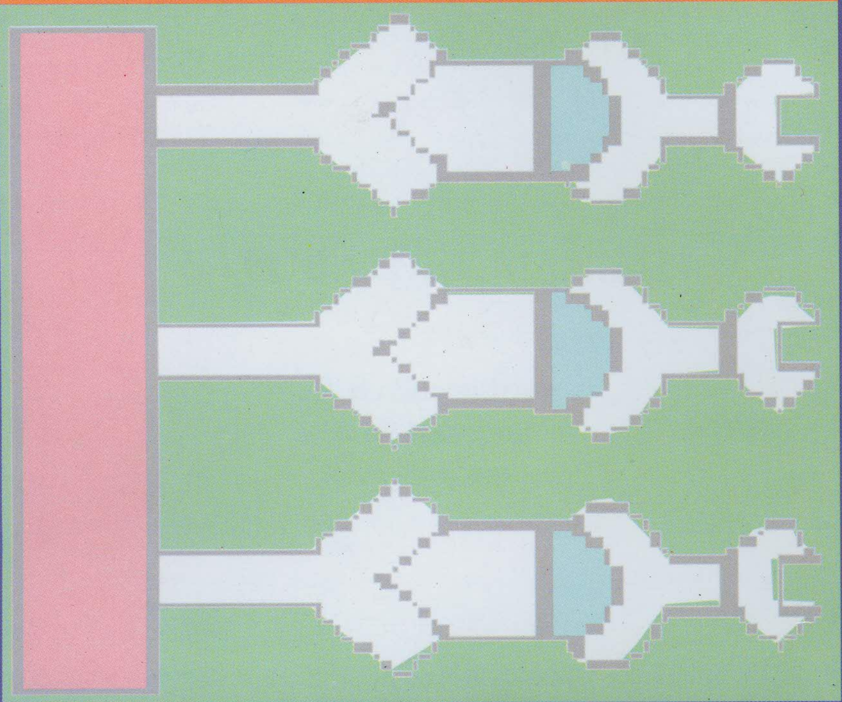
ENZYME LINKED IMMUNO SORBANT ASSAY (ELISA) FOR CHLORAMPHENICOL RESIDUE IN SHRIMP

LABORATORY MANUAL



Central Institute of Fisheries Technology, (ICAR)
Matsyapuri, P.O., Cochin-682 029
2002

Enzyme Linked Immuno Sorbant Assay (ELISA) for Chloramphenicol Residue in Shrimp

LABORATORY MANUAL

P. K. Surendran
V. N. Nambiar
Rakesh Kumar

Central Institute of Fisheries Technology,
(Indian Council of Agricultural Research)
Matsyapuri, P.O., Cochin-682 029
2002

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Preface

Shrimp is today the largest single foreign exchange earning export item for India in the agriculture and related sectors. Shrimp, which constituted around 10% of our marine landings, accounted for almost 75% of our earnings from export of seafoods. Of late, of course, farmed shrimp has become the major share accounting for almost 60% of the total shrimp processed for export. Coastal aquaculture and freshwater shrimp culture has changed the picture totally. We are today facing acute competition from neighbouring countries like Taiwan and Vietnam. Unless we are vigilant and quality conscious we are likely to be pushed back from our prominent position in the shrimp market. Japan and USA had been our traditional markets, but now, diversification of products as well as markets has become a necessity for the industry. European Union (EU) offers a good market. But the stringent quality standards of the EU are posing problems for our exporters. Detection of pathogenic or toxigenic bacteria in the processed products was the main problem so far. But now detection of traces of residues of some common antibiotics is causing problems. EU rejects products in which even ppb level of antibiotics like chloramphenicol are detected. EU's quality standards in general have been a blessing in disguise for us in as much as they compelled our processors to adopt high standards of hygiene and cleanliness. But analysis for ppb levels of antibiotics need highly sophisticated and costly analytical methods, equipment and expertise. Liquid chromatography-Mass spectrometry (LC-MS-MS) is the only answer to this problem. However, for quick detection and confirmation, other techniques like ELISA are routinely used in labs the world over.

