Laboratory Procedure Manual

Analyte: Syphilis-G

Matrix: Serum

Method: Captia Syphilis-G Enzyme Immunoassay (EIA)

Method No.: 

Revised:

as performed by: Division of HIV, STD, and TB Laboratory Research
National Center for Infectious Diseases

Contact: Dr. Vicki Pope

Important Information for Users
NCHSTP, 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:

<table>
<thead>
<tr>
<th>Lab Number</th>
<th>Analyte</th>
<th>SAS Label</th>
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<tbody>
<tr>
<td>I36_c</td>
<td>LBXSY1</td>
<td>Syphilis-G</td>
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</table>
1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The Captia Syphilis-G enzyme immunoassay (EIA) is an indirect method for the detection of IgG antibodies to *Treponema pallidum* (1–5). Currently, CDC recommends that the test be used in the clinical laboratory as a confirmatory test for the diagnosis of syphilis. However, the test may be used as a screening test in blood banking. *T. pallidum* antigens are coated onto the wells of a 96-well microtiter plate. A dilution of the patient’s serum is added to the well to allow any *T. pallidum*-specific antibodies present to bind to the treponemal antigens. Biotinylated anti-human IgG labeled with strepavidin-peroxidase is used to detect the patient’s antibody. After rinsing off the excess antibodies, an enzyme substrate is added for detection. If the patient has antibodies to *T. pallidum*, a color reaction takes place. The intensity of the color development is proportional to the amount of antibody present. This color change can then be read using a plate reader, which eliminates subjective interpretation of the results.

2. SAFETY PRECAUTIONS

The risk of infection due to an occupational exposure to blood depends upon the prevalence of blood-borne pathogens in the population supplying the blood specimens, the probability of infection given a particular type of exposure to a blood-borne pathogen, and the frequency of exposures (6, 7).

*T. pallidum* is present in circulating blood during primary and secondary syphilis. The minimum number (LD50) of *T. pallidum* organisms needed to infect by subcutaneous injection is 23 (8). The concentration of *T. pallidum* in patients’ blood during early syphilis, however, has not been determined. The ability of blood inoculated with *T. pallidum* to infect animals is reduced by refrigerated storage (9, 10). Although multiple instances of transmission of *T. pallidum* due to transfusion of an infected donor’s blood were reported prior to the introduction of penicillin for treatment of syphilis and of refrigeration for blood storage (9). Subsequent reports have been rare (9, 10). Infection of a health care or laboratory worker following exposure to *T. pallidum* infected blood has, apparently, not been reported.

Authoritative sources focus attention on infection with hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) as the principal concerns associated with exposure to blood (7, 12-15). The prevalence of these infections varies greatly among patient populations tested for *T. pallidum* infection. HBV infection is most common. HBV viremia is indicated by tests for HBV surface antigen (HBSAg) in serum. Prevalence of anti-HBsAg, from published studies of patients in hospitals and emergency rooms cited in a recent review, ranged from 0.9% to 6% (5, 16–19). Unlike initial HBV infection, in which only a minority of individuals continues to be viremic, initial HCV and HIV infections lead to persistent viremia in most individuals. Consequently, serum antibody to HCV and HIV are indicators of potential infectiousness. Seroprevalences of antibody to HCV in studies of patients in hospitals and emergency rooms cited in a recent review ranged from 2% to 18% (15, 18–21). HIV prevalence ranged from 0.1% to 5.6% in patients enrolled in a national hospital surveillance system (6, 22). All three infections are more common among patients at increased risk for syphilis, especially patients with a history of illegal drug use. For example, seroprevalences of antibody to HCV were 10% among non-drug-using attendees at sexually transmitted diseases clinics and 60% among injection-drug users (23–25).

