

# Phoenix Bio-Tech Corp.

## Trep-Chek™ Syphilis Test Kit

96 Tests Catalog No. TP-96

960 Test Kit Catalog No. TP-960

### SUMMARY OF PROCEDURE

1. Prepare 1:20 dilutions of Cut-Off Calibrator, Controls and patient samples in Sample Diluent. Mix well.
2. Add 100 µl of Cut-Off Calibrator, Controls and samples into the wells.
3. Incubate at room temperature (18-30° C) for 30 ± 2 min.
4. After incubation, discard the contents of the wells. Wash the wells 4 times with Wash Solution.
5. Add 100 µl of Conjugate to each well.
6. Incubate at room temperature for 30 ± 2 min.
7. Wash the wells as in #4 above.
8. Add 100 µl Substrate Solution to each well.
9. Incubate at room temperature for 15 ± 2 min.
10. Add 100 µl Stop Solution to each well.
11. Read the absorbances at 450/610-630 nm.

### INTENDED USE

The *Syphilis* TREP-CHEK™ Test Kit is a confirmatory *in vitro* enzyme immunoassay for the qualitative detection of *Treponema pallidum* IgG antibodies in human serum or plasma. This product is not cleared (approved) by the U.S Food and Drug Administration (FDA) for use in screening blood or plasma donors.

### SUMMARY AND EXPLANATION

Serological tests for syphilis were first used in 1906 with the development of the nontreponemal (reagin) test by Wasserman.<sup>1</sup> Soon, many tests were developed, each with its own modification of a lipoidal antigen, a crude alcohol extract of liver, heart, or other animal organs. The earliest tests were based on the complement fixation procedure of Bordet and Gengou.<sup>2</sup> All nontreponemal tests measure both immunoglobulin G (IgG) and M (IgM) anti-lipid antibodies formed by the host in response both to lipoidal material released from damaged host cells early in infection and to lipid from the cell surfaces of the treponeme itself.<sup>3</sup> The nontreponemal tests for syphilis in use today are flocculation tests using the basic antigen formula of the Venereal Disease Research Laboratory (VDRL) test, which contains cardiolipin, cholesterol and lecithin.<sup>4</sup> The rapid plasma reagin (RPR) test uses a stabilized suspension to which charcoal particles have been added.<sup>4</sup> The antigen is not attached to these particles, but the charcoal is trapped in the lattice formed by the antigen-antibody complex when reactive serum is added. Because of either the lipid nature of the antigen or some unusual property of the antibodies, the antigen-antibody reaction remains suspended, and flocculation occurs, rather than agglutination or precipitation as in most other serologic tests.<sup>4</sup>

Currently two treponemal tests are considered the standard for syphilis testing. The FTA-ABS test is an indirect immunofluorescent antibody test. The FTA-ABS test is sensitive and must be well controlled. Hemagglutination (MHA-TP) tests using treponemal antigen for *T. pallidum* have gained acceptance since their emergence in the mid-1960's (5-7) as a confirmatory procedure following a reactive nontreponemal assay or as a screening procedure.

The assay methods described all require visual interpretation and are limited to subjective interpretation by the operator. The use of the more specific enzyme immunoassay has received widespread acceptance due to the colorimetric results in a 96 well microplate format that can be automated and read photometrically.

The *Syphilis* TREP-CHEK Test Kit uses recombinant antigens in an enzyme immunoassay format to yield a more specific result with objective reading of absorbances compared to a cut-off calibrator.

### PRINCIPLE OF THE PROCEDURE

The *Syphilis* TREP-CHEK is an enzyme immunoassay utilizing specific recombinant treponemal antigens immobilized to microwells. Anti-treponemal specific antibodies in the patient's serum or plasma bind to the antigens. Non-bound proteins are removed during the washing step. Horseradish peroxidase-labeled goat anti-human IgG is added to bind with the human antibodies on

the well. The unbound conjugate is removed by a second washing step. Then the chromogenic substrate TMB is added. After an appropriate incubation period the reaction is stopped by the addition of acid and the absorbance of the resulting color is measured photometrically with an EIA reader (450 nm). Color intensity is proportional to the amount of specific antibody present in the patient's sample. The whole assay can be performed in less than 2 hours.

