FLUMEQUINE ELISA

5101FLUM[7]06.20

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

EUROPROXIMA FLUMEQUINE ELISA

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TABLE OF CONTENTS

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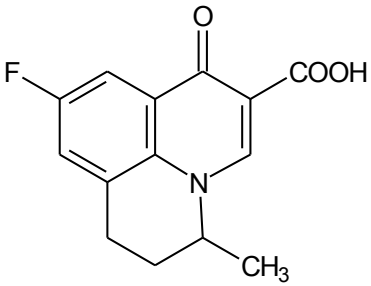
BRIEF INFORMATION

The flumequine ELISA is a competitive enzyme immunoassay for measurement of the concentration of flumequine in various matrices. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that a total of 40 samples can be analyzed.
The ELISA kit contains all reagents to perform the assay. Reagents for sample preparation are not included in the kit.

1. INTRODUCTION



Chemical structure of Flumequine

Flumequine is a second generation quinolone antibiotic and is mainly active against Gram negative bacteria. Flumequine is used in bovine, ovine, poultry, rabbits, goats, horses and salmonidae. As all other (fluoro)quinolones, flumequine acts by inhibition of bacterial DNA-gyrase, abolishing its activity by interfering with the DNA rejoining reaction. Since gyrase is an essential enzyme in prokaryotes, but is not found in eukaryotes, bacteria are an ideal target for these antibiotics.
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 flumequine in the different matrices are as follows:

Animal species	MRLs	Target tissues
Bovine, ovine, caprine, porcine	200 µg/kg 300 µg/kg 500 µg/kg 1500 µg/kg 50 µg/kg	Muscle Fat (porcine; + skin) Liver Kidney Milk
Poultry *	400 µg/kg 250 µg/kg 800 µg/kg 1000 µg/kg	Muscle Skin + fat Liver Kidney
Fin fish	600 µg/kg	Muscle and skin in natural proportions
All food producing species except bovine, ovine, caprine, porcine, poultry and fin fish	200 µg/kg 250 µg/kg 500 µg/kg 1000 µg/kg	Muscle Fat Liver Kidney

* Not for use in animals from which eggs are produced for human consumption

2. PRINCIPLE OF THE FLUMEQUINE ELISA

The microtiter plate based flumequine ELISA consists of one precoated plate (12 strips, 8 wells each). Antibody, horseradish peroxidase (-HRP) labeled flumequine and standard solution or sample are added to wells. Free flumequine from the samples or standards and flumequine -HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation step of one hour, the non-bound reagents are removed in a washing step. The amount of bound flumequine-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H_2O_2/TMB). Bound flumequine-HRP conjugate transforms the colourless 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. The optical density is inversely proportional to the flumequine concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The flumequine ELISA utilizes polyclonal antibodies raised in rabbit to protein conjugated flumequine. The reactivity pattern of the antibody is:
Cross-reactivity:

Flumequine	100%
Enrofloxacine	< 0.1%
Ciprofloxacin	< 0.1%
Pefloxacin	< 0.1%
Enoxacin	< 0.1%
Lomefloxacin	< 0.1%
Levofloxacin	< 0.1%
Fleroxacin	< 0.1%
Difloxacin	< 0.1%
Ofloxacin	< 0.1%
Danofloxacin	< 0.1%
Benofloxacin	< 0.1%
Orbifloxacin	< 0.1%
Norfloxacin	< 0.1%

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)
Tissue	8.1	< 10
Tissue	8.1.1	< 0.5
Egg	8.2	3.5
Honey	8.3	< 10
Milk	8.4	13
Urine	8.5	5.5
Serum	8.6	1
Feed	8.7	10
Water	8.8	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:

