Version 26-08/24

User's Manual

POD (Peroxidase) Assay Kit

REF **DEIA-JY2420**

Σ **96T**

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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 POD in serum, plasma, cell Lysis and other biological samples.

Principles of Testing

This kit is based on enzyme assay method. POD can oxidize TMB to blue solution (max absorption peak: 630 nm). The kit offers POD standard and diluent buffer. The standard and diluted sample were added to the wells subsequently. TMB substrates were added into each well to to visualize the enzyme-substrate reaction(blue), which is stopped by adding sulfuric acid solution later. Read the O.D. absorbance at 450 nm by spectrophotometry. Compare the OD450 value with standard curve using the curve fitting software, and then the concentration of POD in the sample can be calculated.

Reagents And Materials Provided

1. Microplate: 8×12 RT
2. 10×POD Standard (liquid): 2 nmol/ml 120 µL, 2-8°C
3. Diluent buffer: 20 µL, 2-8°C
4. TMB Substrate: 10 mL, 2-8°C (Avoid Direct Light)
5. Stop Solution: 10 mL, 2-8°C
6. Plate Sealer: 5 pieces
7. Product Description: 1 copy

Materials Required But Not Supplied

1. Microplate reader (wavelength: 450 nm)
2. 37°C incubator (CO_2 incubator for cell culture is not recommenced.)
3. Automated plate washer or multi-channel pipette/5 ml 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

The sealed kit can be stored at 2-8 °C.

Specimen Collection And Preparation

1. **Serum**

Place whole blood sample at room temperature for 2 hours or at 2-8°C overnight. Centrifuge for 20 min at 1000 ×g and collect the supernatant to detect immediately. Or you can aliquot the supernatant and store it at -20°C or -80°C for future's assay.

2. **Plasma**

EDTA-Na_2/K_2 is recommended as the anticoagulant. Centrifuge samples for 15 minutes at 1000 ×g 2-8°C within 30 minutes after collection. Collect the supernatant to detect immediately. Or you can aliquot the supernatant and store it at -20°C or -80°C for future's assay. For other anticoagulant types and uses, please refer to the sample preparation guideline.

3. **Tissue Sample**

Generally tissue samples are required to be made into homogenization. Protocol is as below:

3.1. Place the target tissue on the ice. Remove residual blood by washing tissue with pre-cooling normal saline (0.9% NaCl). Then weigh for usage.

3.2. Use normal saline (0.9% NaCl) to grind tissue homogenates on the ice. The adding volume of normal saline (0.9% NaCl) depends on the weight of the tissue. Usually, 9 mL normal saline (0.9% NaCl) would be appropriate to 1 gram tissue pieces. Some protease inhibitors are recommended to add into the PBS (e.g. 1mM PMSF).

3.3. Do further process using ultrasonic disruption or freeze-thaw cycles (Ice bath for cooling is required during ultrasonic disruption; Freeze-thaw cycles can be repeated twice.) to get the homogenates.

3.4. Homogenates are then centrifuged for 5 minutes at 5000 ×g. Collect the supernatant to detect immediately. Or you can aliquot the supernatant and store it at -20°C or -80°C for future's assay.

3.5. Determine total protein concentration by BCA kit for further data analysis. Usually, total protein concentration for Elisa assay should be within 1-3 mg/ml.

Note: The use of other lysates is not recommended, which can cause precipitation and non-color development during TMB incubation.

4. **Cell Culture Supernatant**

Collect the supernatant: Centrifuge at 2500 rpm at 2-8°C for 5 minutes, then collect clarified cell culture supernatant to detect immediately. Or you can aliquot the supernatant and store it at -80°C for future's assay.

5. **Cell Lysate**

5.1. Suspension Cell Lysate: Centrifuge at 2500 rpm at 2-8°C for 5 minutes and collect cells. Then add precooling normal saline (0.9% NaCl) into collected cell and mix gently. Recollect cell by repeating centrifugation. Add 0.5-1 mL normal saline (0.9% NaCl) and appropriate protease inhibitor (e.g. PMSF, working concentration: 1 mmol/L). Disrupt the cell by ultrasonic disruption on ice. (3~5mm probe, 150-300W, 3~5 s/time, 30s intervals for 1~2s working).

5.2. Adherent Cell Lysate: Absorb supernatant and add pre-cooling normal saline (0.9% NaCl) to wash three times. Add 0.5-1 mL normal saline (0.9% NaCl) and appropriate protease inhibitor (e.g. PMSF, working concentration: 1 mmol/L). Scrape the adherent cell with cell scraper. Disrupt the cell by ultrasonic disruption on ice. (3~5mm probe, 150-300W, 3~5 s/time, 30s intervals for 1~2s working).

