Ovine contagious Epididymitis
(*Brucella ovis*)
Complement Fixation Test
(EU RL cold technique)
Standard Operating Procedure

SAFETY PRECAUTIONS

The laboratory shall take all precautions in order to guarantee the necessary safety, for both the operator and the environment, against the biological and chemical hazards due to the activities conducted according to this document.

1 Scope

The present document describes a standard technique aiming at detecting antibodies specific of *Brucella ovis* by the **complement fixation test** in ovine sera.

2 Normative references

- Ovine Epididymitis (*Brucella ovis*), *In*: The OIE Terrestrial Manual, 2018, Chapter 3.8.7, OIE, Paris. https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.08.07_OVINE_EPID.pdf
- Regulation (EU) 2017/625 of the European Parliament and of the Council of 15 March 2017 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products. Official Journal of the European Union 17.03.2017, L95/1-142. Text with EEA relevance. ELI: http://data.europa.eu/eli/reg/2017/625/oj
- ISO/IEC 17025, General requirements for the competence of testing and calibration laboratories.
- French Standard NF U47-008 - Animal health analysis methods - Detection of antibodies against Ovine epididymitis by the Complement Fixation Test, December 2012, AFNOR, France.

3 Definitions

- **Complement (C)**

A serum molecular complex, some components of which may fix themselves to specific antigen-antibody immune complex.

- **Haemolysin (H)**

Serum from a hyper-immunised animal against heterologous red blood cells (RBC, erythrocytes), with a high titre of anti-red blood cells antibodies and causing *in vitro* the lysis of specific corresponding erythrocytes whenever the complement is present.

- **Sensitised RBC**

A mixture of pre-determined quantities of a suspension of RBC and of a specific haemolysin.

- **Series of tests**

Implementation of all of the analytical phases of a technique carried out continuously or intermittently, separated by short interruptions, by the same operator(s), in the same location, with the same equipment and the same reagents.

4 Principle and reaction

4.1 Principle

The heterologous complement is set in a mixture of antigen-test serum. Once the specific antibody-antigen immune complexes are formed, the complement fixes to these complexes. This reaction is revealed by adding a second immune system: erythrocytes-haemolysin (sensitised RBC).

Indigenous complement naturally present in the test serum is destroyed via heat inactivation.

The heterologous complement that was not fixed to the first complexes, will fix to the sensitised RBC, thus causing the lysis of RBC to an extent that depends on the quantity of the complement that was not used on the first stage.

The degree of haemolysis, observed through the colouring of the reaction medium (after centrifugation or sedimentation), is inversely proportional to the titre of specific antibodies originally present in the serum.

4.2 Reaction

4.2.1 Method

The method used is the cold fixation in micro-titration plates.

4.2.2 Antigen (Ag)

The antigen is a water-soluble hot-saline (HS) extract from *Brucella ovis*, strain REO 198 (OIE Manual)[1].

The supplier must certify that the activity of the antigenic preparation has been standardised against the International Standard anti-*Brucella ovis* Serum (ISaBoS - International Standard 1985[2]) or a National secondary standard, with the technique established in this document and according to OIE requirements. Antigen is used at a volume of 25 µl.

4.2.3 Test sera

Sera are tested, once inactivated, at a 25-µl volume at a 1/10 dilution or in any other dilution according to the specifications requested for the test. If a prozone phenomenon, together with a high titre of antibodies in the serum is suspected (*i.e.*: clinical orchi-epididymitis), it is advisable that all sera be tested at the following dilutions 1/10, 1/20, and 1/40 (9.1.6). On the other hand, if titres under 50 international units per millilitre (IU/ml) need to be identified, it is suggested that the serum be tested as of dilution 1/5.

[1] Obtainable from the EU/OIE/FAO Reference Laboratory for Brucellosis at ANSES, 94706, Maisons-Alfort, France
[2] Obtainable from the OIE Reference Laboratory for Brucellosis at AHVLA Weybridge, Addlestone, Surrey KT15 3NB, UK

4.2.4 Control sera

Control sera are used, once inactivated, at a volume of 25 µl, at a 1/10 dilution for the negative control serum and at dilutions around the expected titre for the positive control serum.

