactions were started by adding 30 µl of the starting reagent of the kit to the well with the instrument dispenser. The faster flash type luminescence kinetics was measured with 1 second integration time and zero interval time for 10 minutes. The more stable glow type luminescence was measured with 500 ms integration time and 1 minute interval time for 60 minutes. The assays were performed on white Microlite 1+ 384 well plates (Thermo Scientific).

One example of an extremely fast luminescent reaction is the reaction between Aequorin and calcium. Aequorin is a photoprotein complex which emits blue light upon binding calcium. When loaded inside cells it can be used as an intracellular Ca2+ indicator. The luminescence of AquaLite® Recombinant Aequorin (Invitrogen, USA) was detected by adding 1 pmol of Aequorin to the microplate well and the luminescence reaction was started by dispensing 10µl of 500 mM CaCl2 solution to the well with maximum dispensing speed. The luminescence kinetics was measured with 10 ms integration time and zero intervals, for about 10 seconds. The assay was performed on white Microlite 1+ 96 well plates (Thermo Scientific).

Results

Fluorometric AmplexRed kinetics was measured by starting the reaction by dispensing H2O2 either at the first or fifth kinetic reading. Figure 1 presents these two different ways of combining reagent dispensing and signal detection in kinetic measurement. The reading of dispensing can be selected from the software (Figure 2). If the starting reagent is dispensed at the first reading, the detected signal represents only the fluorescence after reagent addition. In case the reagent is dispensed e.g at the fifth reading, the baseline fluorescence can be detected separately. The software creates the kinetic curves automatically and the average baseline signal of each

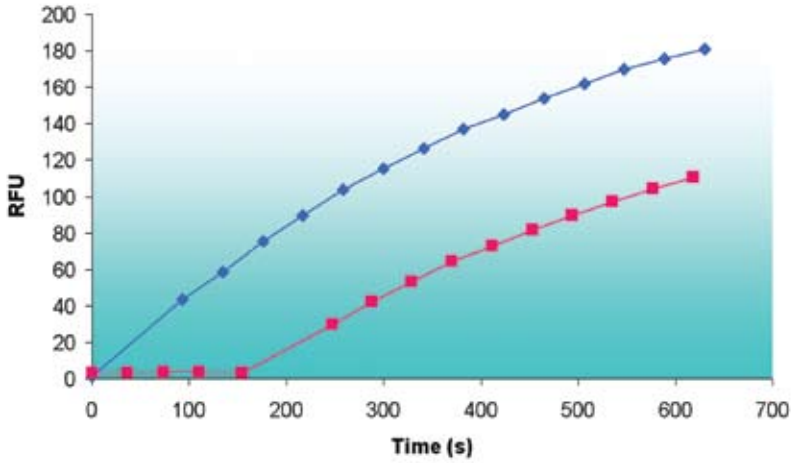


Figure 1. Fluorescence kinetics of resorufin formation of Amplex Red hydrogen peroxide / peroxidase assay (RFU = relative fluorescence unit). The two curves show how reagent dispensing can be done at any stage of the kinetic measurement, depending on which parts of the signal progression need to be detected. The blue curve presents the measurement with dispensing at the first kinetic reading and the red curve presents the measurement with dispensing at the fifth kinetic reading

Figure 2. The dispensing inside kinetic measurement can be performed at any kinetic reading.

sample can then be subtracted from the kinetic curve.

The reaction speed of flash and glow type ATP reactions were compared by measuring the luminescence kinetics of both reactions (Figure 3). The flash type luminescence of the ATP reaction reaches its peak maximum in about 4 seconds after reagent addition. After that the light decays rapidly with a signal half-life of about two minutes. This clearly indicates that the flash type luminescence reaction can not be measured without instrument dispensers. Starting the reaction by manual reagent addition would decrease the assay sensitivity significantly as the peak signal would never be detected. Also the signal instability indicates that the signal of each well must be detected with constant time difference between the reagent addition and measurement.

The glow type luminescence of the ATP reaction on the other hand is quite stable. The peak maximum is reached in about a minute after reagent addition and after that the signal decays slowly. With this type of reaction kinetics it's possible to add the reagents manually to the whole plate at a time. The moderate delay time between reagent addition and starting the measurement won't affect the assay sensitivity remarkably. But compared to manual pipetting reagent addition with instrument dispensers helps to achieve the most reliable and reproducible assay results also in glow type assays.

Because of the difference in reaction speed, different software measurement protocols should be used for glow and flash type kinetic reactions (Figure 4). For the flash type reaction the interval of the kinetic readings must be kept short and therefore the kinetic readings must be done by one well, for which purpose the well loop step with well count 1 is selected to the measurement protocol. It means that all the steps under the well loop are completed for one well before proceeding to go through the same steps for the next well. For the glow type reaction the dispensing and consecutive kinetic readings can be done by plate, as the interval of the kinetic readings can be long enough to measure the readings of the other wells during the interval times.

