STANDARD OPERATING PROCEDURE

Dourine serological diagnosis by complement fixation test

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This SOP is an OIE-based method used at the EURL, all OIE-CFT based methods validated and used successfully in the PT can be used for this essay.

This document describes the method for the detection of antibodies specific to *Trypanosoma equiperdum*, the agent of dourine, by the microtitre complement fixation test (CFT) according to the world organisation for animal health (OIE) international standard: OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals – Chapter 3.5.3, dourine (https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.05.03_DOURINE.pdf). It is applicable to the serological diagnosis of dourine from serum samples of any equid (horse, donkey, mule...).

1. MATERIAL TO BE EXAMINED

1.1. SERUM

The serological diagnosis of dourine (*Trypanosoma equiperdum*) by complement fixation test is performed on equine sera. On receipt, the sample tubes must not be opened or damaged. A volume of serum greater than 100 µl must be provided. The serum must not be hemolyzed or coagulated. Before testing, a serum sample should be kept refrigerated (5±3°C).

1.2. TRANSPORT OF SAMPLES

Sera prepared from the whole blood samples received at the lab must be stored at 5°C ± 3°C. It is advisable for all blood samples to be centrifuged and stored whenever possible without the clot. For long-lasting storage, it is advisable to freeze sera without clot at ≤ -16°C.

2. REAGENTS, PRODUCTS AND APPARATUS

Notice: Trade names or suppliers may be mentioned in the description of the products required for the implementation of this method. This information is provided for the benefit of the users of the method and does not mean that the EU-RL recommends the exclusive use of these products. Equivalent products may be used if it is demonstrated that they lead to the same results.

The conditions for aliquoting the reagents described in this procedure are given for the benefit of the users of the method and each other laboratory is free to choose its own aliquoting conditions.

2.1. ANTIGEN

The antigen is a protein suspension of *T. equiperdum* parasites lysed by hypotonic shock. The antigen should be used at the required concentration (see Annex B) and at a rate of 25 µl per well.

Potential suppliers:
- **USDA** (contact: nicholas.a.ledesma@usda.gov) Dourine antigen (reference 160)
- **ANSES** (contact: laurent.hebert@anses.fr) Dourine antigen (reference: S00656)

2.2 CONTROL SERA: LOW TITRE, HIGH TITRE AND NEGATIVE

Potential suppliers:

USDA (contact: nicholas.a.ledesma@usda.gov)
Low titre serum (reference: 161-L)
High titre serum: (reference: 161-H)
Serum Negative: (reference: 76)

ANSES (contact: laurent.hebert@anses.fr)
Low titre serum (reference: S655)
High titre serum (reference: S654)
Serum Negative: (reference: S653)

2.3 COMPLEMENT (C')

Guinea pig complement is used at 25 µl per test and at to 2 units (see Annex A).

2.4 HEMOLYTIC SERUM (HS)

Hemolytic serum is rabbit anti-sheep red blood cell serum. It is used at to 2 units (see Annex C)

2.5 SHEEP RED BLOOD CELLS (RBC)

Sheep red blood cells are used at concentration of 3%.

2.6 VERONAL BUFFER (VB)

All reagents used in this protocol are diluted in VB at pH 7.4 (± 0.2).

2.7 STORAGE

All reagents should be stored according to the conditions described by the suppliers.

2.8. EQUIPMENT AND PLASTIC/GLASS WARE

Conventional serology laboratory equipment and in particular:

- Temperature-controlled incubator set at 37°C ± 2°C.
- Water bath (circulating water bath if possible) set at 37°C ± 1°C.
- Water bath (circulating water bath if possible) set at 59°C ± 1°C.
- Temperature-controlled refrigerator at 5°C ± 3°C.
- Temperature-controlled freezer at ≤ -16°C.
- Centrifuge, refrigerated if possible (allowing adequate acceleration).
- Distribution and dilution device having a suitable volume range and accuracy.
- Disposable microplates (96 well round U bottomed) with lid or cover (plastic or adhesive).
- Test tubes and racks.
- Light table (eventually).
- Timer or chronometer.

3. PREPARATION OF THE REAGENTS

In order to proceed with the analysis of sera by CFT it is necessary to have previously: titrated: the HS, the C' and the antigen. The procedures for the preparation and titration of the reagents are described in the Annexes:

- Annex A: Titration of complement
- Annex B: Titration of antigen
- Annex C: Titration of hemolytic serum

When a laboratory wants to implement CFT for the first time, please contact the person in charge of dourine activities in the EU-RL for equine diseases (laurent.hebert@anses.fr) to set up the titration procedures.
When dourine CFT if preformed in routine, these procedures can be followed independently depending on the turnover of reagents.

4. DESCRIPTION OF THE CFT PROCEDURE

4.1. Heat inactivation of the sera

To eliminate endogenous complement, sera must be diluted 1:5 in VB (e.g. dilute 30 µl of serum in 120 µl of VB) and then inactivated for 30 min in a water bath at 58±1°C (for horse sera) or 62±1°C (for donkey or mule sera).
This decomplementation must be carried out for all sera included in the test (including HT, LT and, NEG)

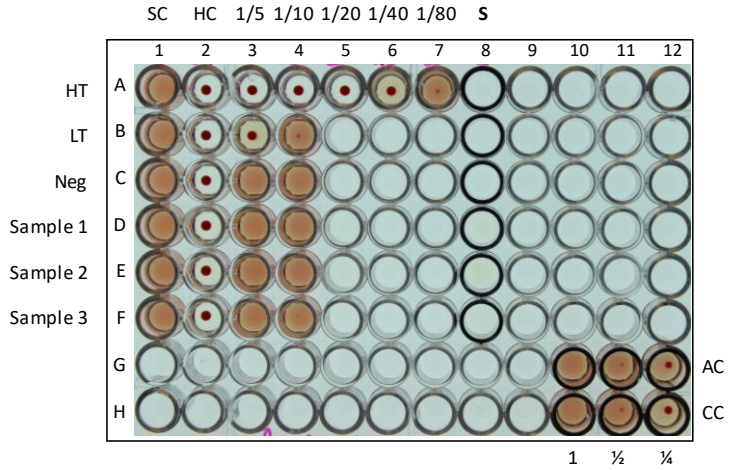
4.2. Resuspension of antigen

The antigen has been previously titrated and aliquoted as described in Annex C.
An aliquot of antigen is thawed on ice and resuspended in VB to a final volume appropriate for the current test (e.g. 1200 µl of antigen).
The solution should be prepared extemporaneously and placed on ice to avoid degradation of the antigen.

