Application Note:
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Characterization of emission properties of Aequorin mutants with rapid, high throughput screening assay

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This application note explains how to perform a rapid, high throughput screening assay of mutated photoprotein aequorin in order to find clones with more intense, slower kinetic, narrow spectral or color-shifted bioluminescent emission. Several examples of bioluminescent emission kinetics and spectra of mutated aequorins are included.

Introduction

Aequorin is a photoprotein isolated from luminescent jellyfish and other marine organisms that can be used to measure intracellular Ca^{2+} levels. Aequorin is composed of two distinct units, the apoprotein apoaequorin (22 kDa) and the prosthetic group coelenterazine (CTZ), which reconstitute spontaneously in the presence of molecular oxygen, forming functional protein (Figure 1).

The protein has several binding sites for Ca^{2+} ions responsible for protein conformational changes. Its prosthetic group, CTZ, is converted via oxidation into excited CTZ and CO_2. As the excited CTZ relaxes to ground state, blue light (wavelength = 469 nm) is emitted and can easily be detected with a luminometer. Due to the extremely fast kinetics involved, special instrumentation needs to be used to screen wild-type and mutant aequorins expressed in recombinant *E. coli* cells. Due to its unique automatic dispensing feature, the Thermo Scientific Varioskan Flash reader is the only available microplate luminometer capable of measuring luminometric spectra. Unlike other instruments, the Varioskan Flash Luminometer can perform the kinetic readings well by well while keeping measurement time very short (10 ms).

The measurement of the kinetic readings must be started simultaneously with automatic reagent dispensing to detect the peak maximum. Alternatively, the dispensing step could be done after five readings to measure background signal prior to dispensing, as in this assay.

Materials required

All the luminometric measurements were performed with the Varioskan® Flash instrument, a spectral scanning multitechnology microplate reader controlled by Thermo Scientific SkanIt Software. The substrate native coelenterazine was NanoFuel® CTZ. The random mutagenesis was performed with the GeneMorph® Random Mutagenesis Kit using a plasmid for the expression of wild-type aequorin as template. An *E. coli* strain (ultra-competent cell XL10 or JM109) was used as the bacterial host for aequorin expression. The assays were performed on a Thermo Scientific Microtiter white 96-well strip plate.

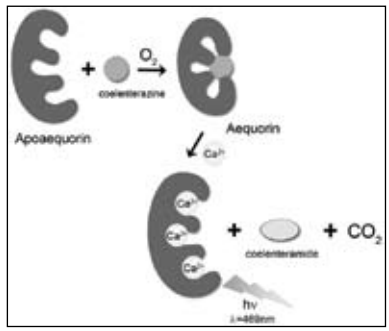

Figure 1. Schematic representation of the bioluminescent mechanism of photoprotein aequorin.

Experimental protocol

Reagent preparation

The CTZ stock solution was prepared at 1 mg/ml concentration in methanol acidified by HCl (to 10 ml of 100% anhydrous methanol 50 µl concentrated HCl was added). Coelenterazine was dissolved in amber microcentrifuge tubes to protect it from light. The CTZ working solution was prepared fresh by diluting the CTZ stock solution to a final concentration of 5 µM in a solution containing 100 mM Tris base pH 8, 90 mM NaCl, and 5 mM EDTA. The triggering solution was composed of 0.75% Triton X-100 and 15 mM $CaCl_2$ in water.

Aequorin reconstitution

Recombinant cells expressing aequorin were grown in selective LB broth to an OD_{600nm} of 0.6, then 150 µl-aliquots of cells were transferred to a white 96-well Microtiter® plate and incubated with 50 µl CTZ-EDTA for 5 hours in the dark at 4°C for aequorin reconstitution.

Instrument preparation

Aequorin bioluminescence is emitted as a rapidly decaying flash upon calcium binding, and a flash-type luminescence reaction can only be measured with an instrument equipped with an automatic dispenser. The Varioskan Flash reader used has three automatic dispensers, but only one was needed. Dispenser 1 was primed with a triggering solution and was set to dispense 10 µl at reading 10 at "High" dispensing speed. The instrument's temperature setting was kept in "Ambient" to maintain an ambient temperature of 23°C.

Kinetic measurements

To monitor the emission decay, the Varioskan Flash reader was programmed to perform the following measurement.

STEP 1
Well loop step, Execution type "by well", Well count 1. This setting enables the instrument to execute all actions to one well before proceeding to the next well.

STEP 2 (inside well loop)
Kinetic loop step, Readings 1000, Interval 0 s. This setting enables the instrument to perform a kinetic assay with 1000 kinetic points. The kinetic reading is performed very rapidly with no interval time between the readings.

