ab83355
ATP Assay Kit
(Colorimetric/
Fluorometric)

For the rapid, sensitive and accurate measurement of ATP in a variety of samples.

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

ATP Assay Kit (Colorimetric/ Fluorometric) (ab83355) is a robust, simple method to quantify total ATP in cell and tissue lysates, biological fluids and blood cells. The assay is based on the phosphorylation of glycerol in order to generate a product that can be easily quantified colorimetrically (OD 570 nm) or fluorometrically (Ex/Em = 535/587 nm). There are other ATP assay kits available which can detect femtomoles or less of ATP by measuring luminescence, but they require specialized luminescence instrumentation and use luciferase, which can be difficult to maintain in active form.

Fluorometric assay is 10-100 fold more sensitive than colorimetric, and it can detect as low as 1 µM of ATP present in the samples.

ATP (adenosine-5’-triphosphate) is a multifunctional nucleotide that is most important in intracellular energy transfer, being the primary energy currency of living systems. Virtually all energy requiring processes utilize the chemical energy stored in the phosphate bond of ATP. ATP is formed exclusively in the mitochondria and a variety of genetic diseases can affect ATP formation in the mitochondria.
2. Protocol Summary

Standard curve preparation

↓

Sample preparation*

↓

Add reaction mix and incubate for 30 minutes

↓

Measure optimal density (OD570 nm) or fluorescence (Ex/Em = 535/587 nm)

*Samples require deproteinization
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 -20°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.

⚠️ Note: Reconstituted components are stable for 2 months.
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>ATP Assay Buffer</td>
<td>25 mL</td>
<td>-20°C</td>
<td>-20°C / 4°C</td>
</tr>
<tr>
<td>ATP Probe</td>
<td>200 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>ATP Converter</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Developer Mix</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>ATP Standard</td>
<td>1 vial</td>
<td>-20°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 absorbance at OD 570 nm (colorimetric) or fluorescence at Ex/Em = 535/587 nm (fluorometric)
- MilliQ water or other type of double distilled water (ddH₂O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with clear flat bottom (for colorimetric assay) / 96 well plate with clear flat bottom, preferably black (for fluorometric assay)
- Dounce homogenizer (if using tissue)
- Deproteinizing Sample Preparation Kit – TCA (ab204708): for deproteinization step in cell or tissue samples
- 10 kD Spin columns (ab93349): for deproteinization step in fluid samples
- (Optional) Red Blood Cell (RBC) Lysis Buffer (ab204733): for lysis of red blood cells.

Optional – for highly metabolic tissues:
- Perchloric acid (2N), Ice cold
- Potassium hydroxide (KOH), 2M
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, standard 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 generating 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 ATP Assay Buffer:
Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C or 4°C.

9.2 ATP Standard (lyophilized, 1 µmol):
Reconstitute in 100 µL of ddH₂O to generate a 10 mM ATP standard stock solution. Keep on ice while in use. Aliquot standard so that you have enough to perform the desired number of assays. Store at -20°C. Avoid repeated freeze/thaw.

9.3 ATP Probe (in DMSO):
Ready to use as supplied. Warm by placing in a 37°C bath for 1 – 5 min to thaw the DMSO solution before use.

△ Note: DMSO tends to be solid when stored at -20°C, even when left at room temperature, so it needs to melt for a few minutes at 37°C. Repeat this step every time probe is needed. Aliquot probe so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light. Once the probe is thawed, use within two months.

9.4 ATP Converter:
Dissolve in 220 µL ATP Assay Buffer. Keep on ice during the assay. Aliquot converter so that you have enough volume to perform the desired number of assays. Store at -20°C. Use within two months.

9.5 Developer Mix (lyophilized):
Dissolve in 220 µL ATP Assay Buffer. Keep on ice during the assay. Aliquot developer so that you have enough volume to perform the desired number of assays. Store at -20°C. Use within two months.
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.

10.1 Colorimetric assay:

10.1.1 Prepare 100 µL of 1 mM ATP standard by diluting 10 µL of the provided ATP Standard (10 mM solution) with 90 µL of ddH₂O.

