Fluoro ATP

Cellular/Tissue Fluorescent ATP Detection Kit

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Notes:

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1. Introduction

Adenosine Triphosphate (ATP) is an organic molecule which is exclusively formed in the mitochondria. Vritually all living systems store energy in the phosphate bonds of ATP and plays a centeral role in cellular metabolism and energy transfer reactions. ATP and be de-phosphorylation to ADP and further de-phosphorylation to AMP. This cycling of ATP is centeral to transferring potential (thermodynamic) energy from one source to another. There are several genetic disorders that effect the generation of ATP in the mitochondria [1-3].

Cell Technology's Fluoro ATP assay provides a reliable, sensitive fluorimetric assay for the quantification of ATP in biological samples.

Applications:

- Detection of ATP in cells or tissue extracts.
- ATP measurment in ATP consuming enzymes like kinases and ATPases.
- Detection of ATP in cell death, energy metabolism, mitochondria function.
- ATP detection in Bacterial, fungal and plant cells.

2. Assay Principle

The Fluoro ATP detection kit utilizes a non-fluorescent detection reagent, which is reduced in the presence of ATP and a coupled enzyme reaction to produce its fluorescent analog. There is a linear relationship of ATP concentration to the fluorescent analog concentration. An ATP standard curve is generated to interpolate sample ATP concentrations. The kit can be used in both endpoint and kenitic modes.

Reaction:

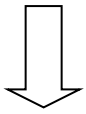
1. ATP + enzyme coupled reaction + non-fluorescent detection reagent ⟶ fluorescent analog + AMP
   Detection: Excitation: 530-570nm and Emission at 590-600nm

3. Storage

Upon arrival store the kit at -20^{0}C.

4. 96 Well Plate Assay

50µL of sample or ATP standard
+
50µL of Enzyme Reaction cocktail



Incubate 30-60 minutes; RT; DARK

Read on plate reader: **Excitation: 530-570nm**
Emission: 590-600nm

5. Warnings and Precautions

1. **For Research use only. Not for use in diagnostic procedures**.
2. Practice safe laboratory procedures by wearing gloves, protective clothing and eyewear.
3. The reaction is not stable in the presence of thiols (DTT or 2-mercaptoethanol).
   See Technical note #1.
4. **Detection reagent Part# 4028 is opened, it is important that low lighting conditions be used while aliquoting as well as performing the experiment. Direct and prolonged light exposure may increase the background, resulting in compromised linearity.**

6. Kit contents Storage and Reconstitution (for 100 assays)

1. **Part #: 6032. Enzyme Mix 25X:** 1 frozen vial 220 µL ready to use. Upon arrival store at -20C^0.
   After use, aliquote and store at -20C^0.
2. **Part# 3065: Substrate Buffer.** 1 bottle 25 mL. Upon arrival store at -20C^0.
   Ready to use.
3. **Part# 4029: Detection Reagent 100X:** 1 vial. Upon arrival store -20C^0
   To reconstitute add 60 µL of andydrous DMSO. Aliquote and store at -20C^0.
4. **Part# 7027: ATP Standard 2.5mM :** 1vial ready to use. Upon arrival store -20C^0.
   After first use aliquote and store at -20C^0.
5. **Part # 7026 : NEM :** 1 vial dried. Reconstitute with 0.5mL of ETOH to make a 1Molar solution. Upon arrival and reconstitution store -20C^0. Dilute as needed.

7. Materials required but not supplied

1. Black 96-well plates (clear bottom optional for bottom reading instruments).
2. Fluorescence or multi mode plate reader.
3. 1X PBB to rinse tissue samples.
4. DMSO Sigma Cat# 276856.

8. Tissue Preparation

Tissue preparation: Prior to tissue extraction, exsanguinate (optional) the animal to remove red blood cells from tissue. Weigh 10-20 mg of tissue and rinse in ice cold PBS. Transfer the tissue into a 1.5mL eppendorf tube and add 100-200µL of **Substrate Buffer** (**Part# 3064**) to the tubes. Proteaus inhibitors and NEM (Part # 7026) can be added at this time. Then using standard techniques homogenize the tissue samples on ice.
See technical notes #1 - #3 Section 11 below before making tissue homogenates.
Vortex the tubes and spin homogenates a 12,000 X g for 5 minutes to clarify the supernatants. Supernatants are ready for the assay (keep on ice until they are ready for the assay). If not using the supernatants immediately, freeze at –70^{0}C.

