Read entire protocol before use.

E2-EASIA

I. INTENDED USE

The DIAsource E2-EASIA is a competitive binding immunoassay for the quantitative determination of estradiol in serum and plasma.

II. GENERAL INFORMATION

A. Proprietary name : DIAsource E2-EASIA Kit

B. Catalogue number : KAP0621 : 96 tests

C. Manufactured by : DIAsource ImmunoAssays S.A.
Rue de l'Industrie, 8, B-1400 Nivelles, Belgium.

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III. INTRODUCTION

A. Estradiol

17-beta-estradiol (E2) is a C-18 steroid hormone (molecular weight 272.4) produced mainly by the ovary and placenta, and in small amounts by adrenals and testes. Estradiol is in equilibrium with estrone, which can be converted to estriol by the liver and placenta.

B. Clinical applications of the measurement of E2 levels

Like LH, FSH and progesterone, measurement of estradiol concentration in serum, peritoneal fluid and follicular fluid is an essential biochemical tool for the investigation of fertility, tumor and sexual diseases and disorders of hypothalamic/pituitary/gonadal axis.

For example:

- to detect the follicular phase;
- to check the effectiveness of the induction of ovulation (with ultrasound) and the level of E2 in follicular fluid. It allows normal detection or dysfunctional ovulation induction (the empty follicle synfrome may reflect a dysfonctional ovulation induction);
- to diagnose the luteinized unruptured follicle (LUF) syndrome (by the estimation of 17 beta-estradiol and progesterone levels in peritoneal fluid);
- to aid in the diagnosis of breast tumors (total estrogens – E1-E2 – and 17 beta-hydroxysteroïd dehydrogenase activity are significantly higher in malignant than in non malignant breast tissues);
- other areas of investigation are: premature adrenarche, gynecomastie and menopausal period.

IV. PRINCIPLE OF THE TEST

E2-EASIA is an enzyme immunoassay performed in a microtiter plate. A fixed amount of estradiol labeled with horseradish peroxidase (HRP) competes with unlabeled estradiol present in calibrators or samples for a limited number of binding sites of a specific antibody. The E2-HRP-antibody complex is simultaneously fixed on the wells of the microtiter plate coated with an excess of anti-rabbit-gammaglobulins.

Neither extraction nor chromatography are required due to the high specificity of the antibody.

After 2 hours incubation at room temperature the microtiter plate is washed to stop the competition reaction.

The substrate solution (tetramethylbenzidine (TMB) – H_20_2) is added and incubated for 30 minutes. The reaction is stopped with H_2S0_4 and the absorbance is measured at the appropriate wavelength. The amount of substrate turnover is inversely proportional to the estradiol concentration in the sample. A calibration curve is plotted and estradiol concentrations in samples are determined by interpolation from the calibration curve.

V. REAGENTS PROVIDED

Reagent	*Quantity*	*Color Code*	*Reconstitution*
Microtiter Plate with 96 wells coated with anti-rabbit IgG.	96 wells	blue	Ready to use
Anti-estradiol; lyophilized.	1 vial	blue	Add 6 mL distilled water
Calibrator 0 pg/mL. Contains human serum with preservatives	1 vial Lyophil.	yellow	Add 4 mL distilled water
Calibrators 1-5. Contains human serum with preservatives (see vial label for exact concentrations)	5 vials Lyophil.	yellow	Add 0.5 mL distilled water
Controls 1 and 2. Contains human serum with preservatives;	2 vials Lyophil.	silver	Add 0.5 mL distilled water
Estradiol-HRP Concentrated Conjugate. Contains phosphate buffer with preservatives	1 vial 0.5 ml	red	Pipette 0.1 mL into 1 vial of conjugate buffer
Conjugate Buffer for dilution of estradiol-HRP conjugate	3 vials 6 ml	red	Ready for use
Wash Buffer	1 vial 10 ml	brown	Dilute 2 mL in 400 mL distilled water or the vial content in 2000 mL distilled water.
Chromogen, TMB (Tetramethylbenzidine)	1 vial 1 ml	green	Pipette 0.2 mL into 1 vial of substrate buffer.
Substrate Buffer. Contains H_2O_2 in acetate/citrate buffer	3 vials 21 ml	white	Ready to use
Stop Reagent. Contains 1.8 N H_2SO_4	1 vial 6 ml	black	Ready to use

Note: Calibrator 0 pg/mL is recommended for sample dilutions.

