



MULTISCREEN[®] Ag ELISA

Enterotoxemia

ELISA kit for detection of *Clostridium perfringens*
and Alpha, Bêta, Epsilon toxins
Sandwich test for culture supernatants and biological fluids
Diagnostic test for all species

I - INTRODUCTION

Enterotoxaemia is a fatal enteric disease that affects all species of domestic animals and is attributable to a toxigenic type of *Clostridium perfringens*. The latter is an anaerobic, strongly gram-positive bacterium that has the ability to form heat-resistant endospores. This bacterium is grouped into five types (types A, B, C, D and E) according to the four major lethal toxins, Alpha, Beta, Epsilon, and iota (α , β , ϵ , ι) produced.

Clostridium perfringens has been shown to be a cause of human diseases such as gas gangrene (clostridial myonecrosis), food poisoning, necrotising enterocolitis of infants, and enteritis necroticans (pigbel). It is also the causative agent of lamb dysentery, ovine enterotoxaemia (struck) and pulpy kidney disease of sheep, and other enterotoxaemic diseases of lambs and calves. Large amounts of toxin in addition to large numbers of *Clostridium perfringens* cells can usually be detected in the intestinal fluid of the diseased or dead animals.

As *Clostridium perfringens* is a natural commensal of human and animal intestines, identifying of the bacterium is not enough. Toxinotyping and quantifying of the isolated strains are essential.

The Bio-X Enterotoxaemia Elisa Kit can detect the Alpha, Beta and Epsilon toxins of *Clostridium perfringens* and reveal the multiplication of the bacterium.

The kit works with culture supernatants as well as biological probes such as liquid intestinal contents and pericardial- or peritoneal fluid.

II - PRINCIPLE OF THE TEST

Specific monoclonal and polyclonal antibodies produced against Alpha, Beta and Epsilon toxins of *Clostridium perfringens* and a monoclonal antibody specific for a structural protein of this bacterium have been immobilized on alternate rows of 8 x 12 well - microtitre plates. These antibodies allow specifically the capture of the corresponding toxins or bacteria that may be present in the samples (intestinal fluid, culture supernatants, body fluids, etc.). Rows A, C, E, G have been sensitised with these antibodies and rows B, D, F and H are coated with aspecific antibodies as controls.

All samples except culture supernatants are diluted in dilution buffer and incubated on the microplate for 1 hour at 21°C +/- 3°C.

After this first incubation step, the plate is washed, then conjugates - peroxidase- labelled anti-pathogen monoclonal or polyclonal antibodies - are added to the wells. The plate is then re-incubated for 1 hour at 21°C +/- 3°C.

After this second incubation step, the plate is washed again and the chromogen (tetramethylbenzidine TMB) is added. This chromogen has the advantages of being more sensitive than the other peroxidase chromogens and not being carcinogenic.

If specific toxins are present in the tested samples, conjugates remain bound to the corresponding microwells and the enzyme catalyses the transformation of the colourless chromogen into a pigmented compound. The intensity of the resulting blue colour is proportionate to the specific pathogen titre in the sample. The enzymatic reaction can be stopped by acidification and the resulting optical density at 450 nm can be read using a photometer. The signals read for the negative control microwells are subtracted from the corresponding positive microwells.

Control antigen is provided with the kit so as to validate the test results.

TOXINOTYPES

Toxinotypes	Alpha	Beta	Epsilon	Iota
A	++	-	-	-
B	+	++	+	-
C	+	++	-	-
D	+	-	++	-
E	+	-	-	++

III - COMPOSITION OF THE KIT

- **Microplates:** two 96-well microtitration plates (12 strips of 8 wells). Rows A, C, E, G are sensitised by specific antibodies, rows B, D, F, H by aspecific antibodies.

Row A: anti-Alpha-toxin
 Row B: control
 Row C: anti-Beta-toxin
 Row D: control
 Row E: anti-Epsilon-toxin
 Row F: control
 Row G: anti-*Clostridium perfringens*
 Row H: control

- **Washing solution:** One 100-ml bottle of 20x concentrated washing solution. The solution crystallises spontaneously when cold. If only part of the solution is to be used, bring the bottle to 21°C +/- 3°C so that all the crystals disappear. Mix the solution well and remove the necessary volume. Dilute the buffer 20-fold with distilled or demineralised water.
- **Dilution buffer:** One 50-ml bottle of 5x colored, concentrated dilution buffer. Dilute the buffer 5-fold with distilled or demineralised water. If a deposit forms at the bottom of the receptacle filter the solution on Whatman filter paper.
- **Conjugates:** Four 6 ml vial of colored conjugates. These solutions are ready to use. Alpha-toxin (red), Beta-toxin (yellow), Epsilon-toxin (blue) and *Clostridium perfringens* (green).
- **Positive Control:** 1 vial of 4 ml. The reagent is ready to use.
- **Single component TMB:** One 25-ml bottle of chromogen tetramethylbenzidine (TMB). Store at + 2°C and + 8°C protected from light. This solution is ready to use.
- **Stopping solution:** One 15-ml bottle of the 1 M phosphoric acid stop solution.

