Total Estrogens ELISA Assay Kit

Catalog Number: ESG31-K01

For Research Use Only. Not for use in diagnostic procedures.

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INTENDED USE

The Eagle Biosciences Total Estrogens ELISA Assay Kit (enzyme-linked immunoassay kit) is intended for the direct quantitative determination of Total Estrogens in human serum. The Eagle Biosciences Total Estrogens ELISA Assay Kit is for research use only and not to be used in diagnostic procedures.

For further information about this kit, its application, or the procedures in this insert, please contact the Technical Service Team at Eagle Biosciences, Inc at www.EagleBio.com or at 866-411-8023.

INTRODUCTION

Total estrogens comprise the total quantity of estrone, estradiol, and estriol. The estrogens are involved in the development of female sex organs and secondary sex characteristics. Before the ovum is fertilized, the main action of the estrogens is on the growth and function of the reproductive tract to prepare it for the fertilized ovum.

During the follicular phase of the menstrual cycle, the total estrogens level shows a slight increase. The production of the total estrogens then increases markedly to peak at around day 13. The peak is of short duration and by day 16 of the cycle levels will be low. A second peak occurs at around day 21 of the cycle. If fertilization doesn't occur, the production of total estrogens decreases.

In post-menopausal women, the concentration of all estrogens decreases substantially and estrone becomes the predominant estrogen. In pregnant women, the concentration of all estrogens escalates and estriol becomes the predominant estrogen.

A total estrogens test is commonly indicated to:

- Aid in diagnosis or delayed puberty, and aromatase and 17 alpha-hydroxylase deficiencies.
- Follow-up female hormone replacement therapy in post-menopausal women.
- Prognose antiestrogen therapy, for example, aromatase inhibitor therapy.

PRINCIPLE OF THE ASSAY

The Total Estrogens ELISA is a competitive immunoassay. Competition occurs between total estrogens (estrone, estradiol, and estriol) present in calibrators, controls, specimen samples and an enzyme-labelled antigen (HRP conjugate) for a limited number of anti-estrogen antibody binding sites on the microplate wells. After a washing step that removes unbound materials, the TMB substrate (enzyme substrate) is added which reacts with HRP to form a blue-colored product that is inversely proportional to the amount of total estrogens present. Following an incubation, the enzymatic reaction is terminated by the addition of the stropping solution, converting the color from blue to yellow. The absorbance is measured on a microplate reader at 450 nm. A set of calibrators is used to plot a calibrator curve from which the amount of total estrogens in specimen samples and controls can be directly read.

PROCEDURAL CAUTIONS AND WARNINGS

1. This kit is for use by trained laboratory personnel (professional use only). For laboratory research use only.

2. Practice good laboratory practices when handling kit reagents and specimens. This includes:
    a. Do not pipette by mouth.
    b. Do not smoke, drink, or eat in areas where specimens or kit reagents are handled.
    c. Wear protective clothing and disposable gloves. d Wash hands thoroughly after performing the test. e Avoid contact with eyes, use safety glasses; in case of contact with eyes, flush eyes with water immediately and contact a doctor.
3. Users should have a thorough understanding of this protocol for the successful use of this kit. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
4. Do not use this kit beyond the expiry date stated on the label.
5. If the kit reagents are visibly damaged, do not use the test kit.
6. Do not use kit components from different kit lots within a test and do not use any component beyond the expiration date printed on the label.
7. All kit reagents and specimens must be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of specimens.
8. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
9. Immediately after use, each individual component of the kit must be returned to the recommended storage temperature stated on the label.
10. A calibrator curve must be established for every run.
11. It is recommended to all customers to prepare their own control materials or saliva pools which should be included in every run at a high and low level for assessing the reliability of results.
12. The controls (included in kit) must be included in every run and their results must fall within the ranges stated in the quality control certificate; a failed control result might indicate improper reagent storage.
13. When dispensing the substrate and stopping solutions, do not use pipettes in which these liquids will come into contact with any metal parts.
14. The TMB Substrate is sensitive to light and should remain colorless if properly stored. Instability or contamination may be indicated by the development of a blue color, in which case it should not be used.
15. Do not use blood contaminated saliva samples.
16. Samples or controls containing azide or thimerosal are not compatible with this kit, they may lead to false results.
17. Samples values above the measuring range of the kit may be reported as >80 pg/mL. If further dilution and retesting is required, only Calibrator A may be used to dilute saliva samples. The use of any other reagent may lead to false results.
18. Avoid microbial contamination of reagents.
19. To prevent the contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, calibrator, and control.
20. To prevent contamination of reagents, do not pour reagents back into the original containers.
21. Kit reagents must be regarded as hazardous waste and disposed of according to local and/or national regulations.
22. Consumables used with the kit that are potentially biohazardous (e.g., pipette tips, bottles or containers containing human materials) must be handled according to