10.1.2 Using 1 mM 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>ATP 1mM Standard (µL)</th>
<th>Assay Buffer (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>End amount ATP in well (nmol/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>150</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>144</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>138</td>
<td>50</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>132</td>
<td>50</td>
<td>6</td>
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<tr>
<td>5</td>
<td>24</td>
<td>126</td>
<td>50</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>120</td>
<td>50</td>
<td>10</td>
</tr>
</tbody>
</table>

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).
10.2 Fluorometric assay:

**Note:** Detection sensitivity of fluorometric assay is 10-100 fold higher than colorimetric assay.

10.2.1 Prepare a 1 mM ATP standard by diluting 5 µL of the provided ATP Standard (10 mM solution) with 45 µL of ddH$_2$O.

10.2.2 Prepare 100 µL of 0.1 mM ATP standard by diluting 10 µL of 1 mM ATP standard with 90 µL of ddH$_2$O.

10.2.3 Using 0.1 mM 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>ATP 0.1mM Standard (µL)</th>
<th>Assay Buffer (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>End amount ATP in well (nmol/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>150</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>144</td>
<td>50</td>
<td>0.2</td>
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<td>3</td>
<td>12</td>
<td>138</td>
<td>50</td>
<td>0.4</td>
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<tr>
<td>4</td>
<td>18</td>
<td>132</td>
<td>50</td>
<td>0.6</td>
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<tr>
<td>5</td>
<td>24</td>
<td>126</td>
<td>50</td>
<td>0.8</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>120</td>
<td>50</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).
11. Sample Preparation

General sample information:
- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you snap freeze your samples in liquid nitrogen upon extraction and store them 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.

\(\Delta\) Note: Endogenous compounds in the sample may interfere with the reaction. To ensure accurate determination of ATP in the test samples, we recommend spiking samples with a known amount of Standard (initial recommendation: 300 pmol).

11.1 Cell lysates:
11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation: 1 x 10^6 cells).
11.1.2 Wash cells with cold PBS.
11.1.3 Resuspend cells in 100 µL of ATP Assay Buffer.
11.1.4 Homogenize cells quickly by pipetting up and down a few times.
11.1.5 Centrifuge 5 minutes at 4°C at 13,000 x g in a cold microcentrifuge to remove any insoluble material.
11.1.6 Collect supernatant and transfer to a new tube.
11.1.7 Keep on ice.
11.1.8 Cells samples may contain enzymes that can interfere with the assay. Remove these enzymes from sample by using Deproteinizing Sample Preparation Kit – TCA (ab204708). Alternatively, you can perform a PCA/KOH deproteinization step following the protocol described in section 11.6.

11.2 Tissue lysates:
For highly metabolically active tissues such as muscle, we recommend preparing tissue samples directly in PCA following the alternative protocol described in Step 11.3. If you don’t have access to PCA, then proceed with the instructions described below.
11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation: 10 mg).
11.2.2 Wash tissue in cold PBS.
11.2.3 Homogenize tissue in 100 µL of ATP Assay Buffer with a Dounce homogenizer or pestle sitting on ice, with 10-15 passes.
11.2.4 Centrifuge sample for 2-5 minutes at 4°C at 13,000 x g using a cold microcentrifuge to remove any insoluble material.
11.2.5 Collect supernatant and transfer to a new tube.
11.2.6 Keep on ice.
11.2.7 Tissue samples may contain enzymes that can interfere with the assay. Remove these enzymes from sample by using Deproteinizing Sample Preparation Kit – TCA (ab204708). Alternatively, you can perform a PCA/KOH deproteinization step following the protocol described in section 11.6.

11.3 Highly metabolically active tissues – alternative protocol:
11.3.1 Harvest the amount of tissue necessary for each assay (initial recommendation: 10 mg tissue).
11.3.2 Wash tissue in cold PBS.
11.3.3 Homogenize tissue in 100 µL cold 2N PCA with a Dounce homogenizer sitting on ice, with 10-15 passes.
11.3.4 Keep samples on ice for 30-45 minutes.
11.3.5 Centrifuge samples at 13,000 x g for 2 minutes at 4°C in a cold centrifuge and transfer supernatant to a fresh tube. Measure volume of supernatant.
11.3.6 After homogenizing the tissue in 100 µL of PCA, dilute the volume to 500 µL with the ATP Assay Buffer.
11.3.7 Precipitate excess PCA by adding ice-cold 2M KOH that equals 10-20% of the total volume (for instance, 50-100 µL of 2 M KOH to 500 µL sample) and vortexing briefly. This will neutralize the sample and precipitate excess PCA.
11.3.8 It is important to test pH during neutralization: pH should equal 6.5-8 (use pH paper to test 5 µL of sample). If necessary, adjust pH with 0.1 M KOH or PCA.
11.3.9 Centrifuge at 13,000 x g for 15 minutes at 4°C and collect supernatant.
11.3.10 Samples are now deproteinized, neutralized and PCA has been removed. The samples are now ready to use in the assay. No additional process is required.
11.3.11 To calculate the dilution factor introduced by the deproteinization step (DDF), simply apply the following formula:

$$DDF = \frac{500 \mu L + volume \ KOH (\mu L)}{initial \ sample \ volume \ in \ PCA}$$

