I'screen Tylosin

Enzyme immunoassay for the detection of tylosin (Cat.nr. HU0050020)

I'screen **Tylosin** is a kit prepared for a competitive immunoenzymatic assay for the quantitative analysis of tylosin.
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).

Analysable samples
animal tissues (muscle, liver and kidney), honey, milk and urines.

Sample preparation
- Tissues: homogenization, extraction in water, centrifugation, filtration
- Milk: centrifugation or filtration, dilution
- Honey: extraction with acetonitrile, centrifugation, evaporation, risospension
- Urine: dilution.

Assay time: 50 minutes (sample preparation not included).

Detection limits
- Tissues, milk: 5 ppb
- Honey: 1 ppb
- Urine: 12,5 ppb

Specificities	
Compound	**Cross-reactivity (%)**
Tylosin A	100
Spyramicin	10
Leucomicin	0.38
Oleandomicin	< 0,01
Tilmycosin	< 0,01
Eritromicin	< 0,01

1. TEST PRINCIPLE

The assay is performed in plastic microwells that have been coated with anti- tylosin antibodies. Tylosin standard solutions or samples, the enzyme conjugate are added to the microwells
During the first incubation, free tylosin in the standard solution /sample and enzyme-labelled tylosin compete for the anti- tylosin antibody binding sites on the solid phase. Any unbound enzyme conjugate and tylosin molecule are then removed in a washing step.
The bound enzyme activity is determined adding a fixed amount of a chromogenic substrate. The enzyme converts the colourless chromogen into a blue product.
The addition of the stop reagent leads to a colour change from blue to yellow. The absorbance is measured with a microplate reader at 450 nm. The colour development is inversely proportional to the tylosin concentration in the standard solution /sample

2. REAGENTS PROVIDED

Microtiter plate: 96 wells (12 strips X 8 wells) coated with anti-tylosin antibodies.
As the strips are breakable, the wells can be used individually. For this purpose, it is sufficient to get out the wells from the frame and to break the joint.
Std Tylosin: 1 plastic vial containing 0.2 ml of 9 mg/ml of tylosin.
Enzyme conjugate: 1 plastic vial containing 0.2 ml.
Enzyme conjugate diluent: 1 plastic bottle containing 12 ml.
Washing-buffer10x: 1 plastic bottle containing 50 ml.
Developing solution: 1 plastic bottle containing 14 ml.
Dilution buffer 10x :1 plastic bottle containing 50 ml.
Stop solution: 1 glass bottle containing 8 ml. White cap.

3. MATERIALS REQUIRED BUT NOT PROVIDED

- Distilled water (tissue, honey)
- Ice (tissue)
- Acetonitrile (honey)
- NaCl (honey)

Equipment
- Tissue homogenizer (tissue)
- Balance (animal tissues, honey)
- Bench centrifuge (milk, animal tissues, honey)
- Filter paper, Whatman n° 4, or equivalent (tissue and milk)
- Evaporator (honey)
- Waterbath incubator (honey)
- Rotatory shaker or rotating wheel (honey)
- 20-200 µl, micropipettes, tips.
- 100-1000 µl, micropipettes, tips.
- 50-250 µl, multichannel micropipette, tips.
- Microplate reader, filter 450 nm.

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.

Eurofins Technologies Hungary Kft., Fóti út 56., 1047 Budapest, Hungary
E-mail: technologies.hu@eurofins.com
Website: www.eurofins-technologies.com

- 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.
- **ATTENTION: It is strongly recommended to be careful to avoid cross-contamination events, especially when performing spiking experiments.**

6. PREPARATION OF SAMPLES

6.1 Milk

1) Refrigerate the sample at +2/+8°C.
2) Centrifuge at +2/+8°C or filtrate it for 10 minutes at 3000 *g*.
3) Separate the fat from the skimmed milk.
4) Dilute the skimmed milk 1:10 with the dilution buffer 1x (for example 100 µl of skimmed milk + 900 µl of dilution buffer 1x).
5) The dilution factor is 10.

6.2 Tissue (muscle, liver, kidney)

1) Grind the defatted sample.
2) Weigh 5 g and add 50 ml of distilled water.
3) Homogenize in a tissue homogenizer (e.g. Ultraturrax) for 1 minute or on a rotating wheel for 30 minutes.
4) Cool at +2/+8°C (on ice or in a refrigerator) for 5 minutes.
5) Centrifuge at 2000 *g* for 5 minutes
6) Recover the supernatant and filter it through a filter paper. Use the clear filtrate directly in the assay.
7) The dilution factor is 10.

6.3 Urine

1) Dilute 1:25x with the dilution buffer (for example 100 µl of urine + 2400 µl of dilution buffer 1x).
2) The dilution factor is 25.