While infections with HBV (24, 26) and HIV (14, 27–29) can occur with skin and mucus membrane exposures to blood, needle stick and percutaneous injury with blood-coated sharp objects are the principal sources of laboratory associated acquisition of these agents. The risk of infection following exposure to blood from an infected patient is greatest for HBV, except for exposed individuals who are immune due to prior HBV infection or vaccination. The risk is highest if the source individual is HBSAg-positive (24, 30–32) and is positive for envelope (E) antigen. A vaccine to prevent HBV infection has been available since 1982 and is strongly recommended for health care workers with potential exposures to blood or other body fluids (30, 33, 34). Individuals with anti-HBV antibody from vaccination or prior infection are considered to be immune to HBV infection.

The risk of HCV infection due to needle stick exposure to blood from an individual with antibody to HCV was 10% in one study (24, 35, 36), but HCV does not appear to survive long in serum held at room temperature (24, 37). A vaccine is not yet available to immunize against HCV infection. Repeated infection with HCV appears to be possible in spite of detectable serum anti-HCV antibody, although the significance of reinfection is unknown (23, 38, 39).
The risk of infection with HIV following a single needle stick exposure to blood from a patient known to be infected with HIV is approximately 0.3% (4). The risks following mucous membrane or skin exposures to HIV-infected blood average approximately 0.1% and <0.1%, respectively (14, 27, 29, 40). The lower rate of transmission for HIV than for HBV or HCV probably reflects a lower concentration of HIV in the blood of infected persons. A vaccine is not available to immunize against HIV infection. The frequency and significance of repeated exposure of individuals with prior anti-HIV antibody is unknown.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT
   A. Each shipment of specimens received from the NHANES III mobile unit contains a corresponding transmittal sheet and an ASCII data file (KOUTPUT.TXT) on a 3 ½ “ high-density floppy diskette. 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 onto the NHANES diskettes by using the program provided by National Center for Health Statistics (NCHS).
   C. After the results are entered on diskettes, backup copies are made and stored in locked areas.
   D. The original diskettes containing analytical results are mailed to NCHS.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION
   A. No special instruction such as special diet or fasting is necessary.
   B. Captia Syphilis-G EIA kits are intended for use with fresh serum specimens. Serum specimens may be collected using regular red-top or serum separator Vacutainers. Specimens are allowed to clot at room temp and centrifuged. Transfer serum to 2-mL polypropylene screw-capped vials. Freeze at <-20°C.
   C. 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.
   D. Serum specimens are stable up to 72 hours at 4–8°C. For longer periods, store the serum at <-20°C in glass or plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.
   E. 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. Heat-inactivated sera may be used (56°C for 30 minutes). Excessive inactivation time or temperature may increase nonspecific background activity which could result in equivocal results.
   F. The optimal amount of serum is 0.5–1.0 mL. Specimen volumes of less than 0.4 mL are not acceptable.
   F. Avoid repeated freeze-thawing cycles, which may compromise specimen integrity.

Specimens should generally arrive frozen.

Residual samples are frozen at <-20°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) Microtiter plate reader with capability of reading at 450 nm.
(2) Micropipettes to deliver 5 to 200 μL.
(3) Adjustable multichannel pipette to deliver reagents (25 to 100 μL per channel)

B. Other Materials

(1) Clean, disposable glass or plastic test tubes, 5 and 10 mL capacities
(2) Parafilm, aluminum foil, or capped test tubes for substrate solution.
(3) Reagent reservoirs to hold reagents dispensed by multichannel pipette.
(4) Wash bottle or microtiter plate washer for washing well strips between reagents.
(5) 1 L graduated cylinder to measure volumes when making working dilutions of reagents.
(6) 2-mL and 10-mL serologic pipettes
(7) Absorbent paper towels.
(8) Timer to accurately measure incubation times.
(9) Discard container and disinfectant.
(10) Incubator, 35–37°C.

C. Reagent Preparation

Each Syphilis-G kit contains enough reagents to test 92 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 4–8°C.

(1) Antigen

Twelve x 8-well strips coated with \textit{T. pallidum} antigen (Nichols strain), ready to use. Strips come sealed in a protective bag with desiccant to keep out excess moisture. Bring to room temp before using.

(2) Conjugate

Anti-human IgG monoclonal antibody labeled with biotin \textit{N}-hydroxysuccinimide ester and strepavidin-horseradish peroxidase (HRP) preserved with 0.01% Thimerosal. For each 8-well row, mix 50 μL of antibody and 50 μL of conjugate with 1.0 mL of dilution buffer.

