South African Veterinary Laboratory Scientific Forum

SAVLSF

SAVLSF Harmonized Serology SOP for Dourine Complement Fixation Test (CFT)

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Prepared by

Dr. A. Potts
Mr. M. Human
Ms. R. Theron
Ms. M. Dreyer
Ms. H. Olivier
Ms. P. Naidoo
Mr. K. Vermeulen

Reviewed by

Dr. Joule Kangumba

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REVISED BY:

Chairperson: SAVLSF:

Date: 25. 11. 2013

APPROVED BY:

Director: Animal Health (DAH): .....

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DISTRIBUTION LIST

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- Northern Cape
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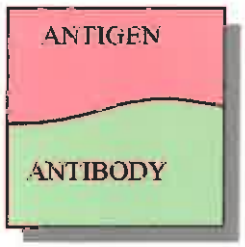
1. PRINCIPLE

The Complement Fixation Test (CFT) is a widely used and accepted confirmatory test for dourine (*Trypanosoma equiperdum*), although it is complex to perform, requires good laboratory facilities and adequately trained staff, to accurately titrate and maintain the reagents. Isolation of the parasite is very difficult, therefore the CFT is used to confirm dourine in equines with clinical symptoms or in latent carriers. The test can give false positive reactions due to cross reactions or anticomplementary activity (15.2).

The complement system consists of a complex series of proteins which, if triggered by an antigen-antibody complex, react in a sequential manner to cause cell lysis. In the first stage of the CFT, antigen and test serum are mixed with normal guinea-pig serum (complement). In the second stage, the indicator or haemolytic system is added. The haemolytic system consists of sheep red blood cells (SRBC), which have been sensitized with anti-sheep red blood cell antibody (amboceptor/haemolysin). If the test serum contains antibodies to *Trypanosoma equiperdum* (positive reaction), complement will be used up or fixed so that it cannot react in the haemolytic system. Thus, no lysis of SRBC will occur and the SRBC will remain intact. If the test serum does not contain *Trypanosoma equiperdum* antibodies (negative reaction) complement will not be fixed and lysis of the SRBC will occur.

Antigen | Antibody | Complement | Sheep Red Blood Cells | Amboceptor

POSITIVE SAMPLE



Formation of Immune Complexes

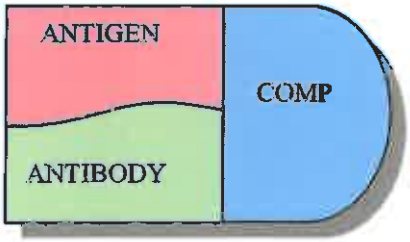
REACTION
Test Reaction:

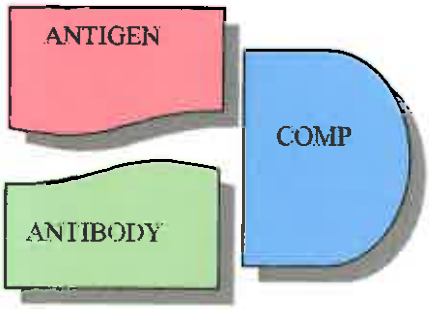
Addition of Test Serum to Test Antigen

NEGATIVE SAMPLE

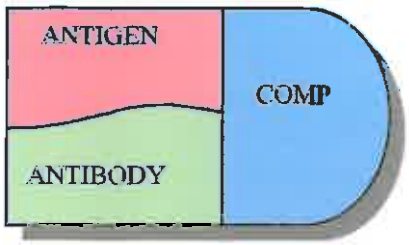
Unbound Antibodies

Addition of Complement





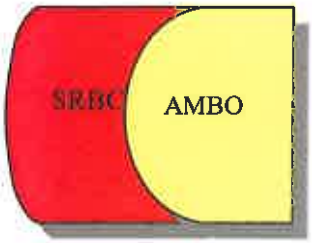
Fixation of Complement by Immune Complexes

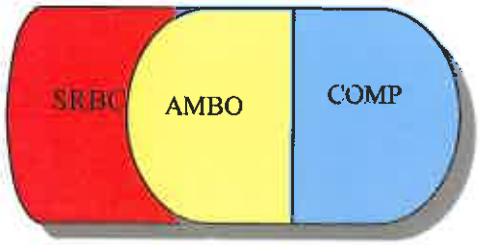


Indicator Reaction:

Unbound Complement

Addition of SRBC and Amboceptor





Sedimentation of SRBC

RESULT

Lysis of SRBC by Activated Complement

The *Trypanosoma equiperdum* antigen and the test serum (its complement inactivated by heating at 58°C ± 2°C for 30 minutes) are brought into contact with each other. If the antibodies in the test serum are specific to the added antigen, an immune complex is formed. If the antibodies are not specific to the antigen, the antibodies and antigen remain unbound.

If these immune complexes are now incubated with normal guinea-pig serum, which provides a source of unbound complement, fixation of the complement with the immune complexes takes place. If there are no complexes for the complement to bind to, the complement remains unbound.

An indicator system consisting of SRBC and amboceptor is added. The SRBC and amboceptor form an immune complex. Since there is no unbound complement in the positive test sample, the SRBC-amboceptor complex remains unaffected, resulting in sedimentation of the SRBC. The negative test sample however, has unbound complement, which will now bind to the SRBC-amboceptor immune complex. This binding initiates the complement cascade leading to haemolysis of the SRBC.

2. PERSONNEL

The person/s performing the procedure shall be declared competent by relevant procedures e.g. witnessing, repeat testing, inter-laboratory/proficiency testing etc.

3. SAFETY/ PRECAUTIONARY MEASURES

3.1 Safety measures

3.1.1 All blood samples should be regarded as potentially capable of transmitting diseases.
3.1.2 Wear personal protective equipment (PPE) at all times i.e. laboratory coat and gloves.
3.1.3 Disinfect the bench before the test is done and after the test is completed.
3.1.4 Spills must be cleaned by spraying over the spill with disinfectant and wiping down with paper towels. Paper towels must be discarded into a biohazard bag.
3.1.5 Amboceptor contains sodium azide as a preservative. Avoid swallowing and contact with the skin or mucous membranes.
3.1.6 Never pipette by mouth.

