**Intended Use**
The AccuDiag Syphilis IgG/IgM ELISA is an enzyme-linked immunosorbent assay for the qualitative detection of antibodies to Treponema pallidum (TP) in human serum/plasma. The Syphilis IgG/IgM ELISA test is intended for diagnosis of syphilis infection and for screening blood donors.

**Test Principle**
Using the antigen sandwich enzyme-linked method (ELISA), this Syphilis IgG/IgM ELISA test can detect anti-TP antibodies. Polystyrene microwell strips are pre-coated with recombinant Treponema pallidum antigens indicated in E. coli. Recombinant TP antigens that are conjugated to horseradish peroxidase (HRP-Conjugate) are incubated in the microwells with the sample. The pre-coated antigens indicate the same epitopes as the HRP-Conjugate antigens, but the hosts are different. If anti-TP is present in the sample during incubation, the conjugated and pre-coated antigens will be bound to the two variable antibody domains, and what's captured on the solid phase, is the specific antibody-antigen complex. It is important that chromogen solutions containing tetramethylbenzidine (TMB) and urea peroxide are added into the wells after washing incubation to remove sample and unbound conjugates. The colorless chromogens are hydrolyzed by the bound HRP conjugate to a blue-colored product when the antigen-antibody-antigen sandwich complex is present. At this point the blue color turns yellow. This occurs after the reaction with sulfuric acid is stopped. What can be measured proportionally at this juncture is the amount of antibody in the sample with the amount of color. Colorless wells indicate negative anti-TP samples.

**Assay Principle Scheme: Double Antigen Sandwich ELISA**

\[ \text{Ag(p)+Ab(s)+(Ag)ENZ} \rightarrow [\text{Ag(p)-Ab(s)-(Ag)ENZ}] \rightarrow \text{blue} \rightarrow \text{yellow (+)} \]

\[ \text{Ag(p) } + (\text{Ag)ENZ} \rightarrow [\text{Ag(p)}] \rightarrow \text{no color (-)} \]

**Incubation Immobilized Complex Coloring Results**

- 15min.
- 60min.

**Specimen Collection and Preparation**

1. **Sample Collection:** Either fresh serum or plasma samples can be used for this assay. Blood collected by venipuncture should be allowed to clot naturally and completely – the serum/plasma must be separated from the clot as early as possible to avoid hemolysis of the RBC. Care should be taken to ensure that the serum samples are clear and not contaminated by microorganisms. Any visible particulate matters in the sample should be removed by centrifugation at 3000 RPM for at least 20 minutes at room temperature, or by filtration on 0.22μ filters. Plasma samples collected into EDTA, sodium citrate or heparin may be tested, but highly lipaemic, icteric, or hemolized samples should not be used as they could give erroneous results in the assay. Do not heat inactivate samples. This can cause sample deterioration.

2. **Transportation and Storage:** Store samples at 2-8°C. Samples not required for assaying within 3 days should be stored frozen (-20°C or lower). Multiple freeze-thaw cycles should be avoided. For shipment, samples should be packaged and labeled in accordance with the existing local and international regulations for transport of clinical samples and ethological agents.

**Materials and Components**

**Materials provided with the test kits**

**MICROWELL PLATE:**
Blank microwell strips fixed on white strip holder. The plate is sealed in aluminium pouch with desiccant.

**CONTROL:**
12x8-well strips per plate. Each well contains recombinant TP antigens.

**STOCK WASH BUFFER:**
Colorless liquid filled in a clear bottle with white screw cap.

**HRP-CONJUGATE REAGENT:**
Red-colored liquid filled in a white vial with red screw cap.

**DILUTE BEFORE USE:** The concentration must be diluted
Materials required but not provided
1. Freshly distilled or deionized water.
2. Disposable gloves and timer.
3. Appropriate waste containers for potentially contaminated materials.
4. Disposable V-shaped troughs.
5. Dispensing system and/or pipette (single or multichannel), disposable pipette tips.
6. Absorbent tissue or clean towel.
7. Dry incubator or water bath, 37 ± 0.5°C.
8. Microshaker for dissolving and mixing conjugate with samples.
9. Microwell plate reader, single wavelength 450nm or dual wavelength 450nm
10. Microwell aspiration/wash system.

