ab65313
ADP/ATP Ratio Assay kit (Bioluminescent)

Instructions for Use

For the rapid, sensitive and accurate measurement of the ratio of ADP/ATP in various samples.

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

Version 8b Last Updated 29 January 2019

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1. BACKGROUND

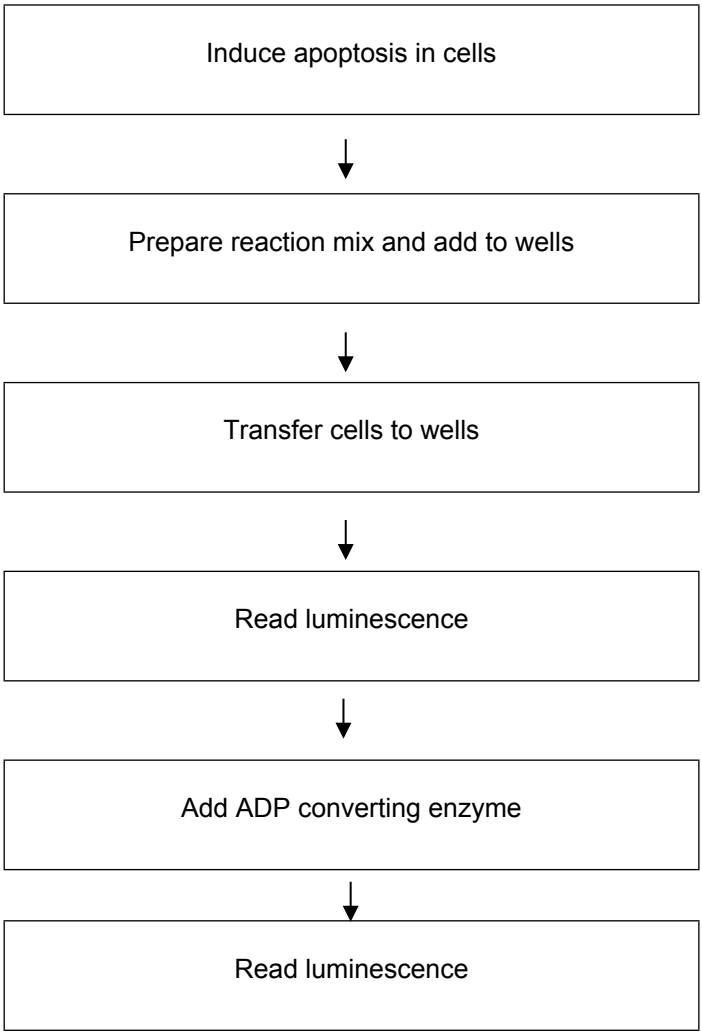
ADP/ATP Ratio Assay Kit (Bioluminescent) (ab65313) is based on the bioluminescent detection of the ADP and ATP levels in the sample for a rapid screening of apoptosis, necrosis, growth arrest, and cell proliferation simultaneously in mammalian cells.

In this assay, luciferase catalyzes the conversion of ATP and luciferin to light, which subsequently can be measured using a luminometer or Beta Counter. ADP level is measured by its conversion to ATP that is subsequently detected using the same reaction. The assay can be fully automatic for high throughput and is highly sensitive (detects 100 mammalian cells/well).

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

The changes in ADP/ATP ratio have been used to differentiate the different modes of cell death and viability. Increased levels of ATP and decreased levels of ADP have been recognized in proliferating cells. In contrast, decreased levels of ATP and increased levels of ADP are recognized in apoptotic cells. The decrease in ATP and increase in ADP are much more pronounced in necrosis than apoptosis.

2. ASSAY SUMMARY



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. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at -20ºC 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 section 5.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
Nucleotide Releasing Buffer	50 mL	-20°C	-20°C
ATP Monitoring Enzyme	1 vial	-20°C	+4°C
ADP Converting Enzyme	1 vial	-20°C	+4°C
Enzyme Reconstitution Buffer	2.15 mL	-20°C	-20°C

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- MilliQ water or other type of double distilled water (ddH_2O)
- Luminometer
- 96 well plate – white walled luminometer plate
- Microcentrifuge
- Pipettes and pipette tips
- Heat block or water bath
- Vortex
- Dounce homogenizer or pestle (if using tissue)

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- 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.

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 will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Keep enzymes and heat labile components and samples on ice during the assay.
- Make sure all buffers and developing solutions are at room temperature before starting the experiment.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the appropriate type of plate for the detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **Enzyme Reconstitution Buffer:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2 **Nucleotide Releasing Buffer:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.3 **10x ADP Converting Enzyme:**

Reconstitute ADP Converting Enzyme with 220 µL of Nucleotide Releasing Buffer and mix gently by inversion. Store at +4°C. Keep on ice while in use, protected from light as much as possible.

