Version 25-09/25

User's Manual

Mouse/Rat Autotaxin/ENPP2 ELISA Kit

REF **DEIA-XY2185**

Σ **96T**

RUO

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

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

For quantitative detection of ENPP2 in serum, plasma, tissue homogenates and other biological fluids.

Principles of Testing

This kit was based on sandwich enzyme-linked immune-sorbent assay technology. Capture antibody was pre-coated onto 96-well plates. And the biotin conjugated antibody was used as detection antibodies. The standards, test samples and biotin conjugated detection antibody were added to the wells subsequently, and washed with wash buffer. HRP-Streptavidin was added and unbound conjugates were washed away with wash buffer. TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the target amount of sample captured in plate. Read the O.D. absorbance at 450nm in a microplate reader, and then the concentration of target can be calculated.

Reagents And Materials Provided

1. ELISA Microplate: (Dismountable) 8×12, 2-8°C/-20°C
2. Lyophilized Standard: 2 vial, 2-8°C/-20°C
3. Biotin-labeled Antibody (Concentrated, 100×): 1 vial, 120 µl, 2-8°C (Avoid Direct Light)
4. HRP-Streptavidin Conjugate (SABC, 100×): 120 µl, 2-8°C (Avoid Direct Light)
5. TMB Substrate: 10 ml, 2-8°C (Avoid Direct Light)
6. Sample Dilution Buffer: 20 ml, 2-8°C
7. Antibody Dilution Buffer: 10 ml 2-8°C
8. SABC Dilution Buffer: 10 ml, 2-8°C
9. Stop Solution: 10 ml, 2-8°C
10. Wash Buffer (25×): 30 ml, 2-8°C
11. Plate Sealer: 5 pieces
12. Product Description: 1 copy

Materials Required But Not Supplied

1. Microplate reader (wavelength: 450 nm)
2. 37°C incubator (CO2 incubator for cell culture is not recommended.)
3. Automated plate washer or multi-channel pipette/5ml pipettor (for manual washing purpose)
4. Precision single (0.5-10 µL, 5-50 µL, 20-200 µL, 200-1000 µL) and multi-channel pipette with disposable tips (calibration is required before use.)
5. Sterile tubes and Eppendorf tubes with disposable tips
6. Absorbent paper and loading slot

7. Deionized or distilled water

Storage

2-8°C/-20°C

Stability

Standard (n=5) 37°C for 1 month: Average 80%

2-8°C for 6 months: Average 95% - 100%

Specimen Collection And Preparation

Serum: Place whole blood sample at room temperature for 2 hours or put it at 2-8°C overnight and centrifugation for 20 minutes at approximately 1000×g, Collect the supernatant and carry out the assay immediately. Blood collection tubes should be disposable, non-pyrogenic, and non-endotoxin.

Plasma: Collect plasma using EDTA-Na_2 or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000 ×g at 2-8°C within 30 minutes of collection. Collect the supernatant and carry out the assay immediately. Avoid hemolysis, high cholesterol samples.

Tissue Homogenates: As hemolysis blood has relation to assay result, it is necessary to remove residual blood by washing tissue with pre-cooling PBS buffer (0.01 M, pH = 7.4). Mince tissue after weighing it and get it homogenized in PBS (the volume depends on the weight of the tissue. Normal, 9mL PBS would be appropriate to 1 gram tissue pieces. Some protease inhibitors are recommended to add into the PBS) with a glass homogenizer on ice. To further break the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5 minutes at 5000 ×g to get the supernatant. The total protein concentration was determined by BCA kit and the total protein concentration of each well sample should not exceed 0.3 mg.

Adherent and Suspension Cell Culture: Use three T25 flasks or one T75 flask for cell culture, the number of cells (1 x 10^7);

1. Suspension cell: centrifuge at 2500 rpm at 2-8°C for 5 minutes; collect clarified cell culture supernatant;
2. Adherent cell: collect supernatant directly; centrifuge at 2500 rpm at 2-8°C for 5 minutes; collect clarified cell culture supernatant for immediate detection or store it separately at -80°C.

Cell Lysate Preparation: Two types of cell lysates are specified below.