ELISA is a simple general method used for detection of antibiotics, viruses, pathogenic and toxigenic strains of bacteria etc. It is a simple, relatively less expensive, quick and dependable method. But it needs expertise and trained manpower.

The Microbioligy, Fermentation and Biotechnology Division of CIFT has been assisting the industry in the detection of these undesirable residues/bacteria in seafoods. Besides analysing samples of prdoucts they have been training technologists of the industry in techniques like ELISA also. This booklet gives the ELISA methodology for the detection of chloramphenicol in processed shrimp. I am sure it is going to be a very useful information to all technologists/students and researchers in this field. I congratuate the scientists of the Microbiology, Fermentation and Biotechnology Division of CIFT for this informative and useful compilation of information and the lucid style of presentation. I hope the end users in the labs will find it useful.

Cochin
Dated 5-12-2002

Dr. K. Devadasan
(Director)

Chapter-I

Enzyme Linked Immuno Sorbant Assay (ELISA) - An Introduction

ELISA is a sensitive technique to quantitate the antibody or antigen of interest. It is a very easy, and simple assay. The assay for an antigen depends on being able to couple with highly specific antibody, enzyme and a solid substrate such as plastic beads or plates (the inner walls of the wells where assay is done). The antigen is immobilized on a solid surface. The antibody specific to this antigen is added to the plates, which binds to the antigen. Then a secondary antibody conjugated with enzyme is added. This binds to the first or primary antibody. This complex can be detected by an enzyme substrate reaction. The intensity of this chromogenic reaction is measured.

ELISA technique is now used in the pharmaceutical industry with application in drug discovery, animal studies, and clinical trials. ELISA method was initially developed to detect serum antibodies, and they have been used in environmental studies for the direct analysis of the herbicides and antibiotics. Food industry uses ELISA in the analysis of pathogens and toxins. Agriculture and medical industries employ this method in the quantification of hormones and drugs. It is also used in the detection of viruses and their proteins in blood or body fluids.

ELISA assay is used for the quantitative analysis of chloramphenicol residues in shrimp. This test operates on the basis of competition between a chloramphenicol enzyme conjugate and the chloramphenicol in the extracted sample for a limited number of binding sites on the antibody coated plate.

There are various modification of the basic ELISA technique and are as follows:

1. Direct ELISA

Antigen is coated on the walls of the microtitre plate wells. The specific antibody itself is conjugated with an enzyme, which binds specifically to the

antigen. This complex is then assayed with the help of a chromogenic substrate reaction (Fig..A).

2. Indirect ELISA

The microtitre plate wells are coated with antigen. The antibody conjugated with enzyme is directed against another antibody, which specifically binds to the antigen in use. This is in common use as it avoids the unnecessary wastage of the primary antibody-enzyme conjugate. (Fig.B)

3. Sandwich ELISA

The plate is first coated with the specific antibody which binds through its Fc receptors. The sample containing the antigen is then added. The antigenic receptors of the coated antibody bind to the antigen added. The second antibody conjugated with enzyme is added. This labeled antibody also specifically binds to the same antigen. The complex is then assayed using chromogenic reaction. (Fig. C)