4.3 Resuspension of complement

The complement has been previously titrated and aliquoted as described in Annex B.
An aliquot of complement is thawed on ice and then resuspended in VB to a final volume appropriate for the current test (e.g. 1500 µl of complement).
The solution should be prepared extemporaneously and placed on ice to avoid complement degradation.

4.4. PLATE LAYOUT AND ABBREVIATIONS



- SC (serum control): controls to ensure that the serum has no anticomplementary activity,
- HC (hemolytic control): control for the stability of red blood cells,
- S: Sample well for sample dilution,
- HT: high titre serum,
- LT: low titre serum,
- Neg: negative serum,
- AC: Antigen Control (undiluted, diluted ½ and diluted ¼), control for the absence of antigen anticomplementary activity,
- CC: Complement control (undiluted, diluted ½ and diluted ¼), control for proper complement activity.

4.5. PREPARATION OF THE CFT plate (see general scheme of CFT procedure below)

Addition of VB

- Add 100 µl of VB to the columns 8 (**S**),
- Add 25 µl of VB to the columns 2 and 4 (HC and 1/10),
- Add 25 µl of VB to the wells A5, A6 and A7.

Preparation of 1/5 dilution of inactivated sera

- Add 25 µl of inactivated HT serum to the well A8 mix well,
- Add 25 µl of inactivated LT serum to the well B8 mix well,
- Add 25 µl of inactivated NEG serum to the well C8 mix well,
- Add 25 µl of inactivated serum sample to the well D8 mix well,
- (repeat this for each sample to test).

Distribution of diluted sera
- Add 25 µL from the wells of the column 8 (**S**) to the wells of the column 1 (SC),
- Add 25 µL from the wells of the column 8 (**S**) to the wells of the column 2 (HC),
- Add 25 µL from the wells of the column 8 (**S**) to the wells of the column (1/5),
- Add 25 µL from the wells of the column 8 (**S**) to the wells of the column 4 (1/10), mix and then aspirate 25 µl,
- Add the aspirated 25 µl to the column 5 (1/20) Mix and then aspirate 25 µl,
- Add the aspirated 25 µl to the column 6 (1/40) Mix and then aspirate 25 µl,
- Add the aspirated 25 µl to the column 7 (1/80) Mix and then discard 25 µl.

Addition of antigen
The antigen has to be prepared according to its corresponding titre and then added to the plate as follow:
- Add 25 µl of antigen to the wells of the column 3 (1/5),
- Add 25 µl of antigen to the wells of the column 4 (1/10),
- Add 25 µl of antigen to the wells A5, A6 and A7.

Addition of complement
The complement has to be prepared according to its corresponding titre and then added to the plate as follow:
- Add 25 µl of complement to the wells of the column 1 (SC),
- Add 25 µl of complement to the wells of the column 3 (1/5),
- Add 25 µl of complement to the wells of the column 4 (1/10),
- Add 25 µl of complement to the wells A5, A6 and A7.

Preparation of the antigen and complement controls
- Add 25 µl of VB to the wells G11, G12, H11 and H12,
- Add 25 µl of complement to the wells G10 and H10,
- Add 25 µl of complement to the well G11, mix well, aspirate 25 µl, add the aspirated 25µl to the well G12, mix well and discard 25 µl,
- Add 25 µl of complement to the well H11, mix well, aspirate 25 µl, add the aspirated 25µl to the well H12, mix well and discard 25 µl,
- Add 25 µl of antigen to the wells G10, G11 and G12,
- Add 25 µl of VB to the wells G10, G11 and G12,
- Add 50 µl of VB to the wells H10, H11 and H12.

Cover the plate with a lid and shake in the microshaker for 10 seconds at 900 rpm, then place in the incubator at 37 ± 2°C for 1 hour.

4.6. PREPARATION OF THE HEMOLYTIC SYSTEM

- Prepare the hemolytic system approximately 20 minutes before the end of the one-hour incubation of the plate. Depending on the hematocrit % and the hemolysin titre of your reagents and according to the table 1 in Annex C determine the appropriate volume of blood, hemolysin and VB needed for the preparation of the hemolytic system.

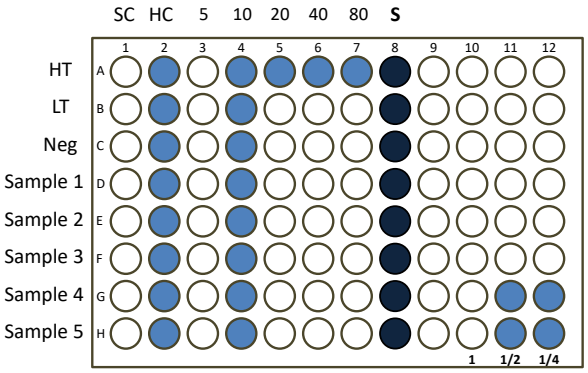
Prepare the solution in a hemolysis tube. Resuspend the blood carefully by agitating the bottle gently 5 to 10 times. Aspirate and expel blood slowly to avoid damaging the red blood cells with the tip of the cone.

- Incubate the hemolysis tube in a water bath at 37 ± 2°C for 10 min.

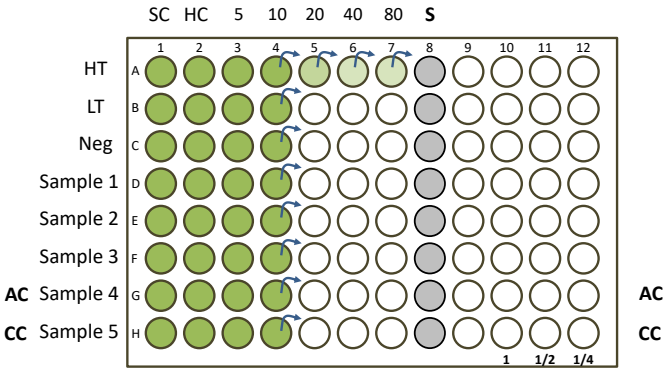
4.7. ADDITION OF THE HEMOLYTIC SYSTEM

- Homogenate carefully the hemolytic system and add 50 µl in each well.
- Cover the plate with a lid and shake in a microshaker for 10 seconds at 900 rpm, then place in the incubator at 37 ± 2°C for 30 min (after 15 min shake again the plate for 10 seconds at 900 rpm).
- Centrifuge the plate for 2 minutes at 500 g and read the results.

