PULSE SCIENTIFIC INC.
Unit 18, 5100 South Service Road
Burlington, Ont. Canada L7L 6A5
Tel: (905) 333-8188
Fax: (905) 333-0500
Toll Free: 1-800-363-7907

CF - Fungal Antigens and Positive Controls

INTENDED USE

The fungal antigens and positive controls are used to detect antibodies in patient serum by the complement fixation (CF) procedure to aid in the diagnosis of four specific fungal diseases:Histoplasmosis,blastomycosis, coccidioidomycosis, and aspergillosis.

EXPLANATION

Patient sera should be tested with each of the antigens, since there is some overlap in antigenicity between the various fungi and the symptoms of the diseases are very similar. Higher CF titers are usually observed on patient sera when they are tested against the same antigen as the etiologic agent of their infections.

PRINCIPLES

The test is based on the Laboratory Branch Complement Fixation (LBCF) Test Procedure (as published by the Centers for Disease Control, Atlanta, GA)[5]. The principal of the CF test is that antibodies present in patient sera, when mixed with the corresponding antigens will "fix," or bind, complement (a component of fresh serum). This "fixation" of complement is determined by using an assay system consisting of sheep red blood cells (SRBC) sensitized with anti-SRBC (hemolysin) and measuring the percentage of lysis of the SRBC (unbound complement initiates lysis). If all complement has been "fixed" the indicator SRBC's will not be lysed.

The CF test involves two basic principles:

1. Complement (C') is irreversibly bound (fixed) by certain classes of antibody-antigen complexes (certain classes of antibodies do not fix complement). The degree of fixation is governed by the relative concentration of antibody or antigen.
2. The lysis of SRBC that have been sensitized with hemolysin is dependent upon the presence of unbound complement.
   The CF test (serum + antigen + C' — incubate — + sensitized SRBC) is interpreted as follows:
   Antibody present = **NO HEMOLYSIS**
   Antibody absent = **HEMOLYSIS**

MATERIALS AVAILABLE

1. **Optimally Diluted Antigens:** These antigens are prediluted to the optimal concentration for use in the LBCF procedure and are ready for use as supplied. The optimum dilutions for these antigens have been determined by "box-titration" against sera from proven cases of the mycoses with known titers. The optimally diluted antigens are available as follows:

DESCRIPTION	REF #	SIZE
Histoplasma Mycelial CF Antigen Dilute	14115M	5 ml
Histoplasma Yeast CF Antigen Dilute	14115Y	5 ml
Blastomyces CF Antigen Dilute	13515	5 ml
Coccidioides CF Antigen Dilute	13115	5 ml
Aspergillus CF Antigen Dilute	13715	5 ml

2. **Antibody Controls:** The positive controls are from hyperimmunized goats. Each positive control should give a 1:32 titer (+/- 1 dilution) with its optimally diluted homologous antigen. The negative control should be negative in the CF test with all antigens.

DESCRIPTION	REF. #	SIZE
Histoplasma Mycelial CF Positive Control	14121M	1 ml
Histoplasma Yeast CF Positive Control	14121Y	1 ml
Blastomyces CF Positive Control	13521	1 ml
Coccidioides CF Positive Control	13121	1 ml
Aspergillus CF Positive Control	13721	1 ml
Negative Control	N80110	1 ml

MATERIALS NOT PROVIDED
(Some vendors are suggested.)

1. Guinea pig complement (Lonza, Cat.# 30-956J)
2. Hemolysin Rabbit Anti-Sheep Erythrocyte Stroma (Lonza, Cat.# 55-402J)
3. Sheep Blood Alsevers (SRBC) (Colorado Serum Co., Cat.# CS1112 #7833 [neutered sheep blood recommended])
4. Veronal Buffered Diluent (VBD, 5X concentrate) (Lonza, Cat.# 12-624E)
5. Gelatin (Sigma, Cat. # G2500)
6. 5% phenol solution in saline
7. pH meter
8. 15-ml graduated centrifuge tubes
9. Centrifuge
10. Refrigerator (2-8 ˚C)
11. Pipettes (1-, 5-, & 10-ml)
12. Glass serological tubes (15 X 125-mm & 12 X 75- mm)
13. Incubator, 37 °C
14. Water baths or heat block (37 °C & 56 °C)
15. Microtiter equipment:
    a. 25-50 µl multichannel micropipetter

b. "U"-bottom polystyrene microtiter plates
c. Reading mirror (concave, 1.5 X mag)

