Chapter 10

RAPID PLASMA REAGIN (RPR) 18-MM CIRCLE CARD TEST

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TEST PRINCIPLES

The rapid plasma reagin (RPR) 18-mm circle card test is a macroscopic, nontreponemal flocculation card test used to screen for syphilis.[1-4] The antigen is prepared from a modified Venereal Disease Research Laboratory (VDRL) antigen suspension containing choline chloride to eliminate the need to heat inactivate serum, ethylenediaminetetraacetic acid (EDTA) to enhance the stability of the suspension, and finely divided charcoal particles as a visualizing agent. In the test, the RPR antigen is mixed with unheated or heated serum or with unheated plasma on a plastic-coated card. **The RPR test measures IgM and IgG antibodies to lipoidal material released from damaged host cells as well as to lipoprotein-like material, and possibly cardiolipin released from the treponemes.[5,6] The antilipoidal antibodies are antibodies that are produced not only as a consequence of syphilis and other treponemal diseases, but also in response to nontreponemal diseases of an acute and chronic nature in which tissue damage occurs.[7]** If antibodies are present, they combine with the lipid particles of the antigen, causing them to agglutinate. The charcoal particles coagglutinate with the antibodies and show up as black clumps against the white card. If antibodies are not present, the test mixture is uniformly gray. The test can be purchased in kit form or in component parts from many commercial sources. **Without some other evidence for the diagnosis of syphilis, a reactive nontreponemal test does not confirm *T. pallidum* infection.**

SPECIMEN COLLECTION AND HANDLING

Specimen

1. Avoid accidental infection when collecting and processing samples by observing universal precautions (Chapter 2).

2. Serum and plasma are both suitable specimens for the qualitative test; however serum is the preferred sample for the quantitative test.[8] Test plasma samples within 48 hrs after collection.[8]

3. An acceptable specimen should not contain particulate matter that would interfere with reading test results. Specimens that are excessively hemolyzed, grossly contaminated with bacteria, chylous or otherwise extremely turbid are unsatisfactory. A specimen is too hemolyzed for testing when printed matter cannot be read through it.

   Note: Hemolysis may be caused by transporting blood in freezing or extremely hot weather without proper insulation.

4 Not all unsuitable specimens should be discarded or not analyzed. When an unsatisfactory sample is received in the laboratory, notify the requesting physician and discuss whether that specimen should be tested. If the ordering physician still desires a

test result, then the condition of the sample must be stated on the report, and a notation made of any limitation on interpretation of the test result.[9]

Collection

The procedures for the collection and processing of venous blood is given in detail in Chapter 3.

1. **Serum**- Collect whole blood into a clean, dry tube without an anticoagulant.

2. **Plasma**- Collect blood in a tube containing EDTA as an anticoagulant. Completely fill the tube or collect blood until the vacuum in the collection tube has been exhausted.

3. Label each specimen with patient identifier, and date.

Handling

A. Serum

1. Allow sufficient time (approximately 20 minutes) at room temperature for the specimen to clot.

2. Centrifuge the specimen at room temperature at 1000 to 1200 x g for at least 5 minutes to sediment cellular elements (see Chapter 3).

3. Keep serum specimens in the original collection tube if testing will be performed within a few hours. Remove serum from clot and store at refrigerator temperature (2° - 8°C) if testing is to be delayed. If a delay of more than 5 days is anticipated before testing, freeze the specimen at -20°C or lower. Avoid repeated freeze-thawing of specimens. Although unheated serum specimens may be used, serum may be heated at 56°C for 30 minutes without affecting test outcome. Specimens must be at room temperature (23° - 29°C; 73° - 85°F) at the time of testing.

4. If serum samples are to be shipped to a testing site, specimen containers must be leakproof and placed within a leakproof plastic bag. Paperwork should be submitted in a separate plastic bag, if included with the sample.[10]

B. Plasma

1. Centrifuge the specimen at room temperature at 1000 to 1200 x g for at least 5 minutes to sediment cellular elements. Plasma may be retained in the original collection tube if the test is to be performed immediately. If not, plasma should be removed from cellular elements.