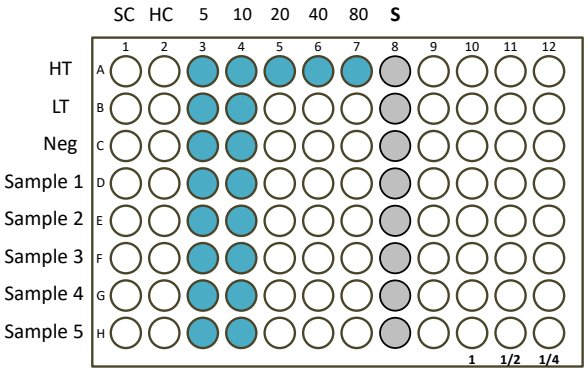
General scheme for CFT



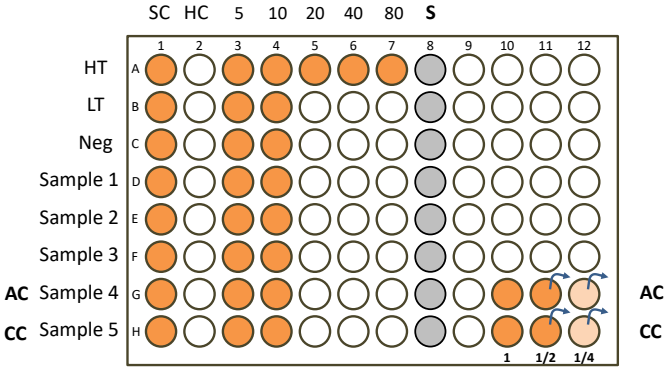
100 µl of Veronal buffer
25 µl of Veronal buffer



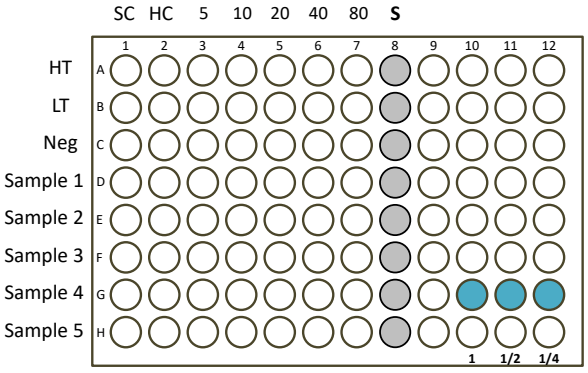
25 µl of the 1/5 diluted serum from the S column



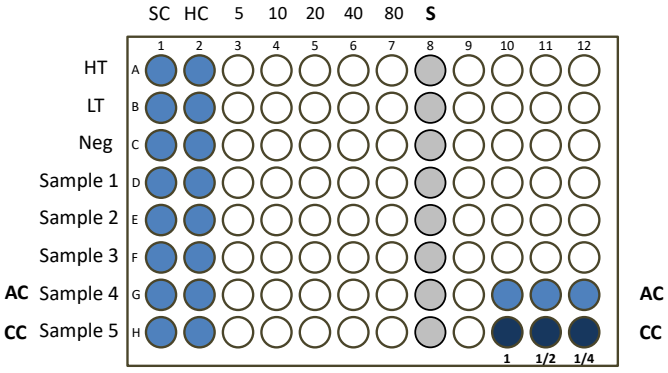
25 µl of antigen



25 µl of complement



25 µl of antigen



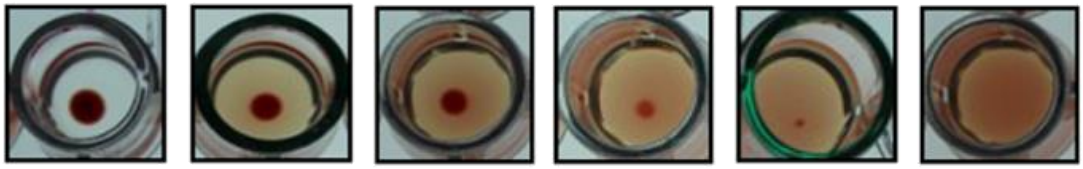
25 µl of veronal buffer
50 µl of veronal buffer

5. ANALYSIS OF CFT RESULTS

5.1. Reading of the plate

Read the results by looking the plate from above with a light source beneath it.
The fixation in every well is assessed by estimating the proportion of cells not lysed. The degree of fixation is expressed as 0, 1+, 2+, 3+, 4+ (0%, 25%, 50%, 75% or 100% cells not lysed). Reactions are interpreted as follows:

- 4+, 3+, 2+ = positive,
- 1+ = suspicious,
- Complete hemolysis or trace = negative.



Hemolysis %	0%	25%	50%	75%	≈100%	100%
Degree of fixation	4+	3+	2+	1+	Trace	0
Result	**Positive**			**Suspicious**	**Negative**	

5.2. Validation of the CFT

- Expected results for each analysis series (*an analysis series consists of analyses carried out at the same time, by one operator, with the same equipment and reagents*):

- Complement Control (undiluted well): complete hemolysis (0),
- Antigen Control (undiluted well): complete hemolysis (0),
- Low Titre serum (LT): ≥ 1+ at 1/5;
- High Titre serum (HT): Absence of hemolysis (4+) and titre conforms to supplier indications.
- Negative serum (NEG) complete hemolysis (0).

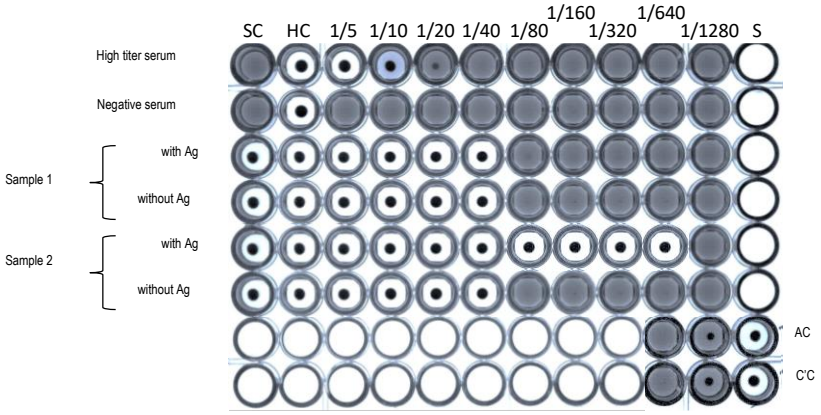
- Expected results for control wells of each tested sera (including control sera):

- Serum Control wells (SC), complete hemolysis (0),
- Hemolytic control wells (HC) absence of hemolysis (4+),

If the results of all the controls are not validated, a new analysis must be performed.

