CELLBIO™ in service of your CELL

Technical Datasheet

EZcount™ ATP Cell Assay Kit

Product Code: CCK051

1. Introduction

Cell proliferation and death are ATP-dependent essential processes for tissue generation and regeneration, organ development etc. in mammals and are usually under stringent control of extra and intracellular factors. Non-physiological alterations in levels of these factors lead to anomalous cytogenetic behavior of cells which in turn leads to cell transformation, uncontrolled cell growth - the initiating event for cancer development. Pharmaceutical research is hence largely focused on effects of drugs, cytotoxic agents and biologically active compounds which affect cytogenetics.

The cellular ATP content plays an important role in determining the fate of a cell. A fairly constant level of ATP in cells maintains them in viable state, or otherwise leads to apoptosis. Multiple procedures are available for determination of cell proliferation and cytotoxicity. Simple and cheap methods for estimating cell viability (or death) are Trypan Blue exclusion and Erythrocin B staining. However, these methods are not sensitive enough and cannot be used for high throughput screening. Measuring the uptake of radioactive substances, usually tritium-labeled thymidine, is accurate but it is also time-consuming and involves handling of radioactive substances. The ATP-based cell assay is a simple, accurate and reproducible assay for determining cellular proliferation and cytotoxicity. The results can be read on a luminometer.

2. About the Assay

The EZcount™ ATP Cell Assay Kit is designed for determination of cell viability and cell proliferation and/or effect of cytotoxic agents. The kit is based on the generation of luminescence as ATP catalyzes the reaction between D-luciferin and enzyme luciferase. The reaction is as follows –

$$\text{D- luciferin} + \text{ATP} + O_2 \xrightarrow[\text{Luciferase}]{Mg^{2+}} \text{Oxyluciferin} + \text{AMP} + \text{Pyrophosphate} + CO_2 + \text{Light}$$

In the above reaction, the reaction between enzyme luciferase and D-luciferin is dependent on presence of ATP. ATP is ubiquitously present in all viable, metabolically active cells. On the other hand, cell death is characterized by a significant decrease in the ATP level.

3. Applications

- Toxicity of physical agents either alone or in combination with chemicals and in particular phototoxicity
- Antibody dependent complement cell damage
- Tumoral cell growth and chemotherapy effectiveness
- Ranking chemicals according to their toxic potencies or hazard
- Toxicity prevention and treatment effectiveness
- The investigation of metabolism mediated cytotoxicity (detoxification and toxification process)
- The study of temperature-dependent toxicity

4. Kit contents

The reagents supplied in this kit are sufficient for 1000 assays (for ten 96 well plates).

Code	Description	CCK051 -1000
CCK051(A)	Reaction Buffer	1×15ml
CCK051(B)	Lysis Solution	3×10ml
CCK051(C)	ATP Standard	1×5mg
CCK051(D)	D-Luciferin	1vial
CCK051(E)	Luciferase	1x25µl
CCK051(F)	ATP enhancer	1vial

Note: Quantities supplied in excess to compensate operational losses

5. Materials required but not provided in the kit

- Ca^{2+} and Mg^{2+} free PBS (TL1006)
- Cells in appropriate medium
- Adjustable pipettes and a repeat pipettor
- Flat-bottom 96-well microtiter plate (**white**) for culturing the cells
- Luminometer

6. Reagent Preparation (Read the instructions carefully)

6.1 Preparation of 1X Reaction Buffer

Make 10.0 mL of 1X reaction buffer by adding 0.5ml of 20X Reaction buffer to 9.5 mL of deionized water.

6.2 Preparation of D-Luciferin

Note: D-Luciferin is extremely sensitive to light. Switch off the lights in working area while handling D-Luciferin.

1. Add 10ml of 1X reaction buffer made in step 6.1, directly in a bottle containing D-Luciferin CCK051(D). Pipette gently up and down to dissolve the powder completely.
2. Aliquot into ten 1.0mL volumes in amber colored vials. D-Luciferin stock solution is reasonably stable for several weeks if stored at -20ºC, protected from light.

