Importance of Fluoroquinolone Determination

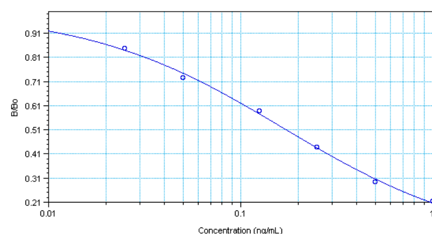
Antibiotic residues in foods pose a serious threat to public health. This is especially true of the Fluoroquinolones, a class of broad-spectrum antibiotics whose use in both humans and animals is restricted in many countries due to the quantity and severity of potential adverse effects. These effects occur during or even long after use and include central nervous system toxicity, peripheral neuropathy, blood disorders, and brain, liver, endocrine, musculoskeletal and gastrointestinal dysfunction. The FDA has recommended black box warnings for all Fluoroquinolone antibiotics due to the risk of tendonitis and tendon rupture. Several Fluoroquinolones have been removed from clinical use due to human fatalities. Side effects are most severe among the elderly and in children. Fluoroquinolones are not approved or are severely restricted for use in children in many countries and should not be taken by women who are pregnant or breastfeeding. Although banned for use in all food animals in Australia, as well as poultry and fish in the United States (due to the sharp increase in Ciprofloxicin resistant *Campylobacter* infections transmitted to humans), Fluoroquinolones are routinely used for veterinary treatment in a variety of food animals in many countries, including China, most countries in the EU and Japan. Fluoroquinolones are administered to treat infections and are also given prophylactically in feed or drinking water. The monitoring of water sources and food products such as meat and milk for antibiotic residues is necessary to ascertain that these compounds are not misused and do not present a danger to human or animal health.

The Abraxis Fluoroquinolones ELISA allows the determination of 41 samples in duplicate determination. Only a few grams or milliliters of sample are required. The test can be performed in less than 2 hours.

Performance Data

Test sensitivity: The limit of detection for Fluoroquinolones, calculated as 90% B/Bound (B/B_0), is approximately 0.016 ng/mL.

Standard Curve: The concentration of residue necessary to cause 50% inhibition (50% B/B_0) is approximately 0.19 ng/mL. Determinations closer to the middle of the calibration range of the test yield the most accurate results.



For demonstration purposes only. Not for use in sample interpretation.

Test reproducibility: Coefficients of variation (CVs) for standards: <10%; CVs for samples: <15%.

Selectivity/ Cross-reactivity: This ELISA recognizes various Fluoroquinolones with varying degrees:

Compound	Cross-reactivity
Enrofloxacin	100%
Danofloxacin	100%
Ciprofloxacin	66%
Fleroxacin	25%
Enoxacin	17%
Pefloxacin	12%
Ofloxacin	11%
Norfloxacin	7%
Marbofloxacin	6%
Levofloxacin	5%
Sarafloxacin	<1%
Oxolinic Acid	<1%

Samples: To eliminate matrix effects in fish, shrimp, or honey samples, sample clean-up is required. See Preparation of Samples, Section C.

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R103117

Fluoroquinolones ELISA, Microtiter Plate

Enzyme-Linked Immunosorbent Assay for the Determination of Fluoroquinolones in Contaminated Samples

Product No. 52250BA

1. General Description

The Fluoroquinolones ELISA is an immunoassay for the detection of Fluoroquinolones. This test is suitable for the quantitative and/or qualitative detection of Fluoroquinolones in contaminated samples including water, fish, shrimp and honey (please refer to Section C, Preparation of Samples for extraction/dilution procedures). Samples requiring regulatory action should be confirmed by HPLC, GC/MS, or other conventional methods.

2. Safety Instructions

The standard solutions in this test kit contain small amounts of Enrofloxacin. In addition, the substrate solution contains tetramethylbenzidine and the stop solution contains diluted sulfuric acid. Avoid contact of stopping solution with skin and mucous membranes. If these reagents come in contact with the skin, wash with water.

3. Storage and Stability

The Fluoroquinolones ELISA Kit should to be stored in the refrigerator (4–8°C). The solutions must be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the expiration date on the box. The conjugate is supplied in lyophilized form (3 vials). Before each assay, the required volume of lyophilized conjugate must be reconstituted with the appropriate diluent (see Section C, Test Preparation). Reconstitute only the amount needed for the samples to be run, as the reconstituted solution will only remain viable for one week (store refrigerated).

