FLUOROQUINOLONES ELISA

(Generic test)

5101FLUQG[16]04.20

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

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TABLE OF CONTENTS

PAGE:

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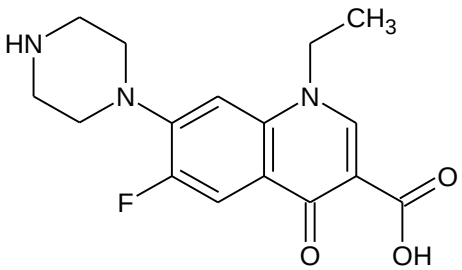
BRIEF INFORMATION

The fluoroquinolones ELISA is a competitive enzyme immunoassay for measurement of the concentration of a broad group of fluoroquinolone antibiotics. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that in total 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 norfloxacin

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 a generic ELISA kit for screening for a broad range of fluoroquinolones. In this ELISA kit an antiserum is used that was raised against a protein bound norfloxacin, the conjugate is a horseradish peroxidase labelled norfloxacin. In the ELISA kit a norfloxacin standard is used, so the fluoroquinolones are expressed as norfloxacin-equivalents.

2. PRINCIPLE OF THE FLUOROQUINOLONES ELISA

The microtiter plate based fluoroquinolones ELISA kit consists of one precoated plate (12 strips, 8 wells each). Antibody, horseradish peroxidase (-HRP) labeled norfloxacin and norfloxacin standard solution or sample are added to the wells. Free fluoroquinolones from the samples or standards norfloxacin-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).
After incubation step of one hour, 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 (peroxide/tetramethylbenzidine, 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 generic fluoroquinolones ELISA utilizes polyclonal antibodies raised in rabbit to protein conjugated norfloxacin. The reactivity pattern of the antibody as tested in buffer is:

Cross-reactivity:	Enrofloxacine	92%
	Ciprofloxacin	124%
	Norfloxacin	100%
	Nadifloxacin	85%
	Pefloxacin	70%
	Enoxacin	57%
	Piromidic acid	62%
	Lomefloxacin	40%
	Ofloxacin	18%
	Danofloxacin	89%
	Fleroxacin	40%
	Oxolinic acid	57%
	Marbofloxacin	16%
	Pipemidic acid	5%
	Sarafloxacin	4%
	Difloxacin	1%
	Levofloxacin	3%
	Gatifloxacin	5%
	Flumequine	2%
	Pazufloxacin	1%
	Cinoxacin	< 0.1%
	Tosufloxacin	< 0.1%
	Nalidixic acid	< 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
Tissue	8.1.1	12 ng/g
Tissue	8.1.2	0.3 ng/g
Egg	8.2	6 ng/g
Tissue and whole egg	8.3	0.5 ng/g
Milk	8.4	3 ng/ml
Water	8.5	2 ng/ml
Honey	8.6	2 ng/g
Serum	8.7	2.5 ng/ml
Urine	8.8	7 ng/ml

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.

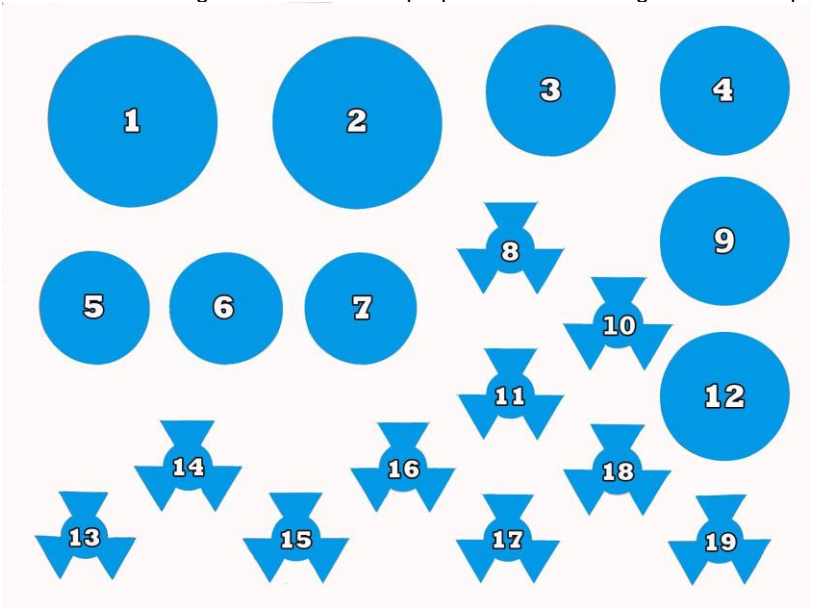
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