biosafety practices to minimize the risk of infection and disposed of according to local and/or national regulations relating to biohazardous waste.

23. This kit contains 1 M sulfuric acid in the stopping solution component. Do not combine acid with waste material containing sodium azide or sodium hypochlorite.
24. The use of safety glasses, and disposable plastic, is strongly recommended when manipulating biohazardous or bio-contaminated solutions.
25. Proper calibration of the equipment used with the test, such as the pipettes and absorbance microplate reader, is required.
26. If a microplate shaker is required for the assay procedure, the type and speed of shaker required is stated in the REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED section. Both the type and speed of saker used can influence the optical densities and test results. If a different type of shake and/or speed is used, the user is responsible for validating the performance of the kit.
27. Do not reuse the microplate wells, they are for SINGLE USE only.
28. To avoid condensation within the microplate wells in humid environments, do not open the pouch containing the microplate until it has reached room temperature.
29. When reading the microplate, the presence of bubbles in the wells will affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.

SAFETY CAUTIONS AND WARNINGS

POTENTIAL BIOHAZARDS MATERIAL

Human serum that may be used in the preparation of the standards and controls has been tested and found to be nonreactive for Hepatitis B surface antigen and has also been tested for the presence of antibodies to HCV and Human Immunodeficiency Virus (HIV) and found to be negative. No testing method, however, can offer complete assurance that HIV, HCV and Hepatitis B virus or any infectious agents are absent. The reagents should be considered a potential biohazard and handled with the same precautions as applied to any blood specimen.

CHEMICAL HAZARDS

Avoid direct contact with any of the kit's reagents. Specifically avoid contact with the TMB substrate (contains tetramethylbenzidine) and Stopping Solution (contains sulfuric acid). If contacted with any of these reagents, wash with plenty of water and refer to the SDS for additional information.

SPECIMEN COLLECTION AND STORAGE

Approximately 0.15 mL of serum is required per duplicate determination. Collect 4–5 mL of venous blood into an appropriately labelled tube and allow it to clot. Centrifuge at room temperature and carefully transfer the serum into a new storage tube or container. Serum samples may be stored at 2-8^{o}C for up to 24 hours or at -10^{o}C or lower if the analyses are to be done later.

Consider all human specimens as possible biohazardous materials and take appropriate precautions when handling.

SPECIMEN PRETREATMENT

This assay is a direct system; no specimen pretreatment is necessary.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated single-channel pipette to dispense 50 µL
2. Calibrated multi-channel pipettes to dispense 50 µL and 150 µL
3. Calibrated multi-channel pipettes to dispense 350 µL (if washing manually)
4. Automatic microplate washer (recommended)
5. Microplate shaker:
    a. Orbital shaker (3mm diameter) set to 600 rpm or
    b. Reciprocating shaker (1.5" stroke length) set to 180 oscillations/min
6. Disposable pipette tips
7. Distilled or deionized water
8. Calibrated absorbance microplate reader with 450 nm filter and an upper OD limit of 3.0 or greater.

MATERIALS PROVIDED

1. **Microplate** — Ready To Use

    Contents: One 96-well (12x8) anti-estrogens polyclonal antibody-coated microplate in a resealable pouch with desiccant.
    Storage: Refrigerate at 2–8°C
    Stability: Unopened: Stable until expiration date on kit. After Opening: Stable for three weeks.