4. Test Principle

The test is a direct competitive ELISA based on the recognition of Fluoroquinolones by specific antibodies. Fluoroquinolones, when present in a sample, and a Fluoroquinolone-enzyme conjugate compete for the binding sites of rabbit anti-Fluoroquinolone antibodies in solution. The Fluoroquinolone antibodies are then bound by a second antibody (goat anti-rabbit) immobilized on the microtiter plate. After a washing step and addition of the substrate solution, a color signal is produced. The intensity of the blue color is inversely proportional to the concentration of Fluoroquinolones present in the sample. The color reaction is stopped after a specified time and the color is evaluated using a microplate ELISA photometer. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

5. Limitations of the Fluoroquinolones ELISA, Possible Test Interference

Numerous organic and inorganic compounds commonly found in samples have been tested and found not to interfere with this test. However, due to the high variability of compounds that might be found in samples, test interferences caused by matrix effects can not be completely excluded.

Mistakes in handling the test can also cause errors. Possible sources for such errors can be: inadequate storage conditions of the test kit, incorrect pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the immune and/or substrate reaction, exposure to direct or indirect sunlight during the substrate reaction, or extreme temperatures during the test performance (lower than 10°C or higher than 30°C).

The Abraxis Fluoroquinolones ELISA kit provides screening results. As with any analytical technique (GC, HPLC, etc.), samples requiring regulatory action should be confirmed by an alternative method.

Working Instructions

A. Materials Provided

1. Microtiter plate coated with a second antibody (goat anti-rabbit)
2. Fluoroquinolone Standards (7): 0, 0.025, 0.05, 0.125, 0.25, 0.5, and 1.0 ng/mL, 1 mL each
3. Fluoroquinolone-HRP Conjugate, 3 vials (lyophilized), 3 mL/vial after reconstitution, see Test Preparation (Section D)
4. Conjugate Diluent, 12 mL
5. Rabbit Anti-Fluoroquinolone Antibody Solution, 6 mL
6. Sample Diluent, 25 mL, use to dilute samples
7. Wash Buffer (5X) Concentrate, 100 mL, must be diluted before use, see Test Preparation (Section D)
8. Substrate (Color) Solution (TMB), 12 mL
9. Stop Solution, 12 mL.

B. Additional Materials (not included with the test kit)

1. Micro-pipettes with disposable plastic tips (10-200 and 200-1000 µL)
2. Multi-channel pipette (10-250 µL), stepper pipette (10-250 µL), or electronic repeating pipette with disposable plastic tips
3. Deionized or distilled water
4. Container with 500 mL capacity (for diluted 1X Wash Buffer, see Section D, Test Preparation)
5. Paper towels or equivalent absorbent material
6. Microtiter plate reader (wave length 450 nm)
7. Timer
8. Tape or Parafilm
9. Methanol (see Section C, Preparation of Samples)
10. Appropriate size glass vials with Teflon lined caps (see Section C, Preparation of Samples)
11. Centrifuge capable of spinning at 2000 x g (for fish/shrimp extraction, see Section C, Preparation of Samples)

C. Preparation of Samples

Water

Water samples should be collected in glass vessels with Teflon lined caps. Prior to analysis, each sample must be diluted with methanol (HPLC grade) to a 10% v/v final concentration of methanol (i.e. 100 µL of methanol into 900 µL of sample). The Fluoroquinolones concentration in the sample is determined by multiplying the ELISA results of the prepared water sample by 1.1

Fish/Shrimp

1. Weigh 1.0 g of homogenized fish or de-shelled shrimp (should have a paste-like consistency) into a 10 mL or larger appropriately labeled glass vial with a Teflon lined cap.
2. Add 3 mL of 80% Methanol to the vial. Vortex thoroughly. Mix using an overhead tube rotator for 20 minutes.
3. Centrifuge vial for 10 minutes at 2000 x g. Pipette 2 mL of the supernatant (top layer) into a clean vial.
4. Centrifuge extract for 10 minutes at 2000 x g (centrifuging the 2 mL of supernatant separately removes a greater amount of matrix interference from the extract to be analyzed).
5. Pipette 1 mL of the supernatant (top layer) into a clean vial.
6. Add 100 µL of the final extract to 900 µL of Sample Diluent and vortex thoroughly. This will then be analyzed as sample (Section F, Assay Procedure, Step 1).

The Fluoroquinolones concentration in the sample is determined by multiplying the ELISA results of the diluted extract by a factor of 40 (range of detection of the assay will be 1.0 – 40.0 ng/mL).

Samples of shrimp and fish, spiked with Enrofloxacin and extracted using the above procedure, recovered between 85-103%.

Honey

1. Weigh 1.0 g of honey into an appropriately labeled glass vial with a Teflon lined cap.
2. Add 1 mL of Sample Diluent (10% methanol in 1X PBS, pH7.0-7.4) to vial. Vortex vigorously for 2 minutes.
3. Add 50 µL of the sample (step 2) to 575 µL of Sample Diluent and vortex thoroughly. This will then be analyzed as sample (Section F, Assay Procedure, Step 1).

