DEVELOP®

INSTRUCTION MANUAL

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General Adenosine Diphosphate (ADP) ELISA Kit

Catalog No: DL-ADP-Ge

96 Tests

FOR RESEARCH USE ONLY; NOT FOR THERAPEUTIC OR DIAGNOSTIC APPLICATIONS!
PLEASE READ THROUGH ENTIRE procedure BEFORE BEGINNING!

INTENDED USE

The kit is a competitive inhibition enzyme immunoassay technique for the in vitro quantitative measurement of ADP in general tissue homogenates, cell lysates or other biological fluids.

REAGENTS AND MATERIALS PROVIDED

Reagents	Quantity	Reagents	Quantity
Pre-coated, ready to use 96-well strip plate	1	Plate sealer for 96 wells	2
Standard	2	Diluent Buffer	1×45mL
Detection Reagent A	1×70μL	TMB Substrate	1×9mL
Detection Reagent B	1×120μL	Stop Solution	1×6mL
Wash Buffer (30 × concentrate)	1×20mL	Instruction manual	1

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Microplate reader with 450 ± 10nm filter.
2. Precision single or multi-channel pipettes and disposable tips.
3. Eppendorf Tubes for diluting samples.
4. Deionized or distilled water.
5. Absorbent paper for blotting the microtiter plate.
6. Container for Wash Solution.

STORAGE OF THE KITS

1. For unopened kit: All the reagents should be kept according to the labels on vials. The **TMB Substrate, Wash Buffer (30 × concentrate)** and the **Stop Solution** should be stored at **4°C** upon receipt while **the others** should be at **-20°C**.
2. For opened kits: Once the kit is opened, the remaining reagents still need to be stored according to the above storage conditions. In addition, return the unused wells to the foil pouch containing the desiccant pack and reseal along entire edge of zip-seal.

Note:

For the expiration date of the kit, please refer to the label on the kit box. All components are stable until this expiration date.

It is highly recommended to use the remaining reagents within 1 month of opening.

SAMPLE COLLECTION AND STORAGE

- **Tissue homogenates** - The preparation of tissue homogenates will vary depending upon tissue type. For this assay, tissues should be

rinsed in ice-cold PBS(0.01mol/L,pH 7.0-7.2) to remove excess blood thoroughly and weighed before homogenization. Mince the tissues to small pieces and homogenize them in 5-10 mL of PBS with a glass homogenizer on ice (Micro Tissue Grinders also work). The resulting suspension should be sonicated with an ultrasonic cell disrupter or subjected to two freeze-thaw cycles to further break the cell membranes. After that, the homogenates are centrifugated for 5 minutes at 5000×g. Remove the supernate and assay immediately or aliquot and store at ≤-20°C.

- **Cell Lysates** - Cells must be lysed before assaying according to the following directions.

1. Adherent cells should be detached with trypsin and then collected by centrifugation (suspension cells can be collected by centrifugation directly).
2. Wash cells three times in cold PBS.
3. Resuspend cells in PBS (1×) and subject the cells to ultrasonication 4 times (or Freeze cells at ≤-20°C. Thaw cells with gentle mixing. Repeat the freeze/thaw cycle 3 times.).
4. Centrifuge at 1500×g for 10 minutes at 2-8°C to remove cellular debris.

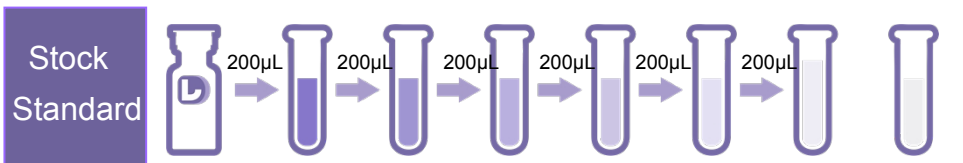
- **Other biological fluids** - Centrifuge samples for 20 minutes at 1000×g. Remove particulates and assay immediately or store samples in aliquots at -20°C or -80°C. Avoid repeated freeze/thaw cycles.

Note:

1. Samples to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination.
2. Sample hemolysis will influence the result, so hemolytic specimen can not be detected.
3. When performing the assay, bring samples to room temperature.

REAGENT PREPARATION

1. **Bring all kit** components and samples to room temperature (18-25°C) before use.
2. **Standard** - Reconstitute the Standard with 1.0mL of Diluent Buffer, keep for 10 minutes at room temperature, shake gently (not to foam). The concentration of the standard in the stock solution is 20ng/mL. Prepare 7 tubes containing 0.2mL Diluent Buffer and use the diluted standard to produce a double dilution series according to the picture shown below. Mix each tube thoroughly before the next transfer. Prepare a dilution series with 7 points; for example: 20ng/mL, 10ng/mL, 5ng/mL, 2.5ng/mL, 1.25ng/mL, 0.625ng/mL, 0.312ng/mL, and the last EP tube with Diluent Buffer is the blank as 0ng/mL.



