SEROLOGIC TESTS FOR SYPHILIS

1955 MANUAL

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

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This manual was prepared with the assistance of the originators of each test technique and the Serology Section, Venereal Disease Research Laboratory, Chamblee, Ga. Director, Dr. Sidney Olansky; Deputy Director, Mr. Ad Harris.

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Preface

At the Assembly of Laboratory Directors and Serologists which met in Hot Springs, Ark., in October 1938, the Committee on the Need of Adherence to Conventional Technique in the Performance of Reliable Serologic Tests for Syphilis made the following recommendation:

"One of the most important factors responsible for nonadherence to the originator's technique is the failure of many serologists and serologic technologists to keep abreast of new developments. It is imperative that obsolete methods be abandoned. One source of difficulty has been the publication of desirable new modifications in technique by the originators of reliable serologic methods in a number of journals or books, some or all of which are not read by the serologist. To overcome this deficiency, this committee recommends the publication, in complete detail, of the technical procedures to be observed in the performance of each of the reliable, evaluated serologic tests for syphilis now commonly employed in this country. It is recommended that such a publication should be issued by the United States Public Health Service in collaboration with the originators of reliable methods as a supplement to *The Journal of Venereal Disease Information*."

These recommendations have been carried out by the issuance of manuals of Serologic Tests for Syphilis as Supplement 9 (1939), Supplement 11 (1940), and Supplement 22 (1949) to *The Journal of Venereal Disease Information*, and VD-Graphic 85 (1944). It is for the purpose of continuing these services that the Venereal Disease Research Laboratory of the U. S. Public Health Service has collaborated with test author-serologists in assembling the data for this new manual. Since *The Journal of Venereal Disease Information* has been discontinued, this manual is now designated as a Public Health Service publication.

Contents

General Information

A test technique is composed of equipment, reagents, volumes, time periods, temperatures, and orders of procedure. Each of these factors may be of equal importance and none can be disregarded with impunity. Technicians who make arbitrary changes in recommended techniques must assume full responsibility for the test results.

Although serologic tests for syphilis are not absolutely specific and some serums and spinal fluids have the capacity to react positively in one test and negatively in another, analyses of conflicting serologic results in terms of diagnosis or prognosis should not be made by the technician. These decisions lie only within the province of the physician. Valid serologic test findings are, however, obtained when (*a*) standardized reagents and adequate controls are used, (*b*) technique recommendations are strictly adhered to, and (*c*) results are reported as specified for each procedure.

Each technician should test every new lot of reagent in parallel with one that is being used and that has acceptable reactivity before the new lot of reagent is placed in routine use. This procedure is recommended regardless of the source from which the new reagent is obtained. Other factors to be considered in the control of serologic tests are discussed in the following paragraphs.

Equipment

Water bath temperatures should be checked each time during the day that they are used. Refrigerator temperatures should be checked daily with a thermometer placed in the part of the refrigerator occupied by the test racks. Temperature again should be noted when the refrigerator is first opened in the morning if complement-fixation tests (16- to 18-hour fixation) are stored within.

The speed of shaking and rotating machines should be checked by the technician each time they are used, and marked variation from prescribed speeds should not be tolerated.

Centrifuges should be equipped with tachometers so that speed may be checked and controlled. The inside of centrifuges should be cleaned occasionally to prevent dust particles from being blown into specimens.

Automatic pipetting machines should be checked daily for correct volume delivery. Should readjustment be found necessary, a volume of 25 or 50 deliveries may be collected and measured in a certified graduated cylinder.

Glassware

Only chemically clean glassware should be used in the serology laboratory. Tubes and pipettes from which protein solutions have not been completely removed will acquire a brown film. This film usually can be removed by submersion in sulfuric-acid-dichromate cleaning solution.

Whether alkaline or acid solutions are used for cleaning, rinsing should be adequate to remove all traces of cleaning solutions. Daily spot testing of glassware with indicator paper or solutions will insure against the release of chemically contaminated glassware to the testing laboratory.

Tubes, slides, or pipettes that become etched or scratched to a degree that will interfere with test readings should be discarded and replaced.

Reagents

Antigens

1. All of the serologic tests for syphilis described in this manual, with the exception of the Kahn procedures, employ cardiolipin-lecithin antigens. Cardiolipin and purified lecithin referred to in this text are products that have been isolated and purified by published methods (*1*) and that meet prescribed chemical and serologic assay standards of the World Health Organization.[1] In most instances it has been found to be more practical and economical to purchase cardiolipin antigens rather than to assemble them from their several components and do the necessary serologic checking.

2. Antigens should produce reactions in qualitative and quantitative tests with serum and spinal fluid that are comparable to those obtained with a standard antigen. Each new lot of antigen should be tested in parallel with one that is being used and has acceptable reactivity before the new lot of antigen is placed in routine use. Parallel testing should be accomplished on more than 1 day, and the differences in reactivity between the two antigens being tested should not be greater than those obtained with duplicate emulsions of standard antigen.

Chemicals

Chemicals such as sodium chloride, cholesterol, etc. should be of reagent quality and meet the specifications of the procedure technique. Adverse reactions may be encountered when substandard chemicals are employed.

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[1] International Pharmacopoeia.

Distilled Water

Distilled water of poor quality may result from failure to clean the still or storage container as frequently as needed. The kind of tap water used and the number of hours per day that the still is in operation will determine the frequency of cleaning. Periodic determinations of pH and conductivity serve as reliable indications of the purity of distilled water. If stored, distilled water should be placed in hard-glass containers that are tightly stoppered to avoid changes due to ion transfer from the glass and absorption of gases present in the laboratory. The use of freshly distilled water is preferred.

Saline Solutions

Sodium chloride for use in saline solutions should be dried in the hot-air oven for 30 minutes at 160° to 180° C. to remove absorbed moisture. Heating at higher temperatures should be avoided since it may result in decomposition of the salt. The sodium chloride may then be weighed and stored in corked test tubes to avoid daily weighing. Dissolve salts in distilled water. Shake solution thoroughly to assure complete mixing. If stored, the solution should be placed in hard-glass, tightly stoppered containers to avoid contamination.

Temperature

Serologic tests for syphilis are influenced to varying degrees by the temperature at which they are performed. Some tests are performed at prescribed water bath or refrigerator temperatures. For uniformity of results it is recommended that other tests be performed at room temperatures between 73° to 85° F. (23° to 29° C.).

Reporting Serologic Test Results

The use of new terminology for reporting results of serologic tests for syphilis was recommended in the 1953 report of the National Serology Advisory Council to the Surgeon General of the U. S. Public Health Service. The new system would provide uniformity of reporting and avoid diagnostic connotations. In accordance with these recommendations, the terms *Reactive, Weakly Reactive,* and *Nonreactive* are being substituted for the terms *Positive, Weakly Positive* or *Doubtful,* and *Negative* in reporting test results.

In reporting results of quantitative reactions, it is recommended (*2*) that the end-point titer be reported in terms of the greatest dilution in which the tested specimen produces a Reactive (positive) result, and that the term *dils,* a contraction of the word dilution, be used to identify these dilution reactivity end points. By this means, reactions of identical intensity will receive the same report, in terms of *dils,* when different testing methods are employed.

Control of Test Performance

Some of the factors which influence test performance have already been described. In addition, inter- and intralaboratory checks are strongly recommended.

These include the daily use of controls of graded reactivity, periodic check readings to maintain uniform reading levels among the laboratory personnel, and comparison of results obtained on control serums with those of a reference laboratory.

Serum controls should be included in each run of all serum-testing procedures. With slide flocculation tests, each preparation of antigen emulsion should first be examined with these control serums. In other instances, these controls should be included in the test run. The results obtained with the controls should reproduce an established pattern of reactivity. In the event that these results are not acceptable, further testing should be delayed until the optimal reactivity has been reestablished.

The daily use of prepared serum controls in all serologic tests for syphilis is a practice of the Venereal Disease Research Laboratory that has been valuable for detecting variations in test reactivity levels that might otherwise have escaped notice. Dr. W. A. Hinton does not concur in recommending their use in the Hinton test (*3*).

Serum controls of graded reactivity may be prepared in the following manner:

1. a. Collect serums, giving Reactive (not Weakly Reactive) reactions in daily test runs, in containers suitable for storage by freezing.

   b. Collect Nonreactive serums in a similar manner.

2. Store the pooled serums in a deep-freeze unit or freezing compartment of a refrigerator.

3. When control serums are to be prepared, allow the frozen serums to thaw at room temperature or in the 37° C. water bath.

4. Filter the pools of serums through a Seitz filter to remove particles.

5. Measure the serum in each pool and add 1 mg. of Merthiolate powder [2] for each milliliter of serum.

6. Prepare preliminary dilutions of Reactive serum in Nonreactive serum. Serial twofold dilutions may be used or a scheme similar to the one outlined in table 1 may be selected.

7. Perform all the tests regularly employed in the laboratory on these serum dilutions and record the results.

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[2] Eli Lilly and Co., Indianapolis, Ind.

Table 1. Results obtained with serum dilutions prepared for use as daily controls

Dilutions	Reactive serum	Nonreactive serum	Kahn test	VDRL slide test	Kline test	Mazzini test	Hinton test	Kolmer test
	(*ml.*)	(*ml.*)						
1	1.0	1.0	R4+	R	R4+	R	R	R4+
2	0.8	1.2	R4+	R	R4+	R	R	R4+
3	0.7	1.3	R4+	R	R4+	R	R	R4+
4	0.6	1.4	R3+	R	R4+	R	R	R4+
5	0.5	1.5	R2+	R	R3+	R	R	R4+
6	0.4	1.6	WR1+	R	R2+	R	R	R3+
7	0.3	1.7	WR±	WR	WR1+	R	R	R2+
8	0.2	1.8	N	WR	WR1+	WR	WR	WR±
9	0.1	1.9	N	N	N	N	N	N
10	0.0	2.0	N	N	N	N	N	N

R=Reactive. WR=Weakly Reactive. N=Nonreactive.

Based upon the results indicated in table 1, a set of dilutions suitable for Kahn, VDRL slide, and Kolmer tests might be selected as follows:

Control 1—Dilution 2
Control 2—Dilution 5
Control 3—Dilution 7
Control 4—Nonreactive Pool

OR

If the tests to be performed included the VDRL slide, Mazzini, and Hinton tests, a set of dilutions might be selected as follows:

Control 1—Dilution 2
Control 2—Dilution 6
Control 3—Dilution 8
Control 4—Nonreactive Pool

8. Select, for control serums, a dilution that is Reactive or 4+ in all tests, and one or more dilutions that show intermediate reactivity. If several tests are employed, two or more dilutions may be required to obtain critical readings in all tests.

9. Calculate the amount of each serum dilution to be prepared. This will be determined by the quantity needed for each day's testing, the period of time during which the controls will be used, and the type of storage facilities available.

Control serums, properly stoppered, may be stored for approximately 60 days in a deep-freeze unit, 30 days in the freezing compartment of a refrigerator, and from 7 to 10 days in a liquid state in a refrigerator.

10. Prepare the calculated volumes of each serum dilution and mix thoroughly.

11. Retest each serum mixture in all the tests in which it is to be used as a control.

12. Dispense aliquots of each dilution, sufficient for one testing period, into properly labeled tubes and stopper tightly with paraffin-coated corks. Arrange in sets and refrigerate. After 24 hours, reset the corks and seal with plastic seals or adhesive tape as further protection against evaporation.

13. For daily use, remove one set of controls from storage, thaw at room temperature, mix thoroughly, centrifuge, decant serum, and heat for 30 minutes at 56° C.

14. New lots of control serums should be tested in parallel with the one currently in use and the pattern of reactivity established before being placed into routine use.

References

(*1*) Pangborn, M. C.; Maltaner, F.; Tomkins, V. N.; Beecher, T.; Thompson, W. R.; Flynn, M. R.: Cardiolipin Antigens. World Health Organization: Monograph Series No. 6, 1951.

(*2*) Harris, A.: Quantitative serologic tests for syphilis. I. Standard method of reporting. J. Ven. Dis. Inform., 28: 249–252, November 1947.

(*3*) Hinton, W. A.: Personal communication.

General Equipment

The following items of equipment and glassware, commonly found in the serology laboratory, have been omitted from the lists appearing in the text under each test heading in order to avoid repetitious listing.

Equipment

1. Centrifuge, with tachometer.
2. Filter pump, for water connection.
3. Interval timers.
4. Microscope, monocular or binocular.
5. Microscope lamp.
6. Racks, wooden or wire, for specimen tubes.
7. Refrigerator, 6° to 10° C., with freezing compartment.
8. Stopwatch.
9. Thermometers, appropriate for water baths and refrigerator.
10. Water baths, 37° C. and 56° C.

Glassware

1. Bottles, glass-stoppered, Pyrex, 100-ml. to 1-liter capacities.
2. Cylinders, graduated, 50-ml. to 1-liter capacities.
3. Flasks, Erlenmeyer, 25-ml. to 2-liter capacities.
4. Pipettes, serologic, graduated to tip:
   - 0.1 ml., graduated in $\frac{1}{100}$ ml.
   - 0.2 ml., graduated in $\frac{1}{100}$ ml.
   - 1.0 ml., graduated in $\frac{1}{100}$ ml.
   - 5.0 ml., graduated in $\frac{1}{10}$ ml.
   - 10.0 ml., graduated in $\frac{1}{10}$ ml.
5. Tubes, specimen, of suitable sizes for blood and spinal fluid collection.

APHA Reference Test

Before performing this test, the technologist should become familiar with contents of chapters General Information and General Equipment.

Equipment

1. Rotating machine, adjustable to 180 r. p. m., circumscribing a circle ¾ inch in diameter on a horizontal plane.
2. Ringmaker to make paraffin rings approximately 14 mm. in diameter.
3. Slide holders. Made of any convenient material to accommodate 2- x 3-inch slides.
4. Hypodermic needles.

    a. 23-gage needles, calibrated to deliver 100 drops of saline solution per milliliter. (Holding the syringe almost vertically will usually deliver the required amount.)

    b. 25-gage regular bevel needles, calibrated to deliver 75 drops of antigen emulsion per milliliter. (Holding syringe horizontally, bevel down.)

    Note: Other means of delivery may be employed, but it is of primary importance that the proper amount (1/15 ml.) of antigen emulsion be used in each test.

Glassware

1. Pipettes, 0.2-ml., graduated in 0.01 ml.
2. Bottles, glass-stoppered or screw-capped (tinfoil-lined), round, 30-ml. capacity.
3. Glass slides, 3- x 2-inch.
4. Syringes, Luer-type, 1- or 2-ml.

Reagents

1. Antigen

    Antigen for this test is an alcoholic solution containing 0.03 percent cardiolipin, 0.9 percent cholesterol (Pfanstiehl-precipitated from absolute ethyl alcohol or equal quality), and purified lecithin in quantity necessary to reproduce the reactivity level of standard antigen. Joint studies (*1*) by the American Public Health Association and the Laboratory Section of the World Health Organization are in progress to determine the optimal lecithin concentration for this antigen.

2. Saline solutions

a. 0.9-percent saline solution

Carefully weigh 900 mg. of previously oven-dried sodium chloride and add the dried sodium chloride to 100 ml. of distilled water. Filter the saline solution before use.

b. Buffered saline solution

Formaldehyde, neutral, reagent grade, ml.	0.5
Secondary sodium phosphate ($Na_2HPO_4+12H_2O$), gm.	0.093
Primary potassium phosphate (KH_2PO_4), gm.	0.170
Sodium chloride, A. C. S., gm.	10.000
Distilled water, ml.	1,000.0

The solution yields potentiometer readings of pH 6.0 $\pm$0.1 and is stored in screw-capped or glass-stoppered bottles.

Preparation of Serums

1. Clear serum is heated in the 56° C. water bath for 30 minutes. All serums containing visible particles after the heating period should be recentrifuged.

2. Serums tested more than 4 hours after the original heating period should be reheated for 10 minutes in the 56° C. water bath.

Preparation of Slides

1. Paraffin rings are prepared by means of the ringmaker (see "Equipment" p. 9).

2. All slides are cleaned as follows:

a. New slides are cleaned with Bon Ami. The Bon Ami is allowed to dry and then is removed with a soft cloth.

b. Used slides are cleaned by first removing the paraffin with hot, soapy water, then washed with soap and water, and Bon Ami applied as described for cleaning of new slides.

3. If slides are cleaned properly, serum will spread unaided when placed within the paraffin ring.

4. If serum does not spread, do not use the slide, as it has not been sufficiently cleaned.

Preparation of Antigen Emulsion

1. Pipette 0.4 ml. of buffered saline solution to the bottom of a 30-ml. round, glass-stoppered or screw-capped bottle.

2. Add 0.5 ml. of antigen (from the lower half of a 1.0-ml. pipette

graduated to the tip) directly onto the saline solution while continuously but gently rotating the bottle on a flat surface.

Note: Antigen is added drop by drop, but rapidly, so that approximately 6 seconds are allowed for each 0.5 ml. of antigen. Pipette tip should remain in upper third of bottle and rotation should not be vigorous enough to splash saline solution onto pipette.

3. Blow last drop of antigen from pipette without touching pipette to saline solution.

4. Continue rotation of bottle 10 seconds more.

5. Add 4.1 ml. of buffered saline solution from a 5.0-ml. pipette.

6. Place top on bottle and shake vigorously for approximately 10 seconds.

7. Antigen emulsion then is ready for use and may be used during 1 day.

All antigen emulsions should be tested with control serums of graded reactivity, as well as with the saline solution. Reactions with control serums should reproduce the reactivity pattern established for them. The particles of the antigen emulsion in the Nonreactive serum and the saline solution control must not appear too large. If the antigen particles are not of satisfactory size (as determined by experience), the emulsion should be discarded.

The Serum Test

1. Pipette 0.04-ml., 0.02-ml., and 0.01-ml. quantities of heated serum to the first, second, and third rings of the paraffin slides, using a 0.2-ml. pipette.

2. Add with needle and syringe two and three drops of 0.9-percent saline solution to the second and third rings, respectively. (Each drop must contain 0.01 ml.; this is accomplished by using the 23-gage needle as explained in the section on "Equipment," p. 9.)

3. Add one drop of antigen emulsion to all three rings. (Each drop contains $\frac{1}{75}$ ml., using the 25-gage needle, holding syringe horizontally, bevel down, as explained in the section on "Equipment.")

4. Rotate the slide for 4 minutes at 180 r. p. m. on the mechanical rotator.

5. Read test immediately after rotation.

6. If clumps are present in the third ring, then dilute the serum 1:8 (1 part serum to 7 parts 0.9-percent saline solution) and repeat the test as with undiluted serum.

Outline of APHA Reference Microflocculation Test (2)

	Paraffin Ring No.					
	1	2	3	4	5	6
Serum, undiluted, ml.	0.04	0.02	0.01	. . .	. . .	. . . .
Serum, diluted 1:8, ml.	. . .	. . . .	. . . .	0.04	0.02	0.01
Saline solution (each drop, 1/100 ml.).	0	2 drops	3 drops	0	2 drops	3 drops
Antigen emulsion (each drop, 1/75 ml.).	1 drop	1 drop	1 drop	1 drop	1 drop	1 drop
Serum dilution . . .	1:1	1:2	1:4	1:8	1:16	1:32

Note: If clumps are present in ring 6 (1:32 dilution), dilute serum 1:64 and repeat test. A Nonreactive result must be obtained before test is concluded.

Reading and Reporting Test Results

1. Read tests microscopically, at 100 X magnification.

2. The result of the reaction between the serum and the antigen is considered to be either Reactive or Nonreactive.

3. Definite clumping of the antigen particles in the serum and/or serum dilution tests is reported as Reactive.

4. No clumping or slight roughness of the antigen particles is reported as Nonreactive.

5. All Reactive results are reported by indicating the highest dilution of serum which caused the antigen particles to clump. For example:

Reactive, undiluted only, or 1 dil
Reactive, 1:2 dilution, or 2 dils
Reactive, 1:4 dilution, or 4 dils

References

(*1*) World Health Organization, Laboratory Section. Personal communication.

(*2*) Subcommittee to Develop Reference Methods for Syphilis Serology. Am. J. Pub. Health, Part 2, Year Book, 42: 78-82, May 1952.

Hinton Tests

Before performing this test, the technologist should become familiar with contents of chapters General Information and General Equipment.

Equipment

1. Kahn shaking machine (275 to 285 oscillations per minute, with 1½-inch stroke).

Glassware

1. Test tubes,[1] 100- x 11¼-mm. outside dimensions, hereafter called Hinton tubes.

2. Flasks, Erlenmeyer, 125-ml. or 250-ml. capacity, with inverted V-shaped ridge in bottom that produces two semicircular compartments (Hinton flasks).

Reagents

1. Hinton indicator

   Stock indicator for this test is an alcoholic solution containing 0.0884 percent cardiolipin, 0.6188 percent purified lecithin, and 0.24 percent cholesterol. Each lot of antigen must be serologically standardized by proper comparison with an antigen of known reactivity (*1–3*).

   Note: Dr. Hinton recommends that laboratories do not depend solely upon this formula, but purchase the indicator from manufacturers who sell it, only if its potency is of the highest grade as determined by serologic and clinical tests.

2. 5-percent sodium chloride solution

   a. Weigh 5 gm. of previously dried sodium chloride (A. C. S.).

   b. Add sodium chloride to 100 ml. of distilled water and heat solution in an autoclave at 15 pounds pressure for 15 minutes.

   c. Store saline solution in glass-stoppered bottles at room temperature (73° to 85° F.).

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[1] Catalog No. 45060/S73, Kimble Glass Co., Vineland, N. J.

3. 0.85-percent sodium chloride solution

Add the required amount (8.5 gm. to each liter) of dried sodium chloride to distilled water. This solution need not be heated and should be prepared on the day used.

4. 50-percent solution of glycerin

Mix equal volumes of Baker and Adamson's glycerin [2] (reagent grade) and distilled water. This solution keeps indefinitely.

HINTON TESTS WITH SERUM

Preparation of Serums

1. Remove serums from clots by centrifuging and pipetting or decanting.

2. Heat the serums in the 56° C. water bath for 30 minutes. Serums should not be heated before the day of testing. If it is necessary to retest a specimen, use serum freshly separated from the clot, if available.

3. Recentrifuge any specimen in which visible particles have formed during heating.

Preparation of Glycerinated Hinton Indicator

1. Pipette one part of Hinton stock indicator into one compartment of a Hinton flask.

Note: Not less than 1 ml. nor more than 5 ml. of Hinton indicator should be mixed at one time.

2. Pipette 0.8 part of 5-percent sodium chloride solution into the other compartment of the flask. Care should be used in pipetting the saline solution into the flask in order to avoid premature mixing of the solutions.

3. Mix contents by shaking the flask very rapidly from side to side for exactly 1 minute.

4. Let the mixture stand for exactly 5 minutes.

5. Add 13.2 parts of 5-percent sodium chloride solution and shake flask vigorously.

6. Add 15 parts of 50-percent glycerin solution and shake flask until the suspension is homogeneous.

7. Store in a glass-stoppered bottle or flask in the refrigerator. This suspension, referred to as glycerinated indicator solution, remains usable for at least 3 weeks.

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[2] General Chemical Division, Allied Chemical and Dye Corp., New York, N. Y.

Hinton Standard Qualitative Test With Serum

1. Arrange Hinton tubes in suitable racks so that there is one tube for each serum to be tested (and also for control serums of graded reactivity).[3] Number tubes to correspond to the identifying numbers of serums.

