

APPLICATION NOTE

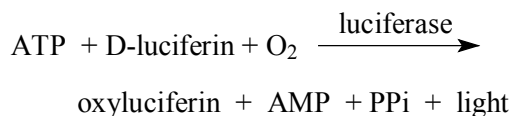
BMG Microplate Luminometer LUMIstar

THE USE OF AN ATP BIOLUMINESCENCE ASSAY TO QUANTIFY CELL CYTOTOXICITY

The quantification of viable cells in culture is a very important problem. However, the technique becomes much more valuable when the effect of growth regulatory substances and also cytotoxic agents are under study. Measurement of viable cell numbers in culture is a critical aspect in the functional in vitro bioassay of a wide range of growth factors. The nucleotide adenosine triphosphate (ATP) plays an important role in energy exchange in biological systems. It serves as the principal immediate donor of energy and is present in all metabolically active cells. ATP has been used as a tool for the functional integrity of living cells since all cells require ATP to remain alive and carry out their specialized function. Most ATP is found within living cells and links catabolic and anabolic processes. Cell injury or oxygen / substrate depletion results in a rapid decrease in cytoplasmic ATP. Measurement of ATP is therefore fundamental to the study of living processes.

A lot of methods have been used for ATP determination, but far the most successful technique is the bioluminescent method, because of its sensitivity and the wide dynamic range. ATP bioluminescence has been used for determining levels of ATP in a number of different cell types.

The reaction is catalyzed by the enzyme luciferase obtained from the firefly (*Photinus pyralis*). The $MgATP^{2-}$ converts the luciferin into a form which is capable of being catalytically oxidized by the luciferase in a high quantum yield chemiluminescent reaction, according to the following equation :



Under optimum conditions, light intensity is lineary related to ATP concentration. Cellular ATP can be measured by direct lysis of the cells with suitable detergent, the released ATP is then free to react with the luciferin-luciferase and leading to light emission at 562 nm. Due to the good sensitivity of the assay, ATP can be measured in concentrations down to 50 attomol.

In this study ATP bioluminescence was used to determine whether there is a linear relationship between the number of HaCaT cells present in the culture and measured luminescence. It was attempted to use ATP-bioluminescence as a measure of proliferation and /or cell cytotoxicity depending of two different substances and avoid the use of radioisotopes. These investigations have been carried out using the keratinocyte cell line HaCaT.

Materials and Methods

Reagents and Instrumentation

HaCaT cells, propagated in 75 square cm tissue culture flasks with weekly passage in DMEM (Seromed, FG 0435, Biochrom KG, Germany) containing 10 % fetal calf serum and 1 % antibiotic /antimycotic solution at 37°C and 6 % CO₂. Cells were seeded in microtitre plates (Greiner 96-well plates) at a density of 5000 cells.

One day after plating in the serum-free medium (Gibco-Defined Keratinocyte-SFM, No. 10785-012), in SFM supplemented hyaluronic acid, hyaluronan or medium was added over a period of 24 h or 48 h to the cells, respectively.

24 h or 48 h later, cell proliferation was measured.

ATP bioluminescence

ATP bioluminescence reagents were supplied by microplate luminometer (BMG, LUMIstar, Germany) and included ATP releasing agent (Somalyze), Tris-acetate buffer, ATP-monitoring reagent (firefly luciferase, D-luciferin) and ATP-standard (EG+G. Wallace, Turku, Finland). Briefly, 100 µL of ATP releasing agent was added to each well of a 96 well microtitre tissue culture plate (Greiner, Germany), containing 100 µL of HaCaT cells, and incubated for 5 minutes at room temperature. Then 180 µL of the cell lysate were added to wells of a 96 well white opaque microtitre plate.

This plate was loaded into the luminometer and 20 µL of the luciferin-luciferase reagent (reconstituted with 10 mL of 0.01 M Tris-acetate buffer, 2 nM EDTA buffer pH 7.75) were added to each well by means of an injection pump, and the samples were measured.

Immediately after addition of luciferin-luciferase luminescence was monitored over a period of 10 s (Fast kinetic method). Light output was given as the integral relative light units (RLUs). ATP measurements were carried out at room temperature.

Results

Cell numbers and the degree of cell proliferation / cytotoxicity were determined by ATP bioluminescence and in comparison to a conventional method, the staining with dye Hoechst 33342.

HaCaT cells were initially used to determine whether there was a correlation between cell numbers and measured ATP. The standard curve of ATP was determined in several groups of experiments (Fig.1) ($r = 0.998$; $p < 0.0001$). From Figure 2 it can be seen that after incubation of increasing cell numbers with ATP releasing agent (100 µL) for 5 min at room temperature an increase of released ATP ($r = 0.99$; $p < 0.0001$) can be observed.