VI. STORAGE AND STABILITY

- Store the kit at 2 - 8°C until expiration date printed on the kit label.
- Once opened, store the concentrated estradiol-HRP conjugate vial at 2 - 8°C. Diluted estradiol-HRP conjugate is stable for 4 hours at room temperature or 24 hours at 2 - 8°C when protected from direct exposure to sunlight.
- After reconstitution, store anti-estradiol, calibrators and controls at 2 - 8°C for 1 week maximum. For prolonged storage they must be frozen. Three freezing-thawing cycles are allowed.
- Store the unused strips at 2 - 8°C in the closed bag containing the desiccant until expiration date.
- The concentrated wash solution is stable at room temperature until expiration date. Prepare fresh diluted wash solution each day.
- The freshly prepared substrate solution is stable for a maximum of 15 minutes at room temperature and must be discarded after use.

VII. SUPPLIES REQUIRED BUT NOT PROVIDED

1. Microtiter plate reader capable of measurement at or near 450 nm.
2. Calibrated adjustable precision pipettes, preferably with disposable plastic tips. (A manifold multi-channel pipette is desirable for large assays.)
3. Distilled or deionized water.
4. Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).
5. Data analysis and graphing software. Graph paper: linear (Cartesian), log-log, or semi-log, as desired.
6. Calibrated beakers and graduated cylinders in various sizes.

VIII. SAFETY

- For in vitro diagnostic use only.
- The human blood components included in this kit have been tested and found non reactive for HBsAg and anti-HIV. Nevertheless, no known method can offer complete assurance that human blood derivatives will not transmit hepatitis, AIDS or other infections. Therefore, handling of reagents, serum or plasma specimens should be in accordance with local safety procedures, e.g., CDC/HIH Health Manual: "Biosafety in Microbiological and Biomedical Laboratories" 1984.
- Avoid any skin contact with H_2SO_4, H_2O_2 and TMB. In case of contact, wash thoroughly with water.
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used.
- Do not pipette liquids by mouth.

IX. PROCEDURAL NOTES/LAB QUALITY CONTROL

- Do not use kit components beyond the expiration date.
- Do not mix materials from different kit lots.
- Do not mix strips from different plates.
- Bring all the reagents and specimens to room temperature (18 - 30°C) prior to use.
- Thoroughly mix the reagents and samples before use by gentle agitation or swirling.
- Use a clean disposable plastic pipette tip for each reagent, calibrator, control or specimen addition in order to avoid cross-contamination. When dispensing H_2SO_4 and substrate solution, avoid pipettes with metal parts.
- Use a clean plastic container to prepare the wash solution.
- The TMB solution in substrate buffer should be colorless. A dark blue color indicates that the reagent is unusable and must be discarded.
- During incubation with substrate solution, avoid direct sunlight on the microtiter plate.
- Follow the incubation times described in the assay procedure.
- Dispense the substrate solution within 15 minutes following the washing of the microtiter plate.

X. SAMPLE COLLECTION AND PREPARATION

- No special pretreatment of the sample is necessary. Prior to use, all the samples should be at room temperature. It's recommended to vortex the samples before use.
- Do not use hemolyzed samples.
- Serum or plasma samples must be kept at 2 - 8°C.
  If the test is not run within 24 hrs, storage in aliquots at -20°C is recommended. Avoid successive freezing and thawing.
- Serum, heparinized plasma or EDTA plasma provide similar results.

  Y (serum) = 1.00 x (HEP) - 3 r = 0.99 n = 48
  Y (serum) = 0.98 x (EDTA) - 9 r = 0.98 n = 48

XI. REAGENT PREPARATION

- **Calibrators and Controls:**
  Reconstitute the lyophilized calibrators and controls to the volume specified on the vial label with distilled water (4 mL for the zero calibrator and 0.5 mL for the other calibrators and controls). Allow them to remain undisturbed until completely dissolved, then mix well by gentle inversion.
- **HRP-Estradiol Conjugate:**
  Pipette 0.1 mL of the concentrated HRP-estradiol solution into one of the vials of conjugate buffer. Prepare immediately prior to use. Maximum stability is 4 hours at room temperature or 24 hours at 2 - 8°C when protected from direct exposure to sunlight.
- **Wash Buffer:**
  Dilute 2 mL in 400 mL distilled water or the content of the entire vial in 2000 mL distilled water (use a magnetic stirrer).
- **Substrate Solution:**
  Pipette 0.2 mL of the chromogen (TMB) into one of the vials of substrate buffer (H_2O_2 in acetate/citrate buffer). Prepare immediately prior to use. Maximum stability is 15 minutes at room temperature when protected from direct exposure to sunlight.

XII. ASSAY PROCEDURE

A calibration curve must be run with each assay.

It is recommended that the assays be performed in duplicate and that instructions for the assay procedure be followed exactly to obtain reliable results.

