1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Estradiol Concentration in Human Serum or Plasma by a Microplate Enzyme Immunoassay, Colorimetric

2.0 SUMMARY AND EXPLANATION OF THE TEST

Measurement of estradiol in serum or plasma is considered to be the most reliable way to assess its rate of production. Estradiol (17β)-estradiol is a steroid hormone (molecular weight of 272.3 daltons), which circulates predominantly protein-bound. In addition to estradiol, other steroid hormones include estrone, estriol, and their metabolites. Natural estrogens are hormones secreted primarily in men and women, as well as by the adrenals, corpus luteum, and placenta and, in males, by the testes. Exogenous estrogens (natural or synthetic) elicit the variable degree of estrogenic responses usually produced by endogenous estrogens.

Estrogenic hormones are secreted at varying rates during the menstrual cycle throughout the period of ovarian activity. During pregnancy, the placenta becomes the main source of estrogenic hormones. At menopause, ovarian secretion of estrogens declines at varying rates. The gonadotropins of the anterior pituitary regulate secretion of the ovarian hormones, estradiol and progesterone, hypothalamic control of pituitary gonadotropin production is in turn regulated by plasma concentrations of the estrogens and progestins. The complex feedback system results in the cyclic phenomenon of ovulation and menstruation.

Estradiol determinations have proved of value in a variety of contexts, including the investigation of precocious puberty in girls and gynecomastia in men. Its principal uses have been in the differential diagnosis of amenorrhea and in the monitoring of ovulation induction.

This kit uses a specific anti-estradiol antibody, and does not require prior sample extraction of serum or plasma. Cross-reactivity to other naturally occurring and structurally related steroids is low.

The employment of several serum references of known estradiol concentration provides a more reliable determination of a graph of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with estradiol concentration.

3.0 PRINCIPLE

Delayed Competitive Enzyme Immunoassay (TYPE II):

The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen conjugate and native antigen. Upon mixing the biotinylated antibody with a serum containing the antigen, a reaction results between the antigen and the antibody. The interaction is illustrated by the following equation:

\[\text{AgAbBtn} \rightarrow \text{Streptavidin immobilized on well}\]

Upon mixing the biotinylated antibody with a serum containing the antigen, enzyme-antigen conjugate and native antigen.

3.1 Essential Reagents

- 1. Pipette capable of delivering 0.025ml (25 µL) and 0.050ml (50 µL) with a precision of better than 1.5%.
- 2. Dispenser(s) for reagent deliveries of 0.100ml (100 µL) and 0.350ml (350 µL) volumes with a precision of better than 1.5%.
- 3. Microplate washer or a squeeze bottle (optional).
- 4. Pipette 0.050ml (50 µL) of Estradiol Enzyme Reagent to all wells.
- 5. Add 0.050 ml (50 µL) of rAbBtn = Biotinylated antibody which has not reacted in first incubation.
- 6. Pipette 6.0 ml/vial of Estradiol Enzyme Reagent – 6.0 ml/vial.
- 7. Add 1.0 µg/ml streptavidin and purified rabbit IgG conjugate in buffer, green dye and H2O2.

4. Absorbent Paper for blotting the microplate wells.

5.0 PRECAUTIONS

**For In Vivo Diagnostic Use Not for Internal or External Use in Humans or Animals

All products that contain human serum have been found to be negative for the presence of Hepatitis B Surface Antigen, HIV 1/2 and HCV. Antibodies for FDA required tests. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control and Prevention’s “Laboratory Guidelines for Safe Handling of Blood and Blood Products.”

Investigating the specimen by utilizing the enzyme activity in the antibody bound fraction after decantation or aspiration.

The enzyme activity in the antibody bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

5.0 REAGENTS

Materials Provided

A. Estradiol Calibrators – 1ml/vial - Icons A-G

These calibrators are provided for estradiol concentrations at 0, 20, 100, 250, 500 and 1500 pg/ml. Store at 2-8°C. A preservative has been added to these calibrators and the antigen concentrations are expressed in molar concentrations (µM) by multiplying by 3.67. For example: 1.0 µg/ml x 3.67 = 3.67 µM.

B. Estradiol Enzyme Reagent – 6.0 ml/vial

The enzyme reagent is stored at 2-8°C. Store at 2-8°C. A preservative has been added to the enzyme reagent. The enzyme reagent contains 1.0µg/ml estradiol in a buffer solution.

C. Biotin Biotin Reagent – 6.0 ml - Icon C

One (1) bottle of reagent contains anti-estradiol biotinylated purified rabbit IgG conjugate in buffer, green dye and preservative. Store at 2-8°C.

D. Streptavidin Coated Plate – 96 wells – Icon D

One (1) well of Streptavidin Conjugate is coated in a protein-stabilizing matrix red with dye. Store at 2-8°C.

E. Streptavidin Coated Plate – 96 wells – Icon F

One (1) well of Streptavidin Conjugate is coated in a protein-stabilizing matrix red with dye. Store at 2-8°C.

F. Substrate Reagent – 12ml/vial - Icon SN

The substrate reagent contains hydrogen peroxide (H2O2) in buffer. Store at 2-8°C. A preservative has been added to the substrate reagent. The substrate reagent contains 1.0µg/ml estradiol in a buffer solution.