Figure 5 presents the luminescence kinetics of Aequorin reaction. As can be seen, the peak maximum is reached instantly in 0.5 second after addition of calcium solution and the signal decays in half in about two seconds. After 10 seconds there's less than 10 % of the signal left. This type of extremely fast reaction kinetics requires special features from the

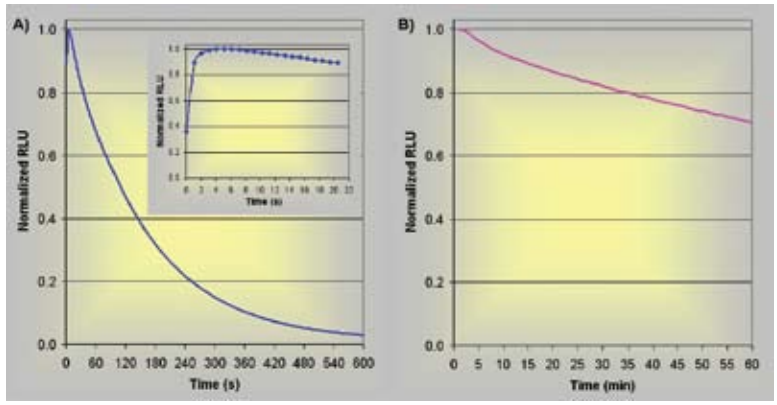
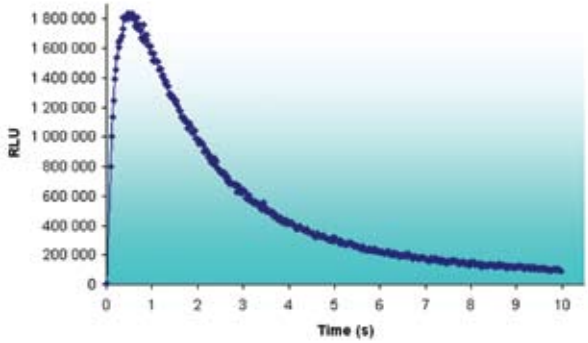


Figure 3. Comparison of the kinetics of flash and glow type luminescence reactions of two ATP detection kits. Picture A represents the faster flash type luminescence and picture B the slower glow type luminescence.

Figure 4. Skanlt software measurement protocol steps for glow and flash type reactions. The protocol on the left can be used for the slower kinetics of glow type ATP reaction and the protocol on the right is optimal for the fast flash type kinetics, where the interval time of kinetic readings must be short.



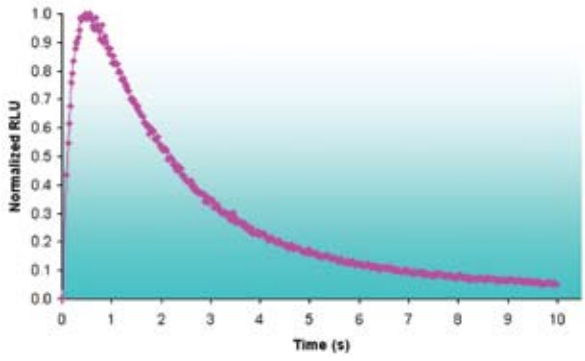


Figure 5. The luminesecence kinetics of the extremely fast reaction between Aequorin and calcium. The blue curve presents the kinetics as RLU (relative luminescence units) values and the purple curve presents the same measurement as normalized RLU values.

instrumentation and measurement settings. The measurement of the kinetic readings must be started simultaneously with automatic reagent dispensing or otherwise the real peak maximum will never be detected. So the kinetic measurements must obviously be done by one well. The reagent must be dispensed at a high enough speed (Figure 6) so that the reagents will get mixed fast and thoroughly. The dispenser head must point directly to the well which is at the measurement position under the optics, as after dispensing there's no time to move the plate to a different measurement position. Also the measurement integration time of the kinetic readings must be very short, like 10 ms with these curves. With too long integration time the real peak of the curve may be lost.

Figure 6. The SkanIt software includes the setting for dispensing speed, which can be changed according to the liquid type and reaction requirements.

Conclusions

The reaction kinetics of fluorometric and luminometric reactions affects the way the measurements must be performed to get the best possible results. The optimization of the instrument settings and measurement parameters can improve the assay sensitivity considerably. Using the instrument dispensers helps to achieve reliable and reprocucible results and decreases the inaccuracy caused by manual pipetting. As shown above, several microplate assays are based on such fast reactions, which can not even be measured without automatic instrument dispensers without losing the assay sensitivity at the same time. When an instrument with in-built dispensers is in use, the protocol parameters for the measurement can be optimized by many different settings.

With Thermo Scientific Varioskan Flash it's possible to have up to three dispensers in the instrument for multiple reagent addition. The flexible SkanIt software allows the user to create the kinetic measurement protocols according to the reaction speed. The measurement can be started simultaneously with the dispensing, which is essential in reactions following really fast kinetics. Also the time of the dispensing inside the kinetic measurement protocol can be selected. The dispensing speed can easily be optimized based on the liquid type and reaction requirements. The order of measuring the kinetic readings of the wells can be freely selected based on the reaction speed. Fast reactions must be measured by one well at a time and the more stable kind of reactions allow the measurements to be performed by the whole plate or in a groups of wells. All these choises help the user to achieve maximal efficiency and high result quality of all types of kinetic microplate assays.

In addition to these offices, Thermo Fisher Scientific maintains a network of representative organizations throughout the world.

North America:

USA / Canada
+1 800 522 7763

Europe:

Austria
+43 1 801 40 0

Belgium
+32 2 482 30 30

France
+33 2 2803 2000

Germany national toll free
08001-536 376

Germany international
+49 6184 90 6940

Italy
+39 02 02 95059 434-254

Netherlands
+31 76 571 4440

Nordic countries
+358 9 329 100

Russia/CIS
+7 (812) 703 42 15

Spain/Portugal
+34 93 223 09 18

Switzerland
+41 44 454 12 12

UK/Ireland
+44 870 609 9203

Asia:

China
+86 21 6865 4588 or
+86 10 8419 3588

India
+91 22 6716 2200

Japan
+81 45 453 9220

Other Asian countries
+852 2885 4613

Countries not listed:
+49 6184 90 6940 or
+33 2 2803 2000

www.thermo.com/readingroom

www.thermo.com/mpi