1. Select sufficient strips to accommodate calibrators, controls and all test samples.
2. Fit the strips into the holding frame.
3. Dispense 50 µL of each calibrator, control or sample into the appropriate wells. Vertical alignment is recommended.
4. Dispense 50 µL of estradiol-HRP conjugate into all wells.
5. Dispense 50 µL of anti-estradiol into each well.
6. Incubate for 2 hours at room termperature on a horizontal shaker set at 700 ± 100 RPM.
7. Wash the plate by:
   a) aspirating the liquid from each well;
   b) dispensing 0.4 mL of wash solution into each well;
   c) aspirating the contents of each well. Repeat steps b) and c) 4 times.
8. Dispense 200 µL of the freshly prepared substrate solution into each well immediately after the washing step.
9. Incubate the plate for 30 minutes at room temperature, protecting from direct sunlight, on a horizontal shaker set at 700 ± 100 RPM.
10. Dispense 50 µL of stop reagent into each well.
11. Read the absorbances of each well at 450 nm (reference wavelength at 650 nm) within 1 hour after addition of stop reagent.

XIII. CALCULATIONS AND RESULTS INTERPRETATION

- Calculate the mean absorbance of duplicate determinations, rejecting obvious outliers.
- For each calibrator and sample calculate the percent bound:

  $$B/Bo \times 100 = \frac{OD\ (calibrator\ or\ sample)}{OD\ (zero\ calibrator)} \times 100$$

- Using either linear-linear or semi-log graph paper, plot the (B/BO x 100) values for each calibrator point as a function of the estradiol concentration of each calibrator point.
- By interpolation of the samples (B/Bo x 100) values, determine the estradiol concentrations of the samples from the reference curve.

If using curve fitting software, the four-parameter algorithm provides the best curve fit.

XIV. EXAMPLE OF TYPICAL REFERENCE CURVE

The following data are for demonstration purposes only and can not be used in place of data generated at the time of assay.

Calibrator	OD units	B/Bo x 100
0 pg/mL	1.790	100
13 pg/mL	1.424	80
50 pg/mL	1.013	57
100 pg/mL	0.762	43
270 pg/mL	0.447	25
935 pg/mL	0.221	12

XV. EXPECTED VALUES

Identification	Number of subjects	Range (pg/ml)
Males	50	10 - 45
Postmenopausal females	30	10 - 45
Ovulating females: (day 0 = LH peak)	14	
Day: - 10		13 – 80
- 4		20 – 165
- 1		73 – 410
0		119 – 417
+2		22 – 154
+5		44 – 174
+10		13 - 146
Pregnant women:		
1st trimester	88	40 – 3100
2nd trimester	39	1600 – 14000
3rd trimester	100	4200 - 32000

pg/ml x 3.67 = pmol/l
pmo/l x 0.272 = pg/ml

XVI. PERFORMANCE CHARACTERISTICS

SENSITIVITY

Minimum detectable concentration (MDC) of estradiol in 10 different assays was 5 ± 2 pg/mL (mean ± SD). MDC is defined as the concentration of estradiol corresponding to 95% of maximum binding.

SPECIFICITY

The percentage of cross-reaction was estimated under physiological conditions in serum by comparison of the concentration yielding a 50% binding inhibition:

Substances	Cross-reactivity (%)
17-b estradiol	100
Estrone	2
Estriol	1.9
E2-3-Glucuronide	0.6
E2-17-Glucoronide	0.56
E2-17-Valerate	0.1
Cartisol	<0.001
Progesterone	0.03
Dhea-sulfate	<0.0001
Testosterone	<0.001
Androstenediol	<0.001
Norgestrel	0.01
Premarin	0.06
Equilin	0.1

PRECISION

Intra-assay				Inter-assay			
Serum	N	<X> ± SD (pg/mL)	CV %	Serum	N	<X> ± SD (pg/mL)	CV %
A	20	131 ± 6	4.6	C	15	101 ± 6	6.0
B	20	257 ± 10	3.9	D	15	196 ± 12	6.1

ACCURACY

Recovery				Dilution test			
Sample	Added (pg/mL)	Recovery (pg/mL)	Recovery (%)	Serum dilution	Theoretical conc. (pg/mL)	Measured conc. (pg/mL)	Recovery (%)
Serum	916	719	78.4	1/1	997	997	100
	516	430	83.3	1/2	498	485	97
	316	304	96.2	1/4	249	252	101
	166	176	106.0	1/8	125	109	87

XVII. BIBLIOGRAPHY

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SUMMARY OF ASSAY PROCEDURE		
	Calibrators (mL)	Controls or samples (mL)
Calibrators	50	-
Controls-samples	-	50
Estradiol-HRP	50	50
Anti-estradiol	50	50
Incubate for 2 hours at RT with continuous shaking (700 RPM) Aspirate the content of each well Wash 5 times with 0.4 mL of wash solution and aspirate		
Substrate solution	200	200
Incubate 30 minutes at RT with continuous shaking (700 RPM)		
H_2SO_4	50	50
Read the microtiter plate 450 nm (versus 650 nm)		

DIAsource Catalogue Nr : KAP0621	P.I. Number : 1700483/en	Revision nr : 100519/1