5.3. In case of anticomplementary serum

Sample with pellet in the well SC (≥1+) after 2 CFT is considered as anticomplementary.
For all other anticomplementary sera, the activity must be titrated. A duplicate series of dilutions is made and the sample is retested using *T. equiperdum* antigen in the first row and VB only in the second. The second row gives the titre of the anticomplementary reaction. **If the first row shows an end-point that is at least three dilutions greater than the second, the anticomplementary effect may be ignored and the sample rated as positive. If the results are any closer, a fresh sample of serum must be requested.**

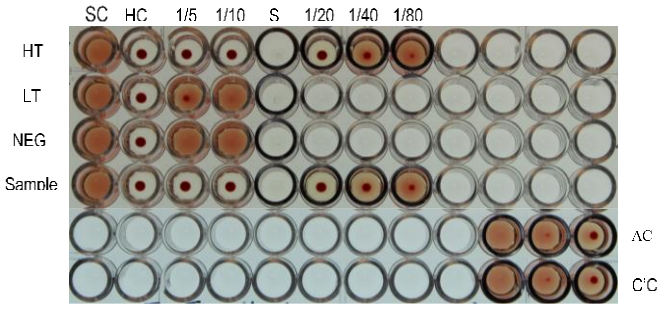


In this example, the first row of sample 1 does not show an end-point that is at least 3 dilutions greater than the 2nd. In consequence, the complementary effect cannot be ignored for the sample 1.
Concerning the sample 2 the first row shows an end-point 3 dilutions greater than the 2nd, the anticomplementary effect may be ignored and the sample rated as positive.

Other strategies which involved dilution of the serum (1/2) and/or heat inactivation at 60–63°C for 30 minutes are not recommended as they may result in reduction or removal of the positive signal.

5.4. In case of positive result:

All sera positive at 1/5, are diluted (two fold serial dilutions ranged to 1/5 to 1/320) to define the titre of the positive sample.



According to the recommendations of the **OIE terrestrial manual chapter 3.5.3 (2018)**:
"*In case of a serologically positive result and after clinical examination: repeat serological tests two times at 15 to 20 days of interval and perform an accurate epidemiological investigation. A confirmed case of dourine is defined as an animal having a positive result with CFT or IFA or PCR and (i) showing clinical signs compatible with dourine or (ii) showing an increase in serological CFT titre in two consecutive tests or (iii) epidemiologically linked with a confirmed case of dourine (Calistri et al., 2013).*"

As a consequence, we recommend in this context to:
- isolate as far as possible the animal from other animals from other equids,
- program 2 more blood sampling, each with 15 to 20 days interval to evaluate the evolution of the dourine CFT titre,
- control the clinical evolution of the animal during the 30 to 40 days between the first and the third serum sample,
- proceed to an epidemiological study to determine if the animal is susceptible to have an epidemiological link with a confirm case of dourine.
- Contact the person in charge of dourine activities in the EU-RL for equine diseases (laurent.hebert@anses.fr).

5.5. Summary of CFT results interpretation

<table>
<tr><th>Condition of the sample on receipt</th><th>Result of the CFT</th><th>Conclusion</th><th colspan="2">Result of the 2nd CFT</th><th>Conclusion</th></tr>
<tr><td>Hemolyzed or coagulated serum, insufficient volume or degraded sample</td><td>Sample rejected</td><td>Recommendation: request a new sample</td><td colspan="3"></td></tr>
<tr><td rowspan="8">Compliant serum</td><td rowspan="4">Anticomplementary TS ≥ +1</td><td rowspan="4">Carry out a new decomplementation and perform a second CFT.</td><td rowspan="3">No anticomplementary effect</td><td>Negative (result = trace or 0)</td><td>Result: negative</td></tr>
<tr><td>Suspicious (result = +1)</td><td>Result: suspicious sample
Recommendation: request a new sample</td></tr>
<tr><td>Positive (result ≥ 2+)</td><td>Result: Positive
Recommendation: see bellow</td></tr>
<tr><td>Anticomplementary TS ≥ +1</td><td>Uninterpretable</td><td>Result: uninterpretable sample
Recommendation: see § 5.3 and request a new sample.</td></tr>
<tr><td>Negative (result = trace or 0)</td><td>Result: negative</td><td colspan="3"></td></tr>
<tr><td rowspan="3">Suspicious (result = +1)</td><td rowspan="3">Carry out a new decomplementation and perform a second CFT.</td><td colspan="2">Negative</td><td>Result: negative</td></tr>
<tr><td colspan="2">Suspicious</td><td>Result: suspicious sample
Recommendation: request a new sample</td></tr>
<tr><td colspan="2">Positive</td><td>Result: positive
Recommendation: see § 5.4</td></tr>
<tr><td>Positive (result ≥ 2+)</td><td>Result: Positive
Recommendation: see § 5.4</td><td colspan="3"></td></tr>
</table>

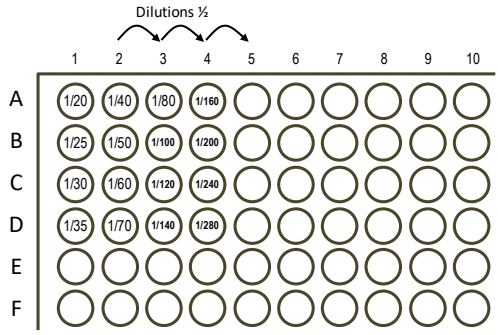
ANNEX A: TITRATION OF THE COMPLEMENT

1. PREPARATION OF 500 µL OF NEGATIVE SERUM
Dilute negative serum to 1/5 (e.g., 100 of serum in 400 µl of VB) and inactivate 30 min at 58°C

2. PREPARATION OF THE ANTIGEN SOLUTION
Prepare at least 500 µl of antigen diluted in VB according to the titre determined during antigen titration.

3. PREPARATION OF THE COMPLEMENT DILUTIONS (in microtubes)
- **dilution 1/20**: 10 µl of complement + 190 µl of VB,
- **dilution 1/25**: 10 µl of complement + 240 µl of VB,
- **dilution 1/30**: 10 µl of complement + 290 µl of VB,
- **dilution 1/35**: 10 µl of complement + 340 µl of VB**.**

4. PLATE PREPARATION (see lay out and General scheme for complement titration below)
- Add 25 µl of VB in columns 2, 3 and 4,
- Add 25 µl of 1/20 complement dilution in wells A1 and A2,
- Add 25 µl of 1/25 complement dilution in wells B1 and B2,
- Add 25 µl of 1/30 complement dilution in wells C1 and C2.
- Add 25 µl of 1/35 complement dilution in wells D1 and D2.



