FLUOROQUINOLONE II ELISA

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

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12. LITERATURE

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2. A-C. Huet, C. Charlier, S.A. Tittlemier, G. Singh, S. Benrejeb, P. Delahaut. Simultaneous determination of (fluoro)quinolone antibiotics in kidney, marine products, eggs, and muscle by Enzyme-Linked Immunosorbent Assay (ELISA). J. Agric. Chem. 2006, **54**, 2822-2827

13. ORDERING INFORMATION

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

14.LAST MUTATIONS

Updated "lay out"

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BRIEF INFORMATION

The fluoroquinolone II ELISA is a competitive enzyme immunoassay for measurement of the concentration of a broad group of fluoroquinolone antbiotics 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

Fluoroquinolones are a synthetic class of antibiotics, which all act by inhibition of 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. Fluoroquinolones are mainly active against Gram negative bacteria and have found wide application in both human and veterinary clinical practice. However, the use of fluoroquinolones in animals used for meat production and its use in aquaculture has also generated concern, as fluoroquinolones have contributed to an increasing bacterial resistance for these antibiotics in man, e.g. *Staphylococcus aureus* (MRSA, MRSE), *Campylobacter jejuni* and others. For this reason, effective screening methods for the presence of fluoroquinolones in animal products as well as food products are required.
EuroProxima has developed two fluoroquinolone ELISA kits, 5101FLUQG and 5101FLUQII. Each kit can be used for screening of its own broad group of fluoroquinolones [1, 2]. In the fluoroquinolone II ELISA, norfloxacin is used as a standard, whereas the conjugate is a horseradish labeled norfloxacin [1, 2]. Thus, the detected fluoroquinolones are expressed as norfloxacin-equivalents.

2. PRINCIPLE OF THE FLUOROQUINOLONE II ELISA

The microtiter plate based fluoroquinolone II ELISA consists of one precoated plate (12 strips, 8 wells each). Antibody, Horseradish peroxidase (-HRP) labeled norfloxacin and standard solution or sample are added to wells. Free fluoroquinolones from the samples or standards and norfloxacin-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation step of 30 minutes, the non-bound reagents are removed in a washing step. The amount of bound norfloxacin-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H_2O_2/TMB). Bound norfloxacin-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 fluoroquinolones concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The fluoroquinolone II ELISA utilizes antibodies raised in rabbit against a broad group of fluoroquinolones. The reactivity pattern of the antibody is:

Cross-reactivities:	Norfloxacin	100%
	Enrofloxacine	84%
	Ciprofloxacin	91%
	Enoxacin	119%
	Ofloxacin	62%
	Fleroxacin	92%
	Marbofloxacin	113%
	Sarafloxacin	92%
	Difloxacin	105%
	Levofloxacin	173%
	Danofloxacin	47%
	Tosufloxacin	67%
	Gatifloxacin	16%

The limit of detection (LOD) is calculated as: Xn + 3SD and is determined under optimal conditions.

Matrix	Procedure	LOD
Shrimps	8.1.1	4 ng/g
Muscle	8.1.1	6 ng/g
Tissue	8.1.2	0.6 ng/g
Honey	8.2	0.1 ng/g
Serum	8.3	3 ng/ml
Urine	8.4	1.5 ng/ml
Feed	8.5	16 ng/ml

The recovery (norfloxacin spiked to various samples) was found to vary between 70% and 120%, dependent on the matrix used.

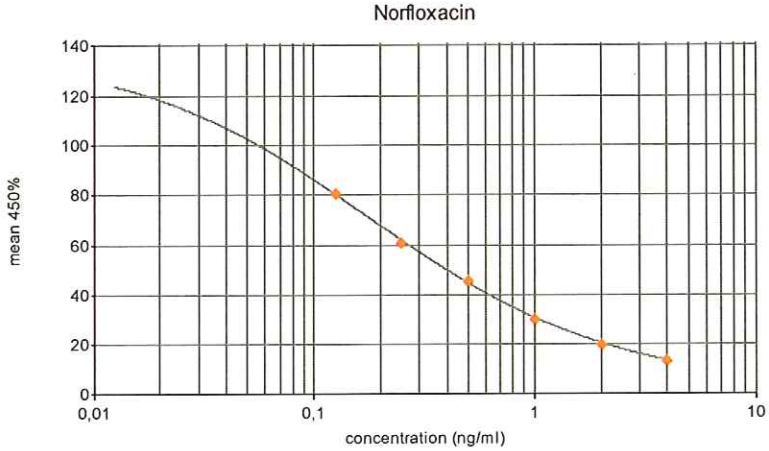


Figure 1 : Example of a calibration curve

The Fluoroquinolones concentration in the extracts (ng/ml) corresponding to the % maximal absorbance of each extract can be read from the calibration curve.

