Version 11-07/24

User's Manual

EE(Ethinylestradiol) ELISA Kit

REF **DEIA06009NS**

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 EE in serum, plasma, tissue homogenates, cell lysates, cell culture supernates and other biological fluids.

Principles of Testing

This assay employs the competitive inhibition enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with EE protein. Standards or samples are added to the appropriate microtiter plate wells then with a biotin-conjugated antibody specific to EE. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of EE in the samples is then determined by comparing the OD of the samples to the standard curve.

Reagents And Materials Provided

1. Pre-coated Microplate, 12×8 wells, 4°C/-20°C (6 months)
2. Standard (lyophilized), 2 vials, 4°C/-20°C (6 months)
3. Standard/Sample Diluent Buffer, 20 mL, 4°C
4. Biotinylated-Conjugate (100×), 60 µL, 4°C/-20°C (6 months)
5. Biotinylated Conjugate Diluent, 10 mL, 4°C
6. Streptavidin-HRP (100×), 120µL, 4°C/-20°C (6 months)
7. HRP Diluent, 12 mL, 4°C
8. Wash Buffer (25×), 20mL, 4°C
9. TMB Substrate Solution, 10 mL, 4°C (avoid light)
10. Stop reagent, 6mL, 4°C
11. Plate Covers, 2

Materials Required But Not Supplied

1. Microplate reader (wavelength:450nm)
2. 37°C incubator
3. Automated plate washer
4. Precision single and multi-channel pipette and disposable tips
5. Clean tubes and Eppendorf tubes
6. Deionized or distilled water
7. Vortex mixer
8. Centrifuge

Storage

1. After opening the package, please check whether the items are complete in time.
2. Kit storage: -20°C (for long-term storage, the reagents should be stored at the temperature indicated on the label); 2-8°C (use within one week); avoid repeated freezing and thawing, and do not use after expiration.
3. When the concentrated washing solution is stored at low temperature, there will be salt precipitation. When diluting, it can be heated in a water bath to help dissolve it.
4. There may be a small amount of water-like substances in the wells of the newly opened microtiter plate, which is a normal phenomenon and will not have any impact on the experimental results.
5. All kit components have been tested for formulation and quality control to function successfully as a kit. Do not mix or substitute reagents or materials from other kits.

Specimen Collection And Preparation

1. 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. Store at -20°C or -80°C, and avoid repeated freezing and thawing.

2. 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. Store at -20°C or -80°C, and avoid repeated freezing and thawing.

3. 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 (0.01M, pH=7.0-7.2). 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 5-10 minutes at 5000×g to get the supernatant.

4. Cell culture supernatant: collect supernatant directly; centrifuge at 1000×g at 2-8°C for 20 minutes; collect clarified cell culture supernatant for immediate detection or store it separately at -20°C or -80°C. Avoid repeated freezing and thawing.

5. Urine: Please collect the first morning urine (midstream urine), or 24-hour urine, centrifuge at 2000×g for 15 minutes and collect the supernatant. Store specimens at -20°C and avoid repeated freezing and thawing.

6. Saliva: Collect the sample with a saliva sample collection tube, then centrifuge at 1,000×g for 15 minutes at 2-8°C, take the supernatant for detection, or store it at -20°C after aliquoting. Avoid repeated freeze-thaw cycles.

7. 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 1 week can be stored at 4°C. Otherwise, please aliquot the sample according to the amount used once, and freeze it at -20°C (test within 1 month), or -80°C (test within 3-6 months). Avoid

multiple freeze-thaw cycles. Bring samples to room temperature before use.

Samples should be clear and transparent. Suspended solids should be removed by centrifugation. Hemolytic samples are not suitable for this test.

The user should estimate the concentration of target protein in the test sample, and select a proper dilution factor to make the diluted target protein concentration fall in the optimal detection range of the kit. Dilute the sample with the provided dilution buffer, and several trials may be necessary. The test sample must be well mixed with the dilution buffer. And also standard curves and sample should be making in pre-experiment.

