TPHA

Σ 200 REF 72503

Σ 500 REF 72504

KITS FOR THE QUALITATIVE AND SEMI-QUANTITATIVE DETECTION OF ANTIBODY TO *TREPONEMA PALLIDUM* IN HUMAN SERUM OR PLASMA BY PASSIVE HAEMAGGLUTINATION

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BIO-RAD

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1. INTENDED USE

TPHA kits are intended for use for the qualitative and semi-quantitative detection of antibodies to *Treponema pallidum* in human serum and plasma. The product may be used for the screening of blood donors, and to aid in the diagnosis of patients where syphilis infection is suspected.

2. SUMMARY AND EXPLANATION OF THE TEST

Syphilis is a chronic infection that progresses through distinct stages of infection: primary, secondary, tertiary, and quaternary. These stages produce diverse clinical symptoms, typically producing initial sores known as chancres then syphilitic rash followed by long periods of dormancy. Untreated infection may eventually result in cardiovascular problems and neurosyphilis.

The infection is caused by the spirochaete *Treponema pallidum*, and is usually acquired by sexual contact, although the disease may be transmitted by transfusion of infected blood. Intrauterine infection also occurs. The organism has proved virtually impossible to culture in artificial media, and diagnosis of the infection usually depends on the demonstration of antibodies in the blood, which appear soon after initial infection and may persist for many years.

Tests for Syphilis fall into four categories: direct microscopic examination, treponemal antibody tests, non - treponemal antibody tests and direct antigen tests. Because of the long periods of dormancy and the non - specific nature of non - treponemal tests, methods that detect specific anti-treponemal antibodies in blood specimens have become increasingly popular for screening. TPHA is one such test.

3. PRINCIPLES OF THE PROCEDURE

TPHA kits use preserved avian erythrocytes coated with antigens of *T. pallidum* (Nichols strain), which will bind with specific antibody present in patient's serum or plasma. The cells are suspended in a medium containing components to eliminate non - specific reactions. Positive reactions are shown by agglutination of the cells, negative reactions by the settling of the cells to a button or small ring.

Although these kits are intended for use primarily as qualitative tests, antibody levels may be titrated by doubling dilution.

Agglutination patterns may be interpreted by eye or by a plate-reader capable of reading agglutination patterns. Contact the concern company for the adaptations and special procedures.

4. REAGENTS

4.1. Description

Identification on label		Description	Presentation 72503 200 tests	Presentation 72504 500 tests
R1	**Test Cells**	**Test Cells** Suspension of Avian erythrocytes coated with antigens of *T. pallidum*, containing Bovine Serum Albumin (BSA)	2 vials 7.8 ml	2 vials 20 ml
R2	**Control Cells**	**Control Cells** Suspension of Avian erythrocytes, containing BSA	2 vials 7.8 ml	1 vial 20 ml
R3	**Diluent**	**Diluent** Saline solution containing Rabbit serum	2 vials 20 ml	1 vial 125 ml
R4	**Positive Control**	**Positive Control** Human serum containing antibodies to *T .pallidum*, negative for HBs Antigen, anti-HIV1/2, and anti-HCV antibodies diluted in phosphate buffer	1 vial 1 ml	1 vial 1 ml
R5	**Negative Control**	**Negative Control** Rabbit serum in phosphate buffer	1 vial 1 ml	1 vial 1 ml

4.2. Storage and handling requirements

This kit should be stored at +2-8°C. **Store bottles up-right**. Do not freeze.

Each item of the kit preserved at +2-8°C can be used up to the expiry date mentioned on the package. After opening and in the absence of contamination, the R1, R2, R3, R4 and R5 reagents preserved at +2-8°C, can be used up to the expiry date shown on the label.

5. WARNING AND PRECAUTIONS

For *in vitro* diagnostic use. For healthcare professional use.

5.1. Health and Safety precautions:

- This test kit should be handled only by qualified personnel trained in laboratory procedures and familiar with their potential hazards. Wear appropriate protective clothing, gloves and eye/face protection and handle appropriately with the requisite Good Laboratory Practices.

- The control materials supplied are derived from human serum. They have been tested at donor level and found negative for HBs Antigen, anti-HIV1/2, and anti-HCV antibodies. No known test method can offer complete assurance that infectious agents are absent. Therefore, all human blood derivatives, reagents and human specimens should be handled as if capable of transmitting infectious disease, following recommended Universal Precautions for blood borne pathogens as defined by local, regional and national regulations.

- Biological spills: Human source material spills should be treated as potentially infectious.