(3) Substrate Solution

Citrate-acetate buffer containing hydrogen peroxide and preserved with 0.005% chlorhexidine, and Tetramethylbenzidine (TMB) chromogen (3,3',5,5'-tetramethylbenzidine in dimethylsulfoxide). Allow reagents to come to room temperature. For each 8-well row, add 10 μL of TMB to 1.0 mL of substrate solution. Hold in capped container at room temperature, and out of direct sunlight, until ready for use. Reagent should be virtually colorless.

(4) Dilution Buffer

Phosphate buffered saline (PBS), pH 7.0–7.2, containing protein stabilizer and 0.05% Tween-20; preserved with 0.01% thimerosal, ready to use. Bring to room temp before use.

(5) Wash Buffer

Stock 20× PBS containing 0.05% Tween-20 which is used to wash unbound reagent from wells between steps. The stock buffer may develop crystals during prolonged storage at 4–8°C. If crystals are present, place the bottle containing the stock buffer in a 37°C water bath until the
crystals disappear. Prepare the working buffer by mixing 1 part stock buffer with 19 parts of distilled water.

(6) Stop Solution
Stop solution of 2 M sulfuric acid is made by adding 6 mL of concentrated sulfuric acid to 48 mL of distilled water.

D. Standards Preparation
Not applicable for this procedure.

E. Preparation of Control Serum Samples
(1) Positive Control Serum
Prepared from human serum samples containing antibodies to *T. pallidum*. Serum is diluted in buffer containing 0.1% sodium azide and is ready to use. Bring to room temp before use.

(2) Cut-Off Control Serum
Prepared from human serum samples containing antibodies to *T. pallidum*. Serum is diluted in buffer containing 0.1% sodium azide and is ready to use. Bring to room temp before use.

(3) Nonreactive Control Serum
Prepared from human serum samples free of *T. pallidum* antibodies. Serum is diluted in buffer containing 0.1% sodium azide and is ready to use. Bring to room temp before use.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A. Working Standards
The cut-off control is used to determine the OD values for reactive, equivocal, and nonreactive. The control must be run at least in duplicate in every run. An average OD of the duplicate samples is determined.

B. 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.

C. Microplate Readers
Most microplate readers manufactured in recent years require no calibration. Wavelengths are set by use of filters, which must be protected from dust and scratches. The reference channel should measure 100% transmission or 0 (zero) optical density (OD). Many plate readers automatically adjust to compensate for any deviations in light intensity.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS
A. Preliminaries
   (1) Bring all reagents and serum samples to room temp before beginning test.
       Kit controls must be included in each assay. The cut-off control should be run in duplicate in each
       assay. The control has been adjusted to approximate the lowest reactivity that may be considered
       clinically significant. The nonreactive and reactive controls should be included in each run.

B. Sample Preparation
   (1) Dilute serum samples 1:21 with the dilution buffer before testing by adding 10 μL of serum to 200
       μL of dilution buffer in a microtiter plate. Alternatively, 50 μL of serum can be added to 1.0 mL of
       dilution buffer in a test tube. An arrangement that corresponds to the 8 X 12 conformation of the
       microtiter plate is ideal, since it facilitates transfer of diluted specimens to the microtiter plate using
       a multichannel pipettor.
   (2) Kits controls are already diluted and should be used as is.

C. Microplate Reader Settings:
   Insert filters that read at 450 nm. Reference channel should measure 100% transmission or 0 (zero)
   optical density (OD).

D. Operation
   Wash Cycle
   Complete rinsing of uncomplexed serum components is a fundamental requirement of all enzyme
   immunoassay procedures. The syphilis IgG EIA uses two rinse cycles of five washes each. Automatic
   plate washers may be used provided they meet the following criteria.
   (1) All wells are completely aspirated.
   (2) All wells are completely filled during the rinse cycle.
   (3) Wash buffer is dispensed at a good flow rate.
   (4) Plate washer must be well maintained to prevent the possibility of contamination from previous
       use.
       Manufacturer’s cleaning procedures must be rigorously followed.

