ETHYNYLESTRADIOL ELISA

5081ESTR[5]01.21

A competitive enzyme immunoassay for screening and quantitative analysis of ethynylestradiol in various matrices

EUROPROXIMA ETHYNYLESTRADIOL ELISA

A competitive enzyme immunoassay for screening and quantitative analysis of ethynylestradiol in various matrices

TABLE OF CONTENTS

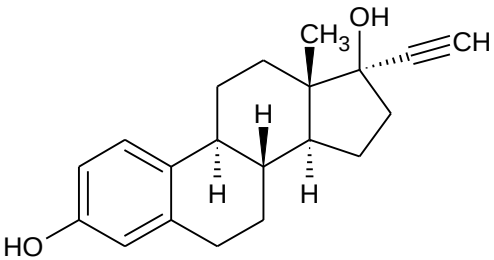
The quality management system of R-Biopharm Nederland B.V. is ISO 9001:2015 certified

5081ESTR[5]01.21

BRIEF INFORMATION

The ethynylestradiol ELISA is a competitive enzyme immunoassay for measurement of the concentration of ethynylestradiol. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that a total of 40 samples can be analyzed.
The ELISA kit contains all reagents to perform the assay. Reagents for sample preparation are not included in the kit.

1. INTRODUCTION



Chemical structure of ethynylestradiol

Ethynylestradiol is a synthetic alkylated estradiol with a 17α-ethynyl substitution. It has high estrogenic potency when administered orally and is often used as the estrogenic component in oral contraceptives.

2. PRINCIPLE OF THE ETHYNYLESTRADIOL ELISA

The microtiter plate based ethynylestradiol ELISA consists of one precoated plate (12 strips, 8 wells each). Antibody, Horseradish peroxidase (-HRP) labeled ethynylestradiol and standard solution or sample are added to wells. Free ethynylestradiol from the samples or standards and ethynylestradiol-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation step of 2 hours the non-bound reagents are removed in a washing step. The amount of bound ethynylestradiol-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H_2O_2/TMB). Bound ethynylestradiol-HRP conjugate transforms the colourless chromogen into a coloured product.
The substrate reaction is stopped by the addition of sulfuric acid. The colour intensity is measured photometrically at 450 nm. The optical density is inversely proportional to the ethynylestradiol concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The ethynylestradiol ELISA utilizes antibodies raised in rabbit against protein conjugated ethynylestradiol. The reactivity pattern of the antibody is:
Cross-reactivity:

Ehynylestradiol	100%
Norethindrone	90%
Norethandrolone	46%
17β-Estradiol	0.4%
Diethylstilbestrol	<0.01%
Estrone	<0.01%

The cross-reactivities are determined in a buffer system. The reported values may be different in samples due to matrix effects.
The test cannot discriminate between analytes and cross-reactive substances

The Limit of detection (LOD) and the detection capability (CCβ) are determined under optimal conditions. Cut-off criteria need critical consideration.

Matrix	Procedure	LOD (ppb)	CCβ (ppb)
Tissue	8.1	0.18	0.2
Tissue SPE	8.2	0.07	0.2
Plasma and Serum	8.3	0.14	0.5
Urine SPE	8.4	0.33	0.5

If the sample is found to be non-compliant, the results shall be verified by re-analysis of the sample using a confirmatory method.

4. HANDLING AND STORAGE

- Kit and kit components should be stored at 2°C to 8°C in a dark place. For repeated use store kit components as specified under chapter 9.
- After the expiry date of the kit and/or components has passed, no further quality guarantee is valid.
- Bring all kit components including the microtiter plate to ambient (room) temperature before use.
- Dilute the kit components immediately before use, but after the components are brought to ambient temperature.
- Avoid condensation in the wells of the plate. Bring the sealed plate to ambient temperature before opening the plate sealing.
- The substrate chromogen solution can be stored in a refrigerator (2ºC to 8ºC) until the expiry date stated on the label.
- Exposure of the chromogen solution to light should be avoided.