The Fluoroquinolones concentration in the sample is determined by multiplying the ELISA results of the diluted sample by a factor of 25 (range of detection of the assay will be 0.625 – 25.0 ng/mL).

Samples of honey, spiked with Enrofloxacin and diluted using the above procedure, recovered between 100-103%.

Highly contaminated samples (those outside of the calibration range of the assay) must be diluted and re-analyzed.

D. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. We recommend using a multi-channel pipette, stepping, or electronic repeating pipette for adding the conjugate, antibody, substrate (color), and stop solutions in order to equalize the incubation periods of the solutions on the entire microtiter plate. Please use only the reagents and standards from one package lot in one test, as they have been adjusted in combination.

1. Adjust the microtiter plate and the reagents to room temperature before use.
2. Remove the number of microtiter plate strips required from the foil bag. The remaining strips are stored in the foil bag and zip-locked closed. Store the remaining kit in the refrigerator (4-8°C).
3. The standard solutions, antibody, substrate and stop solutions are ready to use and do not require any further dilutions.
4. The conjugate provided is lyophilized (3 vials). Before each assay, calculate the volume of conjugate needed (when reconstituted, each vial will provide enough conjugate for approximately 50 wells). Reconstitute only the amount necessary for the samples to be analyzed. Once reconstituted, the conjugate solution will only remain viable for 1 week (store refrigerated). If additional samples are to be analyzed greater than one week from reconstitution, a new vial of conjugate must be prepared. To reconstitute, add 3 mL of Conjugate Diluent to each vial of Conjugate required and vortex thoroughly.
5. Dilute the Wash Buffer (5X) Concentrate at a ratio of 1:5. If using the entire bottle (100 mL), add to 400 mL of deionized or distilled water.
6. The stop solution should be handled with care as it contains diluted H_2SO_4.

E. Working Scheme

The microtiter plate consists of 12 strips of 8 wells, which can be used individually for the test. The standards must be run with each test. Never use the values of standards which have been determined in a test performed previously.

Std 0-Std 6: Standards
0; 0.025; 0.05; 0.125; 0.25; 0.5; 1.0 ppb

Samp1, Samp2, etc.: Samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 0	Std 4	Samp2									
B	Std 0	Std 4	Samp2									
C	Std 1	Std 5	etc.									
D	Std 1	Std 5	etc.									
E	Std 2	Std 6										
F	Std 2	Std 6										
G	Std 3	Samp1										
H	Std 3	Samp1										

F. Assay Procedure

1. Add **50 µL of the standard solutions, samples, or sample extracts** into the wells of the test strips according to the working scheme given. Analysis in duplicate or triplicate is recommended.
2. Add **50 µL of reconstituted enzyme conjugate solution** to the individual wells successively using a multi-channel, stepping, or electronic repeating pipette.
3. Add **50 µL of antibody solution** to the individual wells successively using a multi-channel, stepping, or electronic repeating pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill contents.
4. Incubate the strips for **60 minutes** at room temperature.
5. Remove the covering, decant the contents of the wells into a sink, and blot the inverted plate on a stack of paper towels. Wash the strips **three times** using the diluted wash buffer. Please use at least a volume of **250 µL of 1X wash buffer** for each well and each washing step. ***Blot the inverted plate after each wash step*** on a stack of paper towels. After the last wash/blot, check the wells for any remaining buffer in the wells, and if necessary, remove by additional blotting.
6. Add **100 µL of substrate (color) solution** to the individual wells successively using a multi-channel, stepping, or electronic repeating pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Incubate the strips for **20-30 minutes** at room temperature. Protect the strips from direct sunlight.
7. Add **100 µL of stop solution** to the wells in the same sequence as for the substrate solution using a multi-channel, stepping, or electronic repeating pipette.
8. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

G. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs such as 4-Parameter (preferred) or Logit/Log. For manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the $\%B/B_0$ for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance. Construct a standard curve by plotting the $\%B/B_0$ for each standard on the vertical linear (y) axis versus the corresponding Fluoroquinolones concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for samples will then yield levels in ppb of Fluoroquinolones by interpolation using the standard curve.

The concentrations of the samples are determined using the standard curve run with each test. Samples showing a higher concentration than Standard 6 (1.0 ng/mL) must be diluted further to obtain accurate results. To obtain the final concentration multiply the results by the appropriate dilution factor.

The Abraxis Fluoroquinolones ELISA kit provides screening results. As with any analytical technique (GC, HPLC, etc.), samples requiring regulatory action should be confirmed by an alternative method.