Test Procedure
   (1) Allow all reagents to reach room temperature.
   (2) The kit control sera should be included in each run. The cut-off control should always be run in
       duplicate.
   (3) Select sufficient microtitration well strips to run all test serum specimens and controls. Fit the
       strips into the holding frame. Label wells according to specimen identifier using the letter/number
       cross reference system molded in the plastic.
   (4) Into each well of a separate labeled microtiter plate, place 200 μL of dilution buffer. To this add 50
       μL of patient sample. Mix well 6 to 8 times using the micropipettor.
       Note: Controls are supplied already diluted and are to be used as is.
   (5) Using a fresh pipette tip for each diluted serum sample, dispense 100 μL of each diluted serum
       into the correspondingly labeled well. Mix each sample 6–8 times using the 100 μL pipettor prior
       to transferring the sample to the plate. Dispense 100 μL of each control sera in to the designated
       wells.
   (6) Seal the strips and, holding the frame, place in the resealable plastic bag provided, and incubate
       at 37°C for 30 minutes.
(7) During this incubation prepare the mixture of antibody and conjugate. Do not mix more than is necessary for the number of wells being used. For each row of 8 wells in use, mix 50 μL Antibody and 50 μL of conjugate with 1.0 mL dilution buffer. Cover the tube and hold the tracer mix at room temperature (18–25°C) until ready for use.

(8) Aspirate diluted serum from the wells and wash the plate as described in the Rinse Cycle section.

(9) Pipette 100 μL Antibody/Conjugate mix into each well using a multichannel micropipette.

(10) Place the assembly in the resealable plastic bag and incubate at 37°C for 60 minutes.

(11) During the final 10 minutes of this incubation, prepare the substrate. Ensure reagents are at room temperature. Prepare a volume of substrate which is sufficient for immediate needs only. For each row of 8 wells add 10 μL TMB with 1.0 mL of substrate solution and mix well. Hold in a capped container at room temperature (18–25°C) and protect from direct sunlight until ready for use. The substrate should remain virtually colorless, otherwise contamination must be suspected and fresh substrate prepared using clean equipment and containers.

(12) Aspirate the conjugate from the wells and wash the plate as described in the Rinse Cycle section.

(13) Immediately dispense 100 μL of substrate mixture into each well using a multichannel pipette. Incubate at room temperature (18–25°C), protected from direct sunlight, for 30 minutes.

(14) Stop the reaction by adding 25 μL of 2 M sulfuric acid (stop reagent) to each well using a multichannel micropipette. Gently mix the well contents, preferably using a mechanical plate shaker or by gently tapping the sides of the plate, until the blue solution changes to a uniform yellow color. Ensure that the undersides of the wells are dry and that there are no air bubbles in the well contents.

(15) Within 30 minutes of adding the acid, read the absorbance values at 450 nm using a plate reader blanked on air unless the manufacturer specifically recommends otherwise.

E. Recording of Data

(1) Quality Control Data

(a) The absorbance of the nonreactive control should be <0.25.

(b) The absorbance of the reactive control should be >0.8.

(c) The mean absorbance of the cutoff Control should be > 2.5 x the absorbance of the nonreactive control.

(2) Analysis

Calculate the mean absorbance value of the duplicate cutoff controls. This is the cutoff value for the Captia Syphilis-M and was derived from clinical trials as the value giving optimum discrimination between serum samples which are reactive or nonreactive for IgM antibodies to T. pallidum.

Specimen absorbance values within ±10% of the mean of the low-titer reactive controls are considered equivocal results and the test should be repeated. If the repeat result is equivocal, a new specimen should be taken and tested.

F. Replacement and Periodic Maintenance of Key Components

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

Microplate washer should be rinsed thoroughly after use to prevent saline from drying and depositing salt crystal in the system. Care must be taken to ensure that all wells are being completely filled and emptied during the wash cycle. Any problems may indicate blockage by salts. Waste container must be emptied at the end of the day to prevent overflow. Full strength bleach should be added before running the wash cycle to decontaminate any infectious agents that may be in the serum samples.

