

Read entire protocol before use.

E2-EASIA

INTENDED USE

The GenWay Biotech, Inc. E2-EASIA is a competitive binding immunoassay for the quantitative measurement of estradiol in serum and plasma (96 determinations).

GENERAL INFORMATION

- A. **Proprietary name :** GenWay Biotech, Inc. E2-EASIA Kit
- B. **Catalogue number :** 40-056-205004: 96 tests
- C. **Manufactured by :** GenWay Biotech, Inc.
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1. INTRODUCTION

A. Estradiol

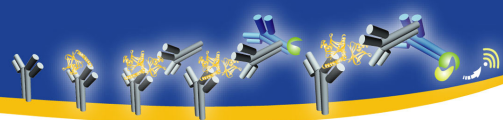
17-beta-estradiol (E2) is a C-18 steroid hormone (molecular weight 272.4) produced mainly by the ovary and placenta, and in small amounts by adrenals and testes. Estradiol is in equilibrium with estrone, which can be converted to estriol by the liver and placenta.

B. 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 research 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 syndrome may reflect a dysfunctional ovulation induction);
- to research the luteinized unruptured follicle (LUF) syndrome (by the estimation of 17 beta-estradiol and progesterone levels in peritoneal fluid);
- to aid in the research of breast tumors (total estrogens – E1-E2 – and 17 beta-hydroxysteroid dehydrogenase activity are significantly higher in malignant than in non malignant breast tissues);
- other areas of investigation are: premature adrenarche, gynecomastie and menopausal period.



C. 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 anti-rabbit-gammaglobulins in excess.

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 revelation solution (tetramethylbenzidine (TMB) – H₂O₂) is added and incubated for 30 minutes. The reaction is stopped with H₂SO₄ and the absorbance is measured at the appropriate wavelength. The amount of substrate turnover is determined colorimetrically by measuring the absorbance which is inversely proportional to the estradiol concentration. A standard curve is plotted and estradiol concentrations in samples are determined by interpolation from the standard curve.

2. REAGENTS PROVIDED

Reagent	Quantity	Color Code	Reconstitution
Microtiter Plate with 96 anti-rabbit IgG coated wells	8 x 12 wells	blue	Ready to use
Anti-estradiol	1 vial 6ml	blue	Add 6 mL distilled water
Standard 0 pg/ml in human serum with preservatives.	1 vial Lyophil.	yellow	Add 4 mL distilled water
Standards 1-5 in human serum with preservatives (see vial label for exact concentrations)	5 vials Lyophil.	yellow	Add 0.5 mL distilled water
Controls 1 and 2 in human serum with preservatives	2 vials Lyophil.	silver	Add 0.5 mL distilled water
Concentrated estradiol-HRP conjugate in 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
Washing 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. H ₂ O ₂ in acetate/citrate buffer	3 vials 21 ml	white	Ready for use
H ₂ SO ₄ 1.8 N Stopping reagent	1 vial 6 ml	black	Ready for use

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

3. STORAGE AND STABILITY

- Store the kit at 2 - 8°C until expiration date mentioned on the kit label.
- Once opened, store the concentrated estradiol-HRP conjugate vial at 2 - 8°C. Stability of diluted estradiol-HRP conjugate is 4 hours at room temperature or 24 hours at 2 - 8°C when protected from direct exposure to sunlight.
- After reconstitution, store anti-estradiol, standards 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. In order to avoid obstructions of the washerheads, it is recommended to prepare each day a freshly diluted washing solution.
- The freshly prepared substrate solution is stable before use for maximum of 15 minutes at room temperature and must be discarded afterwards.

4. MATERIAL REQUIRED BUT NOT PROVIDED

- Distilled water
- Pipettes: 50ul, 100 ul, 200 ul, 500 ul, 2 mL and 4 mL.
- Vortex mixer and magnetic stirrer

5. WARNINGS

Safety

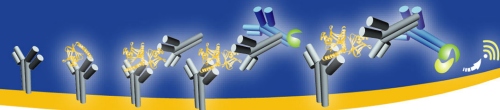
- For research use only.
- The human blood components included in this kit have been tested and found non reactive for HBsAg and anti-HIV. Nevertheless, no known method can offer complete assurance that human blood derivatives will not transmit hepatitis, AIDS or other infections. Therefore, handling of reagents, serum or plasma specimens should be in accordance with local safety procedures, e.g., CDC/HHI Health Manual: "Biosafety in Microbiological and Biomedical Laboratories" 1984.
- Avoid any skin contact with H₂SO₄, H₂O₂ 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.