3.2 Precautionary measures

3.2.1 All reagents must be stored at 4°C ± 2°C at all times when not in use.
3.2.2 CFT buffer, antigen, complement, SRBC suspension, test and control sera must be mixed well before use.
3.2.3 Remove only a sufficient amount of reagents from refrigerator for use.
3.2.4 New tips must be used for each test serum.

4. LIMITATIONS AND PRECISION OF THE TEST METHOD

4.1 Complement factors and other factors inducing anti-complementary effects inherent in the test sera have to be inactivated before performing the CFT.
4.2 The various biological reagents used in the CFT must all be standardized in order to yield optimal results.
4.3 Only serum samples should be tested.

5. EQUIPMENT

5.1 Multi-channel micropipette (5 µl to 50 µl/300 µl) and single channel micropipette (10 µl to 100 µl/1 000 µl; 1-10 ml)
5.2 Incubator (37 °C ± 2 °C)
5.3 Timer
5.4 Refrigerator (4 °C ± 2 °C)
5.5 Water bath **or** Hot Air Oven/Incubator (37 °C and 58 °C ± 2 °C)
5.6 Centrifuges (one standard for blood and one to spin microtitre plates at 1 000x g)
5.7 Dispenser (25 µl to 50 µl)
5.8 Shaker
5.9 X-ray viewing box **or** magnifying mirror

5.10 Spectrophotometer **or** Wintrobe Tubes
5.11 Biological safety cabinet
5.12 Balance
5.13 Thermometers
5.14 Ultra-freezer (-70 °C to -80 °C) or liquid nitrogen canister (-196 °C) for storage of antigen
5.15 Magnetic stirrer

6. MATERIALS

6.1 Permanent marker pen
6.2 Test tubes
6.3 Pipette tips
6.4 Beaker
6.5 96-well microtitre plate filled with 1.2 ml polypropylene cluster tubes
6.6 96-well U-bottom microtitre plates
6.7 Reagent bottles and reservoirs
6.8 Centrifuge tubes
6.9 Measuring cylinders
6.10 Wintrobe tubes

7. REAGENTS

NB: Reagents should be re-constituted according to manufacturer's instructions. Any deviations should be validated.

7.1 *Trypanosoma equiperdum* complement fixation antigen supplied by Onderstepoort Biological Products (OBP) is very labile and should be stored at -70 °C to -80 °C or in liquid nitrogen (-196 °C). Just before use the vial is removed from the ultra-freezer and diluted according to the manufacturer's instructions (usually 1/125 i.e. 0.1 ml antigen + 12.4 ml CFT buffer).

Note:

If a large number of tests are to be done, equal volumes of diluted complement and diluted antigen (C'/Ag) can be mixed so that 50 µl of C'/Ag can be added to the wells instead of 25 µl of the complement and 25 µl of the antigen. In the same way equal volumes of diluted complement and CFT buffer (C'/2) can be mixed for 50 µl to be added to the anti-complementary control row.

7.2 A dourine positive control serum, (e.g. supplied by ARC-OVI Bacterial Serology) should be reconstituted as per instruction for use (certificate of analysis). A volume of this serum should be inactivated together with the test sera every time the test is done.
7.3 Dourine negative sera (Any serum from a horse that consistently tests negative.)
7.4 Guinea-pig complement for CFT (e.g. Siemens or Alere), according to titration, see 8.2.4.
7.5 Amboceptor from rabbit (e.g. Siemens or Alere) use 5 Minimum Haemolytic Doses (MHD) in the CFT (See 8.2.2).
7.6 CFT buffer (Alere). The diluent is prepared freshly every day and stored at 4 °C ± 2 °C. The CFT buffer can also be made up according to the procedure in Appendix 1a or 1b.
7.7 SRBC suspension (3% see 8.2.1).
7.8 Test sera, positive and negative control sera must be inactivated together at 58°C ± 2°C for 30 minutes.

7.9 Cyanmethaemoglobin standard can be obtained from BDS International Diagnostics (spectrophotometric method).

7.10 Drabkin's reagent can be obtained from Sigma (spectrophotometric method).

8. PROCEDURE

8.1 Collection and preservation of SRBC

8.1.1 Select sheep that give red blood cells which consistently react well in the test. At least two sheep should be kept for this specific purpose. (Use the 10 minute haemolysis test for selection, see Appendix 3.
8.1.2 A maximum of 500 ml blood can be drawn at six week intervals.
8.1.3 Blood is collected at a ratio of 50% blood to 50% Alsever's solution (See Appendix 2) or in Fenwal Blood packs – to be used as per manufacturer's instructions (e.g. Adcock Ingram). Mix thoroughly when collecting by gently rotating the container. Work in a biological safety cabinet when dispensing into smaller volumes. Label bottles with the sheep number, as well as collection and expiry dates.
8.1.4 Blood is stored at 4 °C ± 2 °C for a period of no longer than six weeks.
8.1.4 Blood can only be used at least 5 days after collection. (Alton *et al.* 1988:89)

8.2 Preparation of reagents

Reagents must reach room temperature (22 °C ± 3 °C) before use, with the exception of the complement, which is heat labile and should be kept at 4 °C ± 2 °C until needed for dilution. The antigen is also very labile and should be stored at (-70 °C to -196 °C) until just before use.

8.2.1 Preparation of 3% SRBC suspension

There are two methods: the Wintrobe tube method (see 8.2.1.2) and the spectrophotometric method (see 8.2.1.3).
Both methods start with the washing of SRBC.

