Importance of Tetracyclines Determination

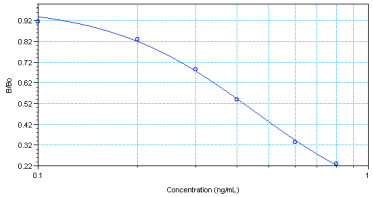
Antibiotic residues in foods pose a serious threat to public health. Tetracycline is a broad spectrum polyketide antibiotic produced by the *Streptomyces* genus of Actinobacteria. It is used for the treatment and prevention of many bacterial infections. Tetracycliness are widely used in food production, 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 ug/Kg (muscle), 300 ug/Kg (liver), 600 ug/Kg (kidney); 100 ug/L in cattle and sheep milk; 200 ug/Kg in egg (poultry). An MRL of 100 ug/Kg for oxyTetracycline in muscle of giant prawn. Europe has proposed an MRL of 10 ug/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.



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 LLC.

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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 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.
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).
6. Conjugate Diluent, 2 bottles, 12 mL each.
7. Wash Solution (5X) Concentrate, 100 mL.
8. Color (Substrate) 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) or stepper pipette with plastic tips (10-250 µL)
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. Vortex mixer

C. 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 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 standards, 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). Before each assay, calculate the volume of conjugate needed (when reconstituted, each vial will provide enough conjugate for 1 plate. Once reconstituted, the conjugate solution will only remain viable for 4 weeks (store –20 °C). If additional samples are to be analyzed greater than one week from reconstitution, a new vial of conjugate must be prepared. To reconstitute, add 1 mL of Conjugate Diluent to each vial of Conjugate required and vortex thoroughly then dilute at a ratio of 1:20 in the same Conjugate Diluent (i.e. 250 uL of HRP and 4.75 mL of Conjugate Diluent).
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/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 gm of homogenized meat into a 15 mL plastic centrifuge tube.
2. Add 3.0 mL of a 1:1 Methanol:McIlvaine pH 7.0 buffer, 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 uL of supernatant and 950 uL 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 uL into 2.0 mL) 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 80. 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 gm of homogenized meat 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.
4. Dilute supernatant 1:10 (i.e. 100 uL of supernatant and 900 uL 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%

NOTE: Highly contaminated samples (those outside of the calibration range of the assay) must be diluted further in 1X Sample Diluent and re-analyzed. Samples with values below the first standard should not be multiplied and reported as < 0.10 ppb.

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

1. Prepare a 0.2M Sodium 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. Adjust pH to 7.0 with 6N Sodium Hydroxide
4. Dilute 1:1 with Methanol before use.

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

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

F. Assay Procedure

1. Add 50 µL of **assay buffer solution** to the individual wells successively using a multi-channel pipette or a stepping 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. We recommend using duplicates or triplicates.
3. Add 50 µL of **enzyme conjugate solution** to the individual wells successively using a multi-channel pipette or a stepping 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. After incubation, remove the covering and vigorously shake the contents of these wells into a sink. Wash the strips **four times** using the 1X washing buffer solution. Use 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.
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 20-30 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 [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. Samples showing lower concentrations of Tetracyclines compared to Standard 1 (0.10 ng/mL) should be reported as containing < 0.10 ng/mL. Samples showing a higher concentration than Standard 6 (0.80 ng/mL) must be diluted further to obtain accurate results.