  Spills not containing acid should be immediately decontaminated, including the spill area, materials and any contaminated surfaces or equipment, with an appropriate chemical disinfectant that is effective for the potential biohazards relative to the samples involved (commonly a 1:10 dilution of household bleach, 70-80% Ethanol or Isopropanol, an iodophor [such as 0.5% Wescodyne™ Plus, etc.), and wiped dry.

  Spills containing acid should be appropriately absorbed (wiped up) or neutralized, the area flushed with water and wiped dry; materials used to absorb the spill may require biohazardous waste disposal. Then the area should be decontaminated with one of the chemical disinfectants.

Note: Do not place solutions containing bleach into the autoclave !

- Dispose of all specimens and material used to perform the test as though they contain an infectious agent. Laboratory, chemical or biohazardous wastes must be handled and discarded in accordance with all local, regional and national regulations.

- The Safety Data Sheet is available on www.bio-rad.com.

5.2. Precautions related to the procedure

5.2.1. Preparing

The reliability of the results depends on correct implementation of the following Good Laboratory Practices:

- Do not mix or associate reagents from different lots within a test run.
- Do not use expired reagents.
- Before use wait for 30 minutes for the reagents to stabilize at room temperature (18-30°C).

5.2.2. Processing

- Do not change the assay procedure.
- Use a new distribution tip for each sample.

6. SPECIMENS

Serum or plasma (EDTA, Sodium Citrate, Heparin and ACD) specimens should be free of blood cells and of obvious microbial contamination. They may be stored at +2-8°C for up to 7 days before testing. Specimens needing longer storage should be frozen at -20°C or lower. Frozen specimens should be thawed and well mixed before testing. Do not repeat more than 5 freeze/thaw cycles. Heated samples at 56°C during 3 hours do not impact the results.

Specimens containing up to 100 mg/L of bilirubin, up to 36 g/L of triglycerid and up to 10 g/L of hemoglobin do not affect the results. However, it is not recommended to use hyperlipaemic or hyperhaemolysed sera or plasma.

If the specimens are to be shipped, they must be packaged in accordance with the regulations in force regarding the transport of etiological agents and preferably transport frozen.

7. PROCEDURE

REMARK: The kit Positive and Negative Controls (R4 and R5) must be run with each run of tests.

7.1. Materials required but not provided

- Distilled water
- Sodium hypochlorite (household bleach) and sodium bicarbonate.
- Absorbent paper
- Disposable gloves
- Safety glasses
- Disposable tubes
- Automatic or semi-automatic, adjustable or preset pipettes or multipipettes to measure and dispense 10 µl, 25 µl, 75 µl and 190 µl.

- Microplate shaker
- U well plate format (96-well microplates) - Code 83375 (5 plates)
- Container for biohazardous waste

7.2. Assay Procedure (Manual technique)

7.2.1. Qualitative assay
Three wells from the U microplate are needed for each specimen.

The TPHA 500 Kit (Product No. 72504) is intended for screening large numbers of specimens and contains only a small volume of Control Cells. It is intended that specimens are screened using only Test Cells in the first instance, and the Control Cells should be used when repeating tests on specimens giving a positive result when first tested.

a. Specimen and Controls Dilution (to 1 in 20)
Add 190 µl of the diluent (R3) to one well.
Add 10 µl of specimen or Positive Control (R4) or Negative Control (R5) to the same well.
Mix thoroughly.

b. Test
Transfer 25 µl of diluted control or diluted specimen from dilution step to test well.
Transfer 25 µl of diluted control or diluted specimen dilution step to control well.
Re-suspend the Test Cells (R1) and the Control Cells (R2) by shaking the vial Examine for complete suspension.
Add 75 µl of Test Cells (R1) to test well and 75 µl of Control Cells (R2) to the control well.
Final specimen dilution is 1: 80.
Mix wells thoroughly.
Incubate at room temperature (15-30 °C) on a vibration-free surface for a minimum of 45 minutes.
Read the settling patterns. Agglutination patterns are stable for at least three hours if undisturbed.

7.2.2. Semi-Quantitative assay
9 wells of the U microplate are needed for each specimen: One well for the specimen or control dilution and 8 wells for the titration.

Note: The Positive and Negative Controls (R4 and R5) must be run with each lot of tests, using the procedure given below.

a. Specimen and Controls Dilution (to 1 in 20)
Add 190 µl of the diluent (R3) to one well.
Add 10 µl of specimen or Negative Control (R5) or Positive Control (R4) to the same well.
Mix thoroughly.

b. Titration
Leaving the first well empty, add 25 µl of diluent (R3) to each of the remaining 7 wells.

