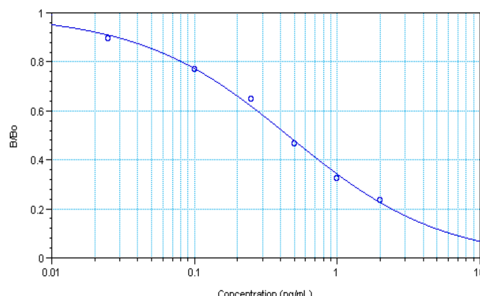
Importance of Chloramphenicol Determination

Antibiotic residues in foods pose a serious threat to public health. Human use of Chloramphenicol, a broad-spectrum antibiotic, is found primarily in developing countries due to its low cost. The use of Chloramphenicol in developed nations is generally limited to topical application for the treatment of eye infections; as chloramphenicol can adversely affect bone marrow, causing aplastic anemia, which is usually fatal. Oral chloramphenicol treatment is therefore only considered appropriate for the treatment of MRSA or other highly antibiotic resistant infections. Chloramphenicol is more frequently used in the veterinary treatment of infections in small mammals and also in amphibians to treat chytridiomycosis; a fungal disease responsible for the loss of one-third of all species of frogs within the past 30 years. The use of Chloramphenicol in food-producing animals is prohibited in many countries including the United States, Canada, the European Union, and Australia due to the high potential risk of severe effects such as aplastic anemia, allergic reactions, and the promotion of antibiotic resistance. The U.S., Canada, and the EU have also imposed bans on all imported foods containing Chloramphenicol residues. 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 Abraxis Chloramphenicol ELISA allows the determination of 40 samples in duplicate determination. Only a few milliliters of sample are required. The test can be performed in less than 2 hours.

Performance Data

Test sensitivity: The limit of detection for Chloramphenicol calculated as Xn +/- 3SD (n=20) or as 90% B/Bound is equal to 0.023 ng/mL. The concentration of residue necessary to cause 50% inhibition (50% B/B_0) is approximately 0.44 ng/mL. Determinations closer to the middle of the calibration range of the test yield the most accurate results.



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

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

Cross-reactivities:

Compound	Cross-reactivity
Chloramphenicol	100%
Chloramphenicol glucuronide	25%
Thiamphenicol	<1%
Florphenicol	<1%

Samples: To eliminate matrix effects in fish, shrimp, honey, and milk samples, sample clean-up may be 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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Chloramphenicol ELISA, Microtiter Plate

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

Product No. 5113B

1. General Description

The Chloramphenicol ELISA is an immunoassay for the detection of Chloramphenicol. This test is suitable for the quantitative and/or qualitative detection of Chloramphenicol 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 Chloramphenicol. 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 Chloramphenicol 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 antibody and conjugate are supplied in lyophilized form (3 vials of each). Before each assay, the required volumes of lyophilized antibody and 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 solutions will only remain viable for one week (store refrigerated).

4. Test Principle

The test is a direct competitive ELISA based on the recognition of Chloramphenicol by specific antibodies. Chloramphenicol, when present in a sample, and a Chloramphenicol-enzyme conjugate compete for the binding sites of monoclonal mouse anti-Chloramphenicol antibodies in solution. The Chloramphenicol antibodies are then bound by a second antibody (goat anti-mouse) immobilized on the 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 Chloramphenicol 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 Chloramphenicol 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 Chloramphenicol ELISA kit provides screening results. As with any analytical technique (GC/MS, HPLC, etc.), positive 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-mouse)
2. Chloramphenicol Standards (7): 0, 0.025, 0.1, 0.25, 0.5, 1.0, and 2.0 ng/mL, 1 mL each
3. Monoclonal mouse anti-Chloramphenicol Antibody, 3 vials (lyophilized). 3.5 mL/vial after reconstitution
4. Antibody Diluent, 15 mL
5. Chloramphenicol-HRP Conjugate, 3 vials (lyophilized). 3.5 mL/vial after reconstitution
6. Conjugate Diluent, 15 mL
7. Sample Diluent concentrate (10X), 25 mL, must be diluted prior to use for sample dilutions, see Test Preparation (Section D)
8. Wash Buffer (5X) Concentrate, 100 mL, must be diluted before use, see Test Preparation (Section D)
9. Substrate (Color) Solution (TMB), 16 mL
10. 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 Test Preparation, Section D)
5. Materials and reagents for sample extraction
6. Microtiter plate reader (wave length 450 nm)
7. Timer
8. Tape or Parafilm

C. Preparation of Samples

Fish/Shrimp Extraction

1. Weigh 3g 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 6 mL of Ethyl Acetate. Vortex thoroughly. Mix using an overhead tube rotator for 10 minutes.
3. Centrifuge vial for 10 minutes at 3000 g. Pipette 4 mL of the supernatant (top layer) into a clean vial.
4. Evaporate to dryness at 40-60ºC under a gentle stream of nitrogen.
5. Add 1 mL of Iso-octane / Trichloromethane (2:3) and vortex thoroughly to re-dissolve.
6. Add 2 mL of 1X Sample Diluent (see Test Preparation, Section D) and vortex thoroughly.
7. Centrifuge vial for 10 minutes at 4000 g.
8. Pipette supernatant (top layer) into a clean vial. This will then be analyzed as sample (Assay Procedure, step 1).