G. Calculations
The mean value of the cut-off control must be calibrated, then the ±10% value for determination of equivocal results must be determined.

**Table 1. Example for determining ranges for reactive, equivocal and nonreactive result**

<table>
<thead>
<tr>
<th>Control</th>
<th>Mean</th>
<th>±10%</th>
<th>Equivocal</th>
<th>Positive Range</th>
<th>Negative Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cut-off</td>
<td>0.300</td>
<td>0.030</td>
<td>0.270–0.330</td>
<td>&gt;0.331</td>
<td>&lt;0.269</td>
</tr>
</tbody>
</table>

H. Special Method Notes

1. Do not cross-contaminate reagents. Always use a new pipette tip when drawing from stock reagent bottles.
2. Always keep the upper surface of the microtitration strips free of excess fluid droplets to prevent possible dilution of well contents. Blot reagent and buffer overspills.
3. Do not allow any well to dry completely during the test procedure.
4. Liquid reagents are clear with no precipitates. Wash buffer may have some precipitate when cold, but this should disappear when the solution reaches room temperature.
5. The chromogen substrate for the enzyme stays clear when diluted, turning color only in the presence of detectable IgG antibodies to *T. pallidum*.
6. Only reagents from the same kit are used. Mixing reagents from different lot numbers of kits may give erroneous results.

9. REPORTABLE RANGE OF RESULTS

Results are reported as Reactive, Nonreactive, or Equivocal.

10. QUALITY CONTROL (QC) PROCEDURES

A. Evaluation of IgG EIA 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 reactive serum samples, three minimally reactive serum samples, and four nonreactive serum samples. If necessary, prepare reactive serum samples of various levels of reactivity by diluting reactive samples with nonreactive serum samples. These pooled samples may be substituted for some of the individual serum samples.

**Testing**

The Syphilis-G reagents from the new and the reference lots are tested on 2 days by using reactive and nonreactive 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. Reconstitute sodium chloride for wash buffer according to directions.
3. Prepare 2 M H₂SO₄ for the stop solution.
4. Prepare dilutions for reactive and nonreactive serum controls and individual serum specimens.
(5) Prepare conjugate and substrate dilutions according to manufacturer’s directions.
(6) Perform the tests on reactive control, nonreactive control and individual serum specimens. Test all serum specimens in parallel, using new and reference (old) reagents.
(7) Read and record test results.
(8) Compare the results obtained with reference and new reagents. Determine whether new Syphilis-G reagents meet the criteria of acceptability.
(9) If results between reagent lots are discordant, additional testing may be necessary.
(10) If the new kit gives the established reactivity patterns for known controls other than the manufacturer supplied controls, further testing can continue.

C. If the new kit gives the established reactivity patterns for known controls other than the manufacturer supplied controls, testing of patient samples can continue.

Daily Control

(1) Temperatures of refrigerators, incubators, and water baths must be recorded daily.
(2) At each routine test run, check expiration date on kit.
(3) Test kit reactivity with control serum specimens of graded reactivity (high titered and low titered reactive, nonreactive controls). Use only if results fall within ±1 standard deviation (SD) of mean OD for each control.

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

If the OD of the reactive control is less than 0.8 or if the OD of the nonreactive control is greater than 0.25, or if the OD of the cutoff control is less than $2.5 \times$ the absorbance of the nonreactive control, the test must be repeated.

If the controls are still 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 cause a false positive due to a high background OD.

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.

Failure to stop the substrate reaction at the proper time may cause false-positive results if the reaction goes too long, or false-negative results if the reaction is stopped too soon.

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.

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 4–8°C to avoid a freeze-thaw cycle.

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

There are no alternative methods for performing this test for NHANES III. In case of system failure, store all specimens at <-20°C until the system is functioning.

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 dat, 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 III 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 result in positive, negative, or borderline/indeterminate values. The absorbance or reactivity values of specimens are compared with a cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.
REFERENCES