Tube	1	2	3	4	5	6	7	8
ng/mL	20	10	5	2.5	1.25	0.625	0.312	0

3. **Detection Reagent A and Detection Reagent B** - Briefly spin or centrifuge the stock Detection A and Detection B before use. Dilute to the working concentration with working Diluent Buffer, respectively (1:100).
4. **Wash Solution** - Dilute 20mL of Wash Solution concentrate (30×) with 580mL of deionized or distilled water to prepare 600 mL of Wash Solution (1×).
5. **TMB substrate** - Aspirate the needed dosage of the solution with sterilized tips. Do not dump the residual solution back into the vial.

Note:

1. Do not perform a serial dilution directly in the wells.
2. Prepare standard within 15 minutes before assay. Do not dissolve the reagents at 37°C directly.
3. Detection Reagent A and B are sticky solutions, therefore slowly pipette them to reduce the volume errors.
4. Carefully reconstitute Standards or working Detection Reagent A and B according to the instruction, and avoid foaming and mix gently until the crystals are completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to pipette more than 10μL at a time to ensure accuracy.
5. The reconstituted Standards, Detection Reagent A and Detection Reagent B can be **used only once**.
6. If crystals have formed in the Wash Solution concentrate (30×), warm to room temperature and mix gently until the crystals are completely dissolved.
7. Any contaminated water or container used during reagent preparation will influence the detection result.

SAMPLE PREPARATION

1. DL Sci & Tech Development Co; Ltd. is only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
2. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments. Samples should be diluted by 0.01mol/L PBS(pH=7.0-7.2).

3. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
4. Tissue or cell extraction samples prepared using a chemical lysis buffer may cause unexpected ELISA results due to the impacts from certain chemicals.
5. Due to the possibility of mismatching between antigens from other origin and antibodies used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.
6. Samples from cell culture supernatant may not be detected by the kit due to influence from factors such as cell viability, cell number and/or sampling time.
7. Fresh samples that have not been stored for extended periods of time are recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and give inaccurate or incorrect results.

ASSAY PROCEDURE

1. Determine wells for diluted standard, blank and sample. Prepare 7 wells for standard, 1 well for blank. Add 50μL each of dilutions of standard (read Reagent Preparation), blank and samples into the appropriate wells, respectively. And then add 50μL of Detection Reagent A to each well immediately. Shake the plate gently (using a microplate shaker is recommended). Cover with a Plate sealer. Incubate for 1 hour at 37°C.
2 Aspirate the solution and wash with 300μL of 1× Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold

dispenser or autowasher, and let it sit for 1-2 minutes. Remove the remaining liquid from all wells completely by tapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

3 Add 100μL of Detection Reagent B working solution to each well. Incubate for 1 hour at 37°C after covering it with the Plate sealer.

4 Repeat the aspiration/wash process for total 5 times as conducted in step 2.

5 Add 90μL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for 15-25 minutes at 37°C (Do not exceed 30 minutes). Protect from light. The liquid will turn blue with the addition of Substrate Solution.

6 Add 50μL of Stop Solution to each well. The liquid will turn yellow with the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.

7 Remove any drops of water and fingerprints on the bottom of the plate and confirm there are no bubbles on the surface of the liquid. Run the microplate reader and conduct measurement at 450nm immediately.

Note:

1. **Assay preparation:** Keep appropriate numbers of wells for each experiment and remove extra wells from microplate. Remaining wells should be resealed and stored at -20°C.
2. **Samples or reagents addition: Please use the freshly prepared Standard.** Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of

all standards and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of standards, samples, and reagents. In addition, use separated reservoirs for each reagent.

3. **Incubation:** To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents are added to the well strips, DO NOT let the strips dry at any time during the assay. Incubation time and temperature must be controlled.
4. **Washing:** The wash procedure is critical. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drops of water and fingerprints on the bottom of the plate. Insufficient washing will result in poor precision and false elevated absorbance reading.
5. **Controlling of reaction time:** Observe the change of color after adding **TMB Substrate** (e.g. observation once every 10 minutes), if the color is too deep, add Stop Solution in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. **TMB Substrate** is easily contaminated. Please protect it from light.
7. The environment humidity may have an effect on the results obtained from the kit. If the humidity in your facility is less than 60%, using a humidifier is recommended.

