l'screen Zeranol

Enzyme immunoassay for the detection of zeranol (Cat.nr. HU0050023)

***l'screen* Zeranol** is a kit prepared for a competitive immunoenzymatic assay for the quantitative analysis of zeranol.
The kit contains the procedure and the materials sufficient for 96 determinations, including standards.
For result evaluation a microtiter plate or a strip photometer is required (manual or automatic ELISA reader).

Type of samples that can be analyzed (matrices)
Urine, muscle, liver.

Sample preparation
- Urine: centrifugation, dilution, enzymatic hydrolysis, purification with C18 columns, evaporation, reconstitution
- Muscle: homogenization, solvent extraction, centrifugation, evaporation, reconstitution, purification with C18 column.
- Liver: homogenization, enzymatic hydrolysis, solvent extraction, centrifugation, evaporation, reconstitution, centrifugation, purification with C18 column.

Assay time: 105 minutes (sample preparation not included).

Detection limit
- Urine: 0.25 ppb
- Muscle, liver: 0.025 ppb

Specificity	
Compound	**Cross-reactivity (%)**
Zeranol	100
Zearalanone	522
Zearalenone	18
β-Zearalanol (taleranol)	110
α-Zearalenol	61
β-Zearalenol	6.8
Hex	< 0.05
Des	< 0.05
Dienestrol	< 0.05
17-β-estradiol	< 0.05
Progesterone	< 0.05
Testosterone	< 0.05
Clenbuterol	< 0.05
Trenbolone	< 0.05
Desametasone	< 0.05

1. TEST PRINCIPLE

The assay is performed in plastic microwells which have been coated with anti-rabbit antibodies. Zeranol standard solutions or samples, the enzyme conjugate and a specific antibody anti-zeranol are added to the microwells
During the incubation, free zeranol molecules and enzyme conjugate compete for the anti- zeranol antibodies binding sites. The anti- zeranol antibodies are simultaneously bound to the solid phase. Any unbound substance is then removed in a washing step.
The bound enzyme activity is determined by adding a fixed amount of a chromogenic substrate.
The enzyme converts the colorless chromogen into a blue product during the second incubation.
The addition of the stop reagent leads to a color change from blue to yellow. The absorbance is measured by a microplate reader at 450 nm. The color development is inversely proportional to zeranol concentration in the sample.

2. PROVIDED REAGENTS

Microtiter plate: 96 wells (12 strips x 8 wells), coated with anti-rabbit IgG.
Wells can also be used individually (the strips are breakable); to separate the wells, extract them from the frame and break the septum between them.
Zeranol Std: 6 plastic vials each containing 1.5 ml of the solution in the following concentrations: 0 ng/ml; 0.025 ng/ml; 0.1 ng/ml; 0.3 ng/ml; 1 ng/ml; 3 ng/ml.
Anti-zeranol antibody: 1 plastic bottle containing 8 ml. Blue solution.
Enzyme conjugate: 1 plastic vial containing 200 µl of enzyme conjugate.
Enzyme Conjugate diluent: 1 plastic bottle containing 12 ml of enzyme conjugate diluent. Red solution.
Dilution buffer 1X:1 plastic bottle containing 50 ml. Orange solution.
Washing-buffer 10 X: 1 plastic bottle containing 50 ml.
Developing solution: 1 plastic bottle containing 14 ml.
Stop solution: 1 glass bottle containing 8 ml. White cap.

3. MATERIALS REQUIRED BUT NOT PROVIDED

- Distilled water
- Methanol
- Sodium acetate buffer 50 mM, pH4.8 (urine)
- Tris/HCl buffer 20 mM pH 8.5 (urine)
- *Helix pomatia* β-glucuronidasi 100.000 units/ml (Sigma cat. n. G7017 o G7770; urine, liver).
- Terbuthylmethylether (muscle, liver)
- Buffer A: 67mM phosphate buffer pH 7.2 + 67mM NaCl (muscle, liver)
- Buffer B: 20mM phosphate buffer pH 7.2 + 20mM NaCl (muscle, liver)
- SPE C_{18} column 100 mg/3 ml

