



High Throughput Bacterial Screening Assay for Emission Properties Characterization of a Library of Aequorin Mutants



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Background and aim

The photoprotein aequorin, isolated from the jellyfish *Aequorea victoria*, is a calcium-sensitive bioluminescent protein formed with the apoprotein apoaequorin and the prosthetic group coelenterazine that emits light upon calcium binding. Aequorin belongs to the family of calcium-dependent luciferase/luciferin systems and because of its high sensitivity to calcium ions has been extensively used as a Ca²⁺ reporter in living cells or as a luminescent label in binding assays. In order to obtain aequorin variants with improved bioluminescence properties for bioanalytical application such as slower emission decay or red-shifted emission spectra, a library of aequorin mutants was generated by random mutagenesis and a rapid and low-cost screening was developed using the Varioskan® Flash multimode reader (Thermo Scientific).

Structural properties of aequorin

Aequorin is a globular molecule of 189aa (MW 22kDa) containing a hydrophobic core cavity that accommodates the imidazopyrazine chromophore coelenterazine. The aequorin molecule contains four helix-loop-helix 'EF-hand' domains, three of which can bind calcium (figure 1).

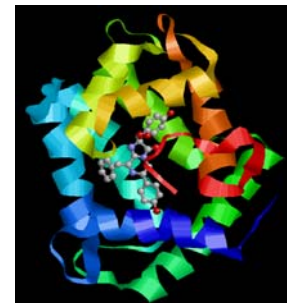


Figure 1. Rasmol display window showing a ribbon representation of aequorin. Coelenterazine is shown as ball&stick.

Bioluminescent reaction mechanism

The luminescent reaction of aequorin is triggered by addition of calcium ion followed by decomposition of AEQ into apoAEQ (apoprotein), coelenteramide, and CO₂, accompanied with spontaneous blue light emission (λ_{max}~470nm) (Figure 2).

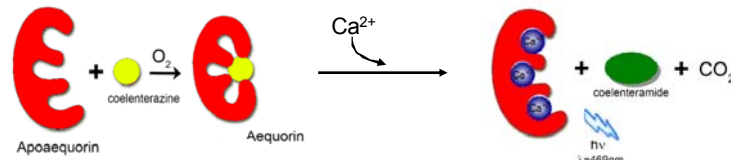


Figure 2. Schematic representation of the bioluminescent mechanism of photoprotein aequorin.

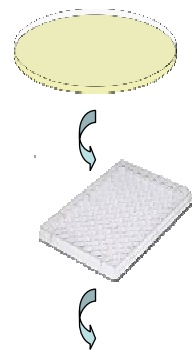
Bacterial Screening Assay with Varioskan Flash

The random mutagenesis was performed with GeneMorph Random mutagenesis kit (Stratagene) using a plasmid encoding wild-type apoaequorin as template. *Escherichia coli* JM109 strain was used for molecular cloning and expression of mutated apoaequorin variants.

Single mutant colonies were transferred manually to a Thermo Scientific Microtiter white 96-well strip plate and grown in selective LB broth for 16 hrs; every plate contained 94 mutant colonies and 2 colonies of WT aequorin as control.

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

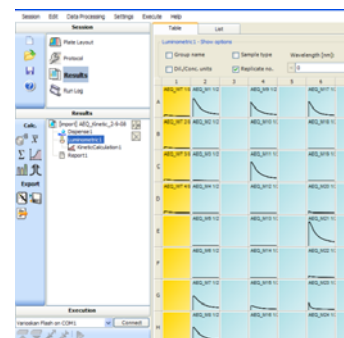
Following equilibration at room temperature for 15 min, aequorin activity (kinetic decay and emission spectra) was measured at 22°C by injecting 10µl of a "triggering solution" containing 50mM Tris (pH 8), 15mM CaCl₂ using the Varioskan® Flash instrument, a spectral scanning multitechnology microplate reader controlled by Thermo Scientific SkanIt Software.



Varioskan Flash spectral scanning multimode reader



Onboard dispensers for automatic reagent dispensing



Results and discussion

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 assay plate was placed into the instrument's plate carrier and the full program was executed automatically by the instrument. The data analysis was performed both with SkanIt® Software and GraphPad Prism 5.0.

Analysis of decay kinetics

The instrument was programmed to perform a kinetic assay with 1000 readings (measurement time 10ms). The instrument's spectral scanning feature was also used to analyze color changes of aequorin mutants (with readings of 100ms to measure aequorin spectra from 400 to 600nm in two nanometer steps). No changes in luminescent emission wavelength were observed in the 500 screened clones.

