SEROLOGICAL DIAGNOSIS OF ENZOOTIC BOVINE LEUKOSIS

BY ELISA METHOD

(SENSITIVITY INDEX: POOL OF 10 SERA)

PLASMA AND SERA

(960 Reactions / 10 plates)

(480 Reactions / 5 plates)

POURQUIER®ELISA Bovine Leukosis Screening
VERSIO N: P02110/14
INTRODUCTION

Enzootic Bovine Leukaemia (EBL) is an infectious lymphoproliferative disease in cattle which occurs throughout the world. The disease is caused by an exogenous C-type retrovirus, bovine leukaemia virus (BLV) which established a persistent infection in a sub-population of B lymphocytes by integration of proviral DNA at a number of sites on the cellular DNA.

The majority of infected cattle remain healthy for life, although approximately 30% of the infected animals develop a persistent lymphocytosis and a small proportion (up to 10%) develops lymphoid tumours.

The disease affects mainly dairy herds and spreads predominantly though horizontal transmission by exposure to blood or secretions containing infected lymphocytes.

As no treatment or vaccine is available, the eradication programs are based on identification and elimination of infected animals, which is realised mainly by detection of anti-viral antibodies. The infected cattle produce specific antibodies to the major viral proteins from an early stage of infection.

Initially, the agar-gel immunodiffusion (AGID) test for detection of antibodies to the viral surface glycoprotein, gp 51, was widely used. However, the sensitivity of AGID test is limited, and cases have been reported were infected animals fails to produce a detectable antibody response.

For this reason, the screening of antibodies is now carried out by ELISA test that is more simple, rapid, and above all more sensitive.

This kit is designed to analyses sera and plasma. It is based on the use of an ultrapurified virus lysate and can be used to demonstrate the presence of all types of antibodies against the virus (envelope proteins, capsid proteins, etc...)

Note: a focused research of specific anti-gP 51 antibodies can be implemented with a blocking ELISA (product reference: P02140).

PRINCIPLE OF THE TEST

The principle of the test is:

1) All the wells of the polystyrene microplates are coated with the viral antigen (BLV).
2) Samples to be tested are diluted to 1/20 (for a short incubation period) and to 1/50 (for a long incubation period) and incubated in the wells. Any antibody specific to BLV present in the sample, will form a BLV-antibody immune-complex and remains bound in the wells.
3) After washing, an anti-bovine antibody immunoglobulin linked to an enzyme is incubated. This conjugate is bound to the immune-complex.
4) After another washing, the enzyme substrate (TMB) is added to the wells. If the conjugate is fixed to the immune-complex, The enzyme transforms the substrate into a blue compound becoming yellow after blocking. The intensity of the colour is a function of the level of antibodies present in the sample to be tested.

The cut-off is determined by using a control serum supplied with the kit ("positive control") which allows the detection of the European standard serum E5 diluted to 1/100 in negative serum. This positive control serum must be added to each microplate.
**KIT CONTENTS and STORAGE**

It is recommended to work with all the components at +21°C (± 5°C). Thus all reagents must be put at room temperature at least one hour before the beginning of the test (except the conjugate).