2. Store plasma specimens at refrigerator temperature (2° - 8°C) and test within 48 hours. Plasma samples must be at 23° - 29°C (73° - 85°F) at the time of testing. Do not heat plasma.

3. Do not use plasma specimens for confirmatory treponemal tests.

MATERIALS

Reagents

Purchased

1. **RPR antigen suspension.** RPR antigen suspension is a stabilized combination of 0.003% cardiolipin, 0.020-0.022% lecithin, 0.09% cholesterol, 10% choline chloride, 0.0125M EDTA, 0.01875% charcoal, 0.01M Na_2HP0_4, 0.01M KH_2P0_4, 0.1% thimerosal in distilled water.[1] The antigen suspension is packaged in ampules. Store unopened ampules at 2° to 8°C; do not store the antigen in bright sunlight or in temperatures above 29°C; do not freeze. An unopened ampule of antigen is stable up to the expiration date.

2. **Control serum samples.** Control serum samples are lyophilized reactive (R), minimally reactive (Rm), and nonreactive (N) control serum specimens on a card, or liquid or lyophilized serum samples of graded reactivity. If quantitative tests are to be performed, a control serum that can be titered to at least a 1:4 dilution should be used. Store control cards or serum samples according to the manufacturer=s directions.

   (**Reagents may be purchased from Ampcor, Bridgeport, NJ; ASI, Arlington, TX; Baxter Healthcare Corp., Miami, FL; Becton-Dickinson Microbiology Systems, Cockeysville, MD; and Remel, Augusta, GA**)

Prepared

1. **0.9% Saline.** Add 0.9 g of dry sodium chloride (ACS) to 100 ml of distilled water.

2. **Diluent.** Prepare a 2% solution of human serum in 0.9% saline, by diluting a human serum nonreactive for syphilis 1:50 in 0.9% saline.

Provided in kit

1. Disposable, calibrated 20-gauge needle without bevel, silicone treated

2. Plastic antigen dispensing bottle, 1 dram

3. Plastic-coated RPR cards, with 10 circles, each approximately 18 mm in diameter. Store cards at room temperature.

4. Dispenstirs, a disposable (plastic) dispensing-stirring device that delivers 50 µl

Not provided in kit

1. Mechanical rotator, fixed-speed or adjustable to 100 ∀ 2 rpm, circumscribing a circle 3/4-inch in diameter on a horizontal plane

2. Humidifying cover

3. High-intensity incandescent lamp

4. Safety pipetting device with disposable tip that delivers 50 µl

5. Calibrated dropper that delivers 50 µl in a single drop (optional)

6. Discard containers and disinfectants

7. Disposable latex gloves, safety glasses, and protective clothing

CALIBRATION

Pipettors and Tips

With the pipettors currently available, the measurement of small serum volumes is routine. Most manufacturers include in the specifications of their pipettors the accuracy for frequently used microliter volumes. Daily use may affect pipettors, making them lose their initial accuracy. The differences in disposable tips from sources other than the manufacturer of the pipettor, 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 and precision of the substitute pipet tips in their test system. Commercial kits to check pipettor 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 the 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's.

Needles

1. Check the calibrated needle each time a new needle is used, when needle has been dropped or wiped, or when the control pattern is not met to ensure the delivery of the correct volume of antigen suspension (60 drops ∀ 2 drops per ml; 17 µl per drop).

2. Place the needle on a 1-ml syringe or on a 2-ml pipette. Fill the syringe or pipette with RPR antigen suspension. Holding the syringe or pipette in a vertical position, count the number of drops delivered in 0.5 ml. The needle is correctly calibrated if 30 drops ± 1 drop is delivered in 0.5 ml.

3. Replace the needle if it does not meet this specification. Be sure to test the calibration of the replacement needle.

Rotator

1. Speed - For rotators without a digital readout, the speed can be estimated by counting the number of rotations made per minute. To count the rotations place your finger next to the rotator and count the number of times the rotator touches your finger in 15 seconds. If the rotator is properly adjusted, the count should be 25. The rotator=s speed should be calibrated each day it is used.