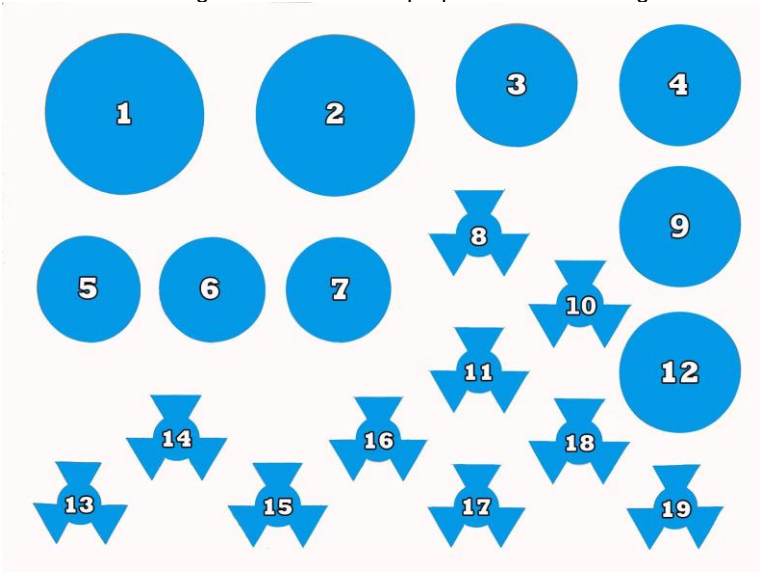
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed (96-wells) microtiter plate (12 strips, 8 wells each), coated with antibody. Plate is ready-to-use.

Position of the reagents in the kit. For preparation of the reagents see Chapter 9.



1. **Dilution buffer** (30 ml, 10x concentrated)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, ready-to-use)
4. **Stop solution** (15 ml, ready-to-use)
5. Not in use
6. Not in use
7. Not in use
8. **Conjugate solution** (100 µl; 100x concentrated)
9. Not in use
10. **Antibody solution** (100 µl; 100x concentrated)
11. Not in use
12. Not in use
13. **Zero Standard Solution** (2 ml, Ready-to-use)
14. **Standard solution 1** (1 ml, Ready-to-use) **0.02 ng/ml**
15. **Standard solution 2** (1 ml, Ready-to-use) **0.05 ng/ml**
16. **Standard solution 3** (1 ml, Ready-to-use) **0.10 ng/ml**
17. **Standard solution 4** (1 ml, Ready-to-use) **0.25 ng/ml**
18. **Standard solution 5** (1 ml, Ready-to-use) **1.0 ng/ml**
19. **Standard solution 6** (1 ml, Ready-to-use) **2.0 ng/ml**

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Evaporation equipment
- Fume hood
- Incubator 55°C
- Homogeniser (vortex, mixer)
- Centrifuge (2000 x g)
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- 4 ml glass tubes
- 20 ml glass tubes
- Methanol 100%
- Ethylacetate
- Tert-butyl-methylether
- Petroleum ether
- SPE C18 100 mg/column
- Helix Pomatia juice
- Sodium acetate

7. PRECAUTIONS

- This kit may contain hazardous substances. For hazard notes please refer to the appropriate safety data sheets (SDS).
- Avoid contact of all biological materials with skin and mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
- Do not use components past expiration date and do not use components from different lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate and rinsing buffer, which crystallize at +4ºC.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain good precision and accuracy.

8. SAMPLE PREPARATIONS

8.1 Tissue direct

- Homogenise approximately 10 grams of sample
- Weigh 1 gram of homogenised sample into a clean tube
- Add 3 ml 100% ethylacetate, vortex
- Mix 15 minutes, head over head (rotor)
- Centrifuge at 2000 x g for 10 minutes
- Pipette 500 µl into a glass tube, evaporate to dryness under a mild stream of nitrogen at 50˚C
- Dissolve the residue with 500 µl sample dilution buffer (see chapter 9)
- Mix thoroughly
- Use 50 µl of this solution in the ELISA

8.2. SPE extraction procedure for Tissue

- Remove fat and grind the sample
- To 1 g of homogenised and ground sample add 2 ml of PBS Buffer (see chapter 9), vortex for 15 seconds
- Extract with 8 ml of tert-butyl-methylether
- Vortex thoroughly and mix head over head for 20 minutes
- Centrifuge at 2000 x g for 10 minutes
- Transfer the supernatant to a 15 ml tube
- Repeat the extraction with 8 ml of tert-butyl-methylether
- Allow the sample to be released from the bottom of the tube Allowing the tert-butyl methyl ether to get the most out of the sample for a second time
- Combine both phases and evaporate under a mild stream of nitrogen at 50°C
- Dissolve the residue in 1 ml methanol/distilled water (80/20)
- Wash the solution with 3 ml of petroleum ether
- Vortex for 15 seconds then centrifuge at 2000 x g for 5 minutes, remove and reject petroleum ether
- Dilute the residue in 3 ml of distilled water

- Activate the SPE column:
  add 3 ml of 100% methanol and after that add 2 ml of PBS Buffer
  Set the speed of the elution at 1 drop/sec.

