

ab83359

ADP Assay Kit

(Colorimetric/Fluorometric)

Instructions for Use

For the rapid, sensitive and accurate measurement of ADP levels in various samples.

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

PLEASE NOTE: With the acquisition of BioVision by Abcam, we have made some changes to component names and packaging to better align with our global standards as we work towards environmental-friendly and efficient growth. You are receiving the same high-quality products as always, with no changes to specifications or protocols.

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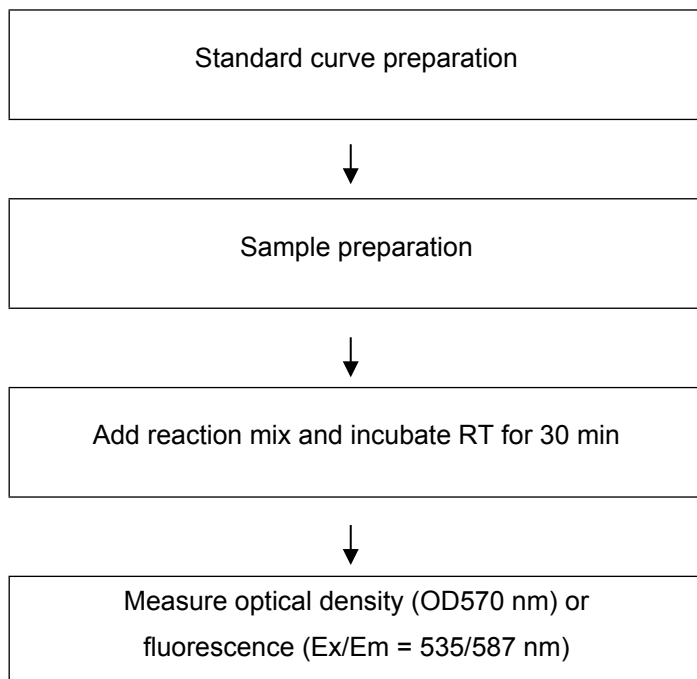
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1. BACKGROUND

ADP Assay Kit (Colorimetric/Fluorometric) (ab83359) provides a convenient colorimetric and fluorometric method to measure ADP level. In the assay, ADP is converted to ATP and pyruvate. The generated pyruvate can be quantified by colorimetric (OD_{max} = 570 nm) or fluorometric method (Ex/Em 535/587 nm). Conventionally, ADP levels are measured by luciferase/luciferin mediated assays after ADP is converted to ATP. However, the luciferase system is unstable and luminescence equipment is not generally available in most laboratories. In comparison, this assay is simple, sensitive, stable and high-throughput adaptable and can be used with conventional microplate readers. The assay can detect as low as 1 μ M ADP in biological samples.

ADP is a product of ATP de-phosphorylation and it can be rephosphorylated to ATP. De-phosphorylation and re-phosphorylation occur via various phosphatases, phosphorylases and kinases. ADP is stored in platelets and can be released to interact with a variety of purinergic receptors. ADP levels regulate several enzymes involved in intermediary metabolism. ADP conversion to ATP primarily occurs within the mitochondrion and chloroplast although several such processes occur in the cytoplasm.

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 -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 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) |
|--|--------|--|---------------------------------------|
| Assay Buffer IV/ADP Assay Buffer | 25 mL | -20°C | -20°C |
| OxiRed Probe/ADP Probe (in DMSO) | 200 µL | -20°C | -20°C |
| Converter Mix II/ADP Converter | 1 vial | -20°C | -20°C |
| Development Enzyme Mix I/ADP Developer Mix | 1 vial | -20°C | -20°C |
| ADP Standard (1 µmole) lyophilized | 1 vial | -20°C | -20°C |

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- MilliQ water or other type of double distilled water (ddH₂O)
- PBS
- Microcentrifuge
- Pipettes and pipette tips
- Colorimetric or fluorescent microplate reader – equipped with filter for OD 570 nm or Ex/Em = 535/587 nm (respectively)
- 96 well plate: clear plates for colorimetric assay; black plates (clear bottoms) for fluorometric assay
- Heat block or water bath
- 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 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.**
- Keep enzymes, heat labile components and samples on ice during the assay.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- 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.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on.