Reagent Preparation

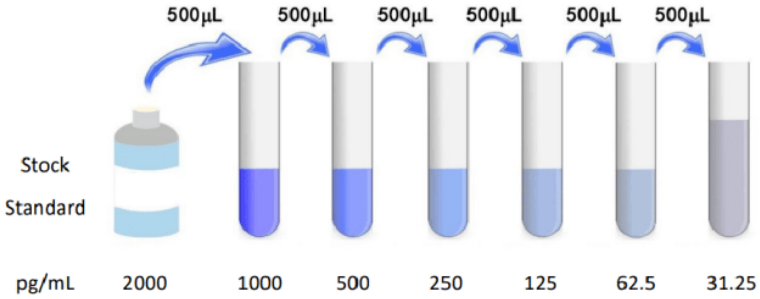
Bring all reagents and samples to room temperature for 30 minutes before use.

1. 1×Wash Buffer:

Dilute the 25× Wash Buffer into 1×Wash Buffer working solution with double distilled water, and return the unused solution to 4°C.

2. Standards:

Standard: Add 1.0 mL of Standard/Sample Diluent Buffer to the lyophilized standard, tighten the cap of the tube, and let it stand for 10 minutes. After it is fully dissolved, mix gently (concentration is 2000pg/mL). Thereafter diluted to 2000pg/mL, 1000pg/mL, 500pg/mL, 250pg/mL, 125pg/mL, 62.5pg/mL, 31.25pg/mL. The Sample Diluent Buffer (0pg/mL) is a blank hole. Configure standard products according to the amount you need for use. It is recommended to add the prepared standard product within 15 minutes, and it is not recommended to store it for a long time.



3. 1×Biotinylated-Conjugate:

Centrifuge before opening the bottle.

a. Calculate required total volume of the working solution: 50µl/well × quantity of wells. (Allow 0.1-0.2ml more than the total volume.)

b. Dilute the Biotinylated-Conjugate with Biotinylated Conjugate Diluent at 1:100 and mix them thoroughly. (i.e. Add 10µl Biotinylated-Conjugate into 990µl Biotinylated Conjugate Diluent.) Prepare within 1 hour before use.

4. 1×Streptavidin-HRP:

a. Calculate required total volume of the working solution: 100µl/well × quantity of wells. (Allow 0.1-0.2ml more than the total volume.)

b. Dilute the Streptavidin-HRP with HRP Diluent at 1:100 and mix them thoroughly. (i.e. Add 10ul of Streptavidin-HRP into 990ul of HRP Diluent.) Prepare within 1 hour before use.

5. TMB Substrate:

Use a pipette to draw up the required dose of solution. Do not pour residual solution back into the reagent bottle.

Assay Procedure

Note: All reagents should be equilibrated to room temperature before starting the experiment. Prepare all reagents in advance. When diluting reagents or samples, they need to be mixed evenly, and try to avoid foaming when mixing. If the concentration of the sample is too high, dilute it with Sample Diluent Buffer so that the sample meets the detection range of the kit. The enzyme label strips in the kit are detachable plates, please use them in batches according to the experimental needs; the remaining kits after use are recommended to be used up within 1 month after the first experiment.

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. Wash plate 2 times before adding standard, sample and control (blank) wells! Add 50 µL of standard or sample to be tested. Be careful not to get air bubbles. Add the sample to the bottom of the well of the microtiter plate, try not to touch the wall of the well. Then add 50 µL of Biotinylated-Conjugate (1×) to each well, shake gently to mix. Cover the microtiter plate and incubate at 37°C for 1 hour.
2. In order to ensure the validity of the experimental results, please use a new standard solution for each experiment
3. Remove the cover, and wash plate 3 times with 200 µl 1×Wash Buffer, and let the wash buffer stay in the wells for 1-2 minute each time, and spin dry.
4. Add 100µL of Streptavidin-HRP (1×) to each well, shake gently to mix. Cover the microtiter plate, and incubate at 37°C for 1 hour.
5. Remove the cover, and wash plate 5 times with 200 µl 1×Wash Buffer, and let the wash buffer stay in the wells for 1-2 minute each time, and spin dry.
6. Add 90µl TMB Substrate into each well, cover the plate and incubate at 37°C in dark within 15-20 minutes. (Note: within 20 minutes, the first 3-4 wells of the standard can be seen with the naked eye at this time. 4 well gradient is not obvious, it can be terminated)
7. Add 50µl 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. In order to ensure the accuracy of the experimental results, the stop solution should be added as soon as possible after the substrate reaction time is up.
8. Read the O.D. absorbance at 450nm in Microplate Reader immediately after adding the stop solution.