### REAGENTS

*Each Syphilis TREP-CHEK Test Kit contains reagents for 96 tests or for 960 tests.*

|                        |   |
|------------------------|---|
| Antigen Wells          | Twelve, 8-well microwell strips, coated with specific recombinant antigen.<br>One hundred and twenty, 8-well microwell strips, coated with specific recombinant antigen.  |
| Negative Control       | One vial with green cap containing 1.0 ml of non-reactive human serum.<br>Three (3) vials with green caps each containing 1.0 ml of non-reactive human serum.<br>Contains 0.05% Proclin® 300.   |
| Cut-Off Calibrator     | One vial with yellow cap containing 1.0 ml human serum.<br>Three (3) vials with yellow caps each containing 1.0 ml human serum.<br>Contains 0.05% Proclin® 300.   |
| Positive Control       | One vial with red cap containing 1.0 ml of reactive human serum.<br>Three (3) vials with red caps each containing 1.0 ml of reactive human serum.<br>Contains 0.05% Proclin® 300.   |
| Sample Diluent (10X)   | One bottle containing 10 ml (10X) concentrate phosphate buffer based Sample Diluent (10X). Shake well before use.<br>One (1) bottle containing 100 ml (10X) phosphate buffer based Sample Diluent (10X).  |
| Wash Concentrate (10X) | One bottle containing 100 ml phosphate buffered saline and Tween 20.<br>Four (4) bottles containing 250 ml phosphate buffered saline and Tween 20.  |
| Conjugate              | One bottle containing 13 ml goat anti-human IgG (γ chain specific) labeled with horseradish peroxidase.<br>One bottle containing 125 ml goat anti-human IgG (γ chain specific) labeled with horseradish peroxidase.<br>Contains Proclin® 300 (0.05% active ingredient). |
| TMB Substrate          | One amber bottle containing 13 ml 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide at 0.03%.<br>One amber bottle containing 125 ml 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide at 0.03%.   |
| Stop Solution          | One bottle containing 13 ml 1N Sulfuric acid.<br>Five (5) bottles containing ≤30 ml 1N Sulfuric acid.   |

**CAUTION:** Solution is toxic. Avoid contact with skin or eyes. If contact is made, flush area with copious amounts of water.

**Store these reagents at 2 to 8° C.**

### OTHER MATERIALS REQUIRED

- Wash bottle or automated microplate washer
- Pipettors capable of dispensing appropriate volumes
- Timer
- One liter graduated cylinder
- One liter wash solution reservoir
- Deionized or distilled water
- Absorbent toweling
- Tubes or microwell plate for sample dilution
- Reader capable of reading absorbance at 450 nm, reference at 610-630 nm

## WARNINGS AND PRECAUTIONS

**REAGENTS:** For *in vitro* Diagnostic Use.

### 1. Reagents Containing Human Source Material

#### Treat as potentially infectious.

- Each serum/plasma donor unit used in the preparation of this product has been tested by a U.S. FDA approved method and found non-reactive for the presence of HBsAg, antibody to HCV, and antibody to HIV 1/2. While these methods are highly accurate, they do not guarantee that all infected units will be detected. This product may also contain other human source material for which there is no approved test. Because no known test method can offer complete assurance that hepatitis B virus (HBV), hepatitis C virus (HCV), Human Immunodeficiency Virus (HIV), or other infectious agents are absent, all products containing human source material should be handled in accordance with good laboratory practices using appropriate precautions as described in the U.S. Centers for Disease Control and Prevention/National Institutes of Health Manual, "Biosafety in Microbiological and Biomedical Laboratories," 4<sup>th</sup> ed., May 1999 or current edition.
- The concentrations of *Treponema pallidum* IgG antibodies in a given specimen determined with assays from different manufacturers can vary due to differences in assay methods and reagent specificity.
  - Never pipette by mouth.
  - Avoid contact with open skin and mucous membranes.
  - Avoid contamination of the TMB substrate solution with conjugate or other oxidants which will cause the solution to change color prematurely.
  - The substrate contains 3,3',5,5' tetramethylbenzidine (TMB) which has shown possible mutagenic effects in laboratory experiments. Substrate is an irritant.
  - All components in this kit have been tested as a unit. Do not intermix components from different kit lots or other manufacturer's kits.
  - Do not use reagents beyond their expiration date.
  - Store unused reagents at 2 to 8° C. Diluted Wash Solution can be stored for up to 1 month at 2 to 8° C.
  - Incubations above or below the recommended temperatures or times may give erroneous results.
  - The EIA method is a very sensitive technique. Maintain consistent pipetting technique, incubation times, and temperature conditions throughout the test procedure. Cross contamination between reagents can invalidate the test. Use separate pipette tips for each sample or reagent. *Do not pour reagents back into vials.*
  - Coated microwells should be stored with the desiccant in the resealable bag provided and returned to the refrigerator immediately after use. Opened and unused strips are stable up to 30 days at 2 to 8° C.
  - The washing procedure is very important and requires special attention. (Please refer to the Procedure section.)