0	1	2	3	4	5	6	7	8
190 µl (R3) + 10 µl Specimen or (R4) or (R5)		25 µl of R3	25 µl of R3	25 µl of R3	25 µl of R3	25 µl of R3	25 µl of R3	25 µl of R3
Specimen or control dilutions	**8 wells for titration**							

Transfer 25 µl of diluted control or specimen to the 1st well and to the 2nd well, then mix.

Then serially dilute along the well sequence, discarding the excess 25 µl from the final well.

0	1	2	3	4	5	6	7	8	Discarding excess 25 µl
190 µl (R3) + 10 µl Specimen or (R4) or (R5)	25 µl of diluted control	25 µl of R3 + 25 µl of diluted control	25 µl of R3	25 µl of R3	25 µl of R3	25 µl of R3	25 µl of R3	25 µl of R3	
Specimen or control dilutions	**8 wells for titration**								

c. Test
Gently mix the Test Cells (R1) to ensure thorough resuspension.
Add 75 µl of Test Cells (R1) to each well.
Final specimen dilution range after addition of cells is 1: 80 – 1 :10240.
Mix thoroughly.

0	1	2	3	4	5	6	7	8
190 µl (R3) + 10 µl Specimen or (R4) or (R5)	25 µl of diluted control + 75 µl of R1	25 µl of dilution + 75 µl of R1	25 µl of dilution + 75 µl of R1	25 µl of dilution + 75 µl of R1	25 µl of dilution + 75 µl of R1	25 µl of dilution + 75 µl of R1	25 µl of dilution + 75 µl of R1	25 µl of dilution + 75 µl of R1
	1:80	**1:160**	**1:320**	**1:640**	**1:1280**	**1:2560**	**1:5120**	**1:10240**
Specimen or control dilutions	**8 wells for titration**							

Incubate at room temperature (15-30°C) on a vibration-free surface for a minimum of 45 minutes.
Read the settling patterns. Agglutination patterns are stable for at least three hours if undisturbed.
The titre of the specimen is the reciprocal of the highest dilution giving agglutination.

7.3. Quality Control
The kit Controls must give the correct result; Negative Control (R5) is negative and Positive Control (R4) is positive.
When the kit Positive Control (R4) is titrated, the expected end point is 1:640 - 1:5120.

7.4. Interpretation of the results and Test Validation criteria

Positive Equivocal Negative

	Test Cells	Control Cells
Positive: Strong	Full cell pattern covering the bottom of the well.	Negative button
Weak Positive	Cell pattern covers approx. 1/3 of the well bottom.	Negative button
Equivocal	Cell pattern shows a distinctly open centre.	Negative button
Negative	Cells settled to a compact button, typically with a small clear centre.	Negative button
Non-specific reaction	Positive reaction.	Positive reaction

A sample where the Test Cell well is non-reactive should be considered as **negative for *T. pallidum***.
Reactivity less than equivocal is considered negative.

Any specimen giving equivocal or positive results should be considered as positive and the test procedure repeated as above, in duplicate, adding the Control Cells (R2) provided to one set of cells and Test cells (R1) to the other.
If at least in one of the well with Test cells (R1), the result is equivocal or positive, the specimen should be considered as **positive for *T. pallidum***.

For non-specific reaction, if the agglutination is greater in the Test Cells (R1) than in the Controls Cells (R2), then the sample is considered positive and should be repeated as above.
When a sample has greater or equal agglutination in the Control Cells (R2) then the sample should be absorbed using the following procedure.

7.5. Absorption of non-specific reactions
(Procedure to be used if agglutination is seen in both Test and Control cells).
1. Add 10 µl of specimen to 190 µl of resuspended Control Cells, mix well and incubate at room temperature for 30 minutes.
2. Centrifuge to deposit the cells at a minimum of 1500g for 3 minutes.
3. Add 25 µl of supernatant from step 2 to each of 2 wells.
4. Gently mix the Test and Control Cells to ensure thorough resuspension.
   Add 75 µl of Test Cells to the 1st well.
   Add 75 µl of Control Cells to the 2nd well.
5. Ensure thorough mixing and incubate at room temperature for a minimum of 45 minutes.
6. Read and interpret the settling patterns as above.

8. TEST LIMITATION

No single test or definitive reference standard is available for every stage of the disease. Thus, Syphilis diagnosis relies predominantly on serological testing, requiring results from both non-treponemal and treponemal methods.

