## INTENDED USE

The Rat Anti- Tetanus Toxoid IgG ELISA Kit detects and quantifies tetanus toxin/toxoid--specific IgG in rat serum or plasma of vaccinated or immunized hosts. This immunoassay is suitable for:

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

This assay is for research use only (RUO), not for diagnostic use.

GENERAL INFORMATION

Tetanus Bacteria emitting toxin → Tetanus Toxin → modified → Tetanus Toxoid
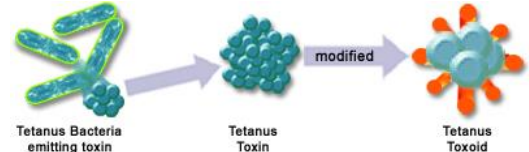
T etanus, also called lockjaw, is a medical condition characterized by a prolonged contraction of skeletal muscle fibers. The primary symptoms are caused by tetanospasmin (also known as **tetanus toxin**), a neurotoxin produced by the Gram-positive, obligate anaerobic bacterium *Clostridium tetani*. Infection generally occurs through wound contamination and often involves a cut or deep puncture wound that produces an anaerobic environment. As the infection progresses, muscle spasms develop in the jaw (thus the name "lockjaw") and elsewhere in the body.

Several tetanus vaccines are available, as single antigen or as multivalent with antigens from other disease-causing microbes. Monitoring the efficacy of vaccines by determining the anti-Tetanus levels in patients, including for clinical trials using new formulation of vaccines, is often required. The ADI Anti- Tetanus Toxoid ELISAs will quantify antibodies produced by vaccines as well as from infection with the toxoid-producing organisms.

PRINCIPLE OF THE TEST

The Rat Anti-Tetanus Toxoid IgG ELISA kit is based on the binding of antibodies in samples to tetanus toxoid immobilized on the microwells, and anti-tetanus toxoid IgG antibody is detected by anti-rat IgG-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-tetanus toxoid 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 rat IgG antibody in samples is calculated relative to anti-tetanus toxoid calibrators.

PRODUCT SPECIFICATIONS

Specificity

Purified tetanus toxoid is used to coat the microwells; thus the assay is specific for antibodies directed to tetanus toxoid or toxin. The anti-rat IgG HRP conjugate reacts with rat IgG antibodies that bind to tetanus toxoid on the plate. IgA, IgM and IgE class antibodies would not be measured above background signals.

KIT CONTENTS

The microtiter well plate and all other reagents, if unopened, are stable at 2-8º 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 4º 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 2-8º C until the kit lot expires or is used up.
Anti-Rat IgG- HRP Conjugate Concentrate (100x) Part: H-RtG.211, 0.15ml	Peroxidase conjugated anti-rat 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 2-8º C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
Tetanus Toxoid Coated Strip Plate	930-111	8-well strips (12)	Coated with tetanus toxoid, and post-coated with stabilizers.
Anti- Tetanus Toxoid IgG Calibrators			
10 U/ml 25 U/ml 50 U/ml 100 U/ml	930212B 930212C 930212D 930212E	0.65ml 0.65ml 0.65ml 0.65ml	Four (4) vials, each containing anti-tetanus toxoid; in buffer with antimicrobial.
Anti-Tetanus Toxoid IgG Positive Control	930-212-PC	0.65ml	Anti-tetanus toxoid; diluted in buffer with protein, detergents and antimicrobial. Net OD > 0.5
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 10 times the initial dilution and performed the same day 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 **10 U/ml Calibrator**. This is usually 1/100 or greater dilution for rat serum with normal levels of IgG and IgM.

- Run the Anti-Tetanus Toxoid IgG Positive Control; net OD > **0.5**.

- 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 a set of **Calibrators**, which validate that the assay was performed to specifications: **100 U/ml** should give a high signal (>1.5 OD); **10 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 (18-30º 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]

o   Add 100 ul of diluent (blank), calibrators, samples and controls each to pre-determined wells.
o   Tap the plate gently to mix reagents and incubate for 60 minutes.
o   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]

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

3.   **Substrate Incubation**   [100ul – 15 min]

o   Add 100ul TMB Substrate to each well. The liquid in the wells will begin to turn blue.
o   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]