2. Pipette 0.5 ml. of each heated serum into its corresponding tube.

Note: Occasionally very strongly Reactive serums will elicit a Non-reactive reaction when 0.5 ml. of serum is employed as the testing quantity. When this type of reaction is suspected, 0.1 ml. of serum should also be tested in addition to the 0.5-ml. quantity of serum.

3. Pipette 0.5 ml. of the glycerinated Hinton indicator into each serum tube.

Note: Flask containing glycerinated Hinton indicator should be shaken when taken from refrigerator. Remove quantity of glycerinated indicator needed and return flask to cold storage immediately.

4. Shake rack of tubes by hand until visual inspection indicates that serums and glycerinated indicator are well mixed.

5. Shake rack of tubes on Kahn shaking machine for 5 minutes.

6. Remove the rack from the shaking machine and place in 37° C. water bath for 16 hours.

Note: The water bath must be uncovered during this period, and temperature should be maintained at 37° C. ± 1°.

Reading and Reporting Test Results

1. Place a shaded cylindrical fluorescent (daylight) lamp 18 or more inches long in front of a darkened background. The lamp tube should be slightly above the level of the eyes.

2. Remove each tube from the rack carefully without disturbing contents.

3. Hold the tube at a 45° angle, at eye level, close to lamp shade.

4. Look for clarification of the fluid and for presence or absence of a ring of white flakes or white coarse granules at the meniscus.

5. Lift the tilted tube slightly above eye level and look through it towards the darkened background to determine the presence or absence of flocculation.

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[3] Dr. W. A. Hinton recommends against the use of control serums of graded reactivity (4), as used at the Venereal Disease Research Laboratory, since he considers them to be "the least stable substances used in the test," and therefore a possible source of confusion.

6. Report findings as follows:

Reactive	White flakes or white coarse granules at the meniscus, and definite flocculation when tube is shaken.
Nonreactive	Absence of ring or band of floccules, and no flocculation or granularity when tube is gently shaken. Hemolyzed or bacterially contaminated serums frequently produce a whitish ring which is strongly adherent to the tube.

7. Centrifuge, at 2,000 r. p. m. for 5 minutes, all tubes in which clear-cut Nonreactive or Reactive readings cannot be made.

8. Remove tubes from centrifuge and read reactions as described previously.

9. Report findings as follows:

Weakly Reactive . . .	Those reactions demonstrating coarse granulation at the meniscus, and definite flocculation when tube is gently shaken.
Nonreactive	All specimens failing to react as described under "Weakly Reactive" above.
Unsatisfactory	Those specimens hemolyzed or bacterially contaminated unless the result is strongly Reactive.

Hinton Rapid Test With Serum

1. Heat fresh serum for 3 minutes at 60° C.

2. Arrange Hinton tubes in suitable racks so that there is one tube for each serum to be tested and for controls of graded reactivity.

3. Pipette 0.5 ml. of serum to be tested into properly numbered test tubes.

4. Add 0.5 ml. of glycerinated Hinton indicator to each serum tube and shake on shaking machine for 10 minutes, after preliminary hand shaking, to mix contents of tubes.

5. Remove rack of tubes from shaking machine and place in the 37° C. water bath for 20 minutes.

6. Remove tubes from water bath and centrifuge at 2,000 r. p. m. for 10 minutes.

7. Remove tubes from centrifuge without agitating contents.

8. Read each tube as described under "Reading and Reporting Test Results" (p. 15).

9. Report observed results as follows:

Reactive	Plainly visible flakes at the meniscus, and well-marked flocculation when tubes are gently shaken.
Nonreactive	Absence of ring or band of floccules at the meniscus, and no flocculation when tubes are gently shaken.
Unsatisfactory . . .	Those specimens hemolyzed or bacterially contaminated unless the reaction is strongly Reactive.

Hinton Standard Quantitative Test With Serum

1. Prepare serum dilutions in the following manner:

   a. Set up Hinton tubes numbered 1 to 8.

   b. Add 0.5 ml. of 0.85-percent sodium chloride solution to tubes 2 through 8.

   c. Add 0.5 ml. of heated serum to tubes 1 and 2.

   d. Mix and transfer 0.5 ml. from tube 2 to tube 3.

   Continue this procedure through the 8th tube and discard 0.5 ml. from tube 8.

2. Add 0.5 ml. of glycerinated indicator to each of the 8 tubes.

3. Shake rack of tubes by hand until visual inspection indicates that serums and glycerinated indicator are well mixed.

4. Place rack on Kahn shaker and shake for 5 minutes.

5. Place rack of tubes in uncovered 37° C. water bath for 16 hours.

6. Read reactions as described under "Reading and Reporting Test Results" (p. 15).

7. Report results in terms of the greatest dilution that produces a Reactive result, i. e., Reactive in 1:8 dilution, Reactive in 1:16 dilution, etc.

Tube No. 1 contains undiluted serum.

DAVIES-HINTON TESTS

Davies-Hinton Microflocculation Test With Serum

Equipment

1. Rubber caps.[4]
2. Rubber bulbs, for capillary pipettes.

Glassware

1. Glass tubes, 80- x 2.5-mm. inside diameter.
2. Capillary pipette (10- x 1-cm. diameter, capillary end being approximately 1 mm. in diameter).

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[4] Listed as No. 3 vial stoppers in No. 68 stock by West Co., 1117 Shakamaxon Street, Philadelphia, Pa.

Preparation of Serums

1. Collect blood in glass tubes (80- x 2.5-mm.), hereafter called collection tubes.

2. Remove cap from one end of tube and use a wire to loosen clot from side of tube.

3. Place capped blood-collection tube into a labeled 13- x 100-mm. test tube and centrifuge at high speed for 10 minutes. Recentrifuge if serum is not well separated.

4. Add water to 13- x 100-mm. test tubes containing capped blood-collection tubes (clot downward) and place in 56° C. water bath for 30 minutes.

5. Remove tubes from water bath and discard water from test tubes.

6. Remove cap from serum end of collection tube and notch tube with a glass file just above junction of clot and serum.

7. Hold the collection tube horizontally, break it, and discard part of tube containing clot.

SERUM MICROFLOCCULATION TEST PROCEDURE

1. Transfer each serum to two glass collection tubes. One tube should contain a column of serum about 2.5 cm. in length and the other a column of serum 0.5-cm. to 1.0-cm. long.

2. Add to the tube containing the 2.5-cm. column an equal amount of glycerinated Hinton indicator. Use capillary pipette for indicator.

Care should be taken not to allow air to separate serum and indicator.

3. Add a column 2.5 cm. to 5.0 cm. in length of glycerinated Hinton indicator to the serum contained in the second tube (0.5-cm. to 1.0-cm. column).

4. Mix the serum and glycerinated Hinton indicator in each tube by tilting the liquid toward alternate ends of the tube 10 times.

5. Cap both ends of the two collection tubes and place them in test tubes (13- x 100-mm.) that are identically numbered.

6. Fill test tubes containing capped collection tubes with water and place in 37° C. water bath for 16 hours.

7. Remove tubes from water bath, pour water from test tubes, and centrifuge test tubes containing capped collection tubes for 5 minutes at approximately 2,000 r. p. m.

Reading and Reporting Test Results

1. Read results under the low-power objective of the microscope, with only enough light so that aggregates at the meniscus are readily visible. The stage of the microscope should be tilted approximately

30° from horizontal and the tube should be placed under the lens with the meniscus uppermost.

2. Observe the degree of clumping, if any, at the meniscus, and report results as follows:

Reactive	Definite, discrete, compact clumps at the meniscus in either tube. (Gentle thumping of the tube may help float clumps into view.)
Nonreactive	No clumps visible in either tube. Amorphous, cloudy, granular particles at the meniscus are also interpreted as Nonreactive.
Weakly Reactive . .	Few small clumps at the meniscus of either tube. In such instances, clumps should be redispersed by thumping the tube with a finger and the tube recentrifuged for 3 minutes. The test is reported Weakly Reactive if small clumps are again visible at the meniscus, but Reactive if large, compact clumps are then present in either tube.

Davies-Hinton Flocculation Test With Spinal Fluid

Reagents

1. Glycerinated Hinton indicator. (See "Preparation of Glycerinated Hinton Indicator," p. 14.)

2. 0.85-percent sodium chloride solution. (See "Reagents" for "Hinton Tests," p. 14.)

3. 3.0-percent sodium chloride solution

Add the required amount (3 gm. to each 100 ml.) of dry sodium chloride to distilled water. This solution should be prepared on the day used.

4. Hinton Nonreactive human serum

Select one or more clear Hinton Nonreactive serums and retest in accordance with the "Hinton Rapid Test With Serum" technique (p. 16), employing the following two indicated quantities:

Tube 1: 0.5 ml. of serum and 0.5 ml. of glycerinated Hinton indicator.
Tube 2: 0.1 ml. of serum and 0.5 ml. of glycerinated Hinton indicator.

Note: When large numbers of spinal fluids are tested, it is convenient to pool and Seitz-filter serums, add Merthiolate to a concentration of 1:10,000, and then to perform Hinton rapid tests as above. Store tested serum at 8° to 10° C. for not more than 3 weeks. Avoid the use of cloudy serum.

5. Gum acacia, 20-percent solution

a. Place 20 gm. of white, powdered gum acacia (U. S. P.) in a 4-ounce bottle.

b. Add 100 ml. of 3-percent sodium chloride solution.

c. Place Bakelite cap with Vinylite liner on bottle loosely (do not screw cap on).

d. Place bottle in the autoclave and heat at 15 pounds pressure for 15 minutes.

e. Remove bottle from autoclave, screw cap on tightly, shake well to completely dissolve the acacia, and maintain in a sterile condition.

Preliminary Test of Hinton Nonreactive Serum and Gum Acacia Solution

1. For every 10 spinal fluids to be tested mix 5 ml. of Hinton Nonreactive serum with 5 ml. of 20-percent gum acacia solution.

2. Perform a rapid test as follows:

a. Into a Hinton tube, pipette 0.6 ml. of 0.85-percent sodium chloride solution, 0.2 ml. of freshly mixed acacia-serum mixture, 0.2 ml. of glycerinated Hinton indicator, and mix well by shaking.

b. Place tube in a 37° C. water bath for 20 minutes.

c. Centrifuge tube at 2,000 r. p. m. for 5 minutes.

d. A satisfactory acacia-serum mixture yields a Nonreactive result.

Preparation of Spinal Fluid

Centrifuge and decant spinal fluid. Fluids which are visibly contaminated or contain gross blood are unsatisfactory for testing.

SPINAL FLUID FLOCCULATION TEST PROCEDURE

1. Arrange Hinton tubes in racks so that there are four tubes (one behind the other) for each spinal fluid to be tested and for Reactive and Nonreactive spinal fluid controls. Number tubes to correspond to the identifying number of each fluid.

2. Pipette 0.6 ml. of each spinal fluid into the correspondingly numbered tube in the first row, 0.4 ml. into the tube in the second row, 0.2 ml. into the tube in the third row, and 0.1 ml. into the tube in the last row.

3. Add 0.2 ml. of acacia-serum mixture to every tube.

4. Add 0.2 ml. of glycerinated Hinton indicator to every tube.

5. Shake racks of tubes vigorously until contents become completely homogeneous.

6. Place racks of tubes in a 37° C. water bath for 16 hours.

7. Remove all tubes from water bath and centrifuge at 2,000 r. p. m. for 5 minutes.

Reading and Reporting Test Results

1. Remove tubes from centrifuge gently, without disturbing contents.

2. Before a suitable artificial light (see "Reading and Reporting Test Results" under "Hinton Standard Qualitative Test With Serum," p. 15), tap each tube gently at the base while holding it near the top.

3. Report as Reactive all spinal fluids that show definite floccules, dispersing downward from the meniscus, in any of the four tubes.

4. Recentrifuge all other tubes at 2,000 r. p. m. for 5 minutes.

5. Remove tubes from the centrifuge and reexamine with tapping as described previously.

6. Report as follows:

Reactive	Definite floccules dispersed downward from the meniscus in one or more of the four tubes.
Weakly Reactive . .	Questionable flocculation in any tube.
Nonreactive	Absence of flocculation and a ground-glass appearance in all tubes.

References

(*1*) STUART, G. O.; GRANT, J. F.; HINTON, W. A.: A note on the use of cardiolipin in the preparation of indicator (antigen) for the Hinton test. J. Ven. Dis. Inform., 29: 27, January 1948.

(*2*) HINTON, W. A.; STUART, G. O.; GRANT, J. F.: The use of cardiolipin-lecithin in the preparation of antigen for the Hinton test. Am. J. Syph., Gonor. and Ven. Dis., 33: 587–592, November 1949.

(*3*) HINTON, W. A.: Personal communication, April 1953.

(*4*) HINTON, W. A.: Personal communication, June 1954.

Kahn Tests

Before performing this test, the technologist should become familiar with contents of chapters General Information and General Equipment.

Equipment

1. Kahn test tube racks.
2. Vial racks.
3. Kahn shaking machine (275 to 285 oscillations per minute, with 1½-inch stroke).
4. Microscope mirror.
5. Gooseneck-type lamp with blue inside-frosted bulb, or fluorescent lamp (adjustable).

Glassware

1. Test tubes, Kahn, 75- x 12-mm. outside dimensions.
2. Pipettes, Kahn, to deliver 0.25 ml., graduated in 0.0125 ml., for antigen suspension.
3. Vials, Kahn antigen-suspension, with flat bottom, 55- x 15-mm. inside diameter.

Reagents

1. Kahn standard antigen. (See "Preparation of Kahn Standard Antigen," p. 36.) The label should give the titer of the antigen.

   a. The antigen should be stored at room temperature (73° to 85°F.) in the dark.

   b. Changes in antigen due to aging are generally reflected in the appearance of Nonreactive tests. If these reactions become too sharply clear or if they become cloudy, the antigen should be retitrated and restandardized. The need for retitration and/or restandardization of this reagent occurs but rarely. Such procedures should be carried out only by a laboratory qualified to make such adjustments.

2. Saline solution

   a. Weigh 9.0 gm. of dried, chemically pure sodium chloride (A. C. S.) for each liter of saline solution.

   b. Dissolve salt in distilled water. Shake solution thoroughly to assure complete mixing. The pH of the 0.9-percent saline solution should not be less than 5.5 nor more than 7.0.

KAHN TESTS WITH SERUM

Preparation of Serums

1. Separate serums from clotted blood that has been centrifuged, by pipetting or decanting.

2. Heat serums in 56° C. water bath for 30 minutes. After removal from water bath, serums should be allowed to remain at room temperature for at least 10 minutes so that all specimens will return to room temperature (73° to 85° F.) before being tested.

When reexamination of the specimen is required, the serums are reheated for 10 minutes if retesting is accomplished within 2 to 24 hours of the initial heating period, or, for 15 minutes, if the retesting is accomplished more than 24 hours after the initial heating period.

If serums have been stored in the refrigerator, they should be allowed to come to room temperature before heating.

3. Any serum in which visible particles have formed during heating or storage should be recentrifuged and decanted.

Kahn Standard Qualitative Test With Serum

1. Arrange test tubes in standard Kahn racks so that there are three tubes for each serum to be tested, including control serums of graded reactivity, and a saline solution control. Number the first row of tubes to correspond to the serums being tested.

2. Prepare standard antigen suspension as follows:

a. Measure into an antigen-suspension vial the amount of saline solution, according to titer, required for the given amount of antigen.

b. Measure into a second antigen-suspension vial the necessary quantity of antigen.

Note: The titer on the bottle of the antigen will state the amount of saline solution that must be mixed with 1 ml. of antigen in order to produce a suspension of standard reactivity. Usually 1 ml. of antigen makes sufficient suspension for 20 tests. Less than 1 ml. or more than 2 ml. of antigen should not be measured in one mixing vial.

c. Pour the saline solution into the antigen and, without stopping, pour the mixture back and forth 12 times. Do not allow vials to drain during mixing period.

d. Allow the antigen suspension to stand for 10 minutes before using. The suspension should not be used beyond 30 minutes from the time of mixing.

3. Place thumb over mouth of mixing vial and shake briskly to suspend antigen particles.

4. Pipette 0.05 ml. of antigen suspension directly to the bottom of each tube of the front row of the Kahn rack, employing a Kahn antigen pipette.

5. Pipette 0.025 ml. of antigen suspension directly to the bottom of each tube of the middle row.

6. Pipette 0.0125 ml. of antigen suspension directly to the bottom of each tube of the back row.

7. Add 0.15 ml. of each serum to the designated set of three tubes containing 0.05 ml., 0.025 ml., and 0.0125 ml. of antigen suspension, respectively.

Note: Complete the addition of antigen suspension and serums to one rack before adding antigen suspension and serums to another rack.

8. Shake rack by hand for 10 seconds, after antigen suspension and serum have been added to all tubes in that rack.

9. Permit serum-antigen-suspension mixture to stand for 3 to 7 minutes at room temperature.

10. Shake rack of tubes for 3 minutes on Kahn shaking machine.

11. Remove rack from shaking machine. Add 1.0 ml. of saline solution to each tube of the front row, and 0.5 ml. of saline solution to each tube of the middle and back rows.

Note: Add saline solution to one rack and complete readings before adding saline solution to another rack.

12. Shake rack by hand gently for a few seconds to mix contents of tubes.

13. Read each tube of the rack immediately (within 2 minutes) after the addition of saline solution. (See "Reading of Results" and "Reporting Results," p. 26.)

14. Reread each tube 15 minutes after the first reading was taken in all instances where a Nonreactive finding was not obtained on the first reading.

Outline of Kahn Standard Qualitative Test With Serum

	Tube 1 (front)	*Tube 2 (middle)*	*Tube 3 (back)*
Ratios of serum : antigen suspension	3:1	6:1	12:1
Antigen suspension, ml.	0.05	0.025	0.0125
Serum, ml.	0.15	0.15	0.15
Shake by hand for 10 seconds. Allow to stand 3 to 7 minutes. Shake for 3 minutes on Kahn shaker.			
Saline solution, ml.	1.0	0.5	0.5
Shake sufficiently to mix ingredients, and examine for presence and absence of precipitates.			

Reading of Results

1. Place a microscope mirror on the work bench with the concave side upward.

2. Adjust a reading lamp (daylight bulb or fluorescent tube) above the mirror so that the bulb image is not visible, but so that the tube can be held within cone of light.

3. Place each tube to be read in a nearly horizontal position with the lower portion of tube about 1 to 2 inches above the mirror.

4. View the image of tube contents in the mirror and note degree of flocculation.

Recording Results

Record degrees of flocculation in accordance with the following outline:

4+	Relatively large floccules.
3+	Medium-sized floccules.
2+	Fine floccules easily distinguishable.
1+	Very fine floccules.
±	Extremely fine floccules just distinguishable.
−	An opalescent medium free from visible floccules.

Reporting Results

Two general types of reactions are observed in the Kahn test:

1. *Typical reactions*—characterized by the occurrence of the *greatest* degree of flocculation with the *lesser* amounts of antigen suspension.

OR

2. *Zonal reactions*—characterized by the occurrence of the *greatest* degree of flocculation with the *larger* amounts of antigen suspension or *equal* degree of flocculation (other than 4 4 4) with *all* three amounts of antigen suspension.

In all instances where these reactions are observed (with exceptions as noted in table 2), both supplementary tests (p. 29) must be performed and evaluated before a report is issued. (See "Reporting of Results" under *Kahn Supplementary Tests*, p. 30.)

Reporting of typical reactions

a. Add the pluses (disregarding ± readings) obtained in the first and second readings.

b. Report test results as Reactive, Weakly Reactive, and Nonreactive as illustrated in table 1.

Table 1. Reporting of typical reactions

Sum of pluses of 1st and 2nd tube readings [1]	Report
22 to 24	Reactive (4+).
16 to 21	Reactive (3+).
10 to 15	Reactive (2+).
5 to 9	Weakly Reactive (1+).
[a] 4	Weakly Reactive (±).
[b] 3 or less	Nonreactive.

[1] ±readings are disregarded.

[a] A sum of 4 pluses should be given a Nonreactive report when the results are −11 and −11 on first and second readings.

[b] A sum of 3 pluses should be given a Weakly Reactive (±) report when the results are −−3 on first reading and −−± on second reading, or when they are −−2 on first reading and −−1 on second reading.

Reporting of zonal reactions is described in tables 2 through 8.

Table 2. Zonal reactions reported "Reactive (4+)" (supplementary tests not necessary)

Serum No.	First reading			Second reading		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
1	4	4	3	4	4	3
2	4	4	2	4	4	2
3	4	4	1	4	4	1
4	4	4	—	4	4	—

Table 3. Zonal reactions reported "Reactive (4+)" provided supplementary tests 1 and 2 are Reactive

Serum No.	First reading			Second reading		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
1	4	3	3	3	3	3
2	4	3	2	4	3	2
3	4	3	—	4	3	—
4	4	1	—	4	—	—
5	3	3	3	3	3	3
6	2	2	2	2	2	2
7	2	—	—	2	—	—
8	1	1	1	1	1	1
9	1	—	—	1	—	—
10	±	±	±	±	±	±
11	±	—	—	±	—	—

Table 4. Zonal reactions reported "Reactive (3+)" provided supplementary tests 1 and 2 are Nonreactive

Serum No.	First reading			Second reading		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
1	4	3	3	3	3	3
2	4	3	2	4	3	2
3	3	3	3	3	3	3

Table 5. Zonal reactions reported "Reactive (2+)" provided supplementary tests 1 and 2 are Nonreactive

Serum No.	First reading			Second reading		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
1	4	2	2	3	2	2
2	4	1	1	4	1	1
3	4	3	±	4	3	±
4	3	3	2	3	3	—

Table 6. Zonal reactions reported "Weakly Reactive (1+)" provided supplementary tests 1 and 2 are Nonreactive

Serum No.	First reading			Second reading		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
1	4	1	±	3	1	±
2	3	2	±	3	—	—
3	3	±	—	3	±	—
4	2	2	2	2	2	2

Table 7. Zonal reactions reported "Weakly Reactive (±)" provided supplementary tests 1 and 2 are Nonreactive

Serum No.	First reading			Second reading		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
1	2	2	1	2	2	±
2	2	2	—	2	1	—
3	2	1	—	2	±	—

Table 8. Zonal reactions reported "Nonreactive" provided supplementary tests 1 and 2 are Nonreactive

Serum No.	First reading			Second reading		
	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
1	2	±	±	2	±	±
2	2	±	±	2	—	—
3	2	±	—	2	—	—
4	2	—	—	2	—	—
5	1	1	±	1	±	±
6	1	±	±	1	—	—
7	±	±	±	±	—	—
8	±	—	—	±	—	—

KAHN SUPPLEMENTARY TESTS WITH SERUM

Supplementary Test 1

1. Pipette 0.05 ml. of antigen suspension (see "Kahn Standard Qualitative Test With Serum," par. 2, p. 24) to the bottom of each of two tubes (numbered 1 and 2).

2. Add 0.05 ml. of serum (previously heated) to tube 1 and 0.10 ml. of serum to tube 2.

3. Shake rack by hand for 10 seconds to mix contents of tubes. Allow to stand 3 to 7 minutes.

4. Shake rack on Kahn shaker for 3 minutes.

5. Remove rack from shaker, add 1.0 ml. of saline solution to each tube, and shake by hand to mix.

6. Read immediately, and again after 15 minutes, as described under "Reading of Results" (p. 26).

Reactive: A 4+ or 3+ reaction in each of the two tubes.
Nonreactive: Less than a 3+ reaction in each of the two tubes.