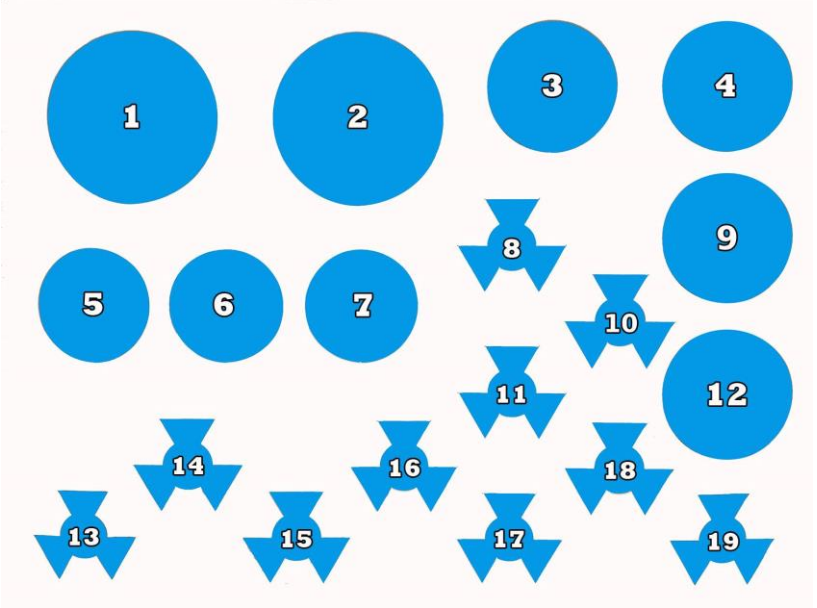
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed (96-wells) microtiter plate (12 strips, 8 wells each), coated with antibody. 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 Solution** (lyophilized, blue cap)
6. **Antibody solution** (lyophilized, yellow cap)
7. not in use
8. **Standard solution 1000 ng/ml** (1ml, Ready-to-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) **0.1 ng/ml**
15. **Standard solution 2** (1ml, Ready-to-use) **0.5 ng/ml**
16. **Standard solution 3** (1ml, Ready-to-use) **1.0 ng/ml**
17. **Standard solution 4** (1ml, Ready-to-use) **5.0 ng/ml**
18. **Standard solution 5** (1ml, Ready-to-use) **10 ng/ml**
19. **Standard solution 6** (1ml, Ready-to-use) **50 ng/ml**

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (vortex, blender, Ultra Turrax, mixer)
- Centrifuge (for 10 - 15 ml test tubes, 2000 x g)
- Vortex
- Automated microplate washer or 8-channel micropipette 100 – 300 µl
- 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
- Aluminum foil or parafilm
- Methanol 100%
- Hexane
- Disodium hydrogen phosphate Na_2HPO_4
- Sodium chloride
- Potassium dihydrogen phosphate KH_2PO_4
- Distilled water

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 Method I tissue samples

- Homogenise approximately 10 g of sample
- Weigh 0.5 g of the homogenised sample and transfer into a test tube
- Add 4.5 ml of 80% methanol in sample dilution buffer (see chapter 9) and mix (head over head) for 30 minutes
- Centrifuge 2000 x g for 10 minutes
- Transfer 100 µl of supernatant into a clean tube and add 900 µl of sample dilution buffer
- Use 50 µl of this solution in the ELISA test.

8.1.1 Method II tissue 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 (head over head) for 15 minutes
- Centrifuge 2000 x g for 10 minutes
- Transfer 2 ml of the supernatant into 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 for 1 minute and centrifuge 2000 x g for 15 minutes
- Use 50 µl of the layer underneath in the ELISA test

8.2 Egg samples

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

8.3 Honey samples

- Transfer 0.5 ml of the homogenised honey into 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 2000 x g for 10 minutes
- Transfer 100 µl from the aqueous part below the fat layer into a clean tube and add 900 µl of 8% methanol in sample dilution buffer
- Mix well and use 50 µl of this solution in the ELISA test.

8.4 Milk samples
- Transfer 0.5 ml of the homogenised milk into 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 2000 x g for 10 minutes
- Use 50 µl of this solution in the ELISA test.

8.5 Urine samples
- Transfer 0.5 ml of the homogenised urine into a test tube and add 4.5 ml of 8% methanol in sample dilution buffer (see chapter 9)
- Vortex the sample for 2 minutes
- Transfer 100 µl into a clean tube and add 400 µl of 8% methanol in sample dilution buffer
- Use 50 µl of this solution in the ELISA test.

8.6 Serum samples
- Transfer 0.5 ml of the homogenised serum into a test tube and add 4.5 ml of 8% methanol in sample dilution buffer (see chapter 9)
- Vortex the sample for 2 minutes
- Transfer 100 µl into a clean tube and add 400 µl of 8% methanol in sample dilution buffer
- Use 50 µl of this solution in the ELISA test.

8.7 Feed samples
- Transfer 0.5 g of the homogenised ground feed in a test tube and add 4.5 ml of 80% methanol in sample dilution buffer (see chapter 9)
- Mix the sample head over head for 30 minutes
- Centrifuge 2000 x g for 10 minutes
- Transfer 100 µl supernatant into a clean tube and add 400 µl of 8% methanol in sample dilution buffer
- Vortex the sample for 1 minute
- Use 50 µl of this solution in the ELISA test.