1. Suspension Cell Lysate:Centrifuge at 2500 rpm at 2-8°C for 5 minutes; Then add pre-cooling PBS into collected cell and gently mix. Recollect cell by repeating centrifugation. Add 0.5-1ml RIPA lysis buffer (NP-40 lysis buffer or Triton X-100 surfactant is not recommended due to the interfering with antigen-antibody reaction). Add suitable protease inhibitor (e.g. PMSF, working concentration: 1 mmol/L). Lyse the cell on ice for 30 min-1 h. During lysate process, use the tip for pipetting or intermittently shake the centrifugal tube to completely lyse the protein. Alternatively, cells are subject to fragmentation by ultrasonic cell disruptor (300 W, 3~5 s/time, 30s intervals, four-five times) or ultrasonic generator (14 μm for 30s). At the end of lysate or ultrasonic disruption, centrifuge at 10000 rpm at 2-8°C for 10 minutes. Then, the supernatant is added into EP tube and stored at -80°C.
2. Adherent Cell Lysate: Absorb supernatant and add pre-cooling PBS once. Then, add 0.5-1 ml RIPA lysis buffer (NP-40 lysis buffer or Triton X-100 surfactant is not recommended due to the interfering with antigen-antibody reaction). Add the suitable protease inhibitor (e.g. PMSF, working concentration: 1 mmol/L).

Scrape adherent cell gently with a cell scraper. Add the cell suspension into centrifugal tube. Lyse the cell on ice for 30 min-1 h. During lysate process, use the tip for pipetting or intermittently shake the centrifugal tube to completely lyse the protein. Alternatively, cells are subject to fragmentation by ultrasonic generator (14 μm for 30s) or ultrasonic cell disruptor (300 W, 3~5 s/time, 30s intervals, four-five times). At the end of lysate/ultrasonic disruption, centrifuge at 10000 rpm at 2-8°C for 10 minutes. Then, the supernatant is added into EP tube and stored at -80°C.

Other Biological Fluids: Centrifuge samples for 20 minutes at 1000 ×g at 2-8°C. Collect supernatant and carry out the assay immediately.

Note: Samples used within 5 days can be stored at 2-8°C; otherwise, they must be stored at -20°C or -80°C or liquid nitrogen to avoid loss of biological activity and contamination. Avoid multiple freeze-thaw cycles. Hemolytic samples are not suitable for this test.

Reagent Preparation

Take the Elisa kit from the fridge around 20 minutes earlier and equilibrate to room temperature(18-25°C). For repeated assays, please just take the strips and standards required for the current assay, store the rest materials according to the relevant condition.

1. **Wash Buffer**

Dilute 30 ml concentrated wash buffer to 750 ml wash buffer with deionized or distilled water and mix well. (The recommended resistivity of ultrapure water is 18 MΩ.) Alternatively, take appropriate amount of concentrated wash buffer according to the assay requirement, then create a 25-fold dilution and mix well. Store the rest solution at 2-8°C.

Crystals formed in the concentrated wash buffer can be heated by water bath at 40°C till complete dissolution. (Heating temperature should be below 50°C.) Mix well for the next step. It's better to use up the prepared wash buffer in one day. Store the rest buffer at 2-8°C within 48h.

