VICH, International Co-operation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products.

WORKING GROUP: Biologicals Quality Monitoring.

TOPIC: Test on the presence of extraneous agents in live viral vaccines

TESTMETHODS TO BE USED FOR MAMMALIAN VETERINARY LIVE VIRUS VACCINES.

1. Introduction

2. Test methods
   - The test method to be used for Master Seed Virus
   - The test method to be used for Master Cell Seed
   - The test method to be used for Bovine Serum
   - The test method to be used for other materials of animal origin if they have not been sterilised by acceptable processes subjected to a suitable validated inactivation procedure
   - The test method to be used for intermediate products
   - The test method to be used for the finished product

3. Special test methods and requirements
   - General test methods
   - Specific test methods

4. Annexes
   - List of viruses
   - Glossary
EXPLANATORY NOTES ON THE AMENDMENTS OF THE TEXT

1. The text in section 1.1. paragraph 2 was changed to allow easier translation into Japanese.

2. It is not clear which decision was taken on the proposed footnote to the Schematic Outline at page 4:

   'Keeping a separate line of control cells that are tested for the presence of extraneous agents and tests for extraneous agents on the harvest (or other intermediate products) and on the finished product are only necessary in cases specified in this guideline'.

   As far as I remember it was agreed that the control cells shall only be examined for the occurrence of cytopathic effect and other abnormalities by microscopic examination. I have included that text in the guideline.

3. The text in section 2.1.1. "The selection of cell lines and tests for specific viruses that potentially may have been introduced by exposure of the MSV to materials of a different origin than that of the MSV will depend on the available documentation on the origin, history and treatment of these materials." is not a new requirement. Based on the 9 CFR 113.3 there is evidence that this is not a new regulation, but rather an interpretation of those regulations and current practises in both the US and the EU.

4. The comment of FEDESA on section 2.1.2:

   "As the neutralising antiserum has to 'free of antibodies against those viruses for which the presence in the Master Seed [Virus], the requirement that the antiserum should be raised against a virus that 'has been passaged on different cells [than the Master Seed Virus] and 'has been adequately purified' are unnecessary and should therefore be omitted.'"

   was considered to be correct. However even so it was decided to leave the text unchanged for the sake of clarity.

5. All volumes in the guideline are specified to the first decimal place in order to prevent unintended rounding misinterpretation e.g. 1 ml shall be stated as 1.0 ml in order to define the volume as between 0.95 ml and 1.04 ml.

6. The comment made in respect of section 2.1.4.3.1.1. that the word "subculture" should be replaced by "passage" was not accepted. The method involves manipulating and examining cells and for that reason the word "subculture" was retained and additional clarifying language incorporated.

7. The fact that MSV shall in principle be produced at a neutralisable titre is based on USDA regulations. It is recognised that in principle there is no difference between diluting the MSV during the test and the dilution of virus suspension before filling the MSV. However for legal reasons it is preferred to ensure that the MSV itself is qualified.
1. INTRODUCTION.

The guideline describes the test methods used to ensure that vaccines are not contaminated with extraneous agents. Such test methods may be used for the starting materials, intermediate products and finished product dependent on the requirements to be met for new vaccines in order to get a marketing authorisation.

1.1 Objective of the guideline.

It is essential that vaccines for veterinary use are free of contaminants, notably viral agents. Potential sources of contamination are the viral strains, the cells and other starting materials of animal origin used in the production of the active ingredient and / or in the assembly of the finished product. Consequently it is necessary to demonstrate that extraneous viruses are not present in veterinary vaccines or in the starting materials required for their manufacture, through the use of accepted testing procedures and sampling methods and subject to the limitations of the test.

The purpose of the guideline is to provide a detailed description of the test methods to detect the presence of extraneous viruses which shall be used on starting materials (MSV, MCS), materials of animal origin used in the vaccine production, intermediate and finished products.

Harmonisation of the test methods will be the first step in the harmonisation of the regulatory requirements for veterinary vaccines.

Explanatory remark:

- The explanatory remark is not part of the text of the guideline
- The wish was expressed by the Working Group that the harmonisation of the regulatory requirements shall be discussed after agreement is reached on the harmonisation of the test methods.