With a multichannel pipette
- Mix the content of the wells of the column 2 and then aspirate 25 µl,
- Add the aspirated 25 µl to the column 3. Mix and then aspirate 25 µl,
- Add the aspirated 25 µl to the column 4. Mix and then **discard** 25 µl,
- Add 25 µl of antigen in all the wells.
- Add 25 µl of negative serum (inactivated and diluted at 1/5) all the wells.

5. PLATE INCUBATION
Cover the plate with a lid and shake in the microshaker for 10 sec at 900 rpm, then place in the incubator (37 ± 2°C, 1 h.)

6. PREPARATION OF THE HEMOLYTIC SYSTEM
- Prepare the hemolytic system approximately 20 minutes before the end of the one-hour incubation of the plate. Depending on the hematocrit % and the hemolysin titre of your reagents and according to the table 1 in Annex C determine the appropriate volume of blood, hemolysin and VB needed for the preparation of the hemolytic system.
Prepare the solution in a hemolysis tube. Resuspend the blood carefully by agitating the bottle gently 5 to 10 times. Aspirate and expel blood slowly to avoid damaging the red blood cells with the tip of the cone.

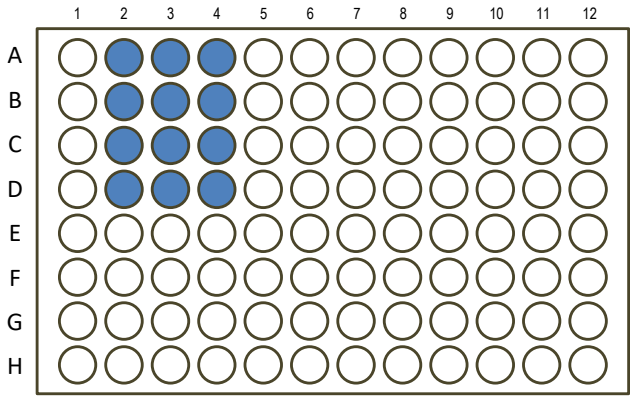
- Incubate the hemolysis tube in a water bath at 37 ± 2°C for 10 min.

7. ADDITION OF THE HEMOLYTIC SYSTEM
- Homogenate carefully the hemolytic system and add 50 µl in each well.
- Cover the plate with a lid and shake in a microshaker for 10 seconds at 900 rpm, then place in the incubator at 37 ± 2°C for 30 min (after 15 min shake again the plate for 10 seconds at 900 rpm).
- Centrifuge the plate for 2 minutes at 500 g and read the results.

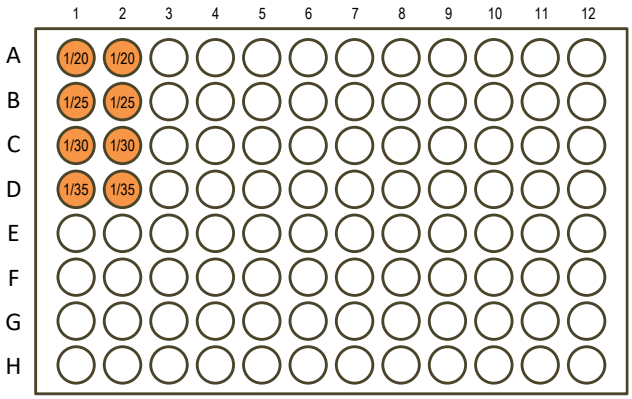
8. RESULT READING
Determine the highest complement dilution with a complete hemolysis. Use 2 complement units during CFT.
For example: if the highest complement dilution giving a total hemolysis is 1/60 (1 hemolytic unit) then the complement is to be used at 1/30 (2 hemolytic units).

11. PREPARATION OF ALIQUOTS
Aliquot the amount of complement to obtain the 2 units of complement for the volume required to perform a CFT and store these tubes at ≤ -65°C. until analysis.
E.g.: For complements to be used at a dilution of 1/30, aliquot 40 µl of complement into a microtube and store at ≤ -65°C. Before analysis, thaw an aliquot on ice and then add 1160 µl of VB to obtain 1200 µl of 2 units complement.

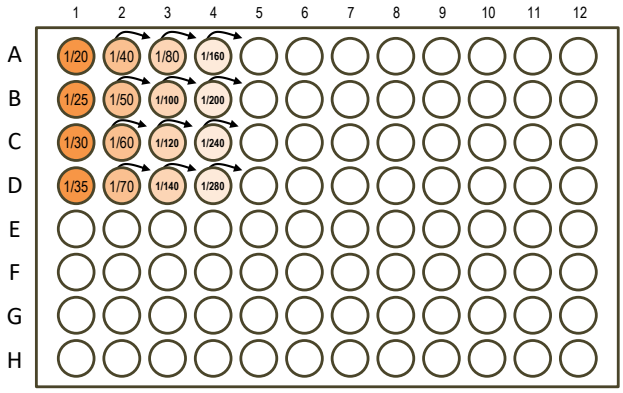
General scheme for complement titration



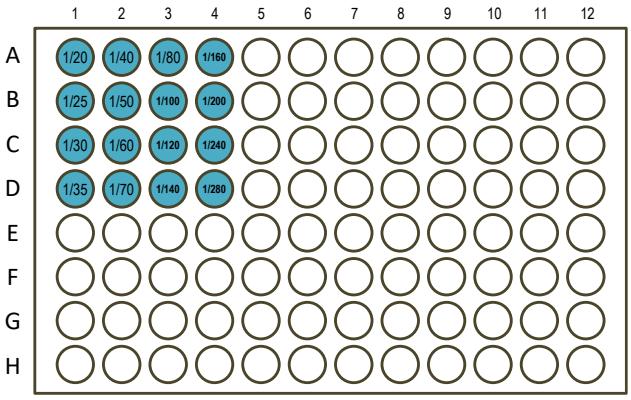
25 µl of Veronal buffer



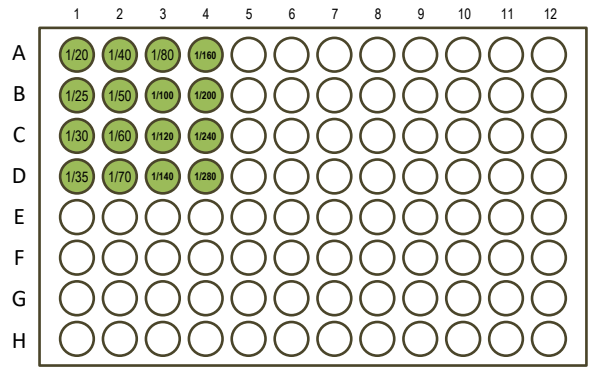
25 µl of the diluted complements



Serial dilution from column 2 to 4 (25 µl)