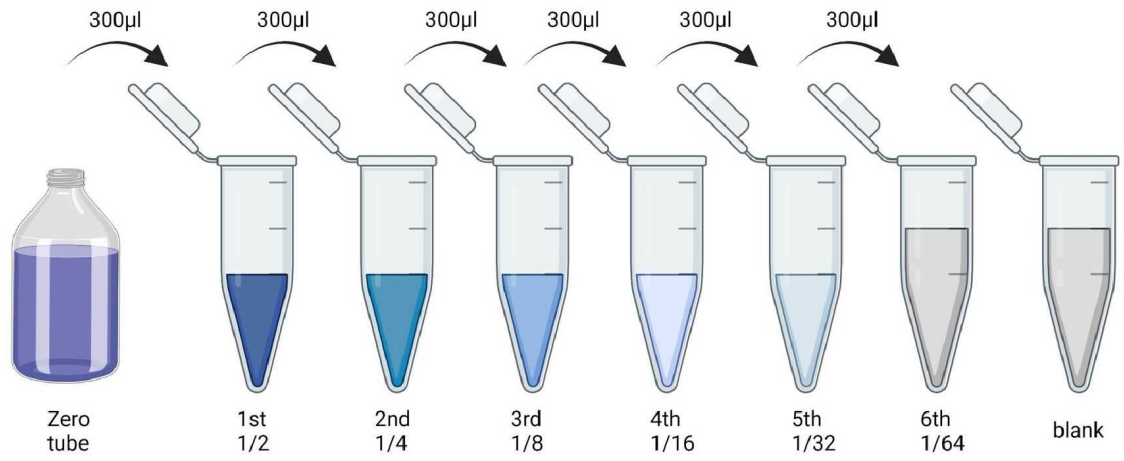
2. **Standards**

2.1. Centrifuge standards tube for 1 min at 10000 xg. Label it as Zero tube. **Note:** If the standard tube concentration higher than the range of the kit,please dilute it and labeled as zero tube.

2.2. Add 1 ml sample dilution buffer into the standard tube. Tighten the tube cap and Let it stand for 2 min at room temperature. Invert the tube several times to mix gently. (Or you can mix it using a low speed vortex mixer for 3-5 seconds.)

2.3. Centrifuge the tubes for 1min at 1000 xg, making the liquid towards the bottom of tube and removing possible bubbles.

2.4. Standard dilution: Label 7 EP tubes with 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and blank respectively. Add 0.3 ml of the sample dilution buffer into each tube. Add 0.3 ml solution from zero tube into 1/2 tube and mix them thoroughly. Transfer 0.3 ml from 1/2 tube into 1/4 tube and mix them thoroughly. Transfer 0.3 ml from 1/4 tube into 1/8 tube and mix them thoroughly, so on till 1/64 tube. Now blank tube only contain 0.3 ml sample dilution buffer.



Notes: It is best to use Standard Solutions within 2 hours.

3. **Preparation of Biotin-labeled Antibody Working Solution**

The working solution should be prepared within 30 min before the assay and can't be stored for a long time.

3.1. Calculate required total volume of the working solution: 100 ul/well x quantity of wells. (It's better to prepare additional 100 µl - 200 µl.)

3.2. Centrifuge for 1min at 1000 xg in low speed and bring down the concentrated biotin-labeled antibody to the bottom of tube.

3.3. Dilute the biotinylated detection antibody with antibody dilution buffer at 1:99 and mix them thoroughly. (e.g. Add 10 µl concentrated biotin-labeled antibody into 990 µl antibody dilution buffer.)

4. **Preparation of HRP-Streptavidin Conjugate (SABC) Working Solution**

The working solution should be prepared within 30 min before the assay and can't be stored for a long time.

4.1. Calculate required total volume of the working solution: 100 ul/well x quantity of wells. (It's better to prepare additional 100 µl - 200 µl.)

4.2. Centrifuge for 1min at 1000 xg in low speed and bring down the concentrated SABC to the bottom of tube.

4.3. Dilute the concentrated SABC with SABC dilution buffer at 1:99 and mix them thoroughly. (e.g. Add 10 µl concentrated SABC into 990 µl SABC dilution buffer.)

Assay Procedure

1. Set standard, test samples (diluted at least 1/2 with Sample Dilution Buffer), control (blank) wells on the pre-coated plate respectively, and then, records their positions. It is recommended to measure each standard and sample in duplicate!
2. Prepare Standards: Aliquot 100 µl of zero tube, 1st tube, 2nd tube, 3rd tube, 4th tube, 5th tube, 6th tube and Sample Dilution Buffer (blank) into the standard wells.
3. Add Samples: Add 100 µl of properly diluted sample into test sample wells.
4. Incubate: Seal the plate with a cover and incubate at 37°C for 90 minutes.
5. Wash: Remove the cover and discard the plate content, and wash plate 2 times with Wash Buffer. Do NOT

let the wells dry completely at any time.