**Note:** 500 µL is final sample volume (PCA + Assay Buffer) before neutralization. Initial sample volume in PCA will be 100 µL.

11.4 Plasma, Serum and Urine (and other biological fluids):
Use heparin when collecting plasma or serum. High concentrations of protein interfere with the assay. Fluid samples containing high levels of protein can be deproteinized with our 10 kD Spin Columns (ab93349) to deproteinize biological fluids. Add sample to the spin column, centrifuge at 13,000 x g for 10 min at 4°C. Collect the filtrate.

The find the optimal values and ensure your readings will fall within the standard values, we recommend performing several dilutions of the sample (initial recommendations: 1:2 – 1:5 – 1:10).

11.5 Red blood cells (RBC):
We recommend harvesting 1 x 10^7 RBCs as they are generally smaller cells.

RBC sample can be prepared using Red Blood Cell (RBC) Lysis Buffer (ab204733) following by a deproteinization step using Deproteinizing Sample Preparation Kit – TCA (ab204708).

If not using RBC Lysis Buffer, red blood cells can be lysed using the following protocol:

11.5.1 Homogenize sample in 100 µL of ATP Assay Buffer.
11.5.2 Lyse cells by snap freeze-thaw cycles.
11.5.3 Centrifuge sample for 2 minutes at 4°C at 13,000 x g using a microcentrifuge to remove any insoluble material.
11.5.4 Collect supernatant and transfer to a clean tube.
11.5.5 Keep on ice.
11.5.6 Remove interfering enzymes from sample by using Deproteinizing Sample Preparation Kit – TCA (ab204708).
11.5.7 Alternatively, you can perform a PCA/KOH deproteinization step following the protocol described in section 11.6.
11.6 Alternative deproteinization protocol:
For this step you will need additional reagents:
- Perchloric acid (PCA) 4M, ice cold
- Potassium hydroxide (KOH), 2M

Prepare samples as specified in the protocol. You should have a clear protein sample after homogenization and centrifugation. Keep your samples on ice.

11.6.1 Add ice cold PCA 4M to a final concentration of 1M in the homogenate solution and vortex briefly to mix well.

Δ Note: high protein concentration samples might need more PCA.

11.6.2 Incubate on ice for 5 minutes.

11.6.3 Centrifuge samples at 13,000 $xg$ for 2 minutes at 4°C in a cold centrifuge and transfer supernatant to a fresh tube. Measure volume of supernatant.

11.6.4 Precipitate excess PCA by adding ice-cold 2M KOH that equals 20-35% of the total volume (sample +PCA) and vortexing briefly. This will neutralize the sample and precipitate excess PCA.

11.6.5 After neutralization, it is very important that pH equals 6.5-8 (use pH paper to test 1 µL of sample). If necessary, adjust pH further with 0.1 M KOH or PCA.

11.6.6 Centrifuge at 13,000 $xg$ for 15 minutes at 4°C and collect supernatant.

11.6.7 Samples are now deproteinized, neutralized and PCA has been removed. The samples are now ready to use in the assay.

11.6.8 To calculate the dilution factor introduced by the deproteinization step (DDF), simply apply the following formula:

$$DDF = \frac{\text{initial sample volume} + \text{volume PCA} + \text{volume KOH (µL)}}{\text{initial sample volume in PCA}}$$

Δ Note: We suggest using different volumes of sample to ensure readings are within the standard curve range.
12. Assay Procedure – Colorimetric Assay

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

**Δ Note:** Glycerol phosphate present in cell or tissue extracts can generate background in this assay. If you suspect your samples contain glycerol phosphate, set up Sample Background Controls.

12.1 Plate Loading:
- Standard wells = 50 µL standard dilutions.
- Sample wells = 1-50 µL samples (adjust volume to 50 µL/well with ATP Assay Buffer).
- Sample Background Control wells = 1-50 µL samples (adjust volume to 50 µL/well with ATP Assay Buffer).

