SEROLOGICAL DIAGNOSIS OF BRUCELLOSIS
BY ELISA METHOD

ANALYSIS OF SERA AND PLASMAS

INDIVIDUAL SAMPLES AND POOLS OF UP TO 10 SERA

(960 reactions)

POURQUIER® Elisa Brucellosis Individual and Pool Serum Screening
VERSION: P04130/10
INTRODUCTION

Brucellosis is a zoonosis found throughout the world with major implications both in the field of public health and farming economy. It is most often caused by Brucella abortus (bovines) or Brucella melitensis (small ruminants). It causes abortions and the bacteria are excreted in the milk.

Man is contaminated by contact with or ingestion of infected products (milk, cheese, meat). The main signs of contamination are undulant fever, with serious and frequent complications during a chronic evolution.

The extent of this disease has led health authorities to implement screening programs to detect infected animals and to eradicate the disease.

Brucellosis screening is mainly done with serological tests (Rose Bengal Test, Wright’s Serum Agglutination Test, Complement Fixation Test, Ring-Test and ELISA), which are the only tests applicable to mass screening programs.

Among the serological techniques used, the ELISA method is the most recent application and is particularly well suited to automation. This test kit allows to analyse individual sera or plasma samples and pools of maximum 10 sera or plasmas.

PRINCIPLE OF THE TEST

The principle of the test is:

1) All the wells of the microplate are coated with the lipopolysaccharide (LPS) of Brucella abortus.
2) The samples to be tested are diluted and incubated in the wells. Any antibodies specific to Brucella present in the sample will form a LPS-antibody immune-complex and remain bound in the wells.
3) After washing, a Peroxidase conjugated anti-ruminant IgG is added to the wells. This conjugate will bind to the immune-complex.
4) After another washing step, the enzyme substrate (TMB) is added to the conjugate, forming a blue compound becoming yellow after blocking. The intensity of the colour is a function of the rate of antibodies present in the sample to test.

The cut-off is set with a positive control serum, which must be added to each microplate.

This kit allows analysing individual sera or plasma samples and pools of maximal 10 sera or plasmas according to the requirements defined in the modification project of the European Directive (CEE 64/432, modifications of Annex C dated on 21/03/2002). It allows analysing individual samples and pools of 10 samples using the same test procedure and only one positive control sample.

PRECAUTIONS FOR USE

1) Do not place the pipette in the mouth when testing reagents.
2) Avoid contact of the substrate (TMB*) with skin, mucous membranes and eyes.
3) "Stop-solution" contains H₂SO₄*(0,5M) acid, which can cause serious burns in case of contact with skin, mucous membranes and eyes.
4) Even if the material in the package does not contain any contaminating element, and that the sera are, in theory, non – infectious, it is nevertheless advised to decontaminate the whole disposable elements, either by immersion for at least 1 hour in freshly prepared 5% sodium
hypochlorite, or by autoclaving them at 120°C for a minimum of 1 hour or by any other method in accordance with the reglementation in force, before discarding.

* The toxicity form of the product is available at the Institut Pourquier.
### KIT CONTENTS AND STORAGE

It is recommended to bring at room temperature (21°C ± 5°C) all the reagents of the kit at least one hour before use (except the conjugate and the control samples).

<table>
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<th>COMPONENTS</th>
<th>QUANTITY</th>
<th>STORAGE AND NOTES</th>
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</table>
| Mono-well Coated microplates | 10 | +5°C (± 3°C)  
  - If a microplate is not entirely used, it may be stored for later use during 3 months if it is immediately closed in an airtight container and stored at +5°C (± 3°C). |
| Concentrated (x 20) wash solution | 2 x 100 ml bottles | +5°C (± 3°C)  
  - May form crystals at +5°C (± 3°C), which rapidly disappear at +21°C (± 5°C). A gentle shaking of the solution will accelerate the dissolution of the crystals.  
  - This solution can also be stored at +21°C (± 5°C) for 1 month, if the vials are closed in an airtight way, in order to be immediately ready to use when needed.  
  - After dilution, the Wash Solution can be stored for 3 days at +5°C (± 3°C).  
  - The same for all the INSTITUT POURQUIER kits and can be used equally in the different kits. |
| Dilution Buffer 2 light green (for samples) | 2 x 120 ml bottles | +5°C (± 3°C) |
| Dilution Buffer 1 light blue (for conjugate) | 1 x 120 ml bottle | +5°C (± 3°C) |
| Positive control | 1 x 1 ml bottle  
  Negative control | 1 x 1 ml bottle | +5°C (± 3°C) |
| Monoclonal anti-ruminant IgG / Peroxidase conjugate | 1 x 1,5 ml bottle | +5°C (±3°C)  
  - The diluted solution of the conjugate cannot be conserved. |
| Revelation solution 3 (TMB) | 1 x 120 ml bottle | +5°C (±3°C)  
  - This solution can be slightly bluish at +5°C (±3°C) and becomes colourless at +21°C (±5°C).  
  - It can be left on the draining board at +21°C (±5°C) up to 1 week (if the vial is well closed in an airtight way), in order to be immediately ready to use when needed. |
| Stop Solution (H₂SO₄ 0.5M solution) | 1 x 120 ml bottle | +5°C (±3°C)  
  - It can be stored at +21°C (± 5°C) up to 1 month (if the vial is well closed in an airtight way), in order to be immediately available when needed.  
  - The same for all the kits of the INSTITUT POURQUIER, it can be used equally in the different kits. |