**NOTE:** *Improperly washed wells may give erroneous results.*

### SPECIMEN COLLECTION

Whole blood should be collected by accepted medical techniques. Separated serum or plasma should remain at 22° C for no longer than 8 hours. If assays are not completed within 8 hours, specimens should be refrigerated (2 to 8° C). If assays are not completed within 48 hours, or the separated sample is to be stored beyond 48 hours, samples should be frozen at -20° C. Do not use frost-free freezers. Do not inactivate plasma. Avoid multiple freeze-thaw cycles. Prior to testing, bring frozen specimens to room temperature slowly and mix gently, avoiding foam formation. Specimens containing visible particulate matter should be clarified by centrifugation before testing. Grossly contaminated, hemolyzed, lipemic, or icteric specimens should not be used. The NCCLS provides recommendations for collecting and storing blood specimens.<sup>8</sup>

### PROCEDURE

Allow all test components and patient samples to warm to room temperature before use. Invert reagent bottles gently several times before use. Return promptly to the refrigerator after use.

Prepare Sample Diluent by diluting 10 ml (100 ml) of Sample Diluent (10X) to a final volume of 100 ml (1000 ml) with deionized or distilled water.

Diluted Sample Diluent can be stored for up to 60 days at 2 to 8°C.

Prepare Wash Solution by diluting 100 ml of Wash Concentrate (10X) to a final volume of 1000 ml with deionized or distilled H<sub>2</sub>O. Diluted Wash Solution can be stored for up to one (1) month at 2 to 8°C.

- Prepare 1:20 dilutions of the Cut-Off Calibrator (in triplicate), controls and patient samples in Sample Diluent (e.g., by addition of 20 ul sample to 380 ul Sample Diluent.).
- Mix sample dilutions gently by withdrawing and expelling in a pipette tip 2 or 3 times or by vortex mixing for 2 or 3 seconds. Transfer 100 ul of diluted Cut-Off Calibrator (in triplicate), Negative Control, Positive Control, and patient samples to subsequent antigen wells. Avoid formation of bubbles when transferring diluted samples.
- Allow the wells to incubate at room temperature (18 to 30°C) for 30 ± 2 minutes.
- Aspirate or discard the contents of the wells. Remove any excess moisture in the wells by tapping on paper toweling if necessary. Wash the wells by rinsing 4 times with at least 300 µl per well of Wash Solution. Remove excess moisture from the wells after washing. When using an automated washer, follow the manufacturer's instructions.
- Place 100 µl of Conjugate into each well, avoiding bubble formation.
- Allow the wells to incubate uncovered at room temperature (18 to 30°C) for 30 ± 2 minutes.
- Wash the wells as described in Step 4 above.
- Place 100 µl of Substrate into each well, avoiding bubble formation.
- Allow the wells to incubate uncovered at room temperature (18 to 30°C) for 15 ± 2 minutes.
- Place 100 µl of Stop Solution into each well, avoiding bubble formation.
- Mix well contents thoroughly and let stand for 5 minutes.
- Read the absorbance of each well at 450 nm. A suitable reference wavelength (e.g., 610-630 nm) reading should be used.

