SEROLOGICAL DIAGNOSIS OF
ENZOOTIC BOVINE LEUKOSIS
BY BLOCKING ELISA METHOD
IN SERUM AND PLASMA SAMPLES
(individual and pool samples)

(SENSITIVITY INDEX: POOL OF 10 SERA)

(960 Reactions)
**INTRODUCTION**

Enzootic Bovine Leukosis (EBL) is an infectious lymphoproliferative disease in cattle which occurs throughout the world. The disease is caused by an exogenous C-type retrovirus, bovine leukemia 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% develop a persistent lymphocytosis and a small portion (up to 10%) develops lymphoid tumors.

The disease affects mainly dairy herds and spreads predominantly through 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 are produced 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 instances have been reported were infected animals failes to produce a detectable antibody response.

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

The principle of this kit is based on the competition between the BLV antibodies of the bovine serum and a peroxidase conjugate monoclonal anti-gp51 BLV antibody. The gp 51 is a major envelope glycoprotein which is well conserved by the BLV.

This kit permits to detect specific antibodies directed to BLV gp51 antigen in both individual and pools of 10 samples.

It is an important tool in complement of the other BLV ELISA indirect which uses an ultrapurified BLV lysate.

**PRINCIPLE OF THE TEST**

The principle of the test is:

1) All the wells of the microplate are coated with the viral antigen (BLV).

2) Samples to be tested are diluted and incubated in the wells. Any antibody specific to BLV gp 51 antigen present in the sample will form a gp51-antibody immune-complex, which effectively masks the gp 51 sites.

3) After washing, an anti-gp51 monoclonal antibody coupled to an enzyme, is incubated in the wells. In presence of specific gp51 antibodies in the sample, the gp51 sites are "masked" and the conjugate cannot bind on the corresponding epitop. On the contrary, the conjugate can bind on the BLV gp 51 antigen if the tested serum do not contain any specific gp51 antibodies.

4) After washing, the enzyme substrate (TMB) is added to the wells. If the conjugate is fixed in the wells (in the case of a negative serum), it transforms the substrate into a blue compound becoming yellow after blocking. The intensity of the colour is an inverse measure of the proportion of anti-gp 51 antibodies present in the serum sample to test.

The cut-off is determined by using a negative control serum supplied with the kit which has to be added to each microplate. This kit allows the detection of the European standard serum E4 diluted to 1/100 in negative bovine serum.
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 and the control samples).

<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)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 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>3 x 100 ml bottles</td>
<td>+5°C (± 3°C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 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.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 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.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 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.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• After dilution, the wash solution can be stored for 3 days at +5°C (± 3°C).</td>
</tr>
<tr>
<td>Positive control</td>
<td>1 x 2 ml bottle</td>
<td>+5°C (± 3°C)</td>
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<tr>
<td>Negative control</td>
<td>1 x 2 ml bottle</td>
<td></td>
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<tr>
<td>Monoclonal anti-gp51 / peroxidase conjugate</td>
<td>1 x 1,5 ml bottle</td>
<td>+5°C (± 3°C)</td>
</tr>
<tr>
<td>Revelation solution 2 (TMB)</td>
<td>1 x 120 ml bottle</td>
<td>+5°C (± 3°C)</td>
</tr>
<tr>
<td>Ready to use</td>
<td></td>
<td>• After preparation, the diluted conjugate solution cannot be stored longer than 8 hours at room temperature.</td>
</tr>
<tr>
<td>Stop Solution (H₂SO₄ 0,5M solution)</td>
<td>1 x 120 ml bottle</td>
<td>+5°C (± 3°C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 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.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 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) «Stop-solution» contains H₂SO₄*(0,5M) acid, that can cause serious burns in case of contact with skin, mucous membranes and eyes.
3) Even if the material delivered in the package does not contain any contaminating element, and that the bovine sera are, in theory, non-infectious, it is nonetheless advised to decontaminate the whole disposable elements used by immersion for a minimum of 1 hour in a freshly prepared 5% sodium hypochlorite, prior eliminating them, or by using the autoclave at 120°C for a minimum of 1 hour.

* 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)

INSTRUCTIONS FOR USE

1) PREPARATION OF WASH SOLUTION
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.

2) DEPOSITING THE SAMPLES
Controls and samples (individual or pool of samples) are diluted to ½ by using the following method:
- Dispense 50 µl of "wash solution" per well.
- Dispense:
  - 50 µl of undiluted positive control sample in A1
  - 50 µl of undiluted negative control sample in B1 and C1 (see notes 2 and 3)
- Dispense, according to figure 1, 50 µl per well of each undiluted sample (individual or pool of samples) to test (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)
- Leave to incubate for 30 minutes (± 3 min) at 21°C (± 5°C)
Notes:
1) The individual filling of the 96 wells is sometimes a long process. In order to standardize the sample incubation time, the controls and samples are prepared in plates with 96 U-shaped-bottom-wells. 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.
2) The position of the controls in A1, B1 and C1 is not important, they may be dispensed anywhere on the plate.
3) In order to check the reaction, the European Standard (E5/10) diluted to 1/10 in negative serum may also be used.

3) WASHING
   a) Empty the content of the plate by «flick-off» or better by a manual or automatic method.
   b) Fill all the wells of the plate with the wash solution; then empty them again.
   c) Repeat the step b) 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.

4) DEPOSITING THE CONJUGATE
   a) Dilute this conjugate to 1/100 with the "Wash Solution".
   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 15 minutes (± 1 min) at 21°C (± 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)

   Note:
   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 2" ready to use per well
   b) Leave to incubate for 10 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.

   Note:
1. The 10-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 pipeting, 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 at 10 minutes ± 5 min.

2. The reading can be done up to 1 hour after having stopped the reaction on condition 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.

Calculate for each serum the S/N percentage in relation to the negative control. This value is obtained in the following manner:

\[
S/N\% = \frac{OD\ 450\ of\ analysed\ sample}{mean\ OD\ 450\ of\ negative\ control} \times 100.
\]

VALIDATION CRITERIA

The reaction is considered as valid, if the following criteria are obtained:

- the negative control has a minimal mean OD 450 value of 0.800
- the S/N percentage of the positive control is equal to or lower than 20%.

INTERPRETATION

- Samples (individual or pools) with S/N percentage equal or greater than 40% are considered coming from an animal that does not carry specific antibodies to the BLV gp51 antigen.
- Samples (individual or pools) with S/N percentage lower than 40% are considered coming from an animal that carries specific antibodies to the BLV gp 51 antigen.

SUMMARY

<table>
<thead>
<tr>
<th>INTERPRETATION</th>
<th>POSITIVE</th>
<th>NEGATIVE</th>
</tr>
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<tbody>
<tr>
<td>SAMPLES</td>
<td>&lt; 40 %</td>
<td>≥ 40 %</td>
</tr>
</tbody>
</table>

LEGEND

 Modification in the using instructions (= modification concerning the “Instruction for use” or “Validation criteria” or “Interpretation”)