2. Time - The rotator=s timer should be checked against another laboratory timer or stop watch. The rotator=s timer should be within ∀ 15 seconds of the set time.

QUALITY CONTROL

It is the responsibility of the laboratorian to ensure that reagents are of good quality and standard reactivity. Chemicals and distilled water should be of high quality, and solutions should be prepared according to the directions specified for each technique.

Test each new lot of RPR antigen for the RPR 18-mm circle card test in parallel with a reference reagent to verify that the two antigens are comparable before placing the new antigen into routine use.

Parallel testing should be performed on at least two testing days, by using different specimens of graded reactivity for each test period. Tests should be performed following the techniques described below. Record the results of all check testing.

Individual specimens of graded reactivity for check testing may be obtained by selecting specimens from the daily test runs and storing them in the freezer. Reactive serum diluted with non-reactive serum to produce various degrees of reactivity may also be used. If possible, fresh serum specimens from routine test runs should be used for the nonreactive specimens.

RPR Test Antigen

Criteria of acceptability

1. Test results on reference control serum specimens of graded reactivity and individual serum specimens in the qualitative and the quantitative tests must be the same as those obtained with the reference antigen suspension.

2. The antigen suspension should show in tests with non-reactive serum specimens the complete dispersion of antigen particles and no more roughness than is seen with the reference antigen suspension.

Procedure for Testing

1. Using controls of graded reactivity, the reactivity of the new antigen suspension and the reference antigen suspension is tested by using the appropriate test technique.

2. If the new antigen suspension shows obvious deviations from the established reactivity pattern of the controls in comparison with the reference antigen suspension, the product is considered unsatisfactory and no further testing is performed.

3. If the new antigen suspension gives the established reactivity pattern of the controls of graded reactivity or shows only a slight deviation in comparison with the reference antigen suspension, specimens may be tested with this reagent.

4. Compare the new antigen suspension and the reference antigen suspension by qualitative testing of individual serum specimens of graded reactivity. Test at least 3 reactive, 10 intermediate (minimally reactive to 1:2 dilution), and 7 nonreactive serum samples. Test serum specimens side by side with the new and the reference antigen suspensions.

5. Select three reactive serum specimens for quantitative testing. Prepare serial dilutions of each serum in 0.9% saline (1:2, 1:4, 1:8, 1:16) in test tubes. Dilutions of 1:32 or greater should be prepared in diluent (2% nonreactive human serum in 0.9% saline). Test each serum dilution side by side with the new and the reference antigen suspensions.

   Note: It is important to make these master serial dilutions in test tubes so that the exact same serum dilutions are being compared between antigens.

6. Record results of all testing.

7. Review test results and determine whether the new antigen suspension meets the criteria of acceptability.

Daily Controls

1. Check room temperature. For reliable and reproducible test results, the RPR Card antigen suspension, controls, and test specimens must be at room temperature (23° - 29°C; 73° - 85°F), when tests are performed.

2. At each routine test run, check the expiration date on the ampule.

3. Determine antigen suspension reactivity with control cards or control serum specimens of graded reactivity (reactive, minimally reactive, and nonreactive). If quantitative tests are to be performed, then a control serum that can be titered to at least a 1:4 dilution should be used.

4. Use only RPR antigens that reproduce the established reactivity pattern of the controls.

PROCEDURES

Qualitative Test

1. To prepare antigen for testing, attach the hub of the dispensing needle to the fitting on the plastic dispensing bottle. Shake the antigen ampule to resuspend the particles. Open the ampule. Squeeze the dispensing bottle to collapse it. Insert the needle into the ampule and withdraw all the antigen suspension into the dispensing bottle.

2. Place 50 µl of serum or plasma onto a 18-mm circle of the RPR test card, using a disposable Dispenstir or a safety pipetting device.

