Creative Diagnostics®

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User's Manual

Human Anti-Cholera Toxin B IgG ELISA kit

REF **DEIA-JY24088**

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RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

The Human Anti-Cholera Toxin B Subunit IgG ELISA Kit detects and quantifies anti-Cholera Toxin B IgG in human serum or plasma of exposed or immunized individuals. This immunoassay is suitable for:

o Determining immune status relative to non-immune controls;

o Assessing efficacy of vaccines, including dosage, adjuvantcy, route of immunization and timing;

o Qualifying and/or standardizing vaccine batches and protocols.

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

General Description

Cholera toxin (sometimes abbreviated to CTX, Ctx, or CT) is a protein complex secreted by the bacterium Vibrio cholerae. CTX is responsible for the harmful effects of cholera infection. The cholera toxin is an oligomeric complex made up of six protein subunits: a single copy of the A subunit and five copies of the B subunit. The two parts are connected by a disulfide bond. The five B subunits, each weighing 12 kDa, form a five-membered ring. The A subunit has two important segments. The A1 portion of the chain (CTA1) is a globular enzyme payload that ADPribosylates G proteins, while the A2 chain (CTA2) forms an extendedm alpha helix which seats snugly in the central pore of the B subunit ring. Once secreted, the B subunit ring of CTX will bind to GM1 gangliosides on the surface of the host's cells. After binding takes place, the entire CTX complex is internalized by the cell and the CTA1 chain is released. CTA1 catalyzes ADP ribosylation leading to the activation of adenylate cyclase. Increased adenylate cyclase activity increases cAMP synthesis causing massive fluid and electrolyte efflux resulting in diarrhea. Neutralizing antibodies against subunit B can prevent the binding of subunit B to epithelial cells preventing the release of Sub A. Currently, recombinantly produced CTB and killed V. cholerae (Serotypes O1 and 0139) are used as a component of internationally licensed oral cholera vaccines (Dukoral® SBL Vaccin AB and Shanchol® Shantha Biotechnics Limited), as the protein induces potent humoral immunity that can neutralize CT in the gut. Additionally, recent studies have revealed that CTB administration leads to the induction of antiinflammatory mechanisms in vivo.

Principles of Testing

The Human Anti- Cholera Toxin B IgG ELISA kit is based on the binding of human anti- Cholera Toxin B IgG in samples to Cholera Toxin B immobilized on the microwells, and anti- Cholera Toxin B IgG antibody is detected by anti-human 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- Cholera Toxin B 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 human IgG antibody in samples is calculated relative to antiCholera Toxin B calibrators.

Reagents And Materials Provided

1. Wash Solution Concentrate (100×): 10ml, Dilute the entire volume 10 ml + 990 ml 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.
2. Sample Diluent Concentrate (20×): 10ml, Dilute the entire volume, 10 ml + 190 ml 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.
3. Anti-Human IgG HRP Conjugate Concentrate (100×): 0.15ml. Peroxidase conjugated anti-human 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 100× to 2-8°C storage.

Ready For Use

4. Cholera Toxin B Subunit Coated Strip Plate: Coated with Cholera Toxin B proteins; postcoated with stabilizers.
5. Anti- Cholera Toxin B Calibrators: 1 U/ml, 2.5 U/ml, 5 U/ml and /10 U/ml. 0.65ml/each. Four (4) vials, each containing antiCholera Toxin B antibodies; in buffer with antimicrobial.
6. Anti-Cholera Toxin B Positive Control: 0.65 ml. Anti- Cholera Toxin B diluted in buffer with protein, detergents and antimicrobial. [Value range on label].
7. Low NSB Sample Diluent: 30 ml. TBTm. Not for HRP Conjugate dilution. Buffer with protein, detergents and antimicrobial. Use as is for sample dilution.
8. TMB Substrate: 12 ml, Chromogenic substrate for HRP containing TMB and peroxide.
9. Stop Solution: 12 ml. Dilute sulfuric acid.

Materials Required But Not Supplied

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

Storage

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

Specimen Collection And Preparation

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 µl serum + 40 µl WSD [or 0.1 ml + 0.4 ml]

Further (1/50): 10 µl initial (1/5) + 90 µl LNSD (1/50)

Assay Design

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 1:100 or greater dilution for human 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-Cholera Toxin B 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).

Plate Preparation

Plate Preparation

1. Bring all reagents to room temperature (18-30º C) equilibration (at least 30 minutes).
2. 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.
3. Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated.
4. Add 200-300ul Working Wash Solution to each well and let stand for about 1-5 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 piate to mix the well contents prior to beginning incubaton.

