Enzyme Immunoassay Kit

Anti-Tetanus Toxoid IgG

Cat # 8900Z

For in-vitro diagnostic use

INTENDED USE
Diagnostic Automation’s Anti-Tetanus is intended for the in-vitro measurement of specific IgG antibodies against tetanus toxoid (T.Tox.) present in serum, in order to determine protective status.

Sufficient materials are supplied to allow a maximum of 39 samples to be tested in duplicate or 87 in single, with a seven point calibration curve and two controls.

The seven point calibration curve can be reduced to five points if measurement below 0.09 IU/mL is not required.

BACKGROUND
Anti-tetanus toxoid antibodies are raised in response to vaccination with tetanus toxoid protein. A patient’s response to the immunisation may be assessed, subsequently, by the serological determination of their anti-tetanus toxoid antibody levels using this quantitative enzyme immunoassay technique. Patients with recurrent infections should be investigated for immunodeficiency due to thymic abnormalities, and the consequent inability to respond to specific bacterial antigens (review 1).

PRINCIPLE OF THE ASSAY
Microwells are pre-coated with tetanus toxoid antigen. The calibrators, controls and diluted patient samples are added to the wells and antibodies recognising the tetanus toxoid antigen bind during the first incubation. After washing the wells to remove all unbound proteins, purified peroxidase labelled rabbit anti-human IgG (γ chain specific) conjugate is added. The conjugate binds to the captured human antibody and the excess unbound conjugate is removed by a further wash step. The bound conjugate is visualised with 3,3',5,5' tetramethylbenzidine (TMB) substrate which gives a blue reaction product, the intensity of which is proportional
to the concentration of antibody in the sample. Phosphoric acid is added to each well to stop the reaction. This produces a yellow end point colour, which is read at 450nm.

**PRECAUTIONS**

**WARNING**

- All human sera supplied have been tested at donor level and found negative for Hepatitis B surface antigens and antibodies to HIV 1 and 2 and Hepatitis C virus. However, these tests cannot guarantee the absence of infectious agents.
- Proper handling and disposal methods should be established and only personnel qualified in handling of potentially infectious materials should be permitted to use this kit.
- Sodium azide may react with lead and copper plumbing to form explosive metal azides. On disposal of reagents flush with a large volume of water, to prevent azide build-up.
- The buffers and serum supplied in this kit contain various enzyme inhibitors as listed below. These are toxic and should be handled with care.

<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>CONCENTRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kathon</td>
<td>0.02%</td>
</tr>
<tr>
<td>Sodium Azide</td>
<td>0.099%</td>
</tr>
<tr>
<td>Proclin 300</td>
<td>0.045%</td>
</tr>
<tr>
<td>Bromonitrodioxane</td>
<td>0.002%</td>
</tr>
<tr>
<td>Methylisothiazzone</td>
<td>0.002%</td>
</tr>
</tbody>
</table>

- Kathon is an irritant and may cause sensitisation by skin contact.
- The stop solution contains 3M phosphoric acid, which is corrosive. To avoid burns do not allow contact with skin or eyes.
- Reagent spills should be cleaned up appropriately, observing local and environmental regulations.

**CAUTION**

- This product should only be used by appropriately trained personnel.
- Strict adherence to the protocol is recommended. Any deviation may affect assay performance, and the results obtained. Pay attention to specific ‘Notes’ and warnings throughout these Instructions for Use.
- Reagents from different batch numbers of kits are **NOT** interchangeable. If large numbers of tests are performed care should be taken to ensure that all reagents are from the **SAME** batch. All strips used must be taken from the same foil pouch. Substitution of any component may lead to incorrect results.
- To avoid reagent contamination, only use new or clean plastic / glassware. **Never** return unused reagents to the bottles.
- Do not leave reagent bottles uncapped; any resulting evaporation or contamination will lead to inconsistent results.
- TMB substrate must not be exposed to light or water.
- Microbially contaminated, haemolysed or lipaemic serum and specimens containing particulate matter should not be used.
- Inaccurate sample dilution cannot be checked, as kit controls are ready to use. The use of calibrated pipettes and appropriate internal QC samples is recommended.
- The use of automated assay systems, sample dilutors and other automated equipment may lead to differences in results when compared to the manual procedure. It is the responsibility of any laboratory to fully validate the system, and ensure the results fall within the limits as defined in this insert and associated QC certificate.
- All equipment used must be calibrated and maintained according to the manufacturer’s instruction.
STORAGE AND STABILITY
• The kit should be stored at 2-8°C and should not be frozen. Inappropriate storage temperatures will affect the results.
• Wash buffer diluted into a clean container can be stored at room temperature for a maximum of 4 weeks.
• The expiry date of the kit is shown on the outer label.