2. **(HRP) Conjugate Concentrate** — Ready To Use

    Contents: One bottle containing Estrogen-Horse Radish Peroxidase (HRP) conjugate in a protein-based buffer with a non-mercury preservative
    Volume: 20 mL/bottle
    Storage: Refrigerate at 2–8°C
    Stability: Unopened: Stable until expiration date on kit. After Opening: Stable for three weeks.

3. **Calibrators** — Ready To Use

    Contents: Eight (8) vials of calibrator containing specified Estrogen concentrations. Protein-based buffer with a non-mercury preservative. Prepared by spiking matrix with defined quantities of Estrogen.

Calibrator	Concentration	Volume/Vial
A	0 pg/mL	2.0 mL
B	25 pg/mL	1.0 mL
C	50 pg/mL	1.0 mL
D	100 pg/mL	1.0 mL
E	250 pg/mL	1.0 mL
F	500 pg/mL	1.0 mL
G	1000 pg/mL	1.0 mL
H	2500 pg/mL	1.0 mL

Storage: Refrigerate at 2–8°C.
Stability: Unopened: Stable until the expiration date on label
After Opening: Stable for three weeks.

4. Controls — Ready to Use

Contents: Two bottles of control containing different Estrogen concentrations. Protein-based buffer with a non-mercury preservative. Prepared by spiking matrix with defined quantities of Estrogen. Refer to QC for target values and acceptable ranges. The concentrations of the controls were verified by a second party with a CDC HoSt certified method.
Volume: 1.0 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: Unopened: Stable until the expiration date on label
After Opening: Stable for three weeks.

5. **Wash Buffer Concentrate** — Requires Preparation x10

Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.
Volume: 50 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: Unopened: Stable until the expiration date on label
After Opening: Stable for three weeks. Prepared buffer is stable for two weeks assuming GLP are adhered to.
Preparation: Dilute the wash buffer concentrate 1:10 in distilled or deionized water to prepare the working wash buffer. If one whole plate is to be used dilute 50 mL of the wash buffer concentrate in 450 mL of water.

6. **TMB Substrate** — Ready To Use

Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.
Volume: 16 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: Unopened: Stable until the expiration date on label
After Opening: Stable for three weeks.

7. **Stopping Solution** — Ready To Use

Contents: One bottle containing 1M sulfuric acid.
Volume: 6 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: Unopened: Stable until the expiration date on label
After Opening: Stable for three weeks.

ASSAY PROCEDURE

Specimen Pretreatment: None.
All reagents must reach room temperature before use. Mix gently by inversion. Calibrators, controls, and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

1. Prepare the Wash Buffer Working Solution *(see Materials Provided for how to dilute Wash Buffer Concentrate)*
2. Plan the microplate wells to be used for calibrators, controls, and samples. Remove the strops from the microplate from that will not be used and place them in the bag with desiccant. Reseal the bag with unused strips and return to proper storage.
3. **Pipette 50 µL** of each calibrator, control, and specimen sample into correspondingly labelled wells in duplicate.
4. **Incubate** the microplate on a microplate shaker for **30 minutes** at room temperature
5. **Pipette 150 µL** of the HRP conjugate into each well
6. **Incubate** the microplate on a microplate shaker for **120 minutes** at room temperature
7. **Wash** the microplate well with an automatic microplate washer (preferred) or manually. Perform 3 wash cycles. Aspirate all wells and then tap the microplate firmly against absorbent paper to remove any residual liquid.
8. **Pipette 150 µL** of TMB Substrate into each well
9. **Incubate** the microplate on a microplate shaker for **30 minutes** at room temperature
10. **Pipette 50 µL** of Stopping Solution into each well in the same order and speed as was used for addition of TMB Substrate. Gently tap the microplate frame to mix the contents of the well.
11. **Measure** the optical density (absorbance) in the microplate wells using an absorbance microplate reader to 450 nm, within 20 minutes after addition of Stopping Solution

CALCULATIONS

1. Calculate the mean optical density for each calibrator, control and specimen sample duplicate.
2. Use a 4-parameter or 5-parameter curve fit with immunoassay software to generate a calibrator curve.
3. The immunoassay software will calculate the concentrations of the controls and specimen samples using the mean optical density values and the calibrator curve.
4. If sample reads more than 2500 pg/mL and needs to be diluted and retested, then dilute with calibrator A not more than 1:10. The rest obtained must be multiplied by the dilution factor.