9. REAGENT PREPARATION

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

9.1 **Assay Buffer IV/ADP Assay Buffer:**

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

9.2 **OxiRed Probe/ADP Probe – in DMSO:**

Ready to use as supplied.

. Warm by placing in a 37°C bath for 1 – 5 minutes 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 few minutes at 37°C.** Aliquot OxiRed Probe/probe so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light and moisture. Keep on ice while in use.

9.3 **Converter Mix II/ADP Converter:**

Reconstitute in 220 µL Assay Buffer IV/ADP Assay Buffer. Pipette up and down to dissolve. Aliquot Converter Mix II/converter so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

9.4 **Development Enzyme Mix I/ADP Developer Mix:**

Reconstitute in 220 µL Assay Buffer IV/ADP Assay Buffer. Pipette up and down to dissolve. Aliquot Development Enzyme Mix I/developer mix so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

9.5 **ADP Standard:**

Reconstitute the ADP Standard (1 µmol) in 100 µL of ddH₂O to generate a 10 mM standard stock solution. Aliquot standard so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be used within 4 hours.

10.1 For the colorimetric assay:

10.1.1 Prepare 100 μL of 1 nmol standard by diluting 10 μL of the reconstituted standard with 90 μL of Assay Buffer IV/ADP Assay Buffer. Mix well.

10.1.2 Using 1 nmol ADP standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

| Standard # | Volume of 1 nmol Standard (μL) | Assay Buffer IV/Assay Buffer (μL) | Final volume standard in well (μL) | End [ADP] in well |
|------------|---|--|---|-------------------|
| 1 | 0 | 150 | 50 | 0 nmol/well |
| 2 | 6 | 144 | 50 | 2 nmol/well |
| 3 | 12 | 138 | 50 | 4 nmol/well |
| 4 | 18 | 132 | 50 | 6 nmol/well |
| 5 | 24 | 126 | 50 | 8 nmol/well |
| 6 | 30 | 120 | 50 | 10 nmol/well |

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

10.2 For the fluorometric assay:

- 10.2.1 Prepare 100 μL of a 1 nmol ADP as described in section 10.1.1.
- 10.2.2 Dilute 10 μL of 1 nmol standard with 90 μL of Assay Buffer IV/ADP Assay Buffer. Mix well.
- 10.2.3 Using 0.1 nmol ADP standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

| Standard # | Volume of 0.1 nmol Standard (μL) | Assay Buffer IV/Assay Buffer (μL) | Final volume standard in well (μL) | End [ADP] in well |
|------------|---|--|---|-------------------|
| 1 | 0 | 150 | 50 | 0 nmol/well |
| 2 | 6 | 144 | 50 | 0.2 nmol/well |
| 3 | 12 | 138 | 50 | 0.4 nmol/well |
| 4 | 18 | 132 | 50 | 0.6 nmol/well |
| 5 | 24 | 126 | 50 | 0.8 nmol/well |
| 6 | 30 | 120 | 50 | 1.0 nmol/well |

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

NOTE: If your sample readings fall out the range of your fluorometric standard curve, you might need to adjust the dilutions and create a new standard curve.

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 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.

11.1 Cell (adherent or suspension) samples:

- 11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = 2×10^6 cells).
- 11.1.2 Wash cells with cold PBS.
- 11.1.3 Resuspend cells in 100 μ L of Assay Buffer IV/ADP Assay Buffer.
- 11.1.4 Homogenize cells quickly by pipetting up and down a few times. Incubate on ice 10 minutes.
- 11.1.5 Centrifuge sample for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.
- 11.1.6 Collect supernatant and transfer to a clean tube.
- 11.1.7 Keep on ice.

11.2 Tissue samples:

- 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 Resuspend tissue in 100 μ L of Assay Buffer IV/ADP Assay Buffer.
- 11.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes. Incubate on ice 10 minutes.
- 11.2.5 Centrifuge samples for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.
- 11.2.6 Collect supernatant and transfer to a clean tube.
- 11.2.7 Keep on ice.
- 11.3 **Plasma, Serum and Urine and other biological fluids:**
Liquid samples can be measured directly.

