SeroPertussis Toxin™ IgG

Enzyme Linked Immunosorbent Assay (ELISA) for the quantitative determination of specific IgG antibodies to Bordetella Pertussis Toxin in human serum

Instruction Manual

Test kit for 96 determinations
(Catalog No.1231-01D)
For In Vitro Diagnostic Use
Store at 2-8°C. Do Not Freeze

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Intended Use
SeroPertussis Toxin™ IgG kit is a quantitative Enzyme Linked Immunosorbent assay (ELISA) for the determination of specific IgG antibodies to Bordetella pertussis Toxin.

For In Vitro Diagnostic Use

Introduction

Whooping Cough (Pertussis) is a highly contagious bacterial respiratory tract infection, caused by Bordetella pertussis – gram-negative bacilli. It is typically manifested in children with paroxysmal spasms of severe coughing; whooping and posttussive vomiting that resides for many weeks.

The disease results in high morbidity and mortality, especially of children.

Pertussis is an endemic disease, but epidemics occur every 3 – 5 years. In the USA, 5000 – 7000 cases are reported each year. The incidence of Pertussis has been greatly reduced by mass vaccination; however, even in countries with high vaccination coverage, the disease is re-emerging (1). Worldwide, nearly 50 million cases of pertussis are diagnosed annually and about 350,000 people die of the disease (2). The incidence of pertussis has increased steadily since 1980 (3). The vaccine induced immunity wanes after 5 to 10 years, making the vaccinated host vulnerable to infection. Infection in vaccinated persons causes a milder non-specific disease, without the classical clinical stages. Whooping cough is seen in only 6% of such cases; instead the illness is characterized by a non-specific, prolonged cough, lasting several weeks to months. Because of these atypical symptoms, Pertussis is under-diagnosed in adults and adolescents, who may be the reservoirs for infection of unvaccinated infants (4). Children who are too young to be fully vaccinated and those who have not yet completed the primary vaccination series are at the highest risk for severe illness.

The disease is highly contagious, with up to 90% of susceptible household contacts developing clinical disease following exposure. Early anti-microbial treatment will reduce the severity of the symptoms and limit the period of transmission. Prompt identification of the cases may help to prevent un-vaccinated or under-vaccinated persons from being infected by vaccination or by anti-microbial prophylaxis.

Laboratory diagnosis of Pertussis can be either direct by culture, DFA or PCR or by indirect serological tests. Since the bacteria reside in the upper respiratory tract during the first two weeks of the infection it can be detected during this period only by direct methods. The preferred specimen for direct detection is naso-pharyngeal sample (aspirates or swabs). Serology tests are helpful in diagnosis of atypical infections with prolonged cough and for epidemiological purposes. Elevated levels of antibodies against Pertussis Toxin (PT) and Filamentous Hemagglutinin (FHA) are regarded as sensitive serological markers for the diagnosis of Pertussis in adults and non-vaccinated children (5). In unvaccinated children increases in the levels of either immunoglobulin G (IgG) or immunoglobulin A (IgA) antibodies to a single or various antigens are required to meet the World Health Organization (WHO) definition of Pertussis. In vaccinated children, a single serum specimen may be diagnostic for Pertussis (6).

ELISA based tests for Pertussis Toxin are recommended for the specific detection of antibodies against B.pertussis, as they avoid detection of parapertussis infections. (7) Concentrations of antibodies to B. pertussis antigens should be quantitatively expressed in International Units per millilitre (IU/ml) as reference preparations are available (8).

SeroPertussis™ Toxin IgG utilizes purified PT as an antigen, allowing quantitative determination of IgG antibodies to Pertussis Toxin according to the first International WHO Standard (WHO International Standard Pertussis Antiserum, human, 1st IS NIBSC Code 06/140).

