INTENDED USE

The **Rabbit Anti-C. perfringens Toxins** (α+β+ε) **IgG** ELISA Kit detects and quantifies IgG directed towards Cp alpha, beta & epsilon toxins in rabbit serum or plasma of exposed or immunized animals. This immunoassay is suitable for:

- Determining immune status relative to non-immune controls;
- Assessing efficacy of vaccines, including dosage, adjuvantcy, route of immunization and timing;
- Qualifying and/or standardizing vaccine batches and protocols.

This kit is for research use only (RUO), not for diagnosis or therapeutic purposes.

INTRODUCTION

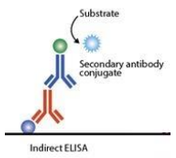
***Clostridium perfringens (Cp)*,** a common anaerobic, rod-shaped Gram-positive bacterium, is the causative agent for food poisoning, gastrointestinal disease, gas gangrene, and other necrotic diseases in mammals, including humans.

This spore-forming bacterium produces 16 different toxins, but only four of them—**alpha, beta, epsilon**, and **iota**—are considered the most important since they are related to the pathogenesis of most of the *C. perfringens*-related diseases. Based on the production profile of these four toxins, *C. perfringens* is classified into five types, from A to E. Thus, **Alpha** toxin, found in group A, causes gas gangrene and hemolysis; **Beta** toxin, found in groups B & C, causes necrosis; **Epsilon** toxin, of groups B & D, cause leaky tissue, mainly in animals and rarely in humans, and is responsible for dysentery and other gut-related conditions; **Iota** toxin is produced solely by group E and can cause tissue death upon infection.

Of these, the **alpha, beta**, and **epsilon** toxins are responsible for causing the most severe *C. perfringens*-related diseases in farm animals. The best way to control these diseases is through vaccination. Commercially available vaccines are based on inactivated toxins and have many production drawbacks, which can be overcome through the use of recombinant antigens.

The ADI **Rabbit Anti-C. perfringens Toxins** (α+β+ε) **IgG** ELISA combines the three recombinant toxins for investigations into the efficacy of these toxin-based vaccines.

PRINCIPLE OF THE TEST



The Rabbit Anti- Cp Toxins IgG ELISA kit is based on the binding of rabbit anti-Cp Toxins IgG in samples to polio immobilized on the microwells, and anti-Cp Toxins IgG antibody is detected by anti-rabbit IgG specific antibody conjugated to HRP (horseradish peroxidase) enzyme. After a washing step, chromogenic substrate (TMB) is added and color is developed by the enzymatic reaction of HRP on the substrate, which is directly proportional to the amount of anti- Cp Toxins IgG present in the sample. Stopping Solution is added to terminate the reaction, and absorbance at 450nm is then measured using an ELISA microwell reader. The activity of rabbit IgG antibody in samples is calculated relative to anti- Cp Toxins calibrators.

KIT CONTENTS

The microtiter well plate and all other reagents, if unopened, are stable at 2-8° C until the expiration date printed on the box label. Stabilities of the working solutions are indicated under Reagent Preparation.

To Be Reconstituted: Store as indicated.

Component	Preparation Instructions
Wash Solution Concentrate (100x) Cat. No. WB-100, 10ml	Dilute the entire volume 10ml + 990ml with distilled or deionized water into a clean stock bottle. Label as **Working Wash Solution** and store at 4° C for long term and RT for short term.
Sample Diluent Concentrate (20x) Cat. No. SD-20T, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample/Conjugate Diluent** and store at 2-8° C until the kit lot expires or is used up.
Anti-Rabbit IgG-HRP Conjugate Concentrate (100x) Part: H-RbG-2a11, 0.15ml	Peroxidase conjugated anti-rabbit IgG in buffer with detergents and antimicrobial. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample/Conjugate Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return 100X to 2-8° C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
Cp Toxins (α,β,ε) Coated Strip Plate	500711	8-well strips (12)	Coated with Cp alpha, beta & epsilon toxin recombinant proteins; post-coated with stabilizers.
Anti- Cp Toxins Calibrators			
1 U/ml 2.5 U/ml 5 U/ml 10 U/ml	500712B 500712C 500712D 500712E	0.65ml 0.65ml 0.65ml 0.65ml	Four (4) vials, each containing anti- Cp Toxins antibodies; in buffer with antimicrobial.
Anti- Cp Toxins Positive Control	500712-PC	0.65ml	Anti- Cp Toxins diluted in buffer with protein, detergents and antimicrobial. [Value range on label]
Low NSB Sample Diluent Reduces non-specific binding	TBTm **Not** for HRP Conjugate dilution	30 ml	Buffer with protein, detergents and antimicrobial. Use as is for sample dilution. See **Assay Design**, page 3.
TMB Substrate	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
Stop Solution	80101	12 ml	Dilute sulfuric acid.

