YALE-NEW HAVEN HOSPITAL	TITLE: *Treponema pallidum* (TP-PA) **Soft Code: TPPA**		**DEPT OF LAB MEDICINE CLINICAL IMMUNOLOGY Policy and Procedure Manual** **DOCUMENT # IMM 180** Page 1 of 9
WRITTEN BY: Voula J. Golfis	**EFFECTIVE DATE:** 5/24/2013	**REVISION:** H-1	**SUPERCEDES:** **New**

I. Introduction:

The identification of *Treponema pallidum* antibodies aids in the diagnosis of syphilis caused by the microorganisms belonging to the genus *Treponema* and provides epidemiological information on syphilis. Nontreponemal tests are usually used for screening and measure anti-lipid antibodies formed by the host in response to lipoidal material released from damaged host cells early in infection and to lipid from the cell surfaces of the treponeme itself. The nontreponemal tests for syphilis in use today are the Venereal Disease Research Laboratory (VDRL) test and the Rapid Plasma Reagin (RPR) test. Treponemal tests have attained standard status for confirmatory syphilis serological testing. Treponemal tests include FTA-ABS, an indirect immunofluorescent antibody test, Hemagglutination (MHA-TP) and TP-PA. Serodia®-TP-PA uses the same treponemal antigen as the MHA-TP, but offers the advantage of gelatin particles, which eliminate non-specific reactions with plasma samples.

At Yale New Haven Hospital (YNHH), TP-PA can be ordered when requested or performed as reflex testing following screening with a nontreponemal test.

II. Principle of the Assay:

This assay is a qualitative gelatin particle agglutination assay intended to be used for the detection of *Treponema pallidum* antibodies in human serum as an aid in the diagnosis of syphilis. The Serodia®-TP-PA test is based on the agglutination of colored gelatin particle carriers sensitized with *T. pallidum* (Nichols Strain) antigen. Serum samples are serially diluted in Sample Diluent in microplate wells. Sensitized Gelatin Particles are added to respective wells and the contents of the plate mixed by hand or on a tray mixer. The mixture is incubated stationary for 2 hours at room temperature. Serum containing specific antibodies will react with the antigen-sensitized colored gelatin particles to form a smooth mat of agglutinated particles in the microtitration tray. A compact button formed by the settling of the non-agglutinated particles characterizes negative reactions. The test is designed to be used exclusively with microtitration techniques. The agglutination patterns and interpretation of the test are read visually or with the aid of a tray viewer.

III. Specimen Collection:

The test should be performed on serum only (from red top tube). Separate serum by

centrifugation, 3000 rpm for 15 minutes. Serum aliquots are routinely frozen at -20°C until testing but can be stored refrigerated for up to 5 days. Sera may be frozen and thawed only once. Heat-inactivation is not necessary for the patient sera. However, previously heat-treated (56°C for 30 minutes) sera may be used. Do not perform the test on grossly hemolyzed or lipemic serum. Lipemic serum should be spun at 10,000 rpm for 20 minutes to remove contaminating lipids.

Standard Aliquot volume = 250 uL
Minimum Aliquot volume = 100 uL
Grossly Hemolyzed: Reject
Grossly Lipemic: Spin at 10,000 rpm for 20 min.
Stability: 5 days refrigerated 6 months at frozen (-20°C) or 5 days refrigerated (2-8°C)

IV. Materials Supplied:

Table 1

	Reagents				
# of assays	Reconstituting Solution (liq) (A)	Sample Diluent (liq) (B)	Sensitized Particles (lyo) (C)	Unsensitized Particles (lyo) (D)	Positive (Reactive) Control (liq) (E)
100 (20X5)	1 vial X 8 mL	1 vial X 29 mL	5 vials X 0.6mL*	5 vials X 0.6mL*	1 vial X 0.5mL

* Reconstitution Volume per vial

1. Reconstituting Solution:
Aqueous solution of 0.05M Phosphate Buffer containing 0.2M NaCl, 0.6% normal rabbit serum, and 0.06% sodium azide, at pH 7.00-7.60. The solution is used for reconstituting the Sensitized and Unsensitized Particles.