Principle of the Test

- SeroPertussis Toxin™ microtiter plates are coated with enriched fraction of Bordetella pertussis Toxin.
- The serum to be tested is diluted 1/101 and incubated in the SeroPertussis Toxin™ plate. In this step B. pertussis Toxin specific antibodies are bound to the immobilized antigens.
- Non-specific antibodies are removed by washing.
- Anti-human IgG conjugated to horseradish peroxidase (HRP) is added. In this step the HRP-conjugate is bound to the prebound antigen-antibody complex.
- Unbound conjugate is removed by washing.
- TMB-substrate is added and is hydrolyzed by the peroxidase, yielding a blue solution of the reduced Substrate.
- Upon the addition of the stop solution, the blue color turns yellow and the absorbance should be read by an ELISA reader at a wavelength of 450/620nm.
- The absorbance is proportional to the levels of the specific antibodies that are bound to the coated antigens.
Summary of Procedure: Manual/Automation*

Add 100µl of each Ready-to-Use (RTU) calibrator C30, C60, C120, and 100µl of Negative Control and specimens diluted 1/101 to microtiter plate wells coated with specific immunodominant B. pertussis Toxin proteins

Cover plate and incubate 1h at 37°C at 100% humidity

Wash 5 times with Wash Buffer

Add 100µl of RTU-HRP Conjugate

Cover plate and incubate 1h at 37°C at 100% humidity

Wash 5 times with Wash buffer

Add 100µl of TMB Substrate

Cover plate and incubate 15min at room temperature

Add 100 µl of Stop Solution

Read absorbance at 450/620nm

Calculate and interpret results

*Automation procedure:

Sample incubation: 25 minutes at RT followed by an additional incubation of 35 minutes at 35°C.

Conjugate incubation: 15 minutes at RT followed by an additional incubation of 35 minutes at 35°C.

Wash steps: 5 wash cycles.

Kit contents: for Manual use and automated use

Test kit for 96 Determinations
Cat. No. 1231-01

1. **B. pertussis** Toxin Antigen Coated Microtiter Plate: 96 break apart wells (8x12) coated with Bordetella pertussis Toxin antigens, packed in an aluminum pouch containing a desiccant card.

   1 Plate

2. Concentrated Wash Buffer (20X): A PBS - Tween buffer. Contains less than 0.05% proclin as a preservative.

   1 bottle, 100 ml

3. Serum Diluent: A ready-to-use buffer solution. Contains less than 0.05% proclin as a preservative.

   2 bottles, 60 ml


   1 Vial, 2 ml

5. C30 Calibrator: A ready-to-use calibrator containing 30 IU/ml (arbitrary international units) of human specific IgG antibodies to B. pertussis Toxin. Contains a mercury-free and azide-free preservatives.

   1 Vial, 2 ml

6. C60 Calibrator: A ready-to-use calibrator containing 60 IU/ml (arbitrary international units) of human specific IgG antibodies to B. pertussis Toxin. Contains a mercury-free and azide-free preservatives.

   1 Vial, 2 ml

7. C120 Calibrator: A ready-to-use calibrator containing 120 IU/ml (arbitrary international units) of human specific IgG antibodies to B. pertussis Toxin. Contains a mercury-free and azide-free preservatives.

   1 Vial, 2 ml

8. HRP-Conjugate: A ready-to-use Horseradish peroxidase (HRP) conjugated anti-human IgG (γ chain specific). Contains a mercury-free and azide-free preservatives.

   1 Bottle, 15 ml

9. TMB-Substrate: A ready-to-use solution. Contains 3, 3', 5, 5' - Tetramethylbenzidine as a chromogen and peroxide as a substrate.

   1 Bottle, 16 ml


    1 Bottle, 16 ml

11. Plate Cover: 1 unit

12. Instruction Manual: 1

Materials Required But Not Supplied

1. Clean test tubes for dilution of patient’s sera.
2. Adjustable micropipettes and multichannel pipettes (5-50, 50-200 and 200-1000µl ranges) and disposable tips.
3. One liter volumetric flask.
4. One 50ml volumetric cylinder.
5. Wash bottle.
6. Absorbent paper.
7. Vortex mixer.
8. A 37°C water bath with a lid, or a moisture chamber placed in a 37°C incubator.
9. ELISA-reader with a 450 and 620nm filters.
10. Distilled or double deionized water.

Warning and Precautions

For In Vitro Diagnostic Use

1. This kit contains human sera which have been tested by FDA and CE approved techniques, and found to be negative for HBsAg and for HCV and HIV antibodies. Since no known method can offer complete assurance that products derived from human blood do not transmit infection, all human blood components supplied in this kit must be handled as potentially infectious serum or blood according to the recommendations published in the CDC/NIH manual “Biosafety in Micro Biological and Biomedical Laboratories, 1988”.

