ab113849
Luminescent ATP Detection Assay Kit

For the measurement of ATP in live cells.

This product is for research use only and is not intended for diagnostic use.
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1. Overview

Luminescent ATP Detection Assay Kit (ab113849) allows the detection of total levels of cellular ATP (adenosine triphosphate). The ATP assay is based on the production of light caused by the reaction of ATP with added firefly’s luciferase and luciferin. The emitted light is proportional to the ATP concentration inside the cell. The reaction can be summarized as follows:

\[ \text{ATP} + \text{D-Luciferin} + \text{O}_2 \rightarrow \text{Oxyluciferin} + \text{AMP} + \text{PPi} + \text{CO}_2 + \text{Light} \]

This kit irreversibly inactivates ATPases (ATP degrading enzymes) during the lysis step, ensuring that the luminescent signal obtained truly corresponds to the endogenous levels of ATP. The major advantages of this assay are:

- Long luminescence signal: the half-life of the luminescent signal is greater than 5 hours.
- Fast: results are obtained in less than 30 minutes.
- Simple: there are no separation steps and there are only 2 reagent additions.
- Homogeneous: No cell-harvesting or centrifugation required.
- Sensitive: detection limit is 5 cells in 100 µL.
- Wide linear dynamic range: from less than 0.001 μM to 10 μM of ATP (depending on instrument sensitivity and settings)

Total levels of cellular ATP can be used to assess cell viability, cell proliferation and cytotoxicity of a wide range of compounds. However, this is not necessarily representative of only the mitochondrial bioenergetic state of the cell, since cultured cells can survive in the absence of an active electron transport chain by supplying all their ATP demands through glycolysis. When cells are cultured with non-fermentable carbon substrates such as galactose, they require an active electron transport chain (ETC) to supply all their ATP demand. Under these conditions, damage to the mitochondrial bioenergetics state of the cell will lead to depletion of cellular ATP, which can be easily measured by luminescence as a decrease in total levels of ATP measured by this product.
2. Protocol Summary

Grow cells

↓

Treat cells with compounds

↓

Add Detergent and incubate 5 minutes at RT

↓

Add Substrate Solution and incubate 5 minutes at RT

↓

Measure luminescence in a luminometer
3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at 4°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.
5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage temperature (before prep)</th>
<th>Storage temperature (after prep)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detergent</td>
<td>20 mL</td>
<td>4°C</td>
<td>4°C</td>
</tr>
<tr>
<td>Substrate Buffer</td>
<td>20 mL</td>
<td>4°C</td>
<td>4°C</td>
</tr>
<tr>
<td>Substrate (lyophilized)</td>
<td>3 vials</td>
<td>4°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>ATP standard (lyophilized)</td>
<td>1 vial</td>
<td>4°C</td>
<td>-20°C</td>
</tr>
</tbody>
</table>
7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring luminescence
- Double distilled water (ddH$_2$O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions: ensure they are ATP-free
- Tubes for the preparation of reagents and buffer solutions
- Sterile 96-well plate with clear flat bottom, preferably white
- General tissue culture supplies
- Orbital shaker
8. Technical Hints

- This kit is sold based on number of tests. A “test” simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.

- Avoid foaming or bubbles when mixing or reconstituting components.

- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.

- Ensure plates are properly sealed or covered during incubation steps.

- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.

- Samples which generate values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.

- Make sure all necessary equipment is switched on and set at the appropriate temperature.
9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 Detergent:
Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C in the dark.

9.2 Substrate:
Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C in the dark.

9.3 Substrate:
Reconstitute each vial of lyophilized substrate in 5 mL of Substrate Buffer (Section 9.2) and mix well by pipetting up and down. Aliquot reconstituted substrate solution so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light.

Δ Note: Once reconstituted, substrate activity will decline up to 30% of the original levels after 1 week storage at 4°C. Therefore, -20°C storage is recommended. After thawing (for second and further use), crystals may appear in the buffer. These crystals can be dissolve by warming the vial at room temperature and mixing by pipetting up and down.