6.4 Honey

1) Weigh 1 g of sample and add 0.5 ml of a 4% NaCl solution in distilled water. Mix until the honey is completely dissolved. If there are difficulties in this step, use a waterbath at 40°C until honey is completely dissolved.
2) Add 2 ml of acetonitrile and mix with a low speed shaker (400rpm) or on a rotating wheel for 10 minutes.
3) Centrifuge at 2000 *g* for 10 minutes.
4) Recover 1 ml of acetonitrile (upper phase) and evaporate it at 50 under a slow air or nitrogen stream.
5) Reconstitute with 1 ml of washing buffer 1x.
6) The dilution factor is 2.

7. WORKING SOLUTIONS PREPARATION

Dilution buffer: dilute 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.

Standard Solutions:

DO NOT VORTEX the solutions, mix gently by inverting the vial.

ATTENTION: In order to reduce the risk of contaminations, it is advisable to prepare the standard curve using a different set of pipettes from that used for the test and to work in an other room different from that used for the assay.

ATTENTION: The standard curve should be prepared fresh for each experiment.

Prepare a first semiconcentrate: 90 µl /ml

- 20 µl of the Tylosin standard solution (9 mg/ml) + 1980 µl of dilution buffer 1x. (1:100)

Prepare a second semiconcentrate: 900 ng/ml

- 20 µl of the first semiconcentrate + 1980 µl of dilution buffer 1x. (1:100).

Proceed with the preparation of standard solutions to be used in the assay:

Std.30 ng/ml: 60 µl of the second semiconcentrate + 1740 µl of dilution buffer 1x;

Std. 10 ng/ml: 200 µl of 30 ng/ml standard + 400 µl of dilution buffer 1x;

Std. 2.5 ng/ml: 250 µl of 10 ng/ml standard + 750 µl of dilution buffer 1x;

Std. 1 ng/ml: 200 µl of 2.5 ng/ml standard + 300 µl of dilution buffer 1x;

Std. 0.5 ng/ml: 100 µl of 1 ng/ml standard + 100 µl of dilution buffer 1x;
Std. 0 ng/ml: 500 µl of dilution buffer 1x.
Enzyme conjugate diluent:
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 concentrated enzyme coniugate + 1980µl of enzyme diluent)
ATTENTION: In order not to take less than 20 µl of concentrate enzyme conjugate make two consecutive dilutions.
DO NOT VORTEX.
Washing buffer: 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.
   - Shake the plate gently with rotatory motion for few seconds.
   - Incubate 30 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 20 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

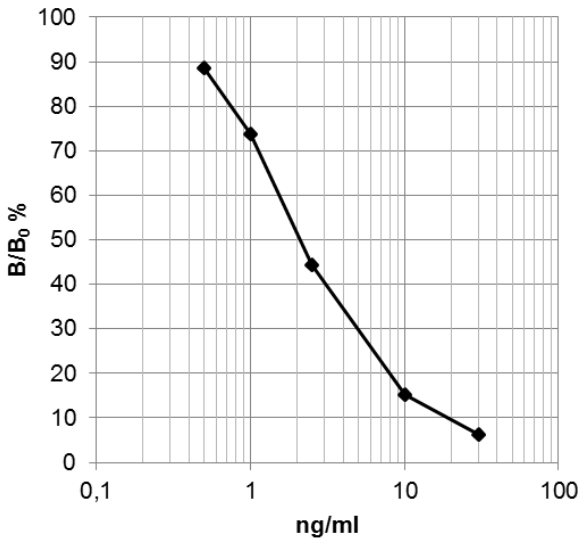
- Calculate the mean absorbance of standard and sample.
- Divide the mean absorbance value of each standard and sample by the mean absorbance of the standard 0 and multiply by 100; the standard 0 is thus made equal to 100% and all the other absorbance values are expressed as percentages:

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

- 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.
- The concentration of tylosin in the sample is to be calculated by multiplying the concentration read on the curve 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. EXAMPLE OF CALIBRATION CURVE



11. RESULTS EVALUATION

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 tylosin 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 positive results, for honey and fish analysis, it is necessary to adopt a decision limit (CCα). It is suggested to adopt an internal CCα. For further information, 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 specifications

Description	Specifications
Mean B_0 absorbance	$\geq$ 0.7 OD_{450nm}
B/ B_0 50 %	0.8 - 4 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		
milk	muscle	honey
25 ppb	50 ppb	5 ppb

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.

14. LITERATURE

Diana F., Puppini B., Bacer V., Postogna E., Persic L. and Paleologo M.. Rapid and sensitive detection of antimicrobial residues in honey by binding assays. Poster presentation at the International Conference on Beekeeping Development and Honey Marketing. 30 October - 1 November, 2010 - Hanoi, Vietnam.