**NOTE:** *The developed color is stable for 30 minutes. Read the absorbances during this time.*

### QUALITY CONTROL

Positive and Negative Controls are supplied with the kit. The controls verify test performance, test integrity and operator reliability. Good laboratory practice dictates running the positive and negative control each time the kit is used. Additional controls must be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations. For guidance on appropriate quality control practices please refer to NCCLS C24-A, Internal Quality Control Testing: Principles and Definitions. If the results of the positive and/or negative control are not within the Index range (as stated on the Quality Control Certificate), the test results are invalid and the assay should be rerun. The Quality Control Certificate is provided in each kit as an aid in verifying the performance of the test by the laboratory.

### RESULTS

#### 1. Calculation

Note that the cut-off for this test is calibrated against a panel of confirmed syphilis positive sera, Calibrators and confirmed syphilis negative sera.

Calculate the mean absorbance of the Cut-Off Calibrator. Note: When calculating the mean absorbance value for the Cut-Off Calibrator exclude any absorbance value that deviates by more than 20% from the mean of the three absorbance values. Use the mean of the remaining two replicates in the calculation. Exclusion of more than one of the three absorbance values invalidates the run. Determine the Index Value for each patient sample or control using the following formula:

$$\frac{\text{Absorbance of Sample}}{\text{Mean Absorbance of Cut-Off Calibrator}} = \text{Index Value of Sample}$$

#### 2. Interpretation

The following is a guide to interpretation of results. Each laboratory is encouraged to establish its own "normal" ranges based on populations encountered.

| Index Value | Interpretation |
|-------------|----------------|
| <0.9        | Negative       |
| 0.9 to 1.1  | *Equivocal     |
| >1.1        | Positive       |

\* Specimens with values in the equivocal range should be retested. If the sample remains equivocal the patient should be considered suspect for disease since a low level of antibody is detected. A new sample should be obtained and retested. If the patient remains inconclusive, the patient should be monitored for antibody status.

### LIMITATIONS

1. The results obtained from this assay are intended to aid in diagnosis only. As with all serological tests for syphilis, interpretation of results obtained with the *Syphilis* TREP-CHEK Test Kit must be used in conjunction with the patient's clinical symptoms, medical history and other clinical and/or laboratory findings to produce an overall clinical diagnosis.
2. The *Syphilis* TREP-CHEK is specific for detecting *Treponema pallidum* antibodies in serum or plasma. It does not detect *T. pallidum* directly.
3. Test instructions must be rigidly followed to obtain optimum results.
4. Use fresh serum or plasma or specimens frozen and thawed only once. Specimens that are improperly stored or subjected to multiple freeze-thaw cycles may yield spurious results.
5. Specimens giving equivocal (inconclusive) results in the assay should be retested. A repeated equivocal (inconclusive) specimen should be reported as equivocal (inconclusive) for follow-up and another specimen drawn in two weeks for testing and/or confirmed by other methods such as FTA-ABS or Western Blot.
6. All treponemal tests tend to remain reactive following treponemal infection; therefore, they should not be used to evaluate response to therapy. Because of the persistence of reactivity, probably for the life of the patient, the treponemal tests are of no value to the clinician in determining relapse or re-infection in a patient who has had a reactive result.
7. Reproducible results depend on careful pipetting, observation of incubation periods and temperature, as well as rinsing the test strips and thorough mixing of all prepared solutions.
8. Do not scratch coated wells during rinsing and aspiration. Rinse and fill all reagents with care. While rinsing, check that all wells are filled evenly with Wash Solution and that there are no residues in the wells.
9. Instructions for appropriate photometers are to be observed; check adjustment of proper wavelength and reference wavelength respectively.