**Using Instructions**
MATERIALS REQUIRED BUT NOT INCLUDED IN THE KIT

1) Microplate spectrophotometer
2) Centrifuge
3) Centrifuge tubes and microtubes
4) Vortex or similar shaker
5) Microplate washing system that distributes 300 µl per well
6) Precision micropipettes and multi-dispensing micropipettes (The precision required must be lower or equivalent to 10% for volumes lower or equivalent to 10 µl and to 5% for all the other volumes indicated)
7) Disposable pipette tips
8) Distilled water: The water used for the reconstitution of the control sera or concentrated wash solution may be produced by a conventional distillation system or any other high-performance water purification system (reverse osmosis, resin or activated charcoal purification ...).
9) Microplate covers (lid, aluminium foil or adhesive)

INSTRUCTIONS FOR USE

1) DEPOSITING THE SAMPLES
For the analysis of individual sera or plasmas, the user can incubate the samples either for 1 hour, or overnight (16 – 24 h). For the analysis of pools of sera or plasmas, only the day incubation has to be used. The dilution of the samples and the temperature of incubation (+21°C (± 5°C)) are the same in both cases.

- Sera (controls and samples) are diluted to 1/20 using the following method:
  - Dispense:
    - 190 µl of “dilution buffer 2” per well.
    - 10 µl of negative control in A1
    - 10 µl of positive control in B1 and C1
    - 10 µl per well of each serum sample to test
  (only one well per sample to test, see fig. 1):
  - Homogenise the contents of the wells by shaking delicately the plate (see note 1)
  - Cover the plate (with a lid, aluminium foil or adhesive)
  - Incubate for 1 hour (±5 min) or overnight (16 – 24 h) at +21°C (± 5°C).

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>P</td>
<td>P</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
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(N = Negative Control
P = Positive Control
1 = Sample n° 1
2 = Sample n° 2
3 = ............................

Figure 1: Distribution of the Samples

Notes:
1) In our laboratories, a microplate shaker originally designed for a Complement Fixation Test micromethod, is used
2) The individual filling of the 96 wells is sometimes a long process. In order to standardize the sera incubation time, the controls and serum samples are prepared in plates with 96 U-shaped-bottom-wells. It is therefore possible to transfer them rapidly (column by column) using a multi-dispensing pipette. It is nevertheless essential to dilute the samples in the same way as for the controls. When the samples are incubated in microtubes, the transfer in the plate can be done directly column by column by using a multi-dispensing pipette.
3) The position of the control sera in A1, B1 and C1 is not very important. They may be dispensed anywhere on the plate.
2) WASHING
a) Dilute a vial of "concentrated (X 20) wash solution" in 1900 ml of distilled water. This solution is hereafter called "wash solution". The dilution can be carried out before the elimination of crystals appeared at \(\pm 5°C\) \(\pm (\pm 3°C)\), so long as the whole 100 ml vial is used.
b) Empty the content of the plate by «flick-off» or better by a manual or automatic method.
c) Fill all the wells of the plate with the wash solution; then empty them again.
d) Repeat the step c) twice (a total of 3 washes).
Note: When several plates are processed at the same time, it is possible (in order to synchronize all the steps) to leave the plates full of "Wash solution" during one hour without modifying the validity of the test.