3. Using the inverted Dispenstir (closed end) or flat toothpicks, spread the serum or plasma to fill the entire circle. Do not spread the specimen beyond the confines of the circle.

4. Gently shake the antigen dispensing bottle to resuspend the particles.

5. Holding the dispensing bottle and needle in a vertical position, dispense several drops to clear the needle of air. Then add exactly 1 free-falling drop (17 µl) of antigen suspension to each circle containing serum or plasma. Do not mix.[2, 3]

6. Place the card on the mechanical rotator under a humidifying cover. Rotate the card for 8 minutes at 100 ∀ 2 rpm.

7. Immediately remove the card from the rotator; briefly rotate and tilt the card by hand (three or four to-and-fro motions) to aid in differentiating nonreactive from minimally reactive results.

8. Perform the quantitative test on serum specimens showing any degree of reactivity (clumping) or Aroughness.@

Reading and Reporting Qualitative Results

1. Read the test reactions in the Awet@ state under a high-intensity incandescent lamp. Read the test without magnification.

2. Report the results as follows.

Reading	**Report**
Characteristic clumping ranging from marked and intense (reactive) to slight but definite (minimally to moderately) reactive	Reactive (R)
Slight roughness or no clumping	Nonreactive (N)

Note: Only two reports with the RPR card test are possible: reactive, no matter how much clumping, or nonreactive.

Quantitative Test[3]

1. Dilute to an endpoint titer all serum specimens with rough nonreactive results in the qualitative test. Test each specimen undiluted (1:1), and in 1:2, 1:4, 1:8, and 1:16 dilutions (see Fig 10:1).

2. Place 50 µl of 0.9% saline in circles numbered 2 through 5. Do not spread the saline.

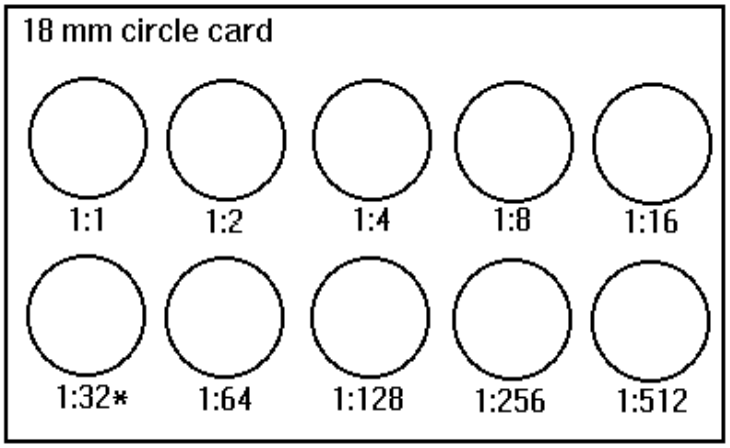
3. Using a safety pipette device, place 50 µl of serum in circle 1 and 50 µl of serum in circle 2 (Fig 10:1).

4. Mix the saline and the serum in circle 2 by drawing the mixture up and down in a safety pipette eight times. Avoid forming bubbles.

5. Transfer 50 µl from circle 2 (1:2) to circle 3, and mix.

6. Transfer 50 µl from circle 3 (1:4) to circle 4, and mix.

7. Transfer 50 µl from circle 4 (1:8) to circle 5 (1:16), mix, and then discard the last 50 µl.



*Begin dilutions in 2% normal human serum

Figure 10:1. Diagram of card for quantitative test

. Using the broad end of a clean Dispenstir, spread the serum dilution to fill the entire surface of circle 5, the highest dilution (1:16). Using the same Dispenstir, repeat for circle 4(1:8), 3(1:4), 2(1:2), and 1 (undiluted).

9. Gently shake the dispensing bottle to resuspend the antigen particles.

10. Holding the antigen dispensing bottle in a vertical position, dispense 1 or 2 drops to clear the needle of air. Then add exactly 1 free-falling drop (17 µl) of antigen suspension in each circle. DO NOT MIX.