Supplementary Test 2

1. Prepare serum dilutions 1:4, 1:8, and 1:16 in the following manner:

a. Pipette into three tubes (numbered 1, 2, and 3) 0.6, 0.4, and 0.4 ml. of saline solution, respectively.

b. Add 0.2 ml. of serum to tube 1 and mix.

c. Transfer 0.4 ml. from tube 1 to tube 2 and mix.

d. Transfer 0.4 ml. from tube 2 to tube 3 and mix.

2. Pipette 0.0125 ml. of antigen suspension (see "Kahn Standard Qualitative Test With Serum," par. 2, p. 24) to the bottom of three Kahn tubes (numbered 1, 2, and 3).

3. Add 0.15 ml. of the 1: 4, 1: 8, 1: 16 dilutions of serum to tubes 1, 2, and 3, respectively, starting with the highest serum dilution.

4. Shake rack by hand 10 seconds to mix contents of tubes. Allow to stand 3 to 7 minutes.

5. Shake rack on Kahn shaker for 3 minutes.

6. Remove rack from shaker, add 0.5 ml. of saline solution to each tube, and shake to mix.

7. Read immediately as described under "Reading of Results" (p. 26).

Reactive: A 4+ or 3+ reaction in at least the 1 : 4 dilution.
Nonreactive: A 2+ reaction, or less, in the 1 : 4 and higher dilutions.

Reporting of Results

1. *Supplementary tests 1 and 2 Reactive.*

Report results of the standard test as Reactive (4+).

2. *Supplementary tests 1 and 2 Nonreactive.*

Report results of the standard test as indicated in tables 4 through 8, (pp. 28–29).

3. *Supplementary test 1 Reactive and supplementary test 2 Nonreactive.*

Prepare 1: 32, 1: 64, and 1: 128 dilutions of the serum, and test as indicated under supplementary test 2 (p. 29). If the result is Reactive in one or more tubes (a 4+ or 3+ reaction), report the results of the standard test as Reactive (4+). If the result is Nonreactive in all tubes (less than a 3+ reaction), report the results of the standard test as indicated in tables 4 through 8 (pp. 28–29).

4. *Supplementary test 1 Nonreactive and supplementary test 2 Reactive.*

Test the undiluted serum in a 1: 2 ratio of serum : antigen suspension (0.05 ml. of antigen suspension plus 0.025 ml. of serum). If the result is Reactive (a 4+ or 3+ reaction), report the results of the standard test as Reactive (4+). If the result is Nonreactive (less than a 3+ reaction), report the results of the standard test as indicated in tables 4 through 8, (pp. 28–29).

Control System for the Kahn Standard Test

1. Control serums of graded reactivity and a saline solution control should be tested with each antigen suspension concurrently with the regular tests.

2. Results obtained with serums or spinal fluids should not be reported if the prescribed pattern of reactivity is not obtained with the controls. Failure to obtain satisfactory results may be caused by

a. improperly prepared saline solution,

b. substandard antigen,

c. incorrectly prepared antigen suspension,

d. by using antigen suspension more than 30 minutes old,

e. by performing tests at cold room temperature.

3. In Dr. Kahn's laboratory, the control system is carried out before performing the regular tests. The antigen suspension for the control is pipetted immediately after it has aged for 10 minutes. After pipetting the controls, the tests are shaken at once on the Kahn shaking machine for 3 minutes, saline solution added, and the results read but once.

Note: This control setup, completed in not more than 5 minutes, permits the reading of the results of the control tests before performing the regular tests, and makes possible the detection of technical error in the preparation of the antigen suspension.

Kahn Standard Quantitative Test With Serum

Quantitative tests are performed on all serums that are 4+ or 3+ in the Kahn standard qualitative test.

1. Prepare serum dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and higher, if necessary, in the following manner:

a. Pipette into each of six (or more) tubes, 0.5 ml. of 0.9-percent saline solution.

b. Add 0.5 ml. of heated serum to the first tube and mix.

c. Transfer 0.5 ml. from the first to the second tube and mix.

d. Continue transferring and mixing from one tube to the next until all dilutions have been made. Allow the mixing pipette to remain in the last tube.

Note: Serum dilutions should be prepared and tested within 2 hours of the heating or reheating of the undiluted serum.

2. Prepare antigen suspension as previously described under "Kahn Standard Qualitative Test With Serum" (p. 24), by mixing Kahn standard antigen with 0.9-percent saline solution.

3. After the antigen suspension has stood 10 minutes (but not more than 30 minutes), place the thumb over the mouth of the mixing vial containing the suspension and shake vial briskly to obtain a uniform suspension.

4. Pipette 0.0125 ml. of antigen suspension to the bottom of six (or more) numbered Kahn tubes.

5. Add 0.15 ml. of the 1:64 dilution of serum to the antigen suspension contained in tube 6.

6. Add 0.15 ml. of the 1:32 dilution of serum to the antigen suspension contained in tube 5.

7. Continue addition of 0.15 ml. of decreasing dilutions of the serum to tubes 4, 3, 2, and 1, respectively.

8. Shake rack by hand for 10 seconds and then allow to stand 3 to 7 minutes.

9. Shake rack on the mechanical shaker for 3 minutes.

10. Remove the rack from the shaking machine, and add 0.5 ml. of 0.9-percent saline solution to each tube.

Note: Add saline solution to one rack and complete reading before adding saline solution to another rack.

11. Shake rack by hand a few seconds to mix contents of tubes.

12. Read each tube of the rack within 2 minutes after the addition of 0.9-percent saline solution.

13. Note the titration end point, i. e., the highest dilution of serum in which a 4+, 3+, or 2+ reaction is observed.

14. Compute the quantitative titer by applying the formula S=4D, where S is the potency of the serum in terms of Kahn units and D is the highest dilution in which a 4+, 3+, or 2+ reaction is observed.

Examples:

a. If the highest dilution in which a 4+, 3+, or 2+ reaction is observed is 1:64, then S=4×64 or 256 Kahn units.

b. If the highest dilution in which a 4+, 3+, or 2+ reaction is observed is 1:16, then S=4×16 or 64 Kahn units.

15. Report results both in terms of Kahn units and the highest serum dilution in which a 4+, 3+, or 2+ reaction is observed.

Examples:

a. 256 Kahn units (1:64 dilution).

b. 64 Kahn units (1:16 dilution).

16. Serums giving 3+, 2+, or 1+ reactions in the Kahn qualitative test may be reported as 3, 2, or 1 Kahn units, respectively, in the quantitative test if serum dilutions are Nonreactive.

Preparation of Saturated Solution of Ammonium Sulfate

1. To 500 gm. of reagent quality ammonium sulfate, add 500 ml. of doubly distilled water in a clean 3- to 5-liter Pyrex flask.

2. Boil until solution becomes clear.

3. Allow solution to cool to room temperature.

4. Filter and store in glass-stoppered bottles at room temperature.

Preparation of Globulin Concentrate of Each Spinal Fluid

1. Centrifuge and decant all spinal fluids to remove cellular debris and particles.

2. Pipette 1.5 ml. of the spinal fluid into a Kahn test tube.

3. Add 1.5 ml. of saturated solution of ammonium sulfate to the 1.5 ml. of spinal fluid.

4. Place thumb (protected with rubber) over mouth of tube and shake vigorously to mix contents.

5. Place mixture in a 56° C. water bath for 15 minutes to hasten precipitation of the globulin.

6. Remove tube from water bath and centrifuge it at 2,000 r. p. m. for 15 minutes. (The globulin precipitate will be found packed at the bottom of the tube.)

7. Decant and discard supernatant fluid by inverting and rotating the tube at the same time. Cover the entire wall of the tube with the supernatant fluid to permit its even drainage and thereby prevent ammonium sulfate from crystallizing on the tube wall.

Note: In rare instances, the amount of globulin is excessive. In such cases, do not decant the supernatant fluid, but, instead, remove it with a capillary pipette.

8. Drain inverted tube for 10 minutes on filter paper, tap tube gently, and use a strip of filter paper to remove any remaining drops of supernatant fluid.

9. Add 0.15 ml. of saline solution to globulin precipitate, holding point of pipette close to bottom of tube to avoid washing down any ammonium sulfate that may be adhering near the mouth of tube.

10. Tap base of tube gently to redissolve the globulin.

Note: When the globulin does not completely dissolve in 0.15 ml. of saline solution, add 0.05 ml. more saline solution and shake tube gently. If globulin is still insoluble, repeat with 0.05 ml. of additional saline solution. In rare instances, the globulin will still be incompletely soluble. Then the clear globulin solution is separated from the insoluble protein by centrifugation. If centrifugation does not clear the supernatant fluid, a trace of talc or kaolin is then added to the mixture and the tube is recentrifugated. The clear supernatant fluid (which is the globulin solution) is removed with a capillary pipette, transferred to a clean tube, and is then ready for testing with the antigen suspension.

Kahn Standard Qualitative Test With Spinal Fluid Globulin Concentrate

1. Arrange Kahn test tubes in a rack so that there is one tube for each spinal fluid globulin concentrate to be tested, including concentrates from Reactive and Nonreactive spinal fluids, and antigen-saline solution controls. Number tubes to correspond to the spinal fluids being tested.

2. Prepare standard antigen suspension as described under "Kahn Standard Qualitative Test With Serum" (p. 24).

3. Place thumb over mouth of mixing vial and shake briskly to suspend antigen particles.

4. Pipette 0.01 ml. of standard antigen suspension directly to the bottom of each tube.

5. Add 0.15 ml. of spinal fluid globulin concentrate to each corresponding tube.

Note: Complete the addition of antigen suspension and globulin concentrate to one rack before adding antigen suspension and globulin concentrate to another.

6. Shake rack by hand for 10 seconds after antigen suspension and spinal fluid concentrates have been added to all tubes.

7. Shake rack of tubes for 4 minutes on Kahn shaker.

8. Remove rack from shaking machine and add 0.5 ml. of 0.9-percent saline solution to each tube.

Note: Add saline solution to one rack and complete reading before adding saline solution to another.

9. Shake rack by hand a few seconds to mix contents of tubes.

10. Read each tube of the rack as described under "Reading of Results" (p. 26), within 2 minutes after the addition of saline solution and again 15 minutes later. For final report, average the results of the two readings.

11. Report in accordance with table 9.

Table 9. Reporting Kahn standard qualitative tests with spinal fluid

Reading	Report
4+	Reactive (4+).
3+	Reactive (3+).
2+	Reactive (2+).
1+	Weakly Reactive(1+).
±	Nonreactive.
−	Nonreactive.

Outline of Kahn Standard Qualitative Test With Spinal Fluid

Antigen suspension, ml.	0.01
Spinal fluid globulin concentrate, ml.	0.15
Shake rack by hand for 10 seconds.	
Shake for 4 minutes on Kahn shaker.	
Saline solution, ml.	0.5
Read within 2 minutes, and again 15 minutes later.	

Kahn Standard Quantitative Test With Spinal Fluid

Quantitative spinal fluid tests are performed on spinal fluids producing Reactive results in the Kahn qualitative test.

1. Prepare dilutions of spinal fluid as indicated in table 10.

2. Prepare standard antigen suspension as described under "Kahn Standard Qualitative Test With Serum" (p. 24).

3. Place thumb over mouth of mixing vial and shake gently to suspend.

Table 10

Tube	Spinal fluid [1]	Saline solution	Designated dilution
	(*ml.*)	(*ml.*)	
1	Quantity available.	None.	1:10
2	0.2	0.1	1:15
3	0.2	0.2	1:20
4	0.1	0.2	1:30
5	0.1	0.3	1:40

[1] Whole spinal fluid is considered a 1:10 dilution since the qualitative test is performed on spinal fluid globulin concentrated 10 times.

4. Pipette 0.01 ml. of the antigen suspension directly to the bottom of a Kahn test tube. One tube is required for each dilution of spinal fluid being tested.

5. Add 0.15 ml. of diluted spinal fluid to each tube, starting with the highest dilution.

6. Shake rack by hand for 10 seconds.

7. Shake rack on Kahn shaker for 4 minutes.

8. Remove rack from shaker, add 0.5 ml. of saline solution to each tube, mix, and read immediately.

9. Note the highest dilution of spinal fluid producing a Reactive result (4+, 3+, or 2+).

10. Calculate the Kahn units according to the formula $S=4D$, where S is the potency of the spinal fluid in terms of Kahn units, and D is the highest dilution in which a 4+, 3+, or 2+ reaction is observed.

Example:

a. Spinal fluid Reactive at 1:10 dilution (designated) $10\times4=40$ Kahn units.

b. Spinal fluid Reactive at 1:40 dilution (designated) $40\times4=160$ Kahn units.

11. Retest spinal fluids that produce only Nonreactive results in the designated dilutions in the following manner:

a. Prepare a spinal fluid globulin concentrate as described under "Kahn Standard Qualitative Test With Spinal Fluid" (p. 34).

b. Add a sufficient quantity of saline solution to the globulin solution to make a 1:5 dilution (0.15 ml. of globulin solution plus 0.6 ml. of saline solution).

c. Perform a 1-tube test as prescribed for testing spinal fluid dilutions.

d. If this 1:5 dilution gives a Reactive result, the quantitative titer is 20 Kahn units; if it gives a Nonreactive result, the titer is equivalent to the reading obtained with the undiluted globulin concentrate.

Preparation of Kahn Standard Antigen

Glassware and Equipment

1. Side-arm filter flask, Pyrex.
2. Buchner filter funnel, porcelain.
3. Filter paper of proper size for Buchner funnel.
4. Tinfoil, thin, high grade.

Reagents

1. Powdered beef heart

   The powdered beef heart may be purchased [1] or prepared in the laboratory as follows:

   a. Remove fat and connective tissue from three or more fresh beef hearts.

   b. Mince tissue by passing through meat grinder three or four times.

   c. Spread in a thin layer on a porcelain platter or glass plate and dry under a current of air from one or more electric fans for 6 hours.

   d. Break material into small pieces and cover with cheesecloth.

   e. Continue drying until small pieces are brittle.

   f. Powder the particles in a mortar or appropriate grinder.

   g. Keep the powder in ice box before use.

2. Ether

   Anesthetic ether free of alcohol should be used.

3. Alcohol

   Ethyl alcohol with an alcohol content of not less than 95 percent.

4. Cholesterol

   High-grade, C. P., ash-free cholesterol should be used.

Method of Preparation of Alcoholic Extract

1. Weigh 100 gm. of powdered beef heart and place in a 1-liter Erlenmeyer flask fitted with a tinfoil-covered cork or glass stopper.

2. Add 400 ml. of anesthetic ether.

3. Stopper the flask and shake it at frequent intervals for 10 minutes.

4. Place filter paper in a Buchner funnel, fitted to a side-arm filter flask by means of a one-hole rubber stopper.

5. Pour the contents of the 1-liter extraction flask into the funnel and filter rapidly by suction.

6. Transfer the moist beef heart to a sheet of filter paper.

7. Break the material into small pieces and return immediately to the 1-liter extraction flask.

8. Add 300 ml. of ether to the flask, stopper, and shake at frequent intervals for 10 minutes.

9. Pour the contents of the flask into the funnel using a fresh filter paper, and filter by suction.

[1] Difco Laboratories, Detroit, Mich.

10. Again transfer the moist beef heart to a sheet of filter paper.

11. Break the material into small pieces and return immediately to the extraction flask.

12. Repeat steps 8, 9, and 10 for a total of four ether extractions.

13. Spread the moist beef heart on a clean sheet of filter paper and dry with the aid of a spatula until the powder is free of ether odor.

14. Invert the flask in which the ether extraction was carried out until the ether odor has disappeared.

15. Weigh the dried, ether-free powder and return it to the flask.

16. Add 5 ml. of 95-percent alcohol for each gram of powder.

17. Stopper the flask and shake it intermittently for 10 minutes.

18. Allow flask to stand for 3 days at room temperature (23° to 29° C.) in the dark.

19. Shake flask intermittently for 10 minutes and filter contents. Refilter, if necessary, to remove all visible particulate material.

20. Store at room temperature in the dark as stock solution.

Cholesterolization of Alcoholic Extract

1. Weigh 6.0 mg. of cholesterol per milliliter of alcoholic extract antigen.

2. Transfer cholesterol to a glass-stoppered bottle or Erlenmeyer flask of suitable size.

3. Add the proper amount of alcoholic extract, measured with a graduated cylinder.

4. Tightly stopper flask and place in a warm water bath to hasten solution of the cholesterol, shaking flask intermittently.

5. After cholesterol is entirely dissolved, allow antigen to cool to room temperature, and filter through fat-free paper (No. 1 Whatman).[2]

Standardization of Antigen

The purpose of standardization is to render a newly prepared antigen comparable to a Kahn standard antigen. The following three steps are employed:

1. *Determination of titer.* Determine the minimum amount of 0.9-percent saline solution to be added to 1 ml. of antigen to produce a suspension of aggregates that completely disperses upon the addition of a designated amount of saline solution, and which reproduces the opalescence of the standard antigen.

[2] Whatman Paper, Springfield Mills, Maidstone, England. Sold by Fisher Scientific Company, Pittsburgh, Pa.

2. *Determination of reactivity level.* Test antigen at its titer with syphilitic and nonsyphilitic serums, employing standard antigen simultaneously as a control.

3. *Correction of antigen.* Correct antigen to standard requirements, when the reactivity is not comparable to that of standard antigen. (See "Antigens" under *General Information,* p. 2.)

Determination of Titer

1. Measure with a 1.0-ml. pipette (graduated in 0.01 ml.) 0.9, 1.1, 1.2, 1.3, 1.4, 1.5, 1.7, and 1.9 ml., respectively, of 0.9-percent saline solution into eight antigen-suspension vials.

2. Measure with a 1.0-ml. pipette into each of eight similar vials, 1 ml. of the 0.6-percent cholesterolized antigen to be titrated (a standard antigen control should be set up at the same time at the titer indicated on the label).

3. Prepare antigen suspensions by mixing the 1-ml. quantities of antigen with the varying amounts of saline solution. Empty the saline solution into the antigen and as rapidly as possible (without waiting to drain the tube) pour the mixture back and forth 12 times. Permit the mixture to stand for 30 minutes instead of the usual 10-minute period.

4. Test for the dispersibility in saline solution of the lipid aggregates present in the antigen-saline solution suspensions as follows:

a. Set up nine series of three Kahn test tubes each.

b. Pipette 0.05-, 0.025-, and 0.0125-ml. quantities of each of the eight antigen suspensions (after thorough agitation) to the bottom of the tubes, in series, using a 0.25-ml. antigen pipette. The same quantities of emulsion, prepared from the standard antigen, should be pipetted into the last three tubes.

Note: A different Kahn antigen pipette should be used for each antigen suspension.

c. With a 1-ml. pipette, add 0.15 ml. of saline solution to each of the 27 tubes.

d. Shake the rack of tubes vigorously for 10 seconds by hand and then for 3 minutes on a shaking machine at a speed of 275 to 285 oscillations per minute.

e. Add 1 ml. of saline solution to the tubes containing the 0.05-ml. amounts of antigen suspension, and 0.5 ml. to the remaining tubes. Shake the rack by hand to mix the ingredients and observe whether the mixtures are opalescent or contain aggregates.

A typical titration picture may show cloudiness in the 3-tube test containing the antigen suspension which was prepared with 0.9 ml. and with 1.1 ml. of saline solution. These suspensions contain aggre-

gates which were not completely redispersed in saline solution. The tests containing the antigen suspension which was mixed with 1.3 ml. of saline solution may appear opalescent, exactly like the standard antigen control. The other four tests with antigen suspensions containing 1.4, 1.5, 1.7, and 1.9 ml. of saline solution may appear clearer than the control. In this case, the titer of the antigen would be 1 ml. of antigen plus 1.3 ml. of saline solution, i. e., 1.3 ml. is the smallest amount of saline solution, which, when added to 1 ml. of antigen, produces aggregates capable of complete dispersion upon the further addition of saline solution and which reproduces the opalescence of the standard antigen. The following tabulation illustrates a typical antigen titration. The "normal" range of titers for standard antigen may vary from 1+1.1 to 1+1.5

Typical antigen titration

Antigen+0.9-percent saline solution (ml.)	*Appearance of mixture*
1+0.9	Cloudy, nondispersible aggregates.
1+1.1	Slightly cloudy, fine, nondispersible aggregates.
1+1.2	Very slightly cloudy.
1+1.3	Opalescent, standard (titer).
1+1.4	Very slightly clearer.
1+1.5	Slightly clearer.
1+1.7	Very clear.
1+1.9	Almost water clear.

Standard antigen control: Opalescent, standard

Antigen suspensions made with progressively increasing volumes of saline solution, i. e., 0.9, 1.1, 1.2, 1.3, 1.4, 1.5, 1.7, and 1.9 ml., usually show a correspondingly progressive increase in the clarity of mixtures of antigen suspension and saline solution. Rarely, however, an antigen suspension will show a "titration zone." That is, cloudiness and nondispersible aggregates may appear again beyond the titer, for example, in the tubes containing 1.7 ml. or 1.9 ml. of saline solution. This factor indicates that this particular antigen has a very short workable range and it is best not to employ this antigen for general use.

Note: After the titer of an antigen has been established, the next step is to determine whether the reactivity level of the antigen is comparable to that of a standard antigen. This is accomplished by testing a number of serums simultaneously with the antigen in question and a standard antigen.

Determination of Reactivity Level of Newly Prepared Antigen

1. Preparation of serums

A series of serums with graded reactivities may be prepared from pooled, Seitz-filtered Reactive and Nonreactive serums by combining selected proportions of these serums so that the desired number of

strongly and weakly reacting specimens are obtained. These prepared specimens may be used for all preliminary testing, but only selected individual serums are adequate for final checking of antigen reactivity. All serums should be heated before being used.

2. Preliminary testing of newly prepared antigen

Prepare antigen suspensions with both antigens in accordance with their respective titers. After both antigen suspensions have stood for 10 minutes, conduct tests with at least 10 serums exactly as described under "Kahn Standard Qualitative Test With Serum" (pp. 24–25). If comparable reactions are obtained with both antigen suspensions, final checking as described below may be accomplished.

3. Final checking of newly prepared antigen

Obtain at least 50 serums that show varying degrees of reactivity in the Kahn test and 50 Nonreactive serums. Perform tests, using both antigen suspensions simultaneously, as described under "Kahn Standard Qualitative Test With Serum" (pp. 24–25).

Each serum should be tested with both antigens in the same rack so that time factors will be constant. Two readings must be made in each instance where recordable reactions are observed during the first reading. The two readings are necessary since some antigens that produce standard first readings may allow readable particles to redisperse at a rate different from that of standard antigen. In this instance, the final report on a given specimen could be less or greater than that obtained with standard antigen. If the results produced by the new antigen are comparable to those of the standard antigen, the newly prepared antigen may be considered to have standard reactivity.

Correction of Antigen

The reactivity level of newly prepared antigen may be greater or less than that of standard antigen. In either case, correction to standard requirements can usually be accomplished. The reagents commonly necessary for antigen correction are listed below.

