►**B**

COMMISSION DECISION

of 15 July 1994

concerning animal health conditions and veterinary certification for the importation of bovine semen from third countries

(94/577/EC)

(OJ L 221, 26.8.1994, p. 26)

Amended by:

		Official Journal		
		No	page	date
►**M1**	Commission Decision 1999/495/EC of 1 July 1999	L 192	56	24.7.1999
►**M2**	Commission Decision 2004/52/EC of 9 January 2004	L 10	67	16.1.2004

Amended by:

►**A1**	Act concerning the conditions of accession of the Czech Republic, the Republic of Estonia, the Republic of Cyprus, the Republic of Latvia, the Republic of Lithuania, the Republic of Hungary, the Republic of Malta, the Republic of Poland, the Republic of Slovenia and the Slovak Republic and the adjustments to the Treaties on which the European Union is founded	L 236	33	23.9.2003

▼B

COMMISSION DECISION

of 15 July 1994

concerning animal health conditions and veterinary certification for the importation of bovine semen from third countries

(94/577/EC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European 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 semen of domestic animals of the bovine species ([1]), as last amended by Directive 93/60/EEC ([2]), and, in particular, Articles 10 and 11 thereof,

Whereas the list of third countries from which Member States authorize importation of semen of domestic animals of the bovine species (hereinafter referred to as 'the third countries list') was established by Commission Decision 90/14/EEC ([3]), as amended by Decision 91/276/EEC ([4]);

Whereas the disease situation in Israel led to a ban on imports from that country by Commission Decision 91/277/EEC ([5]);

Whereas Commission Decisions 91/479/EEC ([6]), 91/549/EEC ([7]), 92/123/EEC ([8]), 92/124/EEC ([9]), 92/125/EEC ([10]), 92/126/EEC ([11]), 92/127/EEC ([12]), 92/128/EEC ([13]), 92/386/EEC ([14]), 92/387/EEC ([15]) and 92/445/EEC ([16]) lay down the animal health conditions and veterinary certification for the importation of bovine semen from the United States of America, Canada, Poland, Finland, New Zealand, Austria, Switzerland, Sweden, Hungary, Norway and Czechoslovakia respectively;

Whereas Directive 93/60/EEC which has to be implemented in the Member States before 1 July 1994 at the latest amends Directive 88/407/EEC thereby necessitating amendment to the above third country Decisions; whereas in order to simplify and clarify Community legislation in this area it is necessary to group together the above Decisions and to repeal those in force for each individual country;

Whereas, given the provisions of the European Economic Area Agreement, it is not necessary to maintain the animal health certificate for importation of bovine semen from Austria, Finland, Norway and Sweden;

Whereas in accordance with the opinion of the Scientific Veterinary Committee, an additional test for epizootic haemorrhagic disease is required;

Whereas only in respect of Canada are routine precollection tests for this disease carried out at intervals of six months; whereas therefore it is necessary to allow a transitional period for introduction of this Deci-

([1]) OJ No L 194, 22. 7. 1988, p. 10.
([2]) OJ No L 186, 28. 7. 1993, p. 28.
([3]) OJ No L 8, 11. 1. 1990, p. 71.
([4]) OJ No L 135, 30. 5. 1991, p. 58.
([5]) OJ No L 135, 30. 5. 1991, p. 60.
([6]) OJ No L 258, 16. 9. 1991, p. 1.
([7]) OJ No L 298, 29. 10. 1991, p. 6.
([8]) OJ No L 48, 22. 2. 1992, p. 1.
([9]) OJ No L 48, 22. 2. 1992, p. 10.
([10]) OJ No L 48, 22. 2. 1992, p. 19.
([11]) OJ No L 48, 22. 2. 1992, p. 28.
([12]) OJ No L 48, 22. 2. 1992, p. 37.
([13]) OJ No L 48, 22. 2. 1992, p. 46.
([14]) OJ No L 204, 21. 7. 1992, p. 13.
([15]) OJ No L 204, 21. 7. 1992, p. 22.
([16]) OJ No L 247, 27. 8. 1992, p. 1.

