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User's Manual

Ethynylestradiol (EE2) ELISA kit

REF **DEIABL456**

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 detection of Ethynylestradiol (EE2) in test samples.

Principles of Testing

The test is based on the recognition of EE2 by specific monoclonal antibodies. EE2 present in the sample and an EE2-enzyme conjugate (i.e. EE2 labeled with a coloring enzyme: HRP) are premixed and added into each well of a microplate, and allowed to compete for limited number of binding sites of specific antibodies immobilized on the surface of the wells. When the EE2 concentration is higher relative to the enzyme conjugate, the EE2 will predominantly bind the antibody and vice versa.

Reagents And Materials Provided

1. Anti-Ethinylestradiol Microplate 96 Wells: (12 × 8 Well strips), -20°C for 6 months.
2. Ethinylestradiol Lyophilized Standard: 2 × 2 ng, -20°C for 6 months.
3. Ethinylestradiol-Biotin Complex: 1 × 120 µL, -20°C for 6 months.
4. 100X Avidin-HRP Conjugate: 1 × 120 µL, -20°C for 6 months.
5. Standard Diluent: 1 × 20 mL, 4°C for 6 months.
6. Biotin Complex Diluent: 1 × 12 mL, 4°C for 6 months.
7. Conjugate Diluent: 1 × 12 mL, 4°C for 6 months.
8. 30X Wash Buffer: 1 × 20 mL, 4°C for 6 months.
9. TMB Substrate: 1 × 9 mL, 4°C for 6 months.
10. Stop Solution: 1 × 6 mL, 4°C for 6 months.

Materials Required But Not Supplied

1. Microplate reader capable of reading absorbance at 450 nm.
2. Automated plate washer (optional).
3. Pipettes capable of precisely dispensing 0.5 µL through 1 mL volumes of aqueous solutions.
4. Pipettes or volumetric glassware capable of precisely measuring 1 mL through 100 mL of aqueous solutions.
5. New, clean tubes and/or micro-centrifuge tubes for the preparation of standards or samples.
6. Absorbent paper or paper toweling.
7. Distilled or deionized ultrapure water.
8. 37°C Incubator (optional)

Storage

Please refer to Reagents And Materials Provided

Specimen Collection And Preparation

• Store samples to be assayed at 2-8°C for 24 hours prior being assayed.

• For long term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.

• Samples not indicated in the manual must be tested to determine if the kit is valid.

• Prepare samples as follows:

1. Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature or overnight at 4°C before centrifugation for 15 minutes at 1,000 x g. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.
2. Plasma - Collect plasma using EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 1,000 x g at 2-8°C within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.
3. Tissue homogenates - Rinse tissue with 1X PBS to remove excess blood, homogenize in 20 mL of 1X PBS and store overnight at ≤ -20°C. Perform two freeze-thaw cycles to break the cell membranes, then centrifuge homogenates for 5 minutes at 5,000 x g. Remove the supernatant and assay immediately or aliquot and store at ≤ -20°C.
4. Cell Lysates - Adherent cells should be washed by cold PBS gently, and then detached with trypsin, and collected by centrifugation at 1,000 x g for 5 minutes (suspension cells can be collected by centrifugation directly). Wash cells three times in cold PBS. Re-suspend cells in fresh lysis buffer at a concentration of 107 cells/mL. If it is necessary, the cells could be subjected to ultrasonication till the solution is clarified. Centrifuge at 1,500 x g for 10 minutes at 2-8°C to remove cellular debris. Assay immediately or aliquot and store at ≤ -20°C.
5. Cell culture supernatants and other biological fluids – Remove particulates by centrifugation and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze/thaw cycles.

Sample Dilution

Target protein concentration must be estimated and appropriate sample dilution selected such that the final target protein concentration falls near the middle of the assay linear dynamic range.

1. Dilute samples using Standard Diluent.
2. Mix diluted samples gently and thoroughly.
3. Pipetting less than 2 µL is not recommended for optimal assay accuracy.
4. Optimal dilution must be determined by the user according to their specific samples.

Reagent Preparation

• Equilibrate all materials to room temperature prior to use and use immediately.

• A. **Ethinylestradiol Assay Standards**

1. Prepare the Ethinylestradiol standards no greater than 2 hours prior to performing experiment. Standards should be held on ice until use in the experiment.
2. Reconstitute one vial of the provided 2,000 pg Lyophilized Standard for each experiment. Prepare a stock

2,000 pg/mL Standard by reconstituting one tube of Lyophilized Standard asfollows:

2.1 Gently spin or tap the vial at 6,000 – 10,000 rpm for 30 seconds to collect all material at the bottom.

2.2 Add 1 mL of Standard Diluent to the vial.

2.3 Seal the vial then mix gently and thoroughly.

2.4 Leave the vial at ambient temperature for 15 minutes.

3. Prepare a set of serially diluted standards as follows:

3.1 Label tubes with numbers 2 – 6.

3.2 Use the undiluted 2,000 pg/mL Standard from step 1.2 as the high standard point (Tube #1).

3.3 Add 600 µL of Standard Diluent to Tube #'s 2 – 6.

3.4 Prepare Standard #2 by adding 300 µL of 2,000 pg/mL Standard (Tube #1) to Tube #2. Mix gently and thoroughly.

3.5 Prepare Standard #3 by adding 300 µL of Standard #2 from Tube #2 to Tube #3. Mixgently and thoroughly.

3.6 Prepare further serial dilutions through Tube #6. Reference the table below as a guide for serial dilution scheme.

3.7 Tube #6 is a blank standard (only Standard Diluent), which should be included with every experiment.

Standard Number (Tube)	Standard To Dilute	Volume Standard to Dilute (µL)	Volume Standard Diluent (µL)	Total Volume (µL)	Final Concentration
1	2,000 pg Lyophilized Standard	NA	1,000	1,000	2,000 pg/mL
2	2,000 pg/mL	300	600	900	666.67 pg/mL
3	666.67 pg/mL	300	600	900	222.22 pg/mL
4	222.22 pg/mL	300	600	900	74.07 pg/mL
5	74.07 pg/mL	300	600	900	24.69 pg/mL
6	NA	0	600	300	0.0 (Blank)

• B. 1× Ethinylestradiol-Biotin Complex

1. Prepare the 1× Ethinylestradiol-Biotin Complex immediately prior to use by diluting the 100× Ethinylestradiol-Biotin Complex 1:100 with Complex Diluent as follows.
2. Briefly and gently mix the 100× Ethinylestradiol-Biotin Complex prior to pipetting.
3. For each well strip to be used in the experiment (8-wells) prepare 1,000 µL 1× EthinylestradiolBiotin Complex by adding 10 µL of 100X Ethinylestradiol-Biotin Complex to 990 µL Complex Diluent.
4. Mix thoroughly and gently. Hold no longer than 2 hours prior to using in procedure.

• C. 1× Avidin-HRP Conjugate

1. Prepare the 1× Avidin-HRP Conjugate immediately prior to use by diluting the 100× AvidinHRP Conjugate1:100 with Conjugate Diluent as follows.

2. Briefly and gently mix the 100× Avidin-HRP Conjugate prior to pipetting.
3. For each well strip to be used in the experiment (8-wells) prepare 1,000 µL 1× Avidin-HRP Conjugate by adding 10 µL of 100× Avidin-HRP Conjugate to 990 µL Conjugate Diluent.
4. Mix thoroughly and gently. Hold no longer than 2 hours prior to using in procedure.

• D. Microplate Preparation

1. Micro-plates are provided ready to use and do not require rinsing or blocking.
2. Unused well strips should be returned to the original packaging, sealed and stored at 4°C.
3. Equilibrate microplates to ambient temperatures prior to opening to reduce potential condensation.

• E. 1× Wash Buffer

1. If crystals have formed in the 30× Wash Buffer concentrate, equilibrate to room temperature and mix gently until crystals have completely dissolved.
2. Add the entire 20 mL contents of the 30× Wash Buffer bottle to 580 mL of ultra-pure water to a clean > 1,000 mL bottle or other vessel.
3. Seal and mix gently by inversion. Avoid foaming or bubbles.
4. Store the 1× Wash Buffer at room temperature until ready to use in the procedure. Store the prepared 1× Wash Buffer at 4°C for no longer than 1 week. Do not freeze.

Assay Procedure

• Take out all the kit contents from the refrigerator and let them reach room temperature (18-25°C) for approximately 30 minutes prior to the assay.

• Do not mix reagents from different kits.

• Store reagents under refrigeration (2-8°C)

• Do not use expired kits.

• Dispose of kit components in accordance with applicable regulations after use.

• Duplicate measurement is recommended for more accurate determination.

• Equilibrate all reagents and materials to ambient room temperature prior to use in the procedure.

• For optimal control of small potential variations in micro well-plate and day to day ambient temperature fluctuations, equilibrate all reagents prior to use and perform all incubation steps at 37°C.

1. Determine the required number of wells and return any remaining unused wells and desiccant to the pouch.
2. Retain at least one well as an absolute Blank without any samples or reagents.
3. Add 50 µL of serially titrated standards, diluted samples or blank into wells of the Anti-Ethinylestradiol Microplate. At least two replicates of each standard, sample or blank is recommended.
4. Immediately add 50 µL of 1× Ethinylestradiol-Biotin Complex to each well (excluding absolute Blank).
5. Cover the plate with the well plate lid and incubate at 37°C for 60 minutes.
6. Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.
7. Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time

8. Wash plate three times with 1× Wash Buffer as follows:

   8.1 Add 350 µL of 1× Wash Buffer to each assay well.

   8.2 Incubate for 2 minutes.

   8.3 Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.

   8.4 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.

