

AquaLite® Recombinant Aequorin (A-6785)

Quick Facts

Storage upon receipt:

- -80°C
- Desiccate

Introduction

In contrast to fluorescent and phosphorescent molecules, bioluminescent molecules generate light through a biochemical reaction and do not require optical excitation. Since bioluminescence is extremely rare in nonaquatic organisms, the use of such molecules as reporters in living cells provides an exquisitely sensitive detection method. Aequorin, a photoprotein complex isolated from luminescent jellyfish and other marine organisms, emits blue light upon binding calcium. Because of this calcium-dependent emission, the aequorin complex has been extensively used as an intracellular Ca^{2+} indicator. For example, aequorin was used for the seminal work demonstrating the massive release of Ca^{2+} during fertilization,¹ for the experiments that clarified the role of Ca^{2+} release in heart muscle damage,² in the analysis of the secretion response of single adrenal chromaffin cells to nicotinic cholinergic agonists³ and in the study of the regulation of the sarcoplasmic reticulum Ca^{2+} pump expression in developing chick myoblasts.⁴

The aequorin complex consists of the 22,000 MW apoaequorin protein, molecular oxygen and the luminophore coelenterazine (Figures 1A and 1B). When Ca^{2+} binds to the aequorin complex, coelenterazine is oxidized to coelenteramide, releasing CO_2 and blue light with an emission maximum of 469 nm.⁵ Since the light emitted by the aequorin complex does not depend upon optical excitation, problems associated with autofluorescence are eliminated and background signal is virtually nonexistent. Thus it is possible to detect less than 10^{-18} mole of the photoprotein over background.⁶ The dynamic range for detection of aequorin itself spans some six orders of magnitude.

Until recently, the primary source of aequorin was the jellyfish *Aequorea victoria*. A typical yield was 125 mg of photoprotein per two tons of jellyfish.⁷ In addition to laborious and expensive extraction procedures, substantial heterogeneity has been observed among different lots of purified aequorin⁸ and lots were sometimes found to be toxic to organisms and cells under study.⁹ Recombinant aequorin, which is produced by purifying apoaequorin from recombinant *E. coli* bacteria^{10,11} followed by reconstitution of the complex *in vitro* with pure coelenterazine, circumvents these problems by providing a product with lot-to-lot consistency and high purity. Moreover, recombinant aequorin is not toxic to cells; the toxicity of crude isolates has been attributed

to unidentified contaminants not present in the recombinant form.⁸ In addition to its usefulness as a Ca^{2+} indicator in living cells, another important application of this pure protein is as a calibration standard for aequorin derivatives and intracellular aequorin measurements.

Aequorin has a number of advantages over other Ca^{2+} indicators. These include the large range of Ca^{2+} concentrations over which aequorin is responsive (from 10^{-7} to 10^{-4} M), its low leakage rate from cells as compared to that of fluorescent dyes (aequorin is not exported or secreted) and its lack of intracellular sequestration or compartmentalization. Also, aequorin does not appear to disrupt cell functions or embryo development. Aequorin luminescence has been detected 26 hours after cell loading using the HOST (hypoosmotic shock treatment) method¹² and the detection of luminescence hours after labeling is common.^{4,13,14} In comparison, fluorescent indicators (for example, fura-2) are normally sequestered in intracellular organelles within hours of labeling,¹⁵ adversely affecting both the accuracy of Ca^{2+} measurements and cellular viability. While the use of dextran conjugates of Ca^{2+} indicators circumvents compartmentalization problems, the phototoxicity of the excitation light may still perturb the experiment. A useful practical overview of bioluminescence and fluorescence methods for monitoring intracellular Ca^{2+} can be found in *Cellular Calcium: A Practical Approach*, edited by J.G. McCormack and P.H. Cobbold, IRL Press at Oxford University Press, New York (1991).