9.4 ATP Standard:
Δ Note: Amount of ATP standard provided is lot-specific and it will be specific in the product label.

Reconstitute ATP standard in ddH$_2$O to make a 10 mM stock solution (ie, if the amount printed on the label is 12 µmol, add 1200 µL ddH$_2$O). Dissolve thoroughly by vortexing the solution. Aliquot that you have enough to perform the desired number of assays. Store at -20°C in the dark.
10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

\(\Delta \text{ Note:}\) The standard dilution described below is a guideline standard and can be adapted if necessary to user’s need. If quantification of ATP levels is not required, you don’t need to prepare the ATP standard and can skip this step.

10.1 Prepare a 100 µM ATP working standard by adding 5 µL of ATP Standard Stock to 495 µL Substrate Buffer.

10.2 Using 100 µM ATP working standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

<table>
<thead>
<tr>
<th>Standard #</th>
<th>Sample to dilute</th>
<th>Volume standard in well (µL)</th>
<th>Cell Culture media (µL)</th>
<th>Prepared ATP standard</th>
<th>End conc ATP in media*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 µM</td>
<td>150</td>
<td>0</td>
<td>100 µM</td>
<td>10 µM</td>
</tr>
<tr>
<td>2</td>
<td>Std #1</td>
<td>15</td>
<td>135</td>
<td>10 µM</td>
<td>1 µM</td>
</tr>
<tr>
<td>3</td>
<td>Std #2</td>
<td>15</td>
<td>135</td>
<td>1 µM</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>4</td>
<td>Std #3</td>
<td>15</td>
<td>135</td>
<td>0.1 µM</td>
<td>0.01 µM</td>
</tr>
<tr>
<td>5</td>
<td>Std #4</td>
<td>15</td>
<td>135</td>
<td>0.01 µM</td>
<td>0.001 µM</td>
</tr>
<tr>
<td>6</td>
<td>Std #5</td>
<td>15</td>
<td>135</td>
<td>0.001 µM</td>
<td>0.0001 µM</td>
</tr>
<tr>
<td>7</td>
<td>Std #6</td>
<td>15</td>
<td>135</td>
<td>0.0001 µM</td>
<td>0.00001 µM</td>
</tr>
<tr>
<td>8 (blank)</td>
<td>0</td>
<td>0</td>
<td>150</td>
<td>0 µM</td>
<td>0 µM</td>
</tr>
</tbody>
</table>

Each dilution has enough amount of standard to set up duplicate readings (2 x 10 µL).

*\(\Delta \text{ Note:}\) ATP standard will be diluted by a factor of 10 when added to the media during Assay Procedure.
11. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- Prepare all reagents and samples as directed in the previous sections.
- Work in subdued lighting, out of direct sunlight or direct bright fluorescence lighting. Bring light may cause plate phosphorescence resulting in higher background levels. Phosphorescence has a half-life of several minutes.
- We recommend growing cells directly on the plate in which the assay will be read for simplicity. If this is not possible, you can transfer cell lysate into the reading plate before the read out.

11.1 Grow cells:

Figure 1 (below) shows a suggested assay layout template to screen dose response for 3 compounds in triplicate.

Plating template suggestions:
- Rows A and H, Columns 1 and 12: do not add any cells (cell culture media only). This will help determine the background luminescence.
- Columns 1 and 12: for setting up ATP standard dilution series.
- Column 2: positive control – diluent control wells to determine the maximal expected signal in the absence of compound.

Figure 1. Suggested assay layout template.
11.1.1 In a sterile white 96-well plate, grow cells in 100 µL media/well (we suggest following layout template in Figure 1).

