# ENROFLOXACIN ELISA

***5101ERFX[10]04.20***

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

# EUROPROXIMA ENROFLOXACIN ELISA

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

TABLE OF CONTENTS

PAGE:


Brief information ........ 2
1. Introduction ........ 2
2. Principle of the ERFX 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. Preparations of reagents ........ 8
10. Assay procedure ........ 9
11. Interpretation of results ........ 11
12. Literature ........ 13
13. Ordering information ........ 13
14. Revision history ........ 13

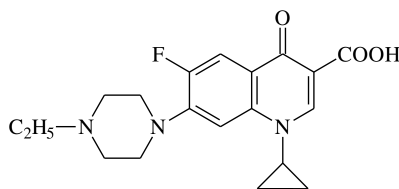

The quality management system of R-Biopharm Nederland B.V. is ISO 9001:2015 certified

5101ERFX[10]04.20

## BRIEF INFORMATION

The enrofloxacin (ERFX) ELISA is a competitive enzyme immunoassay for the screening and quantitative analysis of enrofloxacin in various matrices. The test is based on mouse monoclonal antibodies against ERFX. The ELISA kit contains a 96 well microtiter plate as well as all essential reagents including ready-to-use standards to perform the test. Methods for a fast and efficient extraction of ERFX from different matrices are included in the kit manual.

## 1. INTRODUCTION




ENROFLOXACIN

Enrofloxacin (1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinolone carboxylic acid; ERFX) is a synthetic 6-fluoroquinolone which acts by inhibition of bacterial DNA-gyrase. In veterinary medicine it is administrated by subcutaneous injection to cattle, intramuscularly to pigs, and orally to cattle, pigs, turkeys and chickens for the treatment of infections of the respiratory and alimentary tract.
In Europe, the European Commission, Council Regulation No. 2377/90 and its successive regulations has established the Maximum Residue Limits (MRLs) for drugs employed in veterinary medicine. The MRLs for the sum of ERFX and its active metabolite ciprofloxacin have been stated at 100 µg/kg for muscle tissue and fat (bovine, ovine, caprine, porcine, rabbits, poultry), 200 µg/kg for kidney and 300 µg/kg for liver (bovine, ovine, caprine), 300 µg/kg for kidney and 200 µg/kg for liver (porcine, rabbits, poultry), and 100 µg/kg for milk (bovine, ovine, caprine).

## 2. PRINCIPLE OF THE ERFX ELISA

The kit is based on a microtiter plate (12 strips, each 8 wells), precoated with rabbit antibodies to mouse IgG. Specific antibodies (mouse monoclonal anti-ERFX antibodies), horseradish peroxidase labelled ERFX (enzyme conjugate ERFX-HRP) as well as ERFX standard solutions or samples are added to the precoated wells, followed by a single incubation step. The specific antibodies are bound by the immobilized rabbit anti-mouse antibodies and simultaneously the ERFX-HRP and ERFX present in the standard solutions or in the samples compete for binding to the anti-ERFX antibody (competitive enzyme immunoassay). After incubation for one hour, non-bound reagents are removed in a washing step.
The amount of bound ERFX-HRP is visualized by the addition of enzyme substrate/chromogen (peroxide/tetramethylbenzidine, TMB). Bound enzyme

transforms the 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 and is inversely proportional to the ERFX concentration in the sample.

## 3. SPECIFICITY AND SENSITIVITY

The ERFX ELISA utilizes monoclonal antibodies raised in mouse to protein conjugated ERFX. The reactivity pattern of the antibody is:

| Cross-reactivity: | | |
|---|---|---|
| | Enrofloxacin | 100% |
| | Ciprofloxacin | 0.003% |
| | Benofloxacin | 0.008% |
| | Ofloxacin | 0.16% |
| | Danofloxacin | 0.04% |
| | Benofloxacin | 0.008% |
| | Orbifloxacin | < 0.001% |
| | Norfloxacin | < 0.001% |
| | 1-Ethyl piperazine | 0.45% |
| | 1-Methyl piperazine | 0.15% |
| | Piperazine | < 0.001% |

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) is determined under optimal conditions. Cut-off criteria need critical consideration.

| Matrix | Procedure | LOD (ppb) |
|---|---|---|
| Egg | 8.2 | 9 |
| Tissue | 8.1 | 10 |
| Tissue | 8.1.1 | 4 |
| Milk | 8.3 | 6 |
| Serum | 8.4 | 2.5 |
| Urine | 8.5 | 7 |

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.
- 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.