8.2.1.1 Washing SRBC

1. Pour sufficient blood into two conical graduated centrifuge tubes and centrifuge at 1 000x g to 3 000x g for 5 minutes.
2. Remove supernatant.
3. Re-suspend the cells in CFT buffer, by gentle agitation and centrifuge at 1 000x g to 3 000x g for 5 minutes.
4. Remove supernatant and buffy coat layer.
5. Remove supernatant and repeat Step 3.
6. For final centrifugation centrifuge at 1 000x g to 3 000x g for 10 minutes.
7. Supernatant on final washing must be crystal clear and colourless.
8. Remove the supernatant and fill the centrifuge tubes with CFT buffer. Gently mix the suspension and pour the volumes of the tubes into a graduated measuring cylinder to prepare the 3% suspension from packed red blood cells (SRBC).

8.2.1.2 Wintrobe tube method

1. Prepare a 20% SRBC suspension in CFT buffer from washed cells as in A.
2. Mix thoroughly but gently.
3. With the aid of a Pasteur pipette fill two Wintrobe tubes to the 10-graduation mark.
4. Centrifuge for 15 minutes at 1 000x g.
5. Read packed cell volume (PCV) on the graduated Wintrobe tubes.
6. In order to prepare a 3% SRBC suspension, use the following formula to calculate the volume of CFT buffer to add to a chosen volume of the original 20% suspension e.g.
   PCV = 18
   Chosen volume (20% suspension) = 20 ml

$$\frac{\text{Vol. (20\% suspension)} \times \text{PCV}}{3} - \text{(Vol. 20\% susp.)} = \text{Vol. CFT buffer}$$

$$\frac{20 \times 18}{3} - 20 = 100 \text{ ml}$$

Thus add 100 ml CFT buffer to 20 ml of 20% suspension to give a 3% SRBC suspension.

8.2.1.3 Spectrophotometric method

(i) Calibration of the spectrophotometer

1. A 3% SRBC suspension contains 0.96 g haemoglobin (Hb) per 100 ml (Alton *et al.* 1988:91).
2. All readings are done at 540 nm wavelength.
3. Allow the machine to warm up for 15 minutes.
4. Bring the cyanmethaemoglobin standard to room temperature.
5. Take the reading of this standard, the optical density (OD), on the spectrophotometer using Drabkin's reagent as a blank.
6. Calculate the OD for a 3% SRBC suspension diluted 1/16 in Drabkin's reagent from the following formula:

$$\text{OD (3\% suspension)} = \frac{0.96 \times \text{OD (standard)} \times 1\,000}{\text{Concentration of standard (80 mg/100 ml)} \times 16}$$

7. Prepare a ± 4% SRBC suspension from washed, packed cells.
8. Mix 1 ml of this suspension with 15 ml Drabkin's reagent.
9. Allow to stand for 10 minutes and then read OD.
10. Make a 3% SRBC suspension from the original 4% SRBC suspension by applying the following formula:

$$\text{Vol. (CFT buffer to be added)} = \frac{\text{OD(4\%)} - \text{OD(3\%)}}{\text{OD(3\%)}} \times \frac{\text{Vol.(4\%)}}{1}$$

E.g. If the OD calculated in step 6 above is 0.5 and the OD of the 4% suspension is 0.58 and the volume of the 4% suspension to be diluted is 80 ml then:

$$\text{Vol. (CFT buffer to be added)} = \frac{0.58 - 0.5}{0.5} \times \frac{80}{1}$$

Thus add 12.8 ml CFT buffer to 80 ml of 4% SRBC suspension and this will give a 3% SRBC suspension.

11. Lyse 1 ml of the 3% SRBC suspension with 15 ml Drabkin's reagent. Allow to stand for 10 minutes and read the OD. It should equal the OD calculated in step 6.
12. Distilled water is used routinely to lyse the SRBC suspension (Drabkin's reagent is used to convert haemoglobin to cyanmethaemoglobin which is more stable. "Haemoglobin is unstable in water but this does not materially affect the result provided readings are always made as soon as the laking is complete" Alton *et al.* 1988:92). The final target OD (TOD) is obtained by lysing 1 ml of this 3% SRBC suspension with 15 ml of distilled water, waiting 10 minutes and then reading the OD using distilled water as a blank.
13. This is the target OD (TOD) used in daily calculation. (The target OD is generally near 0.5, Alton *et al.* 1988:93).

(ii) An alternative method for calibration of the spectrophotometer.

In the absence of facilities for the cyanmethaemoglobin method the spectrophotometer may be standardised as follows:

1. Take up to 10 ml of sheep blood, stored in Alsever's solution, and add about 40 ml of CFT buffer: mix well, then centrifuge to pellet the erythrocytes.
2. Discard the supernatant, re-suspend the erythrocytes in about 40 ml of diluent and centrifuge again.
3. Discard the supernatant, re-suspend the erythrocytes, this time in a 15 ml graduated centrifuge tube and centrifuge at 1 000x g to 3 000x g for 10 minutes.
4. Note the volume of the pellet accurately, discard the supernatant, then suspend the whole of the pellet in 32,3 times its volume in CFT buffer to produce a 3% suspension (e.g. if the volume of the pellet is 0.1 ml add 3.23 ml CFT buffer). For reproducible results it is recommended that sufficient erythrocytes be washed to give a final deposit of about 1 to 2 ml.
5. Add 1 ml of this suspension to 15 ml distilled water and mix. Allow to stand for 10 minutes for lysis to complete.
6. As soon as lysis is complete, measure the OD.
7. Repeat steps 1, 2 and 3 several times and calculate the mean OD. This is the target OD used in daily calculation.

(iii) Routine calculation for the preparation of a 3% SRBC suspension

1. Prepare an initial 4% suspension by taking 4 ml packed SRBC (from the washing procedure) and 96 ml CFT buffer.
2. From this 4% suspension, prepare a 1:16 dilution by adding 1 ml of 4% SRBC to 15 ml distilled water. Allow to stand for 10 minutes in order to completely lyse the SRBCs.