**Δ Note:** Optional seeding concentration will depend on the cell type. The table below shows suggested cell line specific seeding numbers. If working with other cell lines, you need to determine in advance the optimal seeding amount by cell titration.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Seeding/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>$1.2 \times 10^4$ cells/well</td>
</tr>
<tr>
<td>HepG2</td>
<td>$2.5 \times 10^4$ cells/well</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>$1.2 \times 10^4$ cells/well</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>$5 \times 10^4$ cells/well</td>
</tr>
</tbody>
</table>

11.1.2 Treat cells with appropriate growth factors, chemical entity or other modifiers as desired (see Figure 1 for experiment design template).

11.2 ATP standard plating:

**Δ Note:** if you are not using the ATP standard to quantify levels of ATP in the sample, proceed to Step 11.3

11.2.1 Add 90 µL complete cell culture media into the empty wells of the plate that have been allocated for the ATP standard dilution series.

11.2.2 Add 10 µL of the ATP standards (Step 10.2) into the appropriate wells to make 100 µL total volume in each well.

11.3 Run ATP assay:

11.3.1 Add 50 µL detergent into each well.

11.3.2 Shake plate for 5 minutes in an orbital shaker at 600-700 rpm: this step lyses the cells and stabilizes the ATP.

11.3.3 Add 50 µL Substrate Solution (Step 9.3) to each of the wells.

11.3.4 Shake the microplate for 5 minutes in an orbital shaker at 600-700 rpm.

11.3.5 Dark adapt the plate by covering it for 10 minutes.

11.3.6 Measure luminescence.
12. Data Analysis

If using ATP standard to quantify signal, samples producing signal greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.

12.1 Assay with ATP Standard:

12.1.1 Average the replicate readings for each standard and for each sample.

12.1.2 Subtract the mean luminescence of the blank (Standard #8) from all standard and sample readings. This is the corrected luminescence.

12.1.3 Plot the corrected luminescence values for each standard as a function of the final concentration of ATP in media.

12.1.4 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).

12.1.5 Interpolate the ATP concentration of experimental samples from within this ATP standard curve.

12.1.6 Concentration of ATP in the test samples is calculated as:

\[
\text{Glutamic acid concentration} = \left( \frac{B}{V} \right) \times D
\]

Where:

- \(B\) = ATP amount in sample well calculated from standard curve (\(\mu\text{M}\))
- \(V\) = sample volume added in the sample wells (\(\mu\text{L}\))
- \(D\) = sample dilution factor

12.2 Assay without ATP Standard

12.2.1 Average the duplicate reading for each sample.

12.2.2 Subtract background (empty wells) from each sample measurement.

12.2.3 Determine ATP levels as relative percentage of positive control.
13. Typical data

Typical standard curve – data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

**Figure 2.** Typical ATP standard curve. ATP standard curve diluted in RPMI media, measured in an opaque white plate (n=6). Relative light units were collected with 0.5 sec integration time.
Figure 3. Rotenone induces cytotoxicity in HepG2 cells. $2.5 \times 10^4$ HepG2 cells were seeded, allowed to adhere and treat for 4 hours with 25 µM rotenone (ETC inhibitor) and vehicle control (DMSO) in glucose-based complete media. After treatment, cells were lysed, exposed to the ATP substrate solution and signal was measured on a luminometer. Mean and standard deviation is plotted for 3 replicates from each condition.
Figure 4. Cellular Energy Flux for HepG2 cells (seeded at 65,000 per well), treated with a combination of drug compounds modulating the ETC (Antimycin A [1 µM] and FCCP [2.5 µM]), shown as a percentage relative to untreated control cells. Comparative measurements with Extracellular Oxygen Consumption Assay (ab197243) [white bars] and Glycolysis Assay [Extracellular acidification] (ab197244), [black bars] show the shift between mitochondrial respiration and glycolysis and the cellular control of energy (ATP); measured 1h post-treatment using Abcam Luminescent ATP Detection Assay kit, (ab113849) [striped bars].
14. Quick Assay Procedure