2. Sample Diluent:
The solution is used for diluting human specimens in the assay. Aqueous solution of 0.05M Phosphate Buffer containing 0.9M NaCl, 2% normal rabbit serum, 0.1% rabbit testicular extract, and 0.1% sodium azide at pH 6.70-7.30.

3. Sensitized Particles:
Lyophilized preparation of colored gelatin particles sensitized with Treponemal pallidum antigen. At the time of use, add the Reconstituting Solution to the volume indicated on the Particles vial label and in the table above. The rehydrated reagent contains a 1% suspension of sensitized gelatin particles and 0.8% (w/v) sodium azide as preservative.

4. Unsensitized Particles:
Lyophilized preparation of colored gelatin particles. At the time of use, add the Reconstituting Solution to the volume indicated on the Particles vial label and in the table above. The rehydrated solution contains a 1% suspension of unsensitized gelatin particles and 0.8% (w/v) sodium azide as preservative.

5. Positive (Reactive) Control Serum:
This liquid serum containing rabbit antibodies to *T. pallidum* should demonstrate a titer of 1:320 final dilution ± one doubling dilution, when tested according to the procedure described below. Control contains 0.099% sodium azide as preservative.

6. Dropper: 2 pcs. in 100 and 220 Test Kits - To dispense approximately 25 µL per well. One dropper to be used exclusively for dispensing reconstituted Sensitized Particles and the other dropper for dispensing the Unsensitized Particles.

7. Non-Reactive Control Serum: Vial and directions for use provided in separate packaging with this kit.

V. Material Required but not Supplied:

1. Polystyrene microplate with "U" shaped wells. Plates should be free from dust, lint, and scratches.
2. Micro-pipette with tips - to dispense 25 µL and 100 µL - for dispensing and diluting serum samples.
3. Pipettes - 1.0 mL serological for reconstitution.

VI. Precautions:

1. All reagents should be brought to room temperature before use.
2. Proper plate mixing, after the addition of all reagents, is important. Use an automatic vibratory plate mixer or tap the plate sharply with your finger to assure proper mixing. The use of a rotator, such as those used for RPR card test, will not provide adequate mixing.
3. During incubation, cover the microplate with an empty plate or a microplate cover and keep free from vibration.
4. Do not intermix reagents from different kit lots.
5. Ideally, the lyophilized reagents in this kit should be used on the same day as reconstituted. However, when stored at 2-10°C they have a reconstituted stability of 7 days.
6. Some reagents contain small amounts of sodium azide as preservative. Sodium azide may react with lead or copper plumbing, which may result in the formation of highly explosive metal azides. If these reagents are to be disposed of in a laboratory sink, flush with generous amounts of water to avoid azide build-up.

VII. Storage:

Store all reagents at 2-10°C both before and after opening or reconstitution. DO NOT FREEZE. **Reconstituted Sensitized and Unsensitized Particles should be used within 7 days**. Liquid reagents are stable through the labeled expiration date. Do not use reagents after the expiration date marked on the kit.

VIII. Preparation of Reagents:

1. Reconstituting Solution, Sample Diluent and Positive (Reactive) Control are liquids ready for use and require no reconstitution.
2. Sensitized Particles and Unsensitized Particles must be reconstituted with the Reconstituting Solution using the volume listed on the vials (See Table 1). Once opened, dispense the appropriate amount of Reconstituting Solution. Mix the reconstituted reagents thoroughly and allow them to stand at room temperature for at least 30 minutes prior to use. Mix particles again prior to dispensing. When the particles are reconstituted, write the date reconstituted plus 7 days out-date on the label. The particles should not be used past this 7 day reconstituted date.

