

II

(Acts whose publication is not obligatory)

COMMISSION**COMMISSION DECISION**

of 29 July 1991

**concerning animal health conditions and veterinary certification for the importation of
bovine semen from the United States of America**

(91/479/EEC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community,

Having regard to Council Directive 88/407/EEC of 14 June 1988 laying down the animal health requirements applicable to intra-Community trade in and imports of deep-frozen semen of domestic animals of the bovine species ⁽¹⁾, at last amended by Directive 90/425/EEC ⁽²⁾, and, in particular, Articles 10 and 11 thereof,

Whereas the United States of America appears in the list, established by Commission Decision 90/14/EEC ⁽³⁾, of third countries from which Member States authorize importation of semen of domestic animals of the bovine species;

Whereas it appears that the animal health situation in the United States of America is good and controlled by well-structured and organized veterinary services as regards diseases transmissible through semen;

Whereas the competent veterinary authorities of the United States of America have confirmed that the United States of America has for at least 12 months been free from

rinderpest, foot-and-mouth disease and contagious bovine pleuro-pneumonia and that no vaccinations have been carried out against those diseases during that time;

Whereas the competent veterinary authorities of the United States of America have undertaken to notify the Commission and the Member States by telex or telefax, within 24 hours, of the conformation of the occurrence of any of the abovementioned diseases or of bluetongue or epizootic haemorrhagic disease or of any change in vaccination policy concerning any of them or, within an appropriate period, of any proposed change in the United States' import rules concerning domestic animals or the semen or embryos thereof;

Whereas the competent veterinary authorities of the United States of America have provided animal health guarantees in respect of bovine tuberculosis and brucellosis which are equivalent to those applicable within the Community;

Whereas the competent veterinary authorities of the United States of America have undertaken to supervise officially the issue of certificates arising from this Decision and to ensure all relevant certificates, derogations and laboratory findings on which certification may have been based remain on official file for at least 12 months following the dispatch of the semen to which they refer;

Whereas the competent veterinary authorities of the United States of America have undertaken to approve officially semen collection centres for the export of bovine semen to the European Economic Community as required by Article 9 of Directive 88/407/EEC;

⁽¹⁾ OJ No L 194, 22. 7. 1988, p. 10.

⁽²⁾ OJ No L 224, 18. 8. 1990, p. 29.

⁽³⁾ OJ No L 8, 11. 1. 1990, p. 71.

Whereas the competent veterinary authorities of the United States of America have undertaken to determine and to communicate without delay to the Commission the date on which, in respect of each approved semen collection centre, all activity of those *Culicoides* species capable of transmitting bluetongue or epizootic haemorrhagic disease ceases and the date on which the activity of such *Culicoides* species is resumed;

Whereas animal health conditions and veterinary certification must be adapted according to the animal health situation of the third country concerned;

Whereas the measures provided for in this Decision are in accordance with the opinion of the Standing Veterinary Committee,

HAS ADOPTED THIS DECISION:

Article 1

1. Until 31 December 1992, Member States may authorize the importation from the United States of America of bovine semen collected from bulls which have given other

than a negative result to the test for bluetongue or the test for epizootic haemorrhagic disease prescribed in Annex I A to this Decision, but shall do so in accordance with the provisions of Annexes II A and II B and shall ensure that such semen does not enter into intra-Community trade. The Commission will review the provisions of this paragraph before the date indicated above.

2. Member States shall authorize the importation from the United States of America of bovine semen which conforms to the conditions listed in the certificate set out in Annex I A and, where relevant, the certificate set out in Annex I B.

Article 2

This Decision is addressed to the Member States.

Done at Brussels, 29 July 1991.

For the Commission

Ray Mac SHARRY

Member of the Commission

for the importation of bovine semen from the United States of America

Competent authority: United States Department of Agriculture — APHIS

Centre approval No:

[illegible]

(Strike out unused entries. This certificate applies only to semen collected at a single approved collection centre.)

Marks on seal applied to transport container:

.....

by:
(means/identification of transport)

Name and address of consignor:

.....