Shrimps, fish and muscle tissue samples

8.1.1 Method I

To obtain the fluoroquinolones content in shrimps, fish and muscle samples, the calculated fluoroquinolones concentration has to be multiplied by a factor 50.

8.1.2 Method II

To obtain the fluoroquinolones content in shrimps, fish and muscle samples, the calculated fluoroquinolones concentration has to be multiplied by a factor 2.

8.2 Honey samples

To obtain the fluoroquinolones content in honey samples, the calculated fluoroquinolones concentration has to be multiplied by a factor 25.

8.3 Serum samples

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

8.4 Urine samples

To obtain the fluoroquinolones content in urine samples, the calculated fluoroquinolones concentration has to be multiplied by a factor 25.

8.5 Feed samples

To obtain the fluoroquinolones content in feed samples, the calculated fluoroquinolones concentration has to be multiplied by a factor 100.

7. Incubate the plate for 30 minutes in the dark at room temperature (20°C – 25°C).

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

9. Add 100 µl substrate/chromogen solution to all wells.

10. Incubate 15 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 (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(Bmax) is thus made equal to 100% (maximal absorbance) and the other OD values are quoted in percentages of the maximal absorbance.

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

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.

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.

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.

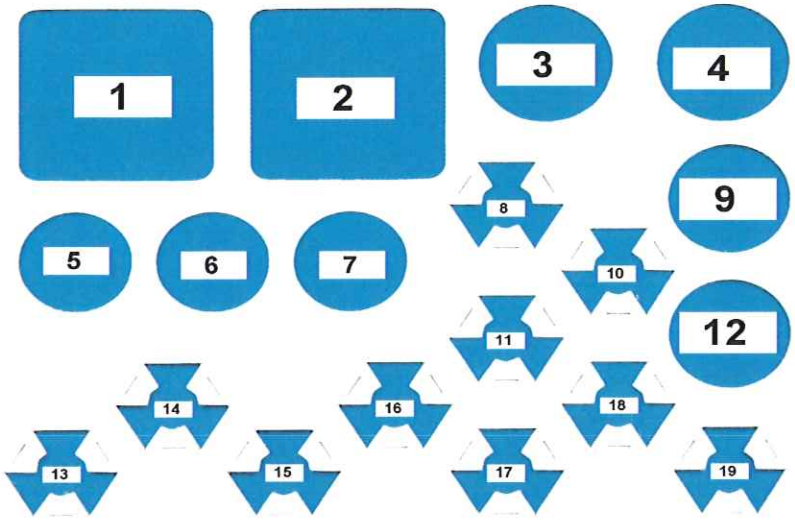
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the substrate solution before transferring it into the wells.
- A weak or absent colour reaction of the zero standard (Bmax, E450nm < 0.8).

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. not in use
6. not in use
7. not in use
8. **Conjugate** (75 µl, 100x concentrated, blue cap)
9. not in use
10. **Antibody** (75 µl, 100x concentrated, yellow cap)
11. **Standard solution 100 ng/ml** (1ml, Ready-to-use)
12. not in use
13. **Zero standard** (2ml, Ready-to-use)
14. **Standard solution 1** (1ml, Ready-to-use) **0.125 ng/ml**
15. **Standard solution 2** (1ml, Ready-to-use) **0.25 ng/ml**
16. **Standard solution 3** (1ml, Ready-to-use) **0.5 ng/ml**
17. **Standard solution 4** (1ml, Ready-to-use) **1.0 ng/ml**
18. **Standard solution 5** (1ml, Ready-to-use) **2.0 ng/ml**
19. **Standard solution 6** (1ml, Ready-to-use) **4.0 ng/ml**

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

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, Bmax).
   Pipette 50 µl of each of the norfloxacin standard solutions in duplicate (wells B1,2 to G1,2 i.e.0.125, 0.25, 0.5, 1, 2 and 4 ng/ml).

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

4. Add 25 µl conjugate (norfloxacin-HRP) to all wells, except wells H1 and H2.

5. Add 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.

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 when used (keep in the dark) and mix the content before pipetting into the wells.