ASSAY PROCEDURE

Step 1 Reagents preparation: Allow the reagents and samples to reach room temperature (18-30°C) for at least 15-30 minutes. Check the Wash buffer concentrate for the presence of salt crystals. If crystals have formed in the solution, resolubilize by warming at 37°C until crystals dissolve. Dilute the stock Wash Buffer to 100 ml with distilled or deionized water. Use only clean vessels to dilute the buffer.

Step 2 Numbering Wells: Set the strips needed in strip holder and number sufficient number of wells including three Negative controls (e.g. B1, C1, D1), two Positive controls (e.g. E1, F1) and one Blank (e.g. A1). Neither samples nor HRP-Conjugate should be added into the Blank well. If the results will be determined by using dual wavelength plate reader, the requirement for use of Blank well could be omitted. Use only number of strips required for the test.

Step 3 Adding HRP-Conjugate: Add 100 µl HRP-Conjugate into each well except the Blank.

Step 4 Adding Sample: Add 20 µl of Positive control, Negative control, and specimen into their respective wells - the HRP-Conjugate-sample mixture in the wells will change the color from GREEN to BLUE after adding of the samples. Note: Use a separate disposal pipette tip for each specimen, Negative, Positive Control to avoid cross-contamination.

Step 5 Incubating: Mix by tapping the plate gently. Cover the plate with the plate cover and incubate for 60 minutes at 37°C. It is recommended to use thermostat-controlled water tank to assure the temperature stability and humidity during the incubation. If dry incubator is used, do not open the door frequently.

Step 6 Washing: At the end of the incubation, remove and discard the plate cover. Wash each well 6 times with diluted Wash buffer. Each time allow the microwells to soak for 30-60 seconds. After the final washing cycle, turn the plate onto blotting paper or clean towel, and tap out any remainders.

Step 7 Coloring: Dispense 50 µl of Chromogen A and 50 µl Chromogen B solution into each well including the Blank, cover the plate with plate cover and mix gently by tapping the plate. Incubate the plate at 37°C for 15 minutes avoiding light. The enzymatic reaction between the Chromogen solutions and the HRP-Conjugate produces blue color in Positive control and anti-TP positive sample wells.

Step 8 Stopping Reaction: Remove and discard the plate cover. Using a multichannel pipette or manually, add 50 µl Stop Solution into each well and mix gently. Intensive yellow color develops in positive control and anti-TP Positive sample wells.

Step 9 Measuring the Absorbance: Calibrate the plate reader with the Blank well and read the absorbance at 450nm. If a dual filter instrument is used, set the reference wavelength at 630nm. Calculate the Cut-off value and evaluate the results. (Note: read the absorbance within 5 minutes after stopping the reaction)

RESULTS

Each microplate should be considered separately when calculating and interpreting results of the assay, regardless of the number of plates concurrently processed. The results are calculated by relating each sample’s optical density (OD) value to the Cut-off value (C.O.) of the plate. If the Cut-off reading is based on single filter plate reader, the results should be calculated by subtracting the Blank well OD value from the print report values of samples and controls. In case the reading is based on dual filter plate reader, do not subtract the Blank well OD from the print report values of samples and controls.

1. Calculation of Cut-off value (C.O.) = *Nc + 0.18
   *Nc = the mean absorbance value for three negative controls

Example:

1. Calculation of Nc:
   Well No  B1  C1  D1
   Negative controls OD value  0.032  0.031  0.027
   Nc=0.030

2. Calculation of Cut-off value (C.O.) = 0.030 + 0.180 = 0.210

If one of the Negative control values does not meet the Quality control range specifications, it should be discarded and the mean value is calculated again using the remaining two values. If more than one negative control OD value does not meet the Quality control range specifications, the test is invalid and must be repeated.

2. Quality control range:

   The test results are valid if the Quality Control criteria are verified. It is advisable that each laboratory must establish appropriate quality control system with quality control material similar to or identical with the patient sample being analyzed.
   1. The OD value of the Blank well, which contains only Chromogens and Stop solution, is less than 0.080 at 450 nm.
   2. The OD value of the Positive control must be equal to or greater than 0.800 at 450/630nm or at 450nm after blanking.
   3. The OD value of the Negative control must be less than 0.100 at 450/630nm or at 450nm after blanking.