Name and address of consignee:

IV. Health information:

I, the undersigned federal veterinarian, certify that:

1. the United States of America has been free, during a period commencing at least 12 months before the first collection of the semen described above and finishing 30 days after the final collection of such semen, from cattle plague (rinderpest);
2. the approved semen collection centre in which the semen described above was collected:
 - (a) has been approved, on the basis that it conforms to all the provisions of this paragraph, by the federal veterinary authority of the United States of America for the export of bovine semen to the European Community;
 - (b) is situated at the centre of an area of 50 km radius in which, during a period commencing at least three months before the first collection of the semen described above and finishing 30 days after the final collection of such semen, there has been no evidence of foot-and-mouth disease, contagious bovine pleuro-pneumonia or vesicular stomatitis;
 - (c) has been free, during a period commencing at least three months before the first collection of semen described above and finishing 30 days after the final collection of such semen, from foot-and-mouth disease and brucellosis;
 - (d) has been free, during a period commencing at least 30 days before the first collection of the semen described above and finishing 30 days after the final collection of such semen, from rabies, anthrax, tuberculosis, enzootic bovine leukosis, or any evidence of infection with *Trichomonas foetus*, *Campylobacter foetus*, *Leptospira canicola*, *Leptospira pomona*, *Leptospira grippotyphosa*, *Leptospira hardjo* or *Leptospira icterohaemorrhagica*;
 - (e) is inspected by a federal veterinarian at least twice a year at which times all matters relating to the conditions set out in this certificate are considered and verified;
 - (f) is under the permanent supervision of a centre veterinarian and is so supervised that:
 - (i) animals are admitted only with the express permission of the centre veterinarian, all movements of animals, in or out, being recorded;
 - (ii) a record is kept of the breed, date of birth, identification and health history of each bovine animal in the centre and of all diagnostic tests and the results thereof, all treatments and all vaccinations carried out on the animals kept therein;
 - (iii) the entry of unauthorized persons is prevented and authorized visitors are required to comply with the conditions laid down by the centre veterinarian;
 - (iv) only technically competent staff are employed, suitably trained in disinfection procedures and hygiene techniques relevant to the control of the spread of disease;
 - (g) contains only bovine animals, except that other domestic animals strictly necessary for the normal operation of the centre may be admitted provided that they present no risk of infection to the bovine species and that they fulfil the conditions laid down by the centre veterinarian;
 - (h) is so constructed that:
 - (i) it has animal accommodation physically separated from the semen processing and semen storage rooms, which are physically separated from each other;
 - (ii) it has an isolation facility for sick animals;
 - (iii) it has a semen collection facility which has a separate room for the cleaning and disinfection or sterilization of equipment;
 - (iv) it has a semen processing room and a semen storage room (which need not necessarily be on the same site);
 - (v) contact with outside livestock is prevented;
 - (vi) the entire centre is capable of being readily cleaned and disinfected;

provided that, where the above conditions are fulfilled, an approved semen collection centre may share a site with one or more other semen collection centres;

3. the bulls present in the approved semen collection centre during the period of collection and storage of the semen described above:

- (a) were in the approved semen collection centre on, and continuously since, 1 January 1990, and have since their arrival been subjected to all the tests described, with the results provided for, at (d) below;

or

- (b) or were transferred from an approved semen collection centre without coming into contact with animals of a lesser health status and, where applicable, in transport which was thoroughly cleansed and disinfected before use;

or

- (c) were admitted, on the authority of the centre veterinarian and showing no clinical signs of disease:

- (i) having originated in herds which were accredited tuberculosis-free by the federal veterinary authority or which were situated in accredited tuberculosis-free areas as defined by the federal veterinary authority and not having been held at any time in herds of lower status;
- (ii) having originated in herds which were certified brucellosis-free by the federal veterinary authority or which were situated in brucellosis class free or class A areas as defined by the federal veterinary authority and not having been held at any time in herds of lower status;

- (iii) — having:

- originated in herds free for at least three years from any evidence for enzootic bovine leukosis, or
- been produced by cows which were subjected, within 30 days before the entry of their sons into the isolation facility, to a serological test for enzootic bovine leukosis carried out in accordance with the procedure laid down in Annex G to Council Directive 64/432/EEC with negative result, or
- been subjected, within 30 days before entry into the officially-approved isolation facility or after having reached the age of two years, whichever is the later, to a serological test for enzootic bovine leukosis carried out in accordance with the procedure laid down in Annex G to Council Directive 64/432/EEC with negative result;

- (iv) having been subjected, within 30 days before entry to the officially-approved isolation facility, to the following tests with negative result in each case:

- a United States Department of Agriculture (USDA) caudal fold tuberculin test,
- a USDA standard serological tube test for brucellosis which is negative at less than 30 IU of agglutination per millilitre and a complement fixation test showing a brucella count lower than 20 EEC units per millilitre (20 ICFT units),
- a serological test for enzootic bovine leukosis carried out in accordance with the procedure laid down in Annex G to Directive 64/432/EEC,
- a serum neutralization test or ELISA test for infectious bovine rhino-tracheitis/infectious pustular vulvo-vaginitis,
- a virus isolation test for bovine viral diarrhoea on blood samples in susceptible cell cultures subsequently subjected to a fluorescent antibody test or an immunoperoxidase test provided that, if at the time of its entry, any bull was less than six months old, the test was delayed until the animal reached that age,

and to the following tests with negative result in each case except where this certificate applies to semen collected and shipped before 1 January 1993 and where the importing Member State has indicated in writing that it is applying the terms of paragraph 1 of Article 1 of Commission Decision 91/479/EEC

- a blocking Elisa test for bluetongue using a group specific monoclonal antibody carried out according to the procedure laid down in Annex IV A to Commission Decision 91/479/EEC,
- an agar gel immunodiffusion test for all serotypes of epizootic haemorrhagic disease found in the United States of America carried out according to the procedure laid down in Annex IV B to Commission Decision 91/479/EEC,

granted that all or any of the above tests may have been carried out while the animals were in the officially approval isolation facility provided that if the result of any test for which this certificate requires a negative result was other than negative the isolation period of 30 days for the other bulls in the isolation facility was not considered to have commenced until after the animal concerned had been removed from the facility and, if applicable, the tuberculosis or brucellosis status of the facility had been restored;

- (v) having, after the completion of the pre-isolation tests described in (iv) above, undergone a period of at least thirty days in an officially approved isolation facility which was, at the date of their entry, at the centre of an area of 10 km radius in which there has been no evidence of foot-and-mouth disease, cattle plague (rinderpest), contagious bovine pleuro-pneumonia or vesicular stomatitis, the centre having been free for at least three months from foot-and-mouth disease and brucellosis and for at least 30 days from rabies, anthrax, tuberculosis and enzootic bovine leukosis and in which they were subjected to the following tests with negative result in each case:

- a USDA standard serological tube test for brucellosis which is negative at less than 30 IU of agglutination per millilitre and a complement fixation test showing a brucella count lower than 20 EEC units per millilitre (20 ICFT units),
- either an immunofluorescent antibody test or a culture test for *Campylobacter foetus* infection on a sample of preputial material or artificial vaginal washings,
- a microscopic examination and a culture test for *Trichomonas foetus* infection on a sample of preputial material or artificial vaginal washings,
- a serum neutralization test or Elisa test for infectious bovine rhino-tracheitis/infectious pustular vulvo-vaginitis,

provided that if the result of any test was other than negative the isolation period of 30 days for the remaining animals was not considered to have commenced until after the animal concerned had been removed from the facility and, where relevant, the brucellosis status of the facility had been restored,

and in which they were treated against Leptospirosis by the injection on two separate occasions, fourteen days apart, of streptomycin or dihydrostreptomycin, or a mixture thereof, at a rate of 25 mg per kg of live body weight;

- (d) were subjected at least once a year to the following tests with negative result in each case:

- (i) a USDA caudal fold tuberculin test;
- (ii) a USDA standard serological tube test for brucellosis that is negative at less than 30 IU of agglutination per millilitre and a complement fixation test showing a brucella count lower than 20 EEC units per millilitre (20 ICFT units);
- (iii) a serological test for enzootic bovine leukosis carried out in accordance with the procedure laid down in Annex G to Directive 64/432/EEC;
- (iv) a serum neutralization test or Elisa test for infectious bovine rhino-tracheitis/infectious pustular vulvo-vaginitis, provided that, in respect of semen collected prior to 31 December 1992 and where the competent authority of the importing Member State has so indicated in writing, the semen of bulls positive to either of these tests may be accepted provided a virus isolation and/or animal inoculation test for the above disease complex has been carried out on such semen with negative result, in this latter case the test having been carried out on the at the laboratory;
- (v) either an immunofluorescent antibody test or a culture test for *Campylobacter foetus* infection on a sample of preputial material or artificial vaginal washings, except that no test under this heading is required in the case of animals which are not being used for the production of semen provided that such a test is carried out before semen production is resumed;
- (vi) a serological test for the *canicola*, *pomona*, *grippotyphosa*, *hardjo* and *icterohaemorrhagica* serotypes of *Leptospira*,

and at least twice a year to the following tests with negative result in each case except where this certificate applies to semen collected and shipped before 1 January 1993 and where the veterinary authority of the importing Member State has indicated in writing that it is applying the terms of paragraph 1 of Article 1 of Commission Decision of 29 July 1991 (C(91) 1541 final):

