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BIOLUMINESCENT DETERMINATION OF ATP WITH LUCIFERASE-LUCIFERIN

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INTRODUCTION AND PRINCIPLE

In 1947 McElroy[1] isolated and purified the heat-stable luciferin and the labile enzyme "luciferase" that were responsible for light production by firefly tails, and showed that adenosine triphosphate (ATP) was also required for the process. The overall reaction proceeds as follows:

$$\text{ATP} + \text{Luciferin} + O_2 \xrightarrow[\text{Magnesium Ions}]{\text{Firefly Luciferase}} \text{Oxyluciferin}$$

$$+ \text{Adenosine 5'-Monophosphate (AMP)} + CO_2 + \text{Pyrophosphate} + \text{Light}$$

The availability of highly sensitive light photomultipliers permits the detection of extremely low concentrations of existing ATP, or ATP as it is being formed in kinetic systems. The assay has been used to determine biomass because of an approximately equal distribution of ATP in all living matter. It is also used as an indicator for coupled reactions which produce ATP as an end product.

Due to the unique sensitivity of the luciferase reaction, great care must be exercised to avoid microbial (biomass) or other trace ATP contamination. The rapid flare and decay of the light flash requires consistent timing of the light measurement to obtain dependable quantitation.

The intensity and duration of the light emitted are a function of reaction conditions, reagent purity and other factors. Instruments ranging from a simple spectrophotometer to a bioluminometer may be employed for light measurement. Sigma offers a number of bioluminescent reagents with various configurations to meet the special needs of the research community.

DURATION OF LIGHT FLASH

Light emission peaks in about one second, and then decays rapidly with a typical half-life of one minute. Arsenate will extend the half-life substantially but with diminished sensitivity for the detection of ATP.

In general, the intensity of light emission may be conveniently recorded after a 20-second time period. If automated sample injection capacity is available, a three-second time period may be appropriate.

It is acknowledged that without the active participation of thousands of children who collect millions of fireflies each year, much research which takes advantage of the remarkable firefly phenomenon would not be possible.

EMISSION SPECTRA

Peak wavelength emission is reported at 560 nm when the reaction is conducted at pH 7.8.[2] Lower pH (i.e., 6.0), will cause a shift in maximum intensity to 620 nm.

INTERFERENCES

Contamination from any bio-source (fingerprints, bacteria, etc.) must be avoided.

Luciferase reagents should be protected from light.

Optimum temperature for the luciferase-luciferin reaction is 28°C. The reaction slows perceptibly at higher temperatures.

Vigorous agitation is to be avoided, as it may denature the luciferase enzyme.

Arsenate is inhibitory, but is sometimes used to prolong the light emission period although at the expense of sensitivity. A concentration of 0.02 M is suggested if arsenate is used for this purpose.

INSTRUMENTS

For greater accuracy, convenience and sensitivity, a bioluminometer is recommended. There are several firms that offer instruments of this type, such as Berthold Analytical Instruments, Inc., M&M Instruments, Packard, and SLM Instruments, Inc. Instruments other than bioluminometers which have been used for bioluminescence measurement include scintillation counters, fluorometers and spectrophotometers.

PREPARATION OF ATP STANDARD SOLUTIONS

Sigma offers ATP Standard, Catalog No. FL-AAS, which contains 2 μmol ATP.

An ATP Standard Stock Solution may be prepared by reconstituting one vial with 2.0 mL distilled water, free of microbial contamination, to yield a 1 millimolar (1×10^{-3} M) solution. The solution is stable when stored frozen (−0°C).

ATP Standard Solutions may be prepared by making serial dilutions of an aliquot of the ATP Standard Stock Solution with distilled water. The extent of the dilutions depends upon the assay sensitivity desired. The dilutions are stable up to 8 hours when stored on ice.

SAMPLE PREPARATION

ATP can be extracted from most biological samples by suspending the sample in buffer (0.02 M glycine, 0.05 M Mg^{2+}, 0.004 M EDTA, pH 7.4 at 25°C) and heating for 45 seconds in a boiling water bath (100°C). Alternatively ATP can be extracted by treating the sample with perchloric acid. The perchlorate supernatant must be neutralized prior to assay. ATP can also be extracted from mammalian cells using 0.05-0.1% Triton X-100.

Detergent extraction of ATP from bacterial cells requires a 0.02% quaternary ammonium salt such as benzalkonium chloride. (The nonionic detergent, Triton X-100, can dismember mammalian cells; however, it may not suitably dismember bacterial cell walls.) Various agents which have been reported to be useful for extraction of bacterial ATP include Tris-EDTA, trichloroacetic acid, perchloric acid, ethanol, butanol, chloroform and DMSO.[3-6] Each carries advantages and disadvantages for the ATP extraction process and subsequent bioluminescent assay.

Lundin and Thore[3] concluded that the choice of extraction technique is governed by the purpose of the study and influenced by practical considerations. The investigator may find it beneficial to empirically determine the most appropriate extraction technique for the assay sensitivity desired.

DETERMINATION OF ATP USING FIREFLY LANTERN EXTRACTS

Firefly Lantern Extracts contain voluminous amounts of luciferase but less than optimal amounts of luciferin. Without fortification, the assay is capable of detecting approximately 10^{-6} to 10^{-11} moles ATP under the described assay conditions. The detection limit may be extended to 10^{-15} moles ATP with the addition of luciferin at a concentration of approximately 1 millimolar.

