Enzyme immunoassay for the detection of furazolidone metabolite (AOZ) (code AB643)

I'screen AOZv2 (Code AB643) is a kit prepared for an immunoenzymatic assay for the quantitative analysis of 3-amino-2-oxazolidone (AOZ) a metabolite of the nitrofuran furazolidone. The kit contains the procedure and the materials sufficient for 96 determinations (including standards). A microtiter plate photometer or a strip photometer is required.

Analysable samples
Crustaceans, bovine and swine muscle, eggs, honey.

Sample preparation
Homogenization, derivatization/hydrolysis, organic solvent extraction, centrifugation, evaporation, reconstitution.

Assay time: 90 minutes (sample preparation not included).

Detection limit
0.05 ppb

<table>
<thead>
<tr>
<th>Specificity</th>
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<tbody>
<tr>
<td>Compound</td>
<td>Cross-reactivity %</td>
</tr>
<tr>
<td>AOZ</td>
<td>100</td>
</tr>
<tr>
<td>FURAZOLIDONE</td>
<td>106±4%</td>
</tr>
<tr>
<td>AMOZ</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>SEM</td>
<td>&lt; 0.01</td>
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<tr>
<td>AHD</td>
<td>&lt; 0.01</td>
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</tbody>
</table>

1 TEST PRINCIPLE
The assay is performed in plastic microwells that have been coated with anti-AOZ antibodies. AOZ standard solutions or samples and the enzyme labelled AOZ-HRP (horseradish peroxidase) are added to the microplate. During the first incubation, free AOZ molecules and AOZ-HRP compete for the binding sites of the anti-AOZ antibodies bound to the solid phase. Any unbound enzyme conjugate is removed in a washing step. The bound enzyme activity is determined by 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 by a microplate reader at 450 nm. The colour development is inversely proportional to the AOZ concentration in the sample.

2 REAGENTS PROVIDED
Microtiter plate: 96 wells (12 strips x 8 wells), coated with specific anti-AOZ IgG.
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.

AOZ Std: 6 amber plastic vials, each containing 1.5 ml of the following concentrations of AOZ equivalents: 0 ng/ml; 0.025 ng/ml; 0.05 ng/ml; 0.125 ng/ml; 0.42 ng/ml; 2.5 ng/ml.

AOZ Spiking solution 50 ppb: 1 amber plastic vial containing 1 ml of 50 ppb of AOZ.

Enzyme conjugate: 1 plastic bottle containing 8 ml.
2-nitrobenzaldehyde 50mM: 1 glass vial containing 3.5 ml of 2-nitrobenzaldehyde 50mM solution.

Washing buffer 20X: 1 plastic bottle containing 50 ml.
Developing solution: 1 amber plastic bottle containing 14 ml.
Stop solution: 1 glass bottle containing 8 ml. White cap.

3 MATERIALS REQUIRED BUT NOT PROVIDED
- Hydrochloric acid (HCl)
- Distilled water
- Potassium phosphate dibasic (K2HPO4)
- Sodium hydroxide
- Ethyl acetate
- Hexane

Equipment
- Homogenizer (Ultraturrax)
- Balance.
- Incubator or water-bath (37°C or 55°C)
- Centrifuge (3000 x g)
- Dry block heater
- 20-200 and 200-1000 µl micropipette with suitable tips.
- 20-300 µl multichannel micropipette with suitable tips.
- Microtiter plate or strip reader equipped with a 450 nm filter.

4 WARNING AND PRECAUTIONS FOR THE USERS
- For in vitro diagnostic use only.
Some reagents contain solutions that may be identified as dangerous substance by the Regulation (EC) No 1272/2008. Please refer to Safety Data Sheet available on Tecna web site.
- Handle the reagents with caution, avoiding contact with skin, eyes and mucous membranes.

**5 HANDLING AND STORAGE INSTRUCTIONS**
- Do not use components after the expiration date.
- Do not intermix components between different kit lots.
- Do not use photocopies of the instruction booklet. Keep always to the instruction booklet included inside the kit.
- Store the kit at +2/+8°C and never freeze any component.
- Do not use components after the expiration date.
- Reseal the unused strips of the microtiter plate in the bag together with the desiccant bag provided.

