Standard Operating Procedure

for

Detection of Quinolones Residues in Tissue by Enzyme-Linked Immunosorbent Assay (ELISA)

(ELISA Kit 5101FLUQG, EuroProxima)

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Issued to	**Lab Manager (LM)**

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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 [1, 2].

2. SCOPE

This Standard Operating Procedure (SOP) describes the procedure for screening of Quinolone residues in tissue samples (chicken, fish, shrimp, mutton, beef), using competitive enzyme immunoassay.

3. OBJECTIVE / PURPOSE

To ensure the accuracy, quality and reproducibility of the procedure / protocol.

4. ABBREVIATIONS

4.1. QNS: Quinolone

4.2. ELISA: Enzyme-Linked Immunosorbent Assay

4.3. HRP: Horseradish Peroxidase

4.4. TMB: Tetra Methyl Benzidine (chromogen)

4.5. O.D.: Optical Density

4.6. LOD: Limit of Detection

4.7. WS: Working Standard

4.8. D.F.: Dilution Factor

5. PRINCIPLE OF THE METHOD

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.

6. 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:

Metabolites	Cross-reactivity (%)	Metabolites	Cross-reactivity (%)

Enrofloxacine	92	Marbofloxacin	16
Ciprofloxacin	124	Pipemidic acid	5
Norfloxacin	100	Sarafloxacin	4
Nadifloxacin	85	Difloxacin	1
Pefloxacin	70	Levofloxacin	3
Enoxacin	57	Gatifloxacin	5
Piromidic acid	62	Flumequine	2
Lomefloxacin	40	Pazufloxacin	1
Ofloxacin	18	Cinoxacin	< 0.1
Danofloxacin	89	Tosufloxacin	< 0.1
Fleroxacin	40	Nalidixic acid	< 0.1
Oxolinic acid	57		

The Limit of detection (LOD) is determined under optimal conditions. It's range lies between 0.3 to 12 ng/g. Cut-off values need critical consideration.

7. HANDLING AND STORAGE

- Store the kit at +2ºC to +8ºC in a dark place.
- 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.
- After the lyophilised enzyme conjugate (Norfloxacin-HRP) and the lyophilized antibodies have been reconstituted, these reconstituted compounds can be stored

in a refrigerator (+2°C to +8°C) for at least half a year. Alternatively, after reconstitution of the conjugate and antibody components, aliquots of these solutions can be prepared. The aliquots can be stored in a freezer (–20°C) until the expiration date has passed (see kit label).

- The substrate chromogen solution can be stored in a refrigerator (+2ºC to + 8ºC) 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, E450 nm < 0.8).

8. EQUIPMENT AND MATERIALS REQUIRED

- Scalpel and blades
- Analytical Balance (5 decimal points)
- Polythene Gloves
- Homogenizer (HG-15D, DIAHAN Scientific, USA)
- Vortex mixer (VM-10, DIAHAN Scientific, USA)
- Refrigerated Centrifuge 5430R, Eppendorf
- Romer's Evap system of dri-block and sample concentrator
- Microtiter plate washer (ELx50™, BioTek)
- Microtiter plate reader (ELx808™, BioTek) with 450 nm filter
- Micropipettes, 100 – 1000 µL
- Commercial ELISA Kit (5101FLUQG, EuroProxima)
- Double Distilled water

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.

9.1 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.

9.2 Rinsing buffer

The rinsing buffer is delivered 20x 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).

9.3 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.

9.4 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.

9.5 Conjugate solution

Reconstitute the vial of lyophilised conjugate (norfloxacin-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 at least half a year.

9.6 Antibody solution

Reconstitute the vial of lyophilised antibodies with 4 ml dilution buffer, mix horoughly and keep in the dark until use. This reconstituted compound can be stored in a refrigerator (+2°C to +8°C) for at least half a year.

9.7 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.

9.8 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.

10. SAMPLE PREPARATION

Two methods are specified: Method I is fast and simple and results in an LOD of 12 ppb. Method II has an evaporation step and results in an LOD of 0.3 ppb.

10.1 Method - I

- Homogenise approximately 10 g of sample.
- Weigh 0,5 g of the homogenised sample and transfer into a test tube.
- Add 1.5 mL of 80% methanol in sample dilution buffer and mix for 30 min.
- Centrifuge (10 minutes, 2000 x g).

- Pipette 100 µL of the supernatant into a clean tube and add 900 µL sample dilution buffer.
- An aliquot of 50 µL is used in the ELISA test.

10.2 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 sample dilution buffer
- 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
- Defat by addition of 1.0 mL hexane
- Vortex for 1 minute and centrifuge (15 minutes, 2000 x g)

11. ASSAY PROCEDURE

11.1 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:

11.2 Manual rinsing

- 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.

- Fill all the wells to the rims (300 µL) with rinsing solution.
- This rinsing cycle (1 and 2) should be carried out 3 times.
- Turn the plate upside down and empty the wells by a firm short vertical
- movement.
- Place the inverted plate on absorbent paper towels and tap the plate firmly to
- remove residual rinsing solution from the wells.
- Take care that none of the wells dry out before the next reagent is dispensed.

11.3 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.

11.4 Assay Protocol

- Prepare samples according and prepare reagents.
- Pipette 100 µL of dilution buffer in duplicate.
- Pipette 50 µL of dilution buffer (zero standard, Bmax) in duplicate.
- Pipette 50 µL of each of the standard in duplicate (0.157, 0.313, 0.625, 1.25, 2.5 and 5.0 ng/mL).
- Pipette 50 µL of each sample solution in duplicate into the remaining wells of the microtiter plate.
- Pipette 25 µL of conjugate (ampicillin-HRP) to all wells.
- Pipette 25 µL of antibody solution to all wells.
- Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

- Incubate for 1 hour in the dark at 4°C.
- Discard the solution from the microtiter plate and wash 3 times with rinsing buffer. Pipette 100 µL of substrate solution into each well.
- Incubate 30 minutes in the dark at 20ºC to 25ºC.
- Add 100 µL of stop solution to each well.
- Read the absorbance values immediately at 450 nm.

12. INTERPRETATION OF RESULTS

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.

$$\text{Relative absorbance (\%)} = \frac{\text{O.D. value of standard (or sample)}}{\text{O.D. value of zero standard/ Bmax}} \times 100\%$$

12.1 Calibration curve (CC):

The values (% maximal absorbance) calculated for the standards are plotted on the Y-axis versus the analyte equivalent concentration on a logarithmic X-axis.

12.2 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. The amount of penicillin in the samples is expressed as penicillin equivalents. The beta-lactam equivalents in the samples (ng/mL) corresponding to the % maximal absorbance of each extract can be read from the CC.

12.3 Method - I

The norfloxacin-equivalents read from the calibration curve have to be multiplied by a D.F. 40 to obtain the fluoroquinolones concentration in ng/g (ppb).

12.4 Method - II

The norfloxacin-equivalents read from the calibration curve have to be multiplied by a D.F. 2 to obtain the fluoroquinolones concentration in ng/g (ppb).

13. PRECAUTIONS

- 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 crystallizes 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.

14. REFERENCES

1. 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.
2. 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.
3. Insert brochure for commercial ELISA Kit 5101FLUQG, EuroProxima.

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