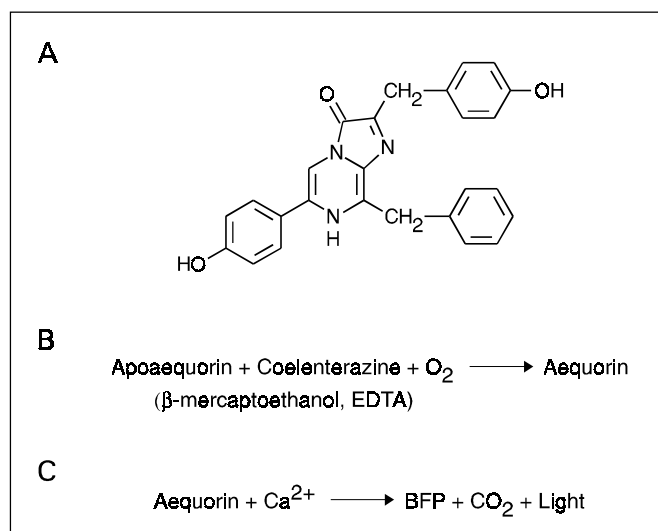


Figure 1. **A)** Structure of the luminophore coelenterazine. **B)** Formation of the aequorin complex (β -mercaptoethanol and EDTA are required for *in vitro* reconstitution). **C)** Light emission by the aequorin complex upon binding calcium. Upon binding calcium, the aequorin complex is consumed, producing the Blue Fluorescent Protein (BFP), carbon dioxide and blue light ($\lambda_{\text{max}} = 469 \text{ nm}$).

Materials

AquaLite® recombinant aequorin is provided in units of 25 µg as a powder lyophilized from a 1 mg/mL solution in 5 mM HEPES, 0.1 M KCl, 30 mM glucose and 5 µM EDTA, pH 7.1. Upon receipt, the product should be stored desiccated at -80°C. When stored properly, AquaLite recombinant aequorin is stable for at least three months.

Once reconstituted in aqueous solution, the aequorin complex will irreversibly bind any available calcium, emitting blue light and consuming the coelenterazine over a period of hours; active aequorin must then be regenerated by adding exogenous coelenterazine.¹⁶ Thus, **all solutions of aequorin should be completely calcium-free until use.** Molecular Probes' Calcium Sponge S (C-3047) is a BAPTA conjugate of water-insoluble polystyrene that can be used to selectively remove Ca²⁺ ions from solutions. Aequorin solutions that are refrigerated at 4°C may be stable for as long as three months. For longer storage, solutions should be divided into aliquots and frozen at -80°C. **AVOID REPEATED FREEZING AND THAWING.**

Protocol

Overview

Aequorin is typically microinjected for intracellular Ca²⁺ measurements because it is a protein complex and thus does not freely enter most cells. Other protocols have been used; for example, human platelets have been made transiently permeable to the aequorin complex with DMSO¹⁷ and monkey kidney cells have been loaded by hypoosmotic shock.¹² Pressure injection is a commonly cited loading method, despite the fact that only large cells can be loaded in this way. Pressure injection has been employed to study the effects of caffeine on mouse diaphragm muscle fibers¹⁸ and the role of Ca²⁺ in the fertilization of sea urchin eggs.¹⁹

Microinjection of Aequorin

Since microinjection methods will depend to a large extent on the type of instrument used and the cell type under study, the following protocol is provided as a general guide only. It is important to observe the precautions in *Application Notes*, particularly those pertaining to the effects of certain ions on aequorin activity.

1.1 Prepare a stock solution of 1–5 mg/mL aequorin in 25–5 µL calcium-free water; refer to section 2 for storage conditions. The maximum solubility of aequorin in aqueous solution is about 7% (70 mg/mL).¹⁶

1.2 A number of buffers have been used to prepare aequorin for microinjection.^{16,20-26} Although the stock solution prepared in step 1.1 can serve as the microinjection solution, it is often desirable to optimize the buffer for the cell type to be studied. Dialysis is frequently used to change the composition of the solution. For example, in their studies of sea urchin oocyte fertilization, Swann and Whitaker dissolved lyophilized aequorin in distilled water and then dialyzed it against 20 mM PIPES, pH 6.7, containing 480 mM NaCl and 100 µM EGTA to yield a protein concentration of 20 mg/mL.²⁰