STEP 3 (inside kinetic loop)
Dispensing step, Dispenser 1, Dispensing volume 10 µl, Dispensing speed "High", Dispensing position "Luminometric 1", Tip priming "Automatic", Dispense at reading 10. This enables the instrument to dispense 10 µl of Ca^{2+} triggering solution into each well at high speed.

STEP 4 (inside kinetic loop)
Measurement step, Luminometric measurement, Luminometric optics "Normal", Dynamic range "AutoRange", Measurement time 10 ms. This enables the reader to a kinetic measure at a sampling speed of 100 points/second (10 ms measurement time). The total kinetic measurement time of the aequorin signal is just 25 seconds. The Step structure of the protocol is shown in Figure 2.

Figure 2. Structure of the measurement protocol used to measure luminescence decay.

Luminometric spectra

The Varioskan Flash reader was programmed to perform the following measurements.

STEP 1
Well loop step, Execution type "by well", Well count 1. This setting enables the instrument to perform the following actions well by well.

STEP 2 (inside well loop)
Dispensing step, Dispenser 1, Dispensing volume 20 µl, Dispensing speed "High", Dispensing position "Luminometric 1", Tip priming "Automatic".

STEP 3 (inside well loop)
Measurement step, Luminometric spectral scanning, wavelength area 400 – 600 nm, Wavelength step 2 nm, Dynamic range "AutoRange", Measurement time 100 ms. This setting enables the reader to measure aequorin spectra from the selected wavelength area in two nanometer steps. The Step structure of the protocol is shown in Figure 3.

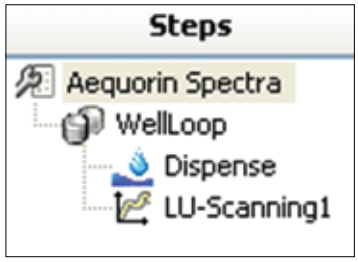

Figure 3. Structure of the measurement protocol used to measure luminescence spectra.

The assay plate was placed into the instrument's plate carrier and the full program was executed. After the measurement, all data was exported from the SkanIt® Software as a Microsoft Excel® file using the "Organized export" function. The data analysis was performed with Excel.

Result and discussion

Figure 4 presents the luminescence kinetics of some mutants of aequorin. Aequorin light emission shows a maximum at about 469 nm and the luminescence peak has about an 80 nm half bandwidth. These results are analogous to previously reported values (Figure 5).

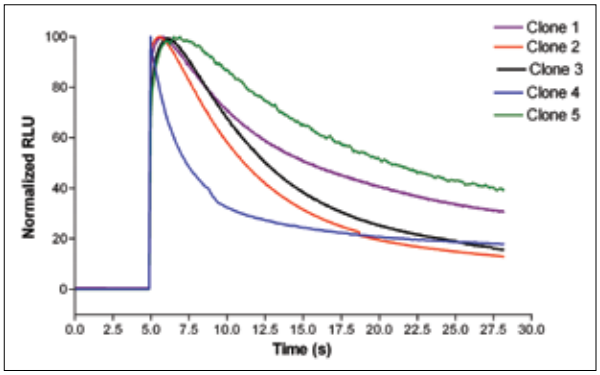

Figure 4. Luminescence kinetics of aequorin mutants (expressed in RLU, relative light unit) values.

Conclusion

The Varioskan Flash reader with a high-sensitivity luminometric module, automatic dispensers and luminometric spectral scanning is a perfect combination to screen a high number of clones generated by random mutagenesis. The instrument dispenser is essential to achieve reliable and reproducible kinetic measurements, and the flexible SkanIt Software allows the user to create and optimize the kinetic measurement protocols. The instrument's spectral scanning feature cananalyze color changes of Aequorin mutants.

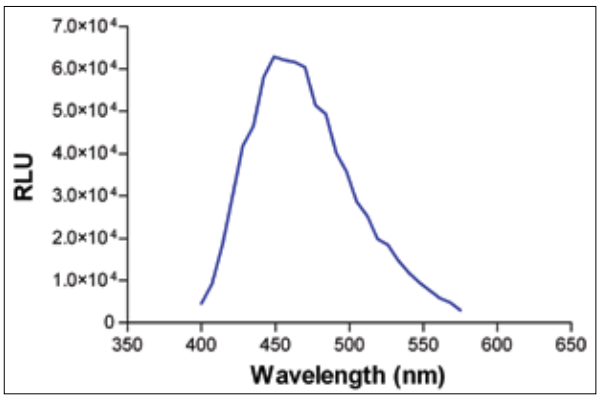

Figure 5. Emission spectrum of wild-type aequorin.

Further information

For further information about the Varioskan Flash reader, please refer to the following web pages:

www.thermo.com/readingroom
www.thermo.com/varioskan

References

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