Equipment
- Centrifuge (urine, muscle) or type 1 Whatman filters.
- Balance (muscle, liver)
- Homogenizer (muscle, liver)
- Glass tubes
- Vortex
- pH indicator
- Incubator (optional; urine, liver).
- Vacuum manifold
- Vacmaster
- Evaporator (Eurofins Tecna cat.nr. AT070)
- Micropipette 20-200 µl, tips.
- Micropipette 100-1000 µl, tips.
- Multichannel micropipette 50-250 µl,tips.
- Microplate reader, filter 450 nm

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4. WARNING AND PRECAUTIONS FOR THE USERS

- The test is for *in vitro* diagnostic use only.
- Some reagents contain solutions that may be identified as dangerous substance by the Regulation (EC) *N° 1272/2008.* Please refer to *Material Safety Data Sheet* available on both the Eurofins Technologies and Eurofins Tecna (*tecna.eurofins-technologies.com)* web site.
- Handle the reagents with caution, avoiding contact with skin, eyes and mucous membranes.

5. HANDLING AND STORAGE INSTRUCTIONS

- Store the kit at +2/+8 °C and never freeze.
- **Bring all reagents to room temperature before use (at least 1 hour). ATTENTION:** Do not unseal the microplate until it reaches the room temperature**.**
- Reseal the unused strips of the microtiter plate in the bag together with the desiccant bag provided.
- Return all reagents to +2/+8 °C immediately after use.
- Do not use components after the expiration date.
- Do not intermix components from different kit lots.
- Do not use photocopies of the instruction booklet. Follow the original instruction booklet that is included with the kit.
- Do not change the assay procedure, in particular:
  - do not prolong the incubation times,
  - do not incubate the plate at temperatures higher than 25°C,
  - do not shake the plate during the incubations.
- Use accurate and precise micropipettes with suitable tips for dispensing.
- Once started, complete all the steps without interruption.
- The reproducibility of ELISA results depends largely upon the efficiency and uniformity of microwells washing; always keep to the described procedure.
- Use a single disposable tip for each standard solution and sample to avoid cross-contamination.
- Do not allow tips to contact the liquid already in the microwells.
- Avoid exposure to direct light during all incubations. It is recommended to cover the microtiter plate without using plate sealers.

6. SAMPLE PREPARATION

6.1 Urine samples

Solid Phase Extraction (SPE)

1) Centrifuge the samples at 2000 g for 5 min.
2) Dilute 0.5 ml of urine with 2.5 ml of sodium acetate buffer 50 mM pH 4.8.
3) Add 10 µl of Helix Pomatia β-glucuronidase, control pH and in case adjust it at 4.8-5; mix.
4) Incubate 2 h at 37°C or 16 h (overnight) at room temperature (20-25°C).
5) Equilibrate C18 columns as follows (flow rate: one drop / sec):
   - 3 ml of methanol 100%.
   - 2 ml of methanol/Tris HCl 20 mM pH 8,5 (20:80 vol/vol).
6) Apply the sample (previously bring it back at room temperature) and allow it to flow down under gravity.
7) Wash the column with:
   - 2 ml of methanol/Tris HCl 20 mM pH 8.5 (20:80 vol/vol).
   - 2 ml of methanol/water (40:60 vol/vol).
8) Dry the column with vacuum for 2 min.
9) Elute with 1 ml of methanol/water (80:20 vol/vol), by gravity, into a glass vial.
10) Evaporate the eluate to dryness by placing it at 50-60°C, under a stream of nitrogen/air.
11) Take up the residue in 0.5 ml of kit Dilution Buffer 1x and thoroughly mix (vortex) for 2 min to resuspend it.
12) Dilute 10x (1+9) with dilution buffer 1X
13) The dilution factor is 10.

6.2 Muscle samples

Solid Phase Extraction (SPE)

1) Remove the fat and homogenize the lean part
2) In a glass tube, add 1ml of buffer A to 1gr of sample. Mix with vortex for 1min.
3) Add 5ml of tert-butyl-methylether.
4) Mix 45 minutes on a shaker (about 400 rpm).
5) Centrifuge 10min. at 3000 g.
6) Transfer 4ml of organic phase (upper) to 10 ml glass tube.
7) Shortly mix the acqueous phase with vortex
8) Repeat the extraction with 5ml of tert-butyl-methylether for 45 minutes
9) Add 4 ml of the organic phase (upper) to the previous one.
10) Evaporate the two extracts (8 ml in total) at +40°C under a stream of nitrogen or air.
11) Dissolve the residue with 0.8 ml of methanol/water (80/20)
12) Dilute with 2 ml of buffer B.
13) Centrifugate 10min. at 3000 g.
14) Purify the extract on SPE C18 column:
15) Rinse C_{18} column with 3ml of methanol (100%)
16) Equilibrate column with 2ml of buffer B.
17) Apply 2ml of clear sample onto the column.
18) Rinse with 2ml of methanol/water (40/60).
19) Dry column for 1min. under vacuum.
20) Elute sample with 1ml of methanol/water (80/20).
21) Evaporate the eluted sample at +60°C under a weak stream of nitrogen or air.
22) Dissolve the residue with 0.57 ml of dilution buffer 1x, by mixing on vortex.
23) The dilution factor is 1.