	BIO K 270/2
Microplates	2
Washing solution	1 X 100 ml (20 X)
Colored Dilution buffer	1 X 50 ml (5 X)
Conjugate	4 X 6 ml (1 X)
Control antigen	1 X 4 ml (1 X)
Single component TMB	1 X 25 ml (1 X)
Stopping solution	1 X 15 ml (1 X)

IV - ADDITIONAL MATERIALS AND EQUIPMENT REQUIRED

Distilled water, graduated cylinders, beakers, plastic tubes, tube rack, dispenser tips, reagent reservoir for multichannel pipettes, lid, adhesive for microplates, graduated automatic (mono- and multichannel) pipettes, microplate reader, and microplate washer and shaker (optional)

V - PRECAUTIONS FOR USE

- This test may be used for “in vitro” diagnosis only. It is strictly for veterinary use.
- The reagents must be kept between +2°C and +8°C. The reagents cannot be guaranteed if the shelf-life dates have expired or if they have not been kept under the conditions described in this insert.
- The concentrated wash solution and dilution buffer may be stored at room temperature. Once diluted, these solutions remain stable for six weeks if kept between +2°C and +8°C.
- Unused strips must be stored immediately in the aluminium envelope, taking care to keep the desiccant dry and the envelope's seal airtight. If these precautions are taken, the strips' activity can be conserved up to the kit's shelf-life date.
- Do not use reagents from other kits.
- The quality of the water used to prepare the various solutions is of the utmost importance. Do not use water that may contain oxidants (e.g., sodium hypochlorite) or heavy metal salts, as these substances can react with the chromogen.
- Discard all solutions contaminated with bacteria or fungi.
- The stop solution contains 1 M phosphoric acid. Handle it carefully.
- All materials and disposable equipment that come in contact with the samples must be considered potentially infectious and be disposed of in compliance with the legislation in force in the country.
- To guarantee the reliability of the results, one must follow the protocol to the letter. Special care must be taken in observing the incubation times and temperatures, as well as measuring the volumes and dilutions accurately.

VI – PROCEDURE

- 1- Bring the kit to 21°C +/- 3°C before use.
- 2- Dilute the concentrated washing solution 20-fold in distilled water. Be sure that all crystals have disappeared before dilution.
Dilute the concentrated dilution buffer 5-fold in distilled water.
Keep these solutions at + 2°C and + 8°C when not in use.
- 3- Dilute the samples volume per volume into dilution buffer prepared as instructed in §2. This is a qualitative dilution only, which must allow the pipetting of biological suspensions. Discard eventual gruds by natural decantation for about 10 minutes. Do not centrifuge the suspensions. Culture supernatants are used undiluted. The best results have been obtained by using liquid TGY under anaerobic conditions (in a tube without shaking) at 37°C. Optimum for Alpha-toxin 4 hours (maximal gaz production). For Epsilon- and Beta-toxin the samples may be cultured for 8 hours or overnight.

Composition of TGY.

- Trypticase (casein tryptic peptone):	30 g
- Yeast extract:	20 g
- Glucose:	1 g
- L-cysteine:	1 g

Dissolve Trypticase and Yeast extract in 950 ml of water and autoclave. Dissolve glucose and L-cysteine in 50 ml of water and sterilise by filtration. Mix the two solutions when the first one is at 21°C +/- 3°C.

- 4- Add 100-µl aliquots of the diluted samples or undiluted supernatants to the wells as follows: sample 1 in wells of column 2, sample 2 in wells of column 3, etc... Add control antigen in wells of column 1.
- 5- Cover with a lid and incubate the plate at 21°C ± 3°C for one hour.
- 6- Rinse the plate with the washing solution prepared as instructed in the section “Composition of the Kit”. To do this, dispose of the microplate's contents by flipping it sharply over a container filled with an inactivating agent. Let the microplate drain upside-down on a sheet of clean absorbent paper so as to eliminate all liquid. Add 300 µl of the washing solution, and then empty the plate once again by flipping it over above the containment vessel. Repeat the entire operation two more times, taking care to avoid the formation of bubbles in the microwells. After the plate has been washed three times go on to the next step.

- 7- Add 100 µl of conjugate solutions per well:
 anti-Alpha-toxin (red) in rows A, B
 anti-Beta-toxin (yellow) in rows C, D
 anti-Epsilon-toxin (blue) in rows E, F
 anti-*Clostridium perfringens* (green) in rows G, H.
 Cover with a lid and incubate the plate at 21°± 3°C for one hour.
- 8- Wash the plate as described in §6 above.
- 9- Add 100 µl of the chromogen solution to each well on the plate. The chromogen solution must be absolutely colourless when it is pipetted into the wells. If a blue colour is visible, this means that the solution in the pipette has been contaminated.
 Incubate at 21°C +/- 3°C and away from light for 10 minutes. Do not cover. This time interval is given as a guideline only, for in some circumstances it may be useful to lengthen or shorten the incubation time.
- 10- Add 50 µl of stop solution per microwell. The blue colour will change into a yellow colour.
- 11- Read the optical densities in the microwells using a plate reader and 450 nm filter. The results must be read fairly soon after the stop solution is added since the chromogen may crystallize in wells with strong signals and thereby distort the results.

VII – INTERPRETING THE RESULTS

Calculate the net optical density for each sample by subtracting from the reading for each sample well the optical density of the corresponding negative control.

Proceed in the same way for the positive control antigen.

The test is validated only if the positive control antigen yields differences in optical density at 10 minutes that are greater than the values given on the QC data sheet.

Divide each resulting value by the corresponding value obtained for the corresponding positive control and multiply this result by 100 to express it as a percentage.

$$\text{Val(ue)} = \frac{\text{Delta OD Sample} * 100}{\text{Delta OD positive}}$$

Using the first table in the quality control procedure, determine each sample's status (positive, negative).

VIII – ORDERING INFORMATION

Multiscreen AgELISA Enterotoxemia

2 X 12 tests

BIO K 270/2