IX. Assay Procedure (See Table 2)

Before Starting

1. Call a Soft pending list by test code TPPA and create a Tasklist by Template TPPA. Refer to the Soft Immunology Procedure (Doc# IMM 120).
2. Fill out a the TPPA worksheet (Doc# IMM 180-B).
3. Allow reagents and samples to come to room temperature before testing.

Procedure

Four wells are required for each patient sample and Control(s) run in this assay. Wells # 1 & 2 are for dilution of sample, Well #3 for Unsensitized Particles and Well # 4 for Sensitized Particles. **The Positive (Reactive) and Non-Reactive Controls should be included in each assay run.**

Table 2

WELL #	1	2	3	4
Sample Diluent (µL) Specimen (µL)	100 25)	25 25)	25 25)	25 25) → Discard
Specimen Dilution	1:5	1:10	1:20	1:40
Unsensitized Particles (µL)			25	
Sensitized Particles (µL)				25
Final Dilution			1:40	1:80
Mix, cover plate, and Incubate for 2 hours				
Interpretation				

1. Place 4 drops (100 uL) of Sample Diluent in Well #1, and 1 drop (25 µL) in Wells #2 through #4 using a calibrated pipette dropper.

2. Using a micropipette, add 25 uL of patient specimen or Positive or Non- Reactive Control Sera into Wells #1.

3. Mix the contents of Well #1 by filling and discharging the micropipette 5 or 6 times. Then, using the micropipette, transfer 25 uL of the diluted solution from Well #1 into Well #2. Mix the contents of Well #2 in the same manner as stated above and transfer 25 uL into Well #3. Following the same procedure, mix the contents of Well #3 and transfer 25 uL into Well #4, mix and discard the 25 uL of solution remaining in the pipette after mixing Well #4.

4. Place 1 drop (25 uL) of Unsensitized Particles in Well #3, and 1 drop (25 uL) of Sensitized Particles in Well #4 using the droppers supplied in the kit. ***Make sure the dropper releases a full drop of the particles as this can affect assay results.***

5. Mix the contents of the wells thoroughly (for approximately 30 seconds). **DO NOT USE A ROTATOR.** Then cover the plate with an empty plate or microplate cover, and let stand at room temperature (15-30°C) for at least 2 hours before reading.
The incubation can be extended to overnight without any perceptible difference in patterns.

X. Interpretation of Results

Place the plate onto a flat surface, preferably with a white background, and visually observe the pattern of agglutination in each well. Observe the agglutination pattern for each patient and Control(s) wells. Ensure each of the Unsensitized Particle wells is non-reactive and interpret the agglutination pattern of the Sensitized Particles using the criteria shown in Table 4:

Table 4

Settling Patterns of Particles	**Reading**	**Interpretation**
Particles are concentrated in the shape of a button at the center of the well with a smooth round outer margin.	(-)	Non-Reactive
Particles are concentrated in the shape of a compact-ring with a **very small** "hole" in the center and a smooth round outer margin.	(-)	Non-Reactive
Particles are concentrated in the shape of a compact ring with a "hole" in the center and a smooth round outer margin.	(+)	Inconclusive
Defined large ring with a rough multiform outer margin and peripheral agglutination.	(+)	Reactive
Agglutinated particles spread out covering the bottom of the Well uniformly, edges sometimes folded.	(++)	

REACTIVE: A specimen showing Non-Reactive with Unsensitized Particles (final dilution 1:40) but demonstrating a reaction of + or ++ at any dilution 1:80 or over with Sensitized Particles is interpreted as Reactive in this test. If a serum sample demonstrates a positive reaction with both the Sensitized and Unsensitized Particles, retest using the Absorption Procedure described below. A reactive treponemal test indicates past or present infection and usually remains reactive for life.

