Importance of Quinolone Determination

Antibiotic residues in foods pose a serious threat to public health. This is especially true of the Quinolones, 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 Quinolone antibiotics due to the risk of tendonitis and tendon rupture. Several Quinolones have been removed from clinical use due to human fatalities. Side effects are most severe among the elderly and in children. Quinolones 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), Quinolones are routinely used for veterinary treatment in a variety of food animals in many countries, including China, most countries in the EU and Japan. Quinolones 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 Quinolones 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

Recovery rate: 70 - 130%

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

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

Compound	Cross-reactivity
Enrofloxacin	100%
Ciprofloxacin	102%
Pefloxacin	85%
Norfloxacin	70%
Danofloxacin	65%
Ofloxacin	60%
Oxolinic acid	55%
Flumequine	50%
Gatifloxacin	47%
Lomefloxacin	31%
Enoxacin	25%
Fleroxacin	15%
Levofloxacin	9%

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ABRAXIS

Quinolones ELISA, Microtiter Plate

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

Product No. 522502

1. General Description

The Quinolones ELISA is an immunoassay for the detection of Quinolones. This test is suitable for the quantitative and/or qualitative detection of Quinolones 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 Quinolones 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 Test Preparation section). 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 Quinolones by specific antibodies. Quinolones, when present in a sample, and a Quinolone-enzyme conjugate compete for the binding sites of anti-Quinolone antibodies immobilized in the wells of a microtiter plate. After an incubation time a washing step in performed followed by the addition of the substrate solution, a color signal is the produced. The intensity of the blue color is inversely proportional to the concentration of Quinolones 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 Quinolones 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, extreme temperatures during the test performance (lower than 10°C or higher than 30°C).

The Abraxis Quinolones 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 anti-quinolones antibody, 12 X 8 strips.
2. Quinolone Standards (6): 0; 0.3; 1.1; 3.3; 10; and 30 ng/mL (ppb), 1 mL.
3. Quinolone-HRP Conjugate (200X) concentrated, 100 uL.
4. Extraction Diluent (10X) concentrated, 2 X 25 mL.
5. Wash Solution (5 X) Concentrate, 100 mL.
6. Color (Substrate) Solution (TMB), 12 mL.
7. 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) or stepper pipette with plastic tips (10-250 µL)
3. Microtiter plate reader (wave length 450 nm)
4. Timer
5. Tape or Parafilm

6. Vortex mixer
7. Mixer (Stomacher, Ultraturax)

C. Additional Reagents (not included with the test kit)

1. Methanol at various dilutions
   a. 80% Methanol: 80 mL of 100% methanol + 20 mL of 1X Extraction Diluent
   b. 70% Methanol: 70 mL of 100% methanol + 30 mL of 1X Extraction Diluent
   c. 60% Methanol: 60 mL of 100% methanol + 40 mL of 1X Extraction Diluent
   d. 55% Methanol: 55 mL of 100% methanol + 45 mL of 1X Extraction Diluent
   e. 25% Methanol: 25 mL of 100% methanol + 75 mL of 1X Extraction Diluent
   f. 8% Methanol: 2.0 mL of 100% methanol + 23 mL of 1X Extraction Diluent
   g. 4% Methanol: 1.0 mL of 100% methanol + 24 mL of 1X Extraction Diluent
   h. 1N NaOH
   i. n-Hexane

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 or a stepping pipette for adding the conjugate, antibody, substrate 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, substrate and stop solutions are ready to use and do not require any further dilutions.
4. The conjugate provided is 200 X concentrated. Before each assay, calculate the volume of conjugate needed. Dilute only the amount necessary for the samples to be analyzed. Once diluted, the conjugate solution must be used immediately, discard after use. To dilute, add the proper amount of HRP conjugate to diluted (1X) Extraction Diluent at a ratio of 1:300, vortex thoroughly (i.e. dilute 25 uL of concentrated HRP to 7.5 mL of 1X Extraction Diluent.
5. Dilute the Wash Solution 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. Assay Procedure

1. Add 100 µL of the standard solutions and samples or sample extracts into the wells of the test strips according to the working scheme given. We recommend using duplicates or triplicates.
2. Add 50 µL of the 1X diluted enzyme conjugate solution to the individual wells successively using a multi-channel pipette or a stepping 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.
3. Incubate the strips for 60 minutes at room temperature.
4. After incubation, remove the covering and vigorously shake the contents of these wells into a sink. Wash the strips three times using the 1X washing buffer solution. Use at least a volume of 250 µL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.
5. Add 100 µL of color (substrate) solution to the wells. 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 minutes at room temperature. Protect the strips from direct sunlight.
6. Add 100 µL of stop solution to the wells in the same sequence as for the substrate solution.
7. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

F. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs (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 Quinolones concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for samples will then yield levels in ppb of Quinolones by interpolation using the standard curve. Samples showing a higher concentration than Standard 6 (30 ng/mL) must be diluted further to obtain accurate results. To obtain final concentration multiply the concentration obtained in the assay by dilution factor (if any).