▼B

sion in respect of Canada to allow the Canadian veterinary authorities sufficient time to introduce the new test in their routine programme;

Whereas, moreover, following recent advances in the understanding of the epidemiology of bluetongue, importation of bovine semen from Australia may now be permitted;

Whereas it appears that the animal health situation in the countries on the third countries list is good and controlled by well-structured and organized veterinary services as regards diseases transmissible through semen;

Whereas the responsible veterinary authorities of the third countries have undertaken to notify the Commission and the Member States by telex or telefax, within 24 hours, of the confirmation of the occurrence of any of the diseases contained in List A of the Office International d'Epizooties (OIE) or of any change in vaccination policy concerning any of them or, within an appropriate period, of the occurrence of diseases in List B of the OIE and any proposed change in the import rules concerning domestic animals or the semen or embryos thereof;

Whereas the responsible veterinary authorities of the countries on the third countries list have undertaken to supervise officially the issue of certificates from this Decision and to ensure that all relevant certificates, derogations and laboratory findings on which certification may have been based remain an official file for at least 12 months following the dispatch of the semen to which they refer;

Whereas the responsible veterinary authorities of the countries on the third countries list 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;

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

Member States shall authorize the importation of bovine semen which conforms to the conditions set out in the certificates in Part 1 of Annexes A, B, C and D and only from those third countries which appear in Part 2 of Annexes A, B, C and D respectively.

However, Member States shall authorize from Canada, imports of bovine semen collected and processed in accordance with Decision 91/549/EEC until 31 December 1994.

Article 2

Decisions 91/479/EEC, 92/123/EEC, 92/124/EEC, 92/125/EEC, 92/126/EEC, 92/127/EEC, 92/128/EEC, 92/386/EEC, 92/387/EEC and 92/445/EEC are repealed.

Decision 91/549/EEC is repealed from 1 January 1995.

Article 3

The provisions in relation to Canada referred to in the first subparagraph of Article 1 shall apply from 1 January 1995.

Article 4

This Decision is addressed to the Member States.

▼B

ANNEX A

PART 1

ANIMAL HEALTH CERTIFICATE

for importation of bovine semen

<table>
<tr><td colspan="2">1. Consignor (name and full address)</td><td>Certificate number
2. Third country of collection</td></tr>
<tr><td colspan="2">3. Consignee (name and full address)</td><td>4. Competent authority</td></tr>
<tr><td colspan="2"></td><td>5. Competent local authority</td></tr>
<tr><td colspan="2">6. Place of loading</td><td>7. Name and address of semen collection centre</td></tr>
<tr><td colspan="2">8. Means of transport
9. Place and Member State of destination
11. Seal number of semen containers</td><td>10. Approval number of semen collection centre</td></tr>
<tr><td colspan="3">12. Identification of semen
fresh or frozen *
(* delete as appropriate)</td></tr>
<tr><td>(a) Number of doses
(d) Identification of donor animal</td><td>(b) Date(s) of collection</td><td>(c) Breed</td></tr>
</table>

▼B

13. I the undersigned official veterinarian have read and am familiar with Council Directive 88/407/EEC as amended and certify that:

(a) .....
(name of exporting country)

has been free from rinderpest and foot and mouth disease during the 12 months immediately prior to collection of the semen for export and until its date of dispatch and no vaccination against these diseases has taken place during the same period;

(b) the semen collection centre at which the semen to be exported was collected was:

(i) approved under the conditions laid down in Annex A, Chapter I of Council Directive 88/407/EEC;

(ii) operated and supervised under the conditions laid down in Annex A, Chapter II of Council Directive 88/407/EEC;

(iii) during the period commencing 30 days prior to the date of collection of the semen to be exported until 30 days after collection (in the case of fresh semen until day of dispatch) have been free from rabies, tuberculosis, brucellosis, anthrax and contagious bovine pleuropneumonia;

(c) the bovine animals standing at the semen collection centre:

(i) come from herds and/or were born to dams which satisfy the conditions at Annex B, Chapter I of Directive 88/407/EEC;

(ii) have, prior to entry into isolation, undergone the tests required by Annex B, Chapter I of Directive 88/407/EEC;

(iii) have satisfied the pre-entry isolation and testing requirements laid down in Annex B, Chapter I of Directive 88/407/EEC;

(iv) if resident for at least one year have undergone the routine tests according to Annex B, Chapter II of Directive 88/407/EEC;

(d) the semen to be exported:

(i) was obtained from donors which have been resident in

.....
(name of exporting country)

for the period of six months immediately prior to collection of the semen for export and which satisfy the conditions laid down in Annex C of Directive 88/407/EEC;

(ii) was obtained from donors which are:

— standing in a semen collection centre in which all the bovine animals have within the 12 months prior to collection of the semen for export been subjected with negative results to a serum neutralization test or an Elisa for infectious bovine rhinotracheitis/infectious pustular vulvo vaginitis (1),

or

— negative to a serum neutralization test or an Elisa for infectious bovine rhinotracheitis/infectious pustular vulvovaginitis carried out within the 12 months prior to collection of semen for export (1),

or

— seropositive having been vaccinated in accordance with Annex B Chapter II of Council Directive 88/407/EEC and having given a negative result to a serum neutralization test or an Elisa carried out in the approved semen collection centre prior to vaccination (1);

(1) Delete as appropriate.

