

[<sup>F1</sup>ANNEX D**Textual Amendments**

- F1** Substituted by [Council Directive 97/12/EC of 17 March 1997 amending and updating Directive 64/432/EEC on health problems affecting intra-Community trade in bovine animals and swine.](#)

[<sup>F2</sup>CHAPTER I**OFFICIALLY ENZOOTIC-BOVINE-LEUKOSIS-FREE HERDS, MEMBER STATES AND REGIONS**

- A. Officially enzootic-bovine-leukosis-free herd means a herd in which:
- (i) there is no evidence, either clinical or as a result of a laboratory test, of any case of enzootic bovine leukosis in the herd and no such case has been confirmed in the previous two years; and
  - (ii) all animals over 24 months of age have reacted negatively during the preceding 12 months to two tests carried out in accordance with this Annex, at an interval of at least four months; or
  - (iii) it meets the requirements of (i) above and is situated in an officially enzootic-bovine-leukosis-free Member State or region.
- B. A herd shall retain officially enzootic-bovine-leukosis-free status provided:
- (i) the condition in A(i) continues to be fulfilled;
  - (ii) any animals introduced into the herd come from an officially enzootic-bovine-leukosis-free herd;
  - (iii) all animals over 24 months of age continue to react negatively to a test carried out in accordance with Chapter II at intervals of three years;
  - (iv) breeding animals introduced into a herd and originating from a third country have been imported in accordance with Directive 72/462/EEC.
- C. The officially leukosis-free status of a herd is to be suspended if the conditions detailed in B are not fulfilled, or where as a result of laboratory tests or on clinical grounds one or more bovine animals are suspected of having enzootic bovine leukosis and the suspect animal(s) are immediately slaughtered.
- D. The status is to remain suspended until the following requirements are complied with:
1. If a single animal in an officially enzootic-bovine-leukosis-free herd has reacted positively to one of the tests referred to in Chapter II, or where infection is otherwise suspected in one animal in a herd:
    - (i) the animal which has reacted positively, and, in the case of a cow, any calf it may have produced, must have left the herd for slaughter under the supervision of the veterinary authorities;
    - (ii) all animals in the herd more than 12 months old have reacted negatively to two serological tests (at least 4 months and less than 12 months apart) carried

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out in accordance with Chapter II three months at least after removal of the positive animal and any possible progeny thereof;

- (iii) an epidemiological inquiry has been conducted with negative results and the herds linked epidemiologically to the infected herd have been subjected to the measures laid down in (ii).

However, the competent authority may grant a derogation from the obligation to slaughter the calf of an infected cow where it was separated from its mother immediately after calving. In this case, the calf must be made subject to the requirements provided for in 2(iii).

- 2. Where more than one animal from an officially enzootic-bovine-leukosis-free herd has reacted positively to one of the tests referred to in Chapter II, or where infection has otherwise been suspected in more than one animal in a herd:

- (i) any animals which have reacted positively and, in the case of cows, their calves, must be removed for slaughter under the supervision of the veterinary authorities;
- (ii) all animals in the herd aged over 12 months must react negatively to two tests carried out in accordance with Chapter II at an interval of at least four months and no more than 12 months;
- (iii) all other animals in the herd must, after identification, remain on the holding until they are aged over 24 months and have been tested in accordance with Chapter II after reaching that age, except that the competent authority may permit such animals to go directly for slaughter under official supervision;
- (iv) an epidemiological inquiry has been conducted with negative results and any herd linked epidemiologically to the infected herd has been subjected to the measures laid down in (ii).

However, the competent authority may grant a derogation from the obligation to slaughter the calf of an infected cow where it was separated from its mother immediately after calving. In this case, the calf must be made subject to the requirements provided for in 2(iii).