The ELISA result will show the Chloramphenicol concentration contained in the fish/shrimp samples (no correction factor is necessary). Highly contaminated samples (those outside of the calibration range of the assay) must be diluted and re-analyzed.

Honey Sample Extraction

1. Add 3 g of honey to a clean glass vial with a Teflon-lined cap.
2. Add 3 mL of distilled or deionized water. Vortex.
3. Add 6 mL of Ethyl Acetate. Vortex. Mix using an overhead tube rotator for 10 minutes.
4. Centrifuge vial for 10 minutes at 3000 g. Pipette 4 mL of the supernatant (top layer) into a clean vial.
5. Evaporate to dryness at 40-60ºC under a gentle stream of nitrogen.
6. Add 1 mL of Iso-octane / Trichloromethane (2:3) and vortex thoroughly to re-dissolve.
7. Add 1 mL of 1X Sample Diluent (see Test Preparation, Section D) and vortex thoroughly. Centrifuge vial for 10 minutes at 3000 g.
8. Pipette supernatant into a clean vial. This will then be analyzed as sample (Assay Procedure, step 1).

The Chloramphenicol concentration contained in honey samples is then determined by dividing the ELISA result by the concentration factor of 2. Highly contaminated samples (those outside of the calibration range of the assay) must be diluted and re-analyzed.

Milk

No sample extraction is necessary for the analysis of milk samples. Proceed to Assay Procedure, step 1. Highly contaminated samples (those outside of the calibration range of the assay) will need to be diluted and re-analyzed.

For additional extraction procedures for various matrices please contact Abraxis, Inc.

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 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 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 antibody provided is lyophilized (3 vials). Before each assay, calculate the volume of antibody needed (when reconstituted, each vial will provide enough antibody for approximately 65 wells). Reconstitute only the amount necessary for the samples to be analyzed. Once reconstituted, the antibody 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 antibody will need to be prepared. To reconstitute, add 3.5 mL of Antibody Diluent to each vial of antibody required and vortex thoroughly.
5. 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 65 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 will need to be prepared. To reconstitute, add 3.5 mL of Conjugate Diluent to each vial of conjugate required and vortex thoroughly.
6. 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.
7. Dilute the Sample Diluent concentrate (10X) at a ratio of 1:10. If using the entire bottle (25 mL), add to 225 mL of deionized or distilled water.
8. 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; 0.1; 0.25; 0.5; 1.0; 2.0 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 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 reconstituted enzyme conjugate** solution to the individual wells successively using a multi-channel, stepping pipette, or electronic repeating pipette.
3. Add **50 µL of reconstituted antibody solution** to the individual wells successively using a multi-channel, stepping pipette, 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 about 30 seconds. Be careful not to spill contents.
4. Incubate the strips for **60 minutes** at room temperature.
5. After incubation, remove the covering and vigorously shake the contents of these wells into a sink. Wash the strips **three times** using the diluted wash buffer. Use at least a volume of **250 µL of 1X wash buffer** for each well and each washing step. Remaining buffer in the wells should be removed by patting the inverted plate dry on a stack of paper towels.
6. Add **150 µL of substrate (color) solution** to the wells successively using a multi-channel, stepping pipette, 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 about 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 (color) solution using a multi-channel, stepping pipette, 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 Chloramphenicol concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for samples will then yield levels in ppb of Chloramphenicol by interpolation using the standard curve. Results can also be determined by 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 Chloramphenicol compared to Standard 1 (0.025 ng/mL) are considered as negative. Samples showing a higher concentration than Standard 6 (2.0 ng/mL) must be diluted further to obtain accurate results. Honey sample extract results need to be divided by the concentration factor of 2 to obtain the sample concentration.

Semi-quantitative results can be derived by simple comparison of the sample absorbances to the absorbances of the standards. Samples with lower absorbances than a standard will have concentrations of Chloramphenicol greater than that standard. Samples which have higher absorbances than a standard will have concentrations of Chloramphenicol less than that standard.

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