9.4 **ATP Monitoring Enzyme:**

Reconstitute ATP Monitoring Enzyme with 2.1 mL of Enzyme Reconstitution Buffer and mix gently by inversion. Store at +4°C. Keep on ice while in use, protected from light as much as possible.

10. SAMPLE PREPARATION

General Sample information:

- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.
- We recommend setting up the samples in culture plates and not culture bottles to allow easy transfer to assay plate.
- Induce apoptosis in cells by desired method. Concurrently incubate a control culture without induction.

10.1 Suspension cell samples:

10.1.1 Harvest the amount of cells (untreated and treated with the desired apoptosis inducer) necessary for each assay (initial recommendation = 1 x 10^4 – 1 x 10^6 cells).

Ensure that the density of your sample is 10^3 – 10^4 cells/10 µL culture.

10.2 Adherent cell samples:

10.2.1 Grow cells and incubate with desired apoptosis inducer (include a control culture without treatment).

10.2.2 Remove culture medium from plate.

10.2.3 Add Nucleotide Releasing Buffer (50 µL Buffer per 10^3 – 10^4 cells) and incubate for 5 minutes at room temperature with gentle shaking. ***NOTE****: Nucleotide Releasing Buffer helps gently loosen the membrane so that ATP will leak out the cell without complete cell lysis.*

10.3 **Tissue samples:**

10.3.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg).

10.3.2 Wash tissue in cold PBS.

10.3.3 Prepare a single cell suspension by any method that does not disrupt membrane integrity (cells have to stay intact).

10.3.4 Add Nucleotide Releasing Buffer (50 µL Buffer per 10^3 – 10^4 cells) and incubate for 5 minutes at room temperature with gentle shaking. ***NOTE****: Nucleotide Releasing Buffer helps gently loosen the membrane so that ATP will leak out the cell without complete cell lysis.*

NOTE: *Avoid contamination with ATP from exogeneous biological sources e.g. bacteria or fingerprints.*

11. ASSAY PROCEDURE and DETECTION

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all controls and samples in duplicate.**

11.1 **Reaction Mix:**

Prepare Reaction Mix for each reaction:

Component	Reaction Mix Samples (µL)
ATP Monitoring Enzyme	**10 µL**
Nucleotide Releasing Buffer	**90 µL**

Mix enough reagents for the number of assays (samples, and background control) to be performed. Prepare a Master Mix of the Reaction Mix to ensure consistency. We recommend the following calculation:

X µL component x (Number samples + 1)

11.2 Add 100 µL of the Reaction Mix to control wells and read the background luminescence (**Data A**).

Optional: To ensure that there is no microbial contamination in the plates which can give high background, let the reaction sit at room temperature for a while (no longer than 1 hour) before addition of samples

11.3 **Cell Sample Set up:**

a) **For suspension cells:** Transfer 10 µl of the cultured cells (10^3 – 10^4 cells) into luminometer plate.

b) **For adherent and tissue cells:** Transfer 50 µl of cells (10^3 – 10^4 cells) treated with Nucleotide Releasing Buffer into luminometer plate.

11.4 After approximately 2 minutes read the sample in a luminometer or luminescence capable plate reader (**Data B**).

11.5 Dilute 10x ADP-Converting enzyme 10-fold with Nucleotide Releasing Buffer. To measure ADP levels in the cells, read the

samples (step 11.4) again (**Data C**), then add 10 µL of 1x ADP Converting Enzyme.

11.6 Read the samples again after approximately 2 minutes (**Data D**).

NOTE: *The results can be analyzed using cuvette-based luminometers or Beta Counters. When Beta Counter is used it should be programmed in the "out of coincidence" (or Luminescence mode) for measurement. The entire assay can directly be done in a 96-well plate*. It can also be programmed automatically using instrumentation with injectors (When using injector the ATP Monitoring Enzyme and the ADP Converting Enzyme can be diluted with the Nuclear Releasing Buffer at 1:50 for injector).*

**The assay utilizes a "glow-type" luciferase which has replaced the original "flash-type" luciferase. While still sensitive to sub-picomole amounts of ATP, the glow-type reactions can still be read an hour later. This means that ATP & ADP levels are now determined by quasi-steady-state light output levels. This makes the reading of an entire 96-well (384-well) plate much more feasible.*

12. CALCULATIONS

- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

ADP/ATP ratio = [Data D – Data C] / [Data B – Data A]

Where:

Data D = sample signal~2 min after addition of 10 µL 1X ADP Converting Enzyme to cells.

Data C = sample signal prior addition of 1X ADP Converting Enzyme to cells.

