

ATP Bioluminescence Assay Kit CLS II

Reagent set for the quantitative detection of ATP by luciferase driven bioluminescence

Cat. No. 1 699 695

1600 MTP-assays

800 tube-assays

Version 3, July 1999

Store at -15 to -25°C

1. Introduction

The determination of ATP using bioluminescence is a well established technique. It uses the ATP dependency of the light emitting luciferase catalyzed oxidation of luciferin for the measurement of extremely low concentrations of ATP (1). The ATP Bioluminescence Assay Kit CLS II is especially optimized for the easy use in tube-luminometers as well as in MTP-format luminometers. The kit exhibits a constant light signal sustaining for several minutes. The kit is especially suited for kinetic studies and ATP determinations in coupled enzymatic reactions. The preparation of an ATP calibration curve is facilitated using the stabilized ATP standard stock provided. The exact ATP content of the standard as indicated on the label, is determined individually for each lot by HPLC and spectroscopy.

2. Kit features

2.1 Kit contents

1. 8×10 ml luciferase reagent, lyophilized (white screw-cap)
2. $4 \times$ ATP standard, approx. 10 mg, lyophilized (red screw-cap)

2.2 Stability and storage conditions

Store kit at -15 to -25°C . All components are stable until expiry date (see lot-specific label imprint).

2.3 Working range and detection limit

The working range of the ATP Bioluminescence Assay Kit CLS II is between 10^{-6} and 10^{-11} M ATP (see fig. 1). The detection limit for ATP, using a Berthold-type microplate luminometer LB-96-P, is in the range of 10^{-11} M (10^{-15} moles) (see fig. 1).

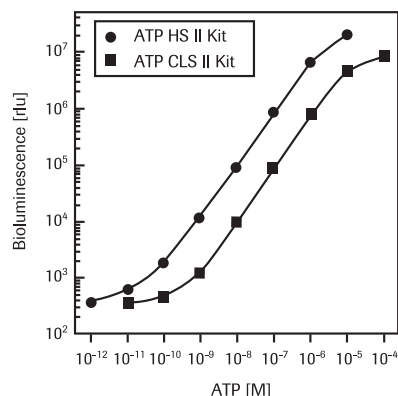


Figure 1: Sensitivity range of the ATP Bioluminescence Assay Kit HS II and the ATP Bioluminescence Assay Kit CLS II. ATP dilutions in a volume of $50 \mu\text{l}$ were assayed with $50 \mu\text{l}$ luciferase reagent in a black microtiter plate on a Berthold LB 96 P luminometer. The light signal was integrated for 10 s after a delay of 1 s.

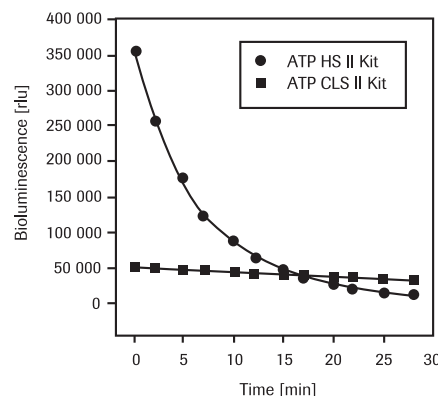


Figure 2: Kinetics of light generation of the ATP Bioluminescence Assay Kit HS II and the ATP Bioluminescence Assay Kit CLS II. 10 pmol ATP in a volume of $50 \mu\text{l}$ was assayed with $50 \mu\text{l}$ luciferase reagent in a black

2.4 Limitations of the kit

The ATP Bioluminescence Assay Kit CLS II is especially developed for applications, where constant light signals are required, e.g. for kinetic studies of enzymes, for metabolic studies or if coupled enzymatic assays are applied. If ATP-determinations are started manually, the CLS-kit provides high reproducibility due to the constant signal generation (see sections 4.1, 4.2 and 4.3). However, the sensitivity of the kit is by a factor of 10 lower as compared with the ATP Bioluminescence Assay Kit HS II (fig.1), which is especially recommended for determinations in the high sensitivity range. The ATP Bioluminescence Assay Kit HS II contains also an efficient cell lysis reagent and can be used for the detection of ATP in microorganisms or animal cells.

3. Standard protocol

3.1 Preparation of working solutions and stability

3.1.1 Luciferase reagent
Dissolve the whole content of one bottle 1 by carefully adding 10 ml of redist. water. Incubate for 5 min at 0–4° C without stirring or shaking. Mix for a homogeneous solution by carefully rotating the bottle. Do not shake. The reagent is stable for one day at 15–25° C or for one week when stored at 0–4° C. However, set up a standard curve each day, because a slightly loss of light activity occurs during this time (approx. 20% after 5 days). Reconstituted luciferase reagent may be stored frozen at –15 to –25° C for longer periods of time. Each freeze/thaw-cycle reduces the luciferase activity to a certain degree, depending on the freezing conditions (shock freezing is most considerate). Therefore, avoid repeated freezing and thawing.