- E. In accordance with the procedure in Article 17 and on the basis of information supplied in accordance with Article 8, a Member State or part of a Member State may be considered officially enzootic-bovine-leukosis-free if:

- (a) all the conditions of paragraph A are fulfilled and at least 99,8 % of the bovine herds are officially enzootic-bovine-leukosis-free;

or

- (b) no case of enzootic bovine leukosis has been confirmed in the Member State or the part of the Member State for the past three years, and the presence of tumours suspected of being due to EBL is compulsorily notifiable, with investigations of cause being carried out, and

in the case of a Member State, all animals aged over 24 months in at least 10 % of the herds, selected randomly, have been tested with negative results in accordance with Chapter II in the previous 24 months, or

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in the case of a part of a Member State, all animals aged over 24 months have undergone a test provided for in Chapter II with negative results in accordance with Chapter II in the previous 24 months;

or

- (c) any other method which demonstrates to a confidence rating of 99 % that less than 0,2 % of herds were infected.
- F. A Member State or a region of a Member State is to retain officially enzootic-bovine-leukosis-free status if:
  - (a) all animals slaughtered within the territory of that Member State or region are submitted to official post-mortem examinations at which all tumours which could be due to the EBL virus are sent for laboratory examination,
  - (b) the Member State reports to the Commission all cases of enzootic bovine leukosis that occur in the region,
  - (c) all animals which react positively to any of the tests provided for in Chapter II are slaughtered and their herds remain subject to restrictions until re-establishment of their status in accordance with Section D, and
  - (d) all animals more than two years old have been tested, either once in the first five years after the status is granted under Chapter II or during the first five years after the grant of the status under any other procedure demonstrating with a certainty level of 99 % that less than 0,2 % of herds have been infected. However, where no case of enzootic bovine leukosis has been recorded in a Member State or in a region of a Member State in a proportion of one herd out of 10 000 for at least three years, a decision may be taken in accordance with the procedure laid down in Article 17 that routine serological tests may be reduced provided that all bovine animals more than 12 months old in at least 1 % of herds, selected at random each year, have been subjected to a test carried out in accordance with Chapter II.
- G. The officially enzootic-bovine-leukosis-free status of a Member State or part of a Member State is to be suspended, in accordance with the procedure in Article 17 if, as a result of investigations carried out in accordance with paragraph F above, there is evidence of a significant change in the situation as regards enzootic bovine leukosis in a Member State or part of a Member State which has been recognised as officially enzootic-bovine-leukosis-free.

The officially enzootic-bovine-leukosis-free status may be restored in accordance with the procedure in Article 17 when the criteria laid down by the same procedure are fulfilled.]

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#### **Textual Amendments**

- F2** Substituted by [Council Directive 98/46/EC of 24 June 1998 amending Annexes A, D \(Chapter I\) and F to Directive 64/432/EEC on health problems affecting intra-Community trade in bovine animals and swine.](#)

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## CHAPTER II

### TESTS FOR ENZOOTIC BOVINE LEUKOSIS

Tests for enzootic bovine leukosis shall be carried out by the immune-diffusion test under the conditions described in A and B or by the enzyme-linked immunosorbent assay (Elisa) under the conditions described in C. The immune-diffusion method may only be used for individual tests. If test results are the subject of a duly-substantiated challenge, an additional check shall be carried out by means of the immune-diffusion test.

A. Agar gel immune-diffusion test for enzootic bovine leukosis

[<sup>F3</sup>1. The antigen to be used in the test must contain bovine leukosis virus glycoproteins. The antigen must be standardised against a standard serum (EI serum) supplied by the Danish Institute for Food and Veterinary Research, Copenhagen.

#### Textual Amendments

**F3** Substituted by [Commission Decision of 5 December 2006 amending Council Directives 64/432/EEC, 90/539/EEC, 92/35/EEC, 92/119/EEC, 93/53/EEC, 95/70/EC, 2000/75/EC, 2001/89/EC, 2002/60/EC and Decision 2001/618/EC as regards lists of national reference laboratories and State institutes \(notified under document number C\(2006\) 5856\) \(Text with EEA relevance\) \(2006/911/EC\).](#)

2. The official institutes indicated below must be responsible for calibrating the standard working antigen of the laboratory against the official EEC standard serum (EI serum) supplied by the Danish Institute for Food and Veterinary Research, Copenhagen.