1.2. Background.

The materials used in the manufacture of veterinary viral vaccines include:

- Viral strains
- Cell substrates
- Starting materials of animal origin which may be used in the production of the active ingredient and / or in the assembly of the finished product.

Present methods of testing for extraneous viruses are described in the European Pharmacopoeia, the USA Code of Federal Regulations, the Minimum Requirements of Japan and the OIE Manual of Standards for Diagnostic Tests and Vaccines.

This guideline deals specifically with the test methods to detect the presence of extraneous viruses. The tests define an acceptable level of sensitivity necessary. However, other test methods may be used provided they are validated and have at least an equal sensitivity as the test method described in the guideline and are approved by the competent authority.

Present methods of testing for extraneous viruses of substances of animal origin are described in the European Pharmacopoeia monograph 62 (1995), the USA Code of Federal Regulations 9.CFR 113, the Minimum Requirements of Japan and the OIE Manual of Standards for Diagnostic Tests and Vaccines. Other test methods may be used provided they are validated.
and have at least an equal sensitivity as the test method described in the guideline and are approved by the competent authority.

1.3. Scope of the guideline.

The scope of the guideline is to provide guidance on the methods to determine the presence of extraneous viruses in:

- Master Seed Virus c.q. Working Seed Virus
- Master (and Working?) Cell Seed c.q. Working Cell Seed
- Bovine serum
- Other starting materials of animal origin not sterilised by acceptable processes subjected to a suitable validated inactivation procedure.
- Intermediate products (in-process controls)
- Finished product

where such testing is required by the competent authority. Inclusion of a test method in the guideline does not imply that it will necessarily be required by the competent authority.

The scope of the guideline is restricted to the test methodology. If and when the materials listed above have to be tested for extraneous viruses is not part of this guideline.
SCHEMATIC OUTLINE

OF THE STAGES OF PRODUCTION AT WHICH TESTING OF VETERINARY
VIRAL VACCINES FOR THE PRESENCE OF EXTRANEOUS VIRUSES
MAY BE REQUIRED

SUBSTRATE
Established cell lines
or
Primary cells

TEST FOR EXTRANEOUS AGENTS

MATERIALS OF ANIMAL ORIGIN

TEST FOR EXTRANEOUS AGENTS

PRODUCTION CELLS
Incubation as stated in Outline of Production

CONTROL CELLS
Incubation under conditions similar to those stated in Outline of Production; examination for cytopathic effect and abnormalities

TEST FOR EXTRANEOUS AGENTS

VIRUS SEEDS

TEST FOR EXTRANEOUS AGENTS

HARVEST

TEST FOR EXTRANEOUS AGENTS

FINISHED PRODUCT
2. TEST METHODS.

2.1. TEST FOR THE PRESENCE OF EXTRANGEOUS VIRUSES IN VIRUS SEEDS.

2.2. TEST FOR THE PRESENCE OF EXTRANEOUS VIRUSES IN CELL SEEDS.

2.3. TEST FOR THE PRESENCE OF EXTRANEOUS VIRUSES IN BOVINE SERUM.

2.4. TEST FOR THE PRESENCE OF EXTRANEOUS VIRUSES IN OTHER MATERIALS OF ANIMAL ORIGIN NOT STERILISED BY ACCEPTABLE PROCESSES.

2.5. TEST FOR THE PRESENCE OF EXTRANEOUS VIRUSES IN INTERMEDIATE PRODUCTS (IN-PROCESS CONTROLS)

2.6. TEST FOR THE PRESENCE OF EXTRANEOUS VIRUSES IN FINISHED PRODUCT.
2.1. TEST FOR THE PRESENCE OF EXTRANEOUS VIRUSES IN VIRUS SEEDS

SCHEMATIC OUTLINE OF TEST FOR EXTRANEOUS VIRUSES

MASTER SEED VIRUS

MSV SAMPLE

- PRIMARY CELLS
  - of source species or, when justified, cells that are sensitive to a suitable range of viruses that may be present in the source species

- CELLS LINE
  - Susceptible to viruses of target animal species and to viruses that may be present in substances of animal origin used during the establishment of the seed (except where the substances of animal origin have been subjected to a suitable validated inactivation procedure)

  - Susceptible to Pestiviruses

(7 DAYS)

- SUBCULTUR E 1

(7 DAYS)

- SUBCULTUR E 2

(7 DAYS)

GENERAL TESTS

- CYTOPATHIC EFFECT

- HAEMADSORPTION

SPECIFIC TESTS

- IMMUNO_STAINING OR IMMUNOCHEMICAL TESTS
  - e.g. FA TEST AND/OR ELISA TEST
2.1.1. General.