1. 1st Incubation [100µl-60 min; 4 washes]

Add 100µl of calibrators, samples and controls each to pre-detemined 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 [100µl-30 min; 5washes]

Add 100µl of diluted Anti-Human IgG HRP to each well.

Incubate for 30 minutes.

Wash wells 5 times as in step 1.

3. Substrate Incubation [100µl-15 min]

Add 100µl 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 405-410nm (results are valid).

4. Stop Step [Stop: 100µl]

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.

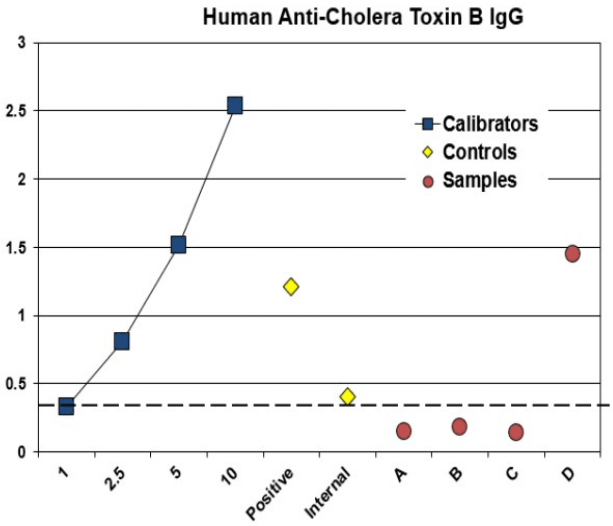
Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.

Interpretation Of Results

A. Antibody Activity Threshold Index

Compare Samples to 1 U/ml Calibrator or Internal Control = Positive/Negative Cut-off.

Example 1:



Results:

The sensitivity of the assay to detect anti- Cholera Toxin B 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 human serum

diluted to 1:100 or greater. Visual inspection of the data in the above graph shows the following:

Calibrators – dilution curve of antiserum from Cholera Toxin B 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 Cholera Toxin B; 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 human 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 (1:100) (A, B, C) are negative: below the threshold; 1 sample (D) is positive: clearly above the threshold.

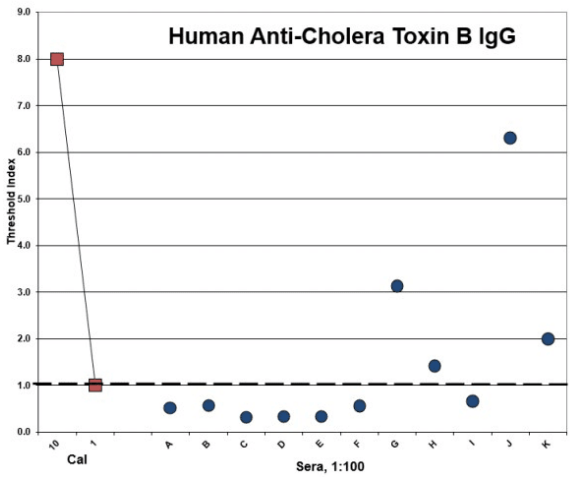
The 1 U/ml Calibrator can be used to calculate a Threshold Index that numerically discriminates Positive/Negative:

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.

Example 2:

Human Serum IgG

A panel of sera from humans of unknown history was tested for anti- Cholera Toxin B IgG (1:100 dilution in Low NSB Sample Diluent). Threshold Index was calculated using the 1 U/ml Calibrator.



Results

Anti-Cholera Toxin B IgG:

Seven human sera were negative (below 1.0 TI); three sera (G,J,K) were positive; one serum (H) was borderline positive.

Notes:

1. Positives may be due to prior encounter with the organism, from exposure to an antigen 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., 1:200) to lower the signals of borderline positives to negative; b) decrease dilution (e.g., 1:50) 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.

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/Nonimmune 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.

A sample value would be Positive if significantly above the value of the pre-immune serum sample or a suitably determined nonimmune 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

Performance Characteristics

Specificity

Purified cholera toxin B subunit from Vibrio Cholerae is used to coat the microwells; thus the assay is specific for antibodies directed to cholera toxin B. The Anti-Human IgG-HRP conjugate reacts specifically with human

IgG class antibodies; IgA, IgM and IgE antibody would not be measured above background signals.

Assay Sensitivity

The Cholera Toxin B coating level, HRP conjugate concentration and Low NSB Sample Diluent are optimized to differentiate antiCholera Toxin B IgG from background (non-antibody) signal with human serum samples diluted 1:100.

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Precautions

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.