Standardization of Kahn standard antigen

Antigen reactivity	*Method of adjustment*
Less reactive than standard	1. Addition of cholesterolized alcohol. 2. Addition of sensitizing reagent. 3. Addition of sensitizing reagent plus cholesterolized alcohol. 4. Addition of an overreactive antigen.
More reactive than standard	1. Addition of cholesterolized alcohol. 2. Addition of a less reactive antigen. 3. Lecithin correction solution.

1. Preparation of cholesterolized alcohol

To 100 ml. of 95-percent alcohol in a 250-ml. glass-stoppered Erlenmeyer flask or bottle add 600 mg. of cholesterol. Rotate the flask in a warm water bath until all cholesterol is dissolved. Filter when cool.

2. Preparation of sensitizing reagent

a. Refilter the ether filtrate obtained in the preparation of antigen to remove the traces of powdered muscle. Place in an evaporating dish and evaporate the ether with the aid of an electric fan. During the evaporation period a few cubic centimeters of water may condense in the evaporating dish. This water will appear at the bottom of the evaporating dish, and it is suggested that it be removed with a capillary pipette as it is formed to avoid emulsification of the lipids. The lipid residue is brownish, semitransparent, and viscous.

b. When the volume has been reduced to a point where ether odor is no longer detectable, the residue is transferred to a small, previously weighed evaporating dish and weighed.

c. A volume of absolute alcohol equivalent to 10 ml. per gram of residue is added and the lipid mixture is transferred to a flask.

d. Extraction is allowed to take place for 30 minutes at room temperature with frequent shaking of the flask. Comparatively little of the residue is soluble in alcohol, lipid masses being distributed throughout the mixture.

e. The mixture is placed in the refrigerator (4° to 9° C.) for 3 hours.

f. The mixture is filtered while cold and the flask containing the clear filtrate placed in the incubator at 37° C. for 24 hours.

g. The clear filtrate should stand for 3 days at room temperature. If a precipitate forms during this period, the solution is refiltered.

h. The filtrate is cholesterolized with 6 mg. of cholesterol per milliliter, according to the usual technique.

i. The cholesterolized extract, known as sensitizing reagent, is filtered and then is ready for use. It should be kept in the dark at room temperature.

3. Correction of antigens more reactive than standard antigen may be accomplished

a. *By mixing the more reactive antigen with a less reactive antigen.*

(1) Add equal amounts of the more reactive and less reactive antigens (for example, 10 ml. each) to a small bottle and mix well.

(2) Perform titration (according to "Determination of Titer" pp. 39-40).

(3) Set up preliminary comparative tests at the determined titer, using a standard antigen as control.

(4) If results are comparable, final checking (p. 41) may be accomplished.

(5) If antigen reactivity is not comparable to that of the standard antigen, try different proportions of the two antigens.

(6) Final checking (p. 41) should be done after the entire lot of antigen has been corrected.

b. *By addition of "lecithin correction solution" to the antigen.*[3]

Preparation of stock lecithin correction solution from egg lecithin

(1) Add 5 gm. of commercial egg lecithin to 100 ml. of 0.6-percent cholesterolized absolute alcohol.

(2) Extract for 1 hour in a 56° C. water bath.

(3) Cool and filter off clear solution.

(a) Add a given amount, such as 0.2 ml., of the stock lecithin solution to 10 ml. of the overreactive antigen.

(b) Titrate antigen (according to "Determination of Titer," pp. 39-40).

(c) Test this antigen with 10 serums, most of which are known to give Weakly Reactive results, employing standard antigen as a control.

(d) If the modified antigen gives results comparable to those obtained with standard antigen, the entire amount of the more reactive antigen may be adjusted and submitted to final checking (p. 41).

c. *By dilution with cholesterolized alcohol.*

See "Correction of antigens less reactive than standard," section **4a** below. The technique described is applicable also to the correction of antigen more reactive than standard.

4. Correction of antigens less reactive than standard antigen may be accomplished

a. *By dilution of antigen with cholesterolized alcohol.*

(1) To a ½-oz. bottle add 10 ml. of the less reactive antigen (0.6-percent cholesterolized) and 1 ml. of the 0.6-percent

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[3] This method, recommended by Dr. Kahn, has not been used at the Venereal Disease Research Laboratory for antigen correction.

cholesterolized alcohol; to another ½-oz. bottle add 10 ml. of the 0.6-percent cholesterolized antigen and 2 ml. of 0.6-percent cholesterolized alcohol—thus making 10-percent and 20-percent dilutions, respectively.

(2) Titrate these two antigens (according to "Determination of Titer," pp. 39–40).

(3) Perform comparative tests with Weakly Reactive serums using standard antigen simultaneously as a control.

(4) If neither 10-percent nor 20-percent dilution with cholesterolized alcohol brings the antigen to standard reactivity, try other dilutions not exceeding 30-percent.

b. *By the addition of sensitizing reagent to the antigen.*

Some less reactive antigens can be brought to the standard level of reactivity by the addition of a trace of sensitizing reagent, i. e., 0.2 percent to 0.7 percent. In some cases, sensitizing reagent in addition to dilution with cholesterolized alcohol is necessary.

c. *By mixing of less reactive antigen with one of greater reactivity.*

This method is essentially the same as described under section **3a** (pp. 42–43), in the correction of a more reactive antigen by mixing with a less reactive one.

Notes on Titration Characteristics of Different Kahn Standard Antigens and Their Importance in Serologic Results

There are two basic titration characteristics of Kahn standard antigens. Antigens may show "sloping" titers or "flat" titers.

1. Antigens showing "sloping" titrations.

These antigens show titration pictures of increasing clarity with the increase in the amounts of saline solution employed in the preparation of the antigen suspensions. The following tabulation illustrates a "sloping" titration picture.

"Sloping" antigen titration

Antigen+0.9-percent saline solution (ml.)	*Titration results*
1+0.9	Cloudy, aggregates.
1+1.1	Slightly cloudy, fine aggregates.
1+1.3	Opalescent (titer), no aggregates, standard opalescence.
1+1.5	Slightly too clear, no aggregates.
1+1.7	Too clear, no aggregates.
1+1.9	Water-clear, no aggregates.

2. Antigens showing "flat" titrations.

These antigens show titration pictures of similar clarity with the increase in the amounts of saline solution employed in the preparation of the antigen suspensions. The following tabulation illustrates a "flat" titration picture.

"Flat" antigen titration

Antigen+0.9-percent saline solution (ml.)	*Titration results*
1+0.9	Cloudy, aggregates.
1+1.1	Slightly cloudy, fine aggregates.
1+1.3	Trace cloudy, questionable aggregates.
1+1.5	Opalescent (titer), standard opalescence.
1+1.7	Opalescence same, no aggregates.
1+1.9	Opalescence same, no aggregates.

Antigens showing a sloping titration are the most desirable for use in the Kahn standard test. Some antigens which may show a flat titration before standardization may show a sloping titration after standardization. Antigens that continue to show a flat titration after standardization are safe for use provided they do not begin to show a "reversed" sloping titration, namely, increased cloudiness following an increase in the amount of saline solution added to the antigen.

References

(*1*) KAHN, R. L.: Technique of Standard Kahn Test and of Special Kahn Procedures. Rev. and enl. ed. University of Michigan Press, June 1945.

(*2*) KAHN, R. L.: Serology With Lipid Antigens. Williams and Wilkins, Baltimore, 1950.

Kline Tests

Before performing this test, the technologist should become familiar with contents of chapters General Information and General Equipment.

Equipment

1. Rotating machine, adjustable to 180 r. p. m. circumscribing a circle ¾ inch in diameter on horizontal plane.
2. Ringmaker, to make paraffin rings approximately 14 mm. in diameter.
3. Mold set,[1] for spinal fluid tests, consisting of a steel mold (3⅛- x 2$^{3}/_{16}$- x ⅛-inch, with two wells 1$^{9}/_{16}$ inches in diameter) and two metal disks (1$^{5}/_{16}$ inches in diameter x $^{3}/_{16}$-inch thick) with central screws.
4. Slide holders for 2- x 3-inch slides.
5. Hypodermic needles, 22- and 26-gage, with filed-off bevels.

Glassware

1. Pipettes, 0.2-ml., graduated in $^{1}/_{100}$ ml. to the tip.
2. Centrifuge tubes, round-bottomed, 3- x 1-inch.
3. Bottles, round, glass-stoppered, 30-ml. capacity.
4. Glass slides,[2] 3- x 2-inch, plain, for paraffin rings.
5. Syringe, glass, hypodermic, 1.0- or 2.0-ml. capacity.

Reagents

1. Antigen

   Antigen [1] for the Kline standard tests is composed of cardiolipin (0.2 percent) and purified lecithin (1.6 to 2.6 percent) in absolute alcohol. This reagent should be assembled from chemically standardized components and be serologically standardized by comparison with an antigen of standard reactivity. Store at refrigerator temperature (6° to 10° C.).

2. Cholesterol solution

   Dissolve 1.0 gm. of cholesterol (Pfanstiehl, ash-free, precipitated from alcohol) in 100 ml. of absolute alcohol and store in glass-stoppered bottle at room temperature (73° to 85° F.).

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[1] LaMotte Chemical Products Co., Towson 4, Md.

[2] Dr. B. S. Kline has withheld approval of some glass concavity slides for this test and has approved others from the same commercial source. Glass concavity slides are not used at the Venereal Disease Research Laboratory for the Kline tests.

3. Distilled water

Distilled water suitable for the Kline tests should have pH 6.0, approximately, and have a minimum of positive ions or other electrolytes.

4. Sodium chloride solution (0.85-percent)

Add the required amount of dry, reagent quality (A. C. S.), sodium chloride (850 mg.) to 100 ml. of distilled water. This solution should preferably be prepared on the day of use. It may be satisfactory for as long as 1 week if kept in a clean glass-stoppered bottle.

KLINE TESTS WITH SERUM

Preparation of Serums

1. Remove serums from clots by centrifuging and pipetting or decanting.

2. Heat serums in 56° C. water bath for 30 minutes. When reexamination of the serum on another day is required, serum should be reheated at 56° C. for 5 minutes.

3. Recentrifuge any serum in which visible particles have formed during heating.

Preparation of Paraffin-Ring Slides

1. Clean 3- x 2-inch glass slides with Bon Ami.

2. Place 12 paraffin rings (14 mm. in diameter) on each slide using a hand-operated or an electrically heated ringmaking machine. Paraffin or a mixture of two parts of paraffin and one part of Vaseline, heated to about 120° C., may be used. Care should be exercised to produce rings of the prescribed diameter.

Preparation of Antigen Emulsion

1. Pipette 0.85 ml. of distilled water to the bottom of a 30-ml., glass-stoppered bottle.

2. Add 1.0 ml. of 1-percent cholesterol solution. This is accomplished by allowing the cholesterol solution to drop slowly from a pipette while the bottle, held at an angle, is vigorously and continuously rotated on a flat surface.

3. Continue rotation of the bottle for an additional 20 seconds.

4. Add 0.1 ml. of antigen against the side of the neck of the bottle from a 0.2-ml. pipette, avoiding the ground-glass area.

5. Place stopper in bottle and shake vigorously for 1 minute, throwing fluid from bottom to stopper and back.

6. Add 2.45 ml. of 0.85-percent sodium chloride solution rapidly to the bottle and shake less vigorously for 30 seconds.

The emulsion is now ready for use, and, if refrigerated, may be used for 48 hours. Double quantities of antigen emulsion may be prepared in 30-ml. bottles.

Preliminary Test With Serum

1. Check the delivery of the hypodermic needle (22-gage attached to a glass syringe). Adjustments should be made so that approximately 125 drops are obtained (0.008 ml. per drop) from each milliliter of antigen emulsion.

2. Complete tests with control serums of graded reactivity as described under "Kline Standard Qualitative Test With Serum."

3. Reactions with control serums should reproduce the reactivity pattern. The Nonreactive serum should show complete dispersion of antigen particles and the optimum number of particles per microscopic field.

Kline Standard Qualitative Test With Serum

1. Pipette 0.05 ml. of heated serum into a paraffin ring on a glass slide.

2. Add one drop (0.008 ml.) of antigen emulsion to each serum.

3. Rotate slides on a rotating machine at 180 r. p. m. for 4 minutes.

Note: When tests are performed in a hot, dry climate, slides may be covered with a box lid containing a moistened blotter, during rotation, to prevent excess evaporation.

4. Examine the reactions microscopically, employing a 100 X magnification.

5. Report observed results in accordance with the following outline:

a. *Typical reactions*

Nonreactive	Antigen particles dispersed, no clumping.
Weakly Reactive (± or 1 +)	Antigen particles in small but definite clumps.
Reactive (2+, 3+, or 4+)	Antigen particles in medium-sized or large clumps.

b. *Atypical reactions*

Atypical reactions are characterized by irregular feathery clumping in which smaller clumps predominate. Atypical reacting serums should be retested, in dilutions from 1:2 to 1:64, as described under "Kline Standard Quantitative Test With Serum" (p. 50). A Reactive report is rendered if a Reactive result is obtained with one or more serum dilutions.

Kline Standard Quantitative Test With Serum

1. Add 0.5 ml. of 0.85-percent sodium chloride solution to each of six or more tubes.

2. Add 0.5 ml. of heated serum to the first tube and mix.

3. Transfer 0.5 ml. from first to second tube and mix.

4. Continue transferring 0.5 ml. from each tube to the next, and mixing, until the last tube contains 1.0 ml.

5. Pipette 0.05 ml. of undiluted serum and of each serum dilution into separate paraffin rings on a glass slide.

6. Add one drop of antigen emulsion (0.008 ml.) to each serum dilution.

7. Rotate slide at 180 r. p. m. for 4 minutes.

8. Read and record reactions as described under "Kline Standard Qualitative Test With Serum" (p. 49).

9. Report results in terms of the highest dilution producing a Reactive (2+, 3+, or 4+) result.

Example:

Undiluted serum	*Serum dilutions*						*Report*
1:1	*1:2*	*1:4*	*1:8*	*1:16*	*1:32*	*1:64*	
4	4	3	1	—	—	—	Reactive, 1:4 dilution or 4 dils.
4	4	4	4	2	—	—	Reactive, 1:16 dilution or 16 dils.
4	4	4	4	4	2	—	Reactive, 1:32 dilution or 32 dils.

KLINE TESTS WITH SPINAL FLUID

Preparation of Spinal Fluid

1. Centrifuge spinal fluid at 2,000 r. p. m. for 5 minutes and remove supernatant fluid by decanting. Spinal fluids that are contaminated or that contain blood are unsatisfactory for testing.

2. Test each spinal fluid for the presence of sugar.

   a. Pipette 5 ml. of Benedict's solution into a test tube.

   b. Place tube in boiling water for 5 minutes.

   c. Remove tube from bath. Copper reduction should not occur during this heating period.

   d. Add 0.5 ml. of spinal fluid. Shake tube to mix contents.

   e. Return tube to boiling water for 5 minutes.

   f. Remove tube from water bath and examine for precipitate indicating presence of sugar. Spinal fluids giving a negative reaction for sugar are unsatisfactory for testing.

3. Place spinal fluids in a 56° C. water bath for 5 minutes immediately before testing.

Preparation of Double-Ring Slides

1. Clean 3- x 2-inch glass slides with Bon Ami.

2. Place steel mold and two center disks on slide.

3. Fill spaces between disks and outer mold with hot paraffin mixture (one part of paraffin plus two parts of Vaseline).

4. Remove mold and disks from slide after paraffin has cooled. Disks may be loosened by turning the central screw to the right. The mold is removed by inserting a knife blade between the slide and mold.

Preparation of Antigen Emulsion

1. Pipette 0.6 ml. of 1-percent cholesterol solution to the bottom of a 3- x 1-inch, round-bottomed test tube.

2. Add 0.4 ml. of distilled water rapidly, by removing finger from pipette and blowing in the last drop, while vigorously rotating the tube on a flat surface.

3. Continue rotation of tube for an additional 10 seconds.

4. Add 0.1 ml. of antigen and rotate tube vigorously for 1 minute.

5. Add 1.4 ml. of 0.85-percent sodium chloride solution and rotate the tube on a flat surface for 30 seconds.

6. Centrifuge tube of antigen emulsion at 1,100 r. p. m. for 5 minutes.

Note: Experimentation will be required to determine the time and speed of centrifuging to obtain a sediment with the optimum number of particles. The sediment should be in such an amount that, when resuspended in 0.6 ml. of saline solution, one drop (0.008 ml.) of the emulsion in 0.3 ml. of spinal fluid will contain the optimum number of antigen particles per microscopic field.

7. Decant the turbid supernatant fluid.

8. Add 0.6 ml. of 0.85-percent sodium chloride solution to the sediment and rotate tube vigorously for 30 seconds to uniformly resuspend the antigen particles.

9. Transfer the antigen emulsion to a stoppered 13- x 100-mm. tube. This emulsion, if stored in the refrigerator, may be used for 48 hours after preparation.

Preliminary Tests With Spinal Fluid

1. Check the delivery of the hypodermic needle (26-gage, attached to a glass syringe). Adjustments should be made so that approximately 125 drops are obtained (0.008 ml. per drop) from each milliliter of antigen emulsion.

2. Complete tests with Reactive spinal fluid and Nonreactive spinal fluid controls as described under "Kline Standard Qualitative Test With Spinal Fluid."

3. Clumping of antigen particles will be observed in the Reactive spinal fluid. The Nonreactive spinal fluid should show complete dispersion of antigen particles and the optimum number of particles per microscopic field.

Kline Standard Qualitative Test With Spinal Fluid

1. Place the required number of double-ring slides in a holder while spinal fluids are being heated.

2. Pipette 0.3 ml. of the warm spinal fluid into a ringed chamber. Reactive and Nonreactive spinal fluid controls should be included.

3. Add one drop(0.008 ml.) of antigen emulsion to the spinal fluid in each chamber.

4. Rotate the slides on a flat surface with moderate vigor for 30 seconds to distribute the antigen emulsion.

5. Move the slide holder back and forth rapidly (about three complete movements a second), a linear distance of one-fourth to one-half inch, for 8 minutes.

6. Examine reactions microscopically at 100 × magnification and report observed results in accordance with the following outline. For ease in reading, the slide may be tilted.

Nonreactive	Antigen particles dispersed, no clumping.
Weakly Reactive (± or 1+)	Antigen particles in small but definite clumps.
Reactive (2+, 3+, or 4+)	Antigen particles in medium-sized or large clumps.

Kline Standard Quantitative Test With Spinal Fluid

1. Prepare spinal fluid dilutions of 1:2, 1:4, 1:8, 1:16, etc., using Nonreactive spinal fluid or 0.9-percent saline solution as the diluent.

2. Test undiluted spinal fluid and each spinal fluid dilution as described under "Kline Qualitative Test With Spinal Fluid."

3. Report results in terms of the highest dilution producing a Reactive (2+, 3+, or 4+) result as described under "Kline Quantitative Test With Serum" (p. 50).

Kolmer Tests

Before performing this test, the technologist should become familiar with contents of chapters General Information and General Equipment.

Equipment

1. Racks, test tube, galvanized wire, for 72 tubes.

Glassware

1. Test tubes, Pyrex, 15- x 85-mm. outside dimensions.
2. Tubes, centrifuge, graduated, 15-ml. capacity, Pyrex.
3. Tubes, centrifuge, round-bottom, 50-ml. capacity.

Reagents

1. Antigen

   Antigen for the Kolmer tests is an alcoholic solution containing 0.03 percent cardiolipin, 0.05 percent lecithin, and 0.3 percent cholesterol. Each new lot of antigen should be tested in parallel with a standard antigen in both qualitative and quantitative tests on Reactive, Weakly Reactive, and Nonreactive serums before being placed into routine use.

2. Saline solution

   a. Weigh 8.5 gm. of dried sodium chloride (A. C. S.) and 0.1 gm. of magnesium sulfate for each liter of saline solution.[1]

   b. Dissolve salts in distilled water, and filter into flasks having glass or gauze-covered cotton stoppers. Freshly prepared saline solution should be used for each test run.

   c. Place portion of saline solution sufficient for diluting complement, to be used for completing the tests, into refrigerator, allowing remainder to stand at room temperature (73° to 85° F.).

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[1] When unusually low complement titers are obtained, Dr. Kolmer recommends that 40 mg. of $CaCl_2.2H_2O$ be added to each liter of saline solution. Under these circumstances, the complement should not be used at a dilution higher than 1:37 (for 2 full units).

3. Sheep red cells. (See "Collection and Preservation of Sheep Blood," *Appendix*, p. 97.)

Freshly collected sheep blood should be refrigerated for 48 hours before being used.

4. Hemolysin. (See "Preparation of Hemolysin," *Appendix*, pp. 100–102.)

5. Complement serum. (See "Preparation and Preservation of Complement," *Appendix*, pp. 102–103.)

a. Cell-free guinea pig serum can be obtained by centrifuging the tubes of blood and decanting serum from the clots when the laboratory practice is to bleed guinea pigs the day before the complement-fixation tests are performed. The serum from three or more guinea pigs should be pooled and returned to the refrigerator.

b. Dehydrated complement serum should be restored to *original serum volume* by dissolving in the proper amount of buffered diluent or distilled water and storing in the refrigerator.

c. Complement serum stored in the frozen state should be returned to liquid state by remaining at room temperature or at 37° C. only long enough to melt. As protein content of these serums will tend to collect at the bottom of the tube during thawing, these tubes of serum should be adequately mixed by inversion and returned to the refrigerator (6° to 10° C.).

Preparation of Serums

1. Centrifuge blood specimens and separate serum from the clot by pipetting or decanting.

2. Heat serum at 56° C. for 30 minutes. Previously heated serums should be reheated for 5 minutes at 56° C. on day of testing.

3. Recentrifuge any serum in which visible particles have formed during heating.

Note: If complement-fixation reactions of maximum sensitivity are desired in the Kolmer quantitative test, it is necessary to remove the natural antisheep hemolysins from serums. This may be accomplished in the following manner:

a. Pipette 1 ml. of each serum into a small (12- x 75-mm.) tube and place in the refrigerator for 15 or more minutes.

b. Add one drop of washed, packed, sheep red cells to each serum and mix well.

c. Return all tubes to the refrigerator for 15 minutes.

d. All tubes then are centrifuged and the serums separated by

decanting. Avoid carrying over cell residue from side walls or bottom of tubes.

e. These serums then are heated at 56° C. for 30 minutes. Previously heated, absorbed serums should be reheated for 5 minutes.

Preparation of Spinal Fluid

1. Centrifuge and decant all spinal fluids to remove cellular and particulate debris. Spinal fluids which are visibly contaminated or contain gross blood should not be tested.

2. Heat all spinal fluids received through the mail, or stored for three or more days, at 56° C. for 15 minutes to remove thermolabile anticomplementary substances.

3. Fresh spinal fluids are tested without preliminary heating.

Preparation of Sheep Red Cell Suspension

1. Filter an adequate quantity of preserved sheep blood through gauze into a 50-ml., round-bottom centrifuge tube.

2. Add two or three volumes of saline solution to each tube.

3. Centrifuge tubes at a force sufficient to throw down corpuscles in 5 minutes (I. E. C.[2] centrifuge No. 1 at 2,000 r. p. m., I. E. C. centrifuge No. 2 at 1,700 r. p. m.).

4. Remove supernatant fluid by suction through a capillary pipette, taking off upper white cell layer.

5. Fill tube with saline solution and resuspend cells by inverting and gently shaking tube.

6. Recentrifuge tube and repeat the process for a total of three washings. If supernatant fluid is not colorless on third washing, cells are too fragile and should not be used.