TEST PRINCIPLE

This assay employs the competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific to ADP has been pre-coated

onto a microplate. A competitive inhibition reaction is launched between biotin labeled ADP and unlabeled ADP (Standards or samples) with the pre-coated antibody specific to ADP. After incubation the unbound conjugate is washed off. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. The amount of bound HRP conjugate is **reverse** proportional to the concentration of ADP in the sample. After addition of the substrate solution, the intensity of color developed is **reverse** proportional to the concentration of ADP in the sample.

CALCULATION OF RESULTS

This assay employs the competitive inhibition enzyme immunoassay technique, so there is an inverse correlation between ADP concentration in the sample and the assay signal intensity. Average the duplicate readings for each standard, control, and samples. Create a standard curve on log-log or semi-log graph paper, with the log of ADP concentration on the y-axis and absorbance on the x-axis. Draw the best fit straight line through the standard points, or it can be determined by regression analysis. Using ploting software, (for instance, curve expert 1.30), is also recommended. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

DETECTION RANGE

0.312-20ng/mL. The standard curve concentrations used for the ELISA's were 20ng/mL, 10ng/mL, 5ng/mL, 2.5ng/mL, 1.25ng/mL, 0.625ng/mL, 0.312ng/mL.

SENSITIVITY

The minimum detectable dose of ADP is typically less than 0.101ng/mL.
The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

SPECIFICITY

This assay has high sensitivity and excellent specificity for detection of ADP.
No significant cross-reactivity or interference between ADP and analogues was observed.

Note:

Limited by current skills and knowledge, it is impossible to perform all possible cross-reactivity detection tests between ADP and all analogues, therefore, cross reactivity may still exist.

PRECISION

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level ADP were tested 20 times on one plate, respectively.
Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level ADP were tested on 3 different plates, 8 replicates in each plate.
CV(%) = SD/meanX100
Intra-Assay: CV<10%

Inter-Assay: CV<12%

STABILITY

The stability of ELISA kit is determined by the loss rate of activity. The loss rate of this kit is less than 5% within the expiration date under appropriate storage condition.

Note:

To minimize unnecessary influences on the performance, operation procedures and lab conditions, especially room temperature, air humidity, and incubator temperatures should be strictly regulated. It is also strongly suggested that the whole assay is performed by the same experimenter from the beginning to the end.

ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards;
2. Add 50μL standard or sample to each well.
3. And then add 50μL prepared Detection Reagent A immediately.
4. Shake and mix. Incubate 1 hour at 37°C;
5. Aspirate and wash 3 times;
6. Add 100μL prepared Detection Reagent B. Incubate 1 hour at 37°C;
7. Aspirate and wash 5 times;
8. Add 90μL Substrate Solution. Incubate 15-25 minutes at 37°C;
9. Add 50μL Stop Solution. Read at 450nm immediately.

IMPORTANT NOTES

1. Limited by the current conditions and scientific technology, it is impossible to conduct comprehensive identification and analysis tests

on the raw materials provided by suppliers. As a result, it is possible there are some qualitative and/or technical risks.

2. The final experimental results will be closely related to validity of the products, operation skills of the end users and the experimental environments. Please make sure that sufficient samples are available.
3. Kits from different batches may be a little different in detection range, sensitivity and color developing time. Please perform the experiment exactly according to the instruction manual included in your kit. Electronic ones on our website are for reference only.
4. Do not mix or substitute reagents from one kit lot to another. Use only the reagents supplied by manufacturer.
5. Protect all reagents from strong light during storage and incubation. All bottle caps of reagents should be closed tightly to prevent evaporation of liquids and contamination by microorganisms.
6. There may be a foggy substance in the wells when the plate is opened at the first time. It will not have any effect on the final assay results. Do not remove microtiter plate from the storage bag until needed.
7. Incorrect procedures during the reagents preparation and loading, as well as incorrect parameter setting for the plate reader may lead to incorrect results. A microplate plate reader with a bandwidth of 10nm or less and an optical density range of 0-3 O.D. or greater at 450 ± 10nm wavelength is acceptable for use in absorbance measurement. Please read the instruction carefully and adjust the instrument prior to the experiment.
8. Even the same experimenter may get different results from two separate experiments. In order to get better reproducible results, the operation of every step in the assay should be controlled. Furthermore, a preliminary experiment before the general assay for each batch is recommended.

9. Each kit has undergone several rigorous quality control tests. However, results from end users might be inconsistent with our in-house data due to some unexpected transportation conditions or different lab equipment. Intra-assay variance among kits from different batches could arise from the above factors as well.
10. Kits from different manufacturers with the same item might produce different results, since we have not compared our products with other manufacturers.
11. Validity period: 12 months.
12. The instruction manual also works with the 48T kit, but all reagents in the 48T kit are reduced by half.

PRECAUTION

The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this reagent.