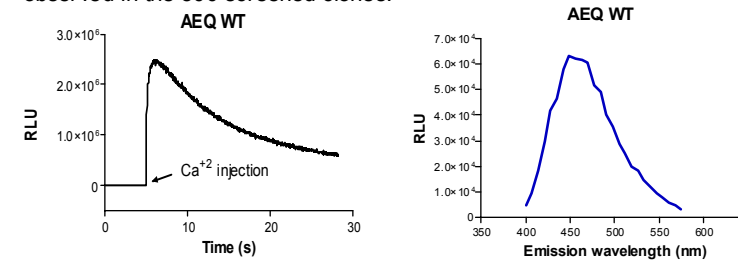


Figure 3. Decay kinetics and emission spectrum of aequorin wild-type

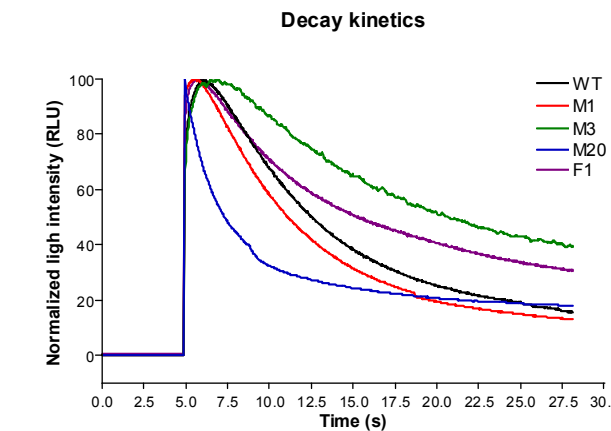


Figure 5. Normalized kinetic decays of selected aequorin mutants relative to wild type kinetic (black line)

Conclusion

A library of aequorin mutants was obtained by random mutagenesis and a rapid bacterial screening assays was developed to measure emission decay kinetics and emission spectra directly in culture media. This screening resulted in the selection of aequorin mutants characterized by an increased luminescence in bacterial cells or by slow decay kinetics.

The results were compared to data obtained with the purified aequorins and confirmed the suitability of this first-level screening to investigate emission properties of mutant aequorins. The high sensitivity of the Varioskan Flash reader, together with the automatic dispenser, allowed to screen a high number of clones and obtain reliable and reproducible kinetic measurements. Further experiments will be performed using purified mutant aequorins selected with the bacterial screening to investigate kinetic properties (e.g. half life, light integral...) and thermostability.

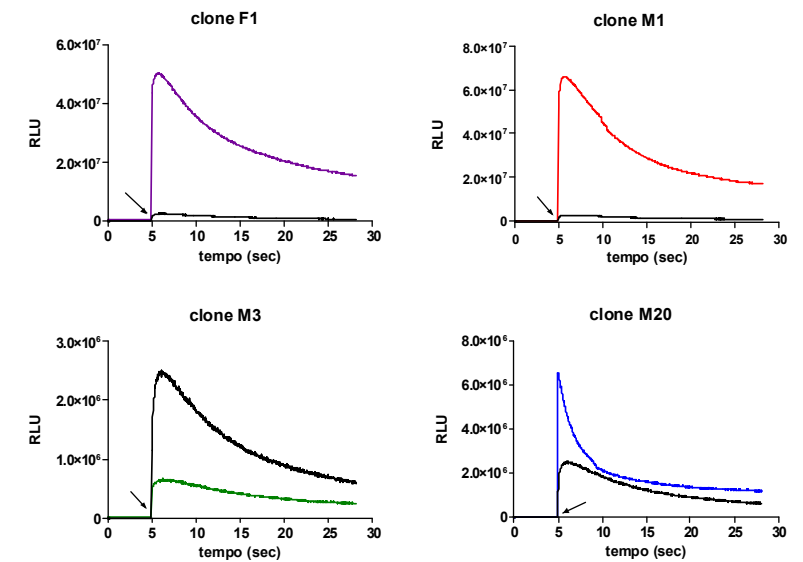


Figure 4. Kinetic decays of selected aequorin mutants relative to wild type kinetic (black line)

Table 1.

Clone	K (s ⁻¹)	Half life (s)	Tau (s)	R ²
WT	0,1229	5,640	8,136	0,9987
M1	0,1641	4,225	6,095	0,9998
M3	0,0976	7,099	10,24	0,9967
M20	0,4334	1,599	2,307	0,9826
F1	0,1573	4,406	6,356	0,9992

The SkanIt® Software was used to analyse kinetic measurements and calculate kinetic decay parameters for each clone. The results for mutants with slow kinetic decays or higher activity are shown in table 1. preliminary sequencing showed that mutations neighbored the His-16 or His-169 coelenterazine binding residues or were located in the first EF-hand

Acknowledgments: we are very grateful to **Thermo Fisher Scientific** for the opportunity to test **Varioskan® Flash** instrument and in particular to Dr. **Jorma Lampinen** for his help with instrumentation settings, suggestions and assistance.

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

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