AT	AGES: Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH — Institut für veterinärmedizinische Untersuchungen Mödling (Austrian Agency for Health and Consumer Protection-Institute for veterinary investigations Mödling) Robert Koch-Gasse 17 A-2340 Mödling Tel.: +43 (0) 505 55-38112 Fax: +43 (0) 505 55-38108 E-mail: <a href="mailto:vetmed.moedling@ages.at">vetmed.moedling@ages.at</a>
BE	CODA — CERVA — VAR Veterinary and Agrochemical Research Centre Groeselenberg 99 B-1180 Brussels
[ <sup>F4</sup> BG	Национален диагностичен научноизследователски ветеринарномедицински институт, Проф. д-р Георги Павлов, бул. Пенчо Славейков 15, София 1606 (National Diagnostic Veterinary Research Institute Prof. Dr. Georgi Pavlov, 15, Pencho Slaveykov Blvd., 1606 Sofia)]

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CY	State Veterinary Laboratory Veterinary Services 1417 Athalassa Nicosia
CZ	Státní veterinární ústav Praha – Lysolaje Sídlištní 136/24 165 03 Praha 6 – Lysolaje
DE	Friedrich-Loeffler-Institut Bundesforschungsinstitut für Tiergesundheit Standort Wusterhausen Seestraße 55 16868 Wusterhausen Tel.: +49 33979-80-0 Fax: +49 33979-80-200
DK	Danish Institute for Food and Veterinary Research, Department of Virology, Lindholm, DK-4771 Kalvehave
EE	Veterinaar- ja Toidulaboratoorium Kreutzwaldi 30, 51006 Tartu, Estonia Tel.: +372 7 386 100 Faks: +372 7 386 102 E-post: info@vetlab.ee
ES	Laboratorio Central de Sanidad Animal de Algete Carretera de Algete, km 8 Algete 28110 (Madrid) Tel.: +34 916 290 300 Fax: +34 916 290 598 E-mail: lcv@mapya.es
FI	Finnish Food Safety Authority Animal Diseases and Food Safety Research Mustialankatu 3 FI-00790 Helsinki, Finland E-mail: info@evira.fi Tel.: +358 20 772 003 (exchange) Fax: +358 20 772 4350
FR	Laboratoire d'études et de recherches en pathologie bovine et hygiène des viandes AFSSA site de Lyon — LERP BHV 31 avenue Tony Garnier 69364 Lyon Cedex 07 FRANCE
GB	Veterinary Laboratories Agency New Haw, Addlestone, Weybridge Surrey KT15 3NB, UK Tel. (44-1932) 341111

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	<p>Fax (44-1932) 347046          Immunodiagnosics Department          Veterinary Sciences Division          Stoney Road Stormont          Belfast BT4 3SD, UK</p>
GR	<p>Hellenic Ministry of Rural Development and Food          Centre of Athens Veterinary Institutions          Institute of Foot and Mouth Disease and exotic diseases          25 Neapoleos Street          15 310 Ag. Paraskevi          Tel.: + 30 210 6010903-6007016          Fax: + 30 210 6399477</p>
HU	<p>Országos Állategészségügyi Intézet          Budapest          Tábornok u. 2.          H-1149</p>
IE	<p>Virology Division          Central Veterinary Research Laboratory          Department of Agriculture and Food Laboratories          Backweston Campus          Stacumny Lane          Celbridge          Co. Kildare</p>
IT	<p>Centro di referenza nazionale per i retrovirus correlati alle patologie infettive dei ruminanti c/o Istituto zooprofilattico sperimentale dell' Umbria e delle Marche,          Via G. Salvemini 1,          06126 Perugia          Tel. +39 75 3431          Fax +39 75 35047</p>
LT	<p>Nacionalinė veterinarijos laboratorija,          J. Kairiūkščio g. 10,          LT-2021 Vilnius</p>
LU	<p>CODA — CERVA — VAR          Veterinary and Agrochemical Research Centre          Groeselenberg 99          B-1180 Brussels</p>
LV	<p>Nacionālais diagnostikas centrs          (National Diagnostic Centre)          Lejupes iela 3, Rīga, LV-1076          Tel.: +371 7620526          Fax: +371 7620434          E-mail: ndc@ndc.gov.lv</p>