6.3 Preparation of ATP enhancer

Dissolve ATP enhancer CCK051 (F) in 4.0 mL of deionized water completely, filter sterilize using 0.22µ syringe filter and aliquot into ten 400µL volumes and store frozen at -20ºC. The reconstituted solution is stable for six months to one year if properly stored in -20ºC.

6.4 Preparation of ATP Assay buffer

Combine the following components given in table to make the ATP Assay buffer.

Components	10.0mL	20.0mL	50.0mL
Deionized water	8.9mL	17.8mL	44.5mL
20X Reaction Buffer CCK051(A)	0.5mL	1.0mL	2.5mL
ATP enhancer (from step 6.3)	0.1mL	0.2mL	0.5mL
D-Luciferin (from step 6.2)*	0.5mL	1.0mL	2.5mL
Luciferase CCK051(E)**	1.25µl	2.5µl	6.25µl

*Store remaining aliquots at -20ºC, protected from light.

**Aliquot into small volumes. Avoid frequent freeze thaw.

Mix all the contents by inverting the tube very gently. **Do not vortex** because firefly luciferase easily gets denatured. Prepared ATP Assay buffer should be kept protected from light. Store unused ATP Assay buffer at 0-4 ºC protected from light. However, stored ATP assay buffer exhibits lower sensitivity compared to freshly prepared buffer.

7. General guidelines

It is important to optimize experimental factors like cell density, incubation time, media composition and concentration of the agents under investigation prior to use of EZcount™ ATP Cell Assay Kit. Procedure for optimizing cell density is outlined in section 8.2. **Assay controls**

Include appropriate assay controls i.e.

1. Medium control (medium without cells)
2. Cell control (medium with cells but without the experimental drug/compound)
3. Vehicle control (medium containing the experimental drug or compound but no cells)

Handling

- Protect all reagents from light and high temperatures.
- CCK051(E) contains the enzyme Luciferase. Do not mix the solution vigorously. Doing so will cause frothing and denaturation of enzyme.
- Due to ubiquitous nature of ATP and the high sensitivity of the luciferase-luciferin reaction, avoid contamination from exogenous biological sources (fingerprints, bacteria, etc.) to prevent interfering signals.

Accuracy

- To obtain statistically significant data, perform the assay in triplicates or more.
- Accuracy of the assay depends on pipetting skills of the personnel. Inappropriate addition and mixing practices may result in erroneous and false-positive or false-negative results.
- Use of a repeating pipettor is recommended to pipette reagents. This saves time and helps maintain more precise incubation times. Pipette tip should be equilibrated with the reagent before use.
- This is carried out by slowly filling up the tip with reagent and gently expelling the contents several times.

- Care should be taken so that no bubbles are introduced into the wells during pipetting or mixing of the reagents.

Plating density

- Different cell lines may have different properties such as metabolic activity and doubling time and hence respond differently to the assay conditions. For this reason, plating density for every cell line should be optimized to obtain results in linear range.

Temperature

- The optimum temperature for the reaction is 28°C. It is crucial to run the assay at a uniform temperature to ensure reproducibility across a single plate or among stacks of several plates.

8. Directions for use

Users are advised to review entire procedure before starting the assay

8.1 Preparation of cells

Always use freshly harvested cells for assay. Seed the cells in a cell culture flask or dish in an amount appropriate for the assay and incubate at 37°C in a 5% CO_2 environment. Allow the cells to grow for up to 24 hours or till confluency is reached. Harvest the cells and use for assay.

(Note: The quantity of the cell suspension to be seeded in the medium depends on doubling time of individual cell lines and seeding density to be used in the assay.)

8.2 Pre-assay optimization procedure

Pre-assay optimization procedure needs to be performed once for each cell line to determine optimum plating density and incubation time.