PRECAUTIONS

1. All reagents are intended for in vitro diagnostic use only!
2. Specific standardization is necessary to produce our high quality reagents and materials. Pulse Scientific cannot guarantee the performance of its products when used with materials purchased from other manufacturers.
3. Do not use reagents containing foreign matter, particulates or aggregates, which indicate contamination or improper storage or handling. Note: Histoplasma Yeast Antigen contains a suspension of yeast cells and should be cloudy.
4. Specimens must not contain bacteria, visible lipids, or other obvious signs of contamination.
5. Do not store specimens in a frost-free type freezer. Repeated freezing and thawing of the specimens can affect test results.
6. When handling patient specimens, adequate measures should be taken to prevent exposure to etiologic agents potentially present in the specimen.
7. All reagents are preserved with sodium azide [0.095% (w/w)]; it is therefore recommended that excess reagents simply be discarded in an appropriate waste receptacle.

STABILITY AND STORAGE

Fungal antigens should be stored at 2-8 °C and are stable until the expiration date as long as titers with the positive controls are 1:32 ± 1 dilution. The positive controls may continue to be used as long as the titer with their homologous antigen is 1:32 ± 1 dilution. The positive control is stable until the expiration date when stored at 2-8 °C **PRIOR** to rehydration. The rehydrated positive control will remain stable for up to one month if stored at 2-8 °C. For storage periods longer than one month, the rehydrated positive control should be aliquotted and frozen (NOT in a frost-free freezer) where it will remain stable until the expiration date. Repeated freezing and thawing should be avoided. When the positive controls are in use, the period at room temperature should be kept as short as possible.

REAGENT PREPARATION

Positive Control

The positive controls are rehydrated by adding 1 ml of distilled or DI water to the vial and incubating at room temperature until completely dissolved (2-3 minutes), followed by gentle mixing.

1X VBD Preparation

1. Combine 100 ml DI water and 0.5 g gelatin to a 1- L flask, and bring to a boil.
2. Remove from heat, and cool to room temperature.
3. Add 300 ml DI water (gel-water solution).
4. Add 100 ml 5X VBD, and mix by inverting (1X VBD).
5. Store at 2-8 °C for up to 1 week.

Sensitized, Standardized Sheep Red Blood Cell Preparation (SRBC)

Standardizing SRBC

1. Determine the volume of sensitized, standardized SRBC needed for antigen or serum titration by multiplying the number of plates by 2.4 and then adding 1 ml for pipetting.
2. To standardize SRBC, wash sheep blood (7 ml blood = ~1.5 ml packed cells) with VBD by centrifugation at 600 x g for 10 min., and remove supernatant by suction; repeat. Only use sheep blood that is > 5 days and < 28 days old.
3. Transfer SRBC to a graduated centrifuge tube; centrifuge 600 x g for 5 minutes, and record volume of packed cells. Remove supernatant by suction.
4. Standardize SRBC to 2.8% by using the following table:

Table 1

Packed Cell Volume (ml)	VBD Volume (ml)	Packed Cell Volume (ml)	VBD Volume (ml)	Packed Cell Volume (ml)	VBD Volume (ml)
0.10	3.40	0.90	30.60	1.70	57.80
0.15	5.10	0.95	32.30	1.75	59.50
0.20	6.80	1.00	34.00	1.80	61.20
0.25	8.50	1.05	35.70	1.85	62.90
0.30	10.20	1.10	37.40	1.90	64.60
0.35	11.90	1.15	39.10	1.95	66.30
0.40	13.60	1.20	40.80	2.00	68.00
0.45	15.30	1.25	42.50	2.05	69.70
0.50	17.00	1.30	44.20	2.10	71.40
0.55	18.70	1.35	45.90	2.15	73.10
0.60	20.40	1.40	47.60	2.20	74.80
0.65	22.10	1.45	49.30	2.25	76.50
0.70	23.80	1.50	51.00	2.30	78.20
0.75	25.50	1.55	52.70	2.35	79.90
0.80	27.20	1.60	54.40	2.40	81.60
0.85	28.90	1.65	56.10		