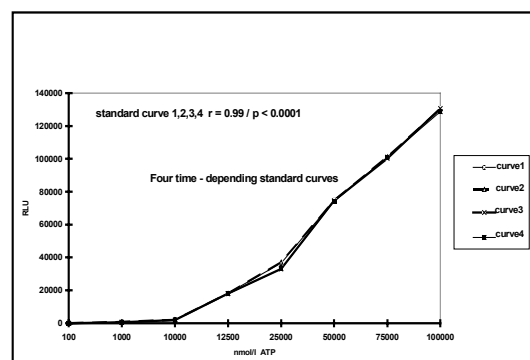


Figure 1 Determination of ATP-standard curves for 24h incubation with several substances

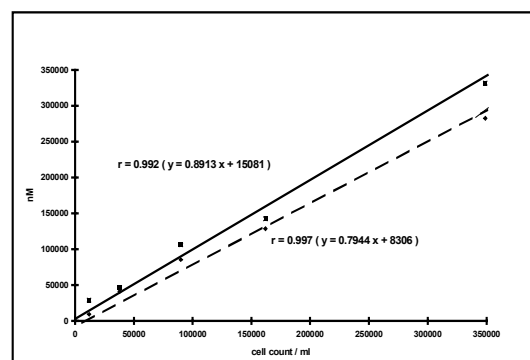


Figure 2 Correlation between cell counts

The correlation was determined from comparison of cell numbers with ATP-bioluminescence readings for each data point and Spearman's rank correlation was determined for the group as whole. Complete medium cultured in the absence of cells served as a control and contained no detectable ATP. Incubation with 100 μ L releasing agent for 5 min at room temperature was optimal for determining the amount of ATP in cultured HaCaT cells. In Figure 3 and 4 the correlation between the cell numbers and the ATP concentrations of several experiments were shown after 24 h and 48 h, respectively.

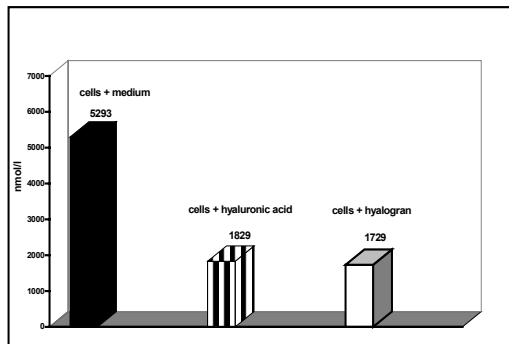


Figure 3 ATP determination in HaCaT cells after 24 h incubation with several substances

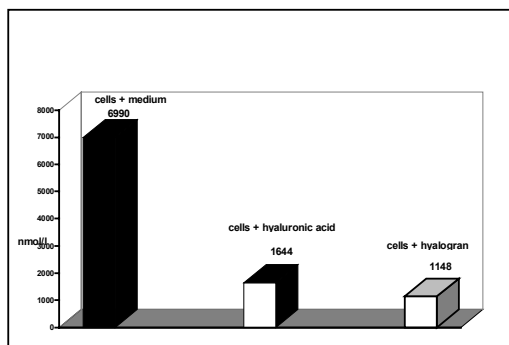


Figure 4 ATP determination in HaCaT cells after 48 h incubation with several substances

and ATP concentrations (standard curve)

The data show that with HaCaT cell line tested there were significant correlation between increased cell number and ATP measured by the luciferin-luciferase reaction. In order to show that ATP is an indicator of proliferation and / or cell cytotoxicity the assay was carried out under addition of two different substances. These substances are hyaluronic acid and hyalogram. Hyaluronic acid (HA) is a major component of extracellular matrix, distributed ubiquitously, with the highest concentration in the soft connective tissue. Especially the influence of hyaluronic acid is in good agreement with the fact that high molecular weight HA in high concentrations inhibits movement, adherence and phagocytosis of several cell types, while low molecular weight HA at low concentrations stimulates cellular migration, proliferation and phagocytosis. HA is a high molecular weight, negatively charged glycosaminoglycan composed of a regular repeating sequence of two non-sulphated monosaccharides, N-acetyl-D-glycosamine and D-glucuronic acid, that interacts with other proteoglycans to provide stability and elasticity to the extracellular matrix. The incubation of HaCaT cells with these two substances leads to the decrease of released ATP.

ATP bioluminescence has been shown by a number of workers to be extremely sensitive to levels of ATP isolated from adipose and muscle tissue as well as platelets and polymorphonuclear leucocytes.

This study confirmed that the concentration of ATP in the HaCaT cell line can be determined by luciferin-luciferase bioluminescence assay, and also that the monitoring of ATP bioluminescence can be used for the measurement of proliferation and / or cytotoxicity.

References

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