25 µl of diluted Antigen



25 µl of decomplemented 1/5 negative serum

ANNEX B: TITRATION OF THE ANTIGEN

1. PREPARATION OF 500 µL OF LOW TITRE SERUM

Dilute low titre serum to 1/5 (e.g., 100 of serum in 400 µl of VB) and inactivate 30 min at 58°C

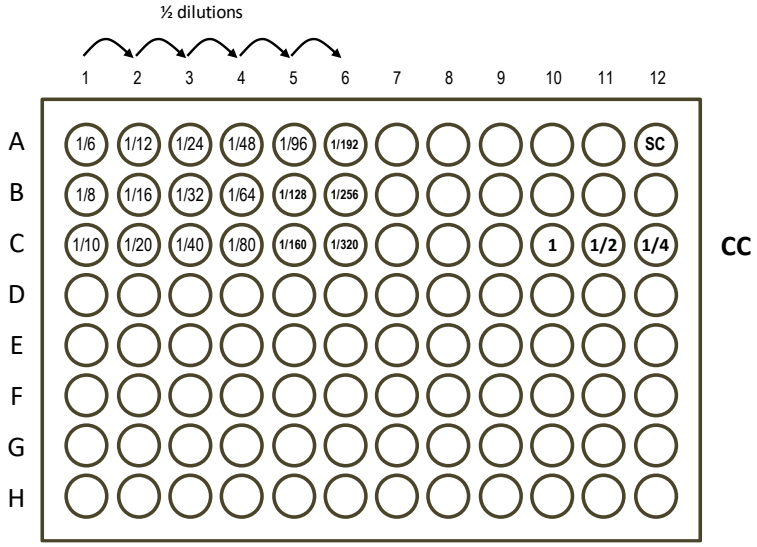
2. PREPARATION OF 3 ANTIGEN SOLUTIONS (in microtubes)

- **dilution 1/6**: (e.g. 20 µl antigen + 100 µl VB),
- **dilution 1/8**: (e.g. 20 µl antigen + 140 µl VB),
- **dilution 1/10**: (e.g. 10 µl antigen + 90 µl VB).

3. PREPARATION OF COMPLEMENT

Prepare about 600 µl of complement at 2 hemolytic units (as determine during complement titration)

4. PLATE LAYOUT



5. PLATE PREPARATION (see lay out and general scheme for antigen titration)

- Add 25 µl of VB in columns 2 to 6,
- Add 25 µl of 1/6 antigen dilution to wells A1 and A2,
- Add 25 µl of 1/8 antigen dilution to wells B1 and B2,
- Add 25 µl of 1/10 antigen dilution to wells C1 and C2.

With a multichannel pipette

- Mix the content of the wells of the column 2 and then aspirate 25 µl,
- Add the aspirated 25 µl to the column 3. Mix and then aspirate 25 µl,
- Add the aspirated 25 µl to the column 4. Mix and then aspirate 25 µl,
- Add the aspirated 25 µl to the column 5. Mix and then aspirate 25 µl,
- Add the aspirated 25 µl to the column 6. Mix and then discard 25 µl,
- Add 25 µl of heat-inactivated low titre serum in all the wells.
- Add 25 µl of complement in each wells.

6. PREPARATION OF SERUM CONTROL (SC) AND COMPLEMENT CONTROL (CC) WELLS

- Add 25 µl of VB to the wells A12, C11 and C12,
- Add 25 µl of heat-inactivated low titre serum in the well A12,
- Add 25 µl of complement to the wells A12, C10,
- Add 25 µl of complement to the well C11, mix well, aspirate 25 µl, add the aspirated 25µl to the well C12, mix well and discard 25 µl,
- Add 50 µl of VB to the wells C10, C11 and C12.

7. PLATE INCUBATION

Cover the plate with a lid and shake in the microshaker for 10 seconds at 900 rpm, then place in the incubator at 37 ± 2°C for 1 hour.

8. PREPARATION OF THE HEMOLYTIC SYSTEM

- Prepare the hemolytic system approximately 20 minutes before the end of the one hour incubation of the plate. Depending on the hematocrit % and the hemolysin titre of your reagents and according to the table 1 in Annex C determine the appropriate volume of blood, hemolysin and VB needed for the preparation of the hemolytic system.
Prepare the solution in a hemolysis tube. Resuspend the blood carefully by agitating the bottle gently 5 to 10 times. Aspirate and expel blood slowly to avoid damaging the red blood cells with the tip of the cone.

- Incubate the hemolysis tube in a water bath at 37 ± 2°C for 10 min.

9. ADDITION OF THE HEMOLYTIC SYSTEM

- Homogenate carefully the hemolytic system and add 50 µl in each well.
- Cover the plate with a lid and shake in a microshaker for 10 seconds at 900 rpm, then place in the incubator at 37 ± 2°C for 30 min (after 15 min shake again the plate for 10 seconds at 900 rpm).
- Centrifuge the plate for 2 minutes at 500 g and read the results.

10. EXAMPLE OF RESULT READING WITH AN ANTIGEN TITRATION PERFORMED WITH LOW TITRE SERUM.

Determine the antigen dilution that allows obtaining 2+ with a low titre serum. Use 2 antigen units during CFT. (Fig. 1)

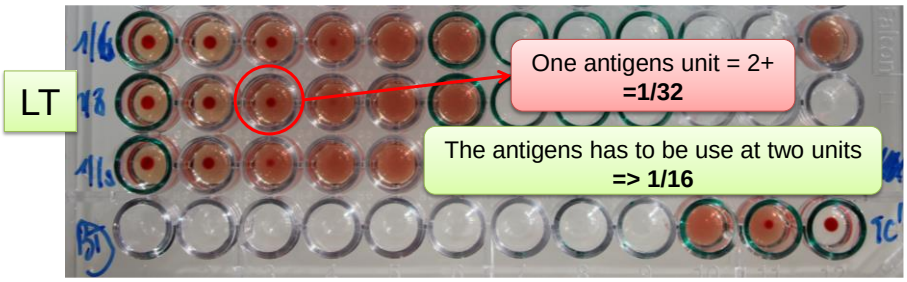
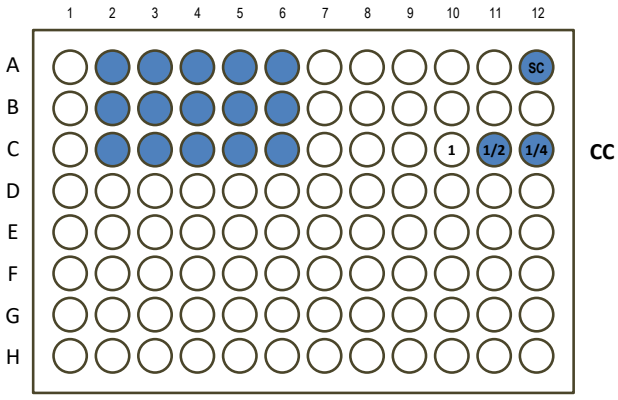


Fig 1. Example of results obtained by titration performed with a low titre serum.