12.2 ATP reaction mix:
12.2.1 Prepare 50 µL of ATP Reaction Mix and Background Control Mix for each reaction. Mix enough reagents for the number of assays to be performed. Prepare a master mix to ensure consistency.

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction Mix (µL)</th>
<th>Background Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP Assay Buffer</td>
<td>44</td>
<td>46</td>
</tr>
<tr>
<td>ATP Probe</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>ATP Converter</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Developer Mix</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

12.2.2 Add 50 µL of Reaction Mix into each standard and sample wells.
12.2.3 Add 50 µL of Background Reaction Mix into the background control sample wells.

**Δ Note:** in absence of ATP converter, the assay detects only glycerol phosphate but not ATP.
12.2.4 Mix and incubate at room temperature for 30 min protected from light.
12.2.5 Measure output on a microplate reader at OD 570 nm.

**Note:** we recommend measure reaction immediately but reaction is stable for at least 2 hours.
13. Assay Procedure – Fluorometric Assay

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.
- For fluorometric assays, use probe 1:10 diluted reduces fluorescence background.

**Note**: Glycerol phosphate present in cell or tissue extracts can generate background in this assay. If you suspect your samples contain glycerol phosphate, set up Sample Background Controls.

### 13.1 Plate Loading:

- Standard wells = 50 µL standard dilutions.
- Sample wells = 1-50 µL samples (adjust volume to 50 µL/well with ATP Assay Buffer).
- Sample Background Control wells = 1-50 µL samples (adjust volume to 50 µL/well with ATP Assay Buffer).

### 13.2 ATP reaction mix:

13.2.1 Prepare 50 µL of ATP Reaction Mix and Background Control Mix for each reaction. Mix enough reagents for the number of assays to be performed. Prepare a master mix to ensure consistency.

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction Mix (µL)</th>
<th>Background Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP Assay Buffer</td>
<td>45.8</td>
<td>47.8</td>
</tr>
<tr>
<td>ATP Probe</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>ATP Converter</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Developer Mix</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
13.2.2 Add 50 µL of Reaction Mix into each standard and sample wells.
13.2.3 Add 50 µL of Background Reaction Mix into the background control sample wells.

**Δ Note:** in absence of ATP converter, the assay detects only glycerol phosphate but not ATP.

13.2.4 Mix and incubate at room temperature for 30 min protected from light.
13.2.5 Measure output on a microplate reader at Ex/Em = 535/587 nm.

**Δ Note:** we recommend measure reaction immediately but reaction is stable for at least 2 hours.
14. Calculations

- Samples producing signals 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.

14.1 If significant, subtract the sample background control from sample reading.

14.2 Average the duplicate reading for each standard and sample.

14.3 Subtract the mean absorbance/fluorescence value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance/fluorescence.

14.4 Plot the corrected absorbance/fluorescence values for each standard as a function of the final concentration of ATP.

14.5 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).

14.6 Apply the corrected sample absorbance/fluorescence reading to the standard curve to get ATP (B) amount in the sample wells.

14.7 Concentration of ATP (nmol/µL or µmol/mL or mM) in the test samples is calculated as:

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

Where:

- \( B \) = amount of ATP in the sample well calculated from standard curve (nmol or mM).
- \( V \) = sample volume added in the sample wells (µL).
- \( D \) = sample dilution factor if sample is diluted to fit within the standard curve range.
- \( \text{DDF} \) = deproteinization dilution factor (from Section 11).

ATP molecular weight = 507.18 g/mol
14.8 For spiked samples, correct for any sample interference by subtracting the sample reading from spiked sample reading.

14.9 For spiked samples, the concentration of ATP in sample well is calculated as:

\[
\text{ATP} = \left(\frac{(\text{Rs cor})}{(\text{Rs} + \text{Bcor}) - (\text{Rs cor})}\right) \times \text{ATP spike (pmol)}
\]

Where:

\( \text{Rs cor} \) = reading sample (OD/RFU) corrected
\( \text{Rs} \) = reading sample (OD/RFU)
\( \text{Bcor} \) = amount of ATP from standard curve corrected
15. Typical Data

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

\[ y = 0.137x - 0.006 \]

Figure 1. Typical ATP standard calibration curve using colorimetric reading.
Figure 2. Typical ATP standard calibration curve using fluorometric reading.

