Diagnostics Biochem Canada Inc.
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TOTAL ESTROGENS ELISA

EU: CE IVD CAN/USA: IVD

REF: CAN-E-630 Version: 5.2 (COMB)
Effective: September 16, 2024

INTENDED USE

For the direct quantitative determination of Total Estrogens in human serum by an enzyme immunoassay. For *in vitro* use only.

PRINCIPLE OF THE TEST

The total estrogens ELISA is a competitive immunoassay. Competition occurs between total estrogens (estrone, estradiol, and estriol) present in calibrators, controls and patient samples and an enzyme-labelled antigen (conjugate) for a limited number of anti-estrogen antibody binding sites on the microplate wells. After a washing step that removes unbound materials, the enzyme substrate is added, and approximately 30 minutes later the enzymatic reaction is terminated by addition of stopping solution. The resulting optical density (OD), measured with a microplate reader, is inversely proportional to the concentration of total estrogens in the sample. A calibrator curve is plotted with a provided set of calibrators to calculate directly the concentration of total estrogens in patient samples and controls.

CLINICAL APPLICATIONS

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 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 does not 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 of sex steroid metabolism related conditions, for example, premature 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.

PROCEDURAL CAUTIONS AND WARNINGS

1. This kit is intended for in vitro use only.
2. Practice good laboratory practices when handling kit reagents and specimens. This includes:
   - Do not pipette by mouth.
   - Do not smoke, drink, or eat in areas where specimens or kit reagents are handled.
   - Wear protective clothing and disposable gloves.
   - Wash hands thoroughly after performing the test.
   - 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. Avoid microbial contamination of reagents.
5. A calibrator curve must be established for every run.
6. It is recommended to all customers to prepare their own control materials or serum pools which should be included in every run at a high and low level for assessing the reliability of results.
7. 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 procedural techniques or pipetting, incomplete washing or improper reagent storage.
8. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
9. All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of specimens.
10. 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.
11. The substrate solution (TMB) is sensitive to light and should remain colourless if properly stored. Instability or contamination may be indicated by the development of a blue colour, in which case it should not be used.
12. When dispensing the substrate and stopping solutions, do not use pipettes in which these liquids will come into contact with any metal parts.
13. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, calibrator and control.
14. 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.
15. Kit reagents must be regarded as hazardous waste and disposed of according to local and/or national regulations.

LIMITATIONS

1. The kit is calibrated for the direct determination of total estrogens in human serum. The kit is not calibrated for the determination of total estrogens in other specimens of human or animal origin.
2. Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
3. Samples or control sera containing azide or thimerosal are not compatible with this kit, they may lead to false results.
4. Only calibrator A may be used to dilute high serum samples. The use of water or any other reagent will lead to false results.
5. The results obtained with this kit shall never be used as the sole basis for a clinical diagnosis. For example, some drugs and the occurrence of heterophilic antibodies in patients regularly exposed to animals or animal products have the potential of causing interferences in immunological tests. Consequently, the clinical diagnosis should comprise all aspects of a patient's background including the frequency of exposure to animals/products.

SAFETY CAUTIONS AND WARNINGS
POTENTIAL BIOHAZARDOUS MATERIAL

The reagents should be considered a potential biohazard and handled with the same precautions applied to blood specimens. All human specimens should be considered a potential biohazard and handled as if capable of transmitting infections and in accordance with good laboratory practices.

CHEMICAL HAZARDS

Avoid contact with reagents containing TMB, hydrogen peroxide and sulfuric acid. If contacted with any of these reagents, wash with plenty of water. TMB is a suspected carcinogen.