7. After supernatant fluid is removed from third washing, cells are poured or washed into a 15-ml. graduated centrifuge tube and centrifuged at previously used speed for 10 minutes in order to pack cells firmly and evenly.

8. Read the volume of packed cells in the centrifuge tube and carefully remove supernatant fluid.

9. Prepare a 2-percent suspension of sheep cells by washing the corpuscles into a flask with 49 volumes of saline solution. Shake flask to insure even suspension of cells.

Example:

2.1 ml. (packed cells) $\times 49 = 102.9$ ml. (saline solution required).

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[2] International Equipment Co., Boston, Mass.

10. Pipette 15 ml. of the 2-percent cell suspension into a graduated centrifuge tube and centrifuge at previously used speed for 10 minutes. A 15-ml. aliquot of a properly prepared cell suspension will produce 0.3 ml. $\pm$ 0.01 ml. of packed cells.

Caution: Use only centrifuge tubes that have been tested for proper calibration in 15-ml. and cell-pack volume zones.

Note: When the packed cell volume is beyond the tolerable limits stated above, the cell suspension concentration should be adjusted. The quantity of saline solution which must be removed or added to the cell suspension to accomplish adjustment is determined according to the following formula:

$$\frac{\text{Actual reading of centrifuge tube}}{\text{Correct reading of centrifuge tube}} \times \text{Volume of cell suspension} = \text{Corrected volume of cell suspension}$$

Example 1:

Volume of cell suspension . . . . . 100 ml.
Centrifuge tube (15 ml.) reading . . . . . 0.27 ml.

$$\frac{0.27 \text{ ml.}}{0.3 \text{ ml.}} \times 100 \text{ ml.} = 90 \text{ ml.}$$

Therefore, 10 ml. of saline solution should be removed from each 100 ml. of cell suspension. Saline solution may be removed by centrifuging an aliquot of the cell suspension and pipetting off the desired volume of saline solution for discard.

Example 2:

Volume of cell suspension . . . . . 100 ml.
Centrifuge tube (15 ml.) reading . . . . . 0.33 ml.

$$\frac{0.33 \text{ ml.}}{0.3 \text{ ml.}} \times 100 \text{ ml.} = 110 \text{ ml.}$$

Therefore, 10 ml. of saline solution should be added to each 100 ml. of cell suspension. An adjusted cell suspension should be rechecked by centrifuging a 15-ml. portion.

11. Place flask of cell suspension in refrigerator when not in use. Always shake before using to secure an even suspension, as the corpuscles settle to the bottom of the flask when allowed to stand.

Preparation of Antigen Dilution

1. Place the required amount of saline solution in a flask and add antigen drop by drop while continuously shaking the flask. The amount needed may be calculated from the number of tubes containing antigen in the test and titrations. The test dose constitutes 0.5 ml. of the antigen dilution indicated on the label of the bottle which is usually 1:150.

2. Antigen dilution is kept at room temperature in a stoppered flask.

3. The diluted antigen should stand at room temperature for at least 1 hour before it is used.

Preparation of Stock Hemolysin Dilution

1. Prepare 1:100 stock hemolysin dilution as follows:

	ml.
Saline solution	94.0
Phenol solution (5 percent in saline solution)	4.0
Glycerinized hemolysin (50 percent)	2.0

Phenol solution should be mixed well with the saline solution before glycerinized hemolysin is added. This solution keeps well at refrigerator temperature but should be discarded when found to contain precipitate.

2. Each new lot of stock hemolysin dilution (1:100) should be checked by parallel titration with the previous stock hemolysin dilution before it is placed into routine use.

3. Dilutions of hemolysin of 1:1,000 or greater are prepared by further diluting aliquots of the 1:100 dilution.

After these reagents are prepared the complement and hemolysin titrations may be assembled.

Complement and Hemolysin Titrations

1. Perform these two titrations simultaneously in the same rack.

2. Place 10 tubes (numbered 1 to 10) in one side of the rack for the hemolysin titration and 8 tubes (numbered 1 to 8) in the other side for the complement titration. Add two other tubes to the rack, one for 1:1,000 hemolysin solution and one for 1:30 complement dilution.

3. Prepare a 1:1,000 dilution of hemolysin by placing 4.5 ml. of saline solution in a test tube and adding 0.5 ml. of 1:100 stock hemolysin solution. Mix well.

4. Pipette 0.5 ml. of 1:1,000 hemolysin solution into the first five tubes of the hemolysin titration.

5. Add the following amounts of saline solution to the hemolysin titration tubes:

	Tube No.									
	1	2	3	4	5	6	7	8	9	10
Saline solution . . .	None.	0.5 *ml.*	1.0 *ml.*	1.5 *ml.*	2.0 *ml.*	0.5 *ml.*	0.5 *ml.*	0.5 *ml.*	0.5 *ml.*	0.5 *ml.*

6. Proceed as follows:

Tube No.	Process	Final hemolysin dilution
1	None.	1:1,000
2	Mix. Discard 0.5 ml.	1:2,000
3	Mix. Transfer 0.5 ml. to tube 6. Discard 0.5 ml.	1:3,000
4	Mix. Transfer 0.5 ml. to tube 7. Discard 1.0 ml.	1:4,000
5	Mix. Transfer 0.5 ml. to tube 8. Discard 1.5 ml.	1:5,000
6	Mix. Transfer 0.5 ml. to tube 9.	1:6,000
7	Mix. Transfer 0.5 ml. to tube 10.	1:8,000
8	Mix. Discard 0.5 ml.	1:10,000
9	Mix. Discard 0.5 ml.	1:12,000
10	Mix. Discard 0.5 ml.	1:16,000

7. Prepare 1:30 dilution of complement by adding 0.2 ml. of guinea pig serum to 5.8 ml. of saline solution, and mixing well.

8. Pipette 0.3 ml. of 1:30 complement into each of 10 tubes of the hemolysin titration.

9. Add the following amounts of 1:30 complement to the complement titration tubes:

	Tube No.							
	1	2	3	4	5	6	7	8
Complement 1:30	0.2 *ml.*	0.25 *ml.*	0.3 *ml.*	0.35 *ml.*	0.4 *ml.*	0.45 *ml.*	0.5 *ml.*	0.0

10. Add 0.5 ml. of antigen dilution to each of the first seven tubes of the complement titration.

11. Add 1.7 ml. of saline solution to each of the 10 tubes of the hemolysin titration.

12. Add the following amounts of saline solution to the complement titration tubes:

	Tube No.							
	1	2	3	4	5	6	7	8
Saline solution	1.3 *ml.*	1.3 *ml.*	1.2 *ml.*	1.2 *ml.*	1.1 *ml.*	1.1 *ml.*	1.0 *ml.*	2.5 *ml.*

13. Add 0.5 ml. of 2-percent sheep red cell suspension to each tube of the hemolysin titration.

14. Shake each tube of the hemolysin titration to insure even distribution of cells and place rack containing the two titrations in the 37° C. water bath for 1 hour.

At this point, the complement titration and the completed hemolysin titration stand as shown in tables 1 and 2.

Table 1. Complement titration (first stage)

Tube No.	Complement 1:30	Antigen dilution	Saline solution
	(*ml.*)	(*ml.*)	(*ml.*)
1	0.2	0.5	1.3
2	0.25	0.5	1.3
3	0.3	0.5	1.2
4	0.35	0.5	1.2
5	0.4	0.5	1.1
6	0.45	0.5	1.1
7	0.5	0.5	1.0
8	0	0	2.5

Table 2. Hemolysin titration (complete)

Tube No.	Hemolysin dilution (0.5 ml.)	Complement 1:30	Saline solution	Sheep cell suspension (2-percent)
		(*ml.*)	(*ml.*)	(*ml.*)
1	1:1,000	0.3	1.7	0.5
2	1:2,000	0.3	1.7	0.5
3	1:3,000	0.3	1.7	0.5
4	1:4,000	0.3	1.7	0.5
5	1:5,000	0.3	1.7	0.5
6	1:6,000	0.3	1.7	0.5
7	1:8,000	0.3	1.7	0.5
8	1:10,000	0.3	1.7	0.5
9	1:12,000	0.3	1.7	0.5
10	1:16,000	0.3	1.7	0.5

15. Remove rack from water bath and read hemolysin titration.

The unit of hemolysin is the highest dilution that gives complete sparkling hemolysis.

Hemolysin for the complement titration and test proper is diluted so that 2 units are contained in 0.5 ml.

16. Prepare a quantity of diluted hemolysin, containing 2 units per 0.5 ml., sufficient for the complement titration in accordance with table 3.

Table 3

Dilution containing 1 unit per 0.5 ml.	Dilution containing 2 units per 0.5 ml.	To prepare 2-unit hemolysin dilution mix	
		1:100 Hemolysin solution	Saline solution
		(*ml.*)	(*ml.*)
1:4,000	1:2,000	0.3	5.7
1:5,000	1:2,500	0.2	4.8
1:6,000	1:3,000	0.2	5.8
1:8,000	1:4,000	0.15	5.85
1:10,000	1:5,000	0.1	4.9
1:12,000	1:6,000	0.1	5.9
1:16,000	1:8,000	0.1	7.9

17. Add 0.5 ml. of diluted hemolysin (containing 2 units of hemolysin) to each of the first seven tubes of the complement titration.

18. Add 0.5 ml. of 2-percent sheep red cell suspension to all eight tubes of the complement titration. The addition of hemolysin and cells to the complement titration should be completed without delay, preferably within 5 minutes after rack is removed from the water bath.

19. Shake each tube of the complement titration to insure even distribution of cells and return to the 37° C. water bath for 30 minutes. The completed complement titration is shown in table 4.

Table 4. Complement titration (complete)

Tube No.	Complement 1:30	Antigen dilution	Saline solution		Hemolysin	Sheep cell suspension (2-percent)	
	(*ml.*)	(*ml.*)	(*ml.*)		(*ml.*)	(*ml.*)	
1	0.20	0.5	1.3	37° C. water bath for 1 hour.	0.5	0.5	37° C. water bath for ½ hour.
2	0.25	0.5	1.3		0.5	0.5	
3	0.30	0.5	1.2		0.5	0.5	
4	0.35	0.5	1.2		0.5	0.5	
5	0.40	0.5	1.1		0.5	0.5	
6	0.45	0.5	1.1		0.5	0.5	
7	0.50	0.5	1.0		0.5	0.5	
8	None.	None.	2.5		None.	0.5	

20. Remove rack from water bath and read complement titration.

The smallest amount of complement giving complete sparkling hemolysis is the exact unit. The full unit is 0.05 ml. more than the exact unit.

For the complement-fixation tests, complement is diluted so that 2 full units are contained in 1.0 ml.

Example:	*ml.*
Exact unit	0.3
Full unit	0.35
Dose (2 full units)	0.7

Dilution of complement to be employed in the test proper may be calculated by dividing 30 by the dose, i. e., $\frac{30}{0.7}$=43 or 1:43 dilution of guinea pig serum.

Table 5 gives additional examples:

Table 5

Exact unit	Full unit	Two full units	Dilution to use	Preparation		
				Complement serum		Saline solution
(*ml.*)	(*ml.*)	(*ml.*)		(*ml.*)		(*ml.*)
0.3	0.35	0.7	1:43	1	+	42
0.35	0.4	0.8	1:37	1	+	36
0.4	0.45	0.9	1:33	1	+	32
0.45	0.50	1.0	1:30	1	+	29

Occasionally hyperactive complement serums are encountered which yield titrations indicating 2 full units per milliliter in dilutions greater than 1:43. These complements should be used at 1:43 dilution to accomplish satisfactory testing.

Note: Tubes of the complement or hemolysin titrations showing complete hemolysis may be removed and placed in the refrigerator for later use as hemoglobin solutions for the reading standards (*see pp. 66–67*).

Kolmer Qualitative Tests With Serum and Spinal Fluid

1. Arrange test tubes in wire racks so that there are two tubes for each serum and spinal fluid to be tested. Control serums of graded reactivity should be included. Number the first row of tubes to correspond to the serum and spinal fluid being tested. Four additional test tubes are included for reagent controls.

2. Pipette 0.5 ml. of saline solution into each tube of the second row.

3. Add the following amounts of saline solution to the four control tubes:

	ml.
Antigen control (for serum tests)	0.2
Antigen control (for spinal fluid tests)	0.5
Hemolytic system control	1.0
Corpuscle control	2.5

4. Pipette 0.2 ml. of each serum to be tested into tubes 1 and 2.

5. Pipette 0.5 ml. of each spinal fluid to be tested into tubes 1 and 2.

6. Pipette 0.2 ml. of each control serum to be tested into tubes 1 and 2.

7. Add 0.5 ml. of the antigen dilution to the first tube of each test, either serum, control serum or spinal fluid, and the two antigen control tubes.

8. Allow test racks to stand for 10 to 30 minutes at room temperature.

9. Prepare diluted complement during this interval. The amount needed is equivalent to 1.0 ml. for each tube of the test plus a slight excess.

Note: The volume of complement serum to be diluted is determined by the amount of diluted complement necessary for the test proper. Dividing the number of milliliters of diluted complement needed by the titration dilution factor (2 full units) will give the number of milliliters of complement serum needed. Calculations may be made in accordance with table 6.

Table 6

Complement titration, 2 full units	Diluted complement needed	Complement serum required	Cold saline solution required
	(*ml.*)	(*ml.*)	(*ml.*)
1:43	43	1	42
1:43	215	5	210
1:37	37	1	36
1:30	210	7	203

10. Add 1 ml. of diluted complement (containing 2 full units) to all tubes of the serum, control serum, and spinal fluid tests, including the antigen control tube and the hemolytic system control tube.

11. Mix the contents of the tubes by shaking the racks well and place in the refrigerator at 6° to 8° C. for 15 to 18 hours.

12. Prepare the volume of diluted hemolysin needed for the test proper, allowing 0.5 ml. (containing 2 units) for each tube. Prepare a slight excess. The following formula may be used for calculating

the amounts of 1:100 hemolysin solution and diluent required to prepare the needed volume of diluted hemolysin.

$$\frac{100}{\text{Hemolysin titration dilution factor (2 units)}} \times \text{Volume of diluted hemolysin needed } (ml.) = \text{ml. of 1:100 hemolysin required}$$

Table 7 gives additional examples:

Table 7

Hemolysin titer, 2 units in 0.5 ml.	Diluted hemolysin needed	1:100 hemolysin required	Saline solution required
	(*ml.*)	(*ml.*)	(*ml.*)
1:3,000	30	1	29
1:4,000	120	3	117
1:2,500	25	1	24
1:2,500	250	10	240

13. Remove racks of tubes from the refrigerator at regular intervals and place immediately in the 37° C. water bath for 10 minutes. The interval will be determined by the length of time necessary to add hemolysin and sheep cell suspension to each rack.

14. Remove each rack from the water bath and add 0.5 ml. of the diluted hemolysin to all tubes of the test except the corpuscle control tube.

15. Add 0.5 ml. of the 2-percent sheep red cell suspension (prepared the previous day) to all tubes. The 2-percent cell suspension should be agitated occasionally to insure even suspension of cells during the period when this reagent is being added to the complement-fixation tests.

16. Mix the contents of the tubes thoroughly by shaking each rack before returning it to the 37° C. water bath for the secondary incubation. Examine the controls at 5-minute intervals. The period of secondary incubation will be determined by the length of time necessary to reproduce the predetermined reactivity pattern of the control serums. In all instances, however, the reading time should be at least 10 minutes more than is required to hemolyze the antigen and hemolytic system controls but should not exceed a total of 60 minutes' incubation. When control serums are not available, the secondary incubation period will be terminated 10 minutes after the hemolytic system control and the antigen control hemolyze. In no instance should this period extend beyond 1 hour.

17. Remove each rack of tubes from the water bath at the end of the secondary incubation period. Record observed hemolysis as de-

scribed under "Preparation of Reading Standards" (pp. 66–67) and "Reading and Reporting Test Results" (pp. 67–68) except in those instances where inhibition of hemolysis is noted in the control tube.

All serums and spinal fluids showing inhibition of hemolysis in the control tube should be returned to the 37° C. water bath for a period sufficient to complete 1 hour of secondary incubation. At the end of this period, these tests are removed from the water bath, and tube readings (including control tubes) are recorded.

Outline of Kolmer Qualitative Tests With Serum and Spinal Fluid

Tube No.		Saline solution	Antigen		Complement, 2 full units		Hemolysin, 2 units	Sheep cell suspension (2-percent)	
	Serum (ml.)	*(ml.)*	*(ml.)*		*(ml.)*		*(ml.)*	*(ml.)*	
1 . . .	0.2	None.	0.5	Shake rack well. Allow to stand at room temperature for 10 to 30 minutes.	1.0	Shake rack well. Primary incubation 15 to 18 hours at 6° to 8° C. followed by 10 minutes in 37° C. water bath.	0.5	0.5	Shake rack well. Secondary incubation in 37° C. water bath.
2 . . .	0.2	0.5	None.		1.0		0.5	0.5	
	Spinal fluid (ml.)								
1 . . .	0.5	None.	0.5		1.0		0.5	0.5	
2 . . .	0.5	0.5	None.		1.0		0.5	0.5	
Controls									
Antigen (serum).		0.2	0.5		1.0		0.5	0.5	
Antigen (spinal fluid).		0.5	0.5		1.0		0.5	0.5	
Hemolytic system.		1.0	None.		1.0		0.5	0.5	
Corpuscle . .		2.5	None.		None.		None.	0.5	

Kolmer Quantitative Tests With Serum and Spinal Fluid

1. Place test tubes in wire racks, allowing 8 tubes for each serum and 6 tubes for each spinal fluid to be tested. Include reagent controls and control serums of graded reactivity.

2. For each serum, pipette 0.9 ml. of saline solution into tube 1 and 0.5 ml. of saline solution into tubes 2, 3, 4, 5, 6, 7, and 8.

3. For each spinal fluid, pipette 0.5 ml. of saline solution into tubes 2, 3, 4, 5, and 6.

4. Pipette the indicated amount of saline solution into each of the following reagent control tubes:

	Saline solution
Antigen control	0.5 ml.
Hemolytic control	1.0 ml.
Corpuscle control	2.5 ml.

5. For each serum proceed as follows:

Tube No.	Process	Serum dilution
1	Add 0.6 ml. of inactivated serum. Mix and transfer 0.5 ml. to tube 8 (control) and to tube 2.	Undiluted.
2	Mix. Transfer 0.5 ml. to tube 3.	1:2
3	Mix. Transfer 0.5 ml. to tube 4.	1:4
4	Mix. Transfer 0.5 ml. to tube 5.	1:8
5	Mix. Transfer 0.5 ml. to tube 6.	1:16
6	Mix. Transfer 0.5 ml. to tube 7.	1:32
7	Mix. Discard 0.5 ml.	1:64
8	. . . . .	Undiluted (Control).

6. For each spinal fluid proceed as follows:

Tube No.	Process	Spinal fluid dilution
1	Add 0.5 ml. of spinal fluid.	Undiluted.
2	Add 0.5 ml. of spinal fluid. Mix. Transfer 0.5 ml. to tube 3.	1:2
3	Mix. Transfer 0.5 ml. to tube 4.	1:4
4	Mix. Transfer 0.5 ml. to tube 5.	1:8
5	Mix. Discard 0.5 ml.	1:16
6	Add 0.5 ml. of spinal fluid.	Undiluted (Control).

7. Add 0.5 ml. of diluted antigen to the first seven tubes of each serum test, to the first five tubes of each spinal fluid test, and to the antigen control tube. Shake the racks to mix thoroughly.

8. Allow racks to stand at room temperature for 10 to 30 minutes.

9. Complete the tests as indicated in paragraphs 9 through 17 of the technique for the performance of the "Kolmer Qualitative Tests With Serum and Spinal Fluid" (pp. 62–64).

Both qualitative and quantitative tests may be conducted in one-half volume. The amounts of complement, antigen, and other reagents needed are in this way reduced by half. Some accuracy of operation may be sacrificed by using these reduced quantities, however, since the relative effects of measuring errors are increased. Hemolysin and complement are titrated at full volume as described for the regular test. The performance of the one-half volume tests is identical with the regular methods except that halved volumes of serum, spinal fluid, and reagents are used.

Outline of Kolmer Quantitative Tests With Serum and Spinal Fluid

Tube No.		Antigen		Complement, 2 full units		Hemolysin, 2 units	Sheep cell suspension (2-percent)	
	Serum (in 0.5 ml.)	(ml.)	Allow to stand at room temperature 10 to 30 minutes.	(ml.)	Primary incubation 15 to 18 hours at 6° to 8° C. followed by 10 minutes in 37° C. water bath.	(ml.)	(ml.)	Secondary incubation in 37° C. water bath.
1	0.2 (undiluted)	0.5		1.0		0.5	0.5	
2	0.1 (1:2)	0.5		1.0		0.5	0.5	
3	0.05 (1:4)	0.5		1.0		0.5	0.5	
4	0.025 (1:8)	0.5		1.0		0.5	0.5	
5	0.012 (1:16)	0.5		1.0		0.5	0.5	
6	0.006 (1:32)	0.5		1.0		0.5	0.5	
7	0.003 (1:64)	0.5		1.0		0.5	0.5	
8	0.2 (undiluted, control).	None.		1.0		0.5	0.5	
	Spinal fluid (in 0.5 ml.)							
1	0.5 (undiluted)	0.5		1.0		0.5	0.5	
2	0.25 (1:2)	0.5		1.0		0.5	0.5	
3	0.125 (1:4)	0.5		1.0		0.5	0.5	
4	0.062 (1:8)	0.5		1.0		0.5	0.5	
5	0.031 (1:16)	0.5		1.0		0.5	0.5	
6	0.5 (undiluted, control).	None.		1.0		0.5	0.5	
	Reagent controls		Shake rack well.		Shake rack well.			Shake rack well.
	Antigen, 0.5 ml. saline solution.	0.5		1.0		0.5	0.5	
	Hemolytic, 1.0 ml. saline solution.	None.		1.0		0.5	0.5	
	Corpuscle, 2.5 ml. saline solution.	None.		None.		None.	0.5	

Preparation of Reading Standards

1. Heat tubes of hemoglobin solution (saved from the titration or obtained from control tubes of current day's tests) in the 56° C. water bath for 5 minutes.

2. Prepare a 1:6 dilution of 2-percent corpuscle suspension by adding 5 ml. of saline solution to 1 ml. of 2-percent suspension.

3. Prepare reading standards by mixing hemoglobin solution and cell suspension in the proportions given in table 8.

Table 8

1:6 corpuscle suspension	Hemoglobin solution	Equivalent complement fixation	
		Percent	Record
(ml.)	(ml.)		
3.0	0.0	100	4+
1.5	1.5	50	3+
0.75	2.25	25	2+
0.3	2.7	10	1+
0.15	2.85	5	±
. . .	3.0	0	−

4. Reading standards are prepared with one-half volumes of cell suspension and hemoglobin solution when performing the one-half volume tests.

Reading and Reporting Test Results

1. All serum and spinal fluid controls should show complete hemolysis.

2. Estimate the individual tube readings by comparison with the reading standards at the end of the secondary incubation period, and record degree of complement fixation noted, except for those specimens showing inhibition of hemolysis in the control tube.