**6 SAMPLE PREPARATION**

**6.1 Muscle (bovine, swine, seafood), eggs: long procedure**
- Homogenize the sample, after removing fat.
- Weigh 1 g of homogenized sample and add 4 ml of distilled water, 0.5 ml of 1 M HCl, 60 µl of 50 mM 2-nitrobenzaldehyde solution.
- Mix and incubate over night at 37°C.
- Add 5 ml of 0.1 M K2HPO4, 0.4 ml of 1M NaOH and 5 ml of ethyl acetate; shake vigorously for 30 seconds.
- Centrifuge at 3000 g for 10 minutes at room temperature (RT).
- Transfer 2.5 ml of the organic upper layer (ethyl acetate) and evaporate at 55°C, under a slow air or nitrogen stream. WARNING: in egg samples, an emulsion could occur in the organic phase; in this case, prior to evaporation, centrifuge at 3000g for 10 minutes.
- Dissolve the residue in 1 ml of hexane and add 1 ml of washing buffer 1x; mix properly.
- Centrifuge at 3000 g for 10 minutes at RT.
- Transfer the aqueous lower phase in a new tube. The extract is ready for the assay. The dilution factor is 2.

**6.2 Muscle (bovine, swine, seafood) eggs: short procedure (alternative)**
- Homogenize the sample, after removing fat.
- Weigh 1 g of homogenized sample and add 4 ml of distilled water, 0.5 ml of 1 M HCl, 60 µl of 50 mM 2-nitrobenzaldehyde solution.
- Mix and incubate 2 hours at 55°C.
- Add 5 ml of 0.1 M K2HPO4, 0.4 ml of 1M NaOH and 5 ml of ethyl acetate; shake vigorously for 30 seconds.
- Centrifuge at 3000 g for 10 minutes at room temperature (RT).
- Transfer 2.5 ml of the organic upper layer (ethyl acetate) and evaporate at 55°C, under a slow air or nitrogen stream. WARNING: in egg samples, an emulsion could occur in the organic phase; in this case, prior to evaporation, centrifuge at 3000g for 10 minutes.
- Dissolve the residue in 1 ml of hexane and add 1 ml of washing buffer 1x; mix properly.
- Centrifuge at 3000 g for 10 minutes at RT.
- Transfer the aqueous lower phase in a new tube. The extract is ready for the assay. The dilution factor is 2.

**6.3. Honey**
- Weigh 1 g of honey sample, add 5 ml of hexane, 0.5 ml of 1M HCl and 4 ml of distilled water.
- Mix on vortex for 1 minute.
- Centrifuge at room temperature for 10 minutes at 3000 xg.
- Take all the lower aqueous phase and add 40 µl of 50mM 2-nitrobenzaldeide solution; vortex for few seconds.
- Incubate at +37°C “overnight”.
- Add 5 ml of 0,1M K2HPO4, 0.4 ml of 1M NaOH and 5 ml of ethylacetate.
- Mix on vortex for 1 minute.
- Centrifuge at room temperature for 10 minutes at 3000 xg.
- Transfer 2.5 ml of the upper organic phase in a glass vial and evaporate at 50 – 60°C under a slow air or nitrogen stream.
- By using vortex, dissolve the residue in 1 ml of washing buffer 1x, for 1 minute. The dilution factor is 2.

**7 WORKING SOLUTIONS PREPARATION**

**AOZ Standards:** ready to use.
**AOZ Spiking solution:** ready to use.
**Enzyme Conjugate:** ready to use
**2-nitrobenzaldeide 50mM:** ready to use
**Washing buffer:** dilute the concentrate 1:20 (1+19) with distilled water.
**Developing solution:** ready to use. The solution is light sensitive and must be stored away from direct light.

**ATTENTION:** in presence of crystals, bring the solution immediately with plenty of water.

**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. The solution is light sensitive and must be stored away from direct light.
**Stop solution:** ready to use. Caution: contains 1M sulphuric acid. Handle with care; in case of contact flush immediately with plenty of water.