SPECIMEN COLLECTION AND STORAGE

Approximately 0.15 mL of serum is required per duplicate determination. Collect 4–5 mL of blood into an appropriately labelled tube and allow it to clot. Centrifuge and carefully remove the serum layer. Store at 4°C for up to 24 hours or at -10°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

No specimen pretreatment is necessary.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED

1. Precision pipette to dispense 50, 150 and 350 µL
2. Disposable pipette tips
3. Distilled or deionized water
4. Microplate shaker:
   a. Orbital (3 mm diameter) set to 600 rpm or
   b. Linear shaker set to 200 iterations per minute
5. Microplate reader with a filter set at 450 nm and an upper OD limit of 3.0 or greater
6. Microplate washer (recommended)

REAGENTS PROVIDED

1. Rabbit Anti-Estrogens Antibody Coated Break-Apart Well Microplate — Ready To Use
Contents: One polyclonal antibody-coated 96-well (12x8) microplate in a resealable pouch with desiccant.
Storage: Refrigerate at 2–8°C
Stability: Unopened: Stable until the expiry date printed on the label. After Opening: Stable for three weeks.

2. Estrogen-HRP Conjugate — Ready To Use
Contents: One bottle containing Estrogen-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 the expiry date printed on the label. After Opening: Stable for three weeks.

3. Calibrators — Ready To Use
Contents: Eight vials containing estrogen in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with defined quantities of an estrogen.

* Listed below are approximate concentrations, please refer to vial labels for exact concentrations.

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

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

4. Controls — Ready To Use
Contents: Two vials containing estrogen in a protein-based buffer with a non-mercury preservative. Prepared by spiking a protein-based buffer with defined quantities of an estrogen. Refer to vial labels for the acceptable ranges.
Volume: 1.0 mL/vial
Storage: Refrigerate at 2–8°C
Stability: Unopened: Stable until the expiry date printed on the 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 expiry date printed on the label. After Opening: Stable for three weeks. Working wash buffer stability: Following preparation, the working wash buffer is stable for up to 2 weeks when stored at 2–8C.
Preparation of working wash buffer: Dilute 1:10 in distilled or deionized water before use. If the 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 expiry date printed on the 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 expiry date printed on the label. After Opening: Stable for three weeks.

ASSAY PROCEDURE

Specimen Pretreatment: **None**.

All kit components and specimen samples must reach room temperature prior to use. Calibrators, controls and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

1. After all kit components have reached room temperature, mix gently by inversion. Prepare the working wash buffer (see wash buffer concentrate under the section REAGENTS PROVIDED).
2. Remove the required number of strips from the microplate and assemble into a plate frame. Reseal the bag and return any unused strips to the refrigerator.
3. Pipette 50 µL of each calibrator, control and specimen sample into correspondingly labelled microplate wells.
4. Incubate the microplate on a microplate shaker** for 30 minutes at room temperature.
5. Pipette 150 µL of the Estrogen-HRP conjugate into the wells containing the previously added calibrators, controls and specimen samples (the use of a multichannel pipette is recommended).
6. Incubate the microplate on a microplate shaker** for 120 minutes at room temperature.
7. Wash the microplate wells 3 times with working wash buffer (350 µL/well for each wash) and tap the plate firmly against absorbent paper to ensure that it is dry. The use of a microplate washer is highly recommended. If a microplate washer is not available, ensure that the wash buffer reaches the top edge of the wells and that no liquid remains in the microplate after the final washing, avoid splashing).
8. Pipette 150 µL of TMB substrate into each microplate well at timed intervals.
9. Incubate the microplate on a microplate shaker** for 30 minutes at room temperature.
10. Pipette 50 µL of stopping solution into each well at the same timed intervals as in step 8 and gently tap the microplate frame to mix the contents of the wells.
11. Read the microplate in a microplate reader at 450 nm, within 20 minutes after addition of the stopping solution.

** See REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED (#4).

CALCULATIONS

1. Calculate the mean optical density of each calibrator, control and sample duplicate.
2. Use a 4-parameter or 5-parameter curve fit with immunoassay software to generate a calibrator curve.
3. Read the values of the unknowns directly off the calibrator curve.
4. If a sample reads more than 2,500 pg/mL dilute it with calibrator A not more than 10-fold. The result obtained must be multiplied by the dilution factor.

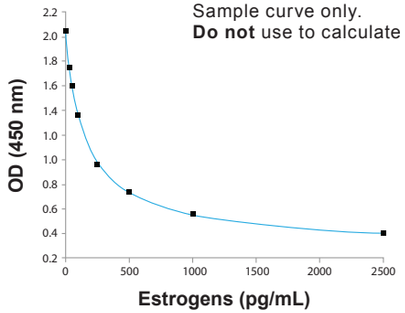
TYPICAL TABULATED DATA

Sample data only. **Do not** use to calculate results.