11. Place the card on the rotator under the humidifying cover and rotate the card for 8 minutes at 100 ∀ 2 rpm.

12. Immediately remove the card from the rotator; briefly rotate and tilt the card by hand (three or four to-and-fro motions) to aid in differentiating nonreactive from minimally reactive results.

13. If the highest dilution tested (1:16) is reactive, continue as follows:

    a. Prepare a 1:50 dilution of nonreactive serum in 0.9% saline to be used for making 1:32 and higher dilutions of the specimen to be tested.

    b. Prepare a 1:16 dilution of the test specimen by adding 0.1ml of serum to 1.5ml of 0.9% saline. Mix thoroughly.

c. Place 50 µl of the 1:50 nonreactive serum diluent in circles 2 through 5 of an RPR card.

d. Using a safety pipetting device with disposable tip, place 50 µl of the 1:16 dilution of the test specimen in circle 1 and 50 µl in circle 2.

e. Using the same pipette and tip, make serial twofold dilutions. Complete test as described in steps 4 through 13 (see AQuantitative Test@). Use a clean tip for each specimen tested. Prepare higher dilutions if necessary in 1:50 nonreactive serum diluent.

14. After completing the day=s tests, remove the needle from the antigen dispensing bottle. Rinse needle in distilled water, and air dry. Do not wipe needle (wiping removes the silicone coating). A satisfactory needle may be retained as a spare for replacement of an unsatisfactory needle.

15. Recap the plastic dispensing bottle containing the antigen suspension and refrigerate at 2° to 8°C. Do not freeze the antigen. Antigen stored in the dispensing bottle will retain its reactivity for 3 months or until the expiration date, whichever is sooner.

Reading and Reporting quantitative results

1. Read the test reaction in a Awet@ state under a high-intensity incandescent lamp as for the qualitative test.

2. Report the results in terms of the highest dilution that has given a reactive result, including a minimally reactive result, as shown in Table 10:1.

Table 10:1. **Reporting quantitative results**

	Serum Dilutions				**Report**
Undiluted (1:1)	1:2	1:4	1:8	1:16	
Rm	N	N	N	N	Reactive, undiluted 1:1, or R 1
R	R	N	N	N	Reactive, 1:2 dilution, or R 2
R	R	R	N	N	Reactive, 1:4 dilution, or R 4
R	R	R	Rm	N	Reactive, 1:8 dilution, or R 8

R=reactive, Rm=minimally reactive, N=nonreactive

CALCULATIONS[11] AND RANGES

$$\textbf{Sensitivity} = \frac{TP}{TP + FN} \qquad \textbf{Specificity} = \frac{TN}{TN + FP}$$

TP = True Positive, the number of persons who test reactive that actually have syphilis
FN = False Negative, the number of persons who test nonreactive that have syphilis
TN = True Negative, the number of persons who test nonreactive that do not have syphilis
FP = False Positive, the number of persons who test reactive that do not have syphilis

Table 10:2. **Performance of the RPR card test by stage of untreated syphilis***

	Sensitivity		Specificity	
Stage	Percent	Range	Percent	Range
Primary	86	77-99	-	-
Secondary	100		-	-
Latent	98	95-100	-	-
Late	73		-	-
Nonsyphilis	-	-	98	93-99

*Results of CDC studies

INTERPRETATION OF RESULTS

1. The RPR card test is an aid in the diagnosis of syphilis. Clinicians combine the RPR card test with results of other serologic tests, darkfield examinations, clinical signs and symptoms, and risk factors in arriving at a syphilis diagnosis. Without some other support for the diagnosis of syphilis, a reactive RPR card test is commonly unrelated to *T. pallidum* infection. The predictive value of a reactive RPR card test in a serologic diagnosis of syphilis is increased when combined with a reactive treponemal test, such as the fluorescent treponemal antibody absorption (FTA-ABS) test or the microhemagglutination assay for antibodies to *T. pallidum* (MHA-TP).