F. Preparation of Samples

Milk

1. Transfer 1.0 ml of milk into a centrifuge tube and centrifuge at 3,500 rpm for 20 minutes.
2. Remove the supernatant layer (top layer) and keep lower layer
3. Pipette 200 uL of the aqueos layer (lower layer) into a tube and add 800 uL of 1X Extraction Diluet. Mix thoroughly. This will then be analyzed as sample (Assay Procedure, step 1). Dilution factor = 5, Sensitivity = 2 ppb.

Fish/Pork Muscle Method I

1. Weigh 2.0 g of homogenized sample and add 4.0 mL of 25% Methanol to vial. Vortex thoroughly for 1-2 minutes.
2. Place in a water bath at 60 °C for 10 minutes.
3. Shake for 10 minutes.
4. Centrifuge vial for 15 minutes at 3500 g.
5. Pipette 200 uL of the supernatant (top layer) into a clean vial and add to 100 uL of 1X Extraction Diluent and mix thoroughly. This will then be analyzed as sample (Assay Procedure, step 1). Dilution factor = 4.5, sensitivity = 1.5 ppb.

Fish/Pork Muscle Method II

1. Weigh 1.0 g of homogenized sample and add 3.0 mL of 80% Methanol to vial. Vortex thoroughly for 1-2 minutes
2. Shake for 10 minutes.
3. Centrifuge vial for 15 minutes at 3000 g.
4. Pipette 1.0 mL of the supernatant (top layer) into a clean glas tube an evaporate to dryness at 50 °C.
5. Dissolve the residue with 0.5 mL of 4% methanol, vortex.
6. Add 1.0 mL of n-Hexane. Vortex thoroughly for 30 seconds.
7. Centrifuge vial for 15 minutes at 3000 g.
8. Remove and discard the top layer
9. The aqueous layer (lower layer) is analyzed as the sample (Assay Procedure, step 1). Dilution factor = 1.5, sensitivity = 0.45 ppb.

Egg (Whole)

1. Weigh 1 g of homogenized egg into a centrifuge tube and add 3.0 mL of 80% methanol. Vortex well for 1 minute.
2. Vortex vigorously for 2 additional minutes
3. Centrifuge vial for 15 minutes at 3500 g.
4. Pipette 200 uL of the supernatant (top layer) into a clean vial and add to 100 uL of 1X Extraction Diluent and mix thoroughly. This will then be analyzed as sample (Assay Procedure, step 1). Dilution factor = 6, sensitivity = 2.0 ppb.

Chicken Muscle, Method I

1. Weigh 2.0 g of homogenized sample and add 4.0 mL of 25% Methanol to vial and 50 uL of 1N NaOH.. Vortex thoroughly for 1-2 minutes.
2. Place in a water bath at 60 °C for 10 minutes.
3. Shake for 10 minutes.
4. Centrifuge vial for 15 minutes at 3500 g.
5. Pipette 200 uL of the supernatant (top layer) into a clean vial and add to 100 uL of 1X Extraction Diluent and mix thoroughly. This will then be analyzed as sample (Assay Procedure, step 1). Dilution factor = 4.5, sensitivity = 1.5 ppb.

Chicken Muscle, Method II

1. Weigh 1.0 g of homogenized sample and add 3.0 mL of 80% Methanol to vial. Vortex thoroughly for 1-2 minutes
2. Shake for 10 minutes.
3. Centrifuge vial for 15 minutes at 3000 g.
4. Pipette 1.0 mL of the supernatant (top layer) into a clean glas tube an evaporate to dryness at 50 °C.
5. Dissolve the residue with 0.5 mL of 4% methanol, vortex.
6. Add 1.0 mL of n-Hexane. Vortex thoroughly for 30 seconds.
7. Centrifuge vial for 15 minutes at 3000 g.
8. Remove and discard the top layer
9. The aqueous layer (lower layer) is analyzed as the sample (Assay Procedure, step 1). Dilution factor = 1.5, sensitivity = 0.45 ppb.

Shrimp, Method I

1. Weigh 2.0 g of homogenized sample and add 4.0 mL of 55% Methanol to vial. Vortex thoroughly for 1-2 minutes.
2. Centrifuge vial for 15 minutes at 3500 g.
3. Shake for 10 minutes.
4. Centrifuge vial for 15 minutes at 3500 g.
5. Pipette 200 uL of the supernatant (top layer) into a clean vial and add to 100 uL of 1X Extraction Diluent and mix thoroughly. This will then be analyzed as sample (Assay Procedure, step 1). Dilution factor = 4.5, sensitivity = 1.5 ppb. **NOTE:** Contact us for a more sensitive alternative procedure for shrimp.