6.3 Liver samples

Solid Phase Extraction (SPE)

1) Homogenize the liver.
2) In a glass tube, add 2 ml of buffer A, 1g of liver homogenate and 10 µl of β-glucuronidase.
3) Mix with vortex for 1min, then incubate for 3 hours at 37°C or overnight at room temperature.
4) To 2 g of the digested homogenate, add 5ml of tert-butyl-methylether.
5) Mix for 45 minutes on a shaker (about 400 rpm).
6) Centrifuge 10min. at 3000 g.
7) Transfer 4ml of the upper organic phase to another glass tube.
8) After shortly mixing the acqueous phase with vortex, repeat the extraction with 5ml of tert-butyl-methylether for 45 minutes.
9) Pool the two organic phases (about 8 ml) and evaporate at +40°C under a stream of nitrogen or air.
10) Dissolve the residue with 0.8 ml of methanol/water (80/20)
11) Dilute with 2 ml of bufferB.
12) Centrifugate 5 minutes at 3000 g.
13) Purify the extract on SPE C18 column:
14) Rinse column with 3ml of methanol (100%)
15) Equilibrate column with 2ml of bufferB.
16) Apply 2ml of clear sample onto the column.
17) Rinse with 2ml of methanol/water (40/60).
18) Dry column for 1 min. under vacuum.
19) Elute sample with 1ml of methanol/water (80/20).
20) Evaporate the eluted sample at +60°C under a weak stream of nitrogen or air.

21) Dissolve the residue with 380 µl of dilution buffer 1x, by mixing on vortex.
22) The dilution factor is 1.

7. WORKING SOLUTIONS PREPARATION

Zeranol Std: ready to use.
Enzyme conjugate diluent: ready to use.
Enzyme conjugate: **ATTENTION:** In order to recover the total amount of the conjugate, before use, centrifuge the vial for some seconds at low speed *(spin-down).*
Calculate and prepare the quantity required for the analytical session. Dilute the conjugate **1/100** in the enzyme diluent. *For example:* take 20 µl of enzyme conjugate concentrate + 1980 µl of enzyme diluent.
DO NOT VORTEX
ATTENTION: In order not to take less than 20 µl of concentrate enzyme conjugate make two consecutive dilutions.
Anti-zeranol antibody: ready to use.
Dilution buffer 1X: ready to use.
Washing buffer 10X: dilute the concentrate 1:10 (1+9) with distilled water. **ATTENTION**: In presence of crystals, bring the solution at room temperature and stir in order to solve them completely.
The diluted washing buffer is stable at room temperature for 24 hours and at +2/+8°C for two weeks.
Developing solution: ready to use; this solution is light sensitive: keep away from direct light.
Stop solution: ready to use. **ATTENTION**: It contains 1M sulphuric acid. Handle with care; in case of contact flush immediately with plenty of water.

8. ASSAY PROCEDURE

1) Predispose an assay layout, recording the standard and samples positions, taking into account that all have to be run in duplicate.
2) First incubation
   - Add 50 µl of each standard/ sample into the corresponding wells.
   - Using the multichannel pipet, add 50 µl of enzyme conjugate in each well.
   - Using the multichannel pipet, add 50 µl of antibody in each well.
   - Shake the plate gently with rotatory motion for few seconds.
   - Incubate 90 minutes at room temperature.
   - Do not prolong the first incubation time and do not use automatic shakers.
3) Washing
   - Pour the liquid out from the wells.
   - Fill completely all the wells with working wash solution using a squeeze bottle. Pour the liquid out from the wells.
   - Repeat the washing sequence four (4) times.
   - Remove the remaining droplets by tapping the microplate upside down vigorously against absorbent paper.

