MP Biomedicals
Diagnostics Division
29525 Fountain Parkway
Solon, OH 44139

17β-Estradiol (E2) ChLIA Kit
Catalog No.: 07M4975A

INTENDED USE:

The MP Biomedicals 17β-Estradiol (E2) ChLIA Kit is intended to be used for the quantitative determination of Estradiol Concentration in Human Serum or Plasma by a Microplate Chemiluminescence Immunoassay (ChLIA). This test is for in vitro diagnostic use only.

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 natural steroidal estrogens include estrone, estriol and their metabolites. Natural estrogens are hormones secreted principally by the ovarian follicles and also by the adrenals, corpus luteum, and placenta and, in males, by the testes. Exogenous estrogens (natural or synthetic) elicit, to varying degrees, all the pharmacologic 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 estrogens. 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 progesterone. This 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 permits construction of a graph of activity(light) and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with estradiol concentration.

PRINCIPLE

### Delayed Competitive Enzyme Immunoassay (TYPE 9):
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:

$$Ag + Ab_{Btn} \rightleftharpoons AgAb_{Btn}$$
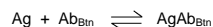
Ab_{Btn} = Biotinylated antibody
Ag = Antigen (Variable Quantity) $AgAb_{Btn}$ = Immune Complex

After a short incubation, the enzyme conjugate is added (This delayed addition permits an increase in sensitivity for low concentration samples). Upon the addition of the enzyme conjugate, competition reaction results between the enzyme analog and the antigen in the sample for a limited number of antibody binging sites (not consumed in the first incubation).

$$^{Enz}Ag + Ag + rAb_{Btn} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{Btn} + {}^{Enz}AgAb_{Btn}$$
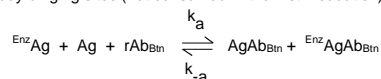
^{Enz}Ag = Enzyme-antigen Conjugate (Constant Quantity)

$^{Enz}Ab_{Btn}$ = Enzyme-antigen Conjugate -Antibody Complex

rAb_{Btn} = Biotinylated antibody not reacted in first incubation

k_a = Rate Constant of Association

k_{-a} = Rate Constant of Disassociation

$K = k_a / k_{-a}$ = Equilibrium Constant

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This affects the separation of the antibody bound fraction after decantation or aspiration.

$$AgAb_{Btn} + {}^{Enz}AgAb_{Btn} + \underline{Streptavidin_{CW}} \Rightarrow \underline{immobilized\ complex}$$

$\underline{Streptavidin_{CW}}$ = Streptavidin immobilized on well

$\underline{Immobilized\ complex}$ = sandwich complex bound to the solid surface

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.

REAGENTS

Materials Provided:
A. Estradiol Calibrators – 1 mL/vial
Seven (7) vials of serum reference for estradiol at concentrations of 0 (**A**), 20 (**B**), 100 (**C**), 250 (**D**), 500 (**E**), 1500 (**F**) and 3000 (**G**) in pg/mL. Store at 2-8°C. A preservative has been added. The calibrators can be expressed in molar concentrations (nM/L) by multiplying by 2.72. For example: 1 pg/mL x 3.67= 3.67 pM/L

B. Estradiol Tracer Reagent – 6.0 mL/vial
One (1) vial of Estradiol (Analog)-horseradish peroxides (HRP) conjugate in a protein-stabilizing matrix with red dye. Store at 2-8°C.

C. Estradiol Biotin Reagent – 6.0 mL
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. Light Reaction Wells – 96 wells
One 96-well white microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E. Wash Concentrate – 20 mL
One (1) vial contains surfactant in buffered saline. A preservative has been added. Store at 2-30°C.

F. Signal Reagent A – 7.0 mL/vial
One (1) vial contains luminol in a buffer. Store at 2-8°C.

G. Signal Reagent B – 7.0 mL/vial
One (1) vial contains hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

H. Product Insert
Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Avoid extended exposure to heat and light. **Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on the label.**

Note 3: Above reagents are for a single 96-well microplate.