▼B

▶(1) ——— ◀

▶(2) ——— ◀

(iii) was processed, stored and transported under conditions which satisfy the terms of Directive 88/407/EEC.

Done at ....., on .....
(place) (date)

(Stamp) (2)

Signature (2): .....

Name in block letters: .....

Official title: .....

▶(2) ——— ◀

(2) The signature and the stamp must be in a colour different to that of the printing.

▶(1) (2) M1

▼A1

PART 2

List of countries authorised to use the model animal health certificate at Part 1 of Annex A

NEW ZEALAND

ROMANIA

SWITZERLAND

▼B

ANNEX B

PART 1

ANIMAL HEALTH CERTIFICATE

for importation of bovine semen from the United States of America

1. Consignor (name and full address)	Certificate number 2. Third country of collection
3. Consignee (name and full address)	4. Competent authority
	5. Competent local authority
6. Place of loading	7. Name and address of semen collection centre
8. Means of transport	10. Approval number of semen collection centre
9. Place and Member State of destination	
11. Seal number of semen containers	
12. Identification of semen	fresh or frozen *

(* delete as appropriate)

(a) Number of doses	(b) Date(s) of collection	(c) Breed
(d) Identification of donor animal		

▼B

13. I the undersigned official veterinarian have read and am familiar with Council Directive 88/407/EEC as amended and certify that:

(a) ..... has been free from rinderpest and
(name of exporting country)

foot and mouth disease during the 12 months immediately prior to collection of the semen for export and until its date of dispatch and no vaccination against these diseases has taken place during the same period;

(b) the semen collection centre at which the semen to be exported was collected was:

(i) approved under the conditions laid down in Annex A, Chapter I of Council Directive 88/407/EEC;

(ii) operated and supervised under the conditions laid down in Annex A, Chapter II of Council Directive 88/407/EEC;

(iii) during the period commencing 30 days prior to the date of collection of the semen to be exported until 30 days after collection (in the case of fresh semen until day of dispatch) have been free from rabies, tuberculosis, brucellosis, anthrax and contagious bovine pleuropneumonia;

(c) the bovine animals standing at the semen collection centre:

(i) come from herds and/or were born to dams which satisfy the conditions at Annex B, Chapter I of Directive 88/407/EEC;

(ii) have, prior to entry into isolation, undergone the tests required by Annex B, Chapter I of Directive 88/407/EEC;

(iii) have satisfied the pre-entry isolation and testing requirements laid down in Annex B, Chapter I of Directive 88/407/EEC;

(iv) if resident for at least one year have undergone the routine tests according to Annex B, Chapter II of Directive 88/407/EEC;

(d) the semen to be exported:

(i) was obtained from donors which have been resident in

.....
(name of country)

for the period of six months immediately prior to collection of the semen for export and which satisfy the conditions laid down in Annex C of Directive 88/407/EEC;

(ii) was obtained from donors which are:

— standing in a semen collection centre in which all the bovine animals have within the 12 months prior to collection of the semen for export been subjected with negative results to a serum neutralization test or an Elisa for infectious bovine rhinotracheitis/infectious pustular vulvo vaginitis (1),

or

— negative to a serum neutralization test or an Elisa for infectious bovine rhinotracheitis/infectious pustular vulvovaginitis carried out within the 12 months prior to collection of semen for export (1),

or

— seropositive having been vaccinated in accordance with Annex B Chapter II of Council Directive 88/407/EEC and having given a negative result to a serum neutralization test or an Elisa carried out in the approved semen collection centre prior to vaccination (1);

(1) Delete as appropriate.

▼B

►(1) ——— ◄

►(2) ——— ◄

(iii) was processed, stored and transported under conditions which satisfy the terms of Directive 88/407/EEC.

