IMMUNOLOGICAL DIAGNOSIS OF ENZOOTIC BOVINE LEUKOSIS IN MILK
BY ELISA METHOD

(SENSITIVITY INDEX: 100 MILKS)

480 REACTIONS
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.

PRINCIPLE OF THE TEST

The principle of the test is:

1) The wells of the polystyrene microplates are coated with viral antigen (BLV) (only the even-numbered columns are coated with the specific antigen).

2) Milk samples to be tested are diluted and incubated in the wells. Any BLV specific antibody present in the milk forms a BLV-antibody immune-complex and remains bound to the wells.

3) After washing, a peroxidase conjugated anti-bovine antibody IgG is added into the wells. This conjugate will bind to the immune-complex.

4) After another washing step, the enzyme substrate (TMB) is added to the wells. If the immune-complex is present, the enzyme of the conjugate forms a blue compound becoming yellow after blocking. The intensity of the colour is a function of the rate of antibodies present in the milk sample to be tested.

The limit of positivity is set by using a positive control supplied in the kit ("positive control milk") which allows detecting the European Standard (E5/250) diluted to 1/100 in bovine milk obtained from uninfected cattle. The positive control must be added into each microplate.

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 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 Pourquier Institute is at your disposal to supply the toxicity form of the product.
**KIT CONTENTS and STORAGE OF REAGENTS**

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

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>QUANTITY</th>
<th>STORAGE AND NOTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biwell 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). • The manufacturing process of the plates can lead to the appearance of deposits or crystals at the bottom of the wells, without modifying the reaction.</td>
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<tr>
<td>Concentrated wash solution (20x)</td>
<td>2 x 100 ml bottles</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) for 1 month, if the vials are closed in a watertight way, in order to be immediately available when needed. • The &quot;Concentrated wash solution (20x)&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 1 (sky blue)</td>
<td>3 x 120 ml bottles</td>
<td>+5°C (±3°C)</td>
</tr>
<tr>
<td>Positive control (freeze-dried)</td>
<td>2 x 2 ml bottles</td>
<td>+5°C (±3°C) • After reconstitution, the positive and the negative control milks must be stored in aliquots at a temperature ≤ -16°C. • They can be frozen and unfrozen up to three times with no loss of activity. • A storage at +5°C (±3°C) will lead to a significant increase of the background noises.</td>
</tr>
<tr>
<td>Negative control (freeze-dried)</td>
<td>2 x 2 ml bottles</td>
<td>+5°C (±3°C)</td>
</tr>
<tr>
<td>Monoclonal anti-bovines IgG / peroxidase conjugate</td>
<td>1 x 1,5 ml bottle</td>
<td>+5°C (±3°C) • The diluted conjugate solution cannot be stored and should be used within the 8 hours</td>
</tr>
<tr>
<td>Revelation solution 2 (TMB) Ready to use</td>
<td>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</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.</td>
</tr>
<tr>
<td>Using Instructions</td>
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MATERIALS REQUIRED BUT NOT INCLUDED IN THE KIT

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 required precision must be ≤ 5% for all the volumes indicated)
7) Disposable pipette tips
8) Distilled water: the water used for the reconstitution of control milks 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 MILK SAMPLES

a) Reconstitution of the controls:
- Reconstitute the negative and the positive control with 2 ml distilled water.

b) Dispense 100 µl of "Dilution Buffer 1" per well.

c) Following figure 1(see notes 1 and 2), dispense:
- 100 µl of undiluted negative control milk in A1 and A2
- 100 µl of undiluted positive control milk in B1 / B2 and C1 / C2
- 100 µl of each undiluted milk sample to test in:
  - 1 coated well (even-numbered columns)
  - 1 uncoated well (odd-numbered columns)

Note: Milk samples may be skimmed or full fat milk.

d) Cover the plate (with a lid, aluminium foil or adhesive)

e) The user can incubate the milks in two ways:

SHORT INCUBATION: 90 minutes (± 5 min.) at 37°C (± 3°C)

LONG INCUBATION AT A LOW TEMPERATURE: overnight (16h to 24h) at +5°C (± 3°C)

ANN = Negative Control
BPP = Positive Control
1 = Sample n° 1
2 = Sample n° 2
3 = ..............................

