Importance of Enrofloxacin Determination

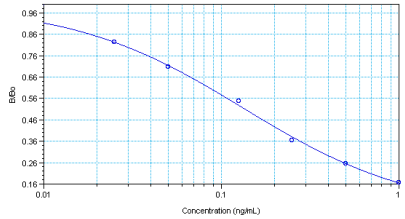
Antibiotic residues in foods pose a serious threat to public health. This is especially true of the Fluoroquinolones, a class of broad-spectrum antibiotics which includes Enrofloxacin. The use of Fluoroquinolones in both humans and animals is restricted in many countries due to the quantity and severity 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 Enrofloxacin ELISA allows the determination of 40 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 Enrofloxacin, calculated as 90% B/Bound, is approximately 0.0125 ng/mL.

Standard Curve: The concentration of residue necessary to cause 50% inhibition (50% B/B_0) is approximately 0.15 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:

Enrofloxacin	100%
Pefloxacin	11%
Fleroxacin	3%
Ciprofloxacin	<1%
Danofloxacin	<1%
Enoxacin	<1%
Levofloxacin	<1%
Marbofloxacin	<1%
Norfloxacin	<1%
Ofloxacin	<1%
Oxolinic Acid	<1%
Sarafloxacin	<1%

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

General Limited Warranty: Abraxis, Inc. warrants the products manufactured by the Company against defects and workmanship when used in accordance with the applicable instructions for a period not to extend beyond the product's printed expiration date. **Abraxis makes no other warranty, expressed or implied. There is no warranty of merchantability or fitness for a particular purpose.**

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R111617

Enrofloxacin ELISA, Microtiter Plate

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

Product No. 522511

1. General Description

The Enrofloxacin ELISA is an immunoassay for the detection of the Fluoroquinolone antibiotic Enrofloxacin. This test is suitable for the quantitative and/or qualitative detection of Enrofloxacin in contaminated samples including water, fish and shellfish (please refer to the appropriate technical bulletins for additional 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 Enrofloxacin 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.

4. Test Principle

The test is a direct competitive ELISA based on the recognition of Enrofloxacin by specific antibodies. Enrofloxacin, when present in a sample, and an Enrofloxacin-enzyme conjugate compete for the binding sites of rabbit anti-Enrofloxacin antibodies in solution. The Enrofloxacin 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 Enrofloxacin 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 Enrofloxacin 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 cannot 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, 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 Enrofloxacin 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 (anti-rabbit), in a re-sealable aluminum pouch with desiccant
2. Enrofloxacin Standards (7): 0, 0.025, 0.05, 0.125, 0.25, 0.5, and 1.0 ng/mL, 1 mL each
3. Enrofloxacin-HRP Conjugate, 6 mL
4. Rabbit Anti-Enrofloxacin Antibody Solution, 6 mL
5. Sample Diluent, 25 mL, used to dilute samples
6. Wash Buffer (5X) Concentrate, 100 mL, must be diluted before to use, see Test Preparation (Section D)
7. Substrate (Color) Solution (TMB), 12 mL.
8. 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 diluting 1X Wash Buffer, see Test Preparation, Section D)
5. Timer
6. Tape or Parafilm
7. Paper towels or equivalent absorbent material
8. Microtiter plate reader (wave length 450 nm)
9. Materials and reagents for sample preparation, see Preparation of Samples (Section C)

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 sample by 1.1. Highly contaminated samples (those outside of the calibration range of the assay) must be diluted with sample diluent and re-analyzed.

Fish/Shrimp

1. Weigh 1g of homogenized fish or de-shelled shrimp (should have a paste-like consistency) into a 10 mL or larger 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 (Assay Procedure, Section F, 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). Highly contaminated samples (those outside of the calibration range of the assay) must be diluted with sample diluent and re-analyzed.

Samples of shrimp and fish were spiked with Enrofloxacin and extracted using the above procedure. Recoveries were between 103-105%.

D. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. We recommend using a multi-channel, 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.

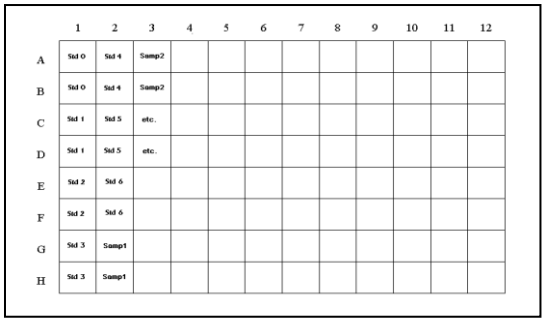
1. Adjust the microtiter plate and the reagents to room temperature before use.
2. The standard solutions, conjugate, antibody, substrate and stop solutions are ready to use and do not require any further dilutions.
3. Dilute the Wash Buffer (5X) Concentrate at a ratio of 1:5. If using the entire bottle (100 mL), add to 400 mL of deionized or distilled water.
4. The stop solution should be handled with care as it contains diluted H_2SO_4.
5. 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.
6. After analysis, store the remaining kit components in the refrigerator (4-8°C).

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



F. Assay Procedure

1. Add **50 µL of the standards, 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 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 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 Enrofloxacin concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for samples will then yield levels in ppb of Enrofloxacin by interpolation using the standard curve. Results can also be determined using a spreadsheet macro available from Abraxis upon request.

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 (see Preparation of Samples, Section C).

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