

FLUMEQUINE ELISA

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

FLUMEQUINE ELISA**A competitive enzyme immunoassay for
screening and quantitative analysis of flumequine
in various matrices**TABLE OF CONTENTS

	PAGE:
Brief information.....	2
1. Introduction.....	2
2. Principle of the flumequine ELISA	3
3. Specificity and sensitivity.....	4
4. Handling and storage.....	5
5. Kit contents.....	5
6. Equipment and materials required but not provided	7
7. Precautions.....	7
8. Sample preparations.....	8
9. Preparations of reagents.....	10
10. Assay procedure.....	11
11. Interpretation of results	12
12. Literature	14
13. Ordering information	14
14. Last mutations	14

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

1. The European Agency for the Evaluation of Medical Products. Veterinary Medicines and Inspections. EMEA/MRL/823/02-Final. January 2002.
2. 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. LAST MUTATIONS

Milk samples treatment.

Water samples is added.

Pipette schedule of the standard curve is adapted.

Alternative for calibration curve:

The value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis.

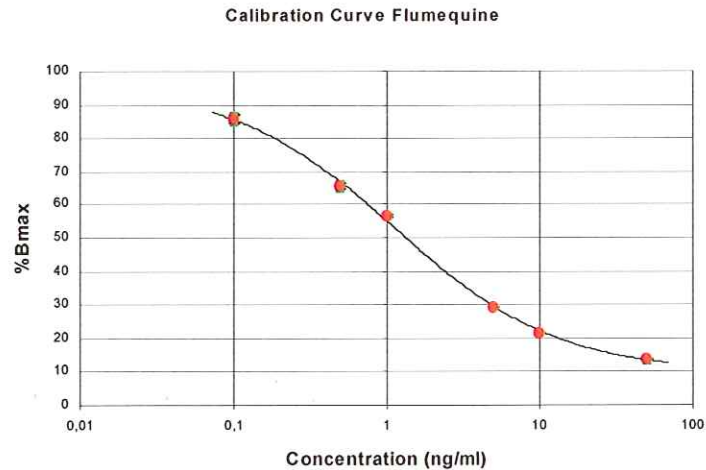


Figure 1 : Example of a calibration curve

The amount of flumequine in the samples is expressed as flumequine equivalents. The flumequine equivalents in the samples (ng/ml) corresponding to the % 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.

8.1.1 Method 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

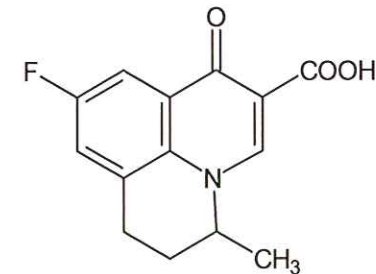
To obtain the flumequine content in milk samples, the calculated flumequine concentration has to be multiplied by a factor 10.

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 (H₂O₂/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.

- 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).
- Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.
- Pipette 25 µl conjugate (Flumequine-HRP) to all wells, except wells H1 and H2.
- Pipette 25 µl antibody solution to all wells, except wells H1 and H2.
- Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.
- Incubate the plate for 1 hour in the dark at 4°C. (2°C - 8°C)
- Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.
- Pipette 100 µl substrate solution into each wells.
- Incubate 30 minutes at room temperature (20°C - 25°C).
- Add 100 µl stop solution to each well.
- Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (OD) of the wells H1 and H2 (Blank) from the individual OD of the wells containing the standards and the samples.

The OD values of the six standards and the samples (mean values of the duplicates) are divided by the mean OD value of the zero standard/Bmax (wells A1 and A2) and multiplied by 100. The zero standard is thus made equal to 100% (maximal absorbance) and the other OD values are quoted in percentages of the maximal absorbance.

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

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.

* Sample dilution buffer

Sample dilution buffer is not provided in the kit. Prepare this buffer as follows:

Quantities indicated are for 1 litre of buffer:

Na ₂ HPO ₄	0.77 g
KH ₂ PO ₄	0.18 g
NaCl	8.94 g
pH	7.4 (7.3-7.5)

10. ASSAY PROCEDURE

Rinsing protocol

In ELISA's, 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:

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 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 (Sample preparation) and prepare reagents according to chapter 9 (Preparations of reagents).

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%
Enrofloxacin	< 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 Limit of detection (LOD) is calculated as: $X_n \pm 3SD$ and is determined under optimal conditions.

Matrix	Procedure	LOD
Tissue	8.1	< 0.1 ng/g
Tissue	8.1.1	< 0.5 ng/g**
Egg	8.2	3.5 ng/g
Honey	8.3	< 10 ng/g
Milk	8.4	13 ng/g
Urine	8.5	5.5 ng/ml
Serum	8.6	1 ng/ml
Feed	8.7	10 ng/ml
Water	8.8	3 ng/ml

** Results of blank samples were equal to a B/B₀ of approximately 100%. The apparent figure obtained was multiplied with the dilution factor and found to be lower than the lowest standard point.