5. Add SRBC to volume of VBD calculated above to a small flask, and swirl gently.
6. To test the accuracy of the SRBC suspension, add EXACTLY 7 ml of the 2.8% SRBC to a graduated centrifuge tube (below 1 ml, graduations should be in 0.1 ml increments), and centrifuge 600 x g for 10 minutes.
7. The packed cells should read exactly 0.2 ml. If not, calculate the CCV using the following formula: CCV = (PCV/0.2)OCV where CCV = corrected cell volume, PCV=packed cell volume, and OCV=original cell volume. If CCV > OCV, calculate the volume of VBD to ADD by CCV-OCV, and repeat step 6. If CCV < OCV, then calculate the volume of VBD to REMOVE by OCV-CCV, and repeat step 6 (NOTE: if VBD is to be added, mix the contents of the centrifuge tube in step 6, and return the contents to the flask of cells before adding the additional VBD).

Sensitizing the Standardized SRBC

8. To a small flask, add volume of standardized SRBC equal to half the volume of sensitized cells required.
9. Prepare an equal volume of optimally diluted hemolysin diluted with VBD, and slowly add to cells while constantly swirling the cells. Make sure to add hemolysin to the cell mixture, not the cell mixture to the hemolysin.
10. Incubate cell mixture for 15 minutes in a 37 °C water bath.
11. Allow to cool to room temperature.
12. Store at 2-8 °C for up to 2 days.

Color Standard Preparation

Prepare 0, 30, 50, 70, 90, and 100% lysis standards.

1. The **hemoglobin** solution is prepared by adding, in order, 1 ml of 2.8% SRBC to a 15 x 125-mm tube, 7 ml

DI water, mix, and then add 2 ml 5X VBD (this final addition returns the solution to an isotonic state).
2. The 0.28% SRBC suspension is prepared by adding 1 ml 2.8% SRBC to a 1 5x 125-mm tube and 9 ml 1X VBD.
3. Label 6 tubes as follows: 0, 30, 50, 70, 90, and 100.
4. Each color standard is prepared by adding hemoglobin and 0.28% SRBC as follows:
5. Mix each tube briefly.

Table 2						
% Lysis	0	30	50	70	90	100
Hemoglobin	0	0.3	0.5	0.7	0.9	1.0
0.28% SRBC	1.0	0.7	0.5	0.3	0.1	0

HEMOLYSIN TITRATION

Hemolysin should be titrated with each new lot number of sheep cells or hemolysin. Upon receiving a new lot number of hemolysin, a stock 1:100 solution should be prepared. The procedure for the preparation of this stock solution is shown below.

Preparation of Stock 1:100 Hemolysin Dilution

1. Add 4.0 ml of 5% phenol solution to a 125-ml Erlenmeyer flask.
2. Add 94 ml of cold VBD to the flask, and mix by swirling.
3. Add 2 ml of glycerinized hemolysin, and mix by swirling. (Note: hemolysin is prediluted 1:2 with glycerine.)
4. Store the 1:100 stock hemolysin at 2-8 ˚C for up to 1 year.

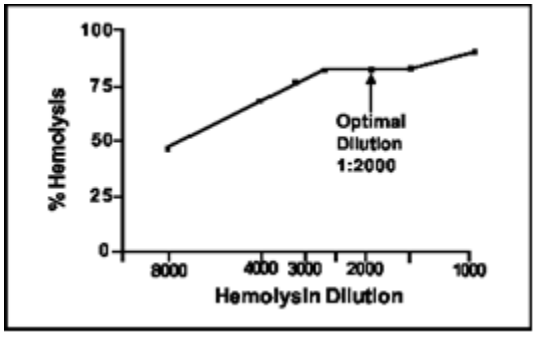
Hemolysin Titration Procedure

1. Label a 15 X 125-mm tube for the 1:1000 hemolysin dilution.
2. Add 9 ml of cold VBD to the tube.
3. Add 1 ml of 1:100 stock hemolysin dilution and mix by pipetting.
4. Label six 15 X 125-mm tubes with the final hemolysin dilutions shown in Table 3.
5. Pipette the volumes of VBD shown in Table 3 into the appropriate tubes.