Calibrator	OD 1	OD 2	Mean OD	Value (pg/mL)
A	2.020	2.068	2.044	0
B	1.752	1.757	1.755	25
C	1.598	1.620	1.609	50
D	1.346	1.389	1.368	100
E	0.969	0.958	0.964	250
F	0.737	0.751	0.744	500
G	0.557	0.565	0.561	1000
H	0.404	0.409	0.407	2500
Unknown	0.786	0.795	0.791	400

TYPICAL CALIBRATOR CURVE

Sample curve only.
Do not use to calculate results.



PERFORMANCE CHARACTERISTICS

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 was determined to be 5.4 pg/mL and the Limit of Detection was determined to be **12.4 pg/mL**.

SPECIFICITY (CROSS-REACTIVITY)

The cross-reactivity was evaluated in relation to estrogens 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
Androstenedione	0.2
Androsterone	0.2
Cholesterol	0
Corticosterone	< 0.01
Cortisol	< 0.1
DHEA	0.3
DHEAS	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 conjugated and unconjugated up to 10 mg/dL, Triglycerides up to 5 mg/mL, Biotin up to 2.4 µg/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 (Faslodex®). This cross-reactivity can cause falsely elevated estrogen levels in patients under Fulvestrant treatment.

The following results were obtained with theTotal 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)	Sample Spiked to 25 ng/mL Fulvestran (pg/mL)
Pool 1	106.8	128.6
Pool 2	87.8	105.8
Pool 3	326.4	377.6

The Cmax has been reported as 11.4 ng/mL (Robertson and Harrison, 2004) and 25.1 ng/mL (AstraZeneca Canada, 2017).

References

1. Faslodex® Product Monograph. AstraZeneca Canada, 2017
2. Robertson JFR and Harrison M. Fulvestran Pharmacokinetics and pharmacology. British Journal of Cancer. 2004; 90:S7–S10.

PRECISION

The experimental protocol used a nested components-of-variance design with 10 testing days, two runs per scientist per day, and two replicate measurements per run (a 10 x 2 x 2 x 2 design) for each sample. The results were analyzed with a two-way nested ANOVA and summarized in the table below.

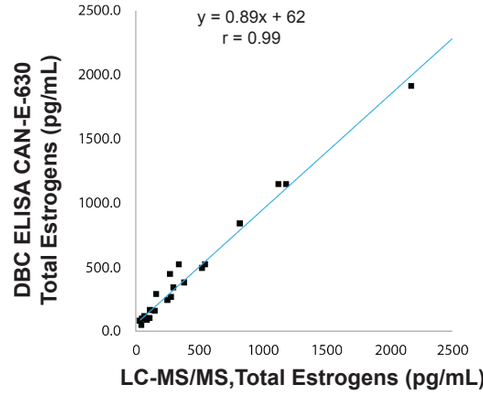
Sample	Mean	Within Run SD	Within Run CV%	Between Run SD	Between Run CV%	Total SD	Total CV%
1	104.6	6.6	6.3	8.3	8.0	11.9	11.4
2	56.5	5.3	9.3	7.0	12.4	8.8	15.5
3	377.2	17.6	4.7	10.8	2.9	24.4	6.5
4	83.3	4.7	5.7	4.2	5.0	7.1	8.5
5	100.2	6.0	6.0	7.5	7.4	9.9	9.9
6	251.8	10.3	4.1	13.3	5.3	17.0	6.8
7	365.9	16.8	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 guideline EP06-A. The samples were diluted in calibrator A at several equidistant concentration levels and up to ten percent (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 DBC 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:
y = 0.89x + 62, r = 0.99



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.

Group	N	Median (pg/mL)	95% Reference 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

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SYMBOLS

CE European Comformity	IVD In vitro diagnostic device	Consult instructions for use
Contains sufficient for <n> tests	Storage Temperature	Legal Manufacturer
Use by	REF Catalogue Number	EC REP Authorized representative
LOT Lot number	X # Dilute 1: # Before use	