Required But Not Provided:
1. Pipette capable of delivering 25 μL and 50 μL with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100 mL and 0.350 mL volumes with a precision of better than 1.5%.
3. Adjustable volume (200-1000 μL) dispenser(s) for conjugate.
4. Microplate washer or a squeeze bottle (optional).
5. Microplate Luminometer.
6. Absorbent Paper for blotting the microplate wells.
7. Plastic wrap or microplate cover for incubation steps.
8. Vacuum aspirator (optional) for wash steps.
9. Timer.
10. Quality control materials.

PRECAUTIONS

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

All products that contain human serum have been found to be non-reactive for Hepatitis B Surface Antigen, HIV 1&2 and HCV Antibodies by 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 / National Institute of Health, "Biosafety in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, HHS Publication No. (CDC) 88-8395.

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood; serum or heparanised plasma in type and taken with the usual precautions in the collection of venipuncture samples. The blood should be collected in a redtop (with or without gel additives) venipuncture tube or for plasma use evacuated tube(s) containing heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050 mL of the specimen is required.

REAGENT PREPARATION

1. **Wash Buffer**
   Dilute contents of Wash Concentrate to 1000 mL with distilled or deionized water in a suitable storage container. Store diluted buffer at room temperature 2-30°C for up to 60 days.
2. **Working Signal Reagent Solution -** Store at 2 - 30°C.
   Determine the amount of reagent needed and prepare by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container. For example, add 1 mL of A and 1 mL of B per two (2) eight well strips (A slight excess of solution is made). **Discard the unused portion if not used within 36 hours after mixing.** If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.

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

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 - 27°C).

**Test Procedure should be performed by a skilled individual or trained professional

1. Format the microplates' wells for each serum reference, 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, control or specimen into the assigned well.
3. Add 0.050 mL (50 μL) of the Estradiol Biotin Reagent to all wells.
4. Swirl the microplate 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 Tracer Reagent to all wells.

Add directly on top the reagents dispensed in the wells

7. Swirl the microplate gently for 20-30 seconds to mix.
8. Cover and incubate for 60 minutes at room temperature.
9. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
10. Add 350 μL of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat four (4) additional times for a total of five (5) washes. **An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat four (4) additional times.**
11. Add 0.100 mL (100 μL) of working signal reagent solution to all wells (see Reagent Preparation Section). **Always add reagents in the same order to minimize reaction time differences between wells.**
12. Incubate at room temperature for five (5) minutes in the dark.
13. Read the relative light units in each well with a chemiluminescence microplate reader for 0.5-1.0 seconds. **The results should be read within 30 minutes after adding the working Signal Reagent.**

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

QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal and elevated ranges for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control records should be maintained and used to monitor batch to batch consistency.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of estradiol in unknown specimens.
1. Record the RLU's obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the RLU's for each duplicate serum reference versus the corresponding estradiol concentration in ng/mL on linear graph paper.
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of estradiol for an unknown, locate the average RLU's for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in ng/mL) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average RLU's (52007) of the unknown intersects the calibration curve at (103) estradiol concentration

Note: Computer data reduction software designed for chemiluminescence assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

Note: Multiply the horizontal values by 1000 to convert into pg/mL.

EXAMPLE 1

Sample I.D.	Well Number	RLU (A)	Mean RLU (B)	Value (pg/mL)
Cal A	A1	100263	100000	0
	B1	99737		
Cal B	C1	87791	87792	20
	D1	87794		
Cal C	E1	52485	52796	100
	F1	53107		
Cal D	G1	28948	28538	250
	H1	28129		
Cal E	A2	15881	15882	500
	B2	15883		
Cal F	C2	7596	7607	1500
	D2	7618		
Cal G	E2	4946	4847	3000
	F2	4748		
Pat# 1	G2	52065	52007	103
	H2	51950		

* The data presented in Example 1 and Figure 1 is for illustration only and **should not** be used in lieu of a dose response curve prepared with each assay. In addition, the RLU's of the calibrators have been normalized to 100,000 RLU's for the A calibrator (greatest light output). This conversion minimizes differences caused by efficiency of the various instruments that can be used to measure light output.