3.1.2 ATP standard
Each bottle contains approx. 10 mg ATP (>98% purity; Mr 605.2). The exact amount of ATP is determined individually for each lot as indicated on the label. Dissolve the content of one bottle 2 by addition of the appropriate volume of redist. water to get a final concentration of 10 mg/ml or 16.5 mM, respectively (e.g. 960 µl to 9.60 mg ATP). The ATP standard solution is stable for one week when stored at 2–8°C (< 5% degradation). When stored at –15 to –25° C, the solution is stable for at least 4 weeks (< 5% degradation). The ATP standard curve is prepared by serial dilutions of one ATP standard with redist. water. Diluted ATP-standards are stable for 8 hours when stored on ice.

3.2 Working procedure
The standard protocols as described are general guidelines and first choice protocols but are open to variations upon special needs. A volume ratio of 50% reagent and 50% sample is optimal. When changing the assay volume it is important that the concentration of the reagent in the assay remains the same.

3.2.1 Determination of free ATP

Step	Procedure/parameter	MTP-format volumes	tube-format volumes
1a	If necessary, dilute samples with redist. water or buffer to an appropriate ATP concentration. The optimal detection range is between 10 ⁻⁷ to 10 ⁻¹⁰ M. The pH of the sample should be in the range of 7.6 to 8.0 (see section 4.3).	50 µl sample	100 µl sample
1b	Dilute ATP standard with redist. water by serial dilution in the range of 10 ⁻⁵ to 10 ⁻¹⁰ M.	50 µl sample	100 µl standard
2	Add luciferase reagent to the samples/standards by automated injection and start measurement after a 1 s delay and integrate for 1 to 10 s.	50 µl reagent	100 µl reagent
3	Subtract the blank (no ATP) from the raw data and calculate ATP concentrations from a log-log plot of the standard curve data.		

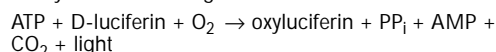
3.2.2 Determination of ATP from biological material (2,3)

Step	Procedure/parameter	required volumes for MTP-format	required volumes for tube-format
1a	<ul style="list-style-type: none"> Dilute cells to a concentration of 10⁹ to 10⁸ cells/ml. If the sample is too dilute, concentrate by centrifugation (bacteria and yeast: 8000 × g, 10 min; somatic cells: 900 × g, 10 min) and discard the supernatant carefully. Resuspend pellet in a minimal volume of dilution buffer (50–100 µl). Concentration from large volumes can be performed by filtration through a 0.2 µm filter. Collect cells in a minimum volume. 	25 µl (minimum sample volume)	50 µl (minimum sample volume)
1b	Dilute ATP standard with redist. water by serial dilution in the range of 10 ⁻⁵ to 10 ⁻¹⁰ M.	25 µl (minimum sample volume)	50 µl (minimum sample volume)
2	Add to the cell suspension 9 volumes of boiling 100 mM Tris, 4 mM EDTA, pH 7.75. Incubate for another 2 min at 100°C. Centrifuge sample at 1000 × g for 60 s and transfer the supernatant to a fresh tube. Keep samples on ice until measurement.		
3	Transfer the appropriate volume of sample/standard prepared in step 2 into a MTP-well or tube.	50 µl	100 µl
4	Add luciferase reagent to the samples/standards by automated injection or by hand, start measurement and integrate for 1 to 10 s.	50 µl reagent	100 µl reagent
5	Subtract the blank (no ATP or no cells) from the raw data and calculate ATP concentrations from a log-log plot of the standard curve data.		

4. Additional informations

4.1 Test principle

The luciferase from *Photinus pyralis* (American firefly) catalyses the following reaction:



The quantum yield for this reaction is about 90%. The resulting green light has an emission maximum at 562 nm. The Michaelis equation has the following form:

$$\text{light intensity} = (V_{\text{max}} \times C_{\text{ATP}}) / (K_m + C_{\text{ATP}})$$

At low ATP concentrations ($C_{\text{ATP}} \ll K_m$) the formula simplifies to

$$\text{light intensity} = V_{\text{max}} \times C_{\text{ATP}} / K_m$$

From this equation it becomes obvious that the light output is directly proportional to the ATP concentration (C_{ATP}) and dependent on the amount of luciferase (V_{max}) present in the assay.