Note:

1. **It is recommended to titrate the cell samples 1:2 or 1:4 so that the concentrations of ADP will fall within the range of the standard curve. These dilutions should be prepared in the Substrate Buffer (Part# 3064) as described in technical note #2.**
2. **It is recommended to make a concentrated sample preperation so that the dilutions may fall within the ADP standard curve.**

9. Mammalian Cell Preparation (Suspension or adherent).

1. Incubate cells according to your experimental conditions
2. Harvest cells using standard protocols and wash in 1X PBS twice. After final wash adjust cells to deliver 1-2 x10^6 cells/sample. Spin down cell pellete, decant and vortex the cell pellet. ***Before next step*** **see technical notes #1 - #3 Section 11 below.**
3. Lysising Cells: Add 200-500µL of Substrate Buffer (Part#3064). Use standard proceedures to lyse the cells (sonication, freeze thaw) and spin down the supernatant to remove cellular debris.

4. The samples are ready for the assay (keep the samples on ice until they are ready for the assay). If not using the supernatants immediately, freeze at –70^{0}C until ready for use.

   Note: Each investigator should optimize the number of cells used per test. Adherent cells can be harvested via scraping or detchement media.

10. Assay Protocol

A. ATP Standard Curve.

1. The ATP standard is supplied at a concentration of 2.5mM. Make a starting dilution of 50µM in Substrate Buffer (Part# 3064). This can be accomplished by making a 1:50 dilution from the stock ATP standard. ***Before next step see technical note #2 below.***
2. Next serially dilute (1:2) the 50µM ADP standard in the Substrate Buffer (see technical note #2) to construct a standard curve as shown in the table below. Keep in mind the actual concentration of the ADP in the well is half of that stated in the table.

Tube #	NADPH Concentration in tubes.
1	50µM
2	25µM
3	12.5µM
4	6.25µM
5	3.125µM
6	1.562µM
7	0.7812µM
8	0

B. Prepare the Enzyme Reaction Cocktail:

To 1mL of **Substrate Buffer (Part# 3064)**, add 40µL of enzyme mix (**Part #: 6031**) and 10µL of Detection Reagent (Part# 4028**)** and vortex gently. This is enough for 10 tests. Make enough Enzyme Reaction cocktail for one day's worth of experiments.

Note: Add the Detection reagent just before use. Light sensitive. Avoid direct and prolonged exposure to light, as this will increase background. Do *not* use tehnical note #2 to modify the Substrate Buffer in this step.

C. Assay:

1. Add 50µL of standard, sample or titrated sample in triplicate to individual wells of a black 96 well plate.

2. Next pipette in 50µL of the **Enzyme Reaction Cocktail** (from step B above) to all the wells.
3. Incubate the plate for 30-60 minutes.
   Each investigator should optimize the incubation time.
4. Measure fluorescence with excitation at 530-570 nm and emission at 590-600nm using a fluorescent plate reader.
5. Graph the data using suitable software (Graph Pad). The concentration of ADP can be determined using the equation generated by the standard curve or using thesample interpolate function in the software.
   Note: Data can be standardized by protein concentration, cell number or tissue mass.

11. Technical Notes

1. **SH groups like DTT or Reduced Glutathione will interfere with the assay. Add NEM to a final concentration of 20-40mM to the samples. Proteaus inhibitors can be added at this time.**

2. **Before constructing the ADP Standard curve or lysising samples, add NEM and other and Proteaus inhibitors to the Substrate Buffer (Part# 3064) so the standard curve matrix will mimic the sample matrix. For example if you use 40mM (final concentration) NEM in your samples add the same NEM concentration to the ADP standard curve. Prepare enough of this buffer to construct standard curve and to process and titrate out your samples several times.**
3. **If using NEM in the Substrate Buffer, use this immediately to make cell lysates or tissue homogenates as NEM will hydrolyze over time.**

12. Data

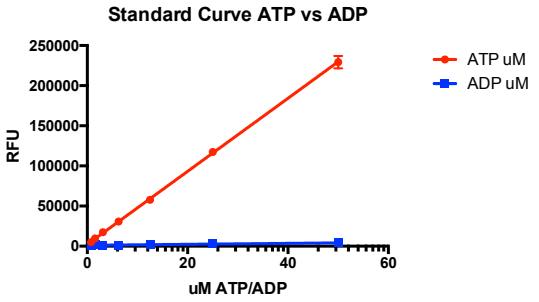


Figure 1. ATP vs ADP Standard Curve fitted with linear regression.

ATP Spike	% Recovery no NEM	% Recovery 40mM NEM
25.5	170	95.85
8.35	230	102
2.154	227	86.75

Table 1. ATP was spiked into Jurkat cell samples in Substrate Buffer (part#3046) with or without 40mM NEM (part# 7026) . % Recovery was determined via linar regression from ATP standard curve. N=3 per sample.

References

1. Mitchell, P., Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature, 191, 144–148 (1961).
2. Alirol, E., and Martinou, J.C., Mitochondria and cancer: is there a morphological connection? Oncogene, 25, 4706–4716 (2006).
3. Carrozzo, R. et al., Infantile mitochondrial disorders. Biosci. Rep., 27, 105–112 (2007).