(iv) was obtained from donor bulls which were subjected on two occasions not more than 12 months apart to the following precollection and post-collection tests with negative results in an approved laboratory (the post-collection test must be performed on a blood sample taken not less than 21 days following the collection of semen for export):

— a competitive Elisa for bluetongue in accordance with Annex E,

— an agar-gel immuno-diffusion test in accordance with Annex E and a virus neutralization test for all serotypes of epizootic haemorrhagic disease (EHD) known to exist in the exporting country

Done at ....., on .....
(place) (date)

(Stamp) (2)

Signature (2): .....

Name in block letters: .....

Official title: .....

►(2) ——— ◄

(2) The signature and the stamp must be in a colour different to that of the printing.

►(1) (2) M1

▼B

PART 2

List of countries authorized to use the model animal health certificate at Part 1 of Annex B

UNITED STATES OF AMERICA

▼B

ANNEX C

PART 1

ANIMAL HEALTH CERTIFICATE

for importation of bovine semen

1. Consignor (name and full address)	Certificate number
	2. Third country of collection
3. Consignee (name and full address)	4. Competent authority
	5. Competent local authority
6. Place of loading	7. Name and address of semen collection centre
8. Means of transport	10. Approval number of semen collection centre
9. Place and Member State of destination	
11. Seal number of semen containers	
12. Identification of semen	fresh or frozen *
	(* delete as appropriate)
(a) Number of doses — (b) Date(s) of collection	(c) Breed
(d) Identification of donor animal	

▼B

13. I, the undersigned official veterinarian have read and am familiar with Council Directive 88/407/EEC as amended and certify that:

(a) .....
(name of exporting country)

has been free from rinderpest and foot and mouth disease during the 12 months immediately prior to collection of the semen for export and until its date of dispatch and no vaccination against these diseases has taken place during the same period;

(b) the semen collection centre at which the semen to be exported was collected was:

(i) approved under the conditions laid down in Annex A, Chapter I of Council Directive 88/407/EEC;

(ii) operated and supervised under the conditions laid down in Annex A, Chapter II of Council Directive 88/407/EEC;

(iii) during the period commencing 30 days prior to the date of collection of the semen to be exported until 30 days after collection (in the case of fresh semen until day of dispatch) have been free from rabies, tuberculosis, brucellosis, anthrax and contagious bovine pleuropneumonia;

(c) the bovine animals standing at the semen collection centre:

(i) come from herds and/or were born to dams which satisfy the conditions at Annex B, Chapter I of Directive 88/407/EEC;

(ii) have, prior to entry into isolation, undergone the tests required by Annex B, Chapter I of Directive 88/407/EEC;

(iii) have satisfied the pre-entry isolation and testing requirements laid down in Annex B, Chapter I of Directive 88/407/EEC;

(iv) if resident for at least one year have undergone the routine tests according to Annex B, Chapter II of Directive 88/407/EEC;

(d) the semen to be exported:

(i) was obtained from donors which have been resident in

.....
(name of exporting country)

for the period of six months immediately prior to collection of the semen for export and which satisfy the conditions laid down in Annex C of Directive 88/407/EEC;

(ii) was obtained from donors which are:

— standing in a semen collection centre in which all the bovine animals have within the 12 months prior to collection of the semen for export been subjected with negative results to a serum neutralization test or an Elisa for infectious bovine rhinotracheitis/infectious pustular vulvo vaginitis (1),

or

— negative to a serum neutralization test or an Elisa for infectious bovine rhinotracheitis/infectious pustular vulvovaginitis carried out within the 12 months prior to collection of semen for export (1),

or

— seropositive having been vaccinated in accordance with Annex B Chapter II of Council Directive 88/407/EEC and having given a negative result to a serum neutralization test or an Elisa carried out in the approved semen collection centre prior to vaccination (1);

(1) Delete as appropriate.

▼B

▶(1) ——— ◀

▶(2) ——— ◀

(iii) was processed, stored and transported under conditions which satisfy the terms of Directive 88/407/EEC.

(iv) was obtained from donor bulls which were either:

— standing in the approved centre on 1 July 1994,

or

— introduced into the approved centre after 1 July 1994 having been subjected to the following tests for bluetongue and epizootic haemorrhagic disease in an approved laboratory with negative results prior to entry and in either case were subjected every six months to the following tests in an approved laboratory with negative results:

— a competitive Elisa for bluetongue in accordance with Annex E;

— an agar-gel immuno-diffusion test in accordance with Annex E and a virus neutralization test for all serotypes of epizootic haemorrhagic disease found in the exporting country.