1. Harvest the cells as explained in section 8.1.
2. Adjust the cell density to 1 x 10^6 cells /ml.
3. Serially dilute the cell suspension from 1 x 10^6 to 1 x 10^3 cells/ml using appropriate culture medium.
4. Seed 100µl of each dilution in 96-well microtiter plate in triplicate.
5. Add medium control in triplicate.
6. Incubate the cells under appropriate conditions depending on the cell line under study.
7. Thaw the Lysis solution CCK051(B) on ice before use.

For adherent cells

Remove media from each well and wash the wells with cold Ca^{2+} and Mg^{2+} free PBS twice.

For suspension cells

Centrifuge the plate at 600 - 700rpm depending on type of cells.
Discard the medium.
Add 200µl cold Ca^{2+} and Mg^{2+} free PBS swirl the plate horizontally for 1 - 2 minutes.
Centrifuge at 600 - 700rpm depending on type of cells. Repeat washing one more time.

8. Add 50µl of cold Lysis Solution CCK051(B) to each well, including controls.
9. Wrap the plate with aluminium foil to avoid exposure to light.
10. Shake the plate manually to mix the contents by rotating clockwise and anti-clockwise for 1-2 minutes.
11. After lysis, add 180µl of ATP Assay buffer (from step 6.4) equilibrated at room temperature to each well and wrap the plate with aluminium foil.
12. Shake the plate manually to mix the contents by rotating clockwise and anticlockwise for 1-2 minutes.
13. Measure luminescence using a luminometer.

8.3 Assay procedure

1. Seed 100µl of cell suspension in a 96-well microtiter plate at the required cell density, with or without a cell growth modifying agent.
   Note:
   a) If the cell growth modifying agent is a cytokine, metabolite, growth factor or any other compound, add its required quantity in the culture system.
   b) If the cell growth modifying agent is any kind of radiation or waves, treat the cells with them for required period of time.
2. Incubate the cells at 37°C in a 5% CO_2 atmosphere for the required period of time.
3. After the incubation period, follow the procedure given in section 8.2, step 7 onwards.
4. Plot the luminescence i.e. light counts on the Y-axis and your experimental parameters on the X-axis.
5. Test values higher than control values indicate increase in cell proliferation and viability and *vice versa*.

8.4 ATP Standard Curve

It is necessary to run standard curve of ATP for each experiment to determine concentration of ATP in test sample.

1. Add 1ml cell culture grade water to the vial containing ATP Standard CCK051(C). Mix well to obtain a solution of 10mM.
2. Dilute 10mM (10000µM) ATP solution 1:1000 to obtain 10µM. Perform 1:10 serial dilutions of 10µM ATP till 0.00001 µM.
3. Seed 10µl of each dilution in 96-well microtiter plate in triplicate.
4. Add culture grade water for control/blank without ATP in triplicate.
5. Add 90µl of ATP Assay Buffer equilibrated at room temperature to each well. Wrap the plate with aluminum foil to avoid exposure to light.
6. Shake the plate manually to mix the contents by rotating clockwise and anticlockwise for 1-2 minutes.
7. Measure luminescence using a luminometer.
8. Extrapolate the concentration of ATP in sample using slope of the curve.

8.5 Calculation

Normalized RLU = Average of Observed RLU – Average of Blank RLU

RLU= Relative luminescence unit

9. Storage

Store the kit at -20ºC away from bright light. Reconstituted ATP Standard solution is stable at room temperature (15-30ºC) for few hours. Aliquots of ATP Standard solution can be stored at -80ºC for several weeks.

Use before expiry date given on the product label.