3. Read the tubes that have been returned to the 37° C. water bath for a full hour's secondary incubation, estimating and recording the degree of complement fixation of each tube and control tube by comparison with the reading standards.

4. Report[3] the results of the qualitative tests in accordance with table 9.

Table 9. Kolmer qualitative test reporting

Test tube reading	Control tube reading	Report	Test tube reading	Control tube reading	Report
4+	—	Reactive.	3+	3+	Anticomplementary.
3+	—	Reactive.	3+	2+	Anticomplementary.
2+	—	Reactive.	3+	1+	Weakly Reactive.
1+	—	Reactive.	3+	±	Reactive.
±	—	Weakly Reactive.	2+	2+	Nonreactive.
—	—	Nonreactive.	2+	1+	Nonreactive.
4+	4+	Anticomplementary.	2+	±	Weakly Reactive.
4+	3+	Anticomplementary.	1+	1+	Nonreactive.
4+	2+	Weakly Reactive.	±	±	Nonreactive.
4+	1+	Reactive.			

5. Quantitative tests are reported in terms of the highest dilution giving a Reactive result (1+, 2+, 3+, or 4+) as illustrated in table 10.

Table 10

Serums or Spinal Fluid							Report
Undiluted	Dilutions						
	1:2	1:4	1:8	1:16	1:32	1:64	
4	3	1	—	—	—	—	Reactive, 1:4 dilution, or 4 dils.
4	—	—	—	—	—	—	Reactive, (4+), undiluted, or 1 dil.
4	4	3	2	—	—	—	Reactive, 1:8 dilution, or 8 dils.
4	4	4	4	4	1	—	Reactive, 1:32 dilution, or 32 dils.
3	1	—	—	—	—	—	Reactive, 1:2 dilution, or 2 dils.
1	—	—	—	—	—	—	Reactive (1+), undiluted, or 1 dil.
2	1	±	—	—	—	—	Reactive, 1:2 dilution, or 2 dils.
±	—	—	—	—	—	—	Weakly Reactive.
—	—	—	—	—	—	—	Nonreactive.
4	4	4	4	4	4	4	See *Note*, p. 68.

[3] See recommendations for "Reporting Serologic Test Results," *General Information* (p. 3).

Note: If a Reactive result is obtained with the highest dilution of the regular quantitative test (1:64), higher dilutions may be prepared and tested.

6. Report the results of the quantitative tests in accordance with table 11, when the full-hour incubation at 37° C. is required.

Table 11. Quantitative test reporting

(After 1-hour secondary incubation at 37° C.)

Test tube reading	Control tube reading	Report [1]
4 4 4 4 4 2 –	4+	Anticomplementary.
4 4 3 – – – –	2+	Reactive.
4 4 1 – – – –	1+	Reactive.
4 4 1 – – – –	±	Reactive.
3 2 – – – – –	±	Reactive.
4 4 1 – – – –	3+	Weakly Reactive.
3 2 – – – – –	1+	Weakly Reactive.
2 1 – – – – –	±	Weakly Reactive.
3 – – – – – –	±	Reactive.
1 – – – – – –	±	Nonreactive.
1 – – – – – –	1+	Nonreactive.
2 – – – – – –	1+	Nonreactive.
2 – – – – – –	2+	Nonreactive.

[1] Quantitative designations of dilution end points or dils are omitted.

Retesting Anticomplementary Serums (Modified Sachs Method)

1. Heat 0.5 ml. of serum in the 56° C. water bath for 15 minutes. If the serum has been previously inactivated, reheat for 5 minutes.

2. Add 4.1 ml. of accurately titrated N/300 hydrochloric acid to the serum and invert several times. Allow to stand for 30 minutes at room temperature.

3. Centrifuge for 10 minutes, save supernatant fluid, and discard sediment.

4. Add 0.4 ml. of 10-percent sodium chloride solution to the supernatant fluid. Neutralization is not necessary.

5. Arrange two rows of five test tubes each and number 1 to 5. The second row contains the serum controls.

6. Pipette 0.5 ml. of saline solution into tubes 3, 4, and 5 of both rows.

7. To both rows:

   a. Add 1.0 ml. of treated serum to tube 1.

   b. Add 0.5 ml. of treated serum to tubes 2 and 3.

   c. Mix tube 3 and transfer 0.5 ml. to tube 4.

d. Mix tube 4 and transfer 0.5 ml. to tube 5.

e. Mix tube 5 and discard 0.5 ml.

The five tubes of each row contain 0.1, 0.05, 0.025, 0.012, and 0.006 ml. of serum, respectively.

8. Add 0.5 ml. of antigen dilution to each tube of the first row.

9. Add 0.5 ml. of saline solution to each tube of the second row.

10. Shake rack of tubes to mix and allow to stand at room temperature for 10 to 30 minutes.

11. Add 1.0 ml. of diluted complement to all tubes of both rows.

12. Shake rack to mix and place in refrigerator for 15 to 18 hours at 6° to 8° C.

13. Complete the test as described under "Kolmer Qualitative Tests With Serum and Spinal Fluid" (pp. 61–64).

14. Record degree of hemolysis in both the test and control tubes.

15. All tubes of the rear row may show complete hemolysis. However, the first and second tubes of the rear row may show slight inhibition of hemolysis.

With Nonreactive serums, the corresponding front tubes show the same degree of inhibition of hemolysis, and if the degree is slight, a Nonreactive report may be rendered. With Reactive serums, inhibition of hemolysis is much more marked in tubes of the front row. Report the results as Reactive, Weakly Reactive, or Nonreactive.

Retesting Anticomplementary Spinal Fluids

1. Heat the spinal fluid for 15 minutes at 56° C.

2. Arrange two rows of five test tubes each and number 1 to 5.

3. Pipette 0.5 ml. of saline solution into tubes 2, 3, 4, and 5 of both rows.

4. To both rows:

a. Add 0.5 ml. of spinal fluid to tubes 1 and 2.

b. Mix tube 2 and transfer 0.5 ml. to tube 3.

c. Mix tube 3 and transfer 0.5 ml. to tube 4.

d. Mix tube 4 and transfer 0.5 ml. to tube 5.

e. Mix tube 5 and discard 0.5 ml.

5. Add 0.5 ml. of antigen dilution to each tube of the first row.

6. Add 0.5 ml. of saline solution to each tube of the second row.

7. Shake rack of tubes to mix and allow to stand at room temperature for 10 to 30 minutes.

8. Add 1.0 ml. of diluted complement to all tubes of both rows, shake rack to mix, place in the refrigerator for 15 to 18 hours at 6° to 8° C., and complete test as described under "Kolmer Qualitative Tests With Serum and Spinal Fluid" (pp. 61–64).

9. Interpret the results according to the following examples:

First row:	4 4 4 1 –	Reactive.
Second row:	4 1 – – –	
First row:	4 3 2 – –	Reactive.
Second row:	4 1 – – –	
First row:	4 1 – – –	Reactive.
Second row:	1 – – – –	
First row:	4 4 2 – –	Nonreactive.
Second row:	4 2 1 – –	
First row:	3 2 – – –	Nonreactive.
Second row:	3 1 – – –	

References

(1) KOLMER, J. A.; LYNCH, E. R.: Cardiolipin antigens in the Kolmer complement fixation test for syphilis. J. Ven. Dis. Inform., 29: 166–172, June 1948.

(2) KOLMER, J. A.: Personal communications.

Mazzini Tests

Before performing this test, the technologist should become familiar with contents of chapters General Information and General Equipment.

Equipment

1. Rotating machine, adjustable to 100 to 180 r. p. m., circumscribing a circle ¾ inch in diameter on a horizontal plane.
2. Slide holder. Made of any convenient material to accommodate from one to four 2- x 3-inch slides.
3. Hypodermic needles, 13- and 21-gage, with bevels removed.

Glassware

1. Glass slides,[1] 2- x 3-inch, having 10 concavities, 16 mm. in diameter by 3 mm. in depth, for serum tests.
2. Glass slides,[1] 2- x 3-inch, having 3 concavities—one, 38 mm. in diameter, and two, 20 mm. in diameter, for spinal fluid tests.
3. Bottles, glass-stoppered or screw-capped, round, 30-ml. capacity.
4. Syringe, Luer-type, 1- or 2-ml. capacity, or an "observation tube" No. 420 LST.[1]

Reagents

1. Antigen

   Antigen (*1*) for this test is an alcoholic solution, containing 0.025 percent cardiolipin, approximately 0.2 percent lecithin, and 0.75 to 0.9 percent cholesterol, that has been serologically standardized against an antigen of known reactivity.

2. Buffered 1-percent saline solution, pH 6.3 to 6.4

   a. Prepare solution according to the following formula:

	gm.
Sodium chloride (C. P.)	8.1
Primary potassium phosphate (KH_2PO_4)	0.2
Secondary sodium phosphate ($Na_2HPO_4+12\ H_2O$)	1.7
	ml.
Distilled water	1,000.0
Normal hydrochloric acid	3.2
Formaldehyde (Merck Reagent)	1.0

   b. Filter and check pH of solution.

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[1] Serological Reagents Co., Indianapolis, Ind.

MAZZINI TESTS WITH SERUM

Preparation of Serums

1. Remove serums from clots by centrifuging and pipetting or decanting.

2. Heat serums in the 56° C. water bath for 30 minutes. Serums should be reheated for 10 minutes if reexamined more than 4 hours after the original heating period.

3. Recentrifuge any specimen in which visible particles have formed during heating.

Preparation of Antigen Emulsion

1. Pipette 0.4 ml. of buffered saline solution to the bottom of a 30-ml. bottle.

2. Measure 0.4 ml. of cholesterolized antigen (reading from the bottom of the pipette) with a 1-ml. pipette graduated to the tip.

3. Hold the bottle in the left hand and impart a rapid rotating motion to it as the antigen is being blown directly and at once into the saline solution from the pipette held in the right hand.

4. Mix by drawing the suspension into the pipette and blowing out exactly six times, returning all of the emulsion left in the pipette into the last mixture.

5. Add 2.6 ml. of the buffered saline solution, cap the bottle, and shake from bottom to top and back *50 times in 15 seconds*.

6. The emulsion then is ready for immediate use and continues usable for the entire day. Shake antigen emulsion gently each time it is used.

Preliminary Testing of Antigen Emulsion

1. Check delivery of the syringe or observation tube, fitted with a 21-gage needle. When held in a vertical position, 0.01 ml. of antigen suspension per drop should be delivered.

2. Test control serums of graded reactivity and buffered saline solution as described under "Mazzini Qualitative Test With Serum." The results obtained should reproduce the reactivity pattern previously established for these serums and should show complete dispersion of the antigen particles in both Nonreactive serum and buffered saline solution.

Mazzini Qualitative Test With Serum

1. Pipette 0.03 ml. of each serum into a separate concavity.

2. Add one drop of antigen emulsion (0.01 ml.) to each serum.

3. Rotate slides on rotating machine for 4 minutes.

Note: When employing a mechanical rotator, the speed should be 160 to 180 rotations per minute. If rotation is by hand, circumscribe a 2-inch circle 120 times per minute.

4. Read each reaction microscopically using the low-power objective (16 mm.) and a 6 X ocular. Record and report all serums that are Nonreactive (no clumping) and all serums that are Reactive (4+).

5. Add one drop (about 0.05 ml.) of 0.9-percent saline solution from a syringe, using a 13-gage needle with cut-off bevel, to each test which gives a 1+, 2+, 3+, or atypical reaction.

6. Rotate the slide for a second 4-minute period on a rotating machine set at 100 to 120 r. p. m., or by hand.

7. Examine microscopically, record, and report the results as follows:

a. *Typical reactions*

Description	Reading	Report
No clumping or coarse reactions	—	Nonreactive.
Very small clumps	1+	Weakly Reactive.
Small clumps	2+	Weakly Reactive.
Medium-sized clumps	3+	Reactive.
Large clumps	4+	Reactive.

b. *Atypical reactions*

Atypical reactions are characterized by irregular particle aggregates of various sizes in which the small clumps and free antigen particles predominate. Atypically reacting serums should be retested, as described under "Mazzini Quantitative Test With Serum."

Mazzini Quantitative Test With Serum

1. Prepare serum dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and higher, if necessary, in the following manner:

a. Pipette 0.5 ml. of buffered saline solution into each of six (or more) tubes.

b. Add 0.5 ml. of heated serum to the first tube and mix.

c. Transfer 0.5 ml. of the diluted serum from the first to the second tube and mix.

d. Continue transferring and mixing from one tube to the next until all dilutions have been made. Allow the mixing pipette to remain in the last tube.

2. Pipette 0.05 ml. of each serum dilution into a respective chamber on a glass slide.

3. Add one drop of antigen suspension (antigen suspension used for the Mazzini qualitative test with serum, p. 73) to each diluted serum on the slide.

4. Rotate the slide on a rotating machine for 4 minutes at 180 r. p. m.

5. Read and record the reactions in the same manner as for the qualitative tests (p. 73).

6. Report results in terms of the highest serum dilution giving a Reactive result (3+ or 4+).

Example:

Serum dilutions						*Report*
1:2	*1:4*	*1:8*	*1:16*	*1:32*	*1:64*	
4	4	4	2	—	—	Reactive, 1:8 dilution, or 8 dils.
4	3	1	—	—	—	Reactive, 1:4 dilution, or 4 dils.
4	4	4	4	1	—	Reactive, 1:16 dilution, or 16 dils.

MAZZINI TESTS WITH SPINAL FLUID

1. Test spinal fluid as soon as possible after collection. Spinal fluids that are visibly contaminated or contain gross blood are unsatisfactory for examination. Spinal fluids are examined without heating.

2. Centrifuge the fresh spinal fluid at 2,000 r. p. m. for 5 minutes.

3. Decant the supernatant fluid into a clean tube.

Mazzini Qualitative Test With Spinal Fluid

1. Pipette 0.05 ml. and 0.2 ml. of spinal fluid, respectively, into each of the two 20-mm. chambers and 0.5 ml. of spinal fluid into the 38-mm. chamber of the special glass slide. Known Reactive and Nonreactive spinal fluids should be included as controls for the spinal fluid series.

2. Add one drop (0.01 ml.) of antigen emulsion (the same emulsion used for the serum test) to each chamber containing spinal fluid.

3. Rotate slides on a mechanical rotator for 10 minutes at 100 to 120 r. p. m.

4. Immediately after rotation, add two drops of 0.9-percent saline solution from a syringe using a 13-gage needle, with cut-off bevel, to chamber containing 0.05 ml. of spinal fluid.

5. Rerotate the slide holder for an additional 20 minutes at 80 to 100 r. p. m.

6. Read and record the results in the same manner as described for the serum tests (pp. 73–74).

7. Report the results of the strongest reactions obtained regardless of which of the three quantities it might be.

Example:

Spinal fluid quantity			*Report*
0.05 ml.	*0.2 ml.*	*0.5 ml.*	
1+	2+	4+	Reactive.
—	1+	3+	Reactive.
4+	2+	2+	Reactive.
2+	4+	3+	Reactive.
—	1+	2+	Weakly Reactive.
—	—	1+	Weakly Reactive.

Mazzini Quantitative Test With Spinal Fluid

Strongly reacting spinal fluid should be further quantitated in the following manner:

1. Prepare serial dilutions of spinal fluid in buffered saline solution at 1:2 to 1:16 and higher, if necessary.

2. Pipette 0.5 ml. of each dilution into respective 38-mm. chambers of the special glass slides.

3. Add one drop (0.01 ml.) of antigen emulsion (the same emulsion used for the serum test) to each chamber containing spinal fluid.

4. Rotate the slides on a mechanical rotator for 10 minutes at 100 to 120 r. p. m.

5. Rerotate for an additional 20 minutes at 80 to 100 r. p. m.

6. Read and record the results of each spinal fluid dilution.

7. Report in terms of the highest dilution giving a Reactive result in the same manner as described for the quantitative test with serum (p. 74).

References

(*1*) MAZZINI, L. Y.: Mazzini cardiolipin microflocculation test for syphilis. J. Immunol., 66: 261–275, February 1951.

(*2*) MAZZINI, L. Y.: Personal communications.

Rein-Bossak Tests

Before performing this test, the technologist should become familiar with contents of chapters General Information and General Equipment.

Equipment

1. Rotating machine, adjustable to 180 r. p. m., circumscribing a circle ¾ inch in diameter on a horizontal plane.
2. Ringmaker, to prepare paraffin rings approximately 14 mm. in diameter.
3. Slide holder. Made of any convenient material, to accommodate three or four 2- x 3-inch slides.
4. Hypodermic needles, 25-gage, beveled, to deliver approximately 120 drops per milliliter when syringe and needle are held vertically.

Glassware

1. Bottles, 30-ml. capacity (1-ounce), round, glass-stoppered, or with tinfoil-covered cork.
2. Glass slides, 2- x 3-inch, with 12 paraffin rings of approximately 14-mm. diameter. Glass slides with ceramic rings also may be used.
3. Centrifuge tubes, 3- x 1-inch, round-bottomed.
4. Syringe, glass, 1- or 2-ml. capacity.

Reagents

1. Antigen

   A cardiolipin-lecithin solution, containing 0.2 percent cardiolipin and approximately 1.3 percent purified lecithin in absolute alcohol, that has been previously standardized against a similar antigen of known reactivity. Store at room temperature (73° to 85° F.) in the dark.

2. 0.9-percent saline solution

   Dissolve 9.0 gm. of reagent quality sodium chloride in 1,000 ml. of distilled water.

3. Distilled water (pH approximately 6.0)

   Boil and cool to room temperature before use.

4. 1-percent cholesterol solution

   Dissolve 1.0 gm. of cholesterol (Pfanstiehl, ash-free) in 100 ml. of absolute alcohol.

Preparation of Serums

1. Separate serum from the clot by centrifuging and pipetting or decanting.

2. Heat in 56° C. water bath for 30 minutes. Cool to room temperature before testing. Serums held over until the following day should be reheated for 10 minutes.

Preparation of Antigen Emulsion

1. Pipette 0.8 ml. of distilled water (boiled and cooled to room temperature) into a 30-ml. round bottle.

2. Hold the bottle with the bottom against the table, and, while rotating rapidly, allow 0.9 ml. of 1-percent cholesterol solution to run directly and at once from a 1.0-ml. pipette into the distilled water.

3. Continue rotation for 15 seconds.

4. Measure 0.1 ml. of the cardiolipin-lecithin antigen with a 0.1-ml. or 0.2-ml. pipette.

5. Add antigen to bottle by blowing contents from pipette.

6. Stopper bottle and shake vigorously for 1 minute by striking the bottom of the bottle rapidly against the palm of the hand.

7. Add 2.5 ml. of 0.9-percent saline solution.

8. Shake the bottle with moderate vigor for 1 minute.

9. Transfer 3.0 ml. of antigen emulsion to a 3- x 1-inch, round-bottomed centrifuge tube.

10. Place tube containing antigen emulsion in the 56° C. water bath for 15 minutes.

11. Remove the tube containing the emulsion from water bath and centrifuge at 1,800 r. p. m. for 15 minutes in I. E. C.[1] No. 1 centrifuge.

12. Remove tube from centrifuge and invert rapidly to pour off turbid supernatant fluid.

13. Wipe the walls of the tube with a piece of gauze or cotton to remove excess liquid.

14. Add 3.0 ml. of 0.9-percent saline solution.

15. Resuspend sediment by gently shaking.

Preliminary Testing of Antigen Emulsion

Each preparation of antigen emulsion first should be examined by testing with control serums of graded reactivity. This is accomplished

[1] International Equipment Co., Boston, Mass.

by adding one drop of antigen emulsion to 0.05 ml. of each serum and completing the tests as described under "Rein-Bossak Qualitative Test With Serum." The results obtained should reproduce the reactivity pattern previously established for these controls.

Rein-Bossak Qualitative Test With Serum

1. Pipette 0.05 ml. of heated serum into one ring of a paraffin-ringed glass slide.

2. Add one drop (approximately $\frac{1}{120}$ ml.) of antigen emulsion to the serum.

3. Rotate for 4 minutes.

4. Examine microscopically immediately after rotation.

5. Report [2] the degree of clumping observed as follows:

Description	Reading	Report
No clumping	−	Nonreactive.
Very small clumps	±	Weakly Reactive.
Small clumps	1+	Weakly Reactive.
Moderate-sized clumps	2+	Weakly Reactive.
Moderately large clumps	3+	Reactive.
Large clumps	4+	Reactive.

Zonal Reactions

Atypical reactions are characterized by a few irregular clumps interspersed with unclumped particles. Retest serums giving atypical reactions in the following manner:

1. Pipette 0.5 ml. of 0.9-percent saline solution into each of five tubes (numbered 1 through 5).

2. Add 0.5 ml. of heated serum to tube 1 and mix.

   a. Transfer 0.5 ml. from tube 1 to tube 2 and mix.

   b. Transfer 0.5 ml. from tube 2 to tube 3 and mix.

   c. Transfer 0.5 ml. from tube 3 to tube 4 and mix.

   d. Transfer 0.5 ml. from tube 4 to tube 5 and mix.

3. Test each serum dilution in the same manner as described under "Rein-Bossak Qualitative Test With Serum."

4. Read microscopically and report as the test reading the strongest reaction obtained with any of the five dilutions.

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[2] See recommendations for "Reporting Serologic Test Results," *General Information* (p. 3).

Rein-Bossak Quantitative Test With Serum

1. Prepare serial dilutions of serum (1:2, 1:4, 1:8, 1:16, etc.) as described under "Zonal Reactions" (p. 79).

2. Test undiluted serum and each serum dilution as described under "Rein-Bossak Qualitative Test With Serum" (p. 79).

3. Report as the quantitative end point the highest dilution giving a 4+ or 3+ reaction. These results are reported in *dils* as illustrated below.

Example:

Undiluted serum	*Serum dilutions*						*Report (dils)*
1:1	*1:2*	*1:4*	*1:8*	*1:16*	*1:32*	*1:64*	
4+	4+	4+	4+	2+	1+	—	8
4+	4+	4+	4+	3+	—	—	16
4+	—	—	—	—	—	—	1

Reference

(*1*) Rein, C. R.; Bossak, H. N.: Cardiolipin antigens in the serodiagnosis of syphilis. A microflocculation slide test. Am. J. Syph., Gonor. & Ven. Dis., 30: 40–46, January 1946.

Before performing this test, the technologist should become familiar with contents of chapters General Information and General Equipment.

VDRL SLIDE FLOCCULATION TESTS WITH SERUM (*1, 2*)

Equipment

1. Rotating machine, adjustable to 180 r. p. m., circumscribing a circle ¾ inch in diameter on a horizontal plane.
2. Ringmaker, to make paraffin rings approximately 14 mm. in diameter.
3. Slide holder, for 2- x 3-inch microscope slides.
4. Hypodermic needles, of appropriate sizes, with or without points.

Glassware

1. Slides,[1] 2- x 3-inch, with paraffin rings approximately 14 mm. in diameter.
2. Bottles,[2] 1-ounce, round, screw-capped (Vinylite or tinfoil-lined) or glass-stoppered, narrow mouth.