SAMPLE COLLECTION AND STORAGE
• Blood samples should be collected by venepuncture allowed to clot naturally and the serum separated.
• The serum may be stored at 2-8°C for up to 48 hours prior to assay, or for prolonged storage kept undiluted at -20°C or below.
• Repeated thawing and freezing should be avoided.
• Serum samples should not be heat-inactivated, as this may give false positive results.

MATERIALS
MATERIALS SUPPLIED
• Instruction Leaflet: Giving full assay details.
• QC Certificate: Indicating the expected performance of the batch.
• T. Tox. Coated Wells: 12 breakapart 8 well strips coated with tetanus toxoid derived from the toxin of Clostridium tetani. The plate is packaged in a re-sealable foil bag containing two desiccant pouches.
• Sample Diluent: 2 bottles containing 50mL of buffer for sample dilution. Coloured yellow, ready to use.
• Wash Buffer: 1 bottle containing 50mL of a 20-fold concentrated buffer for washing the wells.
• T. Tox. IgG Calibrators: 7 bottles each containing 1.2mL of diluted human serum, with the following concentrations of anti-tetanus toxoid antibody: 7, 2.33, 0.78, 0.26, 0.09, 0.03, 0.01 IU/mL. Ready to use.
• T. Tox. IgG High Control: 1 bottle containing 1.2mL of diluted human serum. The expected value is given on the QC certificate. Ready to use.
• T. Tox. IgG Low Control: 1 bottle containing 1.2mL of diluted human serum. The expected value is given on the QC certificate. Ready to use.
• T. Tox IgG Conjugate: 1 bottle containing 12mL of purified peroxidase labelled antibody to human IgG. Coloured red, ready to use.
• TMB Substrate: 1 bottle containing 14mL TMB substrate. Ready to use.
• Stop Solution: 1 bottle containing 14mL of 3M phosphoric acid. Ready to use.

ADDITIONAL MATERIALS AND EQUIPMENT - not supplied
• Automatic microplate plate washer: This is recommended, however, plate washing can be performed manually.
• Plate reader: Capable of measuring optical densities at 450nm referenced on air.
• Distilled or deionised water: This should be of the highest quality available.
• Calibrated micropipettes: For dispensing 1000, 100 & 10μL.
• Multichannel pipette: Recommended for dispensing 100μL volumes of conjugate, substrate and stop solution.
• Glass/plastic tubes: For sample dilution.

PRE-ASSAY STEPS
1. Bring the kit to room temperature
• The kit is designed for room temperature operation (20-24°C).
• Remove the kit from storage and stand at room temperature for approximately 60 minutes. Wells must not be removed from the foil bag until they have reached room temperature.
Note: The kits may be maintained at room temperature for up to 1 week.

2. **Kit components**
   Gently mix each kit component before use.

3. **Wash buffer dilution**
   Add 50mL of the wash buffer concentrate to 950mL of distilled water (1 in 20 dilution) into a clean container and mix. Smaller volumes can be diluted as appropriate.
   Note: Diluted wash buffer can be stored at room temperature for up to 4 weeks, therefore only dilute the appropriate amount.

4. **Sample dilution**
   Dilute 10µL of each sample with 1000µL of sample diluent (1:100) and mix well.
   Note: Diluted sample must be used within 8 hours.