6. Biotin-labeled Antibody: Add 100 µl Biotin-labeled antibody working solution into above wells (standard, test sample and blank wells). Add the solution at the bottom of each well without touching the sidewall, cover the plate and incubate at 37°C for 60 minutes.
7. Wash: Remove the cover, and wash plate 3 times with Wash Buffer, and let the Wash Buffer stay in the wells for 1-2 minutes each time.
8. HRP-Streptavidin Conjugate (SABC): Add 100 µl of SABC Working Solution into each well, cover the plate and incubate at 37°C for 30 minutes.
9. Wash: Remove the cover and wash plate 5 times with Wash Buffer, and let the wash buffer stay in the wells for 1-2 minutes each time.
10. TMB Substrate: Add 90 µl TMB Substrate into each well, cover the plate and incubate at 37°C in dark within 10-20 minutes. (Before adding TMB into wells, equilibrate TMB Substrate for 30 minutes at 37°C.). (Note: The reaction time can be shortened or extended according to the actual color change, but not more than 30 minutes. You can terminate the reaction when apparent gradient appeared in standard wells.)
11. Stop: Add 50 µl Stop Solution into each well. The color will turn yellow immediately. The adding order of Stop Solution should be as the same as the TMB Substrate Solution.
12. OD Measurement: Read the O.D. absorbance at 450 nm in Microplate Reader immediately after adding the stop solution.

Note: If the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

Washing Note:

Manual: Discard the solution in the plate without touching the side walls. Clap the plate on absorbent filter papers or other absorbent material. Fill each well completely with 350ul wash buffer and soak for 1 to 2 minutes, then aspirate contents from the plate, and clap the plate on absorbent filter papers or other absorbent material.

Automatic: Aspirate all wells, and then wash plate with 350ul wash buffer. After the final wash, invert plate, and clap the plate on absorbent filter papers or other absorbent material. It is recommended that the washer shall be set for soaking 1 minute. (Note: set the height of the needles; be sure the fluid can be sipped up completely)

Summary

Step 1: Set standard, test samples, control (blank) wells on the pre-coated plate respectively, and then, records their positions.

Step 2: Add 100 µl standard or sample to each well and incubate for 90 minutes at 37°C.

Wash step: Aspirate and wash plates 2 times.

Step 3: Add 100 µl Biotin-labeled antibody working solution to each well and incubate for 60 minutes at 37°C.

Wash step: Aspirate and wash plates 3 times.

Step 4: Add 100 µl SABC Working Solution into each well and incubate for 30 minutes at 37°C.

Wash step: Aspirate and wash plates 5 times.

Step 5: Add 90 µl TMB Substrate Solution. Incubate 10-20 minutes at 37°C.

Step 6: Add 50 µl Stop Solution. Read at 450nm immediately and calculation.