10. Performance characteristics

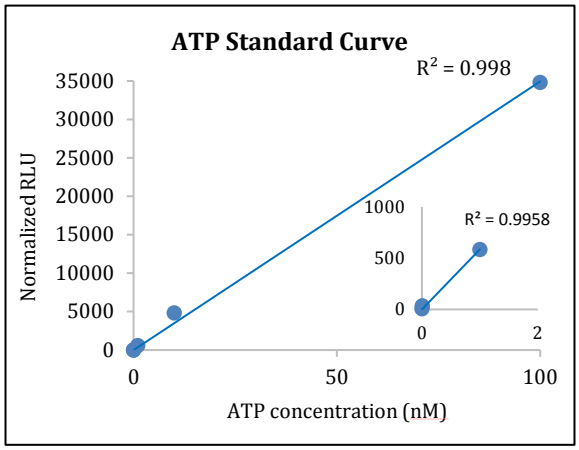

Fig (1): ATP Standard Curve

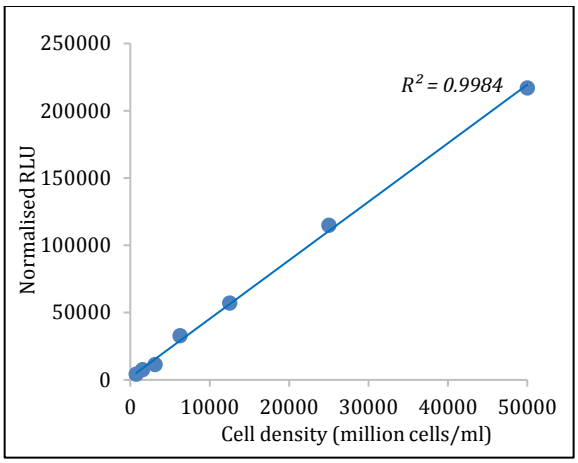

Fig (2): Relationship between cell number and resulting luminescence after ATP cell assay. The sensitivity of ATP assay to detect changes in cell number has been determined by plotting the graph of luminescence as light counts per second versus cell number.

CHO cells were seeded at different densities in 96-well plate and assayed for ATP concentrations using EZcount™ ATP Cell Assay Kit. The emitted light was measured using luminometer.

11. Advantages

- **Time saving:** Ready to use solutions supplied in the kit eliminate the need to prepare separate solutions and save time.
- **Reproducibility:** Entire assay can be performed in a single plate. Cells and reagents need not be transferred. This facilitates reproducibility of the results.
- **Sensitivity and accuracy:** Since ATP is present is all viable cells, there is a linear correlation between cell number and luminescence signal.
- **Safety:** No radioisotopes are involved.
- **Fast:** Luminescence can be measured immediately after addition of reagents; incubation step not required. Also, use of multi-well ELISA plates allows the processing of large number of samples.
- **Flexibility:** The assay can be used for adherent as well as suspension cell lines and can be used for on-line high-throughput systems.

12. Troubleshooting points

Use the following troubleshooting guidelines for technical assistance

Problem	Cause	Solution
No reading on luminometer/ Assay not working	Omission of any step in the protocol	Read the entire procedure before starting the assay and follow appropriately
	Use of partially thawed reagents	Thaw all reagents completely and mix gently before use
	Use of black or clear 96-well plates	Use white plates for measuring luminescence
	Enzyme denatured due to rough handling or multiple freeze-thaw cycles	Handle the enzyme containing reagents gently; do not mix vigorously. Store the reagents in small aliquots.
Samples with variable readings	Cells/tissue samples not homogenized completely	Use appropriate homogenizer and increase number of strokes; check for lysis under microscope.
	Samples used after multiple free/ thaw cycles	Aliquot samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at appropriate temperatures till use
Reading for Standard curve do not follow a linear pattern	Standard stock prepared at the wrong concentration	Read datasheet carefully and prepare standard stock
	Pipetting errors	Avoid pipetting small volumes and avoid vigorous mixing of reagents; Equilibrate with the reagent before use
	Use of partially thawed reagents	Thaw all reagents completely and mix gently before use
	Air bubbles formed in wells	Avoid bubble formation by pipetting gently against the wall of the tubes
	Substituting reagents from older kits/ lots	Use fresh components from the same kit