4.2.5 Complement (C)

Complement (from guinea-pig) is used at a volume of 25 µl and contains 1.25-2 100% haemolytic units of complement (CH100) according to the antigen supplier's instructions.

4.2.6 Sensitised Sheep Red Blood Cells (SRBCs)

The sensitised-SRBCs used at a volume of 50 µl, are a mixture of equal volumes of:

- a sheep red blood cells (SRBCs) suspension at 2.5 %, and of
- a rabbit haemolysin dilution at a titre of twice the minimum concentration required to produce 100 % lysis of SRBCs in the presence of a titrated solution of guinea-pig complement (two 100%-haemolytic-units).

4.3 Controls

4.3.1 Serum Control

The absence of the anti-complementary activity of the test serum is checked in a corresponding "serum-control well". In this well, the test is performed without antigen, its volume being replaced by the same volume of the diluent. This control is performed for each test serum. In this technique, control sera are established at least for the 1/10 dilution (and for the 1/5 dilution if appropriate).

4.3.2 Antigen Control

The absence of the anti-complementary activity of the antigen used is checked in an "antigen-control well". In this well, the test is performed without serum, this latter being replaced by the same volume of diluent. This control is performed once for each series of tests.

4.3.3 Complement control

The activity of the complement on the sensitised SRBC is checked in a "complement-control well". In this well, the test is performed with the complement and the sensitised SRBCs only, the antigen and the serum being both replaced by identical volumes of diluent. This control is performed once for each series of tests.

4.3.4 Sensitised SRBCs control

The sensitised SRBCs' quality is checked in a "sensitised SRBCs-control well". In this well, the test is performed with the sensitised SRBCs only, the complement, the antigen, and the serum being all replaced by identical volumes of diluent. This control is performed once for each series of tests.

4.3.5 Haemolysis standard control

In order to facilitate test reading, the degree of haemolysis is compared with standards corresponding to 0, 25, 50, 75 and 100 % lysis that are systematically created for each test series.

4.3.6 Control sera

Positive and negative control sera are both included in each series of tests.

5 Diluents, culture media, reagents and other products

5.1 Diluents

5.1.1 Magnesium Calcium (MgCa) buffer (pH 7.35)

This may be prepared from tablets available commercially. Otherwise, it may be prepared from a stock solution. The pH is critical and must be adjusted to 7.35 (±0.05). Final solution must be filter (0.22 µm) and stocked at 5°C ± 3°C.

Example:

<u>First solution</u>:
Magnesium ($MgCl_2$) 0,416 mol/L & Anhydrous Calcium Chloride ($CaCl_2$ $2H_2O$) 0,125 mol/L :
$MgCl_2$ .... 9.5 g
$CaCl_2$ .... 3.7 g
Distilled or equivalent quality water .... up to 100 ml

<u>Second solution:</u>
Sodium chloride (NaCl) à 0,85 % :
NaCl .... 8.5 g
Distilled or equivalent quality water .... up to 1 000 ml

<u>Final solution (Calcium Magnesium Buffer):</u>
NaCl 0.85% 1000mL
$MgCl_2$ (0,416 mol/L) & $CaCl_2$ $2H_2O$ (0,125 mol/L) .... 1 ml

5.2 Culture media

Not applicable.

5.3 Reagents

5.3.1 Antigen

The antigen is available commercially, for veterinary use, to be diluted and stored according to the supplier's instructions. If the antigen is supplied freeze-dried, it may be stored at 5° C ± 3°C. Once resuspended in the appropriate volume of distilled (or equivalent quality) water, if the vial content is not entirely used, it may be dispatched in tightly capped microtubes and stored at ≤ – 16°C.

5.3.2 Freeze-dried guinea-pig complement

The complement is available commercially, and should be reconstituted according to the supplier's instructions.

For the CFT, the complement should be diluted according to the result of the complement titration (Annex A). If the complement is not stabilised, it should be titrated at each series of tests. If the complement is stabilised the titration should be performed for each new batch, or better for each new

vial. In any case, complement titration at each series of tests provides the best reliability of the final results.