Table 3 Preparation of Further Hemolysin Dilutions		
Final Hemolysin Dilution	VBD ml	1:1000 Hemolysin Dilution, ml
1:1500	0.5	1.0
1:2000	1.0	1.0
1:2500	1.5	1.0
1:3000	2.0	1.0
1:4000	3.0	1.0
1:8000	7.0	1.0

6. Pipette 1 ml of 1:1000 hemolysin dilution into each tube and mix.
7. Prepare appropriate dilutions of complement using cold VBD. See Table 4 in the *Complement Titration* section for appropriate volumes of VBD and complement.
8. **Note:** Preparing multiple dilutions of complement [i.e. 1:300, 1:350, 1:400, etc.] and running titrations with each simultaneously will frequently save time since some dilutions may not plot satisfactorily.
9. Incubate the complement dilutions at 2-8 °C for 20 minutes, but use within 2 hours.
10. Label 7 tubes, one for each hemolysin dilution shown in Table 3.
11. Sensitize the cells by adding 1 ml of standardized 2.8% SRBC to each tube. (See *Reagent Preparation – Standardizing SRBC*.)
12. Slowly, and with constant swirling, add 1.0 ml of the appropriate hemolysin dilution to each of the tubes.
13. Shake the rack to mix, and incubate in a water bath at 37 °C for 15 minutes.
14. For each dilution of complement, label 7 tubes, one for each hemolysin dilution shown in Table 3, with the appropriate hemolysin and complement dilution.
15. Add 0.4 ml of cold VBD to each tube.
16. Add 0.4 ml of the appropriate complement dilution to each tube and shake the rack to mix.
17. Add 0.2 ml of sensitized SRBC to each of the appropriately labeled tubes, and shake the rack to mix.
18. Incubate tubes in a 37 °C water bath for 1 hour, shaking after 30 minutes.
19. Centrifuge tubes at 600 x g for 5 minutes.
20. Prepare color standards, and record the percent lysis for each tube (See *Reagent Preparation – Color Standards*).
21. Plot the percent (%) hemolysis on the y-axis of standard (linear-linear) graph paper versus the appropriate hemolysin dilution on the x-axis.
22. Examine the graph for a plateau (i.e. the region where increasing the concentration of hemolysin produces no marked increase in lysis).
23. The second dilution on the plateau is the optimum hemolysin dilution to be used to sensitize cells for the complement titrations, antigen titrations, and serum tests. This dilution is termed the 100% hemolytic unit of hemolysin (H'_{50}).

Figure 1



COMPLEMENT TITRATION

Complement must be titrated with each new lot number of hemolysin, complement or SRBCs. Freshly rehydrated complement should be aliquotted into sealed vials or tubes and stored at -60 °C or colder. A thawed aliquot should be used for all titrations. Keep complement, and all dilutions, cold (e.g. refrigerate or ice bath) during and after thawing (2 - 8 ˚C).

Complement Titration Procedure

1. Prepare sensitized, standardized SRBCs as indicated in the "Reagent Preparation" section.
   Note: 3 or 4 different complement dilutions should be titered simultaneously to increase the probability of obtaining values that will give a valid titration curve.

2. For each C' dilution that is to be titered, label two sets of four 12 X 75-mm tubes with the appropriate complement dilution and the numbers 1 through 4. (Each titration is performed in duplicate.)

Table 4 Complement Dilutions		
Complement Dilution	ml Complement	ml VBD
1:200	0.25	49.75
1:225	0.25	56.00
1:250	0.25	62.25
1:275	0.25	68.50
1:300	0.25	74.75
1:325	0.25	81.00
1:350	0.25	87.25
1:375	0.25	93.55
1:400	0.25	99.75

3. Prepare C' dilutions according to Table 4.
4. For each C' dilution that is to be titered, add reagents to the duplicate sets of tubes in the order shown in Table 5.

Table 5 Complement Titration				
Reagent Tube #	1	2	3	4
VBD (ml)	0.60	0.55	0.50	0.40
C' Dilution (ml)	0.20	0.25	0.30	0.40
Sensitized SRBCs (ml)	0.20	0.20	0.20	0.20

5. Shake the tubes and place in a 37 °C water bath for 30 minutes. Shake after 15 minutes of incubation.
6. Remove the tubes from the water bath, and centrifuge to pack the cells.
7. Determine the percent hemolysis in each tube by comparing with the color standards and interpolating to the nearest 5% when a tube does not exactly match one of the standards (See *Reagent Preparation –Color Standards*).
8. Average the percent lysis for each pair of replicate tubes with the same volume and dilution of C'