No diagnostic test provides absolute assurance that a sample does not contain low levels of antibodies to *T. pallidum*, such as those present at a very early stage of infection. Therefore, a negative result at any time does not preclude the possibility of exposure to infection with syphilis.

All treponemal test results tend to remain reactive following treponemal infection therefore they should not be used to evaluate response to therapy. Because of the persistence of reactivity, generally for the life of the patient, the treponemal tests are of no value in determining relapse or re-infection in a patient who had a reactive result. In this case, it is recommended to use other assays: Syphilis Total Ab, Syphilis IgM EIA and RPR.

9. PERFORMANCES CHARACTERISTICS

9.1. Precision Study

Precision study was realized by testing 3 samples (1 negative, 1 low positive and 1 positive) in 10 replicates in the same run (repeatability) and in 2 replicates during 5 days on 2 different lots and reading by 2 operators (Intermediate precision).
Repeatability: The 3 samples gave identical results when repeated 10 times.
Intermediate/ inter-lot precision: The 3 samples gave identical results when tested in the different conditions (40 times).

9.2. Clinical performance

The performances of the TPHA assay have been determined by testing samples from random blood donors, hospitalized patients, patients infected with syphilis and on samples positive for markers unrelated to infection by the *T. pallidum*.
The studies were carried out on 2 blood donor sites and at the Bio-Rad site.

9.2.1. Specificity

A total of 5032 samples from blood donors prospectively collected in 2 different sites were studied.
The samples were either serum (3626), EDTA K2 (539) or Lithium Heparin plasma (867) tested in a period of less than 7 days after sampling and were compared to the screening assay used in the laboratory.

Table 1 : Blood donor population

Site	Number	Initial Reactive samples (IR)	Repeated Reactive samples (RR)	Specificity (%)	95% Confidence Interval
Site # 1	2519	18	7	99.72% (2512/ 2519)	99.43% – 99.89%
Site # 2	2513*	20	7	99.72% (2505 / 2512)	99.43% – 99.89%
Total	**5032**	**38 (8 positive, 30 equivocal)**	**14 (3 positive, 11 equivocal)**	**99.72% (5017/5031)**	**99.53% – 99.85%**

**: one sample (true positive) was withdrawn from the calculation.*

The total specificity on the blood bank population is equal to **99.72% (5017/5031)** with a 95% confidence interval of [99.53% – 99.85%]. Among the 14 false positive samples, 11 were found repeated equivocal.

A retrospective study was also carried out on 201 frozen samples from patients from a Sexually Transmitted Disease Center or from vendors, which were found negative for syphilis.

Specificity on these samples is found at **99.5% (200/201)** with a 95% confidence interval at **[97.3%- 100.0%]**.

9.2.2. Sensitivity

The sensitivity study was studied on 435 retrospective frozen serum samples from the laboratory of Sexually Transmitted Disease Center, or from frozen samples from vendors. These samples were characterized positive by immunoassays, FTA assay, RPR/ VDRL assay or TPHA assays according to their origin.
All the samples were tested first with the CE mark TPHA assay and then with the Bio-Rad TPHA 500 assay (72504).
Sensitivity on this population is found at **100% (435/435)** with a 95% confidence interval at **[99.2%-100.0%]**.

9.3. Analytical sensitivity

Analytical sensitivity was tested against the WHO International Standard for Syphilis NIBSC code 05/132. Using the semi-quantitative protocol, the sensitivity was found at 0.05 IU/mL.

9.4. Analytical Specificity / Cross Reactivity Study

210 potentially interfering samples containing antibodies against pathogens that could lead to infectious illnesses (Cytomegalovirus, Epstein Barr virus, VZV, Rubella virus, Hepatitis C virus, Hepatitis B virus, HIV 1/2, HTLV 1/2, *Toxoplasma gondii*, Dengue, Malaria, Leptospirosis), samples from pregnant women and multiparous women or samples from patients with immune system disorders (autoantibodies (SLE), Rheumatoid factors), were tested with the TPHA assay. Four (4) true positive samples were discarded from the calculation.

One sample was found repeatable positive with the TPHA test.

The specificity observed on this target population of **99.5 % (205/206)** was similar to the specificity of clinical samples.

9.5. Prozone Effect

The existence of a possible prozone effect was studied by testing 3 samples with high titers (≥ 1:20480) at different dilutions. The equivalence of results observed among non-diluted and diluted samples indicates the absence of the prozone effect.

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