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>QUANTITY</th>
<th>STORAGE AND NOTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monowell Coated microplates</td>
<td>10</td>
<td>+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).</td>
</tr>
<tr>
<td>Concentrated (20x) wash solution</td>
<td>2 x 100 ml bottles 1 x 100 ml bottle</td>
<td>+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) up to 1 month, if the vials are closed in an airtight way, in order to be immediately available when needed. • The &quot;Concentrated (x 20) wash solution&quot; is the same for all the kits of the INSTITUT POURQUIER and can be used equally in the different kits. • After dilution, the wash solution can be stored for 3 days at +5°C (±3°C)</td>
</tr>
<tr>
<td>Dilution Buffer 2</td>
<td>2 x 120 ml bottles 1 x 120 ml bottle</td>
<td>+5°C (±3°C)</td>
</tr>
<tr>
<td>Dilution Buffer 1 sky blue</td>
<td>1 x 120 ml bottle 1 x 120 ml bottle</td>
<td>+5°C (±3°C)</td>
</tr>
<tr>
<td>Positive control</td>
<td>1 x 1 ml bottle 1 x 1 ml bottle</td>
<td>+5°C (±3°C)</td>
</tr>
<tr>
<td>Negative control</td>
<td>1 x 1 ml bottle 1 x 1 ml bottle</td>
<td>+5°C (±3°C)</td>
</tr>
<tr>
<td>Monoclonal anti-bovine IgG / peroxidase conjugate</td>
<td>1 x 1,5 ml bottle 1 x 1,5 ml bottle</td>
<td>+5°C (±3°C) • The diluted conjugate solution cannot be stored</td>
</tr>
<tr>
<td>Revelation solution 3 (TMB) Ready to use</td>
<td>1 x 120 ml bottle 1 x 120 ml bottle</td>
<td>+5°C (±3°C) • This solution can be slightly bluish at +5°C (±3°C) and becomes colourless at +21°C (±5°C). • Thus 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 available when needed.</td>
</tr>
<tr>
<td>Stop Solution (H₂SO₄ 0,5M solution)</td>
<td>1 x 120 ml bottle 1 x 120 ml bottle</td>
<td>+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. • Identical for all the kits of the INSTITUT POURQUIER, it can be used equally in the different kits.</td>
</tr>
</tbody>
</table>

Using Instructions
**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 H$_2$SO$_4$*(0,5M) acid, that 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 samples 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 regulation in force, before discarding.

* The Pourquier Institute is at your disposal to supply the toxicity form of the product.

**MATERIALS REQUIRED BUT NOT PROVIDED**

1) Microplates reader
2) Centrifuge
3) Centrifuge tubes and microtubes
4) Vortex or similar
5) Microplate washing system that distributes 300 µl per well
6) Precision Micropipettes and Multi-dispensing micropipettes (the precision required must be ≤ 10% for volumes lower or equivalent to 10 µl and 5% for all the other volumes indicated)
7) Disposable pipette tips
8) Distilled water: the water used for the reconstitution of the controls and of the wash solution can 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)
10) Incubator at +37°C (±3°C)

**INSTRUCTIONS FOR USE**

1) **DEPOSITING THE SAMPLES**

The user can incubate sera in two different ways:

**SHORT INCUBATION: 1 HOUR (± 5 MIN) AT 37°C (± 3°C)**

Sera/plasma (controls and samples) are diluted to 1/20 and incubated for 1 hour (± 5 min.) at 37°C (± 3°C) using the following method (see notes 3 and 4):

- Dispense (following figure 1)
  - 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 of each sample (plasma, serum or pool of sera) to be tested (only one well per sample to test)

- Homogenize the contents of the wells by gently shaking the plate (see note 1)
- Cover the plate (with a lid, aluminium foil or adhesive) and leave to incubate for 1 hour (± 5 min) at 37°C (± 3°C).

This method enables the test to be implemented in less than half a day.
LONG INCUBATION AT LOW TEMPERATURE: OVERNIGHT 16h-24h AT +5°C (± 3°C)

- Prepare a 1/50 dilution in "Dilution buffer 2" of controls and samples (sera, or pool of sera, or plasma) to be tested. (see note 2).
- Dispense (following figure 1)
  - 200 µl of the 1/50 diluted negative control in A1
  - 200 µl of the 1/50 diluted positive control in B1 and C1
  - 200 µl of each sample (serum or pool of sera, or plasma) to be tested
    (only one well per sample to test)
- Cover the plate (with a lid, aluminium foil or adhesive) and leave to incubate overnight at +5°C (± 3°C).

This method allows to dispense the samples in the evening and to develop the assay the day after.