3. Use two cuvettes: one filled with distilled water (to use as a blank), and the second filled with the 1:16 dilution of lysed SRBCs.
4. Read the absorbance value at 540 nm: Each laboratory shall calibrate their instrument with each batch of blood collected (see 8.2.1.3 (i) or (ii)).
5. Use the following formula to calculate the volume of CFT buffer to be added to the suspension:

$$\frac{\text{OD reading of initial 4\% - Target OD}}{\text{Target OD}} \times \text{The volume that is left after 1 ml has been taken out to make 1:16}$$

For example:
If the OD reading was 0.58 and the TOD was 0.5 the formula would be applied as follows:

$$\frac{0.58 - 0.5}{0.5} \times 99 = 15.8 \text{ ml (round off to 16 ml)}$$

Thus, 16 ml CFT buffer must be added to the initial 4% suspension to make a 3% suspension. After the CFT buffer is added, repeat steps 3 and 4. The reading should equal the TOD of the donor used. If this is not the case discard the suspension and start again.

8.2.2 Titration of Haemolysin (Amboceptor). To be done for each new batch.

1. Set up 2 racks of 10 tubes (11 x 75 mm), one for the serial dilution of haemolysin and the other for the titration.
2. Prepare a 3% SRBC suspension in CFT buffer.
3. Prepare a 1/1 000 dilution of haemolysin in CFT buffer (0.1 ml haemolysin in 99.9 ml buffer).
4. Make further dilutions of haemolysin in the first rack according to the following schedule:

Table 1. Dilutions of haemolysin used in the haemolysed titration.

Tube No.	Dilution	1/1000 Master dilution (ml)	CFT buffer (ml)
1	1/1 000	1.0	-
2	1/2 000	1.0	1.0
3	1/3 000	0.5	1.0
4	1/4 000	0.5	1.5
5	1/5 000	0.5	2.0
6	1/6 000	0.5	2.5
7	1/7 000	0.5	3.0
8	1/8 000	0.2	1.4
9	1/9 000	0.2	1.6
10	1/10 000	0.2	1.8

5. In addition 2 control tubes are set up at the start of the test as follows:

Tube No. 11	Complement 1/20	0.4 ml
	3% SRBC suspension	0.4 ml
	CFT buffer	1.2 ml

Tube No. 12	Haemolysin 1/1 000	0.4 ml
	3% SRBC suspension	0.4 ml
	CFT buffer	1.2 ml

These 2 tubes pass through the same incubation (steps 8 and 11) process as the titration tubes and no other reagents are added. Neither must show any sign of haemolysis.

6. Mix each dilution starting with the highest dilution (Tube No. 10) and transfer 0.4 ml to the appropriate tube in the titration rack.
7. Add 0.4 ml of the 3% SRBC suspension to each tube and mix immediately.
8. Shake and place in a water bath at 37 °C for 30 minutes to sensitize the SRBCs.
9. Add 0.4 ml of complement with a final dilution of 1/20 to each tube.
10. Add 0.8 ml CFT buffer to each tube.
11. Shake and incubate for 30 minutes in a water bath a 37 °C ± 2 °C.
12. Remove from the water bath and read.
13. The unit of haemolysin referred to as 1 MHD (minimal haemolytic dose) is the highest dilution showing complete haemolysis.
14. Use 5 MHDs in the CFT.
15. Store as per manufacturer's instructions and avoid contamination.

Note Haemolysin with a titre of less than 1/2 000 should not be used.

Calculation of 5 MHDs

If the haemolysin titration is 1/2 000. Then 1 MHD is contained in a 1/2 000 dilution. Thus 5 MHDs are contained in a 5/2 000 = 1/400 dilution. Therefore 0.25/100 is the correct dilution for 100 ml haemolytic system to contain 5 MHDs. Thus to supply 5 MHDs in 100 ml haemolytic system one must add 0.25 ml of haemolysin with a titre of 1/2 000 to 50ml of CFT buffer.

8.2.3 Preparation of Sensitized Haemolytic System

First determine the volume of blood needed to run the entire test by taking into account the number of test samples, then following the guidance given in 8.2.2 above determine the dilution of the amboceptor to be used.

Add the amboceptor (5 MHDs) to the CFT buffer. Mix the correct volume of the amboceptor-added CFT buffer (5 MHDs) with an equal volume of 3% SRBC to make the haemolytic system. This is achieved by pouring each volume simultaneously into a third container and agitating for a short while. Alternatively, a magnetic stirrer can be used as follows: Place a volumetric flask containing the CFT buffer plus amboceptor on a magnetic stirrer set at a medium speed. Slowly add the 3% SRBC suspension to the flask and stir for 5 minutes.

Incubate the haemolytic system for 30 minutes at 37 °C ± 2 °C in a water bath/incubator before use to allow for the sensitisation of the SRBCs. Gently mix the haemolytic system twice during the incubation i.e. at 10 minutes and 20 minutes.

8.2.4 Titration of complement. To be done for each new batch.

1 Prepare the haemolytic system (sensitized SRBC according to 8.2.3 above).
2 Prepare a 1/40 dilution of complement in cold CFT buffer.
3 Dispense the 1/40 dilution of complement into 10 tubes in the quantities as shown in Table 2.

Table 2. Volumes of complement (1/40) and CFT buffer used in the complement titration.

Tube No.	Complement 1/40 (ml)	CFT buffer (ml)	Incubation	Sensitized sheep red blood cells (ml)
1.	0.03	0.72	30 minutes in the water bath at 37 °C ± 2 °C	0.25
2	0.04	0.71		0.25
3.	0.05	0.7		0.25
4.	0.06	0.69		0.25
5.	0.075	0.675		0.25
6.	0.1	0.65		0.25
7.	0.125	0.625		0.25
8.	0.15	0.6		0.25
9.	0.2	0.55		0.25
10.	0.25	0.5		0.25

4 Add the appropriate quantities of CFT buffer, also shown in the Table.

5 Shake the tubes to mix the contents and place in a water bath at 37 °C ± 2 °C for 30 minutes.

6 Add 0.25 ml of the haemolytic system to all the tubes, mix well and return to the water bath set at 37 °C ± 2 °C for another 30 minutes.

