

# Laboratory Procedure Manual

*Analyte:*       **Syphilis**

*Matrix:*       **Serum**

*Method:*       TREP-CHECK ***Treponema pallidum* (TP-PA) Enzyme Immunoassay Test**

*Method No.:*

*Revised:*

*as performed by:*       *Division of HIV, STD and TB Laboratory Research  
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## **Important Information for Users**

CDC periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

## Public Release Data Set Information

This document details the Lab Protocol for NHANES 2003–2004 data.

A tabular list of the released analytes follows:

<b>Lab Number</b>	<b>Analyte</b>	<b>SAS Label</b>
136_c	LBDSY4	<i>Treponema pallidum</i> EIA (TP-PA)

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The TREP\_CHEK (TP-PA) EIA is a confirmatory in vitro diagnostic enzyme immunoassay for the qualitative detection of *Treponema pallidum* IgG antibodies in human serum or plasma. This product is not cleared by the U.S. Food and Drug Administration (FDA) for use in screening blood or plasma. This test is intended for use by clinical diagnostic laboratories as an aid in diagnosis of syphilis.

Specific, recombinant treponemal antibodies are immobilized on the microplate wells. Participant samples and controls are added to the wells. Anti-treponemal antibodies, if present, will specifically bind to the immobilized antigens; all non-bound proteins are removed during the washing step. The antigen-antibody complex is subsequently reacted with anti-human IgG antibodies conjugated with horseradish peroxidase (HRPO). After a second wash, which removes the unbound conjugate, a chromogenic reaction takes place on the plate as a result of addition of the TMB, a substrate for the peroxidase. The resulting color is measured spectrophotometrically after adding stop solution. Color intensity is proportional to the amount of antibody present in the participant's sample.

## 2. SAFETY PRECAUTIONS

Follow universal precautions. Wear gloves, a lab coat, and safety glasses while handling human serum. Place disposable plastic, glass, and paper (e.g., pipette tips, gloves) that come in contact with serum in a biohazard autoclave bag. Keep these bags in appropriate containers until they are sealed and autoclaved. When work is finished, wipe down all work surfaces where serum was handled with a 10% (v/v) sodium hypochlorite solution.

Avoid skin contact with TMB substrate, stop solution (sulfuric acid), rinsing and dilution buffer. In case of accidental contact, wash thoroughly with tap water.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

- A. Each shipment of specimens received from the NHANES mobile unit contains a corresponding transmittal sheet and an ASCII data file (KOUTPUT.TXT). The data file, containing the specimen ID, collection date, and type of sample (i.e., whole blood, serum, plasma) is checked against the information on the transmittal sheet and specimen label prior to the assay.
- B. After the data is calculated and the final values are approved by the reviewing supervisor for release, all results are entered.
- C. After the results are entered, back up copies are made and stored in locked areas.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

- A. No special instruction such as special diet or fasting is necessary.
- B. Fresh serum samples are the specimens of choice for the TREP\_CHEK TP-PA test. Serum specimens may be collected using regular red-top or serum separator Vacutainers. Specimens are allowed to clot at room temperature and centrifuged.
- C. Transfer serum to 2-mL polypropylene screw-capped vials. Freeze at  $\leq -20^{\circ}\text{C}$ .
- D. Each week, batches of frozen serum samples are placed in a Styrofoam-insulated shipping container with dry ice and sent to the laboratory by an overnight courier.
- E. Serum specimens are stable up to 72 hours at  $2-8^{\circ}\text{C}$ . For longer periods, store the serum at  $\leq -20^{\circ}\text{C}$  in plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.
- F. Excessively hemolyzed, contaminated, or lipemic sera may give aberrant results and should not be used. A specimen is too hemolyzed for testing when printed material cannot be read through it.

- G. The optimal amount of serum is 0.5 to 1.0 mL. Specimen volumes of less than 0.4 mL are not acceptable.
- H. Avoid repeated freeze-thawing cycles, which may compromise specimen integrity.
- I. Specimens should generally arrive frozen.
- J. Residual samples are frozen at  $\leq -20^{\circ}\text{C}$ .