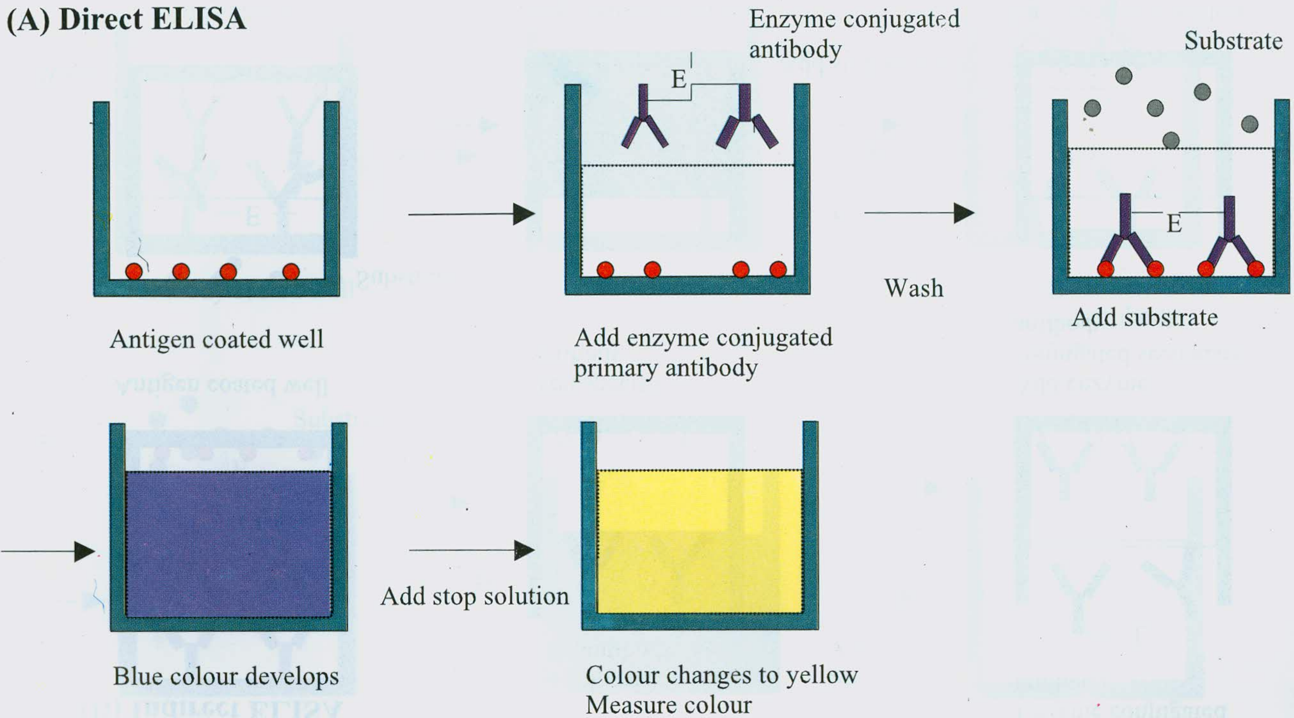
4. Competitive ELISA

In this technique wells are coated with antibodies. Then the antigen and enzyme-conjugated antigen are added to the antibody coated microtitre plate. These two will compete with each other to bind with antibody. The presence of the enzyme-conjugated antigen is detected by chromogenic reaction. (Fig. D)

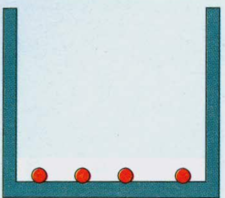
Enzymes and substrates used for labeling:

A number of enzymes have been employed for ELISA for labeling the antigens and antibodies. They are mainly alkaline phosphates, p- nitrophenyl phosphatase and horse radish peroxidase. The substrates p-nitrophenyl phosphate for Phosphatase and Ortho phenylene diamine (OPD) and 3,3',5,5' Teteramethyl benzidine (TMB) are commonly used for Peroxides.

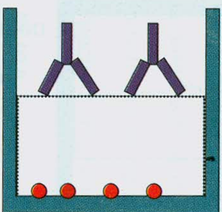
(A) Direct ELISA



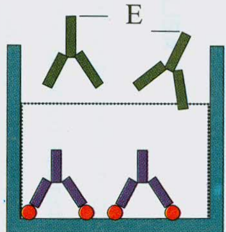
(B) Indirect ELISA


Antigen coated well

Specific antibody


Add specific antibody

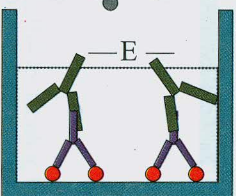
Enzyme conjugated antibody



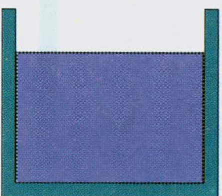
Add enzyme conjugated secondary antibody