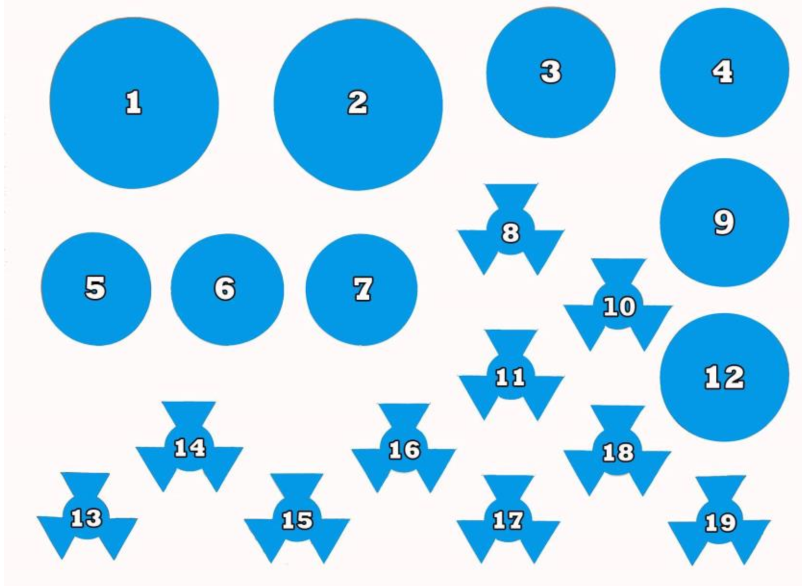
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

## 5. KIT CONTENTS

Manual

One sealed microtiter plate (12 strips, 8 wells each), coated with antibodies to mouse IgG. Plate is ready-to-use.

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




1. **Dilution buffer** (20 ml, ready-to-use)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, ready-to-use)
4. **Stop solution** (15 ml, ready-to-use)
5. **Conjugate** (lyophilized, blue cap)
6. **Antibody** (lyophilized, yellow cap)
7. not in use
8. not in use
9. not in use
10. not in use
11. not in use
12. not in use
13. **Zero standard solution** (2ml, ready-to-use)
14. **Standard solution 1** (1ml, ready-to-use) **0.313 ng/ml**
15. **Standard solution 2** (1ml, ready-to-use) **0.625 ng/ml**
16. **Standard solution 3** (1ml, ready-to-use) **1.25 ng/ml**
17. **Standard solution 4** (1ml, ready-to-use) **2.5 ng/ml**
18. **Standard solution 5** (1ml, ready-to-use) **5.0 ng/ml**
19. **Standard solution 6** (1ml, ready-to-use) **10.0 ng/ml**

## 6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (blender, Ultra Turrax, mixer)
- Centrifuge (for 10 - 15 ml test tubes, with cooling, 2000 x g)
- Vortex
- Automated microplate washer or 8-channel micropipette 100 – 300 µl
- Magnetic stirrer
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Glass test tubes (10 – 15 ml)
- Micropipettes 20 – 200 µl, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Aluminium foil or parafilm
- Methanol 100%
- Hexane
- Disodium hydrogen phosphate
- Potassium dihydrogen phosphate
- Sodium chloride

## 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.

R-Biopharm Nederland makes no warranty of any kind, either expressed or implied, except that the materials from which its products are made are of standard quality. There is no warranty of merchantability of this product, or of the fitness of the product for any purpose. R-Biopharm Nederland shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product.

## 8. SAMPLE PREPARATION

8.1 Method I: Shrimps and muscle tissue (chicken) samples
- Homogenise approximately 10 g of sample.
- Weight 0,5 g of the homogenised sample and transfer into a test tube.
- Add 1.5 ml of 80% methanol in sample dilution buffer (see chapter 9) and mix (30 minutes head over head)
- After centrifugation (10 minutes, 2000 x g), 100 µl of supernatant is pipetted into a tube and 900 µl of sample dilution buffer is added.
- Use 50 µl of this solution in the ELISA test.