6.0 SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum or heparinized plasma in type, and taken with the usual precautions in the collection of venipuncture samples. For accurate comparison to establish normal values, a fasting morning serum sample should be obtained. The blood should be collected in a redtop (with or without gel additives) venipuncture tube(s) or for plasma used evacuated tube(s) containing heparin. Allow the blood to clot for several hours. Centrifuge the specimen to separate the serum or plasma from the cells. The calibrators can be stored at 2-8°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml of the specimen is required.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal and high range for monitoring assay performance. These controls should be run with each set of reagents in the last batch of reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance criteria. Limitations of this batch of reagents or the use of these controls are not indicated with consistent assay performance. Significant deviation from the established performance criteria may indicate unnoticeable change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

8.0 REAGENT PREPARATION

1. Wash Buffer

Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable container. Wash buffer can be stored at 2-10°C for up to 60 days.

**Note:** Do not use reagents that are contaminated or have bacterial growth.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-25°C).

1. Format the microplates’ wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag seal and store at 2-8°C.

2. Pipette 0.025 ml (25 µL) of the appropriate serum reference calibrator, control or specimen into the assigned well.

3. Add 0.050 ml (50 µL) of Enzyme-antigen Conjugate to all wells.

4. Swirl the microwell gently for 20-30 seconds to mix.

5. Cover and incubate for 30 minutes at room temperature.

6. Add 0.050 ml (50 µL) of Estradiol Enzyme Reagent to all wells.

7. Cover and incubate for 30 minutes at room temperature.

8. Add 0.050 ml (50 µL) of wash buffer into each well.

9. Add directly on the top reagents dispensed in the wells.

10. Swirl the microwell gently for 20-30 seconds to mix.

11. Cover and incubate for 30 minutes at room temperature.

12. Discard the contents of the microplate by decantation or aspiration.

13. Decant the washed and repeat the two (2) additional times for a total of three (3) washes. Add 0.050 ml (50 µL) of substrate solution to all wells. Add reagents in the same order to minimize reaction time differences between wells.

**DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION**

14. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm). The results should be read within fifteen (15) minutes of adding the stop solution.

**Note:** Dilute the samples suspected of concentrations higher than 3000pg/ml 1:5 and 1:10 with estradiol ‘0’ pg/ml calibrator or male patient serum pools with a known low value for estradiol.

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of estradiol in unknown specimens. The absorbance values of the calibrator, control and test specimen are plotted on a microplate reader as outlined in Example 1.

2. Plot the absorbance of each serum reference versus the corresponding estradiol concentration in pg/ml on linear graph paper (do not average the duplicates of the serum reference assay). The y-axis should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance criteria. Limitations of this batch of reagents or the use of these controls are not indicated with consistent assay performance. Significant deviation from the established performance criteria may indicate unnoticeable change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

3. Connect the points with a best-fit curve.

4. To determine the concentration of estradiol for an unknown, locate the absorbance of the sample on the graph, and read the concentration of the estradiol in pg/ml on the y-axis. The concentration of the unknown should be read off the graph curve at (160pg/ml) estradiol concentration (See Figure 1).

**Note:** Computer data reduction software designed for ELISA assay may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.
11. It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.

12. Risk Analysis - as required by CE Mark IVD Directive 98/79/EC - for this and other devices, made by Monobind, can be requested via email from Monobind@monobind.com.

12.2 Interpretation
1. Measurement and interpretation of results must be performed by a skilled individual or trained professional.
2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. The reagents for the test system have been formulated to eliminate maximal interference; however, potential interaction between rare serum specimens and test reagents can cause erroneous results. Heterophilic antibodies often cause these interactions and have been known to be problems for all kinds of immunoassays (Boscato LM, Stuart MC. Heterophilic antibodies; a discussion, Clin. Chem. 1988:3427-33). For diagnostic purposes, the results from this assay should be in combination with clinical examination, patient history and all other clinical findings.
4. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
5. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, Monobind shall have no liability.
6. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.

13.0 EXPECTED RANGES OF VALUES
In agreement with established reference intervals for a "normal" adult population and females during gestation the expected ranges for the Estradiol AccuBind® ELISA Test System are detailed in Table 1.


14.1 Precision
The within and between assay precision of the estradiol AccuBind® Monolite ELISA Test System were determined by analysis on three different calibrator day drifts in several (2) different days during the run. The number, mean values, standard deviation and coefficient of variation for each of these control sera are presented in Table 2 and Table 3.

14.2 Sensitivity
The Estradiol AccuBind® ELISA Test System has a sensitivity of 8.2 pg/ml. The sensitivity was ascertained by determining the variability of the 0 pg/ml serum calibrator and using the 2σ (95% certainty) deviation to calculate the minimum dose.

14.3 Accuracy
The Estradiol AccuBind® ELISA Test System was compared with a reference method. Biological specimens from low, normal and relatively high estradiol level populations were used (The values ranged from 10 pg/ml – 4300 pg/ml). The total number of such specimens varied. The least square regression equation and correlation coefficient were computed for this estradiol EIA in comparison with the reference method. The data obtained is displayed in Table 4.

14.4 Specificity
The % cross reactivity of the estradiol antibody to selected substances was evaluated by adding the interfering substance to a serum matrix and using the % cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of estradiol needed to displace the same amount of labeled analog.