- (i) a blocking Elisa test for bluetongue using a group specific monoclonal antibody carried out according to the procedure laid down in Annex IV A to Commission Decision 91/479/EEC;
- (ii) an agar gel immunodiffusion test for all serotypes of epizootic haemorrhagic disease found in the United States of America carried out according to the procedure laid down in Annex IV B to Commission Decision 91/479/EEC.

all the above tests (with the exception of the tuberculin test) being carried out in a laboratory approved for the purpose by the federal veterinary authority of the United States of America;

4. The semen described above

- is classified as bluetongue-free according to the definition contained in Annex II A to Commission Decision 91/479/EEC and, in particular, paragraph 5 thereof;

or

- is classified as bluetongue-tested according to the definition contained in Annex II B to Commission Decision 91/479/EEC and, in particular, paragraph 2 thereof and where the veterinary authority of the importing Member State has indicated in writing that it is applying the terms of paragraph 1 of Article 1 of Commission decision 91/479/EEC (*),

(*) delete whichever does not apply.

and was

- (a) collected, without the use of electro-ejaculation or electro-stimulation techniques, in an approved semen collection centre from bulls:

- (i) which have been continuously on the territory of the United States of America during a period commencing at least six months before the first collection of the semen described above and finishing on the date of its dispatch;
- (ii) which, other than as provided for in a written derogation given in the terms of 3 (d) (iv) above or of Article 1 of Commission Decision 91/479/EEC have not given a positive result to any of the tests referred to in this certificate;
- (iii) which were not, while in the approved semen collection centre, used for natural service;
- (iv) which were kept in the approved semen collection centre for a continuous period of at least 30 days immediately prior to the collection of the semen;
- (v) which showed no clinical signs of disease at the time;

- (b) processed in an approved semen collection centre:

- (i) in which, during the collection of the semen described above, no semen was processed other than semen from bulls in approved centres or semen from bulls having the same health status as bulls in approved centres, provided that in the latter case such processing took place with separate equipment and at different times to the processing of semen from approved centres and that the processing facility was thoroughly cleansed and disinfected before being again used for the processing of semen from bulls in approved centres;
- (ii) in conditions of the strictest hygiene, all implements and equipment coming into contact with the donor bulls or with the semen being properly disinfected or sterilized, as appropriate, before use;
- (iii) using additives, diluents or extenders in which any products of animal origin were obtained from sources which presented no animal health risk or which were so treated prior to use that such risk was prevented;

- (c) protected by the addition of the following antibiotics in such quantities as were necessary to produce the indicated concentrations in the final diluted semen:

not less than:

- 500 IU per ml streptomycin,
- 500 IU per ml penicillin,
- 150 µg per ml lincomycin,
- 300 µg per ml spectinomycin,

and immediately afterwards held at a temperature of not less than 5° C (41° F) for a period of not less than 45 minutes.

or

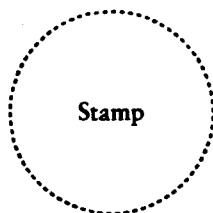
not less than:

- 50 µg per ml tylosin,
- 250 µg per ml gentamycin,
- 150 µg per ml lincomycin,
- 300 µg per ml spectinomycin,

contact between the antibiotic and the undiluted semen being maintained for at least 3 minutes at the temperature at which they were mixed and the semen and the non-glycerol fraction of the diluent being held at a temperature of not less than 5° C (41° F) for at least two hours;

- (d) put into individual containers (straws) each marked with the date of collection, the breed and identity of the donor bull and the identity of the approved centre of collection, provided that such information, or any part of it, may have been marked in code where a full translation of such code has been made available to the competent authority of the importing Member State, and provided that there is a clear correspondence between the marking on each straw and the identification incorporated in this certificate;
- (e) stored, in containers which have been thoroughly cleansed and disinfected or sterilized, as appropriate, before use and using cryogenic agents which have not previously been used for any other product of animal origin, in the approved semen collection centre under the supervision of the centre veterinarian for a period of not less than 30 days prior to dispatch;
- (f) not exported after the date of a positive test on any bull in the centre, other than a test for bluetongue or epizootic haemorrhagic disease as provided for in Article 1 (1) of Commission Decision 91/479/EEC, or a test for infectious bovine rhino-tracheitis/infectious pustular vulvo-vaginitis as provided for in a written derogation given under the terms of 3 (d) (iv) above, or before the health status of the centre has been re-established;
- (g) dispatched in containers which have been thoroughly cleansed and disinfected or sterilized, as appropriate, before use and using cryogenic agents which have not previously been used for any other product of animal origin and which have been sealed under the supervision of the federal veterinarian prior to dispatch from the approved semen collection centre.