### EXPECTED VALUES

To evaluate the prevalence of antibody to syphilis in the *Syphilis* TREP-CHEK and confirm a cut-off for the assay, three hundred and sixty-six (366) serum and plasma samples were obtained. Ninety-one (91) serum samples from healthy asymptomatic adults were obtained from a Canadian Health Center. One hundred and fifty-one (151) matched serum and plasma from donors aged 18 to 78 years were obtained from a blood bank in the Northeast US and one hundred and twenty-four (124) plasma samples from normal blood donors in the Southern US. The samples were tested using the *Syphilis* TREP-CHEK Test Kit. Of these 366 specimens, two (2) were found to be equivocal (inconclusive) (0.5%) and one (1) low positive (0.3%) with the remaining samples negative (98.9%). These results are consistent with published rates for prevalence of antibody in the adult population. Prevalence may vary depending on a variety of factors such as geographical, age, socio-economic status, ethnic background, type of test employed, specimen collection and handling procedures and clinical and epidemiological history.

### PERFORMANCE CHARACTERISTICS

#### 1. Comparison

Studies were conducted which compared the *Syphilis* TREP-CHEK Test Kit to the MHA-TP and RPR using a total of 207 sera specimens from physician referred patients suspected as positive for syphilis or known positive based on history. A total of 366 specimens from healthy adults and blood donors considered as normal population, randomly collected including both males and females, were also evaluated in the *Syphilis* TREP-CHEK Test Kit.

#### Documented Positive Primary and Secondary Samples

An additional panel of 100 documented VDRL and RPR reactive syphilis sera, obtained from patients medically diagnosed with primary and secondary stage infection were tested to assess the performance of the *Syphilis* TREP-CHEK Test Kit. Half of the patients were on penicillin therapy at the time the blood was drawn. This panel consisted of specimens from 27 treated-primary infection, 23 untreated-primary infection, 24 treated-secondary stage, and 26 untreated-secondary stage patients. A small study of 16 primary patients, confirmed by dark field observation, were tested with the *Syphilis* TREP-CHEK Test Kit and Western Blot analysis. One sample was initially negative in the *Syphilis* TREP-CHEK but was positive on retest. The *Syphilis* TREP-CHEK Test Kit identified

all samples reactive and demonstrated 100% agreement to RPR and the reference MHA-TP results. Incubating-stage syphilis samples were not specifically identified and tested in these studies.

### Potential Cross Reactors

A panel of 74 samples was obtained to evaluate potential interference from different disease conditions. These samples were confirmed positive for each respective disease condition and were assayed in the *Syphilis* TREP-CHEK Test kit, TP-PA and MHA-TP tests. These specimens were medically diagnosed for Systemic Lupus Erythematosus (SLE), *Toxoplasma*, *H. pylori*, Arthritis (RF), drug addiction and Lyme Disease. The number of disease condition categories and reactive results obtained is listed in TABLE 1. The *Syphilis* TREP-CHEK showed no cross-reactivity in the samples evaluated, except for one *Syphilis* TREP-CHEK equivocal sample in the Lyme Disease samples. One *H. pylori* sample was 1+ reactive with the MHA-TP and +/- reactive with the TP-PA, but negative in the *Syphilis* TREP-CHEK assay. One *Syphilis* TREP-CHEK reactive patient sample in the drug addiction category was also reactive with the reference MHA-TP and TP-PA assays.

TABLE 1

| Category     | Number Tested | <i>Syphilis</i> TREP-CHEK Number Reactive |
|--------------|---------------|---|
| Drug Users   | 10            | 1*  |
| Toxoplasma   | 10            | 0   |
| SLE          | 10            | 0   |
| Lyme Disease | 24            | 1**                                       |
| H. Pylori    | 10            | 0   |
| Arthritis    | 10            | 0   |

\* This sample was also positive with the reference methods

\*\* This sample had an equivocal result

### Normal Population

A total of 366 specimens from healthy donors considered as a normal population randomly collected including both males and females were evaluated. All the samples were found negative by RPR. Two (2) samples found equivocal (inconclusive) with the *Syphilis* TREP-CHEK were treated as unresolved and not used for the calculations of specificity. One sample found positive with the *Syphilis* TREP-CHEK was also positive by Western Blot. This sample was not corrected as "positive" for the calculation of specificity. The results of testing a normal population yielded Specificity of 99.7%.