Done at ....., on .....
(place) (date)

(Stamp) (2)

Signature (2): .....

Name in block letters: .....

Official title: .....

▶(2) ——— ◀

(2) The signature and the stamp must be in a colour different to that of the printing.

▶(1) (2) M1

▼B

PART 2

List of countries authorized to use the model animal health certificate at Part 1 of Annex C

CANADA: excluding the Okanagan Valley region of British Columbia which is defined as the area enclosed by a line drawn from a point on the Canada/United States border 120º 15′ longitude, 49º latitude northerly to a point 119º 35′ longitude, 50º 30′ latitude north easterly to a point 119º longitude, 50º 45′ latitude southerly to a point on the Canada/United States border 118º 15′ longitude and 49º latitude.

▼B

ANNEX D

PART 1

ANIMAL HEALTH CERTIFICATE

for importation of bovine semen

<table>
<tr><td colspan="2">1. Consignor (name and full address)</td><td>Certificate number
2. Third country of collection</td></tr>
<tr><td colspan="2">3. Consignee (name and full address)</td><td>4. Competent authority</td></tr>
<tr><td colspan="2"></td><td>5. Competent local authority</td></tr>
<tr><td colspan="2">6. Place of loading</td><td>7. Name and address of semen collection centre</td></tr>
<tr><td colspan="2">8. Means of transport
9. Place and Member State of destination
11. Seal number of semen containers</td><td>10. Approval number of semen collection centre</td></tr>
<tr><td colspan="3">12. Identification of semen
fresh or frozen *
(* delete as appropriate)</td></tr>
<tr><td>(a) Number of doses
(d) Identification of donor animal</td><td>(b) Date(s) of collection</td><td>(c) Breed</td></tr>
</table>

▼B

13. I, the undersigned official veterinarian have read and am familiar with Council Directive 88/407/EEC as amended and certify that:

(a) ..... has been free from rinderpest and
(name of exporting country)

foot and mouth disease during the 12 months immediately prior to collection of the semen for export and until its date of dispatch and no vaccination against these diseases has taken place during the same period;

(b) the semen collection centre at which the semen to be exported was collected was:

(i) approved under the conditions laid down in Annex A, Chapter I of Council Directive 88/407/EEC;

(ii) operated and supervised under the conditions laid down in Annex A, Chapter II of Council Directive 88/407/EEC;

(iii) during the period commencing 30 days prior to the date of collection of the semen to be exported until 30 days after collection (in the case of fresh semen until day of dispatch) have been free from rabies, tuberculosis, brucellosis, anthrax and contagious bovine pleuropneumonia;

(c) the bovine animals standing at the semen collection centre:

(i) come from herds and/or were born to dams which satisfy the conditions at Annex B, Chapter I of Directive 88/407/EEC;

(ii) have, prior to entry into isolation, undergone the tests required by Annex B, Chapter I of Directive 88/407/EEC;

(iii) have satisfied the pre-entry isolation and testing requirements laid down in Annex B, Chapter I of Directive 88/407/EEC;

(iv) if resident for at least one year have undergone the routine tests according to Annex B, Chapter II of Directive 88/407/EEC;

(d) the semen to be exported:

(i) was obtained from donors which have been resident in

.....
(name of exporting country)

for the period of six months immediately prior to collection of the semen for export and which satisfy the conditions laid down in Annex C of Directive 88/407/EEC;

(ii) was obtained from donors which are:

— standing in a semen collection centre in which all the bovine animals have within the 12 months prior to collection of the semen for export been subjected with negative results to a serum neutralization test or an Elisa for infectious bovine rhinotracheitis/infectious pustular vulvo vaginitis [1],

or

— negative to a serum neutralization test or an Elisa for infectious bovine rhinotracheitis/infectious pustular vulvovaginitis carried out within the 12 months prior to collection of semen for export [1],

or

— seropositive having been vaccinated in accordance with Annex B Chapter II of Council Directive 88/407/EEC and having given a negative result to a serum neutralization test or an Elisa carried out in the approved semen collection centre prior to vaccination [1];

[1] Delete as appropriate.

▼B

►(1) ——— ◄

►(2) ——— ◄

(iii) was processed, stored and transported under conditions which satisfy the terms of Directive 88/407/EEC.