Do not allow the wells to dry out

4) Developing
   - Using the multichannel pipet, add 100 µl of developing solution to each well.
   - Mix thoroughly with rotatory motion for few seconds.
   - Incubate for 15 minutes at room temperature.
5) Using a multichannel pipet, add 50 µl of stop solution to each well and mix thoroughly with rotatory motion for few seconds.
6) Measure the absorbance at 450 nm.
7) Read within 60 minutes.

9. CALCULATION OF RESULTS

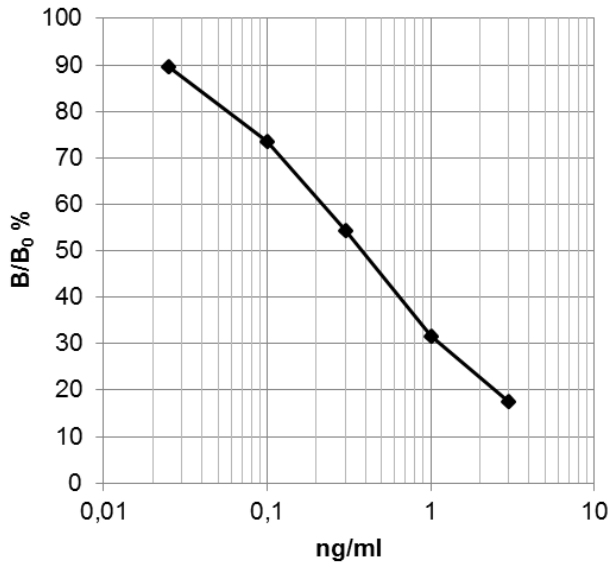
- Calculate the mean absorbance of each control, standard and sample.
- Divide the mean absorbance value of each standard and sample by the mean absorbance of the standard 0 and multiply by 100; the standard 0 is thus made equal to 100% and all the other absorbance values are expressed as percentages:

$$\frac{\text{Standard (or sample) absorbance}}{\text{Standard 0 } (B_0) \text{ absorbance}} \times 100 = \frac{B}{B_0}(\%)$$

- Enter the B/B_0 values calculated for each standard in a semi-logarithmic system of coordinates and draw the standard curve.
- Interpolate the B/B_0 value of each sample to the corresponding concentration from the calibration curve. For dilution applications multiply this concentration for the dilution factor, as reported in chapter 6.

Please note: For results calculation, Excel spreadsheets are available on Eurofins Tecna website tecna.eurofins-technologies.com and can be downloaded directly from the bottom of the product page.

10. CALIBRATION CURVE EXAMPLE



11. EVALUATION OF RESULTS

After processing the results, it is necessary to verify the assay performance. The verification is performed by comparison of obtained data with those given in kit specifications (see chapter 12).
If the values are outside the specifications given, then the results of the test are not assured, therefore the zeranol concentration levels in the samples may not be valid.
In these cases it is advised to check the expiry date of the kit, the wavelength at which the reading was performed, as well as the procedure followed.
If operation errors are not identified as cause, contact our technical assistance.
In order to avoid false no compliant results, it is necessary to adopt a decision limit (CCα).

It is suggested to determine a decision limit for each matrix in your laboratory. In alternative, contact the technical assistance

WARNING: Kit replacement will only be possible in case of return. The kit must be stored in its integral version at +2/+8°C.

12. KIT SPECIFICATIONS

12.1 Assay specification

Description	Specifications
Mean B_0 absorbance	$\geq$ 0.7 OD_{450nm}
B/B_0 50 %	0.3 – 0.8 ng/ml
Std duplicates mean C.V.	$\leq$ 6 %

12.2 Assay performance

The kit performances hereby presented are from an in-house validation; Detection Capability (CCβ) was calculated as requested by of EU Decision 657/2002.

Detection capability or CCβ		
Analyte	**Muscle (ppb)**	**Liver (ppb)**
Zeranol	0,5	
Zeranol+taleranol		0.25+0.25

13. LIABILITY

Samples evaluated as positive using the kit have to be re-tested with a confirmation method.
Eurofins Technologies Hungary shall not be liable for any damages to the customer caused by the improper use of the kit and for any action undertaken as a consequence of results.
Eurofins Technologies Hungary shall not be liable for the unsafe use of the kit out of the current European safety regulations.

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