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** (lyophilized, blue cap)
6. **Antibody** (lyophilized, yellow cap)
7. not in use
8. **Standard solution 100 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) **0.157 ng/ml**
15. **Standard solution 2** (1ml, Ready-to-use) **0.313 ng/ml**
16. **Standard solution 3** (1ml, Ready-to-use) **0.625 ng/ml**
17. **Standard solution 4** (1ml, Ready-to-use) **1.25 ng/ml**
18. **Standard solution 5** (1ml, Ready-to-use) **2.5 ng/ml**
19. **Standard solution 6** (1ml, Ready-to-use) **5.0 ng/ml**

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- General equipment
- 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
- 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
- Methanol 100%
- Hexane
- Di-sodium Hydrogen Phosphate
- Potassium Di-hydrogen Phosphate
- Sodium Chloride
- Oasis HLB Spec disk columns (60 mg/3 ml)
- Ammonia
- Phosphoric acid
- 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 PREPARATIONS

8.1 Methods for shrimps, fish and tissue (chicken) samples

8.1.1 Method I

- Homogenize approximately 10 g of sample.
- Weigh 0,5 g of the homogenized sample and transfer into a test tube.
- Add 1.5 ml of 80% methanol in sample dilution buffer (see Chapter 9) and mix (head over head) for 30 minutes.
- Centrifuge (10 minutes, 2000 x g).
- Pipette 100 µl of the supernatant into a clean tube and add 900 µl sample dilution buffer.
- Pipette 50 µl in the ELISA test

8.1.2 Method II

- Homogenize approximately 10 g of sample
- Weigh 1 g of the homogenized sample and transfer into a test tube
- Add 3 ml of 80% methanol in sample dilution buffer (see Chapter 9)
- 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 50ºC
- Reconstitute the residue with 1 ml of 8% methanol in sample dilution buffer (see Chapter 9)
- 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 Egg samples, raw and pasteurized

- Transfer 0.5 g of the homogenized 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) for 30 minutes.
- After centrifugation (10 minutes, 2000 x g), 100 µl of supernatant is pipetted into a clean tube and 400 µl of sample dilution buffer is added.
- Pipette 50 µl in the ELISA test

8.3 SPE procedure tissue and whole egg

- Weight 1 g homogenized sample into a 50 ml tube
- Add 4 ml of phosphoric acid 0.6% in water/methanol 60/40 (v/v)
- Vortex the muscle sample 30 seconds and then vigorously shaken for 10 minutes, head over head. The egg sample gently swirling otherwise it becomes jelly.
- Centrifuge 10 minutes 2000 x g
- filter the supernatant into a 15 ml tube
- Repeat the extraction step by adding 4 ml of extraction solution, onto the pellet
- The extracts (8 ml) were combined and mixed
- Pipette 4 ml into a glass tube and evaporated the methanol under a mild stream of nitrogen at 50°C. About 1.6 ml of aqueous phase will remain.
- Dilute the aqueous extract further with 4 ml of phosphoric acid 1%.

Solid phase extraction with column
- Add 1 ml of 100% methanol (flow 1ml/minute)
- Add 1 ml distilled water

Note: It is important that the disk is not allowed to dry completely during activation and prior to sample addition! If the disk has become dry, repeat the conditioning procedure.

- Add the diluted aqueous extract onto the column, set flow add 1 ml/minute.

- Add 2 ml of phosphoric acid solution (0.025M, pH 3/methanol 95:5 (v/v))
- Add 2 ml of distilled water let the column become dry.

- Elution of sample:
- Collect the eluent in a clean glass tube.
- Add 2 ml of methanol/ammonia 95:5 (v/v) onto the cartridge
- Let the cartridges to be dry for two minutes.
- Evaporate the eluent under a mild stream of nitrogen at 50°C
- Reconstitute the residue for muscle with 2 ml and for egg with 1 ml of 8% methanol in sample dilution buffer (see Chapter 9)
- Pipette 50 µl in the ELISA test

8.4 Milk samples
- Transfer 0.5 ml of the homogenized milk in a test tube.
- Add 4.5 ml of 8% methanol in sample dilution buffer (see Chapter 9) and mix (head over head) for 30 minutes.
- After centrifugation (10 minutes, 2000 x g), an aliquot of 50 µl from the liquid part below the fat layer is used in the ELISA test.

8.5 Water samples
- Transfer 0.5 ml of the water sample in a test tube.
- Add 1.5 ml of 40% methanol in sample dilution buffer (see Chapter 9) and mix (head over head) for 5 minutes.
- After centrifugation (10 minutes, 2000 x g), 100 µl of supernatant is pipetted into a clean tube and 150 µl of sample dilution buffer (see Chapter 9) is added.
- Pipette 50 µl in the ELISA test

8.6 Honey samples
- Transfer 0.5 ml of the homogenized honey in a test tube.
- Add 4.5 ml of 8% methanol in sample dilution buffer (see Chapter 9) and mix (head over head) for 30 minutes.
- After centrifugation (10 minutes, 2000 x g), an aliquot of 50 µl from the liquid part below the fat layer is used in the ELISA test.