7 Remove the tubes from the water bath and read the result. The quantity of complement in the first tube showing complete haemolysis is 1 exact unit and the quantity in the next tube containing more complement, is 1 full unit. Two (2) full units are used in the CFT. Calculate the dilution of complement to use in the CFT using the formula:

$$\frac{40}{4 \times 2 \; full \; units}$$

For example, if haemolysis is absent or incomplete in tubes 1 to 4, and complete in tubes 5 to 10. The exact unit is 0.075 and the full unit is 0.1, then the dilution factor will be:

$$\frac{40}{4 \times 0.2} = 50$$

i.e. the complement to be used in the test will be diluted 1/50.

8.2.5 Chequerboard titration of *Trypanosoma equiperdum* CFT antigen.

Antigen is commercially available from OBP and is used as per manufacturer's instructions. The working dilution of the antigen may be verified according to the following method:

1. Remove the antigen (0.1 ml) from cold storage (-196 °C to -69 °C) shortly before doing the titration because it is very labile.
2. Make a 1/50 master dilution by adding 4.9 ml CFT buffer to the 0.1 ml antigen.
3. Prepare the antigen dilutions in the following table from the 1/50 master dilution:

Table 3. Dilutions of antigen used in the antigen titration.

Dilution	1/2 Master Dilution (ml)	CFT buffer (ml)
1/50	0.5	0
1/75	0.5	0.25
1/100	0.5	0.5
1/125	0.5	0.75
1/150	0.5	1.0
1/200	0.5	1.5
1/250	0.5	2.0
1/300	0.5	2.5

4. Use a medium titre positive serum (4/20 – 4/40) inactivated at 58 °C for 30 minutes.
5. Pipette 100 µl of inactivated positive serum (diluted 1/5 with CFT buffer i.e. 25 µl serum 100 µl CFT buffer) into each of the first 8 wells of row A of a micro-titre plate. Pipette 25 µl CFT buffer to wells 1 to 8 of rows D-H (See Table 4).
6. Using an 8-channel pipette draw up 25 µl of serum from row A and dispense it into row B wells 1-8. Draw up a further 25 µl of serum from row A and this time dispense it into row C wells 1-8. Draw up a final 25 µl of serum from row A and dispense it into row D wells 1-8.
7. Mix the serum and the CFT buffer in row D (column 1-8). Pick up 25 µl from row D (column 1-8) and dispense it into row E (column 1-8) of the test plate. Draw up 25 µl from row E (column 1-8) of the test plate and dispense it into row F (column 1-8) of the test plate. Continue this serial dilution to row H (column 1-8) of the test plate. Draw up 25 µl from row H (column 1-8) and discard it.
8. Add 25 µl of the correctly diluted complement to wells 1 to 8 of rows B to H.
9. Add 25 µl of the different antigen dilutions, as per Table 4, to columns 1 (1/50) to 8 (1/300) starting from row C to row H. Row B gets no antigen as it acts as the anti-complementary control.

Table 4. Plate layout for the antigen titration indicating volumes of medium titre serum and CFT buffer to be added to each dilution of antigen.

	Column	1	2	3	4	5	6	7	8	9	10	11	12
		Antigen dilutions											
Row	Dilutions of medium titre serum	**1/50**	**1/75**	**1/100**	**1/125**	**1/150**	**1/200**	**1/250**	**1/300**				
A	1/5	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl				
B	1/5	0 µl CFT buffer	0 µl CFT buffer	0 µl CFT buffer	0 µl CFT buffer	0 µl CFT buffer	0 µl CFT buffer	0 µl CFT buffer	0 µl CFT buffer				
C	1/5	0 µl CFT buffer	0 µl CFT buffer	0 µl CFT buffer	0 µl CFT buffer	0 µl CFT buffer	0 µl CFT buffer	0 µl CFT buffer	0 µl CFT buffer				
D	1/10	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer				
E	1/20	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer				
F	1/40	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer				
G	1/80	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer				
H	1/160	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer	25 µl CFT buffer				

10. Add 25 µl CFT buffer to wells 1 to 8 in row B in lieu of antigen.
11. Proceed with the complement fixation test in the routine manner. Record the results, with numbers for each well representing the percentage haemolysis:
    - = 100 % haemolysis
    1 = 75 % haemolysis
    2 = 50 % haemolysis
    3 = 25 % haemolysis
    4 = 0 % haemolysis

See Table 5 below for the results of a typical titration.

Table 5. An example of a chequerboard titration of antigen.

	Column	1	2	3	4	5	6	7	8	9	10	11	12
		Antigen Dilutions											
Row	Dilutions of medium titre serum	**1/50**	**1/75**	**1/100**	**1/125**	**1/150**	**1/200**	**1/250**	**1/300**				
A	1/5	Used for the deposition of a medium titre serum											
B*	1/5	-	-	-	-	-	-	-	-				
C	1/5	4+	4+	4+	4+	4+	3+	2+	2+				
D	1/10	4+	4+	4+	3+	2+	-	-	-				
E	1/20	4+	4+	3+	-	-	-	-	-				
F	1/40	2+	4+	-	-	-	-	-	-				
G	1/80	-	1+	-	-	-	-	-	-				
H	1/160	-	-	-	-	-	-	-	-				

* Anti-complementary row
4+ = 0% haemolysis; 3+ = 25% haemolysis; 2+ = 50% haemolysis; 1+ = 75% haemolysis; - = 100% haemolysis

In the above example, the highest titre is recorded for the antigen dilution of 1/75 and this is the dilution to be used. Any anti-complementary activity in the antigen is detected by a similar titration using a negative serum.

8.3 Inactivation of test serum

1. Prepare a 96-well microtitre plate filled with 1.2 ml polypropylene cluster tubes or test tubes in a test tube rack, in the test procedure format for the day The wells of a 96-well microtitre plate may also be used and the plate sealed with sealing film before inactivation in a water bath or a hot air oven.
2. Test sera should be clear, otherwise, they should be centrifuged before being poured into the cluster tubes. Dilute the sera (including controls) 1/5 before inactivation i.e. 25 µl serum + 100 µl CFT buffer.
3. Inactivate the test and control (positive and negative) sera in a water bath or a hot air oven, at 58 °C ± 2 °C for 30 minutes. It is important when a water bath is used that the water level covers the level of the serum.
4. Mule and donkey sera are inactivated at 62 °C ± 2 °C for 30 minutes.