Arsenate, an endogenous component of Firefly Lantern Extracts, is particularly inhibitory to the bioluminescent reaction. Sensitivity is increased when arsenate is removed.

PROCEDURE I:

1. Prepare an appropriate 10 mg/mL solution of Firefly Lantern Extract in distilled water (supermarket distilled water is satisfactory).
2. Zero the instrument employed.
3. Pipet 100 μL Firefly Lantern Extract Solution prepared in Step 1 into an appropriate tube or cuvet.
4. Place tube in instrument and record the intensity of the background light emitted.
5. Pipet into the tube 20 μL of the sample. Mix gently. Immediately start timer.
6. After 20 seconds measure and record the intensity of the emitted light.
7. Subtract the background light in Step 4 from the emitted light in Step 6 to obtain a corrected value.
8. Repeat Steps 3-7 substituting a solution containing a known amount of ATP (ATP Standard Solution) in place of the sample.
9. Calculate the ATP concentration of the sample by comparing the instrument reading for the sample with that of the ATP Standard. (A 3 point ATP standard curve will generally suffice.)

REAGENT STABILITY:

Firefly Lantern Extract Solutions are usually stable for approximately 2 weeks when stored refrigerated (0-5°C).

DETERMINATION OF ATP USING PURIFIED COMBINED LUCIFERASE-LUCIFERIN PREPARATIONS

These preparations consist of purified luciferase with optimum concentrations of luciferin. In addition, they are free of arsenate and contaminating enzymes (e.g., myokinase). Contaminating enzymes usually present in crude firefly lantern extracts may produce undesirable side reactions.

Buffering is maintained at pH 7.8 for optimal sensitivity. However, any pH between 7.4-7.8 is suitable. Magnesium concentration must be kept between 10^{-3} to 10^{-2} molar. Thiol compounds and traces of EDTA also improve performance. Purified luciferase preparations require the presence of a stabilizing protein such as human albumin.

Sensitivity to 10^{-15} moles ATP may be achieved with the use of purified luciferase-luciferin preparations. Other advantages in using purified luciferase-luciferin preparations, as compared to the use of crude preparations, include greater specificity for ATP and longer light flash duration.

PROCEDURE II:

1. For maximum sensitivity, dissolve luciferase-luciferin at a concentration of 20-40 mg/mL in distilled water. Lower concentrations may be used if less sensitivity is acceptable.
2. Follow Steps 2-9 under "Procedure I" using Firefly Lantern Extracts.

REAGENT STABILITY:

Solutions of purified luciferase-luciferin may be stored frozen. Successive freeze-thaws may result in some reduced sensitivity.

FIREFLY LANTERN EXTRACTS

A. Essentially Arsenate Free

Product Number	Description	Package Sizes
F 3641	FIREFLY LANTERN EXTRACT Glycine buffered with added luciferin.	5 mg 15 mg 50 mg
F 5630	FIREFLY LANTERN EXTRACT Glycine buffered with added luciferin and mannitol.	5 mg
F 5505	FIREFLY LANTERN EXTRACT Glycine buffered with added luciferin and human albumin.	5 mg
F 4005	FIREFLY LANTERN EXTRACT Glycine buffered, luciferin free.	2 mg 5 mg 15 mg 50 mg
F 4255	FIREFLY LANTERN EXTRACT MOPS buffered, essentially luciferin free	5 mg 15 mg 50 mg
B. Containing Arsenate		
FLE-50	FIREFLY LANTERN EXTRACT A crude luciferin and luciferase. Each vial contains soluble extract from 50 mg of dried lanterns. After reconstituting with 5.0 mL H_2O, each vial will contain 0.05 M potassium arsenate and 0.02 M $MgSO_4$ at pH 7.4.	1 vial 5 vials 15 vials

FLE-250	FIREFLY LANTERN EXTRACT A crude luciferin and luciferase. Each vial contains soluble extract from 250 mg of dried lanterns. After reconstituting with 25 mL H_2O, each vial will contain 0.05 M potassium arsenate and 0.02 M $MgSO_4$ at pH 7.4.	1 vial
FLE-1000	FIREFLY LANTERN EXTRACT A crude luciferin and luciferase. Each vial contains soluble extract from 1000 mg of dried lanterns. After reconstitution with 100 mL H_2O, each vial will contain 0.05 M potassium arsenate and 0.02 M $MgSO_4$ at pH 7.4.	1 vial

COMBINED LUCIFERASE-LUCIFERIN

These preparations are suitable for the determination of ATP generated in coupled enzyme systems or in cell extracts. Products L 0633 and L 1761 are suitable for determination of ATP released from blood platelets in platelet aggregation studies.

In addition to luciferase and luciferin, these products also contain $MgSO_4$, EDTA, buffer salts and albumin. They are essentially myokinase-free. The 100 mg package size is sufficient for 50 cell extract assays or for 50 platelet assays. 100 μL of reagent (20 mg/mL) will detect 3 x 10^{-15} moles ATP.

Product No.	Desctiption	Sizes
L 0633	LUCIFERASE-LUCIFERIN In glycine buffer.	20 mg 50 mg 100 mg
L 1761	LUCIFERASE-LUCIFERIN In Tris-aspartate buffer.	20 mg 50 mg 100 mg
L 9134	LUCIFERASE-LUCIFERIN In Tris-succinate buffer.	20 mg 50 mg 100 mg

Please also note that individual luciferase and luciferin preparations are available. Refer to Sigma Catalog under Luciferase and Luciferin.

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