2. A reactive RPR card test may suggest past or present infection with a pathogenic treponeme; however, it may also be a false-positive reaction. False-positive reactions can result from laboratory error as well as serum antibodies unrelated to syphilis infection. Technical errors are detected by a nonreactive RPR card test with a second serum specimen. False-positive RPR card tests from infections with nontreponemal diseases or other disease conditions are identified by an accompanying nonreactive treponemal test.

3. A nonreactive RPR card test without clinical evidence of syphilis may suggest no current infection or an effectively treated infection. A nonreactive RPR card test with clinical evidence of syphilis can be seen in early primary syphilis; in secondary syphilis, as a result of the prozone reaction; and in some cases of late syphilis. A nonreactive RPR card test result does not rule out an incubating syphilis infection.

4. When the quantitative RPR card test is performed on patients with syphilis, a fourfold rise in titer in a repeat specimen may suggest an infection, a reinfection, or a treatment failure; a fourfold decrease, e.g. 1:16 to 1:4, in titer following treatment for early syphilis usually indicates that therapy was adequate.

5. All reactive qualitative RPR card tests should be diluted to an endpoint and the endpoint titer reported. Unusually high RPR card test titers can be seen with concurrent human immunodeficiency virus type 1 (HIV-1) infection. Unusually high false-positive titers may also be seen in patients with lymphomas.

ACCEPTABLE VARIATIONS

1. Prepare a 1:16 dilution for further quantitation, using 50 µl of specimen to 750 µl of 0.9% saline.

2. If, after screening, the serum to be quantitated seems likely to exceed 1:16, prepare all dilutions directly on the card, using a 1:50 nonreactive serum as the diluent beginning with circle 6 (1:32) continuing for the remainder of the card.

SOURCES OF ERROR

1. If the temperatures of the sera, reagents, or testing area are less than 23°C (73°F), test reactivity decreases; if temperatures are greater than 29°C (85°F), test reactivity increases.

2. If the speed of the mechanical rotator is too fast or too slow, improper antigen-antibody interaction will cause unpredictable test results.

3. If the time of rotation is too long test reactivity may be increased, or if too short test reactivity may be decreased.

4. If the card is excessively rotated and tilted (to-and-fro motions) by hand after removal from the rotator, a false-reactive result may occur.

5. If lighting produces a glare on the card, the reactions may be obscured.

6. If the antigen is outdated or not adequately tested for standard reactivity, the results may be inaccurate.

7. If the serum is unevenly spread in the circle, the antigen and antibody may not mix properly.

8. If hemolyzed, contaminated, or improperly collected serum or plasma specimens are tested, the reaction may be masked.

9. If the moistened humidifying cover is not used to cover tests as they are being rotated, proper humidity will not be maintained, and test components may dry on card giving rise to false reactive results.

TEST LIMITATIONS

1. The RPR card test cannot be used to test spinal fluids.[12]

2. A prozone reaction may be encountered occasionally. In a prozone reaction, complete or partial inhibition of reactivity occurs with undiluted serum (maximum reactivity is obtained only with diluted serum). The prozone phenomenon may be so pronounced that only a rough reading is produced in the qualitative test by a serum that will be strongly reactive when diluted. All test specimens producing any degree of roughness or reactivity with the RPR card test antigen in the qualitative test should be retested by using the quantitative procedure. In addition, a specimen should be tested for the prozone phenomenon when the clinician suspects syphilis, but the qualitative RPR is nonreactive.

3. The RPR card test may be reactive in persons from areas where yaws, pinta or nonvenereal syphilis is endemic. Generally, residual titers from these infections will be <1:8.[13, 14]

4. Biological false-positive (BFP) reactions occur occasionally with cardiolipin antigens, mainly in specimens from persons who abuse drugs; who have diseases such as lupus erythematosus, mononucleosis, malaria, leprosy, or viral pneumonia; or who have recently been vaccinated.

5. Nontreponemal test titers of persons who have been treated in latent or late stages of syphilis or who have become reinfected do not decrease as rapidly as do those of the persons in the early stages of their first infection. In fact, these persons may remain Aserofast,@ retaining a low-level reactive titer for life.[15]

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