Materials Required But Not Provided:

- Pipettors and pipettes that deliver 100ul and 1-10ml.
- Disposable glass or plastic 5-15ml tubes
- Stock bottle to store diluted Wash Solution; 0.2 to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- ELISA reader at 450 nm and ELISA plate washer

ASSAY DESIGN AND SET-UP

Sample Collection and Handling
Serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference. For **serum**, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage.

Antibody Stability & Dilution
Initial dilution of serum into **Working Sample Diluent** (WSD) is recommended to stabilize antibody activity. This enhances reproducible sampling, and stabilizes the antibody activity for years, stored refrigerated or frozen. Further dilution into **Low NSB Sample Diluent** (LNSD), which provides the lowest assay background, should be at least 5 times the initial dilution and performed the same week as the assay.
Example: Initial (1/5): **10**ul serum + **40**ul WSD [or 0.1ml + 0.4ml]
Further (1/50): **10**ul initial (1/5) + **90**ul LNSD (1/50)

Assay Design
Review Interpretation of Results (p5-7) before proceeding:

- Select the proper sample dilutions accounting for expected potency of positives and minimizing non-specific binding (NSB) and other matrix effects; for example, net signal for non-immune samples should be lower than the **1 U/ml Calibrator**. This is usually 1:200 or greater dilution for rabbit serum with normal levels of IgG and IgM.
- Run a Sample Diluent **Blank**. This signal is an indicator of proper assay performance, especially of washing efficacy, and is used for net OD calculations, if required. Blank OD should be <0.3.
- Run the Anti-Cp Toxins Positive Control; the value range is on the label.
- Run a set of **Calibrators**, which validate that the assay was performed to specifications: **10 U/ml** should give a high signal (>1.5 OD); **1 U/ml** should give a low signal which can be used to discriminate at the Positive/Negative threshold (see Interpretation of Results, p. 5).

Plate Set-up
Bring all reagents to room temperature (18-30° C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 8 Calibrator wells and 2 wells for each sample control to be assayed.
- Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated.
- Add 200-300ul Working Wash Solution to each well and let stand for about 5 minutes. Aspirate or dump the liquid and pat dry on a paper towel before sample addition.

Assay Procedure

ALL STEPS ARE PERFORMED AT ROOM TEMPERATURE. After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

1. 1st Incubation [100ul – 60 min; 4 washes]

- Add 100ul of calibrators, samples and controls each to pre-determined wells.
- Tap the plate gently to mix reagents and incubate for 60 minutes.
- Wash wells 4 times and pat dry on fresh paper towels. As an alternative, an automatic plate washer may be used. Improper washes may lead to falsely elevated signals and poor reproducibility.

2. 2nd Incubation [100ul – 30 min; 5 washes]

- Add 100ul of diluted Anti-Rabbit IgG HRP to each well.
- Incubate for 30 minutes.
- Wash wells 5 times as in step 2.

3. Substrate Incubation [100ul – 15 min]

- Add 100ul TMB Substrate to each well. The liquid in the wells will begin to turn blue.
- Incubate for 15 minutes in the dark, e.g., place in a drawer or closet.

Note: If your microplate reader does not register optical density (OD) above 2.0, incubate for less time, or read OD at 405-410 nm (results are valid).

4. Stop Step [Stop: 100ul]

- Add 100ul of Stop Solution to each well.
- Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.