**8 ASSAY PROCEDURE**

**8.1 Preliminary comments**
- Wait until all kit components reach room temperature before use at least 2 hours.
- After use, return all reagents at +2/+8 °C. Reseal the unused strips with the desiccant bag provided.
- Do not change the assay procedure, in particular:
  - do not prolong or shorten the first incubation time
  - do not incubate the plate at temperatures higher than 25°C and lower than 18°C
  - Do not shake the plate during incubations
  - use always accurate and precise micropipettes with suitable tips
  - Once started, complete all the steps without interruption.
- The reproducibility of ELISA results largely depends upon the efficiency and uniformity of microwells washing; always keep to the described procedure.
- To avoid cross-contamination, use a single disposable tip for each standard and sample.
- Do not allow tips to contact the liquid already present in the microwells or the inner microwells surface.
- Avoid direct sunlight during all incubations. Covering the microwell plate is recommended.

8.2 Assay procedure
1. Predispose the assay layout, taking into account that standards and samples have to be run in duplicate.
2. Add:
   - 50 µl of each standard/sample into the wells
   - 50 µl of enzyme conjugate into the wells, using a multichannel pipette
Shake the plate gently with rotatory motion for a few seconds.
3. Incubate for 60 minutes at room temperature.
4. Washing sequence:
   - at the end of the incubation, 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.
5. Using the multichannel micropipette, add 100 µl of developing solution to the wells and mix thoroughly with rotatory motion for few seconds.
6. Incubate for 30 minutes at room temperature; protect the plate from direct light.
7. Using a multichannel pipette, add 50 µl of stop solution to each well and shake the plate gently with rotatory motion for a few seconds.
8. Measure the absorbance at 450 nm. Read within 60 minutes.

9 CALCULATION OF RESULTS
- Calculate the mean absorbance of each standard and sample;
- Divide the mean absorbance value of each standard and sample by the mean absorbance of the Standard 0 (B₀) and multiply by 100; the maximum binding (B₀) is thus made equal to 100% and the absorbance values are quoted in percentage:

\[
\text{absorbance of standard (or sample)} - \frac{B}{\text{absorbance of Standard 0 (B₀)}} \times 100 = \% \%
\]

- Enter the B/B₀ values calculated for each standard in a semi-logarithmic system of coordinates and draw the standard curve.
- Interpolate B/B₀ value for each sample to the corresponding concentration from the calibration curve. The concentration of AOZ in the sample in ppb (µg/Kg) is calculated by multiplying the concentration read on the curve for the dilution factor 2 of the extraction procedure.

Please note: For results calculation, Excel spreadsheets are available on website and can be downloaded directly from the bottom of the product page.

10 EXAMPLE OF STANDARD CURVE

11 RESULTS EVALUATION
After results elaboration, it is necessary to verify the assay performance. The verification is performed by comparison of obtained data with those given in kit specifications (chapter 12). If the values are out from the specifications given, it is advised to control the expiry date of the kit, the wavelength of absorbance recording, as well as the procedure employed. If operation errors do not emerge, contact our technical assistance.

In order to avoid false non-compliant results, it is suggested to adopt a decision limit (CCα). The decision limit varies according to the kind of sample. It is suggested to determine a decision limit for each matrix routinely analysed in your laboratory.

WARNING: substitution will be possible just in case of rendered kit. The kit must be conserved in its integral version and at the temperature indicated in this booklet.

12 KIT SPECIFICATIONS
12.1 Assay specifications

<table>
<thead>
<tr>
<th>Mean Bo absorbance</th>
<th>≥ 0.7 OD₄₅₀nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>B/Bo 50%</td>
<td>0.11-0.42 ng/ml</td>
</tr>
<tr>
<td>Std duplicates mean C.V.</td>
<td>≤ 6 %</td>
</tr>
</tbody>
</table>

12.2 Assay performance
The kit performances hereby presented derive from an in-house validation.

Detection capability or CCβ

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Shrimps *</th>
<th>Muscle*</th>
</tr>
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<tbody>
<tr>
<td>AOZ</td>
<td>0.2 ppb</td>
<td>0.2 ppb</td>
</tr>
</tbody>
</table>

* long procedure

13 LIABILITY
Samples evaluated as positive using the kit have to be re-tested with a confirmation method. Tecna 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. Tecna shall not be liable for the unsafe use of the kit out of the current European safety regulations.