I - INTRODUCTION

Enterotoxaemia is a fatal enteric disease that affects all species of domestic animals and is attributable to a toxigenic type of \textit{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 ($\alpha, \beta, \epsilon, \iota$) produced. \textit{C. 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 \textit{C. perfringens} cells can usually be detected in the intestinal fluid of the diseased or dead animals. As \textit{C. 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 kit works with culture as well as biological probes such as liquid intestinal contents and pericardial- or peritoneal fluid. Stools could also be used.

The \textit{C. perfringens} ELISA kit contains a monoclonal antibody specific for a constitutional protein of \textit{C. perfringens}. It gives positive signals when quantities of \textit{C. perfringens} in samples is abnormally high.

II - PRINCIPLE OF THE TEST

The test uses 96-well microtitration plates sensitised by specific monoclonal antibodies for \textit{C. perfringens}. These antibodies allow a specific capture of the corresponding bacterium which is present in the samples at a concentration higher than $10^5$. Rows A, C, E, G have been sensitized with these antibodies and rows B, D, F, H are containing non specific antibodies. These control rows allow the differenciation between specific immunological reaction and non specific bindings.

Biological samples are diluted in dilution buffer and incubated on the microplate for 60 minutes at 21°C +/- 3°C. Culture supernatants are used undiluted. After this first incubation step, the plate is washed and incubated for 60 minutes with the conjugate - a peroxidase labelled anti-\textit{C. perfringens} specific monoclonal antibody. After this second incubation, the plate is washed again and the chromogen (tetramethylbenzidine) is added. This chromogen has the advantages of being more sensitive than the other peroxidase chromogens and not being carcinogenic.

If \textit{C. perfringens} is present at a concentration higher than $10^5$ in the tested samples, the conjugate remains 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 titre of \textit{C. perfringens} in the sample. Enzymatic reaction can be stopped by acidification and resulting optical density at 450 nm can be recorded using a photometer. The signals recorded for the negative control microwells are substraction from the corresponding positive microwells. There is a positive antigen supplied with the kit.
TOXINOTYPES

<table>
<thead>
<tr>
<th>Toxinotypes</th>
<th>Alpha</th>
<th>Bêta</th>
<th>Epsilon</th>
<th>Iota</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

III - COMPOSITION OF THE KIT

- **Microplates**: 96-well microtitration plates (12 Strips x 8 wells). Rows A, C, E, G are sensitised by anti-
  *Clostridium perfringens* specific antibodies, while rows B, D, F, H are sensitized by the non specific
  antibodies.
- **Washing solution**: 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 until disappearance of all
  crystals. Mix the solution well and remove the necessary volume. Dilute the buffer 1:20 with distilled or
demineralised water. Store the diluted solution at 4°C.
- **Dilution buffer**: bottle of 5x concentrated buffer for diluting samples and conjugate. Dilute this concentrated
  dilution buffer 1:5 with distilled or demineralised water. Store the diluted solution at 4°C. If a deposit forms
  at the bottom of the container filter the solution on Whatman filter paper.
- **Conjugate**: vial of anti-*Clostridium perfringens*-peroxidase coloured conjugate. **This solution is ready to
  use.**
- **Control antigen**: bottle containing control antigen. This reagent is ready to use.
- **Single component TMB**: bottle of the chromogen tetramethylbenzidine (TMB). Store at 4°C protected from
  light. **This solution is ready to use.**
- **Stopping solution**: bottle of the 1 M phosphoric acid stop solution.

<table>
<thead>
<tr>
<th></th>
<th>BIO K 269/1</th>
<th>BIO K 269/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microplates</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Washing solution</td>
<td>1 X 100 ml (20 X)</td>
<td>1 X 100 ml (20 X)</td>
</tr>
<tr>
<td>Dilution buffer</td>
<td>1 X 50 ml (5 X)</td>
<td>1 X 50 ml (5 X)</td>
</tr>
<tr>
<td>Conjugate</td>
<td>1 X 2 ml (1 X)</td>
<td>1 X 25 ml (1 X)</td>
</tr>
<tr>
<td>Control antigen</td>
<td>1 X 2 ml (1 X)</td>
<td>1 X 4 ml (1 X)</td>
</tr>
<tr>
<td>Single component TMB</td>
<td>1 X 12 ml (1 X)</td>
<td>1 X 25 ml (1 X)</td>
</tr>
<tr>
<td>Stopping solution</td>
<td>1 X 6 ml (1 X)</td>
<td>1 X 15 ml (1 X)</td>
</tr>
</tbody>
</table>

IV - PRECAUTIONS FOR USE

- This test may be used for in vitro diagnosis only.
- The reagents must be kept between 4 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.
- 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.
- Discard all solutions contaminated with bacteria or fungi.
- The stop solution contains 1 M phosphoric acid. Handle carefully.

V – PROCEDURE

1. Bring all the reagents at 21°C +/- 3°C at least half an hour 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 4°C when not used.

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. The samples may be cultured for 8 hours or overnight.

**Composition of TGY:**

- trypticase (casein tryptic peptone): 30 gm
- yeast extract: 20 gm
- glucose: 1 gm
- L-cysteine : 1 gm

- 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 the non diluted supernatants to the wells as follows: sample 1 in wells A1 and B1, sample 2 in wells C1 and D1, etc. Proceed in the same manner for the positive reference (ex.: G1 and H1)

5- Incubate the plate at 21°C +/- 3°C for 1 hour.

6- Rinse the plate with the washing solution, prepared as instructed in §2, as follows: empty the microplate of its contents by flipping it over sharply over a sink. Tap the microplate upside down against a piece of clean absorbent paper to remove all the liquid. Fill all the used wells with the washing solution using a spray bottle or by plunging the plate in a vessel of the right dimensions, then empty the wells once more by turning the plate over above a sink. 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 proceed to the next step.

7- Distribute the conjugate solution at the rate of 100 µl per well. Incubate at 21°C +/- 3°C for 1 hour. Cover the plate with a lid.

8- Wash the plate as described in Step 6.

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 to each well.

11- Read the optical densities by means of a microplate spectrophotometer with a 450 nm filter. The results must be read as quickly as possible after the stop solution has been applied, for in the case of a strong signal the chromogen can crystallise and lead to incorrect measurements

**VI – INTERPRETING THE RESULTS**

Calculate the net optical density of 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 a difference in the optical density at 10 minutes that is greater than the value given on the QC data sheet. Divide the signal read for each sample well by the corresponding positive control signal and multiply this result by 100 to express it as a percentage. Using the first table in the quality control procedure, determine each sample’s status (positive, negative).

\[
\text{Val} = \frac{\Delta \text{DO spl} \times 100}{\Delta \text{DO pos}}
\]

**VII – ORDERING INFORMATION**

BIO-X CLOSTRIDIUM PERFRINGENS ELISA KIT: 1 X 48 tests BIO K 269/1
2 X 48 tests BIO K 269/2