The reconstituted complement, if not used immediately, must be stored at 5°C ± 3°C, until the performance of the series of tests on the same day, and for any volume left, at ≤ – 16°C or 5°C ± 3°C according to the supplier's instructions.

5.3.3 Sheep erythrocytes at 50 %

Sheep erythrocytes (SRBCs) at 50 % could be prepare as describe on the Annex D and can be diluted to 1/20 in MgCa Buffer (final concentration: 2.5 %) MgCa buffer.

5.3.4 Rabbit haemolytic anti-SRBC serum (haemolysin)

Rabbit anti-SRBC haemolytic serum should be diluted according to the titration performed for each new batch (Annex B).

5.3.5 Control sera

5.3.5.1 Positive control serum with a known titre (commercial or lab. prepared).

5.3.5.2 Negative control serum (commercial or lab. prepared).

5.4 Other products

5.4.1 Water

The chemical and bacteriological quality of the water used to prepare the different reagents shall be verified. It must meet the requirements of the supplier and/or those imposed by the laboratory. These requirements shall enable a satisfactory implementation of the technique described in the present document.

6 Equipment and plastic/glass ware

Conventional serology laboratory equipment and in particular:

6.1 Temperature-controlled incubator set at 37°C ± 2°C.

6.2 Water bath (circulating water bath if possible) set at 59°C ± 1°C.

6.3 Temperature-controlled refrigerator at 5 °C ± 3°C.

6.4 Temperature-controlled freezer at ≤ – 16°C.

6.5 Centrifuge, refrigerated if possible (allowing adequate acceleration)

6.6 Distribution and dilution device having a suitable volume range and accuracy

6.7 Disposable microplates (96 well round U bottomed) with lid or cover (plastic or adhesive)

6.8 Test tubes and racks.

6.9 Plate-reading mirror (eventually).

6.10 Timer or chronometer.

7 Sampling

In epidemiological surveys or in eradication programmes, serum samples are collected from adult males, and, if necessary from females, preferably at a time close to the breeding season.

8 Preparation of the sample for analysis

The preparation of the sample shall comply with the requirements of the OIE Manual.

9 Operating procedure

9.1 Test

9.1.1 Preparation of erythrocytes

SRBC (5.3.3) are diluted to 2.5 % (final dilution) in MgCa Buffer (5.1.1). This suspension should be prepared in a sufficient quantity to perform both the complement titration and the tests.

9.1.2 Preparation of the haemolysin

The haemolytic serum (5.3.4) is diluted according to the results of the titration (Annexe B item B.1) in MgCa Buffer (5.1.1). This suspension should be prepared in a sufficient quantity to perform both the complement titration and the tests.

9.1.3 Preparation of the antigen

Dilute the antigen (5.3.1) according to the supplier's instructions. This suspension should be prepared in a sufficient quantity to perform both the complement titration and the tests.

9.1.4 Complement titration (see Annex A)

9.1.5 Heat-inactivation of test and of control sera

Inactivate test and control sera (5.3.5) undiluted for 30 min in water bath at 59°C ± 1°C.

9.1.6 Dilution of test sera and of control sera

- 10 µl of each inactivated test-serum is mixed to 90 µl of MgCa Buffer (5.1.1) in an intermediate plate to prepare the 1/10 dilution (or 20 µl / 80 µl for a 1/5 dilution).
- 25 µl of this 1/10 dilution (or 1/5 dilution) are placed in the well of the first, second and third rows.
- The first row is an anti-complementary control for each serum.
- Volumes of 25 µl of MgCa Buffer (5.1.1) are added to the wells of the first row (anti-complementary controls) to compensate for lack of antigen.
- Volumes of 25 µl of MgCa Buffer (5.1.1) are added to all other wells except those of the second row.
- Serial doubling dilutions are then made by transferring 25 µl volumes of serum from the third row onwards; 25 µl of the resulting mixture in the last row are discarded.
- Do the same for the positive control serum.

- The negative control serum is tested at 1/10 dilution only.