8.4 **Test Procedure** (Serum dilution, reagent dispensing and incubation)

1. Dispense 25 µl CFT buffer by using an 8-channel pipette into U-bottomed micro-titre plates, beginning with row D (column 1 – 8) and proceeding to row H (column 1-8). See Figure 1, for orientation of the plate.

2. For each microtitre plate row B (column 1) is used for the positive control sera, row B (column 2) is used for the negative control sera and row B (column 3 - 8) is used for the test sera (8 sera on each plate including the positive and negative controls).

3 Using an 8-channel pipette draw up 25 µl of serum from row A and dispense it into row B wells 1-8. Draw up a further 25 µl of serum from row A and this time dispense it into row C wells 1-8. Draw up a final 25 µl of serum from row A and dispense it into row D wells 1-8. Mix the serum and the CFT buffer in row D wells 1-8. Draw up 25 µl from row D wells 1-8 and dispense it into row E wells 1-8. Draw up 25 µl from row E wells 1-8 and dispense it into row F wells 1-8. Continue this serial dilution to row H wells 1-8 of the test plate. Draw up 25 µl from row H wells 1-8 and discard it. This accomplishes a 1/5 serial dilution, leaving dilutions of 1/5, 1/10, 1/20, 1/40, 1/80 and 1/160 in rows C to H.

4 Repeat the procedure for the next batch of sera on a new microtitre plate.

5 Use new tips for each batch of 8 sera (including the positive and negative control sera) and for dispensing the other reagents.

6 Dispense 50 µl complement/CFT buffer using an 8-channel pipette into Row B (wells 1-8).

7 Dispense 50 µl complement/antigen into the test plate using an 8-channel pipette from Row C (wells 1-8) to Row H (wells 1-8).

8 Tap the plates gently to ensure mixing of the test sera and reagents.

9 Incubate the plates at 37 °C ± 2 °C for 30 minutes. Plates must not be stacked during incubation to ensure even distribution of heat to all wells.

10 Dispense 50 µl haemolytic system (mix well before dispensing) with an 8-channel pipette into all wells of the test plates (Row B to H, wells 1-8).

11 Incubate the plate at 37 °C ± 2 °C for 30 minutes on a shaker and again avoid stacking the plates.

12 Remove the plates from the incubator and centrifuge at 1 000x g for 1 minute or leave on work bench at room temperature for 2 to 3 hours to allow the pellets to settle. Stack plates, with one empty plate on the top plate, to avoid moisture loss. Centrifugation is the preferred method.

13 Read the plates over a magnifying mirror or light box as 0 - 4 (0 = 100% haemolysis and 4 = 0% haemolysis) in the last dilution with a reaction and record the results.

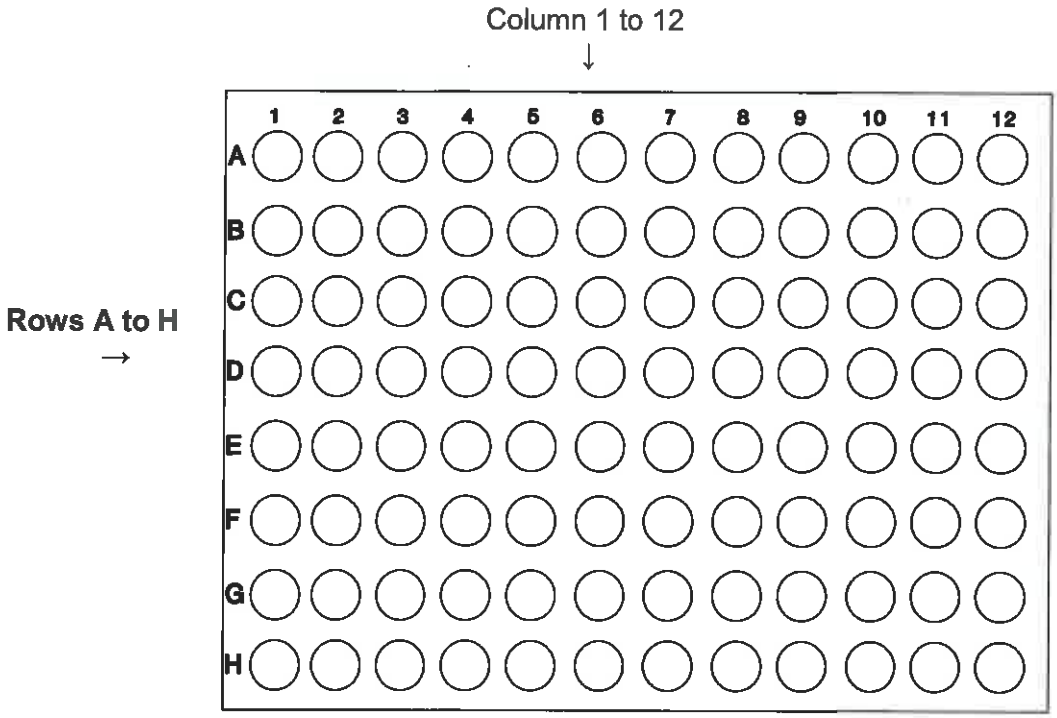


Figure 1. Orientation of the micro-titre plate.

8.5 Results

1 A negative result is where no sedimentation of SRBC is present, i.e. complete haemolysis.

2 A positive result is where sedimentation of SRBC is present as a distinct button at the bottom of the well, and is scored between 1 and 4. The final dilution is determined as the dilution showing more than 0% haemolysis and is recorded as the score/serum dilution e.g. 2/5. Differences of two scores higher and lower in the positive control serum are acceptable. The negative control should always be negative.

3 The anti-complementary well should be negative, otherwise the test for that particular serum must be repeated (see Appendix 4) or the result recorded as anti-complementary. Anti-complementary sera can be inactivated at 62 °C ± 2 °C after diluting 1/5 in CFT buffer.