5.3. At the end of ultrasonic disruption, centrifuge at 10000 rpm at 2-8°C for 10 minutes. Then, the supernatant is added into EP tube to detect immediately. Or you can aliquot the supernatant and store it at - 80°C for future's assay.

Notes: Read notes in tissue sample.

6. **Other Biological Sample**

Centrifuge samples for 15 minutes at 1000 ×g at 2-8°C. Collect the supernatant to detect immediately. Or you can aliquot the supernatant and store it at -80°C for future's assay.

Notes for Samples

1. Blood collection tubes should be disposable and non-endotoxin. Avoid to use hemolyzed and lipemia samples.
2. The best sample storage condition: less than 5 days at 2-8°C; within 6 months at -20°C; within 2 years at -80°C. Stored in liquid nitrogen for a longer storage. When melting frozen samples, rapid water bath at 15 - 25°C can decrease the effect of ice crystal (0°C) on the sample. After melting, centrifuge to remove the precipitate, and then mix well.
3. The detection range of this kit is not equivalent to the concentration of analyze in the sample. For analyses with higher or lower concentration, please properly dilute or concentrate the sample.
4. Pretest is recommended for special samples without reference data to validate the validity.

Recommended Sample Dilution Ratio

Please refer to shipped instructions or contact us for samples, dilution as well background info.

If other dilution ratio for your sample model is required, please refer to the universal dilution ratio below. (The ratio is suitable for single-well assay. For duplicate assay, please follow the calculation: volume of sample and diluent x number of duplicate well)

For 2 fold dilution (1/2): One step dilution. Add 60 µL sample into 60 µL sample diluent and mix gently.

For 5 fold dilution (1/5): One step dilution. Add 24 µL sample into 96 µL sample diluent and mix gently.

For 10 fold dilution (1/10): One step dilution. Add 12 µL sample into 108 µL sample diluent and mix gently.

For 20 fold dilution (1/20): One step dilution. Add 6 µL sample into 114 µL sample diluent and mix gently.

For 50 fold dilution (1/50): One step dilution. Add 3 µL sample and 47 µL normal saline (0.9% NaCl) into 100 µL sample diluent and mix gently.

For 100 fold dilution (1/100): One step dilution. Add 3 µL sample and 177 µL normal saline into 120 µL sample diluent and mix gently.

For 1000 fold dilution (1/1000): Two step dilution. Create a 50-fold dilution first (normal saline is used throughout the dilution). Then, create a 20-fold dilution and mix gently.

For 10000 fold dilution (1/10000): Two step dilution. Create a 100-fold dilution first (normal saline is used throughout the dilution). Then, create the same dilution again and mix gently.

For 100000 fold dilution (1/100000): Three step dilution. Create a 50-fold dilution and 20-fold dilution respectively (normal saline is used in the first two steps.) Finally, create a 100-fold dilution and mix gently.

Notes: The volume in each dilution is not less than 3 µL. Dilution factor should be within 100 fold. Mixing during dilution is required to avoid foaming.

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. Standards

   1.1. Zero tube: add 450 µL Diluent Buffer into one empty EP tube, then add 50ul 10× POD standard and mix well (Working solution after preparation: 1× POD standard, 200 pmol/mL).

   1.2. 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. The standard concentration from zero tube to blank tube is 200 pmol/mL, 100 pmol/mL, 50 pmol/mL, 25 pmol/mL, 12.5 pmol/mL, 6.25 pmol/mL, 3.125 pmol/mL, 0 pmol/mL.

Matrix	Recovery Range (%)	Average (%)
Serum(n=10)	88-103	98
EDTA Plasma(n=10)	86-105	95
Heparin Plasma(n=10)	88-104	96

Assay Procedure

When diluting samples and reagents, they must be mixed completely and evenly. Before adding TMB into wells, equilibrate TMB Substrate for 30 minutes at 37°C. It is recommended to plot a standard curve for each test.

Step 1: Set standard, test samples, 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.

Step 2: Add Samples: Add 100 µL of properly diluted sample or standard into relevant wells.

Step 3: TMB Substrate: Add 100 µL TMB Substrate into each well, cover the plate and incubate at 37°C in dark for 20 minutes.

Step 4: 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.

Step 5: OD Measurement: Read the O.D. absorbance at 450 nm in a microplate reader immediately and calculate.

