OXOLINIC ACID ELISA

5101OXO[1]12.20

A competitive enzyme immunoassay
for screening and quantitative analysis of
Oxolinic acid in fish and shrimp

EUROPROXIMA OXOLINIC ACID ELISA

A competitive enzyme immunoassay for screening and quantitative analysis of Oxolinic acid in fish, shrimp and prawn

TABLE OF CONTENTS

PAGE:

Brief Information 2
1. Introduction 2
2. Principle of Oxolinic acid ELISA 2
3. Specificity and Sensitivity 3
4. Handling and Storage 4
5. Kit contents 5
6. Equipment and materials required but not provided ... 6
7. Precautions 6
8. Sample preparations 7
9. Preparation of reagents 8
10. Assay Procedure 10
11. Interpretation of results 12
12. Literature 13
13. Ordering information 13
14. Revision History 13

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

5101OXO[1]12.20

BRIEF INFORMATION

The EuroProxima Oxolinic acid (OXO) ELISA is a competitive enzyme immunoassay for the screening and quantitative detection of oxolinic acid in fish, shrimp and prawn. Samples and standards are measured in duplicate. A total of 40 samples can be analysed with one kit. OXO ELISA contains all required reagents, including standards, to perform the test. Most of the reagents required for sample preparation are also included.

1. INTRODUCTION

Oxolinic acid is a synthetic antimicrobial agent belonging to the first quinolone class of antibiotics. Oxolinic acid is used in veterinary medicine for treatment of cattle, pigs, poultry, fish and shrimp. After antibiotic treatment, the level of residues in animal derived products should be low to limit human exposure to these drug residues. In the European Union in accordance with Commission Regulation (EU) No 37/2010 the maximum residue limits (MRLs) for oxolinic acid in animal derived products are 100 µg/kg in muscle, 50 µg/kg in fat, 150 µg/kg in liver and 150 µg/kg in kidney.

2. PRINCIPLE OF OXOLINIC ACID ELISA

The microtiter plate-based OXO ELISA consists of one precoated plate with antibody against OXO (12 strips, 8 wells each). Horseradish peroxidase (-HRP) labeled OXO and standard solution or sample are added to the wells. Free OXO from the samples or standards and OXO-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay). After an incubation time of 30 min at room temperature, the non-bound reagents are removed in a washing step. The amount of bound OXO-HRP conjugate is visualized by the addition of a substrate/chromogen solution (tetramethylbenzidine, TMB). During the incubation the colourless chromogen is converted by the peroxidase enzyme into a blue reaction product. This blue colour is inversely proportional to the amount of bound OXO. The more OXO is present in the standard solution or sample, the less colour is developed. The enzymatic reaction is stopped by the addition of sulphuric acid. The colour intensity is measured photometrically at a wavelength of 450 nm.

3. SPECIFICITY AND SENSITIVITY

The cross-reactivity pattern of the antibody is as follows:

(Fluoro)quinolone	Cross-reactivity (%)
Oxolinic acid	100%
Norfloxacin	<0.5%
Cinoxacin	<0.5%
Enrofloxacin	<0.5%
Ciprofloxacin	<0.5%
Flumequine	<0.5%
Ofloxacin	<0.5%
Enoxacin	<0.5%

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.

	LOD (µg/kg)	CCβ (µg/kg)
Fish	2	3
Prawn and Shrimp	2	3

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.