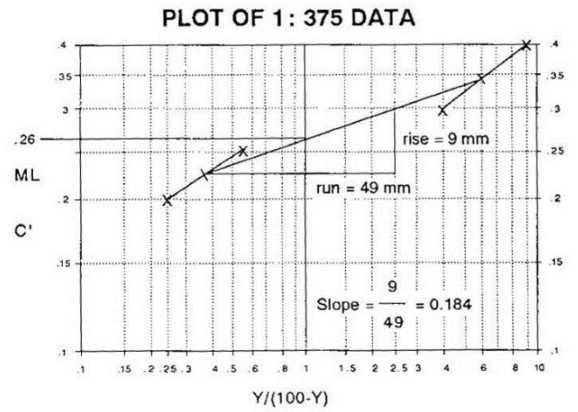
Table 6 Lysed to Unlysed Ratio					
Y	Y (100-Y)	Y	Y (100-Y)	Y	Y (100-Y)
10	0.111	40	0.67	70	2.33
15	0.176	45	0.82	75	3.00
20	0.250	50	1.00	80	4.00
25	0.330	55	1.22	85	5.70
30	0.430	60	1.50	90	9.00
35	0.540	65	1.86		
Do not graph when Y exceeds 90% or when Y is less than 10%					

9. Using Table 6, determine the value of the ratio of lysed to unlysed cells Y/(1 00 – Y) for the average percent lysis of tubes 1-4 for each C' dilution.
   For example, if the average of the percent lysis for a pair of tubes was 35%, the corresponding value of the ratio Y/(100-Y) would be 0.540.
10. Using log-log graph paper, plot the averages for each of the four points for a single C' dilution using the volume of C' plotted against the corresponding Y/(100-Y) value (see Figure 2.)
    Note: To plot satisfactorily, two of the Y/(100-Y) values must be less than 1, and two must be greater than 1. In the example depicted in Figure 2, the 1:375 C' dilution plotted satisfactorily.
11. Join the two points plotted for tubes 1 & 2, and using a ruler, find the midpoint of this line.

FIGURE 2. COMPLEMENT TITRATION

Typical C' Titration Data (duplicate readings):

	1:350 Dilution		1:375 Dilution			1:400 Dilution	
ML C'	Tube	% lysis	Tube	% lysis	Y/(100-Y)	Tube	% lysis
0.2	1	20/20	1	20/20	0.25	1	10/10
0.25	2	50/50	2	30/40	0.54	2	20/20
0.3	3	70/70	3	80/80	4.0	3	40/40
0.4	4	90/90	4	90/90	9.0	4	80/80



12. Repeat this process for the points plotted for tubes 3 & 4.
13. Draw a line connecting the two midpoints for the lines determined in steps 11 & 12 above.
14. Determine the slope of the midpoint line plotted in step 13. To determine the slope, use a millimeter ruler to measure vertically from the point where the midpoint line intersects a vertical line down to the same level where the midpoint line intersects a horizontal line. This length is the "rise." Next, measure horizontally from the point where the midpoint line intersects a horizontal line across the same level where the midpoint line intersects a vertical line. This length is the "run." The slope is calculated by dividing the "rise" by the "run." **The slope must equal 0.2 +/- 0.02 for a satisfactory titration.**
15. From the point where the midpoint line intersects the vertical "1" line (See Figure 2), draw a horizontal line to the vertical axis on the left.
16. Read the volume of the C' dilution in ml. This volume contains 150% hemolytic unit of complement (1 C'H_{50} unit).
17. Determine the volume containing 5 C'H_{50} units by multiplying the volume containing 1 C'H_{50} by 5.
18. Calculate the dilution of C' necessary to give 5 C'H_{50} units in 0.4 ml by using the following formula:

Formula: $$\frac{\text{Dilution of } C' \text{ used in titration}}{\text{Volume containing 5 } C'H_{50} \text{ units}} = \frac{X^*}{0.4}$$

X* = dilution of C' needed for 5 C'H_{50} units

Example: $\frac{375}{(0.26)(5)} = \frac{X^*}{0.4}$ $\qquad X^* = 115$

SPECIMEN PREPARATION

Serum from patients should be obtained from the clotted patient blood (anticoagulants should not be used). All specimens must be free of visible lipids, white blood cells, platelets, fibrin or other contaminants. Platelets, white blood cells and fibrin may be removed by centrifugation. Lipids should be removed by filtration.

Sera to be tested within 72 hours of collection may be stored at 2-8 °C. For longer periods, store at -20 °C or lower. Avoid repeated freeze-thawing.