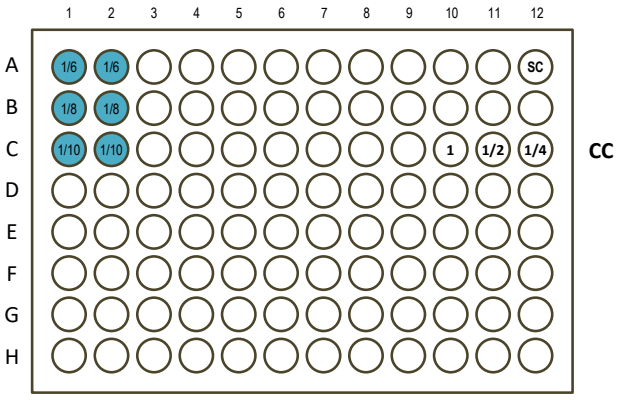
11. PREPARATION OF ALIQUOTS

Aliquot the amount of antigen to obtain the 2 units of antigen for the volume required to perform a CFT and store these tubes at ≤ -65°C until analysis.
E.g.: For antigens to be used at a dilution of 1/16, aliquot 75 µl of antigen into a microtube and store at ≤ -65°C. Before analysis, thaw an aliquot on ice and then add 1125 µl of VB to obtain 1200 µl of 2 units antigen.

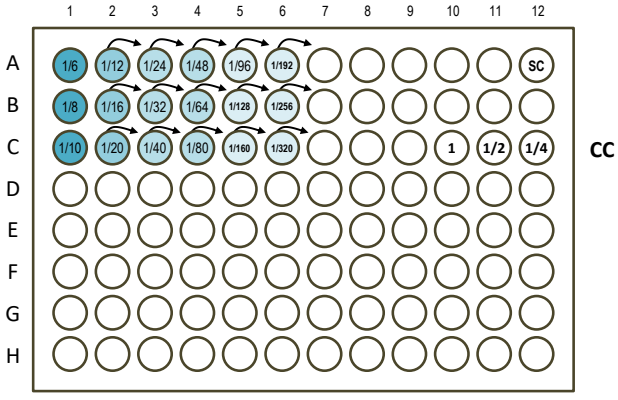
General scheme for antigen titration



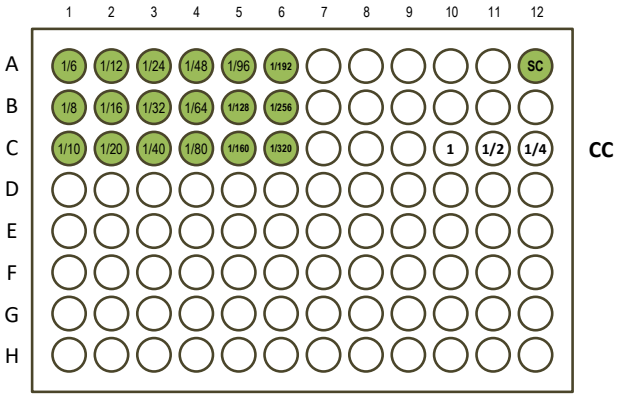
25 µl of Veronal buffer



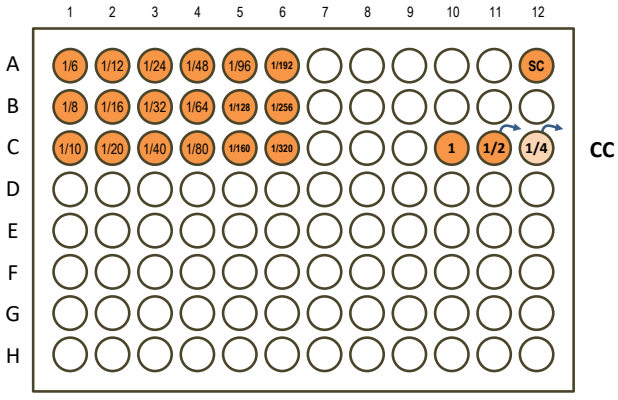
25 µl of the diluted Antigens



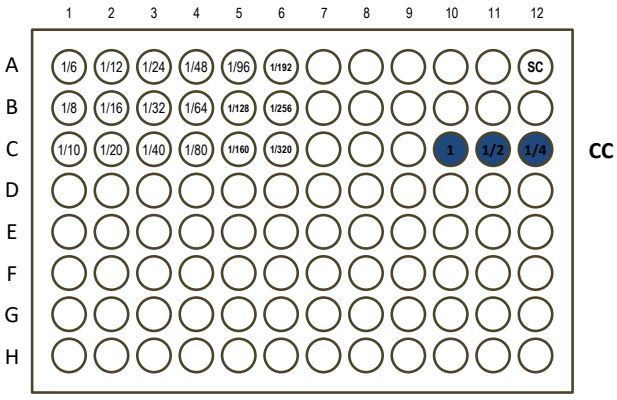
Serial dilution from column 2 to 7 (25 µl)



25 µl of decomplemented low titer serum



25 µl of complement



50 µl of veronal Buffer

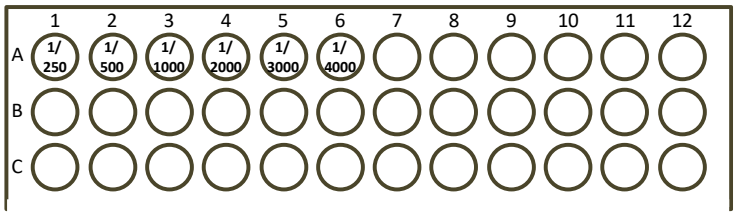
ANNEX C: HEMOLYSIN TITRATION

1. PREPARATION OF 500 µL OF NEGATIVE SERUM
Dilute negative serum to 1/5 (e.g., 40 of serum in 160 µl of VB) and inactivate 30 min at 58°C

2. PREPARATION OF THE ANTIGEN SOLUTION
Prepare at least 200 µl of antigens diluted in VB according to the titre determined during antigen titration.