Done at, on
(place) (date)



Signature

Name in block letters

Official title

ANNEX I B

SUPPLEMENTARY CERTIFICATE

for the transfer of semen from one container to another for shipment from the United States of America to the European Economic Community

I, the undersigned federal veterinarian, certify that:
The semen to which the certificates and seals indicated below refer was transferred, in an approved semen collection centre and under my direct supervision, from the containers in which it was received, seals intact, to the container in which it is to be despatched to the European Economic Community.

Centre of origin	Certificate No	Seal No

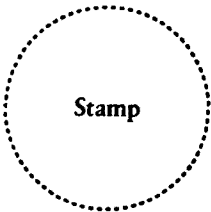
The transfer was carried out at:
.....

Approval No:

The seal applied to the shipment container carries the marks:

The certificates in respect of all the semen in the container are attached.

Done at, on
(place) (date)



Signature
Name in block letters
Official title

ANNEX II A

Procedure for the classification as bluetongue-free of bovine semen intended for export from the United States of America to the European Economic Community

1. All bovine animals in the approved semen collection centre are subjected, within thirty days prior to entry and at six-month intervals thereafter, to a blocking Elisa test for bluetongue virus antibodies and an AGID test for antibodies to all strains of epizootic haemorrhagic disease virus known to exist in the United States of America according to the protocols set out in Annex IV.
2. A bull which has given a negative result to the most recent application of the tests referred to in paragraph 1, is defined for the purpose of this Decision as bluetongue sero-negative unless or until the blood or the semen of the bull gives a positive result to any test referred to in this Annex or in Annex III.
3. The vector-free season as it relates to an approved semen collection centre is defined for the purpose of this Decision as a period commencing 21 days after the date, determined by the United States Department of Agriculture and communicated without delay to the management of the approved semen collection centre concerned and to the Commission of the European Communities, on which all activity of those *Culicoides* species capable of transmitting bluetongue or epizootic haemorrhagic disease virus ceases in the region in which the centre is located, and finishing 21 days before the date, determined and communicated in like manner, on which such *Culicoides* activity resumes.
4. A semen collection period, as it relates to the collection of semen for export to the Community, is defined for the purpose of this Decision as a period not greater than three weeks during which such semen is collected. If the actual time during which such semen is collected is greater than three weeks then this time is divided into two or more collection periods, none of which is greater than three weeks and each of which is treated as a separate collection period in as far as the application of the tests and procedures relating to bluetongue and epizootic haemorrhagic disease referred to in this Decision are concerned.
5. Semen which falls into either of the categories described below is classified as bluetongue-free for the purposes of export of that semen to the European Community:
 - (a) semen collected during the vector-free season from a bluetongue sero-negative bull when a sample of blood from the bull concerned, collected 21 days after the end of the semen collection period, has been submitted, with negative result, to an ELISA test for bluetongue virus antibodies and to an AGID test for antibodies to all serotypes of epizootic haemorrhagic disease virus known to occur in the United States of America according to the protocols set out in Annex IV;
 - (b) semen collected other than during the vector-free season from a bluetongue sero-negative bull when a sample of blood from the bull concerned, collected 21 days after the end of the semen collection period, has been submitted, with negative result, to an Elisa test for bluetongue virus and antibodies and to an AGID test for antibodies to all serotypes of epizootic haemorrhagic disease known to occur in the United States of America according to the protocols set out in Annex IV and when samples of blood collected from the bull on the dates of the collection of the semen intended for export have been submitted, with negative result, to the virus isolation procedure described in Annex III.