Prior to testing, each serum must be heat-inactivated at 56 °C for 30 minutes to destroy any indigenous complement.

SERUM TITRATION PROCEDURE

All testing should be performed as described in the LBCF procedure[5]. All reagents (hemolysin, complement, SRBC, and VBD) must be standardized prior to testing the patient specimens or positive control.

PREPARING 1:8 DILUTIONS OF SERA

1. Label one 12 x 75-mm tube for each 1:8 dilution of the following: patient sera, positive control and negative control.
2. Add 0.7 ml of cold VBD to each tube (See Reagent Preparation – 1 X VBD Preparation).

NOTE: For *Coccidioides*, beginning serum dilutions should be undiluted (see *Expected Values & Performance Characteristics, Coccidioidomycosis*).

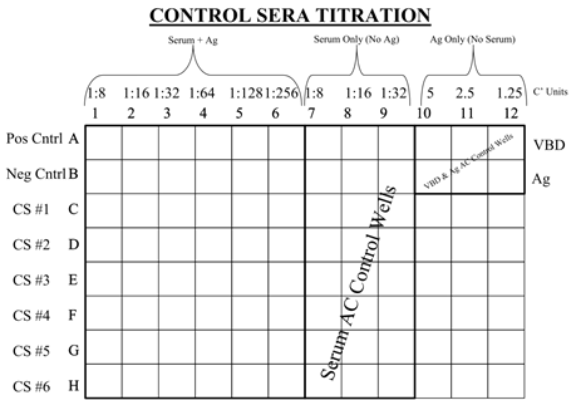
3. Add 0.1 ml of each serum to its respective tube and mix.
4. Heat-inactivate the diluted sera for 30 minutes in a 56°C temperature monitored water bath or heat block.

NOTE: If you are starting your dilutions at any dilution other than 1:8, modify your procedure and labels accordingly.

PREPARING ANTIGEN DILUTIONS

1. Determine the volume of test antigen required by multiplying the number of patient sera and controls by 0.3 (i.e. 0.025 ml/well, 12 wells/serum) and add 0.5 ml excess for pipetting.
2. Prepare the test antigen at the optimum dilution using cold VBD, and mix well.
3. Store antigen at 2-8 °C until use.

Figure 3:



PREPARING TWO-FOLD DILUTIONS OF SERA

1. After heat inactivation, allow sera to cool to room temperature.
2. Mark plates as shown in the "Control Serum Titration" Figure 3 above for serum dilution wells and serum anti-complementary (AC) control wells, and label plate with the antigen type, lot number, and, possibly, a plate number.
3. Pipette 25 µl of cold VBD to all wells in columns 2 through 6 and 8 & 9 (**not in columns 1 and 7, the 1:8 dilution columns).** Pipette 25 µl of VBD into rows A & B, columns 10, 11, & 12 (antigen and VBD controls).
4. Pipette 50 µl of 1:8 diluted positive control to row "A," columns 1 and 7. Repeat for negative control and each case serum in the appropriate row (e.g. CS#1, etc.).
5. Using a micropipetter*, transfer 25 µl of 1:8 diluted serum from the column 1 well, to column 2 (1:16 well); mix well, and repeat the serial dilution process through column 6 (1:256 dilution) for all sera. Discard 25 µl from column 6 (1:256 dilution).
6. Repeat the serial dilution process for the serum AC controls in columns 7 through 9 (1:8 through 1:32) and discard 25 µl from column 9.
7. Pipette 25 µl of optimally diluted antigen to the appropriate wells (columns 1 through 6) for each serum dilution (**NOT** serum AC wells, cols. 7, 8, & 9) and the antigen control wells (row B, col. 10, 11, & 12).
8. Add 25 µl of cold VBD to the serum AC control wells of the test plate (cols. 7, 8, & 9) and to the VBD control wells (row A, cols. 10 through 12).
9. Mix plate on a shaker for 1 minute.
10. Store plates at 2-8 °C while preparing diluted C'.

