Importance of Tetracyclines Determination

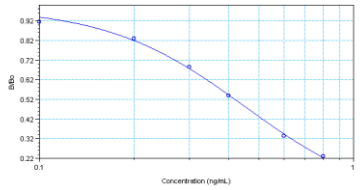
Antibiotic residues in foods pose a serious threat to public health. Tetracycline is a broad spectrum polyketide antibiotic produced by the *Streptomyces* genus of Actinobacteria. It is used for the treatment and prevention of many bacterial infections. Tetracycliness are widely used in food production, however over use can lead to antibiotic resistance. The monitoring of water sources and food products, such as meat, milk and honey, 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 following MRLs for Tetracycline, chlorTetracycline and oxyTetracycline has been recommended by FAO/WHO in cattle, pigs, sheep and poultry: 100 µg/Kg (muscle), 300 µg/Kg (liver), 600 µg/Kg (kidney); 100 µg/L in cattle and sheep milk; 200 µg/Kg in egg (poultry). An MRL of 100 µg/Kg for oxyTetracycline in muscle of giant prawn. Europe has proposed an MRL of 10 µg/Kg for all Tetracyclines in honey.

The Abraxis Tetracyclines ELISA allows the determination of 42 samples in duplicate determination. Only a few milliliters of sample are required. The test can be performed in 90 minutes.

Performance Data

Test sensitivity: The limit of detection for Tetracycline in water calculated as Xn +/- 3SD (n=20) or as 90% B/Bound is equal to <0.10 ng/mL. The concentration of residue necessary to cause 50% inhibition (50% B/B_0) is approximately 0.40 ng/mL. Determinations closer to the middle of the calibration range of the test yield the most accurate results.

The following is the sensitivity in different matrixes: 4.0 ppb in honey; 4.0 ppb in milk; 8.0 ppb in meat; 4.0 ppb in shrimp; 0.11 ppb in water.



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

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

Selectivity: This ELISA recognizes Tetracycline and related compounds with varying degrees:

Cross-reactivities:

Tetracycline	100%
Oxytetracycline	95%
4-epi-tetracycline	95%
Demeclocycline	88%
Rolitetracycline	82%
Chlortetracycline	81%
4-epi-oxytetracycline	71%
Methacycline	60%
Doxycycline	53%
4-epi-chlortetracycline	29%

Samples: To eliminate matrix effects in meat, milk and honey samples, sample dilution is required. See Preparation of Samples section. For additional extraction procedures for various matrices please contact Abraxis, Inc.

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Tetracyclines ELISA, Microtiter Plate

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

Product No. 52254BA

1. General Description

The Tetracyclines ELISA is an immunoassay for the detection of Tetracyclines. This test is suitable for the quantitative and/or qualitative detection of Tetracyclines in contaminated samples. Positive samples 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 Tetracyclines in solution. In addition, the substrate solution contains tetramethylbenzidine and the stop solution contains diluted sulfuric acid. Avoid contact of standard and stopping solutions with skin and mucous membranes. If these reagents come in contact with the skin, wash with water.

3. Storage and Stability

The Tetracyclines ELISA Kit should to be stored in the refrigerator (4–8°C) prior to use. 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. Some reagents need to be stored frozen after reconstitution (Test Preparation, section C).

4. Test Principle

The test is a direct competitive ELISA based on the recognition of Tetracyclines by specific antibodies. Tetracyclines, when present in a sample and a Tetracyclines-enzyme conjugate compete for the binding sites of anti-Tetracyclines antibodies which are immobilized on the wells of 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 Tetracyclines present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

5. Limitations of the Tetracyclines 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 (**or reagents**), 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 assay procedure should be performed away from direct or indirect sunlight.