5. **Strip and frame handling**
   Place the required number of wells in the strip holder. Position from well A1, filling columns from left to right across the plate. When handling the plate, squeeze the long edges of the frame to prevent the wells falling out.
   Note: Return unused wells to the foil bag immediately with the two desiccant pouches and re-seal tightly to minimise exposure to moisture. Take care not to puncture or tear the foil bag, see below.
   WARNING: Exposure of wells to moisture or contamination by dust or other particulate matter will result in antigen degradation, leading to poor assay precision and potentially false results.

**ASSAY METHOD**

Maintain the same dispensing sequence throughout the assay.

1. **Sample addition**
   Dispense 100µL of each calibrator, control and diluted (1:100) sample into the appropriate wells of the plate provided.
   Note: Samples should be added as quickly as possible to the plate to minimise assay drift, and the timer started after the addition of the last sample.
   **Incubate at room temperature for 30 minutes.**

2. **Washing**
   The washing procedure is critical and requires special attention. An improperly washed plate will give inaccurate results, with poor precision and high backgrounds.
   After incubation remove the plate and wash wells 3 times with 250-350µL wash buffer per well. Wash the plate either by using an automatic plate washer or manually as indicated below. After the final automated wash, invert the plate and tap the wells dry on absorbent paper.

   **Plates can be washed manually as follows:**
   a. Flick out the contents of the plate into a sink,
   b. Tap the wells dry on absorbent paper,
   c. Fill each well with 250-350µL of wash buffer using a multichannel pipette,
   d. Gently shake the plate on a flat surface,
   e. Repeat a-d twice,
   f. Repeat a and b.
3. **Conjugate addition**
   Dispense 100µL of conjugate into each well, blot the top of the wells with a tissue to remove any splashes.
   Note: To avoid contamination, never return excess conjugate to the reagent bottle.
   Incubate at room temperature for 30 minutes.

4. **Washing**
   Repeat step 2.

5. **Substrate (TMB) addition**
   Dispense 100µL of TMB substrate into each well, blot the top of the wells with a tissue to remove any splashes.
   Note: To avoid contamination never return excess TMB to the reagent bottle.
   Incubate at room temperature in the dark for 30 minutes.

6. **Stopping**
   Dispense 100µL of stop solution into each well. This causes a change in colour from blue to yellow.

7. **Optical density measurement**
   Read the optical density (OD) of each well at 450nm on a microplate reader, within 30 minutes of stopping the reaction.

**RESULTS AND QUALITY CONTROL**

1. **Quality control**
   In order for an assay to be valid, all the following criteria must be met:
   - Calibrators and controls must be included in each run.
   - The value obtained for each control should be in the range specified on the QC Certificate.
   - The curve shape should be similar to the calibration curve, shown on the QC Certificate.
   If the above criteria are not met, the assay is invalid and the test should be repeated.

2. **Calculate mean optical densities** (For assays run in duplicate only)
   For each calibrator, control and sample calculate the mean OD of the duplicate readings. The percentage coefficient of variation (%C.V.) for each duplicate OD should be less than 15%.

3. **Plot calibration curve**
   The calibration curve can be plotted either automatically or manually as follows by plotting the anti-tetanus toxoid antibody concentration on the log scale against the OD on the linear scale for each calibrator:
   - Automatic - use appropriately validated software, and the curve fit that best fits the data.
   - Manual - using log/linear graph paper, draw a smooth curve through the points (not a straight line or point to point).

4. **Treatment of anomalous points**
   If any one point does not lie on the curve, it can be removed provided that the overall expected curve shape is maintained (this is normally done automatically when using curve fitting software). If the absence of this point means that the curve has a shape dissimilar to that of the sample calibration curve, or more than one point appears to be anomalous, then the assay should be repeated.