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MT	—
NL	Centraal Instituut voor DierziekteControle CIDC-Lelystad Hoofdvestiging: Houtribweg 39 Nevenvestiging: Edelhertweg 15 Postbus 2004 8203 AA Lelystad
PL	Laboratory Departament of Biochemistry Państwowy Instytut Weterynaryjny – Państwowy Instytut Badawczy, Al. Partyzantów 57, 24-100 Puławy Tel.: +48.81.886 30 51 Fax: +48.81.886 25 95 E-mail: sekretariat@piwet.pulawy.pl
PT	Laboratório Nacional de Investigação Veterinária (LNIV) Estrada de Benfica, 701 P-1549-011 Lisboa
[ <sup>F4</sup> RO	Institutul de Diagnostic și Sănătate Animală Strada Dr. Staicovici nr. 63, sector 5 codul 050557, București]
SE	Statens Veterinärmedicinska Anstalt SE-751 89 Uppsala
SI	Univerza v Ljubljani Veterinarska fakulteta Nacionalni veterinarski inštitut Gerbičeva 60, SI-1000 Ljubljana
SK	Štátny veterinárny ústav Pod dráhami 918 SK-960 86 Zvolen]

#### Textual Amendments

**F4** Inserted by [Council Directive 2006/104/EC](#) of 20 November 2006 adapting certain Directives in the field of agriculture (veterinary and phytosanitary legislation), by reason of the accession of Bulgaria and Romania.

3. The standard antigens used in the laboratory must be submitted at least once a year to the EEC reference laboratories listed in 2 for testing against the official EEC standard serum. Apart from this standardization, the antigen in use can be calibrated in accordance with B.
4. The reagents for the test shall consist of:
  - (a) antigen: the antigen must contain specific glycoproteins of enzootic bovine leukosis virus which has been standardized against the official EEC serum;
  - (b) the test serum;

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(c) known positive control serum;

(d) agar gel:

0,8 % agar,

8,5 % NaCl,

0,05 M Tris-buffer pH 7,2;

15 ml of this agar must be introduced into a petri dish of 85 mm diameter, resulting in a depth of 2,6 mm of agar.

5. A test pattern of seven moisture-free wells must be cut in the agar to the bottom of the plate; the pattern must consist of one central well and six wells in a circle around it.

Diameter of central well: 4 mm

Diameter of peripheral wells: 6 mm

Distance between central and peripheral wells: 3 mm

6. The central well must be filled with the standard antigen. Peripheral wells 1 and 4 (see diagram below) are filled with the known positive serum, wells 2, 3, 5 and 6 with the test sera. The wells must be filled until the meniscus disappears.

7. This results in the following quantities being obtained:

antigen: 32 µl

control serum: 73 µl

test serum: 73 µl.

8. Incubation must be for 72 hours at room temperature (20 to 27°C) in a closed humid chamber.

9. The test may be read at 24 and 48 hours but a final result may not be obtained before 72 hours:

(a) a test serum is positive if it forms a specific precipitin line with the BLV antigen and forms a complete line of identity with the control serum;

(b) a test serum is negative if it does not form a specific precipitin line with the BLV antigen and if it does not bend the line of the control serum;

(c) the reaction cannot be considered conclusive if it:

(i) bends the line of the control serum towards the BLV antigen well without forming a visible precipitin line with the antigen;

or

(ii) if it cannot be read either as negative or as positive.

In inconclusive reactions the test may be repeated and concentrated serum utilized.

10. Any other well configuration or pattern may be utilized provided that the E4 serum diluted 1:10 in negative serum can be detected as positive.