Interpretation of CFT results scores 1 - 4

0 = 100% haemolysis of SRBC. No button/pellet of SRBC at bottom, uniformly red supernatant.

1 = 75% haemolysis of SRBC. Small button/pellet of SRBC at bottom of well, lighter red supernatant.

2 = 50% haemolysis of SRBC. Bigger button/pellet of SRBC, medium red supernatant, midway between 0 and 4.

3 = 25% haemolysis of SRBC. Still bigger button/pellet of SRBC, still lighter, almost clear supernatant.

4 = 0% haemolysis of SRBC. Large button/pellet or SRBC, supernatant clear, like water.

9. QUALITY CONTROL

Test only serum samples.
A test plate is valid when the negative control is negative and the positive control falls with the expected range (see Certificate of Analysis of the positive control).

9.1 Sources of Measurement Uncertainty (MU)

When a method is used to test a sample and a result is obtained, the result is likely to have an error associated with it relating to various uncertainties occurring in the sampling and testing process. The significance of these various uncertainties should be assessed. A test result is liable to deviate from the true result for a variety of reasons. The potential sources of measurement uncertainty are the following: sampling, handling and storage of samples, personnel, environmental conditions, limitations of the method, reagents, equipment and reference standards.

1 Test Samples

Blood samples are taken by customers and submitted to the laboratory. Thus sampling and transport procedures are not under the control of the laboratory. Samples should be taken using Vacutest tubes, kept under 10 °C ± 2 °C during transport and reach the laboratory within 2-3 days. Once at the laboratory samples are stored at 4 °C ± 2 °C and tested within 5 days. Poor sample quality such as excessive haemolysis or contamination could be a source of MU.

2 Personnel

Personnel carrying out the test must be trained and deemed competent, preferably after satisfactory witnessing of the test procedure and between analysts comparisons. Potential sources of measurement uncertainty can be identified by repeat testing of the same samples within and between personnel as well as with inter-laboratory and proficiency testing.

3 Environmental conditions

The temperatures under which the test is carried out must be within a consistent range i.e. inactivation temperature of sera 58 °C ± 1 °C, room temperature 22 °C ± 3 °C, incubation temperature of plates and haemolytic system 37 °C ± 1 °C. The temperatures are monitored daily. The plates used in the test should be clean and free of dust.

4 Equipment

All equipment must be calibrated/verified or serviced by approved suppliers using traceable standards where applicable.

5 Reagents

Complement, antigen and amboceptor must be titrated to ensure optimal performance. The SRBC must be standardised according to accepted procedures. The haemolytic system must be prepared carefully ensuring thorough mixing of SRBC and amboceptor.

10. TROUBLESHOOTING

10.1 Inconsistent endpoints

- Incomplete inactivation process of the serum.
- Partial blockage in dispenser, pipette or tip.
- Viscous serum.
- Carry over.
- Stacking of plates during incubation or inactivation in hot-air oven.
- Inaccurate pipetting technique.

10.2 Incomplete haemolysis

- Inadequate sensitization of SRBC e.g. time, temperature, mixing and haemolysin titre.
- Dirty apparatus.
- Insufficient complement.
- Anti-complementary activity (see below).
- Insufficient mixing during incubation periods.
- Insufficient incubation (time, temperature).

10.3 Anti-complementary activity

- Contaminated sera.
- Foreign antibody, antigen or their complexes in any of the reagents.
- Incomplete inactivation process (temperature, time).
- Testing with or without antigen. See appendix 5.
- Where the anti-complementary activity decreases with dilution the serum must be involved as part of the problem. Uniform deviations exclude the involvement of the serum.

11. WASTE DISPOSAL

Laboratory waste is discarded according to the laboratory's waste disposal procedure.

12. FORMS

1 All raw data shall be captured on the relevant forms/worksheets and retained for a minimum of 5 years.
2 SD 1 forms to be used for sample registration.
3 All result entries should be clearly legible in permanent ink.
4 Use Laboratory Information Management System (LIMS) if available.

13. REPORTING

1 Report results on SD 1 forms.
2 Copies of reports are to be kept for 5 years.
3 Original test results are sent to the relevant State Veterinarian.

14. SPECIMEN RETENTION

1 Short term storage: Done according to each laboratory's procedures or samples are kept refrigerated 4 °C ± 2 °C for at least 10 working days, from time of release of results, in case a repeat test is required.
2 Long term storage: Done according to each laboratory's procedures. Some selected samples may be transferred to the serum bank and stored at -20 °C or -70 °C, depending on the duration.

15. REFERENCES

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16. APPENDICES

APPENDIX 1: CFT buffer – Virion - Commercially available from Alere Healthcare and used as per manufacturer's instructions **OR** prepared by using Appendix 1a **OR** a 0.85% solution of Sodium Chloride containing Calcium and Magnesium ions can be used by following Appendix 1b.

APPENDIX 1a: Veronal buffer

			x2 (4 litres)
Barbitone ($C_8H_{12}N_2O_3$) (5,5 Diethylbarbituric acid)	MM 184.20	5.75 g	11.5 g
Barbital-Sodium ($C_8H_{11}N_2NaO_3$)	MM 206.18	3.75 g	7.50 g
Calcium chloride ($CaCl_2.6H_2O$)	MM 219.09	0.33 g	0.66 g
or ($CaCl_2.2H_2O$)	MM 147.02	0.22 g	0.44 g
Magnesium chloride ($MgCl_2.6H_2O$)	MM 203.31	1.02 g	2.04 g
Sodium chloride (NaCl)	MM 58.44	85.00 g	170.00 g

1. Dissolve the barbitone (barbituric acid) in 1 800 ml (or 3 600 ml) hot distilled water near boiling point (80 – 90 °C).
2. Dissolve the remaining reagents.
3. Cool to room temperature and make up to 2 000 ml (or 4 000 ml) with distilled water.
4. The pH should be 7.3 – 7.4. No pH adjustment is permitted. If the pH is not within limits the buffer must be discarded.
5. This stock solution may be stored for up to 3 months at 4 °C ± 2 °C .For use in the CFT dilute the stock solution 1/5 with distilled water (1 part stock + 4 parts cold distilled water). This is prepared freshly every day.