8.8 Water samples
- Transfer 0.5 ml of the water sample into a test tube and add 1.5 ml of 40% methanol in sample dilution buffer (see chapter 9)
- Mix (head over head) for 30 minutes
- Centrifuge 2000 x g for 10 minutes
- Transfer 100 µl of the supernatant into a clean tube and add 150 µl of sample dilution buffer
- Use 50 µl of this solution 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 (Flumequine-HRP) with 4 ml dilution buffer, mix thoroughly and keep in the dark until use.

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

Standard solution
The flumequine standard solutions are ready to use. The standard solutions contain 50, 10, 5, 1.0, 0.5 and 0.1 ng/ml flumequine 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.

Standard solution (1000 ng/ml)
To prepare standards in the appropriate matrix or to prepare spikes use the standard solution containing 1000 ng flumequine per ml. Dilute the standard solution in the appropriate matrix to make a dilution range of 50, 10, 5, 1, 0.5, 0.1 ng/ml. Also the zero standard should be of the same matrix.

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

Sample dilution buffer
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

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 and remove residual liquid by striking the plate against a paper towel.
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. Do not allow the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment

When using automatic plate wash equipment, make sure that all wells can be aspirated completely and 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 of the flumequine standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.1, 0.5, 1.0, 5, 10 and 50 ng/ml).

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

4. Pipette 25 µl conjugate (Flumequine-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 the plate for 1 hour in the dark at 4°C. (2°C - 8°C)

8. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.

9. Pipette 100 µl substrate solution into each wells.

10. Incubate 30 minutes at room temperature (20°C - 25°C).

11. Add 100 µl stop solution to each well.

12. 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 (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.

$$\frac{\text{O.D. of standard (or sample)}}{\text{O.D. of zero standard}} \times 100 = \text{percentage maximal absorbance}$$

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 scale of the Y-axis is logit and the X-axis is logarithmic.

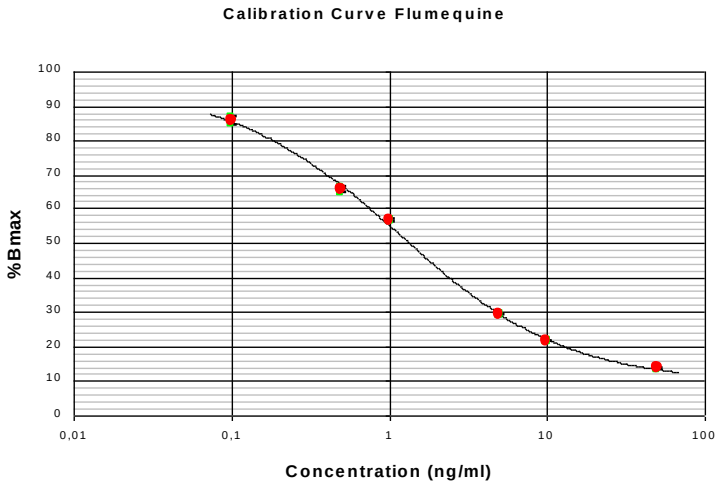


Figure 1 : Example of a calibration curve

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

8.1 Method I tissue samples
To obtain the flumequine content in tissue samples, the calculated flumequine concentration has to be multiplied by a factor 100.

8.1.1Method II tissue samples
To obtain the flumequine content in tissue samples, the calculated flumequine concentration has to be multiplied by a factor 2.

8.2 Egg samples
To obtain the flumequine content in egg samples, the calculated flumequine concentration has to be multiplied by a factor 20.

8.3 Honey samples
To obtain the flumequine content in honey samples, the calculated flumequine concentration has to be multiplied by a factor 100.

8.4 Milk samples
To obtain the flumequine content in milk samples, the calculated flumequine concentration has to be multiplied by a factor 10.

8.5 Urine samples
To obtain the flumequine content in urine samples, the calculated flumequine concentration has to be multiplied by a factor 50.

8.6 Serum
To obtain the flumequine content in serum samples, the calculated flumequine concentration has to be multiplied by a factor 50.

8.7 Feed samples
To obtain the flumequine content in feed samples, the calculated flumequine concentration has to be multiplied by a factor 50.

8.8 Water samples
To obtain the flumequine content in water samples, the calculated flumequine concentration has to be multiplied by a factor 10.

12. LITERATURE

The European Agency for the Evaluation of Medical Products. Veterinary Medicines and Inspections. EMEA/MRL/823/02-Final. January 2002.
R. Verheijen, N. Sajic, I. Hopman and C.J.M. Arts. Detection of fluoroquinolones by Enzyme Immunoassays in biological matrices. VIIth International Conference on Agri-Food Antibodies, Uppsala, Sweden, 11-13 September 2003.

13. ORDERING INFORMATION

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

14. REVISION HISTORY

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

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