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure

6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

A. Instrumentation

- (1) Micropipettes to deliver 5 to 200  $\mu\text{L}$ .
- (2) Automatic vibratory shaker

B. Other Materials

- (3) Disposable, clear plastic trays with 8 rows of 12 U-shaped wells each.
  - (1) 1.0- or 2.0-mL serologic pipettes graduated in 1/100 mL.
  - (2) Safety pipetting devices for serologic pipettes.
  - (3) Tray viewer.
  - (4) 2-mL and 10-mL serologic pipettes
  - (5) Latex gloves, safety glasses, and protective clothing.
  - (6) Discard container and disinfectant.

C. Reagent Preparation

Each TREP\_CHEK TP-PA kit contains enough reagents to test 96 samples and the controls. Reagents should be mixed gently to avoid possible deterioration of the antigen-carrier complex. Reagents are stable until the expiration date printed on the label. All reagents should be stored at  $2-8^{\circ}\text{C}$ .

- (1) Preparation of sample diluent: one diluent buffer (10 x concentrate) 1 bottle of 10 mL.  
Dilute the content of the vial containing concentrated sample diluent buffer with distilled water to a final volume of 100 mL, prior to use.
- (2) Preparation of Wash Buffer(10 x concentrate) 1 bottle of 100 mL  
Dilute the content of the bottle containing concentrated wash buffer containing concentrated wash buffer with diluted water to a final volume of 1 liter.
- (3) Positive control, 1 vial of 500  $\mu\text{L}$ . Stabilized human sera.  
The optical density of the positive control should be higher than the mean OD of cut-off calibrator.
- (4) Negative 1 vial of 500  $\mu\text{L}$  . Stabilized human sera.  
The optical density of the negative control should be lower than 0.25.

(10) Horseradish peroxidase labeled anti-human IgG antibody conjugate 1 vial of 13 mL(ready to use).

(11) Substrate solution TMB(tetramethyl benzidine) 1 vial of 13 mL(ready to use).

D. Preparation of Control Serum Samples

(1) Positive Control Serum

Ready to use. Bring to room temp before use.

(2) Negative Control Serum

Ready to use. Bring to room temp before use.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A. Working Standards

The positive control is used to determine level of reactivity of the test for lot to lot comparison and as an indication of whether reagents are deteriorating.

Pipettors and Tips

With the pipettors currently available, the measurement of small serum volumes is routine. Most manufacturers include in the specifications of the pipettors the accuracy for frequently used microliter volumes. Daily use may affect pipettors, making them lose their initial accuracy. The difference in disposable tips from sources other than the pipette manufacturer is probably the most common error. For budgetary reasons, a less expensive brand of pipette tips may be substituted for those of the manufacturer. Although the less expensive brand may be satisfactory, the laboratory should verify the accuracy of the substitute pipette tips in their system. Commercial kits to check the accuracy are available. Also, manufacturers provide procedures for checking the accuracy of their equipment. Historically, the gravimetric or spectrophotometric procedures, which use the weight of water or absorbance of a substance at a given wavelength, have been the most accepted methods used to calibrate pipettors. These procedures should not be used instead of those specified by the manufacturer nor do they substitute for an annual verification and repair by a company qualified to do this.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Preliminaries

(1) Bring all reagents and serum samples to room temp before beginning test.

(2) The positive and negative controls should be included in every run.

B. Sample Preparation

Samples are ready to be diluted the same as all participant samples.

C. Test Procedure

(1) Allow all reagents to reach room temperature.

(2) The kit control sera should be included in each run.

(3) Dilute all participant sera and controls 1:20, by adding 20  $\mu$ L of participant sample to 380  $\mu$ L of sample diluent.

(4) Dispense 100  $\mu$ L of diluted positive and negative control.

(5) Dispense 100  $\mu$ L of diluted participant samples into respective wells, (Transfer within 5 minutes)

(6) Dispense diluted cut-off calibrator in duplicate at the end of run

(7) Incubate at room temperature for 30 minutes.

- (8) Discard the contents of the wells and wash 4 times with wash buffer.
- (9) Add 100 µL of TMB substrate solution into each well.
- (10) Incubate at room temperature for 15 minutes, protect from light.
- (11) Add 100 uL of stop solution and let stand for 5 minutes.
- (12) Read the optical density at 450 nm. and calculate results. Bi-chromatic measurement with a reference filter at 600-690 nm is recommended.
- (13) Interpretation of Results

The following is intended as a guide to interpretation of the EIA results; each laboratory should establish their own criteria for test interpretation based on sample population.