Calculation

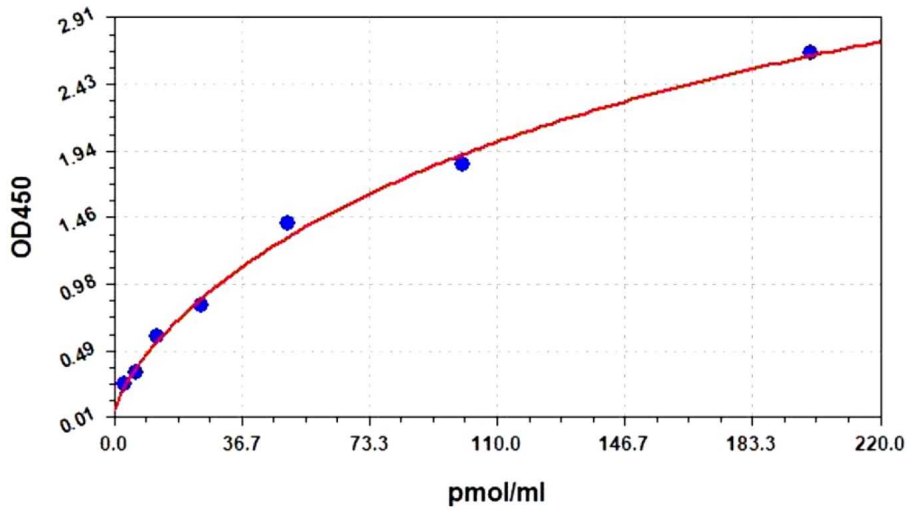
1. Calculate the mean OD450 value of the duplicate readings for each standard, control, and sample . Then, obtain the corrected OD450 by subtracting the OD450 blank.
2. Create a four parameter logistic curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis. (Remove the OD450 blank during plotting.)
3. Calculate the sample concentration by substituting OD450 value into the standard curve. Diluted samples should be multiplied by the relevant dilution ratio.

Typical Standard Curve

This product has been tested by Quality Control Department and meets performance specifications mentioned in the manual. (The humidity in the laboratory is 20%-60%, and the temperature is 18°C - 25°C. TMB was balanced to 37°C before color development, and incubated at 37°C for 15 minutes in the dark after adding the enzyme label plate holes.)

The following assay data are provided for reference, since experimental environment and operation are different. The establishment of standard curve depends on your own assay.

STD.(pmol/ml)	OD-1	OD-2	Average	Corrected
0	0.070	0.066	0.068	0.000
3.125	0.275	0.230	0.252	0.184
6.25	0.333	0.344	0.338	0.270
12.5	0.582	0.609	0.595	0.527
25	0.817	0.820	0.818	0.750
50	1.412	1.435	1.423	1.355
100	1.810	1.892	1.851	1.783
200	2.692	2.615	2.653	2.585



Precision

Intra-assay Precision: samples with low, medium and high concentration are tested 20 times on same plate.

Inter-assay Precision: samples with low, medium and high concentration are tested 20 times on three different plates.

Item	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pmol/ml)	5.88	23.69	102.4	6	23.85	101.41
Standard deviation	0.31	1.21	5.56	0.28	1.49	5.05
CV(%)	5.32	5.12	5.43	4.69	6.23	4.98

Detection Range

3.12-200 pmol/mL

Detection Limit

1.875 pmol/mL

Specificity

Specifically recognize POD, no obvious cross reaction with other analogues.

Linearity

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

Sample	1:2	1:4	1:8
Serum(n=5)	90-98%	88-100%	85-100%
EDTA Plasma(n=5)	84-99%	83-99%	82-96%
Heparin Plasma(n=5)	83-93%	80-93%	86-100%

Recovery

Add a certain amount of POD into the sample. Calculate the recovery by comparing the measured value with the expected amount of POD in the sample.

Matrix	Recovery Range (%)	Average (%)
Serum(n=10)	88-103	98
EDTA Plasma(n=10)	86-105	95
Heparin Plasma(n=10)	88-104	96

Precautions

1. When using different Elisa kits, labeling is required to avoid mixed components and failed assay.
2. After opening the kit, please refer to the table of storage condition for coated plate and standards (Dampness may decrease the activity.). If any component is missing or damaged during the assay or storage, please contact us for ordering a new one to replace.
3. Sterile and disposable tips are required during the assay. After use, the reagents bottle cap has to be tightened to avoid the microbial contamination and evaporation.
4. While manual washing, please keep tips or pipettors for adding wash buffer away from the well. Insufficient washing or contamination easily causes false positive and high background.
5. During the assay, prepare required reagents for next step in advance. After washing, add the reagent into the well in time to avoid dryness. Otherwise, dry plate will result in the failed assay.
6. Before confirmation, reagents from other batches or sources should not be used in this kit.
7. Don't reuse tips and tubes to avoid cross contamination.
8. After loading, seal the plate to avoid the evaporation of the sample during incubation. Complete the incubation process at recommended temperature.
9. Please wear the lab coat, mask and gloves to protect yourself during the assay. Especially, for the detection of blood or other body fluid samples, please follow regulations on safety protection of biological laboratory.