Calculation

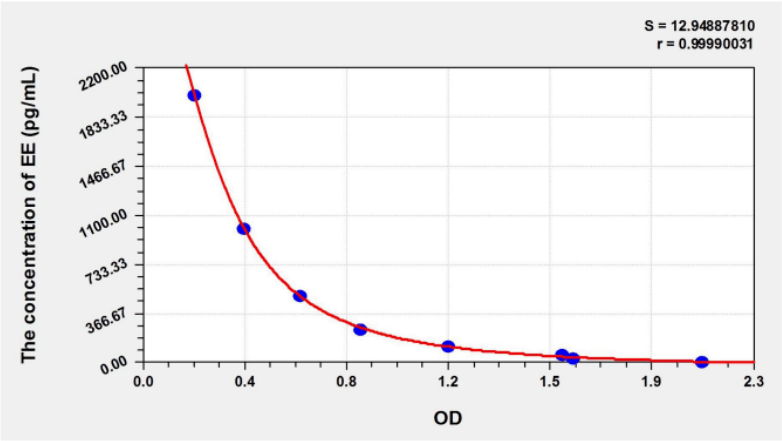
The OD values of competition method standards and samples can be directly substituted into the calculation. If multiple holes are set, the average value should be used for calculation.

Regarding calculation, the standard curve can be plotted as the 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 EE 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)

Concentration (pg/mL)	OD
2000	0.197
1000	0.385
500	0.596
250	0.823
125	1.157
62.5	1.583
31.25	1.624
0	2.112



Precision

Intra-assay Precision (Precision within an assay): CV%<8%

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays): CV%<10%

Three samples of known concentration were tested in forty separate assays to assess inter-assay precision.

Detection Range

31.25-2000 pg/mL

Sensitivity

9.34 pg/mL

Specificity

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

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

Linearity

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of EE and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Matrix	1:2	1:4	1:8	1:16
serum(n=5)	81-94%	96-105%	93-106%	82-95%
EDTA plasma(n=5)	87-98%	92-105%	95-107%	82-96%
Heparin plasma(n=5)	88-101%	93-102%	87-98%	83-97%

Recovery

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

Matrix	Recovery range	Average
serum(n=5)	83-99%	95%
EDTA plasma(n=5)	85-97%	91%
Heparin plasma(n=5)	85-97%	89%

Precautions

1. Please store the kit according to the instruction manual before use. If the reconstituted standard is not used up, please discard it.
2. Biotinylated-Conjugate (100×), concentrated enzyme conjugates are small in volume, and may be dispersed in various parts of the tube during transportation. Please centrifuge at 1000×g for 1 minute before use to allow the liquid on the tube wall or bottle cap to settle to the bottom of the tube . Carefully pipette 4-5 times to mix the solution before use. Please prepare the standard product, Biotinylated-Conjugate working solution, and Streptavidin-HRP working solution according to the required dosage, and use the corresponding diluent to prepare, so as not to confuse them.

3. When the concentrated washing solution is stored at low temperature, there will be salt precipitation. When diluting, it can be heated in a water bath to help dissolve it.
4. The sample addition needs to be done quickly, and it is best to control each sample addition within 10 minutes. In order to ensure the accuracy of the experiment, it is recommended to use duplicate holes. When pipetting reagents, maintain a consistent order of addition from well to well, this will ensure the same incubation time for all wells.
5. The wash buffer remaining in the reaction well should be patted dry on absorbent paper during the washing process. Do not put the filter paper directly into the reaction well to absorb water. Before reading, pay attention to remove the residual liquid and fingerprints at the bottom, so as not to affect the reading of the microplate reader.
6. TMB Substrate should avoid direct exposure to strong light during storage and use. After adding the substrate, pay attention to observe the color change in the reaction well. If the gradient is obvious, please stop the reaction in advance to avoid the effect of too dark color on the microplate reader reading.
7. The test tubes and reagents used in the experiment are disposable, and repeated use is strictly prohibited, otherwise the experimental results will be affected.
8. Please wear lab coats and latex gloves for protection during the experiment, especially when testing blood or other body fluid samples, please follow the national biological laboratory safety protection regulations.
9. Kit components of different batch numbers cannot be mixed (except washing solution and reaction termination solution).