- Transfer the sample onto the activated column
- Wash the column with 2 ml methanol/distilled water (40/60)
- Dry column for 2 minutes under vacuum
- Elute with 1 ml methanol/distilled water (80/20)
- Evaporate under a mild stream of nitrogen at 50°C
- Dissolve the residue in 100 µl of methanol and 900 µl of dilution buffer
- Use 50 µl of this solution in the ELISA.

8.3 Plasma and serum direct

- Pipette 1ml of Plasma/Serum into a clean tube
- Add 3 ml 100% ethylacetate, vortex
- Mix 5 minutes, head over head (rotor)
- Centrifuge at 2000 x g for 10 minutes
- Pipette 500 µl into a glass tube, evaporate to dryness under a mild stream of nitrogen at 50˚C
- Dissolve the residue with 500 µl sample dilution buffer (see chapter 9)
- Mix thoroughly
- Dilute this 4 more times in sample dilution buffer
- Use 50 µl of this solution in the ELISA

8.4 SPE extraction procedure for urine

- To 0.5 ml of homogenised urine samples add 3 ml of Buffer 1 (see chapter 9)
- Add 8 µl of Helix Pomatia juice
- Incubate for 3 hours at 55°C or overnight at room temperature

- Activate the column:
  add 3 ml of 100% methanol and after that add 2 ml of Buffer 1.
  Set the speed of the elution at 1 drop/sec.

- Transfer the hydrolysed sample onto the activated column
- Pipette 2 ml of Buffer 1
- Pipette 3 ml of methanol/distilled water (40/60)
- Dry the column for 2 minutes under vacuum
- Elute slowly with 1 ml of methanol/distilled water (80/20) : 15 drops/minute
- Evaporate the eluent under a mild stream of nitrogen at 50°C
- Dissolve the residue in a mix of dilution buffer/methanol (200 µl of methanol and 1.8 ml of dilution buffer)
- Use 50 µl of this solution in the ELISA

9. PREPARATION OF REAGENTS

Before beginning the test, the reagents should be brought up to ambient temperature. Any reagents not used should be put back into storage immediately at +2ºC to +8ºC. Prepare reagents fresh before use.

Microtiter plate
Return unused strips into the resealable bag with desiccant and store at +2ºC to +8ºC for use in subsequent assays. Retain also the strip holder.

Dilution buffer
This buffer is used for the dilution of conjugate, antibody and samples. The dilution buffer is 10x concentrated. Before dilution (10 ml buffer + 90 ml distilled water) the concentrated buffer should be at room temperature (20ºC to 25ºC) and thoroughly mixed. Concentrated buffer can show precipitates, mix well before dilution. The diluted buffer can be stored at +2°C to +8°C.

Sample dilution buffer
Sample dilution buffer is not provided in the kit. Prepare this buffer as follows: Take 18 ml dilution buffer, add 2 ml 100% methanol, mix and store this buffer at 4ºC until use.

Conjugate
The conjugate is 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 min. 1000 x g). Add 5 µl of the concentrated conjugate to 495 µl dilution buffer. Per 2 x 8 wells 400 µl of conjugate solution is required. Store concentrated conjugate immediately upon use at 2°C - 8°C

Antibody
The antibody is 100x concentrated. Spin down the antibody in the vial by a short centrifugation step (1 min. 1000 x g). Add 5 µl of the concentrated antibody to 495 µl dilution buffer. Per 2 x 8 wells 400 µl of antibody solution is required. Store concentrated antibody immediately upon use at 2°C - 8°C

Rinsing buffer
The rinsing buffer is delivered 20x concentrated. Prepare dilutions freshly before use. For each strip 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Substrate/chromogen solution
The substrate/chromogen solution (ready-to-use) tends to precipitate at +4ºC. Take care that this vial is at room temperature when used (keep in the dark) and mix the content before pipetting into the wells.

Buffer 1
50 mM sodium acetate buffer, pH 4.8:
0.41 g sodium acetate in 100 ml distilled water. Adjust pH to 4.8 with 20% of acetic acid

PBS Buffer

Na_2HPO_4	0.77g
KH_2PO_4	0.18g
NaCl	9.94g

In 1 l water, adjust pH to 7.4.