*PBS
PBS is not provided in the kit. Prepare this buffer as follows:
Quantities indicated are for 1 litre 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)

**Sample dilution buffer
Sample dilution buffer is not provided in the kit. Prepare this buffer as follows:
Add 1 ml methanol 100% to 9 ml of PBS.

***0.2% H_2O_2
Add 2 ml of Hydrogen Peroxide (30%) to 298 ml distilled water.

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, 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
- Methanol 100%
- Hydrogen peroxide H_2O_2, 30%
- n-Hexane

7. PRECAUTIONS

- Fluoroquinolones are toxic compounds. Avoid contact with mouth and skin. Be aware that fluoroquinolones are 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 Methods for shrimps, fish and muscle tissue samples

8.1.1 Method I

- Homogenise approximately 10 g of sample
- Weigh 1.0 g of the homogenised sample and transfer into a test tube
- Add 4.0 ml of 100% methanol and mix head-over-head for 15 minutes
- Centrifuge (5 minutes, 2000 x g)
- Pipette 50 µl of the supernatant into a clean tube and add 450 µl *PBS
- An aliquot of 50 µl is used in the ELISA test.

8.1.2 Method II

- 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 *PBS
- Mix for 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 50°C
- Reconstitute the residue with 1 ml **Sample Dilution Buffer
- Defat by addition of 1.0 ml hexane
- Vortex for 1 minute and centrifuge (15 minutes, 2000 x g)
- Use 50 µl of the layer underneath in the ELISA test

8.2 Honey samples

- Transfer 1 g of the homogenized honey in a test tube
- Add 4.0 ml of *PBS and mix head-over-head for 15 minutes
- Centrifuge (10 minutes, 2000 x g)
- Dilute an aliquot of 50 µl from the upper layer with 200 µl **Sample Dilution Buffer
- An aliquot of 50 µl from the diluted sample is used in the ELISA test.

8.3 Serum samples

- Transfer 0.2 ml of the homogenized serum in a test tube and add 0.8 ml of **Sample Dilution Buffer
- Vortex the sample for 2 minutes
- Centrifuge (10 minutes, 2000 x g)
- Dilute an aliquot of 50 µl from the upper layer with 450 µl **Sample Dilution Buffer
- An aliquot of 50 µl is used in the ELISA test.

8.4 Urine samples

- Transfer 0.2 ml of the homogenized urine in a test tube and add 4.8 ml of **Sample Dilution Duffer
- Vortex the sample for 2 minutes
- An aliquot of 50 µl diluted sample is used in the ELISA test.

8.5 Feed samples

- Transfer 1 g of the homogenized sample in a test tube
- Add 4 ml ***0.2% H_2O_2
- Vortex the sample for 2 minutes and mix head over head for 15 minutes
- Centrifuge (5 minutes, 2000 x g)
- Pipette 25 µl of the supernatant into a clean tube and add 475 µl **Sample Dilution Buffer, vortex
- 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 +2°C to +8°C. 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.

Standard solution

The norfloxacin standard solutions are ready-to-use. The standard solutions contain 4, 2, 1, 0.5, 0.25 and 0.125 ng/ml norfloxacin in 10% 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 (100 ng/ml)

To prepare standards in the appropriate matrix or to prepare spikes use the standard solution containing 100 ng norfloxacin per ml. Dilute the standard solution in the appropriate matrix to make a dilution range of 4, 2, 1, 0.5, 0.25 and 0.125 ng/ml. Also the zero standard should be of the same matrix.

Conjugate solution

The conjugate is delivered 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 min., 1000g). Add 5 µl of the concentrated conjugate solution to 495 µl dilution buffer. Per 2 x 8 wells 400 µl is required. Store unused concentrated conjugate at 2°C - 8°C.

Antibody solution

The antibody is 100x concentrated. Spin down the antibody in the vial by a short centrifugation (1 min. 1000 g). Add 5 µl of the concentrated antibody to 495 µl dilution buffer. Per 2 x 8 wells 400 µl of antibody solution is required. Store concentrated antibody immediately upon use at 2°C - 8°C.

Rinsing buffer

The rinsing buffer is delivered 20x concentrated. Prepare dilutions freshly before use. For each strip 40 ml of diluted rinsing buffer is used (2 ml concentrated rinsing buffer + 38 ml distilled water).