Wash

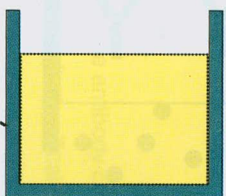
Substrate



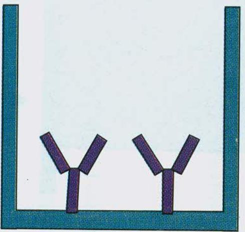
Add substrate


Blue colour develops

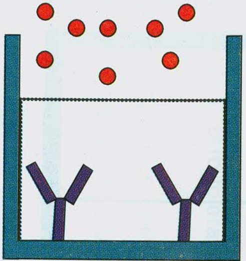
Add stop solution


Colour changes to yellow
Measure colour

(C) Sandwich ELISA

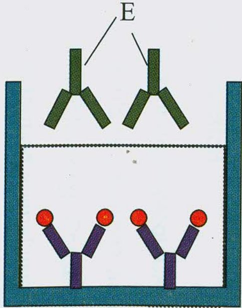


Antibody coated well

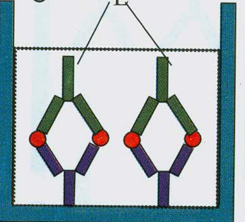


Add antigen

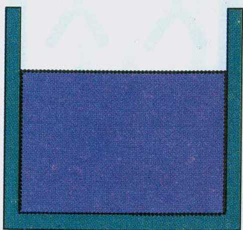
Wash



Add Enzyme conjugated antibody

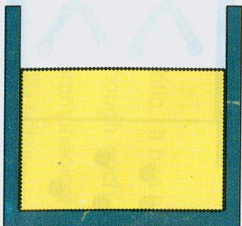


Add Substrate



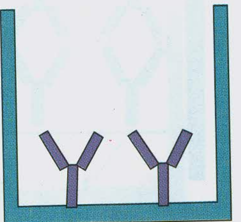
Blue colour develops

Add Stop Solution

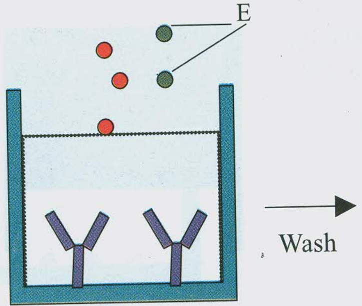


Colour changes to yellow
Measure colour

(D) Competitive ELISA

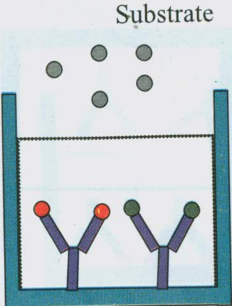


Antibody coated well

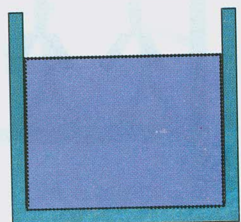


Add antigen & Enzyme congugated antigen

Wash

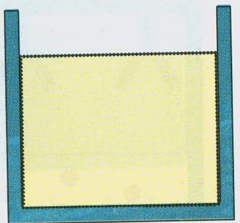


Add substrate



Blue colour develops

Add stop solution



Colour changes to yellow
Measure colour

Chapter-II

ELISA method for estimation of chloramphenicol residues in shrimp tissue

Introduction

Chloramphenicol (CAP) is a broad spectrum antibiotic. It has been used in aquaculture practices as a therapeutic drug, even though such use has no scientific backing. Consequently, small quantities of chloramphenicol are retained in the farmed animal tissues, even if a withdrawal period is followed.

In humans, chloramphenicol causes haematoxic side effects, particularly, the chloramphenicol – induced aplastic anaemia, which is often fatal. For chloramphenicol toxicity, a dosage-effect relationship could not be established so far. So, the chloramphenicol is prohibited for use in animal production. It is notified as a "zero-tolerant substance" in animal products including shrimps.