5. Absorbance Reading

- Use any commercially available microplate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings, and data calculations if available.
- Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.

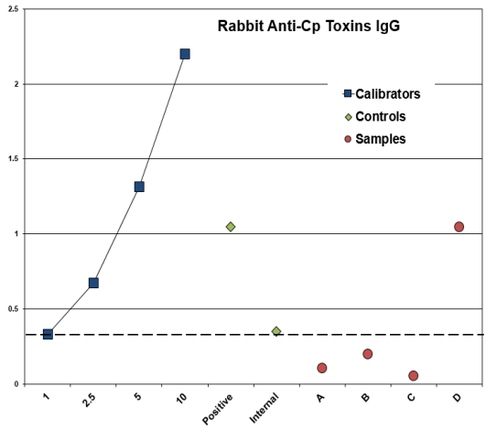
INTERPRETATION OF RESULTS

A. Antibody Activity Threshold Index

Compare Samples to **1 U/ml Calibrator** or **Internal Control**

= Positive/Negative Cut-off.

Example:



Results

The **sensitivity** of the assay to detect anti- Cp Toxins IgG, from either natural infection or vaccination, is controlled so that the **1 U/ml Calibrator** represents a threshold OD for most true positives in rabbit serum diluted to 1:200 or greater. Visual inspection of the data in the above graph shows the following:

Calibrators – dilution curve of antiserum from Cp Toxins immunization, shows the OD range of the assay; high value indicates optimal sensitivity of the assay.
1 U/ml: a 'Cut-off' line has been drawn to indicate a threshold distinguishing between **Positive/Negative**. This is not a clear-cut threshold, rather a low OD area that could represent either low positives or high background negatives.

Positive Control – serum showing reactivity to Cp Toxins; the value range is on the label. This Control may be used to gauge precision and to normalize between-assay variation.

Internal Control – a true positive from an immune rabbit that represents the investigator's experience in distinguishing low positive from negative samples (not in kit). This should be run in each assay to supplement the 1 U/ml Calibrator for Positive/Negative discrimination purposes.

Samples A,B,C,D – 3 samples (1:200) (A, B, C) are negative: below the threshold; 1 sample (D) is positive: clearly above the threshold.

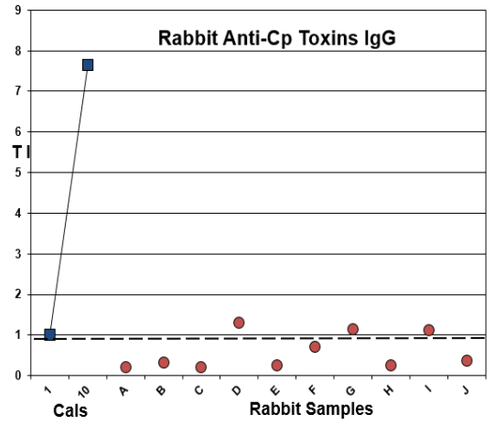
The 1 U/ml Calibrator can be used to calculate a **Threshold Index** that numerically discriminates Positive/Negative:

- Divide each Sample net OD by the 1 U/ml Calibrator net OD. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

INTERPRETATION OF RESULTS (cont)

Example:

Rabbit Serum IgG
A panel of sera from non-immunized rabbits was tested for anti-Cp Toxins IgG (1:200 dilution in Low NSB Sample Diluent). **Threshold Index** was calculated using the **1** U/ml **Calibrator**.



Results

Anti-Cp Toxins IgG:
Six (6) rabbit sera were distinctly negative (below 1.0 TI); 4 sera (D,F,G,I) tested as borderline positive.

Notes:

1. **Positives** may be due to prior encounter with the organism, from exposure to antigen(s) with common epitopes, or from immunization.
2. When the **Positive Index** is **above 5.0,** using a dilution curve to calculate titer is a more accurate quantitation method (see Method C).
3. The **sensitivity** of the assay may be adjusted by changing the sample dilutions: a) increase dilution (e.g., 1:400) to lower the signals of borderline positives to negative; b) decrease dilution (e.g., 1:100) to convert borderline samples to positive. With the latter, the values of negatives may increase, so an alternative threshold should be considered using known negatives to develop a **Positive Index (**see below) or use an **Internal Control** (Page 5).