B. Method for antigen standardization

Solutions and materials required

1. 40 ml of 1,6 % agarose in 0,05 M Tris/HCl buffer, pH 7,2 with 8,5 % NaCl;



2. 15 ml of a bovine leukosis serum, having antibody only to bovine leukosis virus glycoproteins, diluted 1:10 in 0,05 M Tris/HCl buffer, pH 7,2 with 8,5 % NaCl;
3. 15 ml of a bovine leukosis serum, having antibody only to bovine leukosis virus glycoproteins, diluted 1:5 in 0,05 M Tris/HCl buffer, pH 7,2 with 8,5 % NaCl;
4. four plastic petri dishes with a diameter of 85 mm;
5. a punch with a diameter of 4 to 6 mm;
6. a reference antigen;
7. the antigen which is to be standardized;
8. a water bath (56°C).

#### Procedure

Dissolve the agarose (1,6 %) in the Tris/HCl buffer by carefully heating to 100°C. Place in 56°C water bath for approximately one hour. Also, place the bovine leukosis serum dilutions in a 56°C water bath.

Now mix 15 ml of the 56°C agarose solution with the 15 ml bovine leukosis serum (1:10), quickly shake and pour 15 ml into each of two petri dishes. Repeat this procedure with the bovine leukosis serum diluted 1:5.

When the agarose has hardened, holes are made in it as follows:

#### Addition of antigen

- (i) Petri dishes 1 and 3:
  - well A — undiluted reference antigen,
  - well B — 1:2 diluted reference antigen,
  - wells C and E — reference antigen,
  - well D — undiluted test antigen.
- (ii) Petri dishes 2 and 4:
  - well A — undiluted test antigen,
  - well B — 1:2 diluted test antigen,
  - well C — 1:4 diluted test antigen,
  - well D — 1:8 diluted test antigen.

#### Additional instructions

1. The experiment shall be carried out with two serum dilutions (1:5 and 1:10) in order to achieve optimal precipitation
2. If the precipitation diameter is too small with both dilutions, then the serum must be further diluted.
3. If the precipitation diameter in both dilutions is too large and faint, then a lower serum must be chosen.
4. The final concentration of the agarose must be 0,8 %; that of the sera 5 and 10 % respectively.

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5. Plot the measured diameters in the following coordinate system. The dilution of the antigen to be tested with the same diameter as the reference antigen is the working dilution.

C. Enzyme-linked immunosorbent assay (Elisa) for detecting enzootic bovine leukosis

1. The material and reagents to be used are as follows:

- (a) solid-phase microplates, cuvettes or any other solid phase;
- (b) the antigen is fixed to the solid phase with or without the aid of polyclonal or monoclonal catching antibodies. If antigen is coated directly to the solid phase, all test samples giving positive reactions have to be retested against control antigen in the case of EBL. The control antigen should be identical to the antigen except for the BLV antigens. If catching antibodies are coated to the solid phase, the antibodies must not react to antigens other than BLV antigens;
- (c) the biological fluid to be tested;
- (d) a corresponding positive and negative control;
- (e) conjugate;
- (f) a substrate adapted to the enzyme used;
- (g) a stopping solution, if necessary;
- (h) solutions for the dilution of the test samples for preparations of the reagents and for washing;
- (i) a reading system appropriate to the substrate used.

2. Standardization and sensitivity of test

The sensitivity of the Elisa assay must be of such a level that E4 serum is scored positive when diluted 10 times (serum samples) or 250 times (milk samples) more than the dilution obtained of individual samples when these are included in pools. In assays where samples (serum and milk) are tested individually E4 serum diluted 1 to 10 (in negative serum) or 1 to 250 (in negative milk) must be scored positive when tested in the same assay dilution as used for the individual test samples. The official institutes listed in A.2 will be responsible for checking the quality of the Elisa method, and in particular for determining, for each production batch, the number of samples to be pooled on the basis of the count obtained for the E4 serum.

The E4 serum will be supplied by the National Veterinary Laboratory, Copenhagen.

3. Conditions for use of the Elisa test for EBL

The Elisa method may be used on a sample of milk or whey taken from the milk collected from a farm with at least 30 % of dairy cows in milk.

If this method is used, measures must be taken to ensure that the samples taken can be identified with the animals from which the milk or sera examined were taken.]