*** NOTE:** Using an 8-channel micropipetter, all sera can be serially diluted simultaneously.

PREPARING DILUTED C'

1. Determine the volume of diluted 5 C'H_{50} required for the test by multiplying the number of plates by 4.8 (96 wells/plate, 0.050 ml/well), and add 2 ml excess for pipetting and preparation of other dilutions. At the same time, calculate the volume of 2.5 C'H_{50} and 1.25 C'H_{50} required by multiplying the number of wells for each by 0.050.
2. Calculate the volume of VBD and C' stock required to prepare the 5 C'H_{50}.
3. Add the calculated volume of cold VBD to a small flask.
4. Add the calculated volume of C' to the VBD while swirling gently to avoid foaming.
5. Incubate the diluted 5 C'H_{50} at 2-8 °C or in an ice bath for 20 minutes. The diluted C' must be used within 2 hours.
6. After the incubation, prepare the calculated amount of 2.5 C'H_{50} by diluting the 5 C'H_{50} 1:2 with cold VBD. At the same time, prepare 1.25 C'H_{50} by diluting the 5 C'H_{50} 1:4 with cold VBD. Incubate the 2.5 and 1.25 C'H_{50} at 2-8 °C for 20 minutes before use. The diluted C' must be kept cold (2-8 °C, or in an ice bath) and used within 2 hours.

ADDING COMPLEMENT TO THE TEST

1. Pipette 50 µl of 5 C'H_{50} to each titration well, control serum well, and each 5-unit control well, all wells columns 1 through 9 rows A to H and col. 10, rows A & B).
2. Add 50 µl of 2.5 C'H_{50} to each 2.5-unit control wells (col. 11, rows A & B).
3. Add 50 µl of 1.25 C'H_{50} to each 1.25-unit control well

(col. 12, rows A & B).
4. Mix the plates by shaking for one minute.
5. Cover and incubate at 2-8 °C for 15 to 18 hours.

DETECTING FIXATION OF COMPLEMENT

1. Plates should remain at 2-8 °C prior to adding the sensitized, standardized SRBC, then pipette 25 µl of sensitized SRBC (see *Reagent Preparation – Sensitized Standardized SRBC Preparation*) into all wells of the plates, except rows C through H, columns 10 through 12 (these are used for color standards).
2. Mix the plates on a shaker for 2 minutes.
3. Cover or seal plates (do not stack) and incubate in a 37 °C incubator for 30 minutes; **reshake plates at 15 minutes, to resuspend cells, and continue incubation**.

READING AND RECORDING RESULTS:

1. Pipette 0.125 ml of each color standard into empty wells on the plates (*see Reagent Preparation – Color Standard Preparation*).
2. Centrifuge the plates for 3 minutes at 300 X g.
   NOTE: If centrifuge plate carriers are unavailable, incubate plates at 2-8 °C overnight or until cells settle.
3. Read and record the controls (antigen and VBD) by comparison to the color standards.
4. Compare the control readings with those in the table below to determine if they are acceptable. If the controls are not acceptable, the test is invalid; repeat the test.

Table 7:

TYPE CONTROL	5 C' Units	2.5 C' Units	1.25 C' Units
Antigen AC	100%	85-90%	0-75%
VBD	100%	90-100%	40-75%

QUALITY CONTROL

Positive controls against each antigen and a negative control should be run in each batch to indicate that all reagents are satisfactory. Internal controls for the CF test include the following:

1. VBD controls (5, 2.5, & 1.25 U): Contain VBD, complement (units indicated), and sensitized SRBC; used to test the complement and hemolytic system (e.g. 1.25 U control should give 40-75% lysis).
2. Serum anti-complementary controls: Contain serum dilutions, complement, and sensitized SRBC; used to detect complement fixing activity of the serum in the absence of added antigen (e.g. serum anti-complementary activity titer must be at least 4-fold (2 dilutions) lower than the antibody titer for the antibody titer to be valid. Most common serum AC cause: Circulating immune complexes in specimen).
3. Antigen controls (5, 2.5, & 1.25 U): Contain optimally diluted antigen, VBD, complement (units indicated) and sensitized SRBC; used to detect complement binding by the antigen (may indicate antibody present in the complement; 1.25 U should give 0-75% lysis).

NOTE: All controls must read correctly for test results to be valid.

INTERPRETATION OF RESULTS

1. **Negative Test:** Patient sera which fail to "fix" complement in the presence of an antigen should be reported as **"NEGATIVE to named antigen."**
2. **Positive Test:** Patient sera which "fix" complement (have 30% or less lysis with antigen) should be reported as **"POSITIVE to the named antigen" and the titer stated as the highest serum dilution having 30% or less lysis**. Serum anticomplementary activity must be at least 4-fold (e.g. 2 dilutions) below the antibody titer.
3. **Anticomplementary (AC) Serum:** Patient serum which fixes or destroys complement in the absence of any added antigen must be reported as **"Inconclusive test due To anti-complementary activity. Please submit another specimen."** Anti-complementary activity may be due to a number of factors such as circulating immune complexes, detergent, microbial contamination in the specimen, and/or some patient medications.