Note: Some of the 1-ounce, glass-stoppered bottles now available are unsatisfactory for preparing a single volume of antigen emulsion for these tests due to an inward bulging of the bottom that causes the 0.4 ml. of saline solution to be distributed only at the periphery. A satisfactory emulsion may be obtained if the 0.8 ml. of saline solution covers the bottom surface of this type of bottle when double quantities

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[1] Glass slides with ceramic rings may also be used for the VDRL slide test with the following precautions. The rings must be high enough to prevent spillage when slides are rotated at prescribed speeds. Slides must be cleaned after each use so that serum will spread to the inner surface of the ceramic rings. This type of slide should be discarded if or when the ceramic ring begins to flake off, since these particles, in the test serums, may be mistaken for antigen particle clumps thereby causing a false Reactive report.

[2] Catalog No. CA–1, 90525 (plain bottles); CA–1, 90530 (glass-stoppered bottles). Corning Glass Works, Corning, N. Y.

of antigen emulsion are prepared. Round bottles of approximately 35-mm. diameter with flat or concave inner-bottom surfaces are satisfactory for preparing single volumes of antigen emulsion.

The low cost of plastic caps recommends against attempts to clean these for reuse. The use of an unclean stopper or cap can be the cause of unsatisfactory emulsions.

3. Syringe, Luer-type, 1- or 2-ml.

Reagents

1. Antigen

a. Antigen for this test is an alcoholic solution containing 0.03 percent cardiolipin, 0.9 percent cholesterol, and sufficient purified lecithin to produce standard reactivity. During recent years this amount of lecithin has been 0.21 percent ± 0.01 percent.

b. Each lot of antigen must be serologically standardized by proper comparison with an antigen of known reactivity.

c. Antigen is dispensed in screw-capped (tinfoil or Vinylite liners) brown bottles or hermetically sealed glass ampules, and stored at room temperature (73° to 85° F.).

d. The components of this antigen remain in solution at normal temperature so any precipitate noted will indicate changes due to factors such as evaporation or additive materials contributed by pipettes. Antigen containing precipitate should be discarded.

2. Saline solutions

a. Buffered saline solution containing 1 percent sodium chloride

Formaldehyde, neutral, reagent grade, ml.	0.5
Secondary sodium phosphate ($Na_2HPO_4 + 12\ H_2O$), gm.	0.093
Primary potassium phosphate (KH_2PO_4), gm.	0.170
Sodium chloride (A. C. S.) gm.	10.0
Distilled water, ml.	1,000.0

This solution yields potentiometer readings of pH 6.0 ± 0.1 and is stored in screw-capped or glass-stoppered bottles.

b. 0.9-percent saline solution

Add 900 mg. of dry sodium chloride to each 100 ml. of distilled water.

Preparation of Serums

1. Clear serum, obtained from centrifuged, clotted blood, is heated in a 56° C. water bath for 30 minutes before being tested.

2. All serums are examined when removed from the water bath and those found to contain particulate debris are recentrifuged.

3. Serums to be tested more than 4 hours after the original heating period should be reheated at 56° C. for 10 minutes.

Preparation of Slides

1. New slides are cleaned with Bon Ami which is removed with a soft cloth after drying.

2. Previously used slides are first freed of paraffin, washed with soap or detergent, rinsed free of cleaning compound, and then treated as new slides.

3. Slides are handled by the edges, while cleaning, to prevent greasy fingerprints on the testing surfaces.

4. Serums will spread within the circles on clean slides. Failure of the serums to spread is an indication that the slide is unclean and therefore should not be used.

5. Paraffin rings are made by transferring heated paraffin to the slides by means of metal molds.

Note: Glass slides with concavities or glass rings are not recommended for this test.

Preparation of Antigen Emulsion

1. Pipette 0.4 ml. of buffered saline solution to the bottom of a 1-ounce, round, glass- or screw-cap stoppered bottle.

2. Add 0.5 ml. of antigen (from the lower half of a 1.0-ml. pipette graduated to the tip) directly onto the saline solution while continuously but gently rotating the bottle on a flat surface.

Temperature of buffered saline solution and antigen should be in the range of 23° to 29° C. at time antigen emulsion is prepared.

Note: Antigen is added drop by drop, but rapidly, so that approximately 6 seconds are allowed for each 0.5 ml. of antigen. Pipette tip should remain in upper third of bottle and rotation should not be vigorous enough to splash saline solution onto pipette. Proper speed of rotation is obtained when the outer edge of the bottle circumscribes a 2-inch diameter circle approximately three times per second.

3. Blow last drop of antigen from pipette without touching pipette to saline solution.

4. Continue rotation of bottle for 10 seconds more.

5. Add 4.1 ml. of buffered saline solution from 5-ml. pipette.

6. Place top on bottle and shake vigorously for approximately 10 seconds.

7. Antigen emulsion then is ready for use and may be used during 1 day.

Double this amount of antigen emulsion may be prepared at one time by using doubled quantities of antigen and saline solution. A 10-ml. pipette should then be used for delivering the 8.2-ml. volume of saline solution. If larger quantities of antigen emulsion are required, more than one mixture should be prepared. These aliquots may then be tested and pooled.

Testing Antigen-Emulsion Delivery Needles

1. The number of antigen particles per microscopic field is determined by the size of antigen-emulsion drop used. For this reason the needle used each day should be checked.

2. Antigen emulsion is dispensed from a syringe fitted with a 22-gage, regular-bevel, a 23-gage, long-bevel hypodermic needle, or an 18-gage needle without a point.

Note: It is of primary importance that the proper amount (1/60 ml.) of antigen emulsion be used in each qualitative test, so any method of delivery that will produce drops of constant and proper size is adequate (see fig. 1).

3. If the four-fifths volume quantitative VDRL slide method (method B) is employed, a smaller gage needle (with or without point) that will deliver 75 drops of antigen emulsion per milliliter must be employed for this procedure. A 19-gage needle, without point, is used at the Venereal Disease Research Laboratory. Practice will allow rapid delivery of antigen emulsion but care should be exercised to obtain drops of constant size.

4. When allowed to stand, antigen emulsion should be gently mixed before use by rotating the bottle and emptying and refilling the syringe.

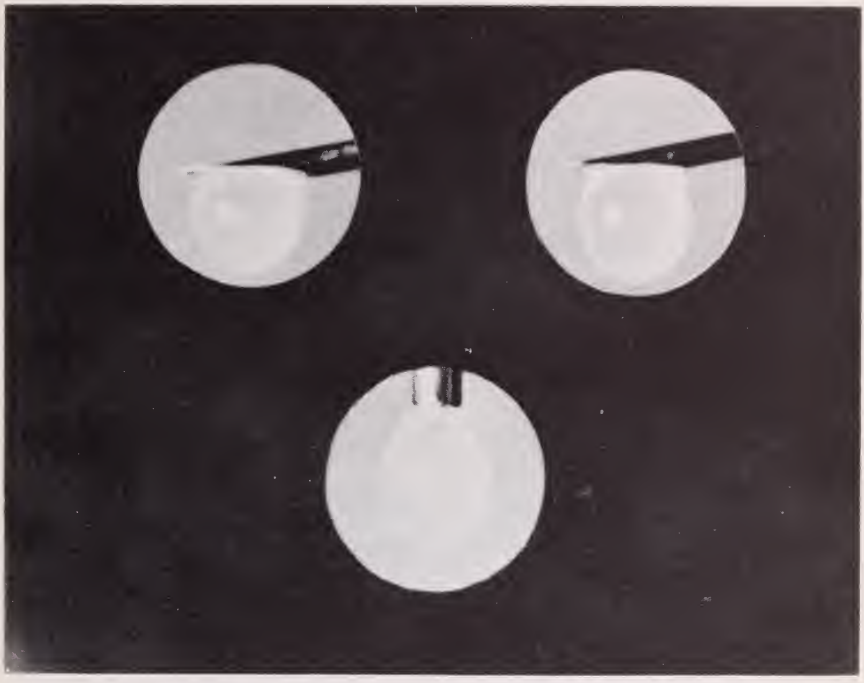

FIGURE 1. Same size drop.

Preliminary Testing of Antigen Emulsion

1. Each preparation of antigen emulsion should first be examined by testing serums of known reactivity in the Reactive, Weakly Reactive, and Nonreactive zones. This is accomplished by the method described under "VDRL Slide Qualitative Test With Serum" (p. 86). These tests should present typical results and the size and number of antigen particles in the Nonreactive serum should be optimum.

2. Only those antigen emulsions that have produced the designated reactions in tests performed with control serums (Reactive, Weakly Reactive, and Nonreactive) should be used. If antigen particles in the Nonreactive serum tests are too large, the fault may be in the manner of preparing antigen emulsion, although other factors may be responsible.

3. An unsatisfactory antigen emulsion should not be used.

VDRL Slide Qualitative Test With Serum

1. Pipette 0.05 ml. of heated serum into one ring of a paraffin-ringed glass slide (see fig. 2).

2. Add one drop (1/60 ml.) of antigen emulsion onto each serum.

3. Rotate slides for 4 minutes. (Mechanical rotators that circumscribe a ¾-inch diameter circle should be set at 180 r. p. m. Rotation by hand should circumscribe a 2-inch diameter circle 120 times per minute.)

4. Read tests immediately after rotation.

Note: Serum controls of graded reactivity (Reactive, Weakly Reactive, and Nonreactive) are always included during a testing period to insure proper reactivity of antigen emulsion at time tests are run.

FIGURE 2. Slides and slide holder for qualitative tests.

Reading and Reporting Slide Qualitative Test Results

1. Read tests microscopically, with low-power objective, at 100 × magnification. The antigen particles appear as short rod forms at this magnification.

Aggregation of these particles into large or small clumps is interpreted as degrees of reactivity.

Reading	*Report*
No clumping or very slight roughness	Nonreactive (N).
Small clumps	Weakly Reactive (WR).
Medium and large clumps	Reactive (R).

2. *Zonal reactions,* due to an excess of Reactive serum component, are recognized by irregular clumping and the loosely bound characteristics of the clumps. The usual Reactive finding is characterized by large or small clumps of fairly uniform size. Experience will allow

differentiation to be made between this type of reaction and the zonal picture wherein large and/or small clumps may be intermingled with free antigen particles. A zonal reaction is reported as Reactive. In some instances, this zoning effect may be so pronounced that a Weakly Reactive result is produced by a very strongly Reactive serum. *It is therefore recommended that all serums producing Weakly Reactive results in the qualitative test be retested using the quantitative procedure before a report of the VDRL slide test is submitted.* When a Reactive result is obtained on some dilution of a serum that produced only a Weakly Reactive result as undiluted serum, the report is Reactive (see "Reading and Reporting Slide Quantitative Test Results," p. 91, under "VDRL Slide Quantitative Tests With Serum").

VDRL Slide Quantitative Tests With Serum

All serums that produce Reactive or Weakly Reactive results in the qualitative VDRL slide test should be quantitatively retested by one of the two methods referred to as quantitative tests A or B. Since both of these procedures, in most instances, provide for direct measurements of serum and saline solution, either method is efficient in its requirement of technician-time and amount of glassware employed. Since quantitative test A uses serum dilutions of 1:2.5, 1:5, 1:10, etc., the alternate quantitative test B has been added for those laboratories desiring the doubling, serum-dilution scheme of 1:2, 1:4, 1:8, 1:16, etc.

VDRL SLIDE QUANTITATIVE TEST A

1. Place four 2- x 3-inch glass slides containing twelve 14-mm. paraffin rings in a 5-place slide holder (see fig. 3, p. 89).

2. Place a glass slide with two parallel strips of masking or adhesive tape in the center space of the slide holder.

Numbers identifying the serums to be tested (four on the two slides above the numbered slide and four on the two lower slides) are written on the adhesive strips.

3. Prepare a 1:10 dilution of each serum to be tested quantitatively by adding 0.1 ml. of the heated serum to 0.9 ml. of 0.9-percent saline solution using a 0.2-ml. pipette graduated in 0.01 ml.

4. Mix the serum and saline solution thoroughly and then allow the pipette to stand in the test tube.

5. Using this 0.2-ml. pipette, transfer 0.05-ml., 0.02-ml., and 0.01-ml. quantities of the 1:10 dilution of the first serum into the fourth, fifth, and sixth rings, respectively.

6. With the same pipette, transfer 0.05-ml., 0.02-ml., and 0.01-ml. quantities of the first serum, undiluted, into the first, second, and third ringed areas, as illustrated in figure 3.

7. Repeat this procedure with each serum and the accompanying 1:10 serum dilution until each of the eight serums are pipetted onto the slides.

8. Add one drop (0.03 ml.) of 0.9-percent saline solution to the second and fifth rings of each serum, by vertical delivery, from a 15-gage [2] hypodermic needle fitted to a glass syringe.

9. Add one drop (0.04 ml.) of 0.9-percent saline solution to the third and sixth rings of all eight serums by vertical delivery from the syringe fitted with the 13-gage [2] needle. The six mixtures of each serum are then equivalent to dilutions of 1:1 (undiluted), 1:2.5, 1:5, 1:10, 1:25, and 1:50.

10. Rotate slides gently by hand for about 15 seconds to mix the serum and saline solution.

11. Add one drop (1/60 ml.) of antigen emulsion to each ring using a syringe and needle as described in the technique for the slide qualitative serum test (p. 86).

This manner of preparing serum dilutions by adding serum and saline solution directly to the slides is outlined in figure 3.

12. Complete tests by rotation of the slides in the manner prescribed for the "VDRL Slide Qualitative Test With Serum" (p. 86).

13. Read results microscopically. The highest serum dilution giving a Reactive result (not Weakly Reactive) is reported as the reactivity end point of the serum, e. g., Reactive—1:25 dilution, or, Reactive—25 dils.

14. If all serum dilutions tested give Reactive results, prepare a 1:100 dilution of that serum by diluting 0.1 ml. of the 1:10 serum dilution with 0.9 ml. of 0.9-percent saline solution.

15. Pipette 0.05-ml., 0.02-ml., and 0.01-ml. amounts of this 1:100 serum dilution onto each ring and add enough saline solution to bring the volumes to 0.05 ml. Serum dilutions of 1:100, 1:250, and 1:500 are thus prepared. Test these dilutions of serum exactly as the lower dilutions are tested.

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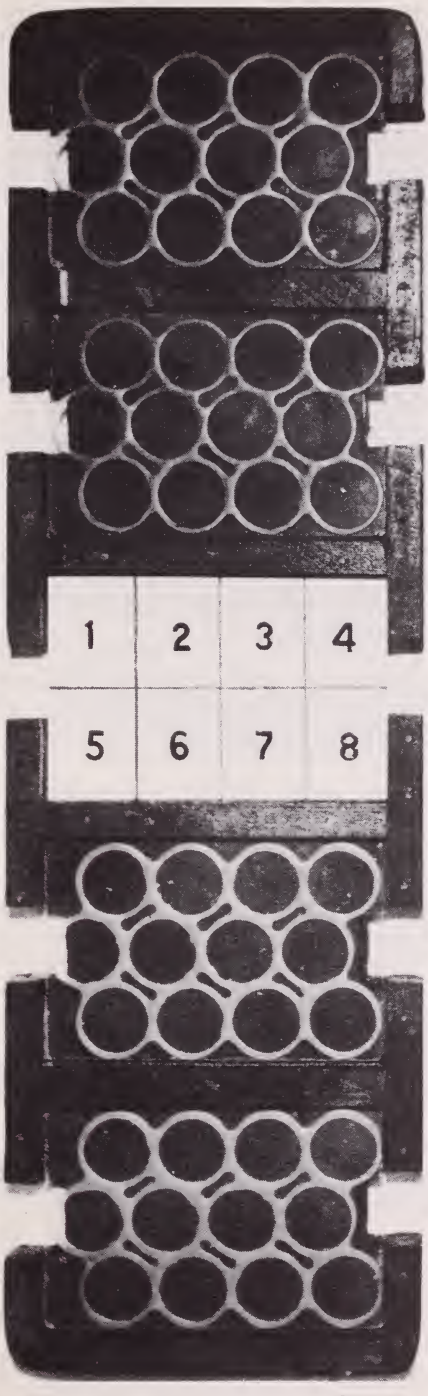
[2] Needles should be checked for proper drop size.



Ring No.	Quantitative Test A			Quantitative Test B		
	Serum	*Saline solution*	*Serum dilutions*	*Serum*	*Saline solution*	*Serum dilutions*
	(*ml.*)	(*ml.*)		(*ml.*)	(*ml.*)	
1	0.05	0	1:1 (*undiluted*)	0.04	0	1:1 (*undiluted*)
2	.02	.03	1:2.5	.02	.02	1:2
3	.01	.04	1:5	.01	.03	1:4
	(*diluted 1:10*)			(*diluted 1:8*)		
4	.05	0	1:10	.04	0	1:8
5	.02	.03	1:25	.02	.02	1:16
6	.01	.04	1:50	.01	.03	1:32
1	.05	0	1:1 (*undiluted*)	.04	0	1:1 (*undiluted*)
2	.02	.03	1:2.5	.02	.02	1:2
3	.01	.04	1:5	.01	.03	1:4
	(*diluted 1:10*)			(*diluted 1:8*)		
4	.05	0	1:10	.04	0	1:8
5	.02	.03	1:25	.02	.02	1:16
6	.01	.04	1:50	.01	.03	1:32

FIGURE 3. Slides and slide holder for quantitative tests.

VDRL SLIDE QUANTITATIVE TEST B

1. Place four 2- x 3-inch glass slides with 12 paraffin rings in a 5-place slide holder (see fig. 3), with a numbered slide in the center space. exactly as described for "Slide Quantitative Test A" (pp. 87–88).

2. Prepare a 1:8 dilution of each serum by adding 0.1 ml. of the heated serum to 0.7 ml. of the 0.9-percent saline solution using a 0.2-ml. pipette graduated in 0.01 ml.

3. Mix the serum and saline solution thoroughly and then allow the pipette to stand in the test tube.

4. Using this pipette, transfer 0.04-ml., 0.02-ml., and 0.01-ml. quantities of the 1:8 serum dilution into the fourth, fifth, and sixth paraffin rings, respectively.

5. With the same pipette, transfer 0.04 ml., 0.02 ml., and 0.01 ml. of the undiluted serum into the first, second, and third paraffin rings, respectively.

6. Repeat this procedure with each serum and the accompanying 1:8 serum dilution until each of the eight serums are pipetted into their respectively numbered places on the slides.

7. Add two drops (0.01 ml. in each drop) of 0.9-percent saline solution to the second and fifth rings of each serum, by vertical delivery from a 23-gage[3] hypodermic needle fitted to a glass syringe.

8. Add three drops of 0.9-percent saline solution (delivered in the same manner) of the same size to the third and sixth rings of each serum.

9. Rotate slides gently by hand for about 15 seconds to mix the serum and saline solution.

10. Add one drop (1/75 ml.) of antigen emulsion to each ring using a syringe and needle of appropriate size. (*Caution.*—Note that the amount of antigen emulsion used in this method has been reduced to 1/75 ml. to correspond with the reduced serum volume of 0.04 ml.)

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[3] Needles should be checked for proper drop size. Saline solutions may be delivered from a 19-gage needle (0.02 ml. per drop) and a 15-gage needle (0.03 ml. per drop).

11. Complete tests in the manner described for the "VDRL Slide Qualitative Test With Serum" (pp. 86–87) and read results microscopically immediately after rotation.

By this method, the dilutions of each serum are 1:1 (undiluted), 1:2, 1:4, 1:8, 1:16, and 1:32.

12. If all serum dilutions tested produce Reactive results, prepare a 1:64 dilution of that serum in saline solution. Add seven parts of saline solution to one part of the 1:8 serum dilution, and test in three amounts as was done with the 1:8 serum dilutions. Dilutions prepared from the 1:64 dilution will be equivalent to 1:64, 1:128, and 1:256.

Reading and Reporting Slide Quantitative Test Results

1. Read tests microscopically at 100 X magnification as described for the qualitative procedure, pp. 86–87.

2. Report results in terms of the greatest serum dilution that produces a Reactive (not Weakly Reactive) result in accordance with the following examples:

Method A

Undiluted serum	*Serum dilutions*				*Report*
1:1	*1:2.5*	*1:5*	*1:10*	*1:25*	
R	WR	N	N	N	Reactive, undiluted only, or 1 dil.
R	R	WR	N	N	Reactive, 1:2.5 dilution, or 2.5 dils.
R	R	R	WR	N	Reactive, 1:5 dilution, or 5 dils.

Method B

Undiluted serum	*Serum dilutions*				*Report*
1:1	*1:2*	*1:4*	*1:8*	*1:16*	
R	WR	N	N	N	Reactive, undiluted only, or 1 dil.
R	R	WR	N	N	Reactive, 1:2 dilution, or 2 dils.
R	R	R	WR	N	Reactive, 1:4 dilution, or 4 dils.

R = Reactive. WR = Weakly Reactive. N = Nonreactive.

Note: Under conditions of high temperature and low humidity which are sometimes present during the summer months in certain areas, antigen emulsion may be stored in the refrigerator but should be restored to room temperature before use. To avoid surface drying under these conditions, tests should be completed and read as rapidly as possible. Slide covers containing a moistened blotter may be employed.

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VDRL TUBE FLOCCULATION TESTS WITH SERUM (3)

Equipment

1. Kahn shaking machine (must be operated at 275 to 285 oscillations per minute).
2. Reading lamp, fluorescent or gooseneck-type.

Reagents

1. Antigen. (VDRL slide flocculation test antigen, see p. 82.)
2. Saline solutions

   a. 1-percent buffered saline solution. (Prepare as for the VDRL slide flocculation tests, p. 82.)

   b. Unbuffered 1-percent sodium chloride solution.

   Add 1 gm. of dry sodium chloride (A. C. S.) to each 100 ml. of distilled water.

Preparation of Serums

1. Clear serum, removed from centrifuged, whole, clotted blood, is heated in a 56° C. water bath for 30 minutes before being tested.

2. All serums are examined when removed from the water bath and those found to contain particulate debris are recentrifuged.

3. Serums to be tested more than 4 hours after being heated should be reheated at 56° C. for 10 minutes.

Preparation of Antigen Emulsion

1. Prepare antigen emulsion as described for the VDRL slide flocculation tests (see pp. 83–84).

2. Add four parts of 1-percent sodium chloride solution to one part of VDRL slide test emulsion. Mix well and allow to stand five or more minutes (not longer than 2 hours) before use. This solution will be referred to as "diluted antigen emulsion." Resuspend diluted antigen emulsion before use.

VDRL Tube Qualitative Test With Serum

1. Pipette 0.5 ml. of heated serum into a 12- x 75-mm. (outside dimension) test tube.

2. Add 0.5 ml. of diluted antigen emulsion to each serum.

3. Shake tubes on Kahn shaker for 5 minutes.

4. Centrifuge all tubes for 10 minutes at force equivalent to 2,000 r. p. m. in No. 1 or 1,700 r. p. m. in No. 2, I. E. C.[4] centrifuge, with horizontal heads.

5. Return tubes to the Kahn shaking machine and shake for exactly 1 minute.

Note: Include Reactive and Nonreactive control serums in each test run.

Reading and Reporting Tube Qualitative Test Results

1. Read test results *as soon as secondary shaking period is completed* by holding tubes close to the shade of a reading lamp with a black background, at approximately eye level. A shaded fluorescent desk lamp or a gooseneck-type lamp with a blue bulb is a satisfactory reading light source.

2. Record results as follows:

Reactive	Visible aggregates in a clear or slightly turbid medium. All borderline reactions, where the observer has doubt regarding visible clumping should be reported as Nonreactive.
Nonreactive	No visible clumping or aggregation of antigen particles. Appearance slightly turbid or granular. Definite silken swirl on gentle shaking.