\[ y = 8383.3x - 37.23 \]

Figure 3. Quantitation of ATP in fish liver (2.5 µL of 10X diluted sample), fish muscle (5 µL of 10X diluted sample) and Jurkat cell lysate (5 µL) using fluorometric assay. Samples were spiked with known amounts of ATP (300 pmol).
16. 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 reagents and aliquot; get equipment ready.
- Prepare ATP standard dilution for your desired detection method: colorimetric [2 – 10 nmol/well] or fluorometric [0.2 – 1 nmol/well].
- Prepare samples (including deproteinization step) in optimal dilutions to fit standard curve readings.
- Set up plate in duplicate for standard (50 µL), samples (50 µL) and background sample control wells (50 µL).
- Prepare a master mix for ATP Reaction Mix and (if appropriate) a master mix for Background Reaction Mix:

<table>
<thead>
<tr>
<th>Component</th>
<th>Col / Bckg Reaction Mix (µL)</th>
<th>Fluo / Bckg Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP Assay Buffer</td>
<td>44 / 46</td>
<td>45.8 / 47.8</td>
</tr>
<tr>
<td>ATP Probe</td>
<td>2 / 2</td>
<td>0.2 / 0.2</td>
</tr>
<tr>
<td>ATP Converter</td>
<td>2 / 0</td>
<td>2 / 0</td>
</tr>
<tr>
<td>Developer Mix</td>
<td>2 / 2</td>
<td>2 / 2</td>
</tr>
</tbody>
</table>

- Add 50 µL Reaction to standard and sample wells.
- Add 50 µL Background Reaction Mix to Sample Background control wells.
- Incubate plate at RT for 30 minutes protected from light.
- Measure plate at OD 570 nm for colorimetric assay or at Ex/Em= 535/587 nm for fluorometric assay.
# Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</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 assay temperature</td>
</tr>
<tr>
<td></td>
<td>Plate read at incorrect wavelength</td>
<td>Check the wavelength and filter settings of instrument</td>
</tr>
<tr>
<td></td>
<td>Use of a different microplate</td>
<td>Colorimetric: clear plates</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluorometric: black wells/clear bottom plates</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Luminometric: white wells/clear bottom plates</td>
</tr>
<tr>
<td><strong>Sample with erratic readings</strong></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>Reason</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>
18. Interferences

These chemical or biological materials will cause interferences in this assay causing compromised results or complete failure:
- Avoid EDTA and other chelators when preparing plasma or serum samples.

19. FAQs

Q. Should perchloric acid be added to the sample after homogenization with assay buffer?
A. No, if you have access to the PCA, it is recommended that you homogenize the samples in PCA. If not, you can use the provided assay buffer.

Q. Do you have any recommendations for the type of 96 well plate to use for the fluorometric assay?
A. For the colorimetric assay, please use a clear plate. For the fluorometric assay, a clear or a white plate can be used. Use flat bottom plates.

Q. If glycerol phosphate causes background in the assay, how can I remove it from the sample?
A. Glycerol phosphate is a natural by-product of glycolysis, especially abundant in highly metabolic tissues, and it is very difficult to remove unless you use analytical techniques. Dilution of the sample in assay buffer will dilute the effect of the glycerol phosphate. Setting up a background control sample will allow to discard any effect caused by the glycerol phosphate.

Q. Is the standard just ATP without any additives (like for example stabilizers)?
A. Yes, this is pure ATP without any additives.
Q. Can we use frozen samples with this assay?
A. Fresh samples are always preferred over frozen samples. However, frozen samples can also be used, provided, they were frozen right after isolation, were not freeze thawed multiple time (for which we recommend aliquoting the samples before freezing) and have been frozen for relatively short periods.

Q. Do I need to run a sample background control for each sample?
A. It will depend largely on the sample type and how high the background is.
If the samples are diverse and the background is high, then you will need to run background from each sample type. However, if the sample is uniform (ie, human serum), then you can run the background for one representative sample only. If you are performing assays at an interval, you will need to run the background again.
Sometimes, long term storage can reduce the activity of interfering enzymes and the background could be lower.

Q. My blood cells have been frozen in glycerol. Will glycerol affect the assay reading?
A. Adding glycerol in cryopreservation of red blood cells should not affect the assay as long as deglycerolization is done properly before proceeding with the assay.
20. Notes