⚠ Note: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Prepare standard, positive control (if using); get equipment ready. [Stock standards produced in section 10.2 are 10-fold higher in concentration than the final concentrations in media shown below].
- Prepare a 9-well plate with cells and treatments in a total volume of 100 µL.
- If quantifying signal, prepare ATP standards [final concentration 0.1 nM – 10 µM in 100 µL].
- Add 50 µL detergent to samples and standards, and incubate 5 minutes in an orbital shaker at 600-700 rpm.
- Add 50 µL of substrate solution, and incubate 5 minutes in an orbital shaker at 600-700 rpm.
- Read 96-well plate in a luminometer.
## 15. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Assay not working</strong></td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at room temperature</td>
</tr>
<tr>
<td></td>
<td>Use of a different 96-well plate</td>
<td>Opaque white plate recommended for maximum sensitivity</td>
</tr>
<tr>
<td><strong>Sample with erratic readings</strong></td>
<td>Samples not lysed</td>
<td>Use provided detergent, incubation and shaking procedure</td>
</tr>
<tr>
<td></td>
<td>Cells/tissue samples not homogenized completely</td>
<td>Use Dounce homogenizer, increase number of strokes</td>
</tr>
<tr>
<td></td>
<td>Samples used after multiple free/ thaw cycles</td>
<td>Aliquot and freeze samples if needed to use multiple times</td>
</tr>
<tr>
<td></td>
<td>Use of old or inappropriately stored samples</td>
<td>Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use</td>
</tr>
<tr>
<td></td>
<td>Presence of interfering substance in the sample</td>
<td>Check protocol for interfering substances; deproteinize samples</td>
</tr>
<tr>
<td><strong>Lower/ Higher readings in samples and Standards</strong></td>
<td>Improperly thawed components</td>
<td>Thaw all components completely and mix gently before use</td>
</tr>
<tr>
<td></td>
<td>Allowing reagents to sit for extended times on ice</td>
<td>Always thaw and prepare fresh reaction mix before use</td>
</tr>
<tr>
<td></td>
<td>Incorrect incubation times or temperatures</td>
<td>Verify correct incubation times and temperatures in protocol</td>
</tr>
<tr>
<td>Problem</td>
<td>Cause</td>
<td>Solution</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>---------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Standard readings do not follow a linear pattern</td>
<td>Pipetting errors in standard or reaction mix</td>
<td>Avoid pipetting small volumes (&lt; 5 µL) and prepare a master mix whenever possible</td>
</tr>
<tr>
<td></td>
<td>Air bubbles formed in well</td>
<td>Pipette gently against the wall of the tubes</td>
</tr>
<tr>
<td></td>
<td>Standard stock is at incorrect concentration</td>
<td>Always refer to dilutions described in the protocol</td>
</tr>
<tr>
<td>Unanticipated results</td>
<td>Measured at incorrect wavelength</td>
<td>Check equipment and filter setting</td>
</tr>
<tr>
<td></td>
<td>Samples contain interfering substances</td>
<td>Troubleshoot if it interferes with the kit</td>
</tr>
<tr>
<td></td>
<td>Sample readings above/ below the linear range</td>
<td>Concentrate/ Dilute sample so it is within the linear range</td>
</tr>
</tbody>
</table>
16. Interferences

These chemicals or biological materials will cause interferences in this assay causing compromised results or complete failure:

- Anionic detergents such as SDS: anionic detergents will inhibit the luciferase reaction
- EDTA will inhibit luciferase reaction – it is a chelating agent and magnesium is needed for the reaction
- Monovalent metal salts such as KCl (concentrations > than 1μM) will affect luciferase activity
- High concentration of divalent metals such as magnesium (> 3 mM), manganese (> 2 mM) or calcium (> 1 mM) will affect luciferase activity

17. FAQs

Q. Can I standardize the results by protein quantification?
A. The mammalian cell lysis solution used in the assay is alkaline and not compatible with protein quantification methods.

Q. Can I pause the experiment at any moment?
A. Although the signal of luciferase is long lasting, there will decay over 5 hours. Theoretically, the plate could be stored in the fridge for a very short period of time (e.g. lunch break, but not overnight) after cell lysis.
18. Notes
Technical Support

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