10. ASSAY PROCEDURE

Rinsing protocol

In ELISA's, between each immunological incubation step, unbound components have to be removed efficiently. This is reached by appropriate rinsing. It should be clear that each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.
Basically, manual rinsing or rinsing with automatic plate wash equipment can be performed as follows:

Manual rinsing

1. Empty the contents of each well by turning the microtiter plate upside down and remove residual liquid by striking the plate against a paper towel.
2. Fill all the wells to the rims (300 µl) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out 3 times.
4. Turn the plate upside down and empty the wells by a firm short vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual rinsing solution from the wells.
6. Take care that none of the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment

When using automatic plate wash equipment, check that all wells can be aspirated completely, that the rinsing solution is nicely dispensed reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute three rinsing cycles.

Assay Protocol

1. Prepare samples according to Chapter 8 and prepare reagents according to Chapter 9.

2. Pipette 100 µl of zero standard in duplicate (wells H1, H2, blank).
   Pipette 50 µl of zero standard (Bmax) in duplicate (wells A1, A2).
   Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.02, 0.05, 0.10, 0.25, 1.0 and 2.0 ng/ml).

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

4. Pipette 25 µl of conjugate (ethynylestradiol-HRP) to all wells, except H1 and H2.

5. Pipette 25 µl of antibody solution to all wells except H1 and H2.

6. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

7. Incubate for 2 hours in the dark at 4ºC.

8. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.

9. Pipette 100 µl of substrate solution into each well.

10. Incubate 30 minutes in the dark at room temperature (20ºC to 25ºC).

11. Add 100 µl of stop solution to each well.

12. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells H1 and H2 (Blank) from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the six standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard/ Bmax (wells A1 and A2) and multiplied by 100. The zero standard/ Bmax is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

$$\frac{\text{O.D. standard (or sample)}}{\text{O.D. zero standard/ Bmax}} \times 100 = \text{percentage maximal absorbance}$$

Calibration curve:
The values (percentage maximal absorbance) calculated for the standards are plotted on the Y-axis versus the analyte equivalent concentration (ng/ml) on a logarithmic X-axis.

Alternative for calibration curve:
The value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis.

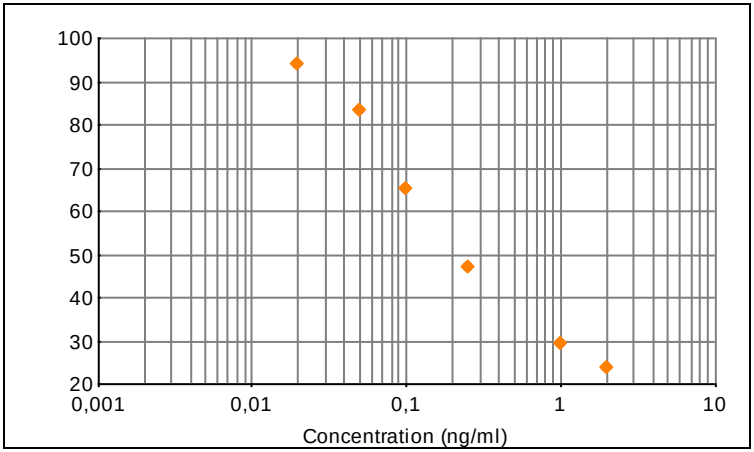


Figure 1 : Example of a calibration curve

The amount of ethynylestradiol in the samples is expressed as ethynylestradiol equivalents. The ethynylestradiol equivalents in the samples (ng/ml) corresponding to the % maximal absorbance of each extract can be read from the calibration curve.

8.1 Tissue direct
To obtain the ethynylestradiol content in tissue samples, the calculated ethynylestradiol equivalents has to be multiplied by a factor 3.

8.2 SPE Tissue
The ethynylestradiol equivalents can be read directly from the standard curve.

8.3.Plasma and Serum
To obtain the ethynylestradiol content in Plasma and Serum samples, the calculated ethynylestradiol equivalents has to be multiplied by a factor 12.

8.4 SPE urine
To obtain the ethynylestradiol content in urine samples, the calculated ethynylestradiol equivalents has to be multiplied by a factor 4.

11. LITERATURE

Not applicable.

13. ORDERING INFORMATION

For ordering the ethynylestradiol ELISA kit, please use cat. code 5081ESTR.

14. REVISION HISTORY

The ELISA is completely revalidated. Sample preparations are updated, LOD values are changed and the test is now validated for plasma/serum.

R-Biopharm Nederland B.V.
Beijerinckweg 18
6827 BN Arnhem
The Netherlands

Tel: + 31 26 3630364
www.europroxima.com
info@r-biopharm.nl