**Table 1. Interpretation of agglutination patterns**

Ratio	Interpretation
Less than 0.9	Negative
0.9-1.1	Equivocal
Greater than 1.1	Positive

The optical density of the calibrator should not vary more than 20%.

D. Recording of Data

(1) Quality Control Data

Record lot number of kit, and date of testing

(2) Analysis

Read and record test results.

E. Replacement and Periodic Maintenance of Key Components

All pipettors should be checked, repaired, and recalibrated at least yearly.

F. Calculations

Calculate the ratio of participant OD over OD mean of cut-off calibrator multiplied by lot specific factor

(f).

$$\frac{\text{OD of samples}}{\{\text{mean OD of cut-off calibrator} \times (f)\}}$$

The value of factor f is lot dependent and is provided in the Quality Control Sheet.

G. Special Method Notes

(1) Always keep the upper surface of the microplates free of excess fluid droplets to prevent possible dilution of well contents. Blot reagent overflows.

(2) Liquid reagents are clear with no precipitates.

- (3) Only reagents from the same kit are used. Mixing reagents from different lot numbers of kits may give erroneous results.
- (4) Do not cross-contaminate reagents. Always use a new pipette tip when drawing from stock reagent bottles.

## 9. REPORTABLE RANGE OF RESULTS

Results are reported as positive, negative, or equivocal.

## 10. QUALITY CONTROL (QC) PROCEDURES

### A. Evaluation of TP-PA kits is the responsibility of the user.

Reagents evaluated as described here must produce results comparable to those obtained with reference reagents. All glassware used must be free of contamination, and distilled water used as diluent must be pure.

### B. Evaluation Procedure

Test 10 individual serum samples of predetermined reactivity on each of 2 days. The recommended distribution is three positive serum samples, three minimally positive serum samples, and four nonpositive serum samples. If necessary, prepare positive serum samples of various levels of reactivity by diluting positive samples with nonpositive serum samples. These pooled samples may be substituted for some of the individual serum samples.

### C. Testing

The TP-PA reagents from the new and the reference lots are tested on 2 days by using positive and nonpositive control serum samples from the new kit and the reference kit and 10 individual serum samples.

- (1) Assemble the 10 individual serum samples described above in B.
- (2) Perform the tests on positive control, nonpositive control and individual serum specimens. Test all serum specimens in parallel, using new and reference (old) reagents.
- (3) Compare the results obtained with reference and new reagents. Determine whether new TP-PA reagents meet the criteria of acceptability.
- (4) If results between reagent lots are discordant, additional testing may be necessary.
- (5) If the new kit gives the established reactivity patterns for known controls other than the manufacturer supplied controls, further testing can continue.

### D. Daily Control

- (1) Temperatures of refrigerators must be recorded daily.
- (2) At each routine test run, check expiration date on kit.
- (3) Test kit reactivity with control serum specimens.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If the controls are out of compliance when repeated, a new kit should be used.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Serum that is excessively lipemic, hemolyzed, or contaminated may interfere with the reaction.

Serum that has been repeatedly frozen and thawed may be falsely negative in the test.

Serum or reagents that have not reached room temperature before performing the test may cause false negative reactions.

Improperly diluting the serum samples will cause erroneous results. If the sample is diluted too much, it may be falsely negative. If not diluted enough, a false-positive result may occur.

Disturbing the microplate during incubation may prevent cells from settling properly leading to erroneous results.

13. REFERENCE RANGES (NORMAL VALUES)

Not applicable to this procedure.

14. CRITICAL CALL RESULTS (PANIC VALUES)

Not applicable to this procedure.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens must be at room temp (18–25°C) during preparation and testing. Otherwise, store the serum at <–20°C. If the sample is going to be retested within 24 hours, store at 2–8°C to avoid a freeze-thaw cycle.

16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

There is no alternate method for performing this test.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable to this procedure.

18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

We recommend that records, including QA/QC data, be retained for 2 years beyond the duration of the survey. Only numerical identifiers (e.g., NCHS ID numbers) should be used.

For the NHANES study, residual samples are stored at <–20°C for 1 year after analysis, then returned to the NCHS serum repository at Rockville, MD.

19. Summary Statistics and QC graphs

Qualitative assays are qualitative assays with a positive, negative or borderline/indeterminate result. The absorbance or reactivity values of specimens are compared with a cutoff value. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

**REFERENCES**

N/A