NOTE: *We suggest using different volumes of sample to ensure readings are within the Standard Curve range.*

12. ASSAY PROCEDURE and DETECTION

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

12.1 Set up Reaction wells:

- Standard wells = 50 μ L standard dilutions.
- Sample wells = 1 – 50 μ L samples (adjust volume to 50 μ L/well with Assay Buffer IV/ADP Assay Buffer).
- Background control sample wells= 1 – 50 μ L samples (adjust volume to 50 μ L/well with Assay Buffer IV/ADP Assay Buffer). **NOTE:** for samples containing pyruvate as it generates background.

12.2 Reaction Mix (COLORIMETRIC ASSAY):

Prepare 50 μ L of Reaction Mix for each reaction

| Component | Colorimetric Reaction Mix (μ L) | Background Control Reaction Mix (μ L) |
|--|--------------------------------------|--|
| Assay Buffer IV/ADP Assay Buffer | 44 | 46 |
| OxiRed Probe/ADP Probe | 2 | 2 |
| Converter Mix II/ADP Converter | 2 | 0 |
| Development Enzyme Mix I/ADP Developer | 2 | 2 |

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

$X \mu\text{L component} \times (\text{Number samples} + \text{standards} + 1)$

12.3 Reaction Mix (FLUOROMETRIC ASSAY):

Prepare 50 μL of Reaction Mix for each reaction

| Component | Fluorometric Reaction Mix (μL) | Background Control Reaction Mix (μL) |
|--|---|---|
| Assay Buffer IV/ADP Assay Buffer | 45.8 | 47.8 |
| OxiRed Probe/ADP Probe | 0.2 | 0.2 |
| Converter Mix II/ADP Converter | 2 | 0 |
| Development Enzyme Mix I/ADP Developer | 2 | 2 |

**For fluorometric readings, using 0.2 μL /well of the OxiRed Probe/probe decreases the background readings, therefore increasing detection sensitivity.*

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

$X \mu\text{L component} \times (\text{Number samples} + \text{Background control} + \text{Standards} + 1)$.

- 12.4 Add 50 μL of appropriate Reaction Mix to each standard, sample and background control wells.
- 12.5 Incubate at room temperature for 30 minutes protected from light.
- 12.6 Measure output on a microplate reader.
 - Colorimetric assay: measure OD570 nm.
 - Fluorometric assay: measure Ex/Em = 535/587 nm.

13. CALCULATIONS

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
 - 13.1 Average the duplicate reading for each standard and sample.
 - 13.2 If the sample background control is significant, then subtract the sample background control from sample reading
 - 13.3 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.
 - 13.4 Plot the corrected absorbance values for each standard as a function of the final concentration of ADP.
 - 13.5 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).
 - 13.6 Concentration of ADP (as nmol/μL, μmol/mL or mM) in the test samples is calculated as:

$$ADP\ concentration = \left(\frac{A}{B}\right) * D$$

Where:

A = Amount of ADP in the sample well from the standard curve (nmol).

B = Sample volume added into the reaction well (μL).

D = Sample dilution factor.

ADP molecular weight: 427.2 g/mol.

14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

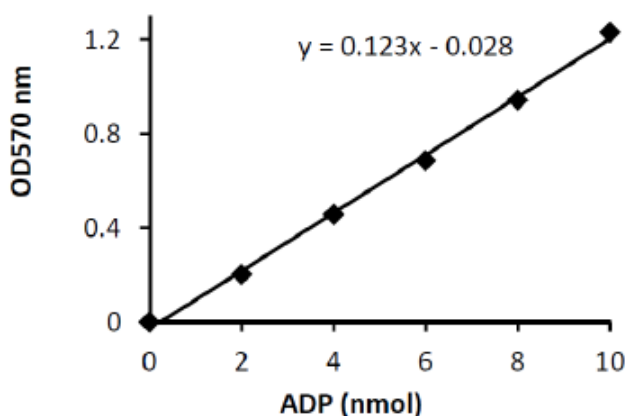


Figure 1. Typical ADP standard calibration curve using colorimetric reading.