Figure 1
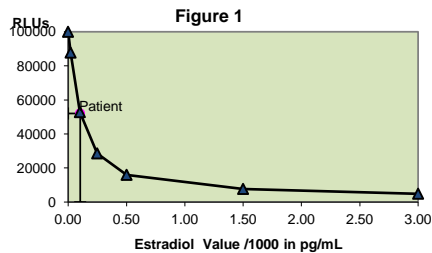
LIMITATIONS OF PROCEDURE

Assay Performance

1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.
4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
5. The addition of signal reagent initiates a kinetic reaction; therefore, the signal reagent(s) should be added in the same sequence to eliminate any time-deviation during reaction.
6. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
7. Use components from the same lot. No intermixing of reagents from different batches.
8. Patient specimens with E2 concentrations above 3000 pg/mL may be diluted (1/2, 1/5 or higher) with E2 '0' calibrator and re-assayed. The sample's concentration is obtained by multiplying the result by the dilution factor.
9. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential.

Any deviation from MP Biomedicals' IFU may yield inaccurate results.

10. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.
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.

Interpretation

1. ***Measurements 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. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
4. 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, MP Biomedicals shall have no liability.
5. 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.

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 ChLIA test are detailed in Table 1.

TABLE I
Expected Values for the Estradiol Test System

	Median	Range
Females	-	-
Follicular Phase	48	9-175
Luteal Phase	103	44-196
Periovulatory	209	107-281
Treated Menopausal	122	42-289
Untreated Menopausal	7.3	ND-20
Oral Contraceptives	13	ND-103
Males	19	4-94

During pregnancy the Estradiol serum levels rise rapidly till the end of third trimester [17].

It is important to keep in mind that establishment of a range of values which can be expected to be found by a given method for a population of "normal" persons is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

PERFORMANCE CHARACTERISTICS

Precision

The within and between assay precision of the Estradiol ChLIA test were determined by analyses on three different levels of pool control sera. The number, mean values, standard deviation and coefficient of variation for each of these control sera are presented in Table 2 and Table 3.

TABLE 2
Within Assay Precision (Values in pg/mL)

Sample	N	X	σ	C.V.
Low	20	119.6	9.2	7.7%
Normal	20	228.0	15.7	6.9%
High	20	420.7	36.9	8.8%

TABLE 3
Between Assay Precision (Values in pg/mL)

Sample	N	X	σ	C.V.
Low	20	138.9	7.0	5.0%
Normal	20	244.7	9.3	3.8%
High	20	444.9	7.3	1.6%

*As measured in ten experiments in duplicate over a ten day period.

Sensitivity

The Estradiol ChLIA test has a sensitivity of 4.162 pg/mL. The sensitivity was ascertained by determining the variability of the 0 pg/ml serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose.

Accuracy

The Estradiol ChLIA test was compared with a chemiluminescence immunoassay 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 was 65. The least square regression equation and the correlation coefficient were computed for this estradiol ChLIA in comparison with the reference method. The data obtained is displayed in Table 4.

TABLE 4

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
This Method (Y)	336.8	Y= 36.5+36.5*(X)	0.989
Reference (X)	293.4		

Only slight amounts of bias between this method and the reference method are indicated by the closeness of the mean values. The least square regression equation and correlation coefficient indicates excellent method agreement.

Specificity

The % cross reactivity of the estradiol antibody to selected substances was evaluated by adding the interfering substance to a serum matrix at various concentrations. 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.

Substance	Cross Reactivity
Androstenedione	0.0003
Dihydrotestosterone	0.0008
Cortisone	<0.0001
Corticosterone	<0.0001
Cortisol	0.0004
Estriol	<0.0001
DHEA sulfate	<0.0001
Estradiol	<0.0001
Estrone	<0.0001
Testosterone	<0.0001

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