ANNEX II B**Procedure for the classification as bluetongue-tested of bovine semen intended for export from the United States of America to the European Economic Community**

1. The definitions set out in Annex II A apply.
 2. Semen which falls into either of the categories described below is classified as bluetongue-tested for the purposes of export of that semen to the European Community:
 - (a) semen collected during the vector-free season from a bull which is other than bluetongue sero-negative when a comparison of a sample of blood collected at the start of the vector-free season with one collected 21 days after the end of the semen collection period shows no evidence of sero-conversion or of a fourfold or greater increase in the level of serum-neutralizing antibodies to any of the serotypes of bluetongue virus or of epizootic haemorrhagic disease virus known to occur in the United States of America and when samples of blood collected from the bull on the dates of the collection of the semen intended for export to the Community have been submitted, with negative result, to the virus isolation procedure described in Annex III;
 - (b) semen collected at any time of year from a bull which is other than sero-negative when a comparison of a sample of blood collected at the start of the semen collection period with one collected 21 days after the end of the same period shows no evidence of sero-conversion or of a fourfold or greater increase in the level of serum-neutralizing antibodies to any of the serotypes of bluetongue virus or of epizootic haemorrhagic disease virus known to occur in the United States of America and when samples of blood collected from the bull on the dates of the collection of the semen intended for export have been submitted with negative result to the virus isolation procedure described in Annex III.
 3. Bluetongue-tested semen must be certified separately from semen defined as bluetongue-free under the terms of Annex II A.
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*ANNEX III***Virus isolation procedure for the detection of bluetongue virus in blood in connection with the export of bovine semen from the United States of America to the European Economic Community**

1. Blood is taken from the bull on the day of each collection of semen intended for export (normally twice weekly) and inoculated subcutaneously and intradermally into a sheep, determined by blocking Elisa test prior to the first such inoculation to be sero-negative, within 48 hours of collection.
2. Blood for virus isolation is collected twice weekly from the sheep for a period of three weeks commencing on the day of the first inoculation referred to in paragraph 1.
3. Blood from the bull and the sheep are inoculated within 48 hours of collection into at least six chicken eggs containing 11-day embryos.
4. If there are no embryo deaths or if embryo deaths occur within two days of inoculation without specific lesions the test is considered negative after seven days.
5. Material from any embryo dying between two and seven days is passaged through chicken eggs containing 11 day embryos, using at least six eggs per dead embryo.
6. If the passage of material from a dead embryo gives rise to an embryo mortality of 50 % or more the test is considered positive. The virus or viruses, after adaption to cell culture, are serotyped to confirm the presence of bluetongue virus.
7. Serum is taken twice weekly from the bull and once weekly from the sheep over a period of three weeks and is subjected to the blocking Elisa described in Annex IV, a serum neutralization test for bluetongue virus antibodies, the AGID test described in Annex IV and a serum neutralization test for epizootic haemorrhagic disease virus antibodies.
8. The sheep is re-submitted to a blocking Elisa 21 days after the last inoculation of blood from the bull.
9. Seroconversion or a fourfold or greater increase in antibody titre in either the bull or the sheep indicates current or recent infection and the test is considered positive.
10. The serological testing is carried out on all samples together at the end of the collection period.
11. Semen is not released for export until all tests have been completed with negative result.

ANNEX IV A

Protocol for a blocking or competitive enzyme-linked immunosorbent assay test using a group specific monoclonal antibody for the detection of bluetongue virus antibodies

THE BLUETONGUE COMPETITIVE ELISA USING MONOCLONAL ANTIBODY 3-17-A3

The competitive Elisa using monoclonal antibody 3-17-A3 is capable of detecting antibodies to all known serotypes of bluetongue virus (BTV).

The principle of the test is the interruption of the reaction between BTV antigen and a group-specific monoclonal antibody (3-17-A3) by the addition of test serum dilutions. Antibodies to BTV present in the test serum block the reactivity of the monoclonal antibody (Mab) and result in a reduction in the expected colour development on addition of enzyme substrate.

Material and Reagents:

1. Flat-bottomed microtitre plates.
2. Antigen: prepared as described below.
3. Blocking buffer: 5 % (w/v) 'Marvel' dried milk powder, 0,1 % (v/v) Tween-20 (supplied as polyoxyethylene sorbiton monolaurate syrup) in PBS.
4. Monoclonal antibody: 3-17-A3 (supplied as hybridoma tissue-culture supernatant) stored at -20°C or freeze-dried, diluted 1/50 with blocking buffer before use, directed against the group-specific polypeptide p7.
5. Conjugate: rabbit anti-mouse globulin (adsorbed and eluted) conjugated to horseradish peroxidase and kept in the dark at 4°C .
6. Substrate and chromogen: 0,2 gm of orthophenylene diamine (OPD) dissolved in a buffer consisting of 2,553 g of citric acid and 4,574 g of di-sodium hydrogenorthophosphate made up to 500 ml with distilled water, divided into 25 ml aliquots and kept in the dark at -20°C , with 12 μl /25 ml of hydrogen peroxide (30 % w/v) added immediately before use.