9.1.7 Distribution of the antigen, the buffer and of the complement

	Diluted serum	MgCa Buffer	Diluted antigen	C
Anti-complementary control well (dilution1/5 or 1/10)	25 µl	25 µl	—	25 µl
Test wells (dilutions 1/5 - 1/40)	25 µl	—	25 µl	25 µl

9.1.8 Preparation of controls

	MgCa Buffer	Diluted antigen	C
Antigen control	25 µl	25 µl	25 µl
Complement control	50 µl	—	25 µl
Sensitised SRBCs control	75 µl	—	—

9.1.9 Reaction

Shake the plates, cover them and place them at 5°C ± 3°C overnight (16-20 h.).

9.1.10 2nd step (haemolysis/ haemolysis inhibition)

9.1.10.1 Preparation of the sensitised SRBCs: mix equal quantities of the solutions (9.1.1) and (9.1.2) that have been prepared beforehand and stored separately at 5°C ± 3°C.

9.1.10.2 Leave the mixture at room temperature for 10 min.

9.1.10.3 Take the plates out of the refrigerator and place them for 10 min. in the incubator at 37°C ± 2°C, if possible without stacking them. The sensitised SRBCs stay then 20 min. at room temperature.

9.1.10.4 Add 50 µl of the sensitised SRBCs in each well, shake the plates, cover them and place them in the incubator at 37°C ± 2°C for 30 min., if possible without stacking them.

9.2 Validation and reading

9.2.1 Centrifugation

Centrifuge plates in order to obtain the SRBCs sedimentation, *e.g.* 3,000-6,000 m.s^{-2} (*i.e. ca.* 300-600 *g*) for 5-10 min. Otherwise, they should be placed at 5°C ± 3°C for 2–3 hours to allow unlysed cells to settle.

9.2.2 Validation

The series of tests is validated by the result of its controls:

- Antigen control: 100 % haemolysis;
- Complement control: 100 % haemolysis;
- Sensitised SRBCs control: 0 % haemolysis;
- Negative serum control: 100 % haemolysis;

- The positive control serum is visualized at the expected titre ± one dilution.

9.2.3 Reading

The reading is made by evaluating the colour of the supernatant in comparison to a haemolysis standard control range that is prepared as follows:

Haemolysis control (%)	100	75	50	25	0
Notation	0	+	++	+++	++++
MgCa Buffer (µl)	0	25	50	75	100
Total haemolysis supernatant (µl)[a]	100	75	50	25	0
[a] *prepared from 100 % haemolysis wells.*					

If needed, read the results using a plate-reading mirror.

9.3 Interpretation

9.3.1 Expression of results

The raw results are expressed according to the percentage of the observed haemolysis:

	Haemolysis	Haemolysis inhibition (%)
++++:	0	100
+++:	25	75
++:	50	50
+:	75	25
0:	100	0

AC: anti-complementary activity (SRBCs not lysed in the 1/10 control well).

9.3.2 Interpretation

The end-point of the reaction is taken as the highest dilution showing 1 + reaction or greater, *i.e.* 25% or more fixation.

Interpretation is done after the conversion of titration results, expressed in international units of complement fixation per millilitre (IU/ml) established as follows:

NOTE: According to OIE and EU requirements, a result of 50 IU/ml (*i.e.* at least 50 % of haemolysis inhibition at 1/10 dilution) is positive.

Serum dilution	**Haemolysis inhibition**			
	25% (+)	50% (++)	75% (+++)	100% (++++)
1/5	20.83	25	29.17	33.33
1/10	41.67	**50***	58.33	66.67
1/20	83.33	100	116.67	133.33
1/40	166,67	200	233.33	266.67
1/80	333.33	400	466.67	533.33
1/160	666.67	800	933.33	1066.67
1/320	1333.33	1600	1866.67	2133.33
** European and OIE threshold of positivity (50 IU/ml).*				

If preferred, the values might be rounded to the nearest integer as follows:

Serum dilution	Haemolysis inhibition			
	25% (+)	50% (++)	75% (+++)	100% (++++)
1/5	21	25	29	33
1/10	42	**50***	58	67
1/20	83	100	117	133
1/40	167	200	233	267
1/80	333	400	467	533
1/160	667	800	933	1067
1/320	1333	1600	1867	2133

** European and OIE threshold of positivity (50 IU/ml).*

10 Storage and disposal of samples

Each laboratory should set up the provisions for the correct storage of samples until their disposal.

