BioThema
LUMINESCENT ASSAYS

BioThema AB
S-136 40 Handen
Sweden
VAT No. SE556303345401

Phone **+46 (0)8 7770020**
Fax **+46 (0)8 7770021**
E-mail: **info@biothema.com**
Website: **www.biothema.com**

Microbial ATP Kit Prod. No. 266-112

Instructions for use

Intended use

The kit is intended for determination of intracellular ATP (adenosine triphosphate) in microbial cells. Most types of somatic cells, e.g. blood cells are lysed with a neutral detergent. The released ATP and other forms of extracellular ATP in the samples is enzymatically degraded. If you are not certain that the Microbial ATP Kit HS is the right one for your application, please check the Kit Selection Guide at www.biothema.com. All living cells contain ATP where it plays the role of energy currency between different cellular processes. When cells die of natural causes, ATP is normally degraded. The intracellular concentration of ATP is carefully regulated to similar levels in all types of cells. ATP is therefore a good estimate of the total intracellular volume. Most bacterial cells contain approx. $2x10^{-18}$ mol ATP per cell, while most eukaryotic cells, as a result of their larger size, contain 10^{-15} mol ATP or more.
The luciferase activity in ATP Reagent HS is so high that if contaminated during reconstitution it will degrade the ATP background by one order of magnitude every 38 min. After mixing sample and ATP Reagent HS the light intensity decays by approx. 6% per min. This is slow enough to allow manual mixing and a tube luminometer without reagent dispenser can be used. The detection limit of the reagent is $10x10^{-18}$ mol ATP with a sensitive luminometer. This corresponds to 5 bacterial cells. We must here differentiate between bacterial cells and CFU, since one CFU may consist of several cells. If the detection limit is not adequate, it is possible to concentrate the sample by microfiltration, a procedure that will also remove most of the extracellular ATP.

Assay principles

Before the assay extracellular ATP and ATP in somatic cells is enzymatically degraded. Subsequently intracellular ATP is released from the cell using Extractant B/S also inactivating the ATP Eliminating Reagent. Finally, ATP is assayed using ATP reagent HS.

$$\text{ATP + D-luciferin + } O_2 \xrightarrow{\text{luciferase}} \text{AMP+ PPi + oxyluciferin + } CO_2 \text{ + light}$$

The intensity of the light is proportional to the amount of ATP and is measured in a luminometer. The light emission is measured before and after addition of a known amount of ATP standard. This makes it possible to calculate the amount of ATP in unknown samples expressing the result in pmol (10^{-12} mol).

Instruments

Any tube or microplate luminometer. It should be noted that tube luminometers are about one order of magnitude more sensitive. If a microplate luminometer is used it should have minimum two dispensers and should be able to shake the microplate.

Kit contents

The kit contains reagents for 150-375 assays (each vial of ATP Reagent HS is enough for 25 assays in a tube luminometer and 62 assays in a microplate luminometer).

1. ATP Reagent HS (6 vials). Lyophilised reagent containing luciferase and luciferin.
2. Diluent B 10 mL (6 vials). Buffer used to reconstitute ATP Reagent HS.
3. Extractant B/S 10 mL (1 vial).
4. ATP standard 5 mL (1 vial). Contains 100 nmol/L ATP.
5. ATP Eliminating Reagent (1 vial)
6. Cell Lysing Reagent 10 mL (1 vial). Used to reconstitute ATP Eliminating Reagent.

Reagent Reconstitution

Extractant B/S and ATP Standard are ready to use. Remove and discard screw lid and rubber stopper from ATP Reagent HS. This can be done by unscrewing the lid and use it to remove the stopper. Remove screw lid from vial containing Diluent B. The content of Diluent B is poured into the ATP Reagent HS. Use the lid from Diluent B to close the ATP Reagent HS vial. Store the reagent in the refrigerator covered from light. The activity may go down to half the initial activity after one week. However, correct ATP results will be obtained, if assays are calibrated with internal ATP standard. ATP Eliminating Reagent is reconstituted by pouring the entire contents of Cell Lysing Reagent into the vial. Use the same procedure with lids and stopper as with ATP Reagent HS. The reconstituted ATP Eliminating Reagent can be stored in the refrigerator up to a week or in the freezer for longer times.