Handling

- 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, standard, control or specimen addition in order to avoid cross-contamination; for the dispensing of H₂SO₄ 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. If a dark blue color develops within a few minutes after preparation, this indicates that the reagent is unusable and must be discarded.
- During incubation with substrate solution, avoid direct sunlight on the microtiter plate.
- Respect the incubation times described in the assay procedure.
- Dispense the revelation solution within 15 minutes following the washing of the microtiter plate.

6. 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.



- Hemolysis has to be avoided.
- Samples may be stored for up to 72 hours at 2 - 8°C prior to testing. Samples held for longer time should be frozen at -20°C prior to assaying.
- Serum, heparinized plasma or EDTA plasma provide similar results.

$$Y(\text{serum}) = 1.00 \times (\text{HEP}) - 3 \quad r = 0.99 \quad n = 48$$

$$Y(\text{serum}) = 0.98 \times (\text{EDTA}) - 9 \quad r = 0.98 \quad n = 48$$

7. REAGENT PREPARATION

- Standards and Controls:**
Reconstitute the lyophilized standards 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 (see section 8). Extemporaneous preparation is recommended. Maximum stability is 4 hours at room temperature or 24 hours at 2 - 8°C when protected from direct exposure to sunlight.
- Washing 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).
- Revelation Solution:**
Pipette 0.2 mL of the chromogen TMB into one of the vials of substrate buffer (H₂O₂ in acetate/citrate buffer). Extemporaneous preparation is recommended. Maximum stability before use is 15 minutes at room temperature avoiding direct exposure to sunlight (see section 8.8).

8. ASSAY PROCEDURE

It is recommended to perform the assays in duplicate and to follow the instructions of the assay procedure to obtain reliable results.

- Select sufficient strips to accommodate calibrators, controls and all test samples.
- Fit the strips into the holding frame.
- Dispense 50 µL of each standard, control or sample into the appropriate wells. Vertical alignment is recommended.
- Dispense 50 µL of estradiol-HRP conjugate into all wells.
- Dispense 50 µL of anti-estradiol into each well.
- Incubate for 2 hours at room temperature on a horizontal shaker set at 700 ± 100 RPM. The use of the EASIA shaker is recommended.
- Wash the plate by:
 - aspirating the liquid from each well;
 - dispensing 0.4 mL of wash solution into each well;
 - aspirating the contents of each well.
 Repeat steps b) and c) 4 times.

The use of the EASIA washer is recommended.

- Dispense 200 µL of the freshly prepared substrate solution into each well immediately after the washing step.
- Incubate the plate for 30 minutes at room temperature, avoiding direct sunlight, on a horizontal shaker set at 700 ± 100 RPM. The use of the EASIA shaker is recommended.
- Dispense 50 µL of stopping reagent into each well.
- Read the absorbances within 1 hour and calculate the results as described in section 9.

9. READING AND RESULTS INTERPRETATION

- Read the microtiter plate at 450 nm (reference filter: 650 nm)
- Calculate the mean of duplicate determinations, rejecting obvious

outliers.

- For each standard or sample calculate

$$B/Bo \times 100 = \frac{OD(\text{standard or sample})}{OD(\text{zero calibrator})} \times 100$$
- Using either linear-linear or semi-log graph paper, plot the (B/BO x 100) values for each standard point as a function of the estradiol concentration of each standard 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.

10. 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

11. 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:		
1 st trimester	88	40 - 3100
2 nd trimester	39	1600 - 14000
3 rd trimester	100	4200 - 32000

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

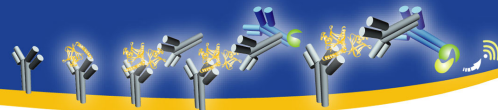
12. PERFORMANCE CHARACTERISTICS

MINIMUM DETECTABLE CONCENTRATION (MDC)

Minimum detectable concentration 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-Glucuronide	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

SUMMARY OF ASSAY PROCEDURE		
	Calibrators (uL)	Controls or samples (uL)
Standards	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 ₂ SO ₄	50	50
Read the microtiter plate 450 nm (versus 650 nm)		

PRECISION

Serum	Intra-assay			Inter-assay			
	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

Sample	Recovery			Dilution test			
	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

13. BIBLIOGRAPHY

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