3. Interpretations of the results:

   (S = the individual absorbance (OD) of each specimen)

   Negative Results (S/C.O. <1) : Samples giving absorbance less than the Cut-off value are negative for this assay, which indicates that no anti-TP antibodies have been detected with this kit. Therefore, the patient is probably not infected and there are no serological indications for past infection with TP.

   Positive Results (S/C.O. ≥1) : Samples giving an absorbance greater than or equal to the Cut-off value are considered initially reactive, which indicates that TP
antibodies have been detected using this anti-TP ELISA kit. Retesting in duplicates of any initially reactive sample is recommended. Repeatedly reactive samples can be considered positive for antibodies to Treponema pallidum and therefore there are serological indications for current or past infection with TP. Any blood unit containing antibodies to Treponema pallidum should be immediately discarded. Borderline (S/CO =0.9-1.1) : Samples with absorbance to Cut-off ratio between 0.9 and 1.1 are considered borderline and retesting of these samples in duplicates is recommended to confirm the results. Repeatedly positive samples could be considered positive for antibodies to TP.

Retesting of these samples in duplicates is recommended. Repeatedly positive samples could be considered positive for Treponema pallidum. Follow-up and supplementary testing any positive with other analytical system is required.

THE PERFORMANCE AND EXPECTED RESULTS
The clinical performances of this assay have been evaluated by a panel of samples obtained from 3400 healthy blood donors from 3 blood banks and by a panel of samples from 192 Syphilis positive patients (comparative study with another commercially available TP diagnostic systems). The evaluation results are given below. Results obtained in individual laboratories may differ.

<table>
<thead>
<tr>
<th>Samples</th>
<th>-</th>
<th>+</th>
<th>Confirmed Positive</th>
<th>Specificity</th>
<th>False positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donors: 3400</td>
<td>3392</td>
<td>8</td>
<td>5</td>
<td>99.91%</td>
<td>3</td>
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<table>
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<th>Syphilis</th>
<th>anti-TP ELISA</th>
<th>TRUST/RPR</th>
<th>TPHA</th>
<th>anti-TP ELISA*</th>
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<tr>
<td>1st period</td>
<td>76</td>
<td>70</td>
<td>6</td>
<td>65</td>
</tr>
<tr>
<td>2nd period</td>
<td>110</td>
<td>110</td>
<td>0</td>
<td>101</td>
</tr>
<tr>
<td>3rd period</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>24</td>
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<table>
<thead>
<tr>
<th>Atrophic Arthritis</th>
<th>Control</th>
<th>panel</th>
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<tbody>
<tr>
<td>24</td>
<td>3</td>
<td>21</td>
</tr>
</tbody>
</table>

Anti-TP ELISA*-another commercially available anti-TP ELISA kit

Analytical Specificity:

No cross reactivity was observed with specimens from patients infected with HAV, HCV, HBV, HTLV, CMV, and HIV. No interference was observed from rheumatoid factors up to 2000U/ml. No high dose hook effect observed during clinical testing. The assay performance characteristics are unaffected from elevated concentrations of bilirubin, hemoglobin, and triolein.

LIMITATIONS OF PROCEDURE
1. Non-repeatable positive result may occur due to the general biological and biochemical characteristics of ELISA method. The test is designed to achieve very high performance characteristics of high sensitivity and specificity.
2. Any positive results must be interpreted in conjunction with patient clinical information and other laboratory testing results.
3. If, after retesting of the initially reactive samples, the assay results are negative, these samples should be considered as non-repeatable (false positive) and interpreted as negative. As with many very sensitive ELISA assays, false positive results can occur due to the several reasons, most of which are related but not limited to inadequate washing step.
4. Common sources for mistakes: kits beyond the expiry date, bad washing procedures, contaminated reagents, incorrect assay procedure steps, insufficient aspiration during washing, failure to add samples or reagents, equipment, timing, volumes, sample nature and quality.
5. The prevalence of the marker will affect the assay’s predictive values.
6. This kit is intended ONLY for testing of individual serum or plasma samples. Do not use it for testing of cadaver samples, saliva, urine or other body fluids, or pooled (mixed) blood.
7. This is a qualitative assay and the results cannot be used to measure antibodies concentrations.