(iv) was obtained from donor bulls which were subjected on two occasions not more than 12 months apart to the following pre-collection and post-collection tests with negative results in an approved laboratory (the post-collection test must be performed on a blood sample taken not less than 21 days following the collection of semen for export):

— a competitive Elisa for bluetongue in accordance with Annex E,

— an agar-gel immuno-diffusion test in accordance with Annex E and a virus neutralization test for all serotypes of epizootic haemorrhagic disease (EHD) known to exist in the exporting country,

— a serum neutralization test for Akabane virus.

Done at ....., on .....
(place) (date)

(Stamp) (2)

Signature (2): .....

Name in block letters: .....

Official title: .....

►(2) ——— ◄

(2) The signature and the stamp must be in a colour different to that of the printing.

▼B

PART 2

List of third countries authorized to use the model animal health certificate at Part 1 of Annex D

AUSTRALIA

▼B

ANNEX E

Protocol for a competitive Elisa ('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 test 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.

MATERIALS 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 phosphate-buffered saline (PBS).
4. Monoclonal antibody: 3-17-A3 (supplied as hybridoma tissue-culture supernatant) stored at − 20 ºC or freeze-dried, diluted 1/50 with blocking buffer before use, directed against the group-specific polypeptide p7.
5. Conjugate: rabbit anti-mouse globulin (absorbed and eluted) conjugated to horseradish peroxidase and kept in the dark at 4 ºC.
6. Substrate and chromogen: 0,2 gm of orthophenylene diamine (OPD) dissolved in a buffer consisting of 2,553 gm of citric acid and 4,574 gm of di-sodium hydrogenorthophosphate made up to 500 ml with distilled water, divided into 25 ml aliquots and kept in the dark at − 20 ºC, 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).

▼B

TEST FORMAT

	H	G	F	E	D	C	B	A	
Blank			Antigen + Conjugate						1
MAB Control			Antigen + MAB + Conjugate						2
+ ve Control	Antigen + Positive Serum + MAB + Conjugate								3
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	
	Test Sera								
	1/2	1/4	1/8	1/16	1/2	1/4	1/8	1/16	4
									5
									6
Test Sera									7
									8
									9
									10
									11
									12

▼B

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 represent 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 ºC for 60 minutes on an orbital shaker. Wash plates three times by flooding and emtying 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 ºC for 60 minutes on an orbital shaker. Wash three times with PBS and blot dry.
6. Dilute rabbit anti-mouse concentrate to 1/5000 in blocking buffer and add 50 µl to all wells of the plate.
7. Incubate at 37 ºC 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.

▼B

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 ºC and examine daily for cytopathic effect (cpe).
3. When cpe is evident in 80 — 90 % of the cell sheet of each roux, harvest the virus by shaking any stillattached cells from the glass.
4. Centrifuge at 2 000 — 3 000rpm to pellet the cells.
5. Discard the supernatant and resuspend the cells in approximately 30 ml of PHS containing 1 % 'Sarkosyl' and 2 ml phenolmethylsulphonyl fluoride (lysis buffer). This may cause the cells to form a gel and more lysis buffer may be added to reduce this effect.

   NOTE: phenylmethylsulphonyl 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 ºC and resuspend the remaining cell pellet in 10 — 20 ml of lysis buffer.
9. Sonicate and clarify, storing the supernatant at each stage, at total of three times.
10. Pool the supernatants and centrifuge at 24 000 rpm for 120 minutes at + 4 ºC 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 resuspend the pellet in PBS by sonication. Store the antigen in aliquots at − 70 ºC.

TITRATION OF BTV ELISA ANTIGEN

Bluetongue Elisa antigen is titrated by the indirect Elisa. Two-fold 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 two-fold dilution series (50 µl/well) using a multichannel pipette.
2. Incubate for 1 hour at 37 ºC 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 1 hour at 37 ºC 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 1 hour at 37 ºC 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.

▼B

Protocol for an agar-gel immunodiffusion test for the detection of epizootic haemorrhagic disease antibodies

The agar-gel immo-diffusion test is carried out according to the following protocol:

MATERIALS AND REAGENTS

1. Antigen

   Precipitating antigen is prepared in any cell culture system that supports the rapid multiplication of the appropriate serotype(s) of epizootic haemorrhagic disease virus. BKH 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.

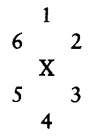
2. Known positive control serum

   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.

3. Test serum.

PROCEDURE

1. 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.
2. 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.



3. 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.
4. 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 precipitation 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.