8.7 Serum samples
- Transfer 0.5 ml of the homogenized serum in a test tube and add 4.5 ml of 8% methanol in sample dilution buffer (see Chapter 9).
- Vortex the sample for 2 minutes.

- Pipette 50 µl in the ELISA test

8.8 Urine samples
- Transfer 0.5 ml of the homogenized urine in a test tube and add 4.5 ml of 8% methanol in sample dilution buffer (see Chapter 9).
- Vortex the sample for 2 minutes.
- Pipette 50 µl 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 +2ºC to +8ºC for use in subsequent assays. Retain also the strip holder.

Rinsing buffer
The rinsing buffer is delivered 20x 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).

Standard solution (100 ng/ml)
To prepare standards in the appropriate matrix or to prepare spikes use the standard solution containing 100 ng FLUOQ per ml. Dilute the standard solution in the appropriate matrix to make a dilution range of 5, 2.5, 1.25, 0.625, 0.313 and 0.157 ng/ml. Also the zero standard should be of the same matrix.

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 (norfloxacin-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 norfloxacin standard solutions are ready-to-use. The standard solutions contain 5, 2.5, 1.25, 0.625, 0.313 and 0.157 ng/ml norfloxacin in 8% methanol solution. A ready-to-use zero standard is enclosed. Keep these standard solutions in the dark and store at +2ºC to +8ºC.

Substrate/chromogen solution
The substrate/chromogen solution (ready-to-use) tends to precipitate at +4ºC. 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

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:

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 rinsing solution from 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 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 norfloxacin standard solutions in duplicate (wells B1,2 to G1,2 i.e.0.157, 0.313, 0.625, 1.25, 2.5 and 5 ng/ml).

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate (40 samples; 80 wells).

4. Pipette 25 µl conjugate (Norfloxacin-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 4ºC (2ºC – 8ºC).

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

9. Pipette 100 µl substrate/chromogen solution into each well.

10. Incubate 30 minutes at room temperature (approximately 20ºC - 25ºC).

11. Pipette 100 µl of 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.

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

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

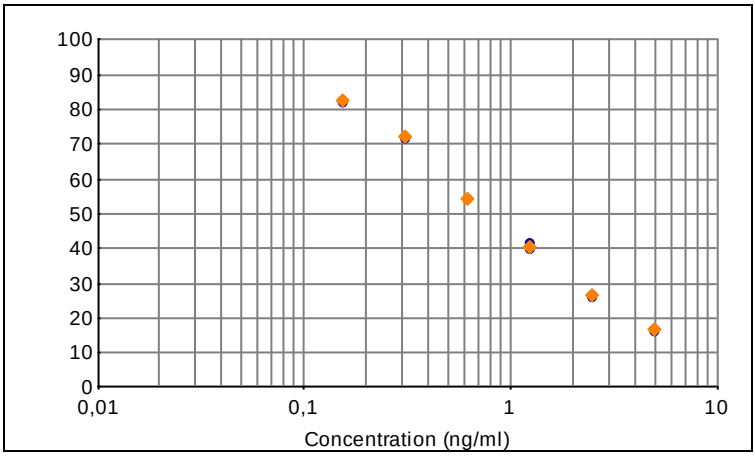


Figure 1 : Example of a calibration curve

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

8.1.1 Method I: Tissue samples (shrimps, fish and chicken)
The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 40 to obtain the fluoroquinolones concentration in ng/g (ppb).

8.1.2 Method II: Tissue samples (shrimps, fish, chicken)
The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 2 to obtain the fluoroquinolones concentration in ng/g (ppb).

8.2 Egg samples, raw and pasteurised
The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 20 to obtain the fluoroquinolones concentration in ng/g (ppb).

8.3 SPE method tissue and whole egg
The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 4.5 for muscle and 2.25 for egg.

8.4 Milk samples
The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 10 to obtain the fluoroquinolones concentration in ng/g or ng/ml (ppb).

8.5 Water samples
The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 10 to obtain the fluoroquinolones concentration in ng/g or ng/ml (ppb).

8.6 Honey samples
The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 10 to obtain the fluoroquinolones concentration in ng/g or ng/ml (ppb).

8.7 Serum samples
The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 10 to obtain the fluoroquinolones concentration in ng/g or ng/ml (ppb).

8.8 Urine samples
The norfloxacin-equivalents read from the calibration curve have to be multiplied by a factor 10 to obtain the fluoroquinolones concentration in ng/g or ng/ml (ppb).

12. LITERATURE

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.
Giampiero Scortichini, Loredana Annunziata, Valeria Di Girolamo, Roberta Buratti, Roberta Galarini. Validation of an enzyme-linked immunosorbent assay screening for quinolones in egg, poultry muscle and feed samples. Analytica Chimica Acta 637 (2009) 273-278.

13. ORDERING INFORMATION

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

14. REVISION HISTORY

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

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