2. TMB-Substrate solution is an irritant material to skin and mucous membranes. Avoid direct contact.

3. All the components of this kit have been calibrated and tested by lot. It is not recommended to mix components from different lots since it might affect the results.

4. Diluted sulfuric acid (1M H₂SO₄) is an irritant agent for the eyes and skin. In case of contact with eyes, immediately flush area with water and consult a physician.

Storage and Shelf-Life of Reagents

1. All the reagents supplied should be stored at 2-8°C. The unopened reagents vials are stable until the expiration date indicated on the kit pack. Exposure of originally stoppered or sealed components to ambient
temperature for a few hours will not cause damage to the reagents. **DO NOT FREEZE!**

2. Once the kit is opened, its shelf life is 90 days.
3. Unused strips must be resealed in the aluminum pouch with the desiccant card, by rolling the open end and sealing tightly with tape over the entire length of the opening.
4. Crystals may form in the 20x concentrated Wash Buffer during cold storage, this is perfectly normal. Redissolve the crystals by warming the buffer to 37°C before diluting. Once diluted, the solution may be stored at 2-8°C up to twenty one days.

**Serum Collection**

Prepare sera from aseptically collected samples using standard techniques. Heat inactivated sera should not be used. The use of lipemic, turbid or contaminated sera is not recommended. Particulate material and precipitates in sera may cause erroneous results. Such specimens should be clarified by centrifugation or filtration prior to the test.

**Specimens Storage**

Specimens should be stored at 2-8°C and tested within 7 days (adding of 0.1% Sodium Azide is highly recommended). If longer storage period is anticipated, aliquot and store at -20°C. Avoid repeated thawing and freezing.

**Test Procedure for Manual Use**

**A. Preparation of Reagents**

1. Bring all components and the clinical specimens to be tested to room temperature. Mix gently the calibrators (C30, C60, C120), Negative Control and the clinical specimens before use.
2. Determine the total number of specimens to be tested. In addition to the specimens, the following must be included in each test: Three wells of calibrators (C30, C60, C120) and one well of Negative Control.
3. Withdraw the microtiter plate from its aluminum pouch by cutting one end near the seal. Leave the required number of strips (according to the number of specimens to be tested) in the 96-well frame. Unused strips must be resealed in the aluminum pouch with the desiccant card, by rolling the open end and sealing tightly with tape over the entire length of the opening.
4. Dilute the Concentrated Wash Buffer 1/20 with double-deionized or distilled water. For example, in order to prepare one liter of wash buffer, add 50ml of the Concentrated Wash Buffer to 950ml of double-deionized or distilled water.

**B. Incubation of sera samples and controls**

5. Dilute each patient serum 1/101 with the supplied Serum Diluent as follows: Add 10 μl of patient serum to 1000μl of Serum Diluent.
6. Dispense 100μl of each of the three calibrators C30, C60, C120, Negative Control and of serum samples into separate wells of the test strip.
7. Cover the strips with a plate cover and incubate for 1h at 37°C in a moisture chamber.
8. Discard the liquid content of the wells.
9. **Washing step:**
   - **Manual Wash:** Fill each well with wash buffer up to the end of the well and discard the liquid, repeat this step twice for a total of 5 washing steps.
   - **Automated Wash:** Fill each well with 350μl of wash buffer and discard the liquid, repeat this step twice for a total of 5 washing steps.
10. Dry the strips and frame by gently tapping them over clean absorbent paper.

**C. Incubation with conjugate**

11. Dispense 100μl of ready-to-use HRP-Conjugate into each well.
12. Cover the strips with a plate cover and incubate for 1h at 37°C in a moisture chamber.
13. Discard the liquid content and wash as described in steps 9-10.

**D. Incubation with TMB Substrate**

14. Dispense 100μl TMB Substrate into each well, cover the strips with a plate cover and incubate at room temperature for 15 minutes.
15. Stop the reaction by adding 100μl of stop solution (1M H₂SO₄) to each well.

**E. Determination of Results**

16. Determine the absorbance at 450/620nm and record the results. Determination should not exceed 30 minutes following stopping of chromogenic reaction.

- **Note:** Any air bubbles should be removed before reading. The bottom of the ELISA plate should be carefully wiped.

