ELISA PRODUCT INFORMATION & MANUAL

ADP Assay Kit (Colorimetric/ Fluorometric)

NBP2-54856

Enzyme-linked Immunosorbent Assay for quantitative detection. For research use only.
Not for diagnostic or therapeutic procedures.

www.novusbio.com - P: 303.730.1950 - P: 888.506.6887 - F: 303.730.1966 - technical@novusbio.com

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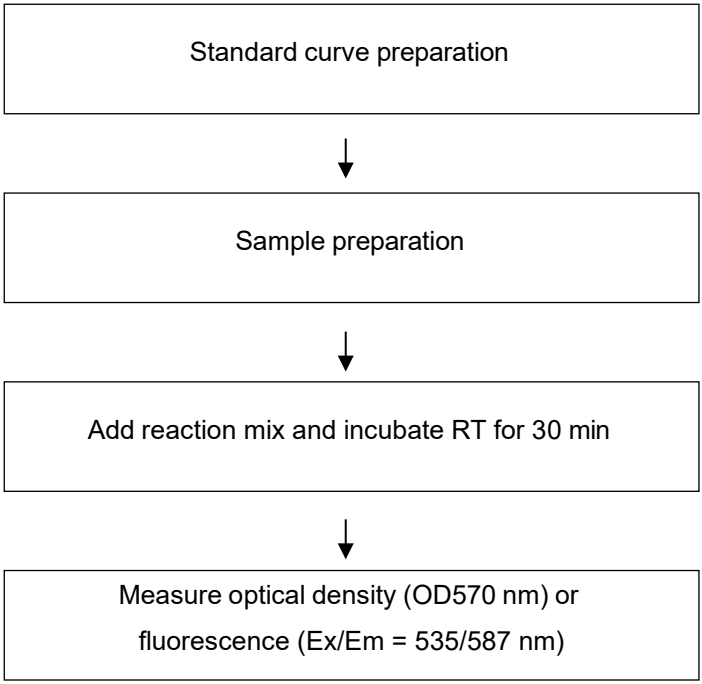
1. BACKGROUND

ADP Assay Kit (Colorimetric/Fluorometric) (NBP2-54856) 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 (ODmax = 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 µM 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 -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)
ADP Assay Buffer	25 mL	-20°C	-20°C
ADP Probe (in DMSO)	200 µL	-20°C	-20°C
ADP Converter	1 vial	-20°C	-20°C
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 (ddH_2O)
- 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 **ADP Assay Buffer:**

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

9.2 **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 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 **ADP Converter:**

Reconstitute in 220 µL ADP Assay Buffer. Pipette up and down to dissolve. Aliquot 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 **ADP Developer Mix:**

Reconstitute in 220 µL ADP Assay Buffer. Pipette up and down to dissolve. Aliquot 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 ddH_2O 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 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 (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 flurometric 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 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 (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 x 10^6 cells).

11.1.2 Wash cells with cold PBS.

11.1.3 Resuspend cells in 100 µL of 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 µL of 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 µL standard dilutions.
- Sample wells = 1 – 50 µL samples (adjust volume to 50 µL/well with ADP Assay Buffer).
- Background control sample wells= 1 – 50 µL samples (adjust volume to 50 µL/well with ADP Assay Buffer). ***NOTE****: for samples containing pyruvate as it generates background.*

12.2 **Reaction Mix (COLORIMETRIC ASSAY):**

Prepare 50 µL of Reaction Mix for each reaction

Component	Colorimetric Reaction Mix (µL)	Background Control Reaction Mix (µL)
ADP Assay Buffer	**44**	**46**
ADP Probe	**2**	**2**
ADP Converter	**2**	**0**
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 µL component x (Number samples + 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)
ADP Assay Buffer	**45.8**	**47.8**
ADP Probe	**0.2**	**0.2**
ADP Converter	**2**	**0**
ADP Developer	**2**	**2**

**For fluorometric readings, using 0.2 µL/well of the 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 µL component x (Number samples + Background control + 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.

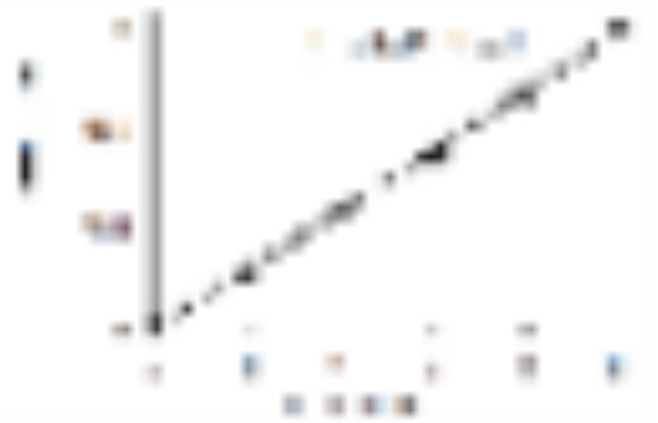


Figure 1. Typical ADP standard calibration curve using colorimetric reading.

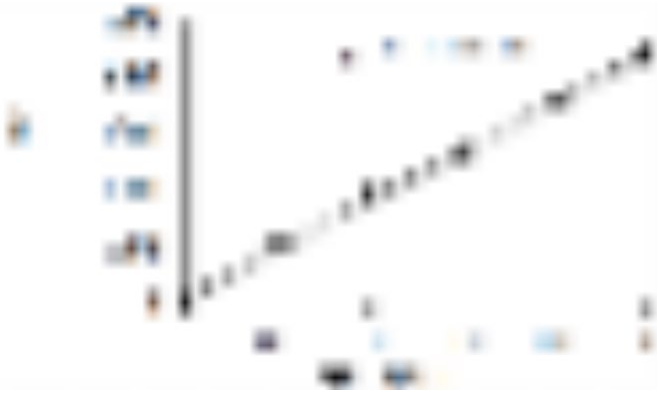


Figure 2. Typical ADP standard calibration curve using fluorometric reading.

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, probe, converter and 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 µL), samples (50 µL) and background control wells (50 µL).
- Prepare ADP Reaction Mix (Number samples + background control + standards + 1).

Component	Colorimetric Reaction Mix (µL)	Background Reaction Mix (µL)
ADP Assay Buffer	**44**	**46**
ADP Probe	**2**	**2**
ADP Converter	**2**	**0**
ADP Developer	**2**	**2**

Component	Fluorometric Reaction Mix (µL)	Background Reaction Mix (µL)
ADP Assay Buffer	**45.8**	**47.8**
ADP Probe	**0.2**	**0.2**
ADP Converter	**2**	**0**
ADP Developer	**2**	**2**

- Add 50 µL 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

Problem	Cause	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) 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 µM 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 or does she need to prepare the samples again just in assay buffer?

For most efficient results, we always recommend that the samples are made in the assay buffer provided with the kit. If that is not possible due to sample limitations, you can dilute it in the 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**