

## **ETHYNYLESTRADIOL ELISA**

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

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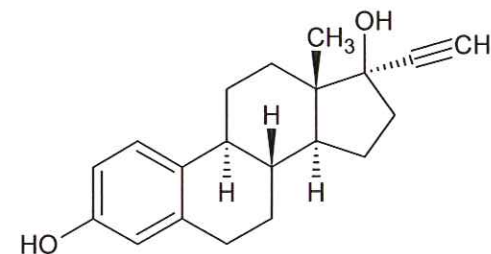
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## BRIEF INFORMATION

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 17 $\alpha$ -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<sub>2</sub>O<sub>2</sub>/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:

Ethynylestradiol	100%
Norethindrone	90%
Norethandrolone	46%
17 $\beta$ -Estradiol	0.4%
Diethylstilbestrol	<0.01%
Estrone	<0.01%

The Limit of detection (LOD) is calculated as:  $X_n+3SD$  and is determined under optimal conditions.

Matrix	Procedure	LOD (ppb)
Tissue	8.1	0.035
Muscle	8.2	0.02
Urine	8.3.1	0.6
Urine	8.3.2	0.04

### 4. HANDLING AND STORAGE

- Store the kit 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 (see kit label) has passed, quality claims are not accepted.
- Before opening the sealed plate, the plate should be at ambient temperature in order to avoid condensation in the ELISA.
- Dilute the kit components immediately before use, but after the components are at ambient temperature.
- The substrate chromogen solution can be stored in a refrigerator (+2°C to +8°C) until the expiry date stated on the label.
- Any direct action of light on the substrate chromogen solution should be avoided.

Degeneration of the reagents may have occurred when the following phenomena are observed:

- A blue colouring of the substrate solution before transferring it into the wells.
- A weak or absent colour reaction of the zero standard ( $B_{max}$ ,  $E_{450nm} < 0.8$ ).

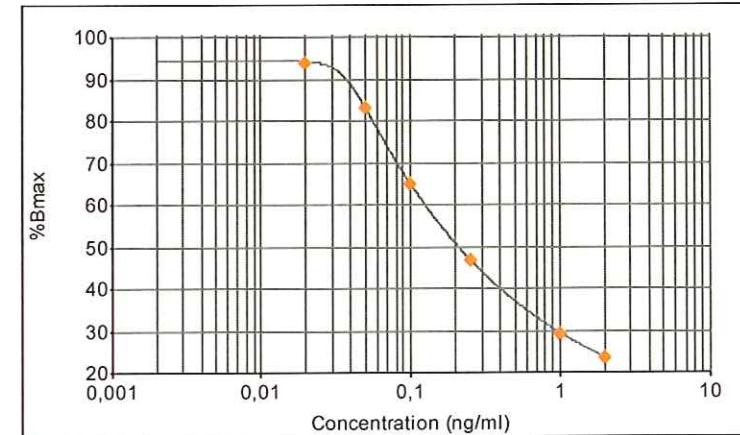


Figure 1 : Example of a calibration curve

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

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

#### 8.2 SPE muscle

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

#### 8.3.1 Urine direct

To obtain the ethynylestradiol content in urine samples, the calculated ethynylestradiol equivalents has to be multiplied by a factor 30.

#### 8.3.2 SPE urine

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

### 12. ORDERING INFORMATION

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

### 13. LAST MUTATIONS

Urine direct method is added

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 4°C.
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 (20°C to 25°C).
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.

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

#### Calibration curve:

The values (% 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:

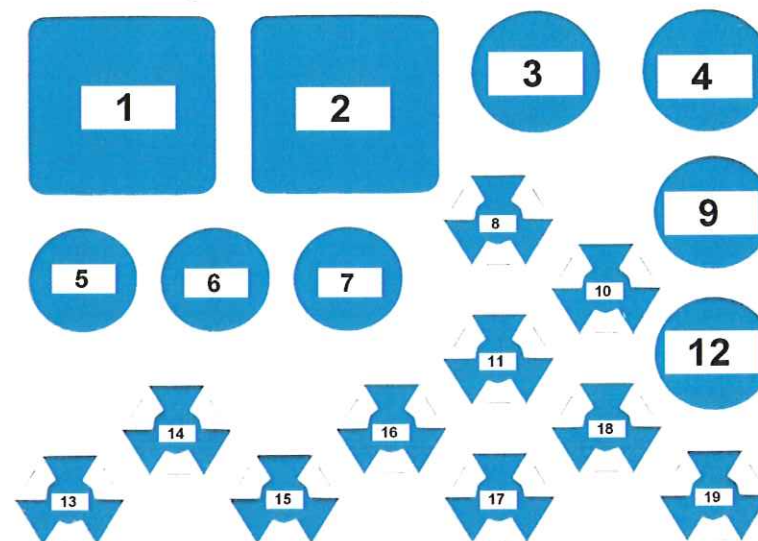
The value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis.

### 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. Not in use
11. Antibody solution (100 µl; 100x concentrated)
12. Not in use
13. Zero Standard (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

### 6.1 General equipment

- 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
- Methanol 100%
- 4 ml glass tubes
- 20 ml glass tubes

### 6.2 Materials and chemicals

The materials and chemicals required for each sample treatment are indicated per chapter.