3) DEPOSITING THE CONJUGATE
a) The dilution of the conjugate depends on the incubation mode used for the samples:

   For a short incubation, 1 hour – incubation, (analysis of individual samples or pools):
   - Dilute the conjugate to 1:100 in dilution buffer n° 1 and dispense 100 µl of the diluted conjugate per well.

   For a night incubation (analysis of individual samples only):
   - Dilute the conjugate to 1:200 in dilution buffer n° 1 and dispense 100 µl of the diluted conjugate per well.

b) Cover the plate (with a lid or aluminium foil) and incubate for 30 minutes \((\pm 3\ min)\) at \(21°C\) \((\pm 5°C)\).

4) WASHING
a) Empty the content of the plate by «flick-off » or by some other way or by an automatic method.
b) Fill all the wells on the plate with the wash solution; then empty them again.
c) Repeat the step b) twice (total of 3 washes)

Notes:
1) Particular care with the last wash is very important in getting a good test result.
2) If the wash is carried out with a manual method, it is possible after the last wash to drum the microplate on an absorbent support in order to empty the wells completely.

5) REVELATION
a) Dispense 100 µl of "Revelation Solution n° 3" ready to use per well.
b) Incubate the plate at +21°C \((\pm 5°C)\) for 20 minutes (away from the light).
c) Dispense 100 µl of "Stop Solution" per well.
d) Shake gently the plate until the coloured solution is homogenised. Wipe carefully the underside of the plate.

Note:
1) The 20 - minute - revelation period, which is indicated in the method, gives the O.D. values provided in the paragraph "Validation Criteria", when performed in our laboratories. However the rate of colour revelation can be slightly modified by different factors (quality of the washes, quality of water used, precision of the pipetting, temperature of the reaction,...). Depending then on these factors, the revelation step may give O.D. values higher or lower than those expected. In this case, the reaction may be stopped at 20 min \((\pm 10\ min)\).
2) The reading can be done, up to one hour after the reaction has been stopped provided that the plates are kept in the dark.

6) READING
Read the optical densities at 450 nm (OD.450). The photometer must first be blanked on air.
VALIDATION CRITERIA

The plates are validated, if:

- a minimal mean OD.350 value of 0.600 for the positive control is obtained.
- and
- the ratio between the mean OD.450 value of the positive controls and OD.450 value of the negative control is greater than or equal to 3.

INTERPRETATION

Calculate for each sample the S/P percentage:

\[
S/P\% = \frac{100 \times (OD_{450\text{ value of the sample}} - OD_{450\text{ value of the negative control}})}{(\text{mean OD}_{450\text{ value of the positive control}} - OD_{450\text{ value of the negative control}})}
\]

INDIVIDUAL SERUM AND PLASMA SAMPLES

- Any sample with a S/P% equal or lower than 110% are considered to be from animals, which do not have any specific antibodies to the Brucella abortus LPS.
- Any sample with a S/P% between 110% and 120% are considered to be doubtful. A second determination is necessary to determine the status of these sera.
- Any sample with a S/P% equal or higher than 120% are considered to be from animals, which have specific antibodies to the Brucella abortus LPS.

POOL SERA:

- Any sample with a S/P% equal or lower than 20% are considered to be from a group of animals, which do not have any specific antibodies to the Brucella abortus LPS.
- Any sample with a S/P% higher than 20% are considered to be from a group of animals, in which at least one animal has specific antibodies to the Brucella abortus LPS.

Note:
1) Since ELISA has a higher sensitivity than other conventional methods, the positive reaction will not always be confirmed by Wright, Rose Bengale or CF tests.
2) Some individual sera showing OD values near the threshold can give divergent results depending on the incubation way used. In case of divergent results, it is advised to take preferentially into account the result obtained with the 1-hour-incubation. Indeed, sporadic increases of background noises in night incubation can classically be observed, particularly in zones where atypical reactions appear.

Samples | Incubation | Interpretation
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<tr>
<td>Individual samples (sera or plasmas)</td>
<td>1 hour (± 5mn) at +21°C (± 5°C) or 1 night et +21°C (± 5°C)</td>
<td>%S/P ≤ 110%</td>
</tr>
<tr>
<td>Pools of sera</td>
<td>1 hour (± 5mn) at +21°C (± 5°C)</td>
<td>%S/P ≤ 20%</td>
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BIBLIOGRAPHY


POURQUIER Institute
POURQUIER® ELISA Brucellosis Individual and Pool Serum Screening - Version P04130/10 - Page 6/5
- Programme n°109 of COFRAC