<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>
</tr>
</tbody>
</table>

Notes:
1) In our laboratories, a microplate shaker originally designed for a complement fixation test micro method is used.
2) In order to facilitate automatic methods, a volume of 250 µl of samples or controls per well (diluted to 1/50) may be used, with no consequence on the quality of discrimination between positive and negative samples.
3) The individual filling of the 96 wells is sometimes a long process. In order to standardize the sample incubation time, the controls and the samples are prepared in 96-well plates with U-shaped bottoms. It is therefore possible to transfer them rapidly (column by column) by using a multi-channel pipette. It is nonetheless essential to make the dilutions of the samples in the same way as for the controls.
4) The position of the control sera in A1, B1 and C1 is not important. They may be dispensed anywhere on the plate.
5) In order to check the reaction, the European Standard (E5/10) diluted to 1/10 in a negative serum may also be used.
2) **WASHING**

a) Dilute a vial of "Concentrated (20x) wash solution" in 1900 ml of distilled water. This solution is hereafter called "Wash solution". The dilution can be carried out before the disappearance of the crystals which previously appeared at +5°C (±3°C) as long as the whole 100 ml vial is used.
b) Empty the content of the plate by returning or by another manual or automatic method.
c) Fill all the wells of the plate with the Wash solution; and then empty them again.
d) Repeat step c) twice (a total of 3 washes).

**Note:**
If a lot of 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» up to one hour without any modification on the validity of the results.

3) **DEPOSITING THE CONJUGATE**

a) Dilute this conjugate to 1/100 with the "Dilution buffer 1".
b) Dispense 100 µl of diluted conjugate in each well.
c) Cover the plate (with a lid or aluminium foil or adhesive) and leave to incubate for 30 minutes (±3 min) at 37°C (±3°C)

4) **WASHING**

a) Empty the content of the plate by returning it or by another manual or automatic method.
b) Fill all the wells of the plate with the wash solution; and then empty them again.
c) Repeat step b) twice (a total of 3 washes).

**Note:**
The care brought to the last washing is essential for a good implementation of the test. If the washing is manual, it is possible, after the last washing, to tap the plate on an absorbent support in order to completely empty the wells.

5) **REVELATION**

a) Dispense 100 µl of "Revelation Solution 3" ready to use per well
b) Incubate 20 minutes at +21°C (±5°C) (away from the light)
c) Add 100 µl of "Stop solution" per well.
d) Gently shake the plate until the coloured solution is homogenized. Wipe carefully the underside of the plate.

**Notes:**
1) The 20 - minute - revelation period, which is indicated in the method, gives the O.D. values provided in the paragraph "INTERPRETATION", when implemented in our laboratories. However the rate of colour revelation can be slightly affected by different factors (quality of the washes, quality of the water used, precision of the pipetting, temperature of the reaction...). Regarding the work conditions, the revelation step may give OD values higher or lower than those expected. So, the user may stop the reaction after 20 minutes (±10 minutes).
2) The reading can be done up to 1 hour after having stopped the reaction on condition that 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 results can be considered reliable if:

- The positive control has a **minimal mean OD 450 value of 0.350** and
- The ratio between the mean OD 450 value of the positive control and the OD 450 value of the negative control is **greater than or equal to 3.**

INTERPRETATION

Calculate for each sample the S/P percentage:

$$S/P\% = 100 \times \frac{OD_{450 \text{ value of the sample} - OD_{450 \text{ value of the negative control}}}{OD_{450 \text{ value of the mean positive control} - OD_{450 \text{ value of the negative control}}}}$$

- Samples (individual or pool or plasma) with a **S/P% lower than or equal to 85%** are considered coming from animals or herds, which have not been in contact with the BLV virus.
- Samples (individual or pool or plasma) with a **S/P% between 85% and 115%** are considered to be doubtful.
- Samples (individual or pool or plasma) with a **S/P% greater than or equal to 115%** are considered coming from animals or herds, in which at least one animal has been in contact with the BLV virus.

**Note:**

1. It is highly recommended to confirm samples found with an S/P% between 85% and 115% by a second determination. If after this second determination, the samples show again a S/P% between 85% and 115%, these samples will be considered as doubtful samples. In this case, it would be better to confirm these results with a new blood sampling or another method.
2. Pools found positive have to be tested in individual serology.
3. Individual samples found positive can also be confirmed by our Elisa Leukosis Serum Blocking kit (P02140)

LEGEND

Φ = Modification in the using instructions