INCONCLUSIVE/INDETERMINATE: A specimen showing Non-Reactive with Unsensitized Particles (final dilution 1:40) but demonstrating a plus/minus reaction with Sensitized Particles at a 1:80 final dilution, is regarded as inconclusive or indeterminate. In such cases, it is recommended that the result be held until the assay is repeated. The result of the repeated test should be reported if found to be Reactive or Non-Reactive. **Repeated inconclusive results should be reported as Inconclusive and the sample will be reflexed for FTA-ABS testing at no charge.** The sample will also be reported with the following comment "The TPPA assay showed an inconclusive result. Further evaluation with an additional treponemal assay, the FTA-ABS, will be performed."

NON-REACTIVE: Regardless of the reaction pattern with Unsensitized Particles, a specimen showing Non-Reactive with Sensitized Particles at a 1:80 final dilution is regarded as Non-Reactive in this test. A Non-Reactive result indicates no past or present infection, but during incubating-stage syphilis, a Non-Reactive result may also occur.

XI. Quality Control:

1. The Positive (Reactive) Control should be processed at least once on the day of testing or when a batch of specimens is run and should yield a positive reaction.
2. A Non-Reactive Control should be run at least once on the day of testing or when a batch of specimens is run and should yield a negative reaction. A separate Non-Reactive Control is provided with the kit. If the Non-Reactive Control is not with the kit or more is needed, the control can be obtained from the Technical Service Dept.
3. Confirm that the reaction with Unsensitized Particles (1:40 final dilution) is Negative (-) for each patient sample.
4. If an assay does not meet Quality Control parameters listed above, the patient results from that assay should not be reported.
5. Enter all Quality Control results on the the TP-PA QC log (Doc# IMM180-Q) located on the L:Drive.

New Kit lots
All new lots are verified by testing both a Reactive and a Non-Reactive patient which have been previous tested on a verified lot. Results are recorded on Reagent Verification logs located on the L:Drive.

XII. Absorption Procedure:

In most cases, test samples do not show agglutination with Unsensitized Particles. However, if a test sample produces agglutination with both Unsensitized and Sensitized Particles, it should be re-tested after using the following absorption procedure:

1. Place 0.95 mL of reconstituted Unsensitized Particles in a small test tube.
2. Add 50 µL of test specimen and mix thoroughly using tube mixer and incubate at room temperature for 20 - 30 minutes (mix manually 1 or 2 times).
3. Centrifuge for 5 minutes at 2,000 rpm. Place 50 µL of supernatant (absorbed at a 1:20 ratio of test specimen to Unsensitized Particles) to Well #3 of the microplate.
4. Add 1 drop (25 µL) of Sample Diluent into Well #4 of the microplate using a micropipette, transfer 25 µL of Well #3 (absorbed 1:20 diluted sample) into Well #4. Mix completely by filling and discharging the micropipette 3 or 4 times with fluid in Well #4, in order to make a doubling dilution, and then discard the 25 µL of solution remaining in the pipette after mixing.
5. Place 1 drop (25 µL) of Unsensitized Particles in Well #3 and 1 drop (25 µL) of Sensitized Particles in Well #4.
6. Follow the original procedure and read the patterns.

XIII. Limitations:

1. As with all serological tests for syphilis, interpretation of results obtained with the Serodia®-TP-PA syphilis Antibody test 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. Specimens giving inconclusive results in the assay should be re-tested. A repeated inconclusive specimen should be reported as Inconclusive for follow-up and another specimen drawn in two weeks for testing and/or confirmed by other methods, such as FTA-ABS.
3. The Serodia®-TP-PA is less sensitive than the fluorescent treponemal antibody absorption (FTA-ABS) test in untreated primary syphilis but compares favorably in all other stages of syphilis.
4. 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 Serodia®-TP-PA result.
5. The Serodia®-TP-PA may be reactive in a small percentage (less than 1%) of normal or healthy persons; these false-positive results are often transient, their cause unknown. False positive Serodia®-TP-PA results may occur in association with other underlying illnesses.
6. The Serodia®-TP-PA may be reactive in persons from areas where yaws or pinta was, or is, endemic.
7. The Serodia®-TP-PA, as do all laboratory tests, performs best in populations at risk for the disease for which the test has been developed.
8. Samples from patients with HIV, Leprosy, Toxoplasmosis, H. pylori, and drug addiction may react, on occasion, with either the sensitized or the unsensitized particles, causing false-positive or inconclusive results.