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.

Decontamination and disposal of samples must be performed in accordance to in-force regulations.

11 Restitution of results

For the laboratory's clients, restitution of the results is made:

- either qualitatively (negative, positive, anti-complementary or un-interpretable) ;
- or semi-quantitatively (titre in IU/ml or anti-complementary or un-interpretable)

12 Precision

The use of positive reference material (secondary or working standard) during each series of tests enables to check the reproducibility of tests conditions. The expected titre of this reference test material must be found at more or less one dilution.

13 Analysis report

The analysis report must comply with the requirements of ISO/IEC 17025.

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Annex A

Titration of the Complement

A.1 Titration of the Complement

A.1.1 Prepare dilutions of the complement (5.3.2) in MgCa Buffer as follows:

Dilutions	**1/15**	**1/20**	**1/25**	**1/30**	**1/35**	**1/40**	**1/45**	**1/50**
MgCa Buffer (µl)	700	950	1 200	1 450	1 700	1 950	2 200	2 450
C (µl)	50	50	50	50	50	50	50	50

Note: the series might be modified according to the batch of complement.

A.1.2 Distribute in a microplate according to the chart as follows:

- 25 µl of each complement dilution in rows A and B;
- 25 µl of MgCa Buffer in rows B and C.

Prepare the dilutions by transferring 25 µl from row B to row C and discarding 25 µl of the resulting mixture in row C. Mix well the mixture obtained at each step.

	Well	1	2	3	4	5	6	7	8	Transfer
Rows A	C (µl)	25	25	25	25	25	25	25	25	
	Dilutions	1/15	1/20	1/25	1/30	1/35	1/40	1/45	1/50	
B	C (µl)	25	25	25	25	25	25	25	25	
	MgCa Buffer (µl)	25	25	25	25	25	25	25	25	
	Dilutions	1/30	1/40	1/50	1/60	1/70	1/80	1/90	1/100	→ 25 µl
C	MgCa Buffer (µl)	25	25	25	25	25	25	25	25	↲
	Dilutions	1/60	1/80	1/100	1/120	1/140	1/160	1/180	1/200	→ Discard 25 µl

Prepare further dilutions if necessary.

A.1.3 Distribute 25 µl of diluted antigen (9.1.3) and 25 µl of MgCa Buffer in all wells. Shake the plate gently. Place the plate (covered) in the incubator at 37°C ± 2°C for 30 min.

A.1.4 Prepare the sensitised SRBCs. Mix the necessary quantities for the complement titration 20 min. before use and leave at lab. room temperature. Store the remaining volumes of haemolysin and SRBCs separately at 5°C ± 3°C until the day after.

A.1.5 Distribute 50 µl of the sensitised SRBCs **per well** and shake the plate gently.

A.1.6 Place the plate (covered) in the incubator at 37°C ± 2°C for 30 min.

A.1.7 Centrifuge the plate in order to obtain the SRBCs sedimentation, *e.g.* 3,000-6,000 m.s^{-2} (*i.e. ca.* 300- 600 *g*) for 5-10 min. Otherwise, the plate should be placed at 5°C ± 3°C for 2–3 hours to allow unlysed cells to settle.

A.1.8 Reading of the titration

Note the highest dilution (lowest concentration) of complement showing a 100% haemolysis (100%-haemolytic-unit)

A.1.9 Interpretation: 1.25 to 2 100%-haemolytic-units must be used in the test according to the antigen supplier instructions.

EXAMPLE 100% haemolysis observed in B6 (confirmed in C2): dilution 1/80.
For 2 100%-haemolytic-units: complement dilution to be used: 1/80 × 2 = 1/40.
For 1.25 100%-haemolytic-units: complement dilution to be used: 1/80 × 1.25 = 1/80 x 5/4 = 1/64

Annex B

Haemolysin titration

B.1 Titration of haemolysin

This titration is performed in microplates, in the operating conditions of the complement fixation test. All dilutions are prepared in MgCa Buffer (5.1.1).