Chloramphenicol residues are detected and determined by (i) GC-MS (ii) HPLC MS-MS (LC-MS-MS) (iii) Radio-immuno Assay and (iv) ELISA (Enzyme Linked Immuno Sorbant Assay).

The first two methods are advanced instrumental methods requiring very expensive equipments. Radio-immuno assay involves the use of radioactive materials, which are not usually advised in ordinary laboratories. ELISA method is simple and easy to operate. It requires specific ELISA-kits for chloramphenicol, as well as ELISA Washer and ELISA Reader.

Principle

The ELISA method currently used for chloramphenicol is a competitive ELISA. Competitive ELISA can be (1). Direct Competitive ELISA or (2). Indirect Competitive ELISA

(1). Direct Competitive ELISA

The wells of the microtitre plates are precoated with purified rabbit antibody (IgG) specific for chloramphenicol. The test operates on the basis of competition

between chloramphenicol residues in the sample extract and chloramphenicol enzyme conjugate (CAP-conjugate) for the limited number of specific binding sites on the antibody precoated wells.

(2). Indirect Competitive ELISA

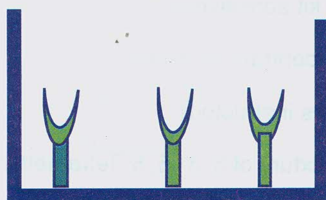
In indirect competitive ELISA, the microtitre plates are coated with purified sheep antibody, directed against rabbit immunoglobulin (IgG), specific for chloramphenicol i.e. against the anti-chloramphenicol antibody. The specific anti-chloramphenicol antibody is added just before the actual ELISA test. The anti-chloramphenicol antibody binds specifically to the precoated sheep antibody, so that, anti-chloramphenicol antibody gets coated on the plate. Now the sample extract and the chloramphenicol enzyme conjugate are added. The rest of the test proceeds as in the case of direct competitive ELSA.

The tests operate on the basis of competition between chloramphenicol residues in the sample extract and chloramphenicol-enzyme conjugate (CAP-conjugate) for the limited number of specific binding sites on the antibody pre-coated well. Usually 'peroxidase' enzyme is used for conjugation (labeling). The presence of the CAP-conjugate is detected by appropriate chemical reaction. A mixture of H_20_2 and 3,3',5,5' Teteramethyl benzidine (TMB), a dye, is added to the well. The peroxidase enzyme decomposes H_20_2. The oxygen released oxidises the TMB, giving rise to a blue colour, which on acidification changes to yellow. The colour is read in an ELISA-reader. The absorbance is inversely proportional to the chloramphenicol concentration in the sample.

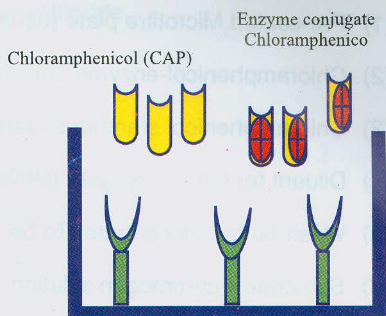
Chloramphenicol ELISA kits

At least, half-a-dozen chloramphenicol ELISA kits are available in the market (List in Appendix-I). Some manufacturers claim that this is a quantitative method for determination of chloramphenicol in tissues, with sensitivity as low as 0.1 ng/g (ie. 0.1 ppb). Others state that this is only semi quantitative and so, all positive results are to be confirmed by GC-MS or LC-MS-MS methods.