XIV. Expected Results:

Studies performed on 175 normal male and female and 171 normal pregnant female populations indicated a reactive rate of 2.6% (9/346) from people located in the Southeast United States. Six of these donors were also reactive on RPR testing. The population ranged in age from 14 to 40 years of age. The rate of reactivity will vary according to geographic location and social demographics of the population.
One hundred percent (100%) of samples tested from both treated and untreated patients in both the primary and secondary stages of the disease were detected by the Serodia®-TP-PA test. Samples from individuals with autoimmune disease, Toxoplasmosis, H. pylori, IV drug users, and HIV demonstrated an 11.4% (18/158) reactive rate. These same samples were also reactive by the MHA-TP Hemagglutination assay.

XV. YNHH Method Validation Summary

Correlation:

Correlation was performed by comparison with ARUP laboratories and showed a 95% agreement. The two samples that varied from the ARUP results tested Reactive at YNHH and Nonreactive at ARUP. These samples were repeated by another method (FTA) and were found to be reactive.

# of samples: 40

Agreement: 95.0 %

Positive Agreement: 100% (20 of 20)

Negative Agreement: 90% (18 of 20)

Precision:

Intrarun Precision: Intra-assay performance was evaluated by testing of 2 specimens, a Nonreactive and a Reactive 4 times on a single run. All the results were in agreement.

Interrun Precision: Inter-assay performance was evaluated by testing 2 specimens, a Nonreactive and a Reactive, on 4 separate runs. All the results were in agreement.

Reference Range Verification:

A reference range of "negative" was obtained through literary research performed by Dr. Brian Smith. From that research, the reference range was verified at YNHH by evaluating 20 random patient samples. Of the 20 samples, 2 were inconclusive and 18 were nonreactive. This falls within the limits for reference range acceptability.

CAP Proficiency Results:

Survey G-C 2011 was tested and all results were acceptable.

XVI. Appendix

IMM 180-A	Treponema pallidum (TP-PA) Training Checklist
IMM 180-B	Treponema pallidum (TP-PA) Worksheet
IMM 180-Q	Treponema pallidum (TP-PA) Quality Control Log

XVII. References:

1. Serodia®-TP-PA Kit insert
2. Larsen S, Hunter E, Kraus S, A Manual of Test for Syphilis. Washington, DC, American Public Health Association,1990.

Treponema pallidum (TP-PA) Training Checklist

Reading:	Assay Procedure		
	Kit Insert		
Reagent Preparation and Storage:			
	Sensitized Particles		
	Unsensitized Particles		
Assay Protocol:		Unassisted/ Assisted	Checked
Controls:	Preparation		
	Storage		
	Positive Control		
	Negative Control		
Results:	Interpretation		
	Absorption Procedure		
	Entering		
	Verification		
Reagents/ Supplies:			
	Inventory		
	Ordering		
	Storage		

Training Completed _____

Signature _____ Verified By _____

Learning Technologist Teaching Technologist

TPPA Worksheet

Sample #				Sample #				Sample #			
Negative QC											
Positive QC											

Date: _____

Tech: _____

TP-PA Kit Lot #: _____

Expiration: _____

Doc# IMM 180-B

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May 24, 2013

Signature Approval for Annual Review

Treponema pallidum (TP-PA)
Document #: IMM 180

Name (Print)	Title	Signature	Date of Review	Revision Page and Section # (Use Procedure Review Log to document staff review)	Issue Date for Training if Applicable	Effective Date for Use
TEODORICO LEE	LAB MANAGER	Teodorico Lee	5/28/13	New procedure.	5/24/2013	5/24/2013
BRIAN SMITH	LAB DIRECTOR	[illegible]	5/24/2013			