Enterotoxaemia is a fatal enteric disease that affects all species of domestic animals and is attributable to a toxigenic type of Clostridium perfringens. Most animal diseases due to C. perfringens are intestinal and involve types B, C or D. Type A has been implicated in rare outbreaks of gastritis and haemolytic disease of ruminants (enterotoxemic jaundice, the yellows, yellow lamb disease) and in hemorrhagic enteritis in cattle, horses, dogs and infant alpacas. Clostridium perfringens type A causes necrotic enteritis in poultry and a mild form of food poisoning in humans. Demonstration of alpha toxin in the contents of the small intestine is the only way to definitively diagnose enterotoxemia. For that purpose, small amounts of clarified fluid are injected into the tail vein of mice. Death after more than a few minutes post injection constitutes presumptive evidence of enterotoxemia. Other toxins produced by C. perfringens have to be neutralized by specific antisera. By using ELISA method, it is possible to detect alpha toxin in biological fluids (intestinal, peritoneal or pericardic fluid) or in culture supernatants in less than 3 hours. The test can be used to type an unknown strain in conjunction with beta and epsilon Elisa test kits.

### Reliable Results
The use of monoclonal antibody as conjugate ensures excellent specificity and very reliable results.

### Ease-of-Use
- Minimal hands-on-time
- Room temperature incubation
- Results available in 140 minutes for single or batch testing

### Flexibility
Results can be read visually or spectrophotometrically.

<table>
<thead>
<tr>
<th>Toxin-types</th>
<th>Alpha</th>
<th>Beta</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>
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</tr>
<tr>
<td>C</td>
<td>+</td>
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</tr>
<tr>
<td>D</td>
<td>+</td>
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<td>++</td>
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<tr>
<td>E</td>
<td>+</td>
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</tr>
</tbody>
</table>

#### EIA Procedure

1. Microplate coated with polyclonal antibody
2. Add samples and positive control.
   Incubate 1 hour at 21°C +/- 3°C
   Wash
3. Add conjugate.
   Incubate 1 hour at 21°C +/- 3°C
   Wash
4. Add chromogen + Substrate
   Add stop solution.
   Wait 10 minutes.
   Read at 450 nm
Example of results

Direct colony hybridation (DCH)

<table>
<thead>
<tr>
<th></th>
<th>+</th>
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</thead>
<tbody>
<tr>
<td>+</td>
<td>235</td>
<td>0</td>
<td>235</td>
</tr>
<tr>
<td>-</td>
<td>3</td>
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</tr>
</tbody>
</table>

Specificity: NA
Sensitivity: 99 %

Typing of strains from various animal using Bio-X Kits

<table>
<thead>
<tr>
<th>Strains</th>
<th>Bovine</th>
<th>Ovine &amp; Caprine</th>
<th>Ovine &amp; Caprine</th>
<th>Porcine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=69</td>
<td>n=35</td>
<td>n=22</td>
<td>n=45</td>
</tr>
<tr>
<td>Alpha</td>
<td>98.55 %</td>
<td>100 %</td>
<td>100 %</td>
<td>100 %</td>
</tr>
<tr>
<td>Beta</td>
<td>0 %</td>
<td>0 %</td>
<td>18.18 %</td>
<td>11.11 %</td>
</tr>
<tr>
<td>Epsilon</td>
<td>0 %</td>
<td>68.57 %</td>
<td>63.64 %</td>
<td>2.22 %</td>
</tr>
</tbody>
</table>

Composition of the kit

BIO K 084 ALPHA TOXIN ELISA

2 plates of 12 strips for a total of 96 tests
2 vials of positive reference
1 vial of conjugate
1 bottle of washing solution
1 bottle of dilution buffer
1 drop-dispenser bottle of chromogen
1 bottle of substrate solution
1 bottle of stopping solution

1 year of stability at 4°C
II - PRINCIPLE OF THE TEST

The test uses 96-well microtitration plates sensitised by specific antibodies for the alpha-toxin. These antibodies allow a specific capture of the corresponding antigen which is present in the samples. 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 differentiation between specific immunological reaction and non specific bindings. Biological samples (for example: contents of the small intestine, peritoneal fluid...) are diluted in dilution buffer and incubated on the microplate for 60 minutes at room temperature. Culture supernatants are used without dilution.

After this first incubation step, the plate is washed and incubated for 60 minutes with the conjugate - a peroxidase labelled anti-alpha-toxin specific polyclonal antibody. After this second incubation, the plate is washed again and the enzyme substrate (hydrogen peroxide) and the chromogen (tetramethyl benzidine TMB) are added. This chromogen has the advantages of being more sensitive than the other peroxidase chromogens and not being carcinogenic.

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.

The limit of positivity for the antigen is 0.150. Any sample that yields a difference in optical density that is greater than or equal to 0.150 is considered positive. Conversely, any sample that yields a difference in the optical density that is less than 0.150 is considered negative.
If alpha-toxin is present 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 alpha-toxin 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 subtracted from the corresponding positive microwells. There is a positive antigen supplied with the kit.

**TOXINS**

<table>
<thead>
<tr>
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<th>Iota</th>
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</tbody>
</table>

**III - COMPOSITION OF THE KIT**

- **Microplates**: Two 96-well microtitation plates (6 Strips x 16 wells). Rows A, C, E, G are sensitised by anti-alpha-toxin specific antibodies, while rows B, D, F, H are sensitised by the non specific antibodies.
- **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 room temperature 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**: One 50-ml 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**: One bottle of anti-alpha-toxin-peroxidase conjugate. Reagent is liquid. The conjugate will keep at 4°C for one year.
- **Positive reference**: Two bottles containing the alpha-toxin reference antigen. Reconstitute this antigen with 0.5 ml of distilled or demineralised water. The reconstituted reagent must be kept at 4°C.
- **Chromogen solution**: One 10-ml bottle of the chromogen tetramethylbenzidine. Store at 4°C.
- **Substrate solution**: One 30-ml bottle of the hydrogen peroxide substrate solution. Store this reagent at 4°C.
- **Stopping solution**: One 15-ml bottle of the 1 M phosphoric acid stop solution.

**IV - PRECAUTIONS FOR USE**

- This test may be used for in vitro diagnosis only.
- The reagents must be kept between 4 and 8°C. Conjugates must be kept at 4°C. Control antigens must be stored at 4°C for conservation. 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 room temperature 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 sample 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 gas production).

**Composition of TGY:**
- trypticine (casein tryptic peptone): 30 gm
- yeast extract: 20 gm
- glucose: 1 gm
- L-cysteine : 1 gm

Dissolve Trypticine 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 room temperature.

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 room temperature 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. Dilute the conjugate 1:50 with the dilution buffer (for example, for one plate dilute 250 µl of the conjugate stock solution in 12.25 ml of diluent). Add 100 µl of the diluted conjugate solution to each well. Incubate at room temperature for 1 hour.

8. Wash the plate as described in §6 above.

9. Prepare 10 ml of indicator solution extemporaneously as follows: add 12 drops (500µl) of chromogen to 9.5 ml of the substrate solution (enough for 1 plate). Mix thoroughly, then apply to the plate immediately in volumes of 100 µl per microwell. At the time of distribution of the chromogen-substrate mixture on the plates the solution must be completely colourless. If a blue colour appears at this stage, this solution must be discarded and a new one made up using clean glassware and equipment.

10. Incubate for 10 minutes at room temperature. This time is given as a guideline only, for in some circumstances it may be useful to lengthen or shorten the incubation time.

11. Add 50 µl of stop solution per microwell.