QUALITY CONTROL

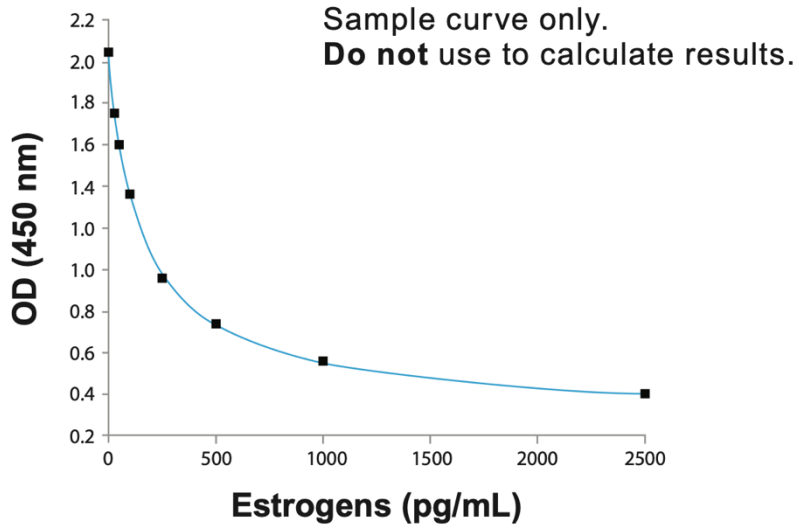
When assessing the validity of the test results, the following criteria should be evaluated.

1. The calibrator A mean optical density meets the acceptable range as stated in the QC certificate.
2. The calibrator with the highest concentration meets the % binding acceptable range as stated in the QC certificate. % Binding = (OD of calibrator/OD of calibrator A) X 100.
3. The values obtained for the kit controls are within the acceptable ranges as stated in the QC Certificate.
4. The results of any external controls that were used meet the acceptable ranges.

TYPICAL TABULATED DATA

Calibrator	Mean OD	% Binding	Value (ng/mL)
A	2.044	100	0
B	1.755	86	25
C	1.609	79	50
D	1.386	67	100
E	0.964	47	250
F	0.744	36	500
G	0.561	27	1000
H	0.407	20	2500
Unknown	0.791	-	400

TYPICAL CALIBRATOR CURVE



SENSITIVITY

The lower detection limit was calculated following EP17-A2. Sixty replicates of the matrix and a low concentration sample were run in independent tests with two lots of the kit. The Limit of Background determined to be 5.4 pg/mL and the Limit of Detection was determined to be 12.4 pg/mL.

SPECIFICITY (CROSS-REACTIVITY)

The following compounds were tested for cross-reactivity in relation to estrogens cross-reacting at 100%

Compound	% Cross-Reactivity
Estrone	100
17β-Estradiol	100
Estriol	100
11-Deoxycorticosterone	0.4
17-Hydroxyprogesterone	0.3
17α-Estradiol	5.3
Aldosterone	0.2
Androsterone	0.2
Cholesterol	0
Corticosterone	<0.01
Cortisol	<0.1
DHEA	0.3
DHEA-S	0.004
DHT	0.5
Equilin	6.3
Estradiol sulfate	0.1
Estrone sulfate	0.07
Prednisone	0
Pregnenolone	<0.1
Pregnenolone sulfate	<0.1
Progesterone	<0.1
Testosterone	0.3

INTERFERENCES

Hemoglobin up to 2 g/L, Bilirubin conjugates and unconjugated up to 10 mg/dL, Triglycerides up to 5 mg/mL, Biotin up to 2.4 g/mL, HAMAS up to 1.2 µg/mL, and Rheumatoid Factor up to 1500 IU/mL did not interfere with the assay.