Note: Turbid or hemolyzed serums may cause completed tests to be too turbid for macroscopic reading and are therefore unsatisfactory specimens for this test.

Zonal Reactions, due to excess of Reactive serum component, may appear to be very weak or, in rare instances, Nonreactive. Whenever a zonal reaction is suspected, another test should be performed using 0.1 ml. of heated serum and 0.4 ml. of saline solution in place of the original 0.5 ml. of serum. If a Reactive finding is obtained with the smaller amount of serum, a Reactive report should be issued.

VDRL Tube Quantitative Test With Serum

1. Pipette 0.5 ml. of freshly prepared 0.9-percent saline solution into each of five or more test tubes (12- x 75-mm.) omitting the first tube.

2. Add 0.5 ml. of heated serum to the first and second tubes. (The first tube may be omitted if the VDRL tube qualitative test has been performed, and if sufficient serum is not available.)

3. Mix and transfer 0.5 ml. from second to third tube.

4. Continue mixing and transferring 0.5 ml. from each tube to the next until the last tube is reached.

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[4] International Equipment Co., Boston, Mass.

5. Mix and discard 0.5 ml. from last tube.

6. Add 0.5 ml. of diluted antigen emulsion to each tube and proceed as described under "VDRL Tube Qualitative Test With Serum", (pp. 92–93).

Reading and Reporting Tube Quantitative Test Results

The greatest serum dilution producing a definitely Reactive result is reported as the reactivity end point as shown in the following examples:

Undiluted serum	*Serum dilutions*				*Report*
1:1	*1:2*	*1:4*	*1:8*	*1:16*	
R	N	N	N	N	Reactive, undiluted only, or 1 dil.
R	R	R	N	N	Reactive, 1:4 dilution, or 4 dils.
R	R	R	R	N	Reactive, 1:8 dilution, or 8 dils.

R = Reactive. N = Nonreactive.

VDRL TESTS WITH SPINAL FLUID (4)

Equipment

1. Kahn shaking machine (must be operated at 275 or 285 oscillations per minute).

Reagents

1. Antigen. (VDRL slide flocculation test antigen, see p. 82.)

2. Saline solutions

   a. 1-percent buffered saline solution. (Prepare as for the VDRL slide flocculation tests, p. 82.)

   b. 10-percent sodium chloride solution

   Dissolve 10 gm. of dry sodium chloride (A. C. S.) in 100 ml. of distilled water.

Preparation of Spinal Fluid

1. Centrifuge and decant each spinal fluid. Spinal fluids which are visibly contaminated or contain gross blood are unsatisfactory for testing.

2. Heat spinal fluid at 56° C. for 15 minutes. Cool to room temperature before testing.

Preparation of the Sensitized Antigen Emulsion

1. Prepare antigen emulsion as described for the VDRL slide flocculation tests (see "Preparation of Antigen Emulsion," pp. 83–84).

2. Add one part of 10-percent sodium chloride solution to one part of VDRL slide test emulsion.

3. Mix well, and allow to stand at least 5 minutes but not more than 2 hours before use.

VDRL Qualitative Test With Spinal Fluid

1. Pipette 1.0 ml. of heated spinal fluid into a 13- x 100-mm. test tube.

Include Reactive and Nonreactive spinal fluid controls in each test run.

2. Add 0.2 ml. of sensitized antigen emulsion to each spinal fluid.

Resuspend the sensitized antigen emulsion immediately before use by inverting container several times.

3. Shake racks of tubes on Kahn shaking machine for 15 minutes.

4. Centrifuge all tubes for 5 minutes at force equivalent to 1,800 r. p. m. in No. 1 or 1,600 r. p. m. in No. 2, I. E. C.[4] centrifuge.

5. Return tubes to Kahn shaking machine and shake exactly 2 minutes.

Reading and Reporting Qualitative Test Results

1. Read test results as soon as possible after the secondary shaking period by holding tubes close to the shade of a desk lamp having a black background.

Note: Each tube may be held motionless or shaken gently during the reading. Excessive agitation should be avoided.

2. Report results as follows:

Reactive.....	Definitely visible aggregates suspended in a water-clear or turbid medium.
Nonreactive	No aggregation, complete dispersion of particles, appearance turbid or slightly granular.

VDRL Quantitative Test With Spinal Fluid

Quantitative tests are performed on all spinal fluids found to be Reactive in the qualitative test.

1. Prepare spinal fluid dilutions as follows:

   a. Pipette 1.0 ml. of 0.9-percent sodium chloride solution into each of five or more tubes.

[4] International Equipment Co., Boston, Mass.

b. Add 1.0 ml. of heated spinal fluid to tube 1, mix well, and transfer 1.0 ml. to tube 2.

c. Continue mixing and transferring from one tube to the next until the last tube contains 2 ml. Discard 1.0 ml. from the last tube. The respective dilution ratios are 1:2, 1:4, 1:8, 1:16, 1:32, etc.

2. Test each spinal fluid dilution as described under "VDRL Qualitative Test With Spinal Fluid" (p. 95).

Reading and Reporting Quantitative Test Results

1. Read each tube as described under "VDRL Qualitative Test With Spinal Fluid."

2. Report test results in terms of the highest dilution of spinal fluid producing a Reactive result. The term "dils" which expresses the same dilution reactivity end point may be applied.

Example:

Spinal fluid dilutions					*Report*
1:2	*1:4*	*1:8*	*1:16*	*1:32*	
N	N	N	N	N	Reactive,[1] undiluted only, or 1 dil.
R	R	R	N	N	Reactive, 1:8 dilution, or 8 dils.
R	R	R	R	N	Reactive, 1:16 dilution, or 16 dils.

R = Reactive. N = Nonreactive.

[1] Reactive finding with undiluted spinal fluid in the qualitative test.

References

(*1*) Harris, A.; Rosenberg, A. A.; Riedel, L. M.: A microflocculation test for syphilis using cardiolipin antigen. Preliminary report. J. Ven. Dis. Inform., 27: 169–174, July 1946.

(*2*) Harris, A.; Rosenberg, A. A.; Del Vecchio, E. R.: The VDRL slide flocculation test for syphilis. II. A supplementary report. J. Ven. Dis. Inform., 29: 72–75, March 1948.

(*3*) Harris, A.; Rosenberg, A. A.; Del Vecchio, E. R.: A macroflocculation test for syphilis using cardiolipin-lecithin antigen. J. Ven. Dis. Inform., 29: 313–316, October 1948.

(*4*) Rosenberg, A. A.; Harris, A.; Harding, V. L.: A macroflocculation spinal fluid test employing cardiolipin-lecithin antigen. J. Ven. Dis. Inform., 29: 359–361, December 1948.

Appendix

Collection and Preservation of Sheep Blood

Sheep cells may be too resistant or too susceptible to the hemolytic action of complement and hemolysin. As the reactivity level of a complement-fixation test is influenced by the quality of sheep cells employed, particular attention should be given to this test component. The effects of bacterial contamination on sheep cell reactivity are unpredictable and only aseptically collected sheep blood in sterile containers is recommended for use in complement-fixation testing.

Red corpuscles from an occasional sheep will be found to be exceptionally resistant to the hemolytic action of complement and hemolysin. Whenever blood from an untested sheep is drawn, comparative complement and amboceptor titrations should be made, employing other sheep cells of acceptable quality.

Sheep cells are too fragile for use when a saline suspension of washed cells, prepared as prescribed by a test technique, shows any degree of hemolysis when stored overnight at 6° to 8° C.

The only recommended solution, in either of these instances, is to discard unsatisfactory cells and to obtain another supply of sheep blood.

The following method for collecting and preserving sheep blood (*1,2*) has been in use at the Venereal Disease Research Laboratory for several years with satisfactory results.

Equipment and Glassware

1. Bleeding flask, Erlenmeyer, 2-liter capacity, fitted with a No. 10 2-hole rubber stopper; filter funnel (1 inch in diameter, 2½-inch stem); rubber and glass tubing; and 13-gage hypodermic needle (fig. 1).

2. Dispensing assembly consisting of rubber tubing, clamp, and filling mantle, plus air-filter tube (fig. 2).

Reagent

1. 3.8-percent sodium citrate solution

   Dissolve 3.8 gm. of sodium citrate (A. C. S.) in each 100 ml. of distilled water. Sixty milliliters of this solution is required for each 50 ml. of sheep blood collected.

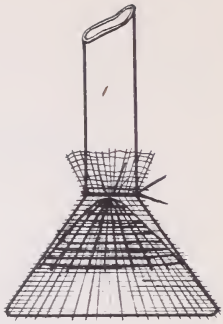


Figure 1a

BLEEDING FLASK

Figure 1

Assembly of Bleeding Flask

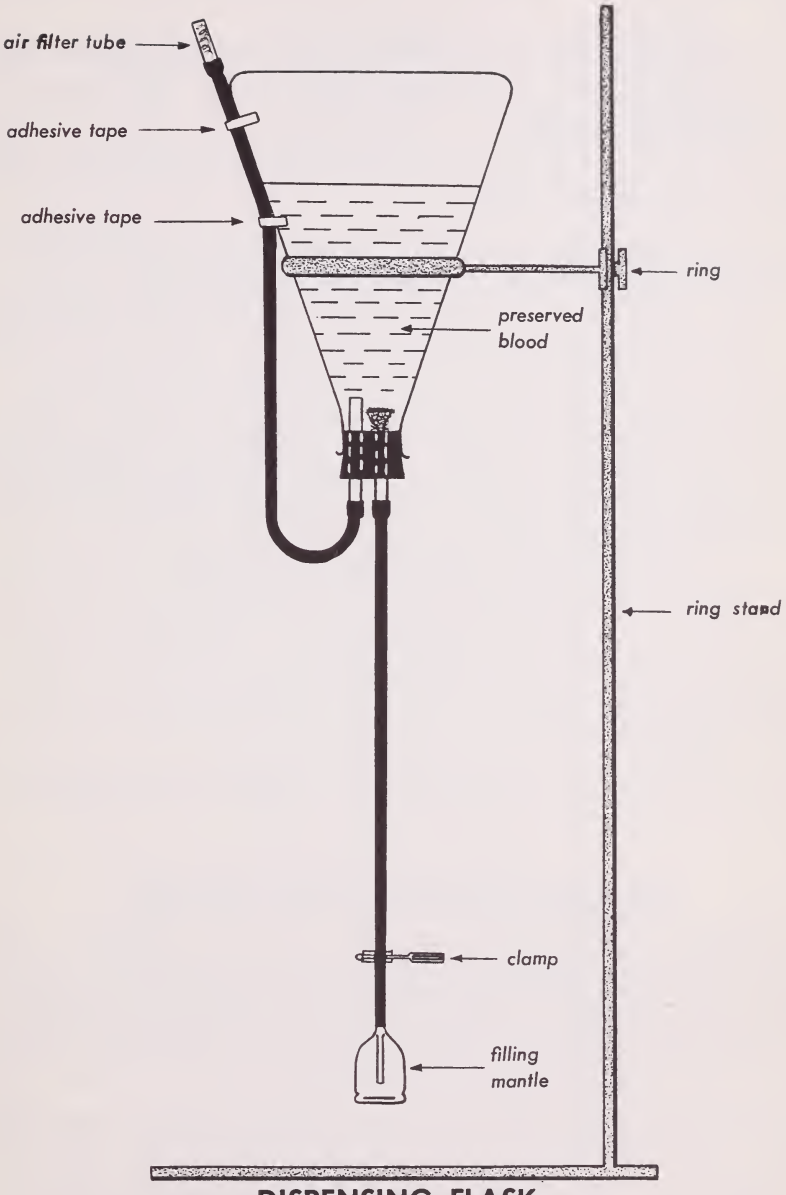
1. Draw a line on the 2-liter flask with a wax pencil marking off 880-ml. volume. This will provide for the collection of 400 ml. of blood in 480 ml. of citrate solution. Proportionally smaller quantities may be provided for.

2. Pour 480 ml. of 3.8-percent sodium citrate solution into the flask.

3. Cut a small piece of wire screening and place in the funnel. Tie a piece of gauze over the mouth of the funnel as illustrated in figure 1a.

4. Assemble bleeding flask, as shown in figure 1, by inserting stopper of bleeding apparatus into flask and securing it with string ties.

5. Sterilize the entire unit at 15 pounds pressure for 20 minutes.



DISPENSING FLASK

Figure 2

Collection of Blood

1. Immobilize the sheep in a standing position.

2. Raise the head until the nose and center of neck form a straight line.

3. Turn the head slightly and clip the wool from the puncture area.

4. Apply digital pressure above the collar bone to cause dilation of the external jugular vein.

5. Sterilize the area directly over the located vein with weak iodine solution or 70-percent alcohol.

6. Apply digital pressure just below the point to be punctured and insert the sterile needle into the skin, then into the vein.

7. Rotate the flask continuously during collection of the blood and for 5 minutes afterwards to prevent clotting.

8. Allow the blood to cool to room temperature (73° to 85° F.).

9. Replace the glass suction mouthpiece with the previously sterilized filling mantle shown in figure 2. Replace the hypodermic needle with a section of sterile glass tubing containing loosely packed sterile cotton (air-filter tube, fig. 2).

10. Invert and suspend the flask as shown in figure 2.

11. Dispense the blood aseptically into sterile rubber-stoppered vaccine bottles and store in the refrigerator.

12. Remove blood mixtures from these bottles, as required, with a sterile syringe and needle.

Note: Sheep blood of good quality, collected and stored in the manner described, has been found satisfactory for use over a 3-month period.

Preparation of Hemolysin (Sheep Red Cells)

The following method (*3*) has been found to give consistently satisfactory results in producing high-titered antisheep hemolysin. Whole sheep blood is injected intracutaneously into rabbits, followed by intravenous inoculation of washed sheep cells. Blood from several sheep is used during the course of injections.

1. Select six or eight medium-sized young rabbits weighing about 2 kg. each.

2. Defibrinate a small amount of sheep blood with glass beads.

3. Give each rabbit a series of five intracutaneous injections of whole sheep blood every other day, beginning with a dosage of 0.5 ml. and increasing this each time by 0.5 ml. See table 1.

Table 1. Schedule of injections

Day	Dosage	Route of inoculation	Material injected
	(*ml.*)		
1	0.5	Intracutaneous	Whole blood.
3	1.0	Intracutaneous	Whole blood.
5	1.5	Intracutaneous	Whole blood.
7	2.0	Intracutaneous	Whole blood.
9	2.5	Intracutaneous	Whole blood.
12	1.0	Intravenous	20-percent washed cells.
15	1.0	Intravenous	20-percent washed cells.
18	Test bleeding		

4. Prepare a 20-percent suspension of sheep cells by washing red cells thoroughly with a 0.85-percent saline solution to which 0.01 percent magnesium sulfate has been added (see "Reagents, saline solution," *Kolmer Tests*, p. 53).

5. Two intravenous injections of 1 ml. of 20-percent sheep cell suspension are given at 3-day intervals after the last injection of whole sheep blood. See table 1.

6. Test bleedings are made, beginning 3 days after the second intravenous injection, by piercing the marginal ear vein with a stylet and collecting approximately 1.0 ml. of blood in a small tube.

7. Serum is separated from these blood samples by centrifuging after clotting is completed.

8. Each rabbit serum is titrated for hemolysin content by the method described in the Kolmer complement-fixation technique (pp. 57–61).

9. Exsanguinate those rabbits having a hemolysin titration unit of 1:5,000 dilution or higher.

10. Separate serums from blood clots, pool, and preserve by the addition of an equal amount of glycerin.

In the Venereal Disease Research Laboratory hemolysin is preserved by lyophilization. Aliquots of 4 ml. are dehydrated, sealed in an atmosphere of nitrogen, and stored in the refrigerator. For use, an ampule is opened and the dried hemolysin is dissolved in 4 ml. of distilled water. The restored material is further diluted with an equal volume of glycerin and stored in the refrigerator.

11. Repeat the intravenous injections of 20-percent washed cells in the rabbits with a low hemolysin titer as described in par. **5**, and continue as outlined in pars. **6**, **7**, **8**, **9**, and **10**.

Stock glycerinated (50-percent) sheep red cell hemolysin can be stored at refrigerator temperature for long periods of time with little loss of reactivity. A more rapid lowering of titer will be noted in the diluted hemolysin solutions. When a marked drop in titer is found

or if precipitate is seen in the diluted hemolysin, this reagent should be discarded and reprepared from stock hemolysin.

A sudden drop in hemolysin titer may also be caused by the complement, sheep cells, or saline solutions used. Comparative tests should be made to determine which reagent is at fault.

Preparation and Preservation of Complement

Guinea pig blood may possess complement activity less than the prescribed minimum or greater than the prescribed maximum. Low complement titers may be caused by improper feeding or housing of guinea pigs or, most commonly, through loss of reactivity during storage of guinea pig serum. Complement serum stored as liquid (with preservative added) at refrigerator temperature or in the frozen state should be adequately protected from partial drying as a result of evaporation. Aliquots sufficient for 1 day's use should be placed in containers to avoid complement destruction due to repeated thawing and refreezing.

Some technicians are deceived by restoring dehydrated complement serum to only one-half or two-thirds of the original serum volume and then omitting the serum concentration factor when calculating complement dilution. Substandard serum may be made to appear adequately reactive in this way. This practice, used to circumvent technique restrictions, is to be discouraged.

Individual guinea pig serums may possess complement in higher titer than is optimum for some techniques. The pooling of serum from several guinea pigs will usually avoid this condition.

Cardiolipin-lecithin antigens are free of the fraction responsible for the nonspecific fixation of complement at low temperature. Pretesting of guinea pig serums and the use of egg white is therefore omitted when this type of antigen is employed.

Preparation

1. Select three or more large, healthy male guinea pigs.

2. With a needle and syringe, remove 5 ml. of blood from the heart and place in individual tubes identified by number.

3. After the blood has clotted at room temperature, ring with applicator stick, and refrigerate for at least 1 hour.

4. Centrifuge and remove clear serum from the clot.

5. Pool serums from all tubes, recentrifuge, and preserve.

OR

6. If animals are to be exsanguinated, stun to anesthetize, cut the external jugular veins, and collect blood in petri dishes or 50-ml. centrifuge tubes.

7. Allow clotting to take place for 1 hour at room temperature.

8. Loosen clot from wall of container, and refrigerate for 1 to 2 hours.

9. Decant and filter serum through gauze, centrifuge, pool clear serum, and preserve.

Preservation

Method 1.—Dehydrate complement serum from the frozen state, in vacuo, by the lyophile or cryochem methods.[1]

Method 2.—Add an equal part of the following solution to the complement serum:

Sodium acetate	12 gm.
Boric acid	4 gm.
Sterile distilled water	100 ml.

For use, dilute 1 ml. of the preserved complement serum with 14 ml. of saline solution to prepare a 1:30 dilution. Store in refrigerator.

Method 3.—Add 1.0 gm. of sodium chloride for every 10 ml. of guinea pig serum. Store in refrigerator.

Method 4.—Freeze complement serum and retain in the frozen state until used.[1]

Use of Merthiolate as a Bacteriostat

Grossly contaminated serums or spinal fluids are unsatisfactory specimens for serologic tests for syphilis. The effects of random bacterial contamination of body humors on serologic results are not predictable.

Although spinal fluid is usually drawn with reasonable attention to sterility, many fluids mailed to central testing laboratories, especially during the warmer months of the year, show evidence of gross bacterial contamination on arrival. Removal of bacteria from contaminated spinal fluids by centrifuging or filtration is ineffective since some of the products of bacterial metabolism are soluble, and changes of original spinal fluid components are not corrected or compensated for.

The use of Merthiolate as a bacteriostatic agent for spinal fluid preservation has been reported (*4*). This compound (sodium ethylmercurithiosalicylate)[2] curtails bacterial growth without interfering

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[1] 2-gm./percent sodium acetate citrate (A.C.S.) is added to complement serum before dehydration or storage in frozen state at the Venereal Disease Research Laboratory.

[2] Eli Lilly and Co., Indianapolis, Ind.

with the mechanisms of the usual serologic tests for syphilis either through chemical action or the introduction of a dilution factor. Furthermore, its presence does not affect the results obtained with the turbidimetric methods for determining total proteins in spinal fluids. However, the *Treponema pallidum* immobilization (TPI) test and certain of the chemical assay methods, such as the micro-Kjeldahl and the tyrosine equivalent, cannot be satisfactorily performed when this compound is present in spinal fluid or serum. The colloidal gold reaction may also be altered by Merthiolate.

Collection tubes containing Merthiolate may be prepared as follows:

1. Prepare the necessary amount of Merthiolate solution on the day used by adding 1.0 gm. of Merthiolate powder to each 100 ml. of distilled water. Do not use commercially prepared tinctures or solutions.

2. Pipette 0.1 ml. of 1-percent aqueous Merthiolate solution to the bottom of 13- x 100-mm. tubes.

3. Place tubes in vacuum desiccator over calcium chloride at room temperature protected from light. Dehydration is completed in 24 hours or less if adequate vacuum is established.

4. Prepare paraffined corks by submerging corks in hot, but not smoking, paraffin for 1 minute and removing excess paraffin from corks by rolling them on cloth while hot.

5. Remove tubes from desiccator and stopper tightly with paraffined corks.

6. Store tubes in the dark. They will remain usable for several months.

The concentration of Merthiolate obtained when 2.0 ml. to 8.0 ml. of spinal fluid is added to these tubes is sufficient to inhibit bacterial growth.

Smaller tubes (12- x 75-mm.) prepared in this manner and containing 1 mg. of Merthiolate are suitable for shipment of 2.0 ml. to 4.0 ml. of serum.

Quantitative Determination of Spinal Fluid Protein (5, 6)

Equipment

1. Photoelectric colorimeter.

2. Photoelectric colorimeter cuvettes, to accommodate 5-ml. volumes or less.

Glassware

1. Test tubes, 13- x 100-mm. outside dimension.

Reagents

1. 10-percent trichloracetic acid solution

   Dissolve 10 gm. of trichloracetic acid (C. P.) in 100 ml. of distilled water. Filter into a glass-stoppered flask and store at room temperature.

2. Standard serum

   Select serum free of gross bacterial contamination or hemolysis and determine the total protein concentration by Kjeldahl analysis.

Preparation of Spinal Fluid

Centrifuge spinal fluid and decant. Spinal fluids containing visible contamination or gross blood are unsatisfactory for testing.

Performance of the Test

1. Pipette 2.5 ml. of spinal fluid[3] into a 13- x 100-mm. test tube. A protein solution of known concentration should be included each time tests are performed.

2. Add 2.5 ml. of 10-percent trichloracetic acid solution.

3. Invert the tube twice to mix contents. Avoid foaming.

4. Allow tube to stand for 10 minutes in 37° C. water bath.

5. Again invert the tube and pour fluid into photoelectric colorimeter cuvette.

6. Determine percentage of light transmission at a wave length of 420 mu. with the unknown, using a water blank at 100-percent transmission.

7. Convert percent transmission of unknown to milligrams-percent total protein by reference to a calibration chart.

Note: If spinal fluids contain concentrations of protein greater than 60-mg. percent they should be appropriately diluted with 0.9-percent sodium chloride solution and retested. Values obtained from the calibration chart are then multiplied by the dilution factor.

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[3] Lesser amounts of spinal fluid may be tested in the same proportion provided that the photoelectric colorimeter employed is adaptable to the use of smaller cuvettes.

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