B.1.1 Prepare an initial 1/250 dilution of the Haemolysin (H). This dilution is the first of the series of dilutions presented below:

	—	1/2	1/4	1/8	1/12	1/16	1/20	1/24
Final dilution	1/250	1/500	1/1 000	1/2 000	1/3 000	1/4 000	1/5 000	1/6 000

Further dilutions may be prepared if necessary.

B.1.2 Prepare a complement dilution (5.3.2) with complement in excess (1/10 for example)

B.1.3 Prepare a SRBCs suspension at 2.5 % (9.1.1).

B.1.4 Each preparation is distributed in duplicate in a microplate following the chart below:

Wells	**1 HC***	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
H dilutions	1/250	1/250	1/500	1/1 000	1/2 000	1/3 000	1/4 000	1/5 000	1/6 000
Diluted H (µl)	25	25	25	25	25	25	25	25	25
SRBCs 2.5 % (µl)	25	25	25	25	25	25	25	25	25
MgCa Buffer (µl)	75	50	50	50	50	50	50	50	50
C 1/10 (µl)	—	25	25	25	25	25	25	25	25
** HC =Haemolysin control.*									

Further dilutions may be prepared if necessary.

B.1.5 Shake the plate, cover it and put it in the incubator pre-set at 37°C for 30 min.

B.1.6 Centrifuge the plate at 3,000-6,000 $m.s^{-2}$ (*i.e. ca.* 300-600 *g*) for 5-10 min. (refrigerated centrifuge if possible).

B.1.7 Results

The haemolysin control (HC) shall not present any haemolysis.

The highest dilution that causes a 100 % haemolysis will determine the 100 % haemolytic unit.

In the complement fixation test, two units are used, that is the double of the quantity determined by the titration.

EXAMPLE The 1/2 000 dilution is the highest one giving a 100 % haemolysis (there is a partial haemolysis for the 1/3 000 dilution). Therefore, the dilution to use in the test is 1/1 000.

Annex C

Inhibition of the serum anti-complementary activity

This technique allows the inhibition of the anti complementary capacity of some sera.

C.1 Inhibition of the serum anti-complementary activity

C.1.1 Prepare a solution of 5 % of serum-albumin (fraction V) in MgCa Buffer.

C.1.2 Prepare the first serum dilution (1/5 or 1/10) to be tested in this solution.

C.1.3 Incubate this dilution at 37°C ± 2°C for 45 min.

C.1.4 Re-start the test at step 9.1.5.

Annex D
Preparation of sheep red blood cells 50%

D.1 Washing of SRBC (commercial product):

Regardless of the origin of the red blood cell suspensions stored in Alsever's solution (prepared from a blood sample or purchased), these are washed before calibration. The commercial red blood cells are transferred (in a volume of 10 mL) into a 15 mL Falcon tube.

D.1.1 Leave the suspension to stand (for a maximum of 24 hours or centrifuge for 10 min at 1500 g for immediate treatment) in order to see a clear demarcation between the red blood cells and the supernatant.

D.1.2 Remove the supernatant (which must not be hemolyzed)

D.1.3 Estimate the volume of the pellet (using the graduations of the Falcon tube) and add the same volume of MgCa Buffer

D.1.4 Mix gently to re-suspend the pellet

D.1.5 Repeat the centrifugation step described below at least 3 times:

- Centrifuge 10 min at 1500 g
- Remove the supernatant
- Estimate the volume of the pellet and add the same volume of MgCa Buffer
- Mix gently to re-suspend the pellet

D.1.6 If supernatant is not translucent, repeat the last steps

D.2 SRBC Standardisation:

SRBC (5.3.3) are diluted to 2.5% (final dilution). The standardization of the SRBC can be carried out according to two methods depending on the equipment available in the laboratory: by determination of the haematocrit or by the determination of haemoglobin using a spectrophotometer.