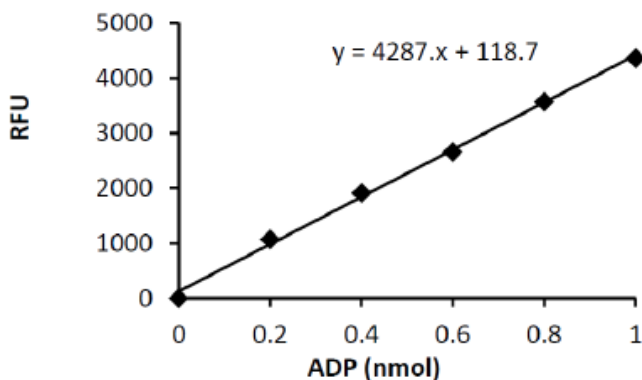


Figure 2. Typical ADP standard calibration curve using fluorometric reading.

15. 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, OxiRed Probe/probe, Converter Mix II/converter and Development Enzyme Mix I/developer mix (aliquot if necessary); get equipment ready.
- Prepare appropriate standard curve for your detection method of choice (colorimetric or fluorometric).
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Set up plate for standard (50 μ L), samples (50 μ L) and background control wells (50 μ L).
- Prepare ADP Reaction Mix (Number samples + background control + standards + 1).

| Component | Colorimetric Reaction Mix (μ L) | Background Reaction Mix (μ L) |
|--|--------------------------------------|------------------------------------|
| Assay Buffer IV/ADP Assay Buffer | 44 | 46 |
| OxiRed Probe/ADP Probe | 2 | 2 |
| Converter Mix II/ADP Converter | 2 | 0 |
| Development Enzyme Mix I/ADP Developer | 2 | 2 |

| Component | Fluorometric Reaction Mix (μ L) | Background Reaction Mix (μ L) |
|----------------------------------|--------------------------------------|------------------------------------|
| Assay Buffer IV/ADP Assay Buffer | 45.8 | 47.8 |
| OxiRed Probe/ADP Probe | 0.2 | 0.2 |
| Converter Mix II/ADP Converter | 2 | 0 |
| Development | 2 | 2 |

| | | |
|---------------------------------------|--|--|
| Enzyme Mix I/ADP Developer | | |
|---------------------------------------|--|--|

- Add 50 μ L of Reaction Mix to the standard, sample and background control wells.
- Incubate plate at RT 30 min protected from light.
- Measure plate at OD 570 nm for colorimetric assay or Ex/Em= 535/587 nm for fluorometric assay.

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 and Standards | 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 |

RESOURCES

| Problem | Cause | Solution |
|--|---|--|
| Standard readings do not follow a linear pattern | Pipetting errors in standard or reaction mix | Avoid pipetting small volumes ($< 5 \mu\text{L}$) and prepare a master mix whenever possible |
| | Air bubbles formed in well | Pipette gently against the wall of the tubes |
| | Standard stock is at incorrect concentration | Always refer to dilutions on protocol |
| 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. FAQ

What is the detection limit of the assay?

This assay can detect as low as 1 μ M ADP. It is specific for ADP and does not detect AMP. Intracellular ADP level is usually in the range of 0.1 – 3 mM.

Does 2.5% perchloric acid interfere with the ADP assay kit? I would like to pre-treat my samples with 2.5% perchloric acid to remove protein prior to testing for ADP.

It is recommended to spin filter over the PCA treatment (you can use 10kD Spin Column (ab93349)). Theoretically the PCA treatment should be fine, but since we have not tested the outcome of this assay with such treated samples, we would recommend using the spin filter to be sure of no adverse effects.

I have sample lysates prepared in Tris, EDTA. 1) Are these compatible with the kit? 2) Can those samples be diluted in Assay Buffer IV/assay buffer or does she need to prepare the samples again just in Assay Buffer IV/assay buffer?

For most efficient results, we always recommend that the samples are made in the Assay Buffer IV/assay buffer provided with the kit. If that is not possible due to sample limitations, you can dilute it in the Assay Buffer IV/assay buffer as a last resort. However, this might compromise the results to a certain extent.

18. INTERFERENCES

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

- Pyruvate.

19. NOTES

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