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

Working Instructions

A. Materials Provided

1. Microtiter plate coated anti-Tetracyclines antibody, in a resealable foil pouch with desiccant.
2. Tetracycline Standards (6): 0, 0.10, 0.20, 0.30, 0.40, 0.60 and 0.80 ng/mL; Control at 0.50 ng/mL. Standard and Control vials supplied lyophilized, 1 mL/vial after reconstitution, see Test Preparation (Section C).
3. Assay Buffer, 6 mL.
4. Sample Diluent (10X) Concentrate, 2 X 25 mL bottles, must be diluted before use. Use to dilute samples.
5. Tetracyclines-HRP Conjugate, 2 vials (lyophilized), must be diluted prior to use, see Test Preparation (Section C).
6. Conjugate Diluent, 2 bottles, 12 mL each.
7. Wash Buffer (5X) Concentrate, 100 mL, must be diluted prior to use, see Test Preparation (Section C).
8. Substrate (Color) Solution (TMB), 16 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 (50-250 µL), stepper pipette (10-250 µL), or electronic repeating pipette with disposable plastic tips
3. Microtiter plate reader (wave length 450 nm)
4. Timer
5. Tape or Parafilm
6. Glass vials with Teflon-lined caps
7. Distilled or deionized water
8. Container with 500 mL capacity (for diluting 1X Wash Buffer, see Test Preparation, Section C)
9. Vortex mixer
10. Materials and Reagents for Preparation of Samples (Section D)

C. 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 assay buffer, conjugate, substrate and stop solutions in order to equalize the incubations 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 should be stored in the foil bag and zip-locked closed. Store the remaining kit in the refrigerator (4-8°C).
3. The assay buffer, substrate, and stop solutions are ready to use and do not require any further dilutions.
4. The conjugate provided is lyophilized (2 vials). One reconstituted lyophilized vial will provide enough (concentrated) conjugate to be diluted for an entire plate assay. Once reconstituted, the (concentrated) conjugate solution will only remain viable for 4 weeks (store at –20°C). If additional samples are to be analyzed greater than four weeks from reconstitution, a new vial of (concentrated) conjugate must be prepared. To reconstitute the concentrated conjugate, add 1 mL of Conjugate Diluent to one vial of lyophilized conjugate and vortex thoroughly. **Before each assay, calculate the volume of diluted conjugate required for the assay then further dilute the concentrated conjugate in Conjugate Diluent according to the notice slip that was included in the kit.** Dilute fresh for each assay.
5. The standards and control are provided lyophilized. To reconstitute, add 1.0 mL of deionized water to each vial and vortex thoroughly. Once reconstituted, the standards and control solutions will only remain viable for 4 weeks if stored at –20°C. Additional vials are available upon request.
6. Dilute the sample diluent (10X) concentrate at a ratio of 1:10. If using the entire bottle (25 mL), add to 225 mL of deionized or distilled water.
7. 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.
8. The stop solution should be handled with care as it contains diluted H_2SO_4.

D. Preparation of Samples

Samples should be analyzed immediately after preparation to prevent adsorption/degradation of the analyte.

Meat (Chicken, Beef)

1. Weigh 1.0 g of homogenized meat into a 15 mL plastic centrifuge tube.
2. Add 3.0 mL of a 1:1 Methanol:McIlvaine buffer pH 7.0*, vortex thoroughly. Mix using an overhead rotator for 40 minutes.
3. Centrifuge for 10 min at 2,000 X g. Save supernatant.
4. Dilute supernatant 1:20 (i.e. 50 µL of supernatant and 950 µL of 1X Sample Diluent). Vortex to mix and analyze as sample (Assay Procedure, step1).

The Tetracyclines concentration contained in meat samples is then determined by multiplying the ELISA result by the dilution factor of 80. Recoveries were 80-110%

Milk

1. Dilute milk sample 1:40 (50 µL into 1950 µL) in 1X sample diluent.
2. Analyze as sample (Assay Procedure, step 1).

The Tetracyclines concentration contained in milk samples is then determined by multiplying the ELISA result by the dilution factor of 40. Recoveries were 100-120%

Honey

1. Add 0.5 g of honey to a clean plastic tube.
2. Add 19.5 mL of Sample Diluent (1X). Vortex until honey is completely dissolved.
3. Analyze as sample (Assay Procedure, step 1).

NOTE: Centrifugation at 3,000 RPM for 5-10 minutes will help with samples exhibiting precipitates.