### Positive Patient Samples

TABLES 2 and 3 demonstrate the performance of the *Syphilis* TREP-CHEK Test Kit compared to the MHA-TP using 257 known/suspected positive and negative patient samples and the overall performance of the *Syphilis* TREP-CHEK Test Kit using all samples (including cross reactors) tested versus the reference methods, i.e. MHA-TP, RPR, Western Blot and dark field observation. Sensitivity and specificity determinations were made with 95% Confidence Intervals (CI) calculated by the Exact Method.

Samples that resulted in Index (ratio) Values in the equivocal (inconclusive) range (0.9-1.1) were considered unresolved and not used in the calculations of sensitivity and specificity.

TABLE 2  
Suspected/Known Positive Syphilis Patients  
MHA-TP

|            | Positive | Negative | Total |
|------------|----------|----------|-------|
| TREP- CHEK |          |          |       |
| Positive   | 208      | 0        | 208   |
| Negative   | 1        | 48       | 49    |
| Total      | 209      | 48       | 257   |

\*\*95% CI

|             |          |               |
|-------------|----------|---------------|
| Sensitivity | = 99.5%  | 97.4 - 100.0% |
| Specificity | = 100.0% | 92.6 - 100.0% |
| Agreement   | = 99.6%  | 97.8 - 100.0% |

**TABLE 3**  
**Total Performance**  
**REFERENCE METHODS**

|            |              |          |          |       |
|------------|--------------|----------|----------|-------|
|            |              | Positive | Negative | Total |
| TREP- CHEK | Positive     | 225      | 1        | 226   |
|            | Negative     | 1        | 433      | 434   |
|            | <i>Total</i> | 226      | 434      | 660   |

\*\*95% CI

|             |         |               |
|-------------|---------|---------------|
| Sensitivity | = 99.6% | 97.6 - 100.0% |
| Specificity | = 99.8% | 98.7 - 100.0% |
| Agreement   | = 99.7% | 98.9 - 100.0% |

**Reproducibility**

Studies were performed to demonstrate the inter-day, inter-lot and intra-assay reproducibility of the *Syphilis* TREP-CHEK Test Kit.

The intra-assay reproducibility study used 6 serum samples: 2 low positive, 2 mid-positive, 1 high positive and 1 negative sample run in twelve wells each within one assay. The inter-day study used 6 serum samples: 1 low positive, 2 mid-positive, 1 high positive and 1 negative. These 6 samples were run on 6 different days to demonstrate the day-to-day reproducibility. The inter-lot assay reproducibility study used 6 serum samples: 3 low positive, 2 mid-positive and 1 negative. These 6 samples were run on 3 different lots of kits. The following table demonstrates the intra-assay, inter-assay and inter-lot reproducibility of the *Syphilis* TREP-CHEK Test Kit.

**TABLE 4**  
Intra-Assay, Interassay and Inter-Lot Precision for the  
*Syphilis* TREP-CHEK

| SAMPLE | INTRA-ASSAY |       |      | INTERASSAY |       |     | INTER-LOT |       |      |
|--------|-------------|-------|------|------------|-------|-----|-----------|-------|------|
|        | MEAN OD     | SD    | CV%  | MEAN OD    | SD    | CV% | MEAN OD   | SD    | CV%  |
| 1      | 0.026       | 0.007 | 26.9 | 0.258      | 0.022 | 8.5 | 0.289     | 0.020 | 7.1  |
| 2      | 0.351       | 0.024 | 6.6  | 0.462      | 0.026 | 5.5 | 0.372     | 0.022 | 6.1  |
| 3      | 0.340       | 0.025 | 7.4  | 0.691      | 0.043 | 6.2 | 0.426     | 0.044 | 10.3 |
| 4      | 0.702       | 0.039 | 5.5  | 2.696      | 0.040 | 1.6 | 0.395     | 0.036 | 9.1  |
| 5      | 0.701       | 0.040 | 5.7  | 1.999      | 0.084 | 4.2 | 1.456     | 0.160 | 11.0 |
| 6      | 2.512       | 0.065 | 2.6  | 1.281      | 0.057 | 4.5 | 1.580     | 0.038 | 2.4  |

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Patent 5,681,934

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