**Test Procedure for Automated Use**

*The vials and reagents’ volume have been adapted for automation applications.*

**A. Preparation of Reagents**

1. Bring all components and the clinical specimens to be tested to room temperature. Mix well the calibrators (C30, C60, C120), Negative Control, and the clinical specimens before use.
2. Determine the total number of specimens to be tested. In addition to the specimens, the following must be included in each test: Three wells of calibrators (C30, C60, C120) and one well of Negative Control.
3. Withdraw the microtiter plate from its aluminum pouch by cutting one end near the seal. Leave the required number of strips (according to the number of specimens to be tested) in the 96 well frame.
4. Dilute the Concentrated Wash Buffer 1/20 with double-deionized or distilled water. For example, in order to prepare one liter of wash buffer, add 50ml of the Concentrated Wash Buffer to 950ml of double-deionized or distilled water.

**B. Incubation of sera samples and controls**

5. Dilute each patient serum 1/101 as follows:
   - Dispense 750μl of Serum Diluent to each sample tube.
   - Aspirate 250μl of Serum Diluent and 10μl of patient serum.
• Add the 260µl (1:26 pre-diluted sample) to each sample tube (final volume of 1010µl in each sample tube).

6. Dispense 100µl each of Negative Control, the three calibrators (C30, C60, C120) and 1:101 diluted serum samples into separate wells of the test strip.

7. Incubate for 25 minutes at room temperature (22-28°C). Time incubation is taken from dispensing the first vial, as indicated in the automation program: "Time incubation from start of previous assay step". Following the incubation at 22-28°C continue to incubate for 35 minutes at 35°C.

8. Eliminate assay drift caused by this operation.


10. Perform 2 aspiration cycles to aspirate residual fluid from the vial, as indicated in the automation program: "Perform 2 aspirate cycles no aspirate sweep. Partial plate mode: maintain full plate time".

C. Incubation with conjugate

Each vial of HRP Conjugate can only be used twice

11. Dispense 100µl of Ready-to-Use HRP-conjugate into each well.

12. Incubate for 15 minutes at room temperature (22-28°C). Time incubation is taken from dispensing the first vial, as indicated in the automation program: "Time incubation from start of previous assay step". Following the incubation at 22-28°C continue to incubate for 35 minutes at 35°C.

13. Wash as described in steps 9-10.

D. Incubation with TMB – Substrate

14. Dispense 100µl of TMB Substrate into each well and incubate at room temperature (22-28°C) for 15 minutes in the dark. Time incubation is taken starting from dispensing the first vial, as indicated in the automation program: "Time incubation from start of previous assay step".

15. Stop the reaction by adding 100µl of Stop Solution (1M H2SO4) to each well.

E. Determination of Results

16. Determine the absorbance at 450/620nm and record the results. Determination should not exceed 30 minutes following stopping of chromogenic reaction.

Please note that each automation machine has specific technical commands. Please implement Savoy’s automation procedure for this kit on the operation protocol of your automation machine.

Calculation of Test Results

1. Manual method, using a squared graph paper:
   i. Plot the absorbance values (OD) of the 3 calibrators (C30, C60 and C120) on X axis versus their concentration (IU/ml) on Y axis.
   ii. Using the standard curve, interpolate the concentration of the tested sample values (in IU/ml) from each absorbance measured (see example 1).

2. Computerized method using MS-Excel:
   i. Open an MS-Excel spreadsheet
   ii. In raw B, mark down the values of the three calibrators (30, 60, 120) and in raw A add the corresponding OD values.
   iii. Click the plot icon ( ) and choose scatter plot. Plot raw B against raw A.
   iv. Right click on one of the plotted symbols and choose “Add Trendline….”.
   v. Under the “Type” tab choose “Power” and under the “Options” tab choose “Display equation on chart”. Click the “OK” button.
   vi. An equation in the form of \( Y = ax^b \) will be displayed where Y corresponds to the PT concentration in IU/mL and X to the OD450/620. Use this equation to calculate the PT concentration according to the OD values for each given sample (see example 1).

Example 1. Interpolation of results:
Read the absorbance value of the sample (X1) and mark it on the X-axis. Draw a vertical line from this point to the calibration curve, and from the intercept draw a horizontal line to the Y-axis.

Read the concentration in IU/ml of the sample.