D.2.1 Determination of the hematocrit (from a suspension of washed SRBC):

- Suspend washed SRBC
- Insert the end of a capillary in an almost horizontal position in the SRBC suspension
- Allow the liquid to rise by capillary action to ¾ of the capillary (Figure 1A)
- Wipe the outside of the capillary with absorbent paper (so as not to dirty the centrifuge)
- Plug the end of the capillary in contact with the blood using the "sealing compound" (or equivalent paste), by pushing the capillary into it (Figure 1B)
- Repeat the action with a second capillary (in order to balance the centrifuge)

- Place the two capillaries face to face in the hematocrit centrifuge, the sealed end outward (Figure 1C)
- Close the cover
- Centrifuge 4 min at 10,000 rpm
- Read the hematocrit (in%) (Figure 1D):
  - Align the cover so that the last notch of the spiral (red arrow) is at the level of the trace of the total volume present in the capillary
  - Reading: the hematocrit, expressed as a percentage, represents the volume occupied by red blood cells in the total volume of blood, thereby visually determining the dark red demarcation (blue arrow). If the values of the two capillaries are different, take the average to determine the final value

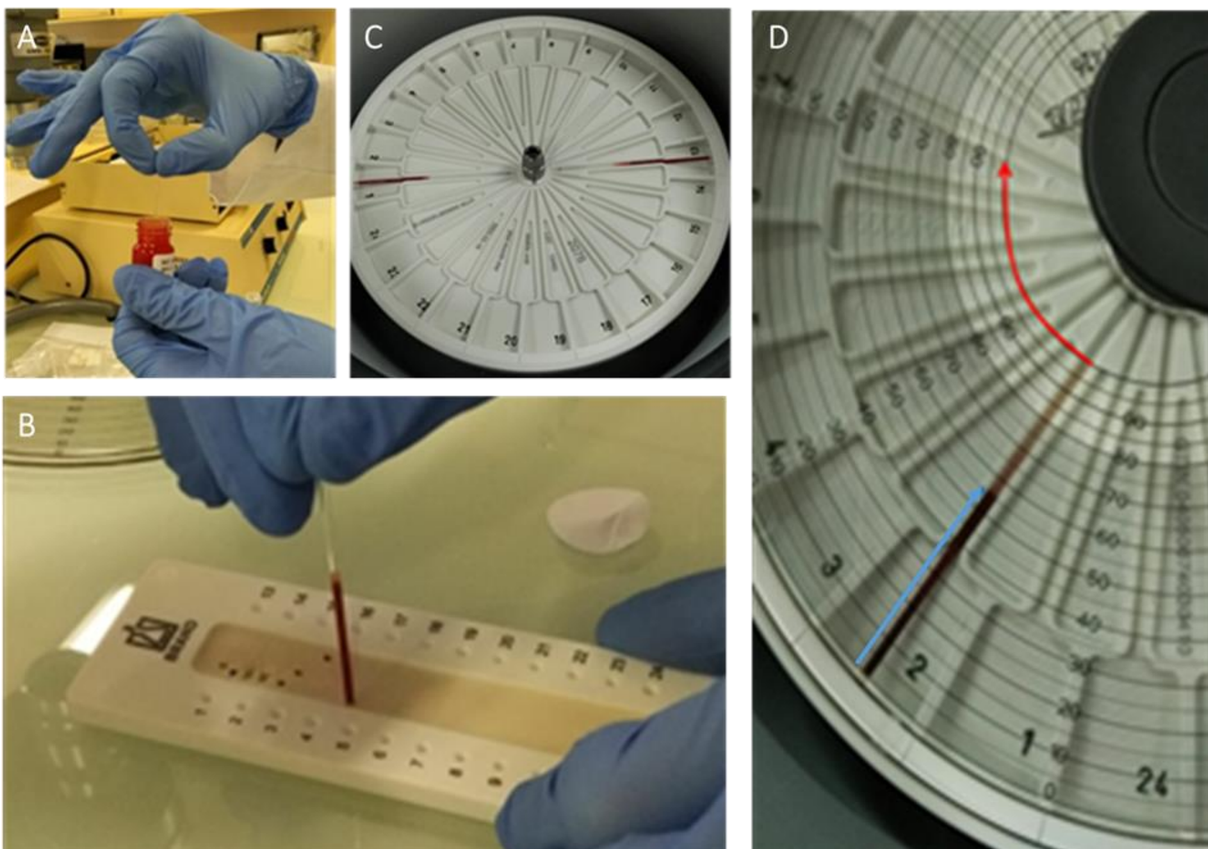

Figure 1: Illustration of the different steps for determining the haematocrit.