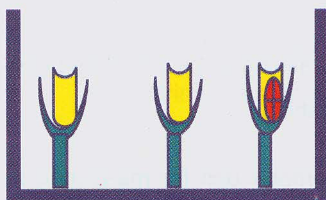
Competitive ELISA for Chloramphenicol



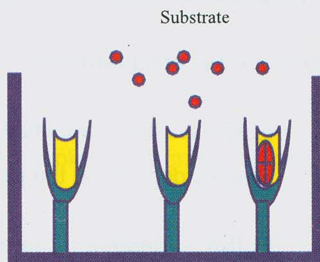
1. Antibody coated well



2. Add CAP and CAP enzyme conjugate

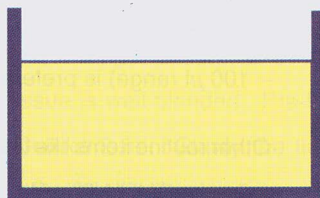


3. CAP and Enzyme conjugate bind with antibody, in competition



4. Add Substrate - TMB & H_2O_2

5. Blue Colour Develops



6. On Acidification colour changes to yellow

Each kit is provided with

(1) Pre-coated Microtitre plate (usually with 8 wells on each strip)

(2) Chloramphenicol-enzyme conjugate (CAP-conjugate)

(3) Chloramphenicol standards (varies with kit sensitivity)

(4) Diluent for CAP-conjugate (either as concentrate or dilute)

(5) Wash-buffer concentrate (To be diluted as instructed)

(6) Substrate – chromogen solution. It is a mixture of 3, 3', 5, 5' Tetramethyl benzidine (TMB) and Hydrogen peroxide (H_2O_2). In some kits, they are supplied separate and so have to be mixed before use as instructed.

Note: Indirect competitive ELISA kits will be provided with anti-chloramphenicol rabbit immunoglobulin also

Other essential requirements

1. ELISA Washer – automatic for uniform washing of the microtitre plates. Results depend on the perfection of washing.

2. ELISA – Reader - It is a spectrophotometer unit for measuring the absorbance and automatic recording/print outs (Filters for 450 nm and 650 nm essential)

Note: ***A combined ELISA Washer and Reader unit is also available***

3. Micropipettes (Fin-pipettes) with disposable tips for 10 microlitre (μl),

4. 100 μl and 1000 μl (1 ml) capacities. An 8-channel micropipette (10 μl – 100 μl range) is preferable.

5. Other routine items like test tubes, measuring cylinder, filter paper, plastic adhesive film (1/2" wide), syringe filters (0.2 μ) etc.

6. 1 Molar Hydrochloric acid AR or 0.2 Molar H_2SO_4 AR.

7. Extraction Buffer

It is phosphate buffer with sodium chloride.

Composition per litre

$Na_2 HPO_4 . 2H_2O$	-	0.96 g
KH_2PO_4	-	0.17 g
NaCl	-	9.00 g
Tween 20	-	0.5 ml
Distilled water	-	1 litre
pH	-	7.2

Alternatively, 0.1 M phosphate buffer, with 0.5M NaCl, pH – 7.2 can also be used.

Extraction of Chloramphenicol

Methods 1 (Neogen method-USA)

1. Thaw shrimp sample completely. A sample of 50 grams of cleaned shrimp is taken.
2. If the shrimp is raw, cook in boiling water for 2 minutes, shell-on.
3. Remove heads, shell and appendages. Remove excess water from the cleaned shrimp with paper towels.
4. Weigh 25 g of the sample
5. Place the weighed sample into a blender. Add extract buffer equal to four times the weight of the sample (100 ml)
6. Blend for 3 minutes at high speed (use a waring blender). Extraction efficiency will be enhanced when the tissue is well blended. Pre-filter through Whatman No.1 filter paper. Collect 2 ml. Clarify the liquid extract by passing through 0.2 μ filter. (Syringe filter)

Note: ***Alternatively, centrifuge the blended tissue for 15 min at 2000 g, collect the supernant. Filter through a syringe filter (0.2 μ).***

Method II (r-biopharm method-EU)