APPENDIX 1b: 0.85% Solution of Sodium chloride containing Calcium and Magnesium ions.

Barbital buffered saline is the standard diluent for the CFT test, but satisfactory results are also obtained with a 0.85% solution of sodium chloride containing calcium and magnesium ions, provided the pH lies between 7.3 and 7.4. This latter solution is prepared by adding 1 ml of the stock solution of calcium and magnesium salts to each litre of saline solution. If 0.25 ml of a 5% solution of sodium azide is added per litre to control bacterial contamination, the solution can be stored at room temperature.

Stock solution

Anhydrous Magnesium Chloride	9.5 g
Anhydrous Calcium Chloride	3.7 g
Made up to 100 ml distilled water	
Store in refrigerator at 4 °C ± 3 °C	

0.85% Solution of sodium chloride

Sodium chloride (NaCl)	8.5 g
Distilled water	1.0 litre

Working solution

Add 1 ml of the stock solution containing calcium and magnesium ions to 1.0 litre of saline solution. Store in the refrigerator at 4 °C ± 3 °C.

APPENDIX 2: Anti-coagulant Alsever's solution

Glucose D+ ($C_6H_{12}O_6$)	MM 180.16	20.50 g
Sodium chloride (NaCl)	MM 58.44	4.18 g
Sodium citrate ($Na_3C_6H_5O_7.2H_2O$)	MM 294.12	8.00 g
Citric acid ($H_3C_6H_5O_7.H_2O$)	MM 210.15	0.55 g
Distilled water		1 litre

1. Mix glucose in 100 ml distilled water.
2. Do not autoclave, instead filter through a 0.20 µm Millipore filter in biological safety cabinet.
3. Add remainder of ingredients to 900 ml distilled water.
4. Autoclave at 70 kPa for 20 min.
5. Allow to cool.
6. Add glucose mixture.
7. Store at 4 °C ± 2 °C.
8. The pH should be 6.1.

The following appendices are practical aids to the test:

APPENDIX 3: 10 minute Haemolysis Test for Reagents used in the CFT

Apparatus

Test tube rack
Test tubes (5)
Pipettes
Water bath at 37 °C ± 2 °C.

Reagents

Complement diluted according to the titration.
Complement diluted 1/10 (1 ml Complement +9 ml distilled water)
Haemolytic system
CFT Buffer

Procedure

Add the reagents to the test tubes as indicated in the table below:

Table 6. Volumes of reagents used in the 10 minute haemolysis test.

REAGENTS	TUBE NO.				
	1	2	3	4	5
Diluted complement (ml)	0.6	0.4	0.2	-	-
Complement 1/10 (ml)	-	-	-	-	0.4
CFT buffer (ml)	0.6	0.8	1.0	1.2	0.8
Haemolytic system (ml)	0.8	0.8	0.8	0.8	0.8
Total volume (ml)	2	2	2	2	2

Incubate for 5 minutes in a water bath at 37 °C ± 2 °C.
Check and incubate for a further 5 minutes if necessary.

Interpretation

Usually complete haemolysis is seen in tubes 1 (1.5 doses of complement), 2 (full dose of complement) and 5 (complement in excess) and partial haemolysis in tube 3 (0.5 dose of complement), after 5 minutes incubation.
If this is not seen, incubate for a further 5 minutes when this pattern will be seen if all the reagents are optimal.
No haemolysis should occur in tube 4 (no complement).
Haemolysis in tube 4 indicates that some factor other than complement is active e.g. i) old SRBC in the H/S, or ii) hypotonicity of the CFT buffer.
Incomplete haemolysis in tubes 1 and 2 but complete in tube 5 indicates that the complement dilution used is too weak.
Incomplete haemolysis in tubes 1, 2 and 5 indicates that there is a fault with either the complement or the H/S e.g. i) complement almost totally inactive; ii) H/S not properly sensitized.

APPENDIX 4: Re-testing of anti-complementary sera

1. Where anti-complementary activity occurs and the titre exceeds 4/20 a retest may be carried out.
2. This retest is done in duplicate excluding the antigen from the one sample and replacing it with 25 µl CFT buffer.
3. The difference in titre of the test without antigen must be three dilutions or greater lower than the test with antigen before the serum can be regarded as positive and the titre is then as read.

Example A

Table 7. Example where the difference between the test with antigen and the test without antigen is more than 3 dilutions.

ROW	Original Test Result	Repeat test with Antigen	Repeat test Without Antigen
B (Anti-complementary Control)	4	4	4
C	4	4	2
D	4	4	-
E	4	4	-
F	4	4	-
G	4	4	-
H	-	-	-
Titre Recorded	A/C	4/80	> 3 dilution difference

A/C = Anti-complementary

Example B

Table 8. Example where the difference between the test with antigen and the test without antigen is less than 3 dilutions

ROW	Original Test Result	Repeat test with Antigen	Repeat test Without Antigen
B (Anti complementary Control)	4	4	4
C	4	4	4
D	4	4	4
E	4	4	4
F	4	4	4
G	4	4	-
H	-	-	-
Titre Recorded	A/C	A/C	Single dilution difference

APPENDIX 5. Guidelines for the interpretation of titres.

Table 9. Guidelines for the Interpretation of serum dilution titres in South Africa (Herr *et al.* 1985)

Serum dilution	Interpretation
< 1/5	Negative
1/5 – 2/20	Suspicious
> 2/20	Positive

In the case of suspicious reactions a further test is carried out ± 1 month after the first test. If the titre stays the same or declines a negative result is recorded. The reason for this is as follows: "The animals with suspicious antibody titres were retested, and half of these had returned to negative status within 1 – 2 months. The rest showed titres in this same range when retested. None of these animals were reported to have any clinical manifestation of the disease." (Herr *et al.* 1985:281).

The EU requirement for export horses is < 1/5.