1.3 Standard microinjection protocols can be used, providing that certain precautions are taken (see *Application Notes*). Pressures

used for pressure injection of aequorin are normally between 0.1 and 10 atmospheres; refer to the instrument manual for details. An informative description of microinjection techniques can be found in *Cellular Calcium: A Practical Approach*.²⁷ Amounts to inject will vary depending on the size of the cell and the exact method used. In the example cited in step 1.2, 2.5–5 pL of a 20 mg/mL aequorin solution were injected into a sea urchin oocyte.²⁰ To investigate the effects of anabolic agents in ventricular myocytes, ~0.2 µL of a 30 mg/mL aequorin solution was microinjected per myocyte.²⁸

1.4 For measuring aequorin luminescence, most researchers construct their own photon detection system consisting of an enclosed chamber containing a photomultiplier; the apparatus must totally exclude outside light.²⁷

Application Notes

Bioluminescence Activity

In vertebrate cytoplasm, recombinant aequorin is most responsive to Ca²⁺ concentrations between ~0.5 and 10 µM. Aequorin has a K_d for Ca²⁺ of ~1 µM²⁹ and a quantum yield of ~0.22 at 25°C.⁵ One mg of pure aequorin emits approximately 4 × 10¹⁵ photons at 25°C⁵ and 10⁻¹⁸ moles of the photoprotein have been detected over background.⁶

Effects of Temperature

Almost all aequorin measurements are done at room temperature because the calcium-independent luminescence background is 10-fold lower at 20°C than that at 37°C.¹² Although apoaequorin is stable at temperatures up to 80°C, the activity of the aequorin complex is dramatically reduced at temperatures above 40°C.¹¹

Other Variables Affecting Aequorin Response

Aequorin is essentially insensitive to changes in pH over the normal physiological range (pH 6.0–8.5). Increasing the concentration of EDTA, EGTA, Mg²⁺ or ionic strength will slow the rate of photon emission, as will decreasing temperature.³⁰ Any Ag⁺ in aequorin solutions will cause a substantial increase in background bioluminescence.³¹ Both Ag⁺ and Hg²⁺ will catalytically destroy aequorin.²⁷ Other ions to avoid include Pb²⁺, Co²⁺, Cu²⁺, Cd²⁺, Sr²⁺ and lanthanides, all of which give rise to significant calcium-independent luminescence. The isoelectric point of aequorin is 4.7.³²

Calibration

Aequorin's response to changes in Ca²⁺ concentration is sigmoidal and, as discussed above, somewhat environment-sensitive. Two calibration methods are commonly employed: the null point method and the use of a standard curve. In the null point method, a small amount of the photoprotein is microinjected and then known amounts of free Ca²⁺ are added until no further change in luminescence is observed. The drawbacks of this approach are that the Ca²⁺ solutions must be injected into the same region of the cell and only a single measurement is made. Use of a standard curve requires that the cells be lysed and that the calibration solutions mimic as closely as possible the intracellular solution under study.³³ A detailed review of techniques for calibrating aequorin measurements can be found in Chapter 33 of *The Heart and Cardiovascular System*.³⁴

Coelenterazine

AquaLite recombinant aequorin is fully “charged” with coelenterazine. However, as coelenterazine is oxidized over time to coelenteramide in the presence of calcium, it may be necessary to replenish it, thereby regenerating the calcium-responsive aequorin complex. This will only be required during extremely

long-term experiments, such as those lasting several hours or longer. Since coelenterazine is cell-permeant,³⁵⁻³⁹ it can be added directly to the extracellular solution. Molecular Probes provides ultrapure coelenterazine (C-2944), as well as several coelenterazine analogs.