LIMITATIONS OF THE PROCEDRUE

The CF test relies on the property that certain immunoglobulin classes "fix" complement following reaction with their specific antigen. However, not all immunoglobulin classes produced in a specific immunologic response have the property of "fixing" complement; most notably, IgA and some sub-classes of IgG do not "fix" complement. For this reason, the CF test may not be positive throughout all stages of an infectious disease.

EXPECTED VALUES & PERFORMANCE CHARACTERISTICS

Histoplasmosis: A positive CF test is presumptive evidence of active or recent infection with *H. capsulatum.* CF titers of 1:32 or greater or rising titers (a 4-fold or greater increase) with yeast antigens, mycelial antigens, or both are strong presumptive evidence of histoplasmosis. Titers of 1:8 or 1:16, with either antigen, are generally considered suggestive of histoplasmosis but are less readily interpreted[6]. Failure to demonstrate CF antibodies does not exclude histoplasmosis, especially if only a single specimen is tested.

In "primary pulmonary infection" CF titers to the yeast antigen occur within 10 to 21 days following exposure to the organism, usually by the time symptoms are present. CF titers with the mycelial antigen are generally lower and develop later than the yeast antigen titers. However, in certain chronic cases, the mycelial antigen titers may be higher or be the only antigen positive[2].

In general, higher titers are associated with more severe disease and increasing titers (4-fold or greater rise) on serial specimens indicate progression of disease. However, persistently low titers may also indicate severe disease (up to 15% of chronic pulmonary cases have low or negative titers).

Following successful therapy, the CF titers will slowly return to negative, usually within 3 months; however, rarely, patients have had persistent titers for up to 6 months following successful therapy.

Sera from patients with other mycoses may cross-react with Histoplasma antigens in the CF test, most notably blastomycosis and rarely, coccidioidomycosis patients. For more complete serologic testing, immunodiffusion and latex agglutination tests should be performed in parallel with the CF tests.

Blastomycosis: CF tests with the purified 'A' antigen of *B. dermatitidis* may only be positive in approximately 40% of confirmed cases[1]. Thus, a negative test has little value and in no way excludes the possibility of active blastomycosis. Titers of 1:8 or greater are presumptive evidence of active or recent infection. The antigen frequently gives titers with confirmed cases of histoplasmosis and coccidioidomycosis. High titers or rising titers are suggestive of blastomycosis, and serologic tests for other fungi should be performed simultaneously. For more complete serologic testing, immunodiffusion tests for blastomycosis should be performed in parallel with the CF tests.

Coccidioidomycosis: A CF titer of 1:2 or greater may be significant and is presumptive evidence of current or recent past infection[3]. Titers greater than 1:16 are usually associated with severe disease. Patients with a limited number of extrapulmonary lesions, e.g. bone, skin, or central nervous system, may have titers below 1:16. Four-fold or greater decreases in titer on serial specimens usually indicate improvement. In chronic residual pulmonary disease, a significant number of patients (>30%) may have negative CF tests[4].

Antibody demonstrable by the CF test usually develops within 4 to 6 weeks after exposure and reaches a maximum response within 2 to 3 months. For those patients with positive reactions, the test is of diagnostic and prognostic value. Low titers are generally associated with mild, localized disease or stable residual lesions, while high or rising titers are usually associated with progressive or severe disease. A negative test does not exclude the possibility of active coccidioidomycosis. For more complete serologic testing, immunodiffusion and latex agglutination tests should be performed in parallel with the CF tests.

Aspergillosis: Less is known about the serological-clinical relationship in aspergillosis than in the previously discussed mycoses. A CF titer of 1:8 or greater is strong suggestion of *Aspergillus* infection or allergy. However, for a definitive diagnosis, the organism must be repeatedly demonstrated by culturing or microscopy.

Approximately 5% of the "normal" population has circulating antibodies against *Aspergillus*. Insufficient data are available for a statement regarding cross-reactions between *Aspergillus* and other fungi. For more complete serologic testing, immunodiffusion tests should be performed in parallel with the CF tests.

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Pulse Scientific Inc.
Burlington, Ontario, Canada