3. PREPARATION OF THE COMPLEMENT
Prepare at least 200 µl of complement diluted in VB according to the titre determined during complement titration.

4. PLATE LAYOUT



5. PLATE PREPARATION (see lay out and general scheme of hemolysin titration procedure)

- Add 25 µl of complement in wells A1 to A6,
- Add 25 µl of heat-inactivated serum diluted at 1/5 in wells A1 to A6,
- Add 25 µl of antigen in wells A1 to A6.

6. PLATE INCUBATION
Cover the plate with a lid and shake in the microshaker for 10 seconds at 900 rpm, then place in the incubator at 37 ± 2°C for 1 hour.

7. PREPARATION OF THE HEMOLYTIC SYSTEMS
- Prepare the hemolytic systems approximately 20 minutes before the end of the one-hour incubation of the plate. In separated tubes prepare the hemolytic system at the dilutions: 1/250, 1/500, 1/1000, 1/2000, 1/3000, 1/4000 according to the table 1.
Prepare the solution in a hemolysis tube. Resuspend the blood carefully by agitating the bottle gently 5 to 10 times. Aspirate and expel blood slowly to avoid damaging the red blood cells with the tip of the cone.

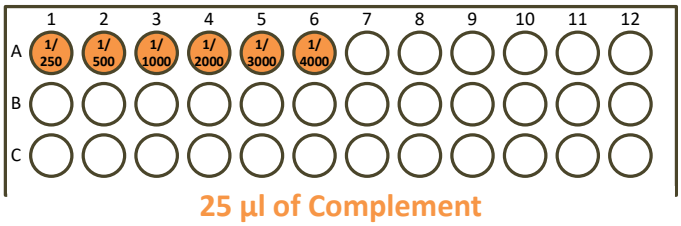
- Incubate the hemolysis tubes in a water bath at 37 ± 2°C for 10 min.

8. ADDITION OF THE HEMOLYTIC SYSTEMS
- Homogenate carefully the hemolytic systems and add 50 µl of each hemolytic system dilutions in the corresponding well.
- Cover the plate with a lid and shake in a microshaker for 10 seconds at 900 rpm, then place in the incubator at 37 ± 2°C for 30 min (after 15 min shake again the plate for 10 seconds at 900 rpm).
- Centrifuge the plate for 2 minutes at 500 g and read the results.

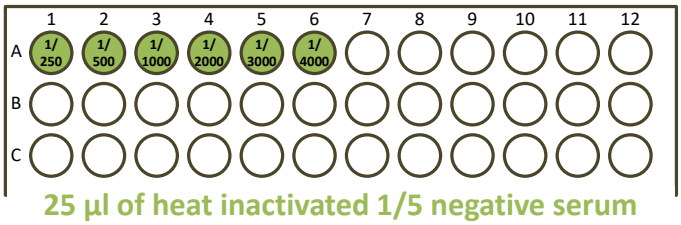
9. RESULT READING
Determine the highest hemolysin dilution with a complete hemolysis (= 1 unit). Use 2 units of hemolysin during CFT.

For example: if the highest hemolysin dilution giving a total hemolysis is 1/1000 (1 hemolytic unit), then the complement should be used at 1/500 (2 hemolytic units).

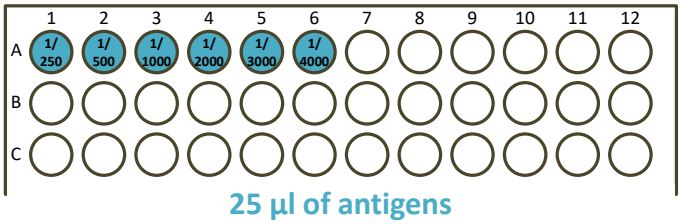
General scheme of hemolysin titration procedure



25 µl of Complement



25 µl of heat inactivated 1/5 negative serum



25 µl of antigens

Table 1: preparation of hemolytic system (HS) solutions

For hemolysin titre of 1/250	Hematocrit (% of red blood cells)											
	45	46	47	48	49	50	51	52	53	54	55	56
Blood (µl)	133	131	127	125	123	120	118	115	113	111	109	107
Hemolysin (µl)	8	8	8	8	8	8	8	8	8	8	8	8
Veronal Buf. (µl)	3859	3861	3865	3867	3869	3872	3874	3877	3879	3881	3883	3885

For hemolysin titre of 1/500	Hematocrit (% of red blood cells)											
	45	46	47	48	49	50	51	52	53	54	55	56
Blood (µl)	133	131	127	125	123	120	118	115	113	111	109	107
Hemolysin (µl)	4	4	4	4	4	4	4	4	4	4	4	4
Veronal Buf. (µl)	3863	3865	3869	3871	3873	3876	3878	3881	3883	3885	3887	3889

For hemolysin titre of 1/1000	Hematocrit (% of red blood cells)											
	45	46	47	48	49	50	51	52	53	54	55	56
Blood (µl)	133	131	127	125	123	120	118	115	113	111	109	107
Hemolysin (µl)	2	2	2	2	2	2	2	2	2	2	2	2
Veronal Buf. (µl)	3865	3867	3871	3873	3875	3878	3880	3883	3885	3887	3889	3891

For hemolysin titre of 1/2000	Hematocrit (% of red blood cells)											
	45	46	47	48	49	50	51	52	53	54	55	56
Blood (µl)	133	131	127	125	123	120	118	115	113	111	109	107
Hemolysin (µl)	1	1	1	1	1	1	1	1	1	1	1	1
Veronal Buf. (µl)	3866	3868	3872	3874	3876	3879	3881	3884	3886	3888	3890	3892

For hemolysin titre of 1/3000	Hematocrit (% of red blood cells)											
	45	46	47	48	49	50	51	52	53	54	55	56
Blood (µl)	133	131	127	125	123	120	118	115	113	111	109	107
Hemolysin (µl)	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67
Veronal Buf. (µl)	3866	3868	3872	3874	3876	3879	3881	3884	3886	3888	3890	3892

For hemolysin titre of 1/4000	Hematocrit (% of red blood cells)											
	45	46	47	48	49	50	51	52	53	54	55	56
Blood (µl)	133	131	127	125	123	120	118	115	113	111	109	107
Hemolysin (µl)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Veronal Buf. (µl)	3867	3869	3871	3873	3875	3877	3880	3882	3885	3889	3891	3893