*Note on Fulvestran

Estradiol immunoassays have been reported to show interference from the drug Fulvestran. This cross-reactivity can cause falsely elevated estrogen levels in samples treated with Fulvestran.

The following results were obtained with the Total Estrogens ELISA kit after pooled serum samples from three cohorts were spiked to a concentration of 25 ng/mL of Fulvestran.

Sample	Unspiked Sample (pg/mL)	% Binding
Pool 1	106.8	128.6
Pool 2	87.8	105.8
Pool 3	326.4	377.6

PRECISION

The experimental protocol used a nested components-of-variance design with 10 testing days, two runs per scientist per day, and two replicate measurments per run (a 10x2x2x2 design) for each sampls. The results were analyzed with a two-way nested ANOVA and summarized in the table below

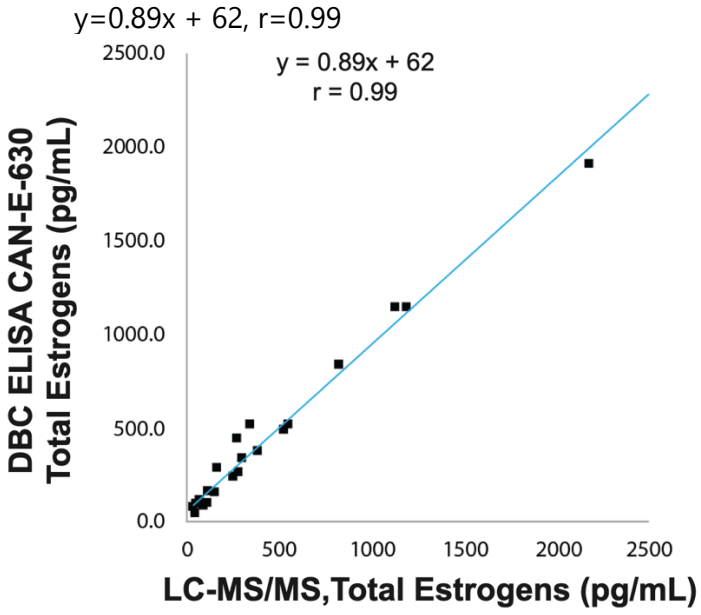
Sample	Mean	Within Run SD (pg/mL)	Within Run CV%	Between Run SD (pg/mL)	Between Run CV%	Total SD (pg/mL)	Total CV%
1	104.6	6.6	6.3	8.3	8.0	11.9	11.4
2	56.5	5.3	9.3	7.0	12.4	8.8	15.5
3	377.2	17.6	4.7	10.8	2.9	24.4	6.5
4	83.3	4.7	5.7	4.2	5.0	7.1	8.5
5	100.2	6.0	6.0	7.5	7.4	9.9	9.9
6	251.8	10.3	4.1	13.3	5.3	17.0	6.8
7	365.9	16.8	4.6	52.2	14.3	54.8	15.0
8	1276.7	78.9	6.2	46.8	3.7	98.0	7.7

LINEARITY

The linearity study was performed with four human serum samples covering the range of the assay and following CLSI guidelines EP06-A. The samples were diluted in calibrator A at several equidistant concentration levels and up to ten (1:10), tested in duplicate, and the results compared to the predicted concentration. The statistical analysis shows that the assay is sufficiently linear up to a 1:10 dilution when using calibrator A as the diluent.

COMPARATIVE STUDIES

The Eagle Biosciences Total Estrogens ELISA kit (y) was compared to Liquid Chromatography-Tandem Mass Spectrometry (x) Estrogens method. The comparison of 27 serum samples yielded the following linear regression results:



REFERENCE RANGES

Reference ranges (95%) were established using samples obtained from individuals of diverse races. Each laboratory shall establish their own range of reference values and ranges.

Group	N	Median (pg/mL)	95% Confidence Range (pg/mL)
Pre-Menopausal females, cycle			
1-10 Days	40	120	16-328
11-20 Days	40	136	34-501
21-30 Days	40	168	48-350
Post-Menopausal females	120	74	40-244
Adult Males	120	104	56-213

WARRANTY INFORMATION

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For further information about this kit, its application or the procedures in this kit, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.