Applications
The sensitivity of this kit allows determination of low ATP levels in a variety of circumstances:

1. Bacteriological control of liquids (e. g. drinking water, process water)
2. Bacterial adhesion to surfaces
3. Quality assurance of laundry
4. Bacterial growth.
5. Antibiotic effects on bacteria

Assay procedure
Assay of intracellular ATP with tube luminometer:

1 Mix 50 µL sample and 50 µL ATP Eliminating Reagent in a cuvette. Incubate at room temperature for 10 min.
2 Add 50 µL Extractant B/S directly into the solution in the cuvette. Subsequently make sure that there are all parts of the ATP Eliminating Reagent has come in contact with Extractant B/S. This can be achieved with a Vortex shaker swirling the solution up on the walls of the cuvette.
3 Add 0.4 mL ATP Reagent HS and measure light emission I_{smp}.
4 Add 10 µL 100 nmol/L ATP Standard, i.e. 1 pmol ATP, and measure light emission, $I_{smp+std}$.

Assay of intracellular ATP with microplate luminometer:
The detection limit with microplate luminometers is about one order of magnitude higher than what can be achieved with tube luminometers. The lower sensitivity with microplate luminometers comes largely from the lower assay volume (less light is created). The procedure with a microplate luminometer with 3 reagent dispensers would be as follows:

1 Add 20 µL ATP Eliminating Reagent to each well.
2 Add 20 µL sample to each well and incubate 10 min with occasional shaking.
3 Inject 20 µL Extractant B/S with dispenser 1 followed by mixing.
4 Inject 160 µL reconstituted ATP Reagent HS with dispenser 2 and measure light emission, I_{smp1}.
5 Measure light emission, I_{smp2}, and immediately inject 10 µL 100 nmol/L ATP Standard, i.e. 1 pmol ATP, with dispenser 3 to all wells.
6 Shake plate and measure light emission, $I_{smp+std}$.

Each step should be performed for all samples in the plate before starting the next step. This is important to avoid too much cross-talk from the addition of ATP Standard. With some luminometers it is not possible to perform step 5. In this case contact BioThema for further advice. The light emission read in step 6 can be corrected for the 6 % decay per minute by dividing the value by $e^{-0.06xt}$ where t is the time between adding the ATP standard and the light being read. If only 2 dispensers are available Extractant B/S may be added manually.

Calculations:
Calculate amount of ATP (pmol) in the cuvette by the equations:
Tube luminometer: $ATP_{smp} = I_{smp} / (I_{smp + std} - I_{smp})$
Microplate luminometer: $ATP_{smp} = I_{smp1} / (I_{smp + std} - I_{smp2})$

Procedural notes

1 Cuvettes, microplates and pipette tips must be ATP free. Use disposable plastics stored well covered only. Autoclaving is a way to release ATP from cells but not to remove it. Use powder-free gloves for your hands and handle e.g. cuvettes with a clean pair of pincers.
2 Some plastics show a delayed fluorescence after exposure to light and luciferin is rapidly destroyed in bright light. Avoid bright light in the laboratory, while working with ATP assays.
3 Run blanks containing the medium in which the bacteria are suspended.
4 Always perform at least triplicate assays, since ATP contamination in cuvettes, microplates or on pipette tips may jeopardise the results.
5 Volumes of sample, ATP Eliminating Reagent and Extractant B/S should be the same.
6 The ratio between volumes of Extractant B/S and ATP Reagent HS should never exceed 1/4 (it may however be lower).
7 The volume of ATP Standard should always be 10 µL.
8 The higher the total assay volume the higher the sensitivity of the assay and the more accurate the results.
9 The ($I_{smp+std}$ - I_{smp}) value should be at least 10x higher than the I_{smp} value. If not, the sample should be diluted.
10 If the cells in all samples are suspended in a similar medium and if this medium is not highly inhibitory to the luciferase reaction, it is possible to perform the assay without internal ATP standardisation of every assay, i.e. step 4 (tube luminometer) or 5 (microplate luminometer) in the procedures described above can be omitted. Instead a single sample or the medium is assayed with and without ATP Standard and the ($I_{smp+std}$ - I_{smp}) value is used for all samples.
11 If there is no extracellular ATP in the samples, we recommend 266-311 ATP Biomass Kit HS. This kit is similar but contains neither ATP Eliminating Reagent nor Cell Lysing Reagent.
12 The degradation of extracellular ATP may be inadequate in the following situations: a) the extracellular ATP level is very high, b) the required detection limit for intracellular is extremely low or c) the sample medium is strongly inhibitory to the ATP degrading enzyme system. In these situations, one may either prolong the 10 min incubation or use a more concentrated ATP Eliminating Reagent. Please contact us for further instructions.

References
Lundin, A. (2014) Optimization of the firefly luciferase reaction for analytical purposes. Adv. Biochem. Engin./Biotechnol. 2014; 145:31-62