## 7. PRECAUTIONS

- Ethynylestradiol is a toxic compound. Avoid contact with mouth and skin. Be aware that ethynylestradiol is not inhaled.
- The stop reagent contains 0.5 M sulfuric acid. Do not allow the reagent to get into contact with the skin.
- 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.
- TMB is toxic by inhalation, in contact with skin and if swallowed; take care when handling the substrate.
- 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, which crystallises at +4°C.
- 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.

## 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 (Sample preparation) and prepare reagents according to Chapter 9 (Preparation of reagents).
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, 0.1, 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.

## 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 +2°C to +8°C. Prepare reagents fresh before use.

### Microtiter plate

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

### Dilution buffer (10x concentrated)

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 (20°C to 25°C) 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 4°C 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 40 ml of diluted rinsing buffer is used (2 ml concentrated rinsing buffer + 38 ml distilled water).

### Substrate/chromogen solution

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

## 8. SAMPLE PREPARATION

### 8.1 Tissue

#### Chemicals required

#### Ethylacetate

- Homogenise approximately 10 gram sample
- Weigh 1 gram homogenized 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
- Mix thoroughly
- Use 50 µl of the sample in the ELISA

\* see chapter 9

### 8.2. SPE extraction procedure for muscle

#### Chemicals required

- Tert-butyl-methylether
- Methanol
- Petroleum ether
- Dichloromethane
- Sodium hydroxide
- Phosphoric acid
- Ethanol

#### Buffers required

- (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
- (2) 67 mM phosphate-buffer, pH 7.2  
1.79 g NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O + 9.61 g Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O + 9.0 g NaCl in 1000 ml distilled H<sub>2</sub>O.
- (3) 20 mM Tris-buffer, pH 8.5  
2.42 g Tris-(hydroxymethyl)-amino methane filled up to 1000 ml with distilled water

#### Materials required

Bakerbond SPE C18 100 mg/column (JT Baker ref. nr 7020-01) (flow rate : 1 drop/sec.).

- Remove fat and grind the sample
- To 1 g of homogenised and ground sample add 2 ml of 67 mM of phosphate buffer pH 7.2 (2)

- 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 an 20 ml tube
- Repeat the extraction with 8 ml of tert-butyl-methylether
- Combine the both phases and evaporate under a mild stream of nitrogen at 50°C
- Dissolve the residue in 1 ml methanol/distilled water (70/30)
- Wash the solution with 3 ml of petroleum ether
- Vortex for 15 seconds centrifuge shortly, aspirate and reject petroleum ether
- Evaporate the solution under a mild stream of nitrogen at 50°C
- Dissolve in 1 ml of dichloromethane
- Extract the solution with 3 ml 1M NaOH
- Centrifuge and transfer the supernatant (NaOH phase) into another tube
- Adjust the pH at 8.5 with 6M phosphoric acid

#### Activation of the column

- Add 3 ml of 100% methanol
- Add 2 ml of 20 mM Tris buffer pH 8.5 (3)/methanol (80/20)
- Transfer the sample onto the activated column
- Pipette 2 ml 20 mM Tris buffer pH 8.5 (3)/methanol (80/20) onto the column
- Pipette 3 ml methanol/distilled water (40/60) onto the column
- 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 ethanol and 900 µl of dilution buffer
- Mix and use 50 µl per well in the ELISA.

#### **8.3.1 Urine direct**

- Pipette 100 µl into a tube
- Add 2900 µl dilution buffer
- Vortex
- Centrifuge at 2000 x g for 10 minutes
- Use 50 µl in the ELISA

#### **8.3.2 SPE extraction procedure for urine**

##### Chemicals required

- Helix Pomatia juice
- Methanol
- Ethanol

##### Buffers required

- (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
- (2) 67 mM phosphate-buffer, pH 7.2  
1.79 g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  + 9.61 g  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  + 9.0 g NaCl in 1000 ml distilled  $\text{H}_2\text{O}$ .

- (3) 20 mM Tris-buffer, pH 8.5  
2.42 g Tris-(hydroxymethyl)-amino methane filled up to 1000 ml with distilled water

##### Materials required

Bakerbond SPE C18 100 mg/column (JT Baker ref. nr 7020-01) (flow rate : 1 drop/sec.).

- To 0.5 ml of homogenised urine samples add 3 ml of 50 mM sodium acetate buffer pH 4.8 (1)
- Add 8 µl of glucuronidase / arylsulfatase of Helix Pomatia juice
- Incubate for 3 hours at 55°C or overnight at room temperature
- The hydrolysed urine is purified by means of Solid Phase Extraction Column

##### Activation of the column

- Add 3 ml of 100% methanol
- Add 2 ml of 50 mM sodium acetate buffer, pH 4.8 (1)
- Transfer the hydrolysed sample onto the activated column (3.5 ml)
- Pipette 2 ml of 50 mM sodium acetate buffer, pH 4.8
- 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 200 µl of ethanol and 1.8 ml of dilution buffer
- Mix and use 50 µl per well in the ELISA