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>OD 450/620</th>
<th>IgG IU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>C30</td>
<td>1.221</td>
<td>30</td>
</tr>
<tr>
<td>C60</td>
<td>1.852</td>
<td>60</td>
</tr>
<tr>
<td>C120</td>
<td>2.731</td>
<td>120</td>
</tr>
<tr>
<td>Sample</td>
<td>X1=2.235</td>
<td>Y1=84</td>
</tr>
</tbody>
</table>

Sero Pertussis Toxin IgG Calibration

Excel: \( Y = 21.10^{x^{1.7214}} \)

M1231-01E 04-07/12
Interpretation of Results

1. Validity of sample results: If OD_{450/620} of a certain sample is ≥ 3.0, it is recommended that the sample will be re-tested at a dilution of 1:404. The result in IU/ml of this particular sample should be then multiplied by a factor of 4.

2. Cut-off: According to recent literature and recommendations from reference laboratories across the EU (Guiso et al, 2010), Savyon Diagnostics recommends the following interpretation of results:

<table>
<thead>
<tr>
<th>IgG IU/ml</th>
<th>Result</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;55 IU/ml</td>
<td>Negative</td>
<td>No indication for acute infection</td>
</tr>
<tr>
<td>≥55 IU/ml &lt;60 IU/ml</td>
<td>Borderline</td>
<td>Equivocal results; Test IgA - if Negative, re-test</td>
</tr>
<tr>
<td>60-125 IU/ml</td>
<td>Positive</td>
<td>To differentiate between recent vaccination, past and current infection re-test IgG in 2-4 weeks</td>
</tr>
<tr>
<td>≥125 IU/ml</td>
<td>Strong Positive</td>
<td>Indication for an acute infection or current vaccination</td>
</tr>
</tbody>
</table>

3. Testing convalescent sera samples: In order to differentiate between recent vaccination, past and acute infection, Savyon Diagnostics recommends to re-test positive and borderline samples (55-125 IU/ml) in a second (convalescent) serum sample taken 2-4 weeks later. Recent literature recommends that ≥2-fold elevation in IgG levels is indicative for current acute infection (Andre, 2008 – Ref 1 from Guiso 2010).

4. IgG/IgA antibody profiling: In order to achieve a comprehensive antibodies profile, Savyon Diagnostics recommends also to test IgA levels:

<table>
<thead>
<tr>
<th>IgG</th>
<th>IgA</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>No indication of B.pertussis infection (see test limitations)</td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>Indication of current infection</td>
</tr>
<tr>
<td>Positive or Negative</td>
<td>Positive</td>
<td>Indication of recent infection</td>
</tr>
<tr>
<td>Positive</td>
<td>Negative or Positive</td>
<td>Indication of recent or past infection or past immunization</td>
</tr>
</tbody>
</table>

Test Limitations

1. No single serological test should be used for final diagnosis. All clinical and laboratory data should be taken into account.
2. Samples obtained too early during primary infection may not contain detectable antibodies. If B.pertussis is suspected, a second sample should be obtained 2-4 weeks later and tested in parallel with the original sample.
3. When infection is suspected in infants under the age of 6 months, another test should be performed (culture, PCR) since children younger than 6 months rarely develop antibodies.

Performance Characteristics

Precision

Intra-assay (within-run) precision:

<table>
<thead>
<tr>
<th>Sample</th>
<th>No of Replicates</th>
<th>Mean OD Value</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>120IU/m</td>
<td>10</td>
<td>119</td>
<td>3.5</td>
</tr>
<tr>
<td>30IU/ml</td>
<td>10</td>
<td>32</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Inter-assay (between-run) precision:

<table>
<thead>
<tr>
<th>Sample</th>
<th>No of Replicates</th>
<th>Mean OD Value</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>120IU/ml</td>
<td>10</td>
<td>122</td>
<td>6.3</td>
</tr>
<tr>
<td>30IU/ml</td>
<td>10</td>
<td>30</td>
<td>9.4</td>
</tr>
</tbody>
</table>

Bibliography

1. Melker H.E. et al., Emerging Infectious Diseases 6(4), 2000. Centers of Disease Control
3. CDC report, February 1998
5. Trollfors B. et al., Clinical Infectious Diseases 1999; 28; 552-9

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