Data B = sample signal ~2 min after addition of cells to reaction mix

Data A = background signal of reaction mix

13. TYPICAL DATA

Interpretation of results:

Cell Fate	ADP Level	ATP Level	ADP/ATP ratio
Proliferation	Very low	High	Very low
Growth Arrest	Low	Slightly increased	Low
Apoptosis	High	Low	High
Necrosis	Much higher	Very low	Much higher

The interpretation of different ratios obtained may vary significantly according to the cell types and conditions used. However, the following criteria may be used as guidelines:

- **Proliferation** = Test shows markedly elevated ATP values with no significant increase in ADP levels in comparison to control cells.
- **Growth arrest** = Test shows similar or slightly higher levels of ATP and little or no change in ADP compared to control cells.
- **Apoptosis** = Test shows lower levels of ATP to control but shows an increase in ADP.
- **Necrosis** = Test shows considerable lower ATP levels than control but greatly increased ADP.

When ADP/ATP ratio increases, cells are going through apoptosis or necrosis but when the ratio decreases, the cells could be in growth arrest or still proliferating.

14. QUICK ASSAY PROCEDURE

***NOTE**: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when initially performing the assay.*

- Prepare enzyme mix; get equipment ready.
- Prepare samples in duplicate.
- Prepare Reaction Mix (Number samples + 1).

Component	Reaction Mix Samples (µL)
ATP Monitoring Enzyme	**10 µL**
Nucleotide Releasing Buffer	**90 µL**

- Add 100 µL of the reaction mix to the background wells and read the background luminescence (Data A). For suspension cells, transfer 10 µL of the cultured cells per well.
- For adherent cells: add 50 µL treated cells per well.
- Read after ~2 min (Data B).
- To measure ADP levels in the cells, read the samples again (Data C).
- Add 10 µL of 1x ADP Converting Enzyme.
- Read the after ~ 2 minutes (Data D).

15.INTERFERENCES

16. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use PCA precipitation protocol for deproteinization
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Lower/ Higher readings in samples	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

Problem	Cause	Solution
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

17. FAQs

Why didn't my ATP Monitoring Enzyme completely dissolve?

The ATP monitor enzyme is hard to dissolve and the final solution is a yellow-green milky solution (not clear solution). This is normal and it is fine to use it.

What kind of Luciferase is used in this assay?

The Luciferase used was expressed in *E. coli* using the cloned luciferase gene from the North American firefly, *Photinus pyralis*. It rapidly loses activity after reconstitution. It should either be used fresh or deep freeze immediately after reconstitution.

Why is our 10 minute reading higher than the 1 minute reading?

The fact that the 10 min value is higher than the 1 min value means that when the lysate settled down, it become clearer for light passing through and thus results in higher readings. We suggest 2 options to try (1) centrifuge the samples to collect and use only the clear portion of the cell lysate. (2), using more cells (e.g., 10 000 cells/assay) may obtain more reliable results.

Does any wavelength limit need to be set while detecting this assay with a luminometer?

No, unlike fluorometric readings where the emission has a specific wave length for reading the excited molecule, and the excitation has an optimum wave length to excite the molecule, the chemiluminescence works on a different principal. The molecule is present at a high energy level. The substrate breaks that constriction and brings it down to the lower and more stable energy level. The difference in energy is released in the form of light. The luminometer captures this light and measures its intensity. There is no wave length setting in this process.

Could you confirm if this kit has ever been used with a Beckman Coulter LS6500 Multipurpose Scintillation Counter? Do you have any advice on how we can set up the instrument to use with this kit? Would this type of reader be suitable?

The read out of this kit is via luminescence and hence if the indicated equipment is a luminometer, the equipment can be used. Theoretically a luminometer and a scintillation counter are different. Conventional scintillation counters can't be used. However, when Beta Counter is used it should be programmed in the "out of coincidence" (or Luminescence mode) for measurement. So if the equipment has this setting which can be programmed, then the beta counter may be used.

We have detected have levels of ATP and ADP. If we want to present the ATP levels independent of the ratio, which concentration unit or how can we convert the levels to concentration?

To get the exact concentration, you would need a standard which is not included in this kit. You can purchase an ATP standard, but you will have to optimize the production of the standard curve.

The protocol suggested that we need to add 50 µL Nucleotide Releasing Buffer to 10^3-10^4 adherent cells, how we can change the volume of buffer when we have 1x10^6 Beas-2B cells?

We would recommend you to take just 10^4 cells for this assay. If the cell number cannot be taken, we would recommend using ~5 mL of the buffer to keep up the proportion.

Why do I need to measure the level of ADP/ATP so many times?

The *Data B* is for the ATP generated by the cells when they are incubated with the NRB. *Data C* is for the total ATP released and present in the sample which keeps rising for a few minutes after the cells are incubated with the NRB and finally plateau off. Before the ADP converter is added you will not know the levels of ADP, since only ATP will be recognized luminometrically. The ADP has to be converted to ATP for recognition. *Data D* is after conversion of ADP from the samples to ATP.

18. NOTES

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