B. Positive Index
Experimental sample values may be expressed relative to the values of Control or Non-immune samples, by calculation of a **Positive Index**. One typical method is as follows:

1. Calculate the net OD mean + 2 SD of the Control/Non-immune samples = **Positive Index**.
2. Divide each sample net OD by the Positive Index. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

INTERPRETATION OF RESULTS (cont)

A sample value would be **Positive** if significantly above the value of the pre-immune serum sample or a suitably determined non-immune panel or pool of samples, tested at the same sample dilution.

This calculation also **quantifies** the positive Antibody Activity level, assigning a higher value to samples with higher Antibody Activity, and vice versa.

Method C. Titers from Sample Dilution Curves
The titer of elevated antibody activity calculated from a dilution curve of each sample is recommended as the most accurate quantitative method. Best precision can be obtained using the following guidelines:

1. Use an OD value Index in the mid-range of the assay (2.0 – 0.5 OD); this provides the best sensitivity and reproducibility for comparing experimental groups and replicates. An arbitrary 1.0 OD is commonly used.
2. Prepare serial dilutions of each sample to provide a series that will produce signals higher and lower than the selected index. With accurate diluting, duplicates may not be required if at least 4 dilutions are run per sample.
3. A 5-fold dilution scheme is useful to efficiently cover a wide range which produces ODs both above and below 1.0 OD. The dilution scheme can be tightened to 3-fold or 2-fold for more precise comparative data.
4. The Calibrator values can be used to normalize inter-assay values.

Calculations
1. On a log scale of inverse of Sample Dilution as the x-axis, plot the OD values of the two dilutions of each positive sample having ODs above and below the OD value of the Index (arbitrary or selected Calibrator).
2. From a point-to-point line drawn between the two sample ODs, read the dilution value (x-axis) corresponding to the OD of the selected Index
   = **IgG Antibody Activity Units**

PRODUCT SPECIFICATIONS

Specificity
Microwells are coated with equal quantities of the three purified recombinant toxins (E.coli): **alpha** – accession# ACF40003.1 (29-398); **beta** – accession# Q46308 (28-336); **Epsilon** – accession# AAA23235 (29-398).

Assay Sensitivity
The Cp Toxins coating level, HRP conjugate concentration and Low NSB Sample Diluent are optimized to differentiate anti- Cp Toxins IgG from background (non-antibody) signal with rabbit serum samples diluted 1:200.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Calibrators, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains dilute sulfuric acid. Follow good laboratory practices, and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. All reagents may be disposed of down a drain with copious amounts of water. MSDS for TMB, sulfuric acid and BND can be requested or obtained from the ADI website: http://4adi.com/commerce/info/showpage.jsp?page_id=1060&category_id=2430&visit=10

Instruction Manual No. M-RB-500700-1

Rabbit Anti-*C. perfringens* Toxins (alpha+beta+epsilon) IgG ELISA Kit

Cat. RB-500700-1, 96 tests

For Quantitation of Anti-*Clostridium perfringens* Alpha, Beta & Epsilon Toxins IgG in Serum, Plasma, Biological Fluids

For research use only, not for diagnostic or therapeutic use.

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ELISA Kit Components	Amount	Part #
Cp Toxins (α,β,ε) Coated Strip Plate	8-well strips (12)	500711
Anti-Cp Toxins Positive Control	0.65 ml	500712PC
Anti-Cp Toxins Calibrator 1 U/ml	0.65 ml	500712B
Anti-Cp Toxins Calibrator 2.5 U/ml	0.65 ml	500712C
Anti-Cp Toxins Calibrator 5 U/ml	0.65 ml	500712D
Anti-Cp Toxins Calibrator 10 U/ml	0.65 ml	500712E
Anti-Rabbit IgG HRP Conjugate (100X)	0.15 ml	H-RbG-2a11
Sample Diluent (20x)	10 ml	SD20T
Low NSB Sample Diluent	30 ml	TBTm
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	M-RB-500700-1