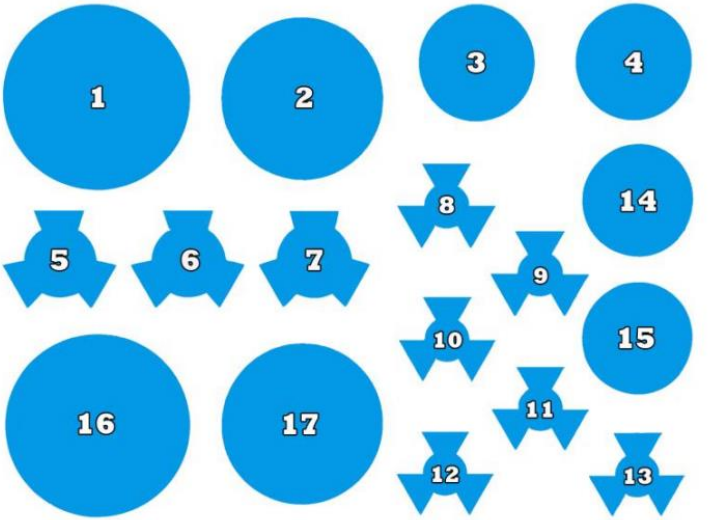
Degeneration of the reagents may have occurred when the following phenomena are observed:

- 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. Ready-to-use.

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



1. **Dilution buffer** (40 ml, ready-to-use)
2. **Rinsing buffer** (30 ml, 20× concentrated)
3. **Substrate solution** (12 ml, ready-to-use)
4. **Stop solution** (12 ml, ready-to-use)
5. **Conjugate** (lyophilized)
6. **Conjugate** (lyophilized)
7. **Standard** (1 ng/ml, lyophilized)
8. Not in use
9. Not in use
10. Not in use
11. Not in use
12. Not in use
13. Not in use
14. **Extraction solution** (10 ml, 10× concentrated)
15. **Spiking solution** (1 µg/ml, lyophilized)
16. **Dilution buffer** (40 ml, ready-to-use)
17. Not in use

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser
- Vortex mixer
- Centrifuge (4000 × g)
- Automated microplate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes 20 – 200 µl, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Aluminium foil or parafilm
- Deionized or distilled water
- Methanol
- 15 ml polypropylene centrifuge tubes

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 PREPARATION

8.1 Fish, shrimp and prawn

- Weigh 1 g of homogenised sample into a 15 ml polypropylene centrifuge tube.
- Add 1 ml of diluted extraction solution (see Chapter 9) and shake the sample intensively or vortex for 1 min.
- Add 3 ml of methanol and shake intensively or vortex for 1 min.
- Mix head over head for 10 min.
- Centrifuge for 5 min at 4000 × g.
- Transfer 20 µl of the upper layer into 780 µl of dilution buffer and vortex
- 50 µl of the solution is used in the ELISA test

9. PREPARATION OF REAGENTS

Before starting the assay, reagents should be brought to ambient temperature (20ºC - 25ºC). Any unused reagents should be stored immediately at +2ºC to +8ºC.
Prepare reagents freshly before use.

Microtiter plate
Bring the plate to ambient temperature before opening, to avoid condensation in the wells. Return unused strips into the zip resealable bag with desiccant and store at +2°C to +8°C for use in subsequent assays. Retain also the strip holder.

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

Dilution buffers
This ELISA kit contains two bottles of dilution buffer which is ready to use . The dilution buffer is used for the dilution of the conjugate, standards and samples.

Standards
Prepare a dilution range of the standards. Add 2 ml of the dilution buffer to the lyophilised standard and mix. This solution contains 1 ng/ml of oxolinic acid. Pipette 0.4 ml of this solution into a clean tube and add 0.6 ml of the dilution buffer. Continue to make a dilution range of 1, 0.4, 0.16, 0.064, 0.026 and 0.010 ng/ml of oxolinic acid. The reconstituted standard 1 ng/ml can be frozen (-20°C) and used again within 6 months.

Conjugate (lyophilized)
Add 4 ml of the dilution buffer to the conjugate vial and mix well. One vial is sufficient to analyse half of the plate. If whole plate is used at the same time, add 4 ml of the dilution buffer to each of 2 vials and combine both conjugate solutions before use. The reconstituted conjugate can be frozen (-20°C) and used again within 6 months.