Handle OPD with care — wear rubber gloves — suspected mutagen.

7. 1 Molar sulphuric acid: 26,6 ml of acid added to 473,4 ml of distilled water.

Remember — always add acid to water, never water to acid.

8. Orbital shaker.
9. Elisa plate reader (the test may be read visually).

Test format

	H	G	F	E	D	C	B	A	
Blank			Antigen + conjugate						1
Mab-control			Antigen + Mab + conjugate						2
+ ve control	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	3
	1/2	1/4	1/8	1/16	1/2	1/4	1/8	1/16	4
Test sera									5
									6
									7
									8
									9
									10
									11
									12

Test protocol:

Blank control:

row 1 A — H is a blank control consisting of BTV antigen and conjugate. This may be used to blank the Elisa reader.

Mab control:

row 2 A — H is the monoclonal antibody control and consists of BTV antigen, monoclonal antibody and conjugate. This represents a negative control. The mean of the optical density readings from this control row represents the 0 % inhibition value.

Positive control:

row 3 A — H is the positive control. This consists of BTV antigen, BTV positive antiserum dilutions, Mab and conjugate. This is included as an indicator that the test is functioning properly and similar levels of inhibition should be obtained from test to test.

Test sera:

in the test format shown above, 18 sera can be tested over a dilution rate of 1/2, 1/4, 1/8 and 1/16. This will give some indication of the titre of antibody in the test sera. The dilution range could be extended further to obtain serum dilution end-point titres. Alternatively, for large-scale serological surveys, sera could be tested at a single dilution (1/4) or two dilutions (1/2 and 1/4) as a rapid screening test.

Procedure:

1. Dilute BTV antigen to pre-titrated concentration in PBS, sonicate briefly to disperse aggregated virus (if sonicator is not available, pipette vigorously) and add 50 µl to all wells of the Elisa plate. Tap sides of plate to disperse antigen.
2. Incubate at 37 °C for 60 minutes on an orbital shaker. Wash plates three times by flooding and emptying the wells with unsterile PBS and blot dry on absorbent paper.
3. Add 50 µl per well of blocking buffer. Add test sera and positive serum to the appropriate wells and dilute across the plate using a multichannel pipette. Do not add sera to the blank control or the Mab control.
4. Immediately after the addition of the test sera, dilute Mab in blocking buffer (to pre-titrated dilution) and add 50 µl to all wells of the plate except for the blank control.
5. Incubate at 37 °C for 60 minutes on an orbital shaker. Wash three times with PBS and blot dry.
6. Dilute rabbit anti-mouse concentrate to 1/5 000 in blocking buffer and add 50 µl to all wells of the plate.
7. Incubate at 37 °C for 60 minutes on an orbital shaker. Wash three times with PBS and blot dry.
8. Thaw the OPD and immediately before use add 12 µl of 30 % hydrogen peroxide to each 25 ml of OPD. Add 50 µl to all wells of the plate. Allow colour to develop for approximately 10 minutes and stop the reaction with 1 M sulphuric acid (50 µl per well). Colour should develop in the Mab control wells and in those wells containing sera with no antibody to BTV.
9. Examine and record the plates either visually or using a spectrophotometric reader.

Analysis of results:

Calculate the mean OD reading from the Mab controls. This represents the 0 % inhibition value. Optical density readings from the test sera are expressed as percentage inhibition values using the following formula:

$$\text{Percentage inhibition value} = 100 \frac{\text{OD in the presence of test serum}}{\text{OD in the absence of test serum}} \times 100.$$

Inhibition values greater than 40 % at a serum dilution of 1/4 are considered positive. Visual reading is possible as 40 % inhibition is the lowest value easily discernible by eye.