1. Homogenize a reasonable amount (25 g) of shrimps in a waring blender.
2. Weigh 3 g of homogenized shrimp and add 6 ml ethyl acetate
3. Mix intensively for 10 min. (use a Vortex mixer)
4. Centrifuge the suspension: 10 min / 2000 g / room temperature
5. Remove 2 ml of the supernatant (corresponding to 1 g of sample) and reduce to dryness by keeping in a water bath (50°C).
6. Dissolve the residue in 1 ml Isooctane/Chloroform (2:3) mixture
7. Add 1 ml of sample dilution buffer to this solution and mix intensively approx. 1 min on a vortex
8. Centrifuge for separation: 10 min / 3000 g / room temperature
9. Use 50 μl of the aqueous (upper) layer per well in the assay

Test Procedures – Direct Competitive ELISA

(For ELISA kits working on direct competition ELISA principle)

Allow all the reagents to attain room temperature, prior to use.

1. Determine the number of wells to be used. Run standards and sample in duplicate
2. Dilute the chloramphenicol enzyme conjugate (as instructed by the manufacturer). Add 1 μl of enzyme conjugate into 50 μl total volume of EIA buffer for each well assayed (Neogen).

Note: ***For whole kit, add 55 μl of the enzyme conjugate into 2.75 ml total volume of EIA buffer. Mix well.***

3. Add 50μl of standards (S) (provided in the kit) or unknown (U) (sample extract) to the appropriate wells in duplicate

4. Add 50 μl of the diluted enzyme conjugate to each well.

   Steps 3 and 4 should be completed within the least possible time.

5. Mix by shaking plate gently

6. Cover plate with plastic film or plate cover and incubate at room temperature for one hour.

7. Dilute concentrated wash buffer with deionized water (Double distilled water). Mix thoroughly.

8. After incubation, dump out the content of the wells. Wash the wells by filling the wells completely with dilute wash buffer. Dump out the wash solution. Repeat the wash step twice for a total of three washes. Thorough washing is critical for dependable results. Following the final wash, tap out contents thoroughly on a clean lint-free towel.

9. Add 150 μl of substrate to each well

   (If H_2O_2 & TMB are given separately, mix equal quantities for use)

10. Allow to stand at room temperature for 30 minutes.

11. Add 50 μl of 1 M HCl to each well to stop enzyme reaction (or 0.2 M H_2SO_4)

12. Gently shake plate before taking reading to ensure uniform colour throughout each well.

13. Read the plate at 450 nm.

Test Procedure – Indirect Competitive ELISA

(For ELISA kits working on indirect competitive ELISA principle)

1. Remove the required number of strip.

2. Rinsing protocol

   - Fill the wells with 300ml of the rinsing solution
   - Turn the plate upside down and empty the wells of their contents.

- The rinsing cycle should be carried out 3 times.
- Knock the microtitre upside down against a piece of clean adsorbent paper to remove all the liquid.

3. Add 50μl of each standard dilution in duplicate and 50ml of each sample dilution in duplicate
4. Add 100μl of dilution enzyme conjugate to all wells
5. Add 100μl of the diluted chloramphenicol antibody to each well.
6. Shake the plate gently by rotatory motion. Incubate for 1 hr. at ambient temperature.
7. At the end of the incubation period, empty all the wells and wash them three times.
8. Add 150 μl of the solution H_2O_2/TMB to each well. Mix thoroughly and incubate for 30 min. in the dark at ambient temperature.
9. Add 50μl of the stop solution to each well. Mix well and measure the absorbance at 450nm within 30 min.