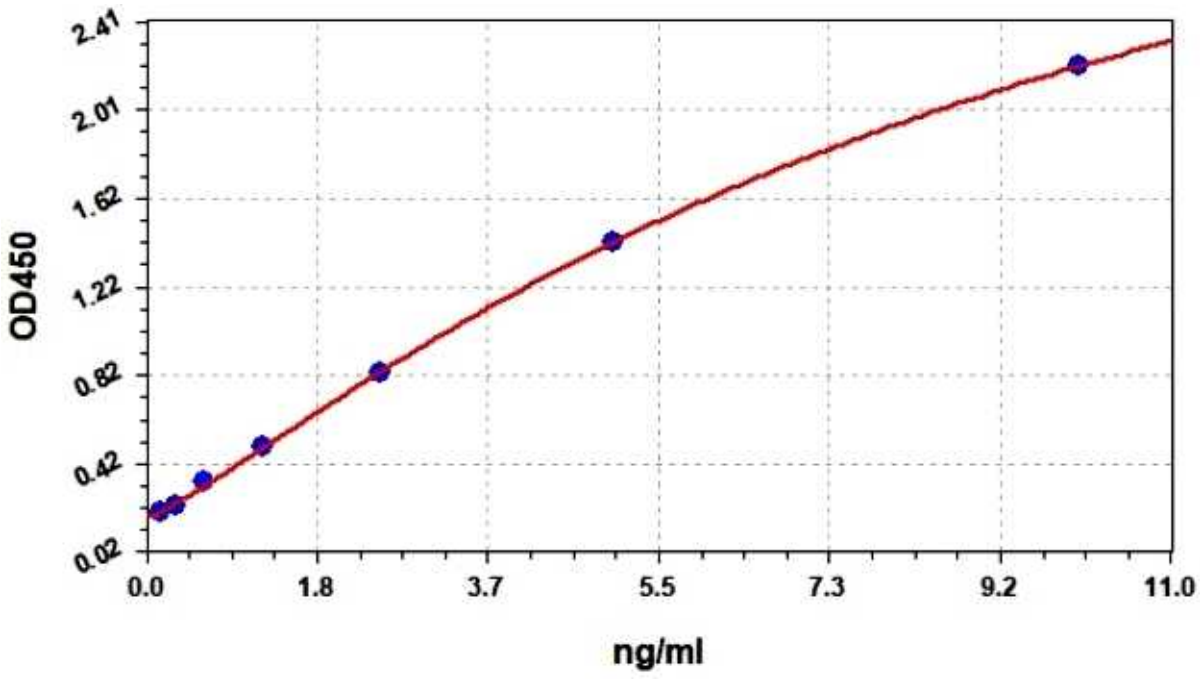
Calculation

Regarding calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of blank well). The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The target concentration of the samples can be interpolated from the standard curve. It is recommended to use some professional software to do this calculation

Typical Standard Curve

Results of a typical standard operation of a Enpp2 ELISA Kit are listed below. This standard curve was generated at our lab for demonstration purpose only. Users shall obtain standard curve as per experiment by themselves. (N/A=not applicable)

STD.(ng/ml)	OD-1	OD-2	Average	Corrected
0	0.179	0.188	0.182	0.09
0.156	0.197	0.207	0.201	0.109
0.312	0.229	0.24	0.233	0.141
0.625	0.328	0.344	0.334	0.242
1.25	0.484	0.508	0.494	0.402
2.5	0.811	0.853	0.828	0.736
5	1.392	1.463	1.42	1.328
10	2.167	2.277	2.211	2.119



Precision

Intra-Assay: CV<8%

Inter-Assay: CV<10%

Detection Range

0.156-10 ng/ml

Sensitivity

0.094 ng/ml

Specificity

This assay has high sensitivity and excellent specificity for detection of Enpp2. No significant cross-reactivity or interference between Enpp2 and analogues was observed.

Note: Limited by current skills and knowledge, it is difficult for us to complete the cross-reactivity detection between Enpp2 and all the analogues, therefore, cross reaction may still exist.

Linearity

Dilute the sample with a certain amount of Enpp2 at 1:2, 1:4 and 1:8 to get the recovery range.

Sample	1:2	1:4	1:8
Serum(n=5)	86-102%	86-103%	85-98%
EDTA Plasma(n=5)	83-99%	92-99%	84-99%
Heparin Plasma(n=5)	89-99%	81-100%	86-99%

Recovery

Matrices listed below were spiked with certain level of Enpp2 and the recovery rates were calculated by comparing the measured value to the expected amount of Enpp2 in samples.

Matrix	Recovery Range (%)	Average (%)
Serum(n=5)	85-103	92
EDTA Plasma(n=5)	86-104	92
Heparin Plasma(n=5)	85-101	93

Precautions

1. To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, pilot experiment using standards and a small number of samples is recommended.

2. After opening and before using, keep plate dry.
3. Before using the kit, spin tubes and bring down all components to the bottom of tubes.
4. Storage TMB reagents avoid light.
5. Washing process is very important, not fully wash easily cause a false positive and high background.
6. Duplicate well assay is recommended for both standard and sample testing.
7. Don't let microplate dry at the assay, for dry plate will inactivate active components on plate.
8. Don't reuse tips and tubes to avoid cross contamination.
9. Please do not mix the reagents in different kits of our company. Do not mix reagents from other manufacturers.
10. To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.