- Dilute the suspension of washed SRBC according to the table below (for a volume of 10mL) e.g 2.5%:

Preparation of SRBC at 2.5 %

Hematocrit (%)	Volume SRBC (mL)	Volume MgCa Buffer (mL)	Hematocrit (%)	Volume SRBC (mL)	Volume MgCa Buffer (mL)
5	5,00	5,00	33	0,76	9,24
6	4,17	5,83	34	0,74	9,26
7	3,57	6,43	35	0,71	9,29
8	3,13	6,88	36	0,69	9,31
9	2,78	7,22	37	0,68	9,32
10	2,50	7,50	38	0,66	9,34
11	2,27	7,73	39	0,64	9,36
12	2,08	7,92	40	0,63	9,38
13	1,92	8,08	41	0,61	9,39
14	1,79	8,21	42	0,60	9,40
15	1,67	8,33	43	0,58	9,42
16	1,56	8,44	44	0,57	9,43
17	1,47	8,53	45	0,56	9,44
18	1,39	8,61	46	0,54	9,46
19	1,32	8,68	47	0,53	9,47
20	1,25	8,75	48	0,52	9,48
21	1,19	8,81	49	0,51	9,49
22	1,14	8,86	50	0,50	9,50
23	1,09	8,91	51	0,49	9,51
24	1,04	8,96	52	0,48	9,52
25	1,00	9,00	53	0,47	9,53
26	0,96	9,04	54	0,46	9,54
27	0,93	9,07	55	0,45	9,55
28	0,89	9,11	56	0,45	9,55
29	0,86	9,14	57	0,44	9,56
30	0,83	9,17	58	0,43	9,57
31	0,81	9,19	59	0,42	9,58
32	0,78	9,22	60	0,42	9,58

D.2.2 Determination of haemoglobin using a spectrophotometer (from a suspension of washed SRBC):

D.2.2.1 Measure of hemoglobin

- Remove the supernatant (which must not be hemolyzed)
- Estimate the volume of the pellet and add MgCa Buffer up to 5 mL (ex: for estimated volume of 1 mL of the red blood cell pellet, add 4 mL of MgCa Buffer)
- Transfer the contents to a 50 mL Falcon tube
- For an estimated volume of 1 mL of red blood cells, add 25 mL of MgCa Buffer. Be careful, for this step, it is better to dilute less than to dilute too much, because it is then easier to adjust the OD541nm from a suspension that is too concentrated

- In a new 15 mL Falcon tube, put 1 mL of the suspension in 9 mL of distilled water - Mix well for complete lysis of red blood cells
- Put 1 mL of this solution in a spectrophotometer cuvette
- **Measure the optical density at the wavelength 541 nm**

D.2.2.1 Preparation of SRBC at 2.5%

The reference optical density (OD) at 541 nm for a 2.5% red blood cell suspension is 0.625 (± 0.005) corresponding to n = 6x108 sheep red cells in 1 mL.

- If $\frac{\text{OD}}{0.625} = 1$ That means = 2.5 p. cent **OK**

- If $\frac{\text{OD}}{0.625} > 1$ That means > 2.5 p. cent **(suspension too concentrated)**

Example: 0.650 / 0.625 = 1.04 ⟶ 1 – 1.04 = - 0.04
We must add 0,04 mL of MgCa Buffer per mL of suspension (e.g.: for 30 mL of suspension, add 1.2 mL of MgCa Buffer)
Measure again OD_{541nm} and readjust if necessary.

- If $\frac{\text{OD}}{0.625} < 1$ That means <2,5 p. cent **(suspension not concentrated enough)**

Example: 0.610 / 0.625 = 0.976 ⟶ 1 – 0.96 = 0,024

We must remove 0,024 mL of MgCa Buffer per mL of suspension. Centrifuge 5min at 1000g.
Remove 0,024 mL of MgCa Buffer per mL of suspension (e.g.: for 30 mL of suspension, remove 0.72 mL of MgCa Buffer)
Measure again OD_{541nm} and readjust if necessary.

This standardized SRBC suspension at 2.5% can be stored for 7 days at 5°C.