Interpretation of results

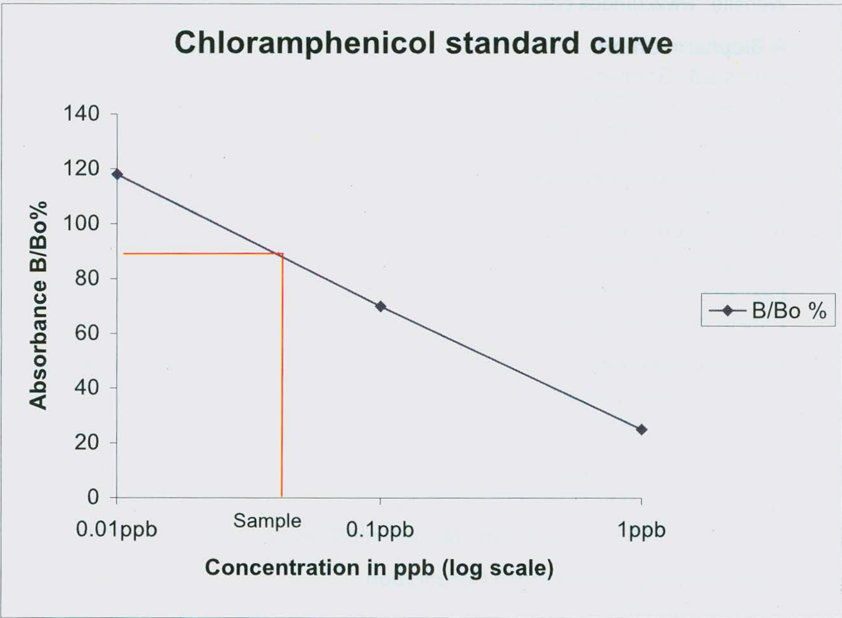
The mean values of the absorbance (at 450 nm) values obtained for the standards and samples are divided by the absorbance value of zero standard (zero chloramphenicol) and multiplied by 100.

$$\frac{\text{Absorbance Standards (or sample)}}{\text{Absorbance (zero standard)}} \times 100 = \text{B/Bo (\%) absorbance}$$

Tabulate the calculated B/Bo % value of each standard and the corresponding chloramphenicol concentration (in ng/g) is plotted on a semi logarithmic graph with concentration on the X -axis and absorbance (B/Bo%) on Y-axis. Draw the standard calibration curve. Take the B/Bo % value of each sample and find out the corresponding concentration of chloramphenicol from the curve. The concentration read from the graph should be further multiplied by the dilution

factor. Here the assumption is that there is 100% recovery of chloramphenicol by the extraction process.

Note: ***In the case of the ELISA kit of the Neogen Corporation, the dilution factor has already been taken care of by the manufacturer. So, the concentration read from the curve directly gives the concentration of chloramphenicol in parts per billion (ppb, ie. nanogram/g (ng/g).***



Appendix

Details of ELISA Kit Manufacturers (for Chloramphenicol detection)

	Manufacturer	Remarks
1.	**Riedel-deHaen** RdH Laborchemikalien GmbH & Co. KG Postfach / P.O. Box 100 262 D-#0918 Seelze Tel.: ++49 (0) 5137 / 8238-0 Fax:++$((0)5137 / 8238-120	Direct Competitive ELISA
2.	**RANDOX Laboratories Ltd.**, Ardmore, Diamond Road, Crumlin, Co. Antrim, United Kingdom, BT29 4QY Tel: Crumlin (028) 94422413 Fax. No.INT.44 (028) 94452912 UK (028) 94452912 Website: www.randox.com	Direct Competitive ELISA
3.	**R-Biopharm GmbH**, Darmstadt, Germany Tel.: +49 (0) 61528102-0 Telefax: +49 (0) 61518102-20	Indirect Competitive ELISA
4.	**Laboratoire d'Hormologie** Rue du Point du Jour, 8 B-6900, MARLORIE (Belgium) Tel:+32 (0) 84310090 Fax: +32 (0) 84316108 E-mail: delahaut.cerdha@skynet.be	Indirect Competitive ELISA
5.	**Neogen Corporation** Corporate Headquarters Food safety Division 620 Lesher Place Lansing MI 48912, USA Tel.: 517/372-9200 Facsimile: 517/372-0108 E-mail: neogen-info@neogen.com	Direct Competitive ELISA
6.	**Indian Distributor for Neogen Corporation** **Tara International** 'Shree Mangal', Office no. 1&2, Plot no. 687-688 T.P.S. III, Ramnagar, Borivali (W), Mumbai-400 092 Tel: 022-861 7001 Fax; 022-861 5319 E-mail: tararp@vsnl.com	