The obtained % recoveries of samples spiked with flumequine all fell into the required range of 70% - 130%.

4. HANDLING AND STORAGE

- Store the kit 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 (see kit label) has passed, quality claims are not accepted.
- Before opening the sealed plate, the plate should be at ambient temperature in order to avoid condensation in the ELISA.
- Dilute the kit components immediately before use, but after the components are at ambient temperature.
- The substrate chromogen solution can be stored in a refrigerator (+2°C to +8°C) until the expiry date stated on the label.
- Any direct action of light on the substrate chromogen solution should be avoided.

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

- A blue colouring of the substrate solution before transferring it into the wells.
- A weak or absent colour reaction of the zero standard (B_{max} , $E_{450nm} < 0.8$).

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 +2°C to +8°C. Prepare reagents fresh before use.

Microtiter plate

Return unused strips into the resealable zip lock 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 40 ml of diluted rinsing buffer is required (2 ml concentrated rinsing buffer + 38 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 lyophilised conjugate (Flumequine-HRP) with 4 ml dilution buffer, mix thoroughly and keep in the dark until use. This reconstituted compound can be stored in a refrigerator (+2°C to +8°C) for one week. For prolonged storage aliquots can be prepared and stored at -20°C till the expiry date.

Antibody solution

Reconstitute the vial of lyophilised antibodies with 4 ml dilution buffer, mix thoroughly and keep in the dark until use. This reconstituted compound can be stored in a refrigerator (+2°C to +8°C) for one week. For prolonged storage aliquots can be prepared and stored at -20°C till the expiry date.

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.

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 and mix (head over head) for 30 minutes
- Centrifugate 2000 x g for 10 minutes
- Use an aliquot of 50 µl 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
- 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 an aliquot of 50 µl 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
- 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 an aliquot of 50 µl 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
- 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 an aliquot of 50 µl in the ELISA test.

8.8 Water samples

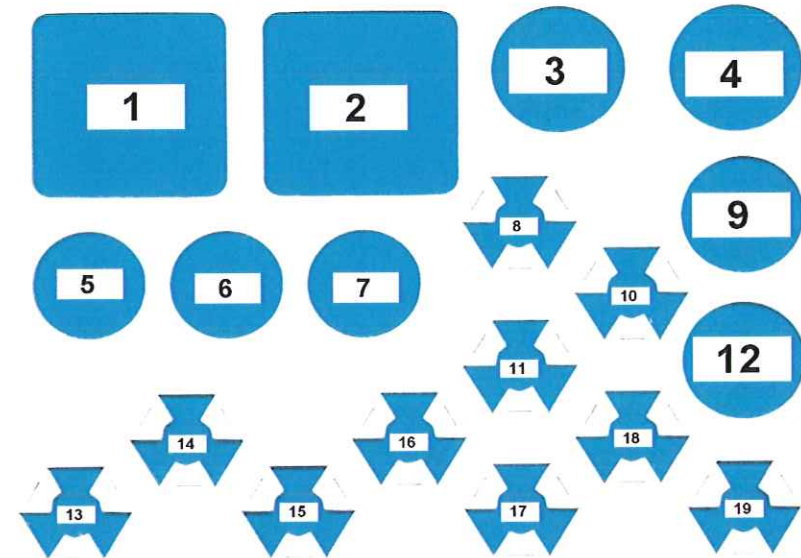
- Transfer 0.5 ml of the water sample into a test tube and add 1.5 ml of 40% methanol in *sample dilution buffer
- Mix (head over head) for 30 minutes
- Centrifuge 2000 x g for 10 minutes
- Transfer 100 µl of the supernatant into a clean tube and add 150 µl of *sample dilution buffer
- Use an aliquot of 50 µl in the ELISA test.

* see Chapter 9, Preparation of reagents

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** (lyophilised, blue cap)
6. **Antibody solution** (lyophilised, 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** (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
- Aluminium foil or parafilm
- Methanol 100%

7. PRECAUTIONS

- Flumequine is toxic compound. Avoid contact with mouth and skin. Be aware that flumequine is not inhaled.
- The stop reagent contains 0.5 M sulfuric acid. Do not allow the reagent to get into contact with the skin.
- 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.
- TMB is toxic by inhalation, in contact with skin and if swallowed; take care when handling the substrate.
- 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, which crystallises at +4°C.
- 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 TREATMENT

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 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 an aliquot of 50 µl 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
- 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 50°C
- 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 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 an aliquot of 50 µl 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 and mix (head over head) for 30 minutes
- Centrifuge 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 an aliquot of 50 µl in the ELISA test.