The Tetracyclines concentration contained in honey samples is then determined by multiplying the ELISA result by the dilution factor of 40. Recoveries were 101-136%.

Shrimp

1. Weigh 1.0 g of homogenized shrimp into a 15 mL plastic centrifuge tube.
2. Add 3.0 mL of 80% Methanol, vortex thoroughly. Mix using an overhead rotator for 20 minutes.
3. Centrifuge for 10 min at 2,000 X g. Pipette 2 mL of the supernatant into clean vial.
4. Centrifuge extract for 10 minutes at 2000 X g. Pipette 1 mL of supernatant into clean vial.
5. Dilute supernatant 1:10 (i.e. 100 µL of supernatant and 900 µL of 1X Sample Diluent). Vortex to mix and analyze as sample (Assay Procedure, step1).

The Tetracyclines concentration contained in shrimp samples is then determined by multiplying the ELISA results by the dilution factor of 40. Recoveries were 114%

Water

Prior to analysis, each sample must be filtered using a 0.2 um polysulfone filter and diluted with 10x Sample Diluent to a 1x final concentration of Sample Diluent (i.e. 100 µL of 10x Sample Diluent into 900 µL of sample).

The Tetracyclines concentration contained in water samples is then determined by multiplying the ELISA results by the dilution factor of 1.11.

***Preparation of McIlvain Buffer pH 7.0**

1. Prepare a 0.2M Sodium Phosphate Dibasic solution: 28.4 g of Na_2HPO_4 to 1 L of deionized water
2. Prepare 0.1M Citric Acid: 29.4 g of Citric Acid Trisodium salt to 1L of deionized water.
3. For 200 mL McIlvain Buffer: mix 164.7 mL 0.2M Na_2HPO_4 and 35.3 mL 0.1M Citric Acid.
4. Adjust pH to 7.0 with Sodium Hydroxide or Hydrochloric Acid if necessary.
5. Mix 1 part McIlvain Buffer and 1 part methanol when preparing for sample extraction.

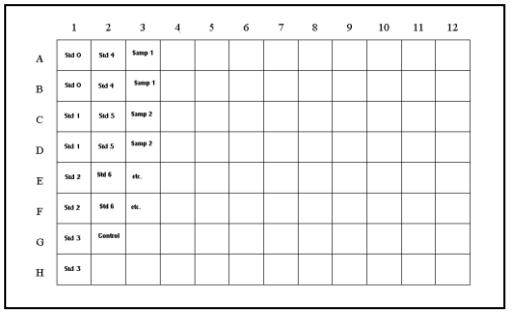
E. Working Scheme

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; 0.10; 0.20; 0.30; 0.40; 0.60 ; 0.80 ppb

Control

Samp1, Samp2, etc.: Samples



F. Assay Procedure

1. Add **50 µL of assay buffer solution** to the individual wells successively using a multi-channel, stepping, or electronic repeating pipette.
2. 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. Analysis in duplicate or triplicate is recommended.
3. Add **50 µL of diluted enzyme conjugate solution** to the individual wells successively using a multi-channel, stepping, or electronic repeating pipette.
4. 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.
5. Incubate the strips for **60 minutes** at room temperature.
6. 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 **four 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.
7. Add **150 µL of substrate (color) 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 30 minutes at room temperature. Protect the strips from direct sunlight.
8. Add **100 µL of stop solution** to the wells in the same sequence as for the substrate solution.
9. 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 Tetracycline concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for samples will then yield levels in ppb of Tetracyclines by interpolation using the standard curve. The results must be multiplied by the appropriate dilution factor to account for the sample extraction/dilution. 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 lower concentrations of Tetracyclines compared to Standard 1 (0.10 ng/mL) should be reported as containing < 8 ng/mL for meat samples, < 4 ng/mL for milk, honey and shrimp samples, and < 0.11 for water samples. Samples showing a higher concentration than Standard 6 (0.80 ng/mL) must be diluted further with 1X Sample Diluent to obtain accurate results.

As with any analytical technique (GC, HPLC, etc.), positive results requiring regulatory action should be confirmed by an alternative method.