Figure 1: Distribution of the milks
Notes:
1. The individual filling of the 96 wells is sometimes a long process. In order to standardize the milk incubation time, we prepare the controls and milk samples in 96 U-shaped-bottom-well plates. It is therefore possible to transfer them rapidly (column by column) using a multi-channel pipette.
2. The position of the control milks in A1, A2, B1, B2 and C1, C2 is not important. They may be dispensed anywhere on the plate. It may be better to add several times control milks to the same plate in order to establish an average threshold value. It is recommended to put a repetition of the controls in the centre of the plate. However, it is essential to dilute the samples in the same way as for the controls.
3. In order to check the reaction, the user may also use the European Standard for Milks (E5/250) diluted at1/100 in negative control milk.
4. If the temperature of incubation in the case of the long incubation period is not respected, the quality of discrimination between negative and positive milks will not be modified, but the final level of OD value will be affected.

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 +5°C (±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).

Notes:
1. If milks are skimmed (or taken under the cream), this type of washing is sufficient. With full fat milk, it can be necessary to modify this method of washing: if a visual control reveals whitish traces in the wells, it is advised to leave the solution of washing in contact 1 to 3 times 3 minutes. Indeed, these contact times allow the elimination of fat particles that are likely to fix the conjugate in the next step in a non-specific way.
2. 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
The dilution of the conjugate and the incubation time depend on the incubation way used for the milk samples:

- If the user has chosen a SHORT INCUBATION PERIOD FOR THE SAMPLES:
  - Dilute the conjugate to 1/100 in "Dilution Buffer 1" and dispense 100 µl per well.
  - Cover the plate (with a lid or aluminium foil) and incubate for 30 minutes (± 3 mn) at 37°C (± 3°C)

- If the user has chosen a LONG INCUBATION PERIOD FOR THE SAMPLES AT LOW TEMPERATURE:
  - Dilute the conjugate to 1/400 in "Dilution Buffer 1" and dispense 100 µl per well.
  - Cover the plate (with a lid or aluminium foil) and incubate for 1 hour (± 5 mn) at 37°C (± 3°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) **REVEALATION**

a) Dispense 100 µl of “Revelation Solution 2” ready to use per well.

b) Incubate the plate at +21° (± 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 homogenized. 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 "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**

a) Read the optical densities at 450 nm (OD.450). The photometer must first be blanked on air.

b) Calculate the **corrected OD.450** for each milk sample:

Subtract the OD.450 value obtained from the uncoated well from the OD.450 of the coated well.

**VALIDATION CRITERIA**

The results can be considered reliable if:

- The positive control has **a minimal mean OD 450 value of 0.300 (uncorrected)**
- and
- The **ratio** between the mean corrected OD.450 value of the positive control and the corrected OD.450 value of the negative control is **equal or greater than 3**

**Note:**

1- The corrected OD.450 of the positive control can be « negative » or equal to zero. In that case, use the absolute value for the validation.

2- If the corrected OD.450 of the negative control is equal to zero, you can use the mean 0.001 to calculate the ratio

**INTERPRETATION**

Calculate for each sample the S/P percentage:

$$S'/P'\% = \frac{\text{corrected. OD 450 of the sample}}{\text{mean corrected. OD 450 of the positive control}} \times 100$$

- Any milk sample (individual or pools) with a **S'/P'\% equal or lower than 60%** is considered coming from herd which has not been in contact with the BLV virus.

- Any milk sample (individual or pools) with a **S'/P'\% between 60% and 70%** is considered as doubtful.

- Any milk sample (individual or pools) with a **S'/P'\% equal or higher than 70%** is considered coming from herd, which has been in contact with the BLV virus.

**LEGEND**

⊕ = Modification in the using instructions