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

5.   **Absorbance Reading**

o   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.
o   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.
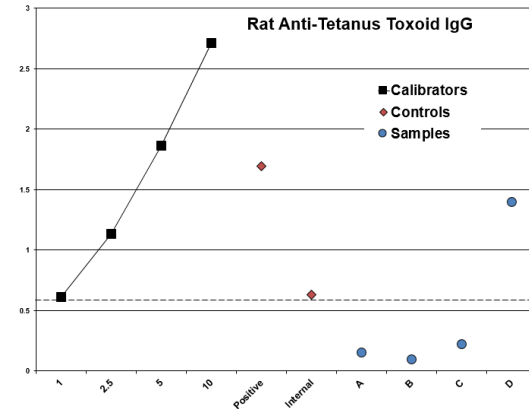
A. Antibody Activity Threshold Index

Compare Samples to **10 U/ml Calibrator** or **Internal Control**

= **Positive/Negative Cut-off**.

Example:

Rat Anti-Tetanus Toxoid IgG

- Calibrators
- Controls
- Samples

Results

The **sensitivity** of the assay to detect anti-Tetanus Toxoid IgG, from either natural infection or vaccination, is controlled so that the **10 U/ml Calibrator** represents a threshold OD for most true positives in rat 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 tetanus toxoid immunization, shows the OD range of the assay; high value indicates optimal sensitivity of the assay.
10 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 – antiserum showing reactivity to tetanus toxoid; net OD > **0.5**. This Control can be used to normalize between-assay variation.

Internal Control – a true low positive from an immune animal that represents the lab's experience in distinguishing low positive from negative samples (not included in the kit). This should be run in each assay to supplement the 10 U/ml Calibrator for Positive/Negative discrimination purposes.

Samples A,B,C,D – 3 samples (1:100) (A, B, C) are <u>negative</u>: below the threshold; 1 sample (D) is <u>positive</u>: clearly above the threshold.

The 10 U/ml Calibrator can be used to calculate a **Threshold Index** that numerically discriminates Positive/Negative:

❖ Divide each Sample net OD by the 10 U/ml Calibrator net OD. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

Example:

Rat Serum IgG

A panel of sera from laboratory rats, was tested for anti-tetanus toxoid IgG (1:100 dilution in Low NSB Sample Diluent). **Threshold Index** was calculated using the **10** U/ml **Cal**.

Rat Anti-Tetanus Toxoid IgG

- Calibrators
- Rat Serum
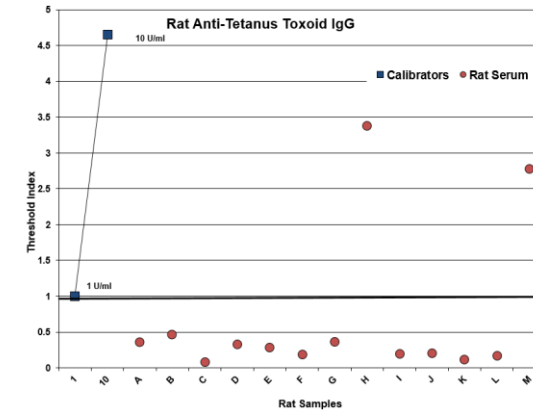
Results

Rat Anti-Tetanus Toxoid IgG: two (2) of the sera were positive (Threshold Index >1.0) at 1:100 dilution; the remaining 11 sera were negative (below the threshold).

Notes:
1. **Positives** may be due to prior infection and/or vaccination with *C. tetani* or tetanus toxin/toxoid.

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** (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.

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 Positive and Sensitivity Control values can be used to normalize inter-assay values.

<u>Calculations</u>

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**

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

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Instruction Manual No. M-930-150-TMG

Rat Anti-Tetanus Toxoid IgG ELISA Kit

Cat. No. 930-150-TMG, 96 Tests

For Quantitation of Anti-Tetanus Toxoid IgG in Serum/Plasma

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

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ALPHA DIAGNOSTIC
I N T E R N A T I O N A L

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ELISA Kit Components	Amount	Part #
Tetanus Toxoid Coated Strip Plate	8-well strips (12)	930-111
Anti-Tetanus IgG Positive Control	0.65 ml	930-212-PC
Anti-Tetanus Calibrator 10 U/ml	0.65 ml	930-212B
Anti-Tetanus Calibrator 25 U/ml	0.65 ml	930-212C
Anti-Tetanus Calibrator 50 U/ml	0.65 ml	930-212D
Anti-Tetanus Calibrator 100 U/ml	0.65 ml	930-212E
Anti-Rat IgG HRP Conjugate (100X)	0.15 ml	H-RtG.211
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-930-150-TMG