INDICATIONS OF INSTABILITY OR DETERIORATION OF REAGENTS
1. Values of the Positive or Negative controls, which are out of the indicated Quality control range, are indicator of possible deterioration of the reagents and/or operator or equipment errors. In such case, the results should be considered as invalid and the samples must be retested. In case of constant erroneous results classified as due to deterioration or instability of the reagents, immediately substitute the reagents with new ones.
2. If after mixing of the Chromogen A and B solutions into the wells, the, the color of the mixture turns blue within few minutes, do not continue carrying out the testing and replace the reagents with fresh ones.

PRECAUTIONS
This kit is intended FOR IN VITRO USE ONLY [IVD]

FOR PROFESSIONAL USE ONLY
The ELISA assay is a time and temperature sensitive method. To avoid incorrect result, strictly follow the test procedure steps and do not modify them.

1. Do not exchange reagents from different lots, or use reagents from other commercially available kits. The components of the kit are precisely matched to achieve optimal performance during testing.
2. Make sure that all reagents are within the validity indicated on the kit box and are of the same lot. Never use reagents beyond the expiry date stated on reagents labels or on the kit box.
3. CAUTION - CRITICAL STEP: Allow the reagents and samples to stabilize at room temperature (18-30°C) before use.
4. Shake reagent gently before, and return to 2-8°C immediately after use.
5. Use only sufficient volume of sample as indicated in the procedure steps. Failure to do so, may cause in low sensitivity of the assay.
6. Do not touch the bottom exterior of the wells; fingerprints or scratches may interfere with microwell reading.
7. When reading the results, ensure that the plate bottom is dry and there are no air-bubbles inside the wells.
8. Never allow the microplate wells to dry after the washing step. Immediately proceed to the next step. Avoid the formation of air-bubbles when adding the reagents.
9. Avoid assay steps long time interruptions. Ensure same working conditions for all wells.
10. Calibrate the pipette frequently to assure the accuracy of samples/reagents dispensing. Always use different disposal pipette tips for each specimen and reagents as to avoid cross-contaminations. Never pipette solutions by mouth. The use of automatic pipettes is recommended.
11. Ensure that the incubation temperature is 37°C inside the incubator.
12. When adding samples, avoid touching thewell’s bottom with the pipette tip.
13. When reading the results with a plate reader, it is recommended to determine the absorbance at 450nm or at 450nm with reference at 630nm.
14. All specimens from human origin should be considered as potentially infectious.
15. Materials from human origin may have been used in the kit. These materials have been tested with tests kits with accepted performance and found negative for antibodies to HIV ½, HCV, TP and HBsAg. However, there is no analytical
method that can assure that infectious agents in the specimens or reagents are completely absent. Therefore, handle reagents and specimens with extreme caution as if capable of transmitting infectious diseases. Strict adherence to GLP (Good Laboratory Practice) regulations can ensure the personal safety. Never eat, drink, smoke, or apply cosmetics in the assay laboratory.

16. Bovine derived sera may have been used in this kit. Bovine serum albumin (BSA) and fetal calf sera (FCS) are derived from animals from BSE/TSE free-geographical areas.

17. The pipette tips, vials, strips and sample containers should be collected and autoclaved for 1 hour at 121°C or treated with 10% sodium hypochlorite for 30 minutes to decontaminate before any further steps for disposal.

18. The Stop solution (2M H₂SO₄) is a strong acid. Corrosive. Use it with appropriate care. Wipe up spills immediately or wash with water if come into contact with the skin or eyes. ProClin 300 used as a preservative can cause sensation of the skin.

19. The enzymatic activity of the HRP-conjugate might be affected from dust, reactive chemical, and substances like sodium hypochlorite, acids, alkalins etc. Do not perform the assay in the presence of such substances.

20. Materials Safety Data Sheet (MSDS) available upon request.

21. If using fully automated microplate processing system, during incubation, do not cover the plates with the plate cover. The tapping out of the remainders inside the plate after washing, can also be omitted.

22. The components of the kit will remain stable through the expiration date indicated on the label and package when stored between 2-8°C. do not freeze. To assure maximum performance of this anti-TP kit, during storage protect the reagents from contamination with microorganism or chemicals.