4.2 Kinetic of the light reaction (constant light signal)

Due to the relatively low luciferase activity present in the assay mix, the ATP concentration can be considered to be constant with time: The reaction produces, for example, at an ATP concentration of 10^{-9} M, a flow of photons of 5×10^7 photons \times ml $^{-1}$ \times s $^{-1}$. Assuming a quantum yield of 100%, this results in a reduction of ATP concentration of 0.5% per min. I. e. under optimum experimental conditions, one obtains an almost constant light signal at a defined ATP concentration (figure 1) (4). Therefore, when performing the assay manually, there is sufficient time for addition of the reagent, placing the sample into the luminometer and starting the measurement.

4.3 pH of the sample

The luciferase reaction has its pH-optimum in the range of 7.75. Samples having extreme pH-values or samples of a different pH containing high buffer concentrations may influence the light reaction in an unpredictable way. To avoid problems, correct the pH to a value between 7.6 and 8.0 or dilute samples in tricine buffer of the appropriate pH. The buffer concentration in the luciferase reagent is 40 mM.

4.4 Equipment

The reagent set can be used with all commercially available luminometers (tube-format or MTP-format). For microplate luminometers use white or black microtiter plates. Automated injection systems provide the option to add reagents directly in measurement position and to start light signal integration after a constant, selectable delay (5). This opens the possibility to process even a large number of samples in a convenient and highly reproducible way.

5. Trouble shooting

Problem	Possible reason	Measure
No signal or signal too weak	ATP content in the sample is very low.	Use ATP Bioluminescence Assay Kit HS II (Cat. No 1 699 709) for detection of ATP with highest sensitivity
	Sample is too diluted.	Minimize volume prior to cell disruption e.g. by filtration or centrifugation
	ATP is destroyed by endogenous ATPases.	Minimize time between cell disintegration and inactivation (if a method other than boiling has to be used). Keep samples at 0–4°C. Test on ATPases by adding exogenous ATP to the sample.
	Excess of chelating agents in the sample capturing Mg ²⁺ -ions necessary for luciferase-activity.	Add an appropriate concentration of Mg ²⁺ sufficient to compensate chelating agents. The luciferase reagent contains 20 mM Mg ²⁺

Problem	Possible reason	Measure
	Luciferase reagent destroyed.	Dissolve a new vial of luciferase reagent. Do not stir or shake during dissolution. Store maximally for 5 days at 2–8°C, once dissolved. Avoid repeated freezing/thawing.
	pH of sample is out of optimal range.	Adapt sample pH to 7.6–8.0.
	Measuring time too short	Prolong the integration time with your luminometer.
	Use of (wrong) filter in the luminometer.	Do not use any filters during measurement.
Non-linear calibration curve	Pipetting error	Pipette the serial dilutions carefully. Check precision of pipettes. Prepare the dilutions in triplicate.
	Cross talk between individual wells of the MTP	Use only white or preferably black MTP; do not use transparent MTP.
	Measuring time for the total MTP is too long: substrate is depleted in lately measured standards.	Ensure that the time between addition of the luciferase reagent and measuring is constant for all samples (maximum 5 min)
Too high background	ATP contaminations in water, buffers, vials used	Use only redist. water for the preparations of the reagents and buffers used for cell harvesting and sample preparation. Check labware for ATP contaminations.
	Measuring time too long.	Decrease the integration time. Minimize volume prior to cell disruption e.g. by filtration or centrifugation.
Strong variations of ATP content between several identical experiments.	Cell are harvested using too harsh conditions	Avoid high centrifugation speeds (for bacteria and yeast maximally 8000 \times g, for somatic cells maximally 900 \times g). Reduce volume flow upon filtration of cells.
	No time-standardized cell harvesting conditions	Standardize experimental time for all harvesting steps.
	Cross talk between individual wells of the MTP.	Use only white or preferably black MTP; do not use transparent MTP's.

6. Related products*

Product	Pack size	Cat. No
ATP Bioluminescence Assay Kit HS II	1000 MTP-assays 500 tube-assays	1 699 709
Luciferase from photinus pyralis (American firefly)	1 mg protein 5 mg protein	411 523 634 409
D(-)-Luciferin synthetic, crystallized	10 mg 50 mg	411 400 1 626 353
ATP, disodium salt, crystallized, < 98%	1 g 5 g 10 g 50 g	126 888 127 523 127 531 635 316
ATP, disodium salt, crystallized, 100%	1 g 5 g	519 979 519 987

* This combination comprises only the most related products. Please refer to our latest catalogue for our current product range or contact your local Roche Molecular Biochemicals representative directly.

References

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