5. **Calculation of the control value**
   Read the level of the anti-tetanus toxoid antibody in the controls directly from the calibration curve. The value should fall within the range given on the QC Certificate.
6. **Calculation of antibody levels in diluted samples**
   Read the level of the anti-tetanus toxoid antibody in the diluted samples directly from the calibration curve.
   **Note:** The calibrator values have been adjusted by a factor of 100 to account for a 1:100 sample dilution. No further correction is required.

7. **Assay calibration**
   The assay is calibrated against the NIBSC tetanus antitoxin reference preparation, 76/589.

8. **Limitations**
   - Pre- and post-vaccination samples should be run simultaneously.
   - This kit may be used to aid diagnosis of immunodeficiency. Results must be confirmed by clinical findings and other serological tests.
   - The results obtained from this assay are not diagnostic proof of lack of protection/ protection against tetanus or the presence or absence of immunodeficiency.

**PROTECTIVE LEVELS**
The level of protective antibody in the normal population has been cited in the literature as between 0.01 and 0.15 IU/mL\(^2,3,4\). The largest study conducted in the United States\(^2\), involved a sample population of 10,618 individuals, ranging from age 6 upwards. Overall 69.7% had protective levels of >0.15 IU/mL, the rate decreased from 87.7% in 6-11 year olds to 27.8% in those 70 years of age and older. The results from two smaller studies\(^3,4\), indicate protective levels of 0.01 IU/mL.

Due to the wide ranges quoted above, it is recommended that each laboratory determine its own normal protective range.

**TYPICAL VALUES**
Anti-tetanus toxoid IgG antibody levels were measured in serum from 100 normal adult blood donors (of unknown vaccination and immune status). The results displayed below, are provided for illustration only, and should not be used to calculate a normal range.
Normal ranges have been established using The Binding Site T.Tox kit (MK010). See reference 5.

**PERFORMANCE CHARACTERISTICS**

1. **PRECISION**
   The intra- and inter-assay precision was measured using three samples within the range of the calibration curve. The concentration and % C.V. for each sample are given below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (IU/mL)</th>
<th>% C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>4.05</td>
<td>2.31</td>
</tr>
<tr>
<td>Sample 2</td>
<td>1.21</td>
<td>2.65</td>
</tr>
<tr>
<td>Sample 3</td>
<td>0.51</td>
<td>5.06</td>
</tr>
</tbody>
</table>

2. **ANALYTICAL SENSITIVITY**
   Sensitivity was determined as the mean concentration + 2 SD given by 16 determinations of the sample diluent. This equates to 0.0093 IU/mL.

3. **MEASURING RANGE**
   The measuring range of the assay is 0.01 - 7 IU/mL.

4. **INTERFERING SUBSTANCES**
   Various serum types were assayed to test the possible effect of interfering substances using an Interference Check A plus kit (Kokusai, Japan).

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin F (Free)</td>
<td>183mg/mL</td>
</tr>
<tr>
<td>Bilirubin C (Conjugate)</td>
<td>190mg/mL</td>
</tr>
<tr>
<td>Haemolysed Haemoglobin</td>
<td>4900mg/mL</td>
</tr>
<tr>
<td>Chyle</td>
<td>19300 Units</td>
</tr>
<tr>
<td>Rheumatoid Factor</td>
<td>500 IU/mL</td>
</tr>
</tbody>
</table>

No interference was observed with free or conjugated bilirubin, haemoglobin, lipid or rheumatoid factor.

**REFERENCES**


PLATE TEMPLATE

Summary of Procedure

1. Add 100µL of each calibrator, control and 1:100 diluted sample to the appropriate wells. Incubate for 30 minutes. Wash.

2. Add 100µL of conjugate to each well. Incubate for 30 minutes. Wash.

3. Add 100µL of substrate to each well. Incubate for 30 minutes.

4. Add 100µL of stop solution to each well. Measure the absorbance at 450nm.

<table>
<thead>
<tr>
<th>Date Adopted</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006-06-07</td>
<td>DA-Anti-Tetanus Toxoid IgG-2008</td>
</tr>
</tbody>
</table>

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