Extraction solution
Extraction solution is provided 10 times concentrated. Prepare the dilution freshly before use, for example by adding 1 ml of the concentrated extraction solution to 9 ml of distilled water. For each sample 1 ml of diluted extraction solution is used.

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.

Spiking solution
Add 2 ml of distilled or deionized water to the lyophilized oxolinic acid spiking solution vial and mix well to obtain 1 µg/ml (1000 ng/ml) solution.

Spike the samples with appropriate volume of the spiking solution to obtain the required spiking level. The recommended spiking volume is 20-100 µL. If needed, dilute the spiking solution further in water to 100 ng/ml or 50 ng/ml to avoid using very small volumes for spiking. The reconstituted spiking solution can be stored frozen (-20°C) and used again within at least 3 months.

10. ASSAY PROCEDURE

Rinsing protocol

Unbound components have to be removed efficiently between each incubation step in ELISAs. This is achieved by appropriate rinsing. Each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.
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 followed by a firm short vertical movement.
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 washing solution in 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 and is 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 the dilution buffer (wells H1, H2, blank). Pipette 50 µl of the dilution buffer in duplicate (wells A1, A2, maximal signal). Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.010, 0.026, 0.064, 0.16, 0.4, 1 ng/ml).

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

4. Pipette 50 µl of the reconstituted conjugate into all wells, except the blank H1 and H2.

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

6. Incubate for 30 minutes in the dark at room temperature (20°C -25°C).

7. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.
8. Pipette 100 µl of substrate solution into each well.
9. Incubate 30 minutes at room temperature (20ºC-25ºC).
10. Add 100 µl of stop solution into each well.
11. 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 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 (wells B1 and B2) and multiplied by 100. The zero standard is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

O.D. standard (or sample)
----------x 100 = Percentage maximal absorbance
O.D. zero standard

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

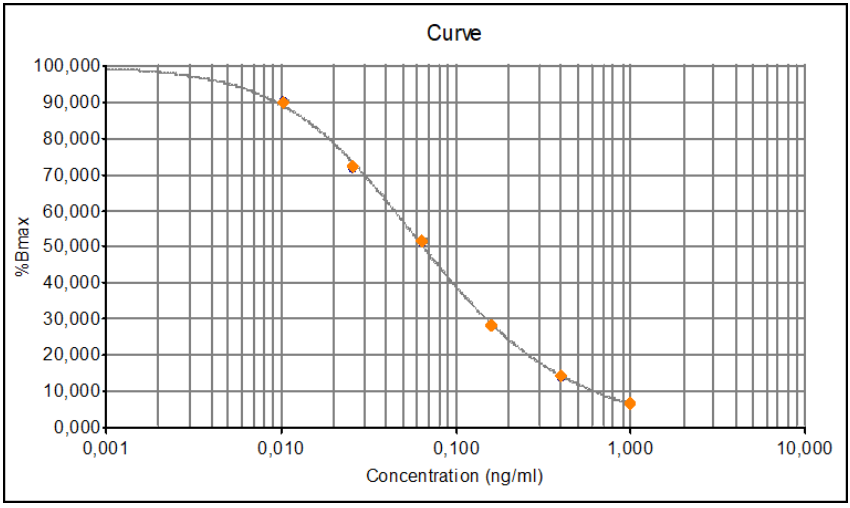


Figure 1: Example of a calibration curve.

Calculation factor

To obtain the total OXO concentration in a sample (µg/kg), the Oxolinic acid equivalent read from the calibration curve has to be multiplied by a factor 200.

12. LITERATURE

Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Official Journal of the European Union L15/1-72.

13. ORDERING INFORMATION

For ordering the EuroProxima Oxolinic acid ELISA, please use catalogue code 5101OXO.

14. REVISION HISTORY

Not applicable

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

TEL: + 31 26 3630364
FAX: + 31 26 3645111
www.europroxima.com
info@r-biopharm.nl