   8.5 Repeat steps 8.1 through 8.4 two more times.

9. Add 100 µL of 1× Avidin-HRP Conjugate to each well.
10. Cover the plate with the plate sealer and incubate at 37°C for 30 minutes.
11. Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.
12. Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.
13. Repeat wash five times as in step 8.
14. Add 90 µL of TMB Substrate to each well, cover with plate sealer and incubate at 37°C in the dark for 10-20 minutes. Wells should change to gradations of blue. If the color is too deep based on the standard, adjust incubation times.

(NOTE: optimal incubation time must be determined by the user. Optimal development can be visualized by blue shading in the bottom four standard wells, while the remaining standards still appear clearer.)

15. Add 50 µL of Stop Solution to each well. Well color should change to gradations of yellow immediately. Add the Stop Solution in the same well order as done for the TMB Substrate.
16. Read the O.D. absorbance at 450 nm with a standard microplate reader within 5 minutes of stopping the reaction in step 15. If wavelength correction is available, set to 540 nm or 570 nm.

Calculation

For analysis of the assay results, calculate the Relative OD_{450} for each test or standard well as follows:

(Relative OD_{450}) = (Well OD_{450}) – (Mean Blank Well OD_{450})

The standard curve is generated by plotting the mean replicate Relative OD_{450} of each standard serial dilution point vs. The respective standard concentration. The Ethinylestradiol concentration contained in the samples can be interpolated by using linear regression of each mean sample Relative OD_{450} against the standard curve. This is best achieved using curve fitting software.

Note: if wavelength correction readings are available, subtract the readings at 540 nm or 570 nm from the readings at 450 nm. This may provide greater reading accuracy.

Note: if the samples measured were diluted, multiply the derived mean sample concentration by the dilution factor for a final sample concentration.

Typical Standard Curve

pg/mL	Absorbance		Mean Absorbance	Log of concentration
	Rep 1	Rep 2		
2,000	0.075	0.101	0.088	3.301
666.7	0.286	0.295	0.291	2.824
222.2	0.602	0.633	0.618	2.347
74.07	1.236	1.192	1.214	1.870
24.69	1.969	1.936	1.953	1.393

Performance Characteristics

Intra-assay reproducibility was evaluated with 20 replicates of 3 samples representing low, middle and high level target. Inter-assay reproducibility was evaluated with 3 samples representing low, middle and high level target using 8 replicates on each of 3 plates.

Sample	Intra-Assay			Inter-Assay		
	1	2	3	1	2	3
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/ml)	71.81	223.24	665.98	72.47	225.28	668.92
SD	2.974	11.984	42.310	3.149	12.836	46.182
CV (%)	4.1	5.4	6.4	4.3	5.7	6.9

Specificity

NA

Linearity

Linearity of the kit was evaluated by spiking Ethinylestradiol into matrices, serially diluted and measured. Observed values were compared to the expected measurements.

Matrix	1:2 Dilution	1:4 Dilution	1:8 Dilution	1:16 Dilution
Serum (n=5)	90-104%	78-89%	87-101%	83-97%
EDTA Plasma (n=5)	92-102%	81-94%	84-98%	79-93%
Heparin Plasma (n=5)	79-92%	90-99%	80-95%	82-96%

Recovery

Matrices were spiked with Ethinylestradiol and recovery rates were calculated by comparing the measured values to the expected concentrations.

Matrix	Recovery Range (%)	Mean Recover (%)
Serum (n=5)	81-97	90
EDTA Plasma (n=5)	90-105	97
Heparin Plasma (n=5)	84-99	92

Precautions

1. Do not mix or substitute components from other kits.
2. To ensure the validity of experimental operation, it is recommended that pilot experiments using standards and a small selection of sample dilutions to ensure optimal dilution range for quantitation.
3. Samples exhibiting OD measurements higher than the highest standard should be diluted further in the appropriate sample dilution buffers.
4. Prior to using the kit, briefly spin component tubes to collect all reagents at the bottom.
5. Replicate wells are recommended for standards and samples.
6. Cover microplate while incubating to prevent evaporation.
7. Do not allow the microplate wells dry at any point during the assay procedure.
8. Do not reuse tips or tube to prevent cross contamination.
9. Avoid causing bubbles or foaming when pipetting, mixing or reconstituting.
10. Completely remove of all liquids when washing to prevent cross contamination.
11. Prepare reagents immediately prior to use and do not store, with the exception of the top standard.
12. Equilibrate all materials to ambient room temperature prior to use (standards exception).
13. For optimal results in inter- intra- assay consistency, equilibrate all materials to room temperature prior to performing assay (standards exception) and perform all incubations at 37°C.
14. Pipetting less than 1 µL is not recommended for optimal assay accuracy.
15. Once the procedure has been started, all steps should be completed without interruption. Ensure that all reagents, materials and devices are ready at the appropriate time.
16. Incubation times will affect results. All wells should be handled in the same sequential order and time intervals for optimal results.
17. Samples containing precipitates or fibrin strands or which are hemolytic of lipemic might cause inaccurate results due to interfering factors.
18. TMB Substrate is easily contaminated and should be colorless or light blue until added to plate. Handle carefully and protect from light.