8.1.1 Method II: Shrimps and muscle tissue (chicken) samples
- Homogenise approximately 10 g of sample
- Weigh 1 g of the homogenised sample and transfer into a test tube
- Add 3 ml of 80% methanol in sample dilution buffer (see chapter 9)
- Mix (15 minutes head over head)
- Centrifuge (10 minutes, 2000 x g)
- Transfer 2 ml of the supernatant to a glass tube (volume tube 4 ml)
- Evaporate under a mild stream of nitrogen at 50ºC
- Reconstitute the residue with 1 ml of 8% methanol in sample dilution buffer
- Defat by addition of 1.0 ml hexane
- Vortex 1 minute and centrifuge (15 minutes, 2000 x g)
- Use 50 µl of the layer underneath in the ELISA test

8.2 Egg powder samples

Reconstitute the egg powder according to the manufacturers instruction. If the instruction is not available follow next preparation: pipette 3 ml of deionized or distilled water to 1 g egg powder. Mix well, but avoid the solution becomes jelly. Take 0.5 g of this solution and continue the egg sample preparation 8.2.1

8.2.1 Egg samples
- Transfer 0.5 g of the homogenised whole egg, the white or yolk, in a test tube.
- Add 1.5 ml of 40% methanol in sample dilution buffer (see chapter 9) and mix (head over head, 30 minutes)
- After centrifugation (10 minutes, 2000 x g), 100 µl of supernatant is pipetted into a tube and 400 µl of sample dilution buffer is added.
- Use 50 µl of this solution in the ELISA test.

8.3 Milk samples
- Transfer 0.5 ml of the homogenised milk in a test tube and add 4.5 ml of 8% methanol in sample dilution buffer (see chapter 9) and mix (head over head) for 30 minutes
- Centrifugate 10 minutes, 2000 x g
- Use 50 µl of the layer underneath in the ELISA test.

8.4 Serum samples

- Transfer 0.5 ml of the homogenised serum in a test tube and add 4.5 ml of 8% methanol in sample dilution buffer (see chapter 9) and vortex the sample for 2 minutes
- Use 50 µl of this solution in the ELISA test.

8.5 Urine samples

- Transfer 0.5 ml of the homogenised urine in a test tube and add 4.5 ml of 8% methanol in sample dilution buffer*** and vortex the sample for 2 minutes
- An aliquot of 50 µl is used in the ELISA test.

## 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 2ºC to 8ºC. 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.

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

Dilution buffer for conjugate and antibody
For reconstitution of the enzyme conjugate and antibody, ready-to-use dilution buffer is delivered with the kit.
The buffer may be stored in a refrigerator (2°C to 8°C) until the expiration date stated on the kit label.

Conjugate solution
Reconstitute the vial of lyophilized conjugate (ERFX-HRP) with 4 ml of dilution buffer, mix thoroughly and keep in the dark until use.

Antibody solution
Reconstitute the vial of lyophilized antibodies with 4 ml of dilution buffer, mix thoroughly and keep in the dark until use.

Standard solution
The Enrofloxacin standard solutions are ready-to-use. The standard solutions contain 10, 5, 2.5, 1.25, 0.625 and 0.313 ng/ml Enrofloxacin in 8% methanol solution. A ready-to-use zero standard is enclosed. Keep these standard solutions in the dark and store at 2°C to 8°C.

<u>Substrate/chromogen solution</u>
The substrate/chromogen solution (ready-to-use) tends to precipitate at 4°C. Take care that this vial is at 20°C to 25°C and mix the content well before use. Avoid direct (sun) light.

<u>Sample dilution buffer</u>
Sample dilution buffer is not provided in the kit. Prepare this buffer as follows:

Quantities indicated are for 1 liter of buffer:

| | |
|---|---|
| $Na_2HPO_4$ | 0.77 g |
| $KH_2PO_4$ | 0.18 g |
| NaCl | 8.94 g |
| pH | 7.4 (7.3-7.5) |

## 10. ASSAY PROCEDURE

<u>Rinsing protocol</u>

In ELISAs, 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:

<u>Manual rinsing</u>

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 <u>to the rims</u> (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 <u>to remove residual washing solution in the wells</u>.
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 and prepare reagents according to chapter 9.
2. Pipette 100 µl of the zero standard in duplicate (wells H1, H2, blank).
   Pipette 50 µl of the zero standard in duplicate (wells A1, A2, maximal signal).
   Pipette 50 µl of each Enrofloxacin standard dilution (Bmax) in duplicate (wells B1,2 to G1,2 i.e. 0.313, 0.625, 1.25, 2.5, 5 and 10 ng/ml).
3. Pipette 50 µl of each prepared sample solution in duplicate into the remaining wells of the microtiter plate.
4. Pipette 25 µl conjugate (ERFX-HRP) to all wells, except wells H1 and H2.
5. Pipette 25 µl antibody solution to all wells, except wells H1 and H2.
6. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.
7. Incubate for 1 hour in the dark at 37°C.
8. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.
9. Pipette 100 µl substrate/chromogen solution to each well.
10. Incubate for 30 minutes in the dark at 20°C - 25°C.
11. Pipette 100 µl stop solution to each well.
12. Read the absorbance value 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.

$$\frac{\text{OD of standard (or sample)}}{\text{OD of zero standard (Bmax)}} \times 100 = \text{percentage maximal absorbance}$$

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 absorption value of the standards is plotted on the Y-axis versus the concentration on the X-axis. The Y-axis is in logit the Y-axis is logarithmic.

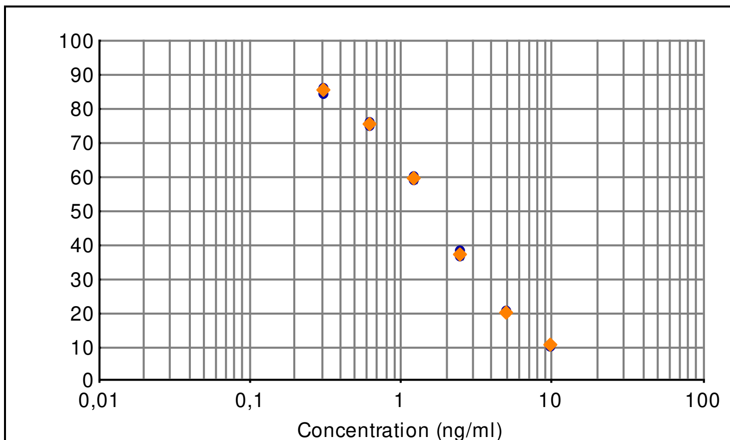



**Figure 1: Example of a calibration curve**

The amount of enrofloxacin in the samples is expressed as enrofloxacin equivalents. The enrofloxacin equivalents in the samples (ng/ml) corresponding to the % maximal absorbance of each extract can be read from the calibration curve.

8.1 Shrimps and muscle tissue (chicken) samples
The Enrofloxacin equivalents, as read from the calibration curve, have to be multiplied by a factor 40 to obtain the Enrofloxacin concentration in shrimps and muscle (chicken) samples.

8.1.1 Shrimps and muscle tissue (chicken) samples
The Enrofloxacin equivalents, as read from the calibration curve, have to be multiplied by a factor 2 to obtain the Enrofloxacin concentration in shrimps and muscle (chicken) samples.

8.2.1 Egg powder and egg samples
The Enrofloxacin equivalents, as read from the calibration curve, have to be multiplied by a factor 20 to obtain the Enrofloxacin concentration in egg powder and egg samples.

8.3 Milk 8.4 Serum and 8.5 Urine samples
The Enrofloxacin equivalents, as read from the calibration curve, have to be multiplied by a factor 10 to obtain the Enrofloxacin concentration in milk, serum and urine samples.

## 12. LITERATURE

Watanabe H., Satake A, Kido Y, Tsuji A. Monoclonal-based enzyme-linked immunosorbent assay and immunochromatographic assay for enrofloxacin in biological matrices. Analyst, **127**, 98-103, 2002.

Watanabe H., Satake A, Kido Y, Tsuji A. Monoclonal-based enzyme-linked immunosorbent assays and immunochromatographic assays for enrofloxacin and salinomycin. In: Residues of veterinary drugs in food. Proceedings of the Euro-Residue IV conference. Edited by L.A. van Ginkel and A. Ruiter. Veldhoven The Netherlands. (2000), 1119-1123.

## 13. ORDERING INFORMATION

For ordering the ERFX ELISA kit, please use cat. code 5101ERFX.

## 14. REVISION HISTORY

The manual is adapted to a new layout of the test kit. Several textual changes are added.

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

Tel: + 31 26 3630364
www.europroxima.com
info@r-biopharm.nl