References

1. Proc Natl Acad Sci USA 74, 623 (1977);
2. Nature 312, 444 (1984);
3. FEBS Letters 211, 44 (1987);
4. Am J Physiol 251, C512 (1986);
5. Symp Soc Exp Biol 30, 41 (1976);
6. W. Ward in *Chemi- and Bioluminescence*, Dekker, New York (1985);
7. Biochemistry 11, 1602 (1972);
8. Biochem J 270, 309 (1990);
9. J Gen Physiol 85, 189 (1985);
10. Proc Natl Acad Sci USA 82, 3154 (1985);
11. Biochemistry 25, 8425 (1986);
12. Am J Physiol 247, C396 (1984);
13. Am J Physiol 246, E198 (1984);
14. Am J Physiol 253, C817 (1987);
15. Cell Calcium 11, 57 (1990);
16. Cell Calcium 12, 635 (1991);
17. Biochem Biophys Res Commun 177, 888 (1991);
18. Neurosci Lett 127, 28 (1991);
19. J Cell Biol 100, 1522 (1985);
20. J Cell Biol 103, 2333 (1986);
21. J Cell Biol 96, 750 (1983);
22. J Cell Biol 101, 1245 (1985);
23. J Cell Biol 105, 1613 (1987);
24. J Cell Biol 106, 1229 (1988);
25. J Cell Biol 99, 2175 (1984);
26. Proc Natl Acad Sci USA 73, 366 (1976);
27. P.H. Cobbold and J.A.C. Lee in *Cellular Calcium: A Practical Approach*, J.G. McCormack and P.H. Cobbold, Eds., IRL Press at Oxford Press, New York (1991);
28. Nature 312, 444 (1984);
29. Biochem Biophys Res Commun 126, 1259 (1985);
30. Annu Rev Biophys Bioeng 12, 91 (1983);
31. Methods Enzymol 57, 292 (1978);
32. J Biochem 105, 473 (1989);
33. J Cell Biol 103, 2333 (1986);
34. J. Blinks in *The Heart and Cardiovascular System*, H.A. Fozzard, Ed., Raven Press, New York (1986);
35. Nature 352, 524 (1991);
36. Biochem Biophys Res Commun 174, 115 (1991);
37. FEBS Lett 282, 405 (1991);
38. Proc Natl Acad Sci USA 88, 6878 (1991);
39. Anal Biochem 209, 343 (1993).

Product List

Current prices may be obtained from our Web site or from our Customer Service Department.

| Cat # | Product Name | Unit Size |
|--------|---|-----------|
| A-6785 | AquaLite® aequorin (aequorin) *recombinant* | 25 µg |

Contact Information

Further information on Molecular Probes' products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Leiden, the Netherlands. All others should contact our Technical Assistance Department in Eugene, Oregon.

Please visit our Web site — www.probes.com — for the most up-to-date information

Molecular Probes, Inc.

PO Box 22010, Eugene, OR 97402-0469
Phone: (541) 465-8300 • Fax: (541) 344-6504

Customer Service: 7:00 am to 5:00 pm (Pacific Time)
Phone: (541) 465-8338 • Fax: (541) 344-6504 • order@probes.com

Toll-Free Ordering for USA and Canada:
Order Phone: (800) 438-2209 • Order Fax: (800) 438-0228

Technical Assistance: 8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 465-8353 • Fax: (541) 465-4593 • tech@probes.com

Molecular Probes Europe BV

PoortGebouw, Rijnsburgerweg 10
2333 AA Leiden, The Netherlands
Phone: +31-71-5233378 • Fax: +31-71-5233419

Customer Service: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5236850 • Fax: +31-71-5233419
eurorder@probes.nl

Technical Assistance: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5233431 • Fax: +31-71-5241883
eurotech@probes.nl

Molecular Probes' products are high-quality reagents and materials intended for research purposes only. These products must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Please read the Material Safety Data Sheet provided for each product; other regulatory considerations may apply.

Several of Molecular Probes' products and product applications are covered by U.S. and foreign patents and patents pending. Our products are not available for resale or other commercial uses without a specific agreement from Molecular Probes, Inc. We welcome inquiries about licensing the use of our dyes, trademarks or technologies. Please submit inquiries by e-mail to busdev@probes.com. All names containing the designation ® are registered with the U.S. Patent and Trademark Office.

Copyright 2001, Molecular Probes, Inc. All rights reserved. This information is subject to change without notice.