Preparation of BTV Elisa antigen:

1. Wash 10 roux of confluent BHK-21 cells three times with serum-free Eagle's medium and infect with bluetongue virus serotype 1 in serum-free Eagle's medium.
2. Incubate at 37 °C and examine daily for cytopathic effect (cpe).
3. When cpe is evident in 80 to 90 % of the cell sheet of each roux, harvest the virus by shaking any still-attached cells from the glass.
4. Centrifuge at 2 000 to 3 000 rpm to pellet the cells.
5. Discard the supernatant and re-suspend the cells in approximately 30 ml of PBS containing 1 % 'Sarkosyl' and 2 ml phenylmethanesulphonyl fluoride (lysis buffer). This may cause the cells to form a gel and more lysis buffer may be added to reduce this effect.

NB: phenylmethanesulphonyl fluoride is harmful — handle with extreme caution.

6. Disrupt the cells for 60 seconds using an ultrasonic probe at an amplitude of 30 microns.
7. Centrifuge at 10 000 rpm for 10 minutes.
8. Store the supernatant at +4 °C and re-suspend the remaining cell pellet in 10 to 20 ml of lysis buffer.
9. Sonicate and clarify, storing the supernatant at each stage, a total of three times.
10. Pool the supernatants and centrifuge at 24 000 rpm for 120 minutes at +4 °C over a 5 ml cushion of 40 % sucrose (w/v in PBS) using 30 ml Beckmann centrifuge tubes and an SW 28 rotor.
11. Discard the supernatant, drain the tubes thoroughly and re-suspend the pellet in PBS by sonication. Store the antigen in aliquots at -70 °C.

Titration of BTV Elisa antigen:

Bluetongue Elisa antigen is titrated by the indirect Elisa. Twofold dilutions of antigen are titrated against a constant dilution (1/50) of monoclonal antibody 3-17-A3. The protocol is as follows:

Procedure:

1. Dilute BTV antigen in PBS across the microtitre plate in a twofold dilution series (50 µl/well) using a multichannel pipette.
2. Incubate for one hour at 37 °C on an orbital shaker.
3. Wash plates three times with PBS.
4. Add 50 µl of monoclonal antibody 3-17-A3 (diluted 1/50) to each well of the microtitre plate.
5. Incubate for one hour at 37 °C on an orbital shaker.
6. Wash plates three times with PBS.
7. Add 50 µl of rabbit anti-mouse globulin conjugated to horseradish peroxidase, diluted to a pre-titrated optimal concentration, to each well of the microtitre plate.
8. Incubate for one hour at 37 °C on an orbital shaker.
9. Add substrate and chromogen as described previously. Stop the reaction after 10 minutes by the addition of 1 Molar sulphuric acid (50 µl/well).

In the competitive assay, the monoclonal antibody must be in excess, therefore a dilution of antigen is chosen which falls on the titration curve (not on the plateau region) which gives approximately 0,8 OD after 10 minutes.

ANNEX IV B**Protocol for an agar-gel immuno-diffusion test for the detection of epizootic haemorrhagic disease antibodies**

The agar gel immuno-diffusion test is carried out according to the following

Antigen:

Precipitating antigen is prepared in any cell culture system that supports the rapid multiplication of a reference strain of bluetongue virus. BHK or Vero cells are recommended. Antigen is present in the supernatant fluid at the end of virus growth but requires 50 to 100-fold concentration to be effective. This may be achieved by any standard protein concentration procedure; virus in the antigen may be inactivated by the addition of 0,3 % (v/v) beta-propiolactone.

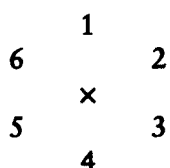
Known positive control serum:

Using the international reference serum and antigen a national standard serum is produced, standardized for optimal proportion against the international reference serum, freeze-dried and used as the known control serum in each test.

Test serum**Procedure:**

1 % agarose prepared in borate or sodium barbitol buffer, pH 8,5 to 9,0, is poured into a petri dish to a minimum depth of 3,0 mm.

A test pattern of seven moisture-free wells, each 5,0 mm in diameter, is cut in the agar. The pattern consists of one centre well and six wells arranged round it in a circle of radius 3 mm.



The central well is filled with the standard antigen. Peripheral wells 2, 4 and 6 are filled with known positive serum, wells 1, 3 and 5 are filled with test sera.

The system is incubated for up to 72 hours at room temperature in a closed humid chamber.

Interpretation:

A test serum is positive if it forms a specific precipitin line with the antigen and forms a complete line of identity with the control serum. A test serum is negative if it does not form a specific line with the antigen and it does not bend the line of the control serum. Petri dishes should be examined against a dark background and using indirect illumination.