Samples of all viral seed materials are tested to the extent possible and if necessary for the presence of extraneous viruses that may originate from:

- the source species of the material.
- those animal species to which the seed material may have been exposed to e.g. during passaging.

and also

- for the presence of possible contaminating viruses to which the target animal species is susceptible to infection.

**NOTE:**

The selection of cell lines and tests for specific viruses that potentially may have been introduced by exposure of the MSV to materials of a different origin than that of the MSV will depend on the available documentation on the origin, history and treatment of these materials. Tests for viruses that may be present in substances of animal origin used in the establishment of the seed are not necessary when these substances have been subjected to a suitable validated inactivation procedure or tested and shown to be free from viruses.

If during one or more of the preparatory stages of the testing for extraneous agents an additional process is used e.g. adsorption it will be necessary to demonstrate that this process does not affect the sensitivity of the test and the test results.

In exceptional cases and when justified inoculation / serology tests in seronegative animals may be used.

2.1.2. Samples

Normally the samples shall be taken from the Master Seed Virus. However, there may be instances in which it should be permitted to use material obtained by a further passage of the MSV. This may be the case when e.g. it is necessary to update the testing of the MSV for a vaccine which is already on the market or for an existing vaccine introduced into a new market and of which the quantity of MSV has become very limited. This material should have the lowest possible passage level necessary to obtain sufficient material for the test and not exceed 5 passages of that MSV.

The Master Seed Virus shall - when necessary - be neutralised with a monoclonal antibody or a polyclonal specific antiserum containing sufficiently high levels of neutralising antibody to the virus present in the Master Seed Virus.

When a polyclonal specific antiserum is used, it shall be free of antibodies against those viruses for which presence the Master Seed is tested. To that end, such an antiserum will be prepared, wherever possible, with an antigen that:

- is not derived from any passage level of the virus isolate giving rise to the Master Seed Virus.
- has been passaged on different cells
- has been adequately purified.
If this is not possible, the antiserum may be prepared with a different isolate or an earlier passage of the virus concerned. Where the antiserum cannot be prepared in this way, the procedure used must be justified.

**NOTE:**

If the MSV cannot be completely neutralised, alternative validated methods to eliminate the MSV may be used when duly justified.

### 2.1.3. Substrates

The substrates used for testing the presence of extraneous agents shall consist of one or more types of sensitive cells, provided that the three following conditions are met:

- primary cells of the source species or, when justified, cells that are sensitive to a suitable range of viruses that may be present in the source species;
- cells sensitive to viruses pathogenic for the target species for which the vaccine is intended;
- cells sensitive to pestiviruses. The sensitivity of these cells shall be demonstrated by the use of positive controls prepared with a non-cytopathic strain of Bovine Viral Diarrhoea virus.

Where relevant, other cells or substrates e.g. eggs, sensitive to other relevant viruses that may potentially be present in the virus seed, as a result of passaging the virus strain in cells of other animal species shall be used.

The tests methods using embryonated eggs are described in the Guideline for Testing for the Presence of Extraneous Viruses in Avian Viral Vaccines.

### 2.1.4. Test method

#### 2.1.4.1. Preparation of the substrate cells

For initial inoculation, the monolayers of cells of the appropriate sensitivity to be used in the test shall cover an area of at least 70 cm$^2$.

#### 2.1.4.2. Inoculum

An inoculum of 1.0 ml of the undiluted MSV combined if necessary with appropriate antiserum shall be used to inoculate each surface area of at least 70 cm$^2$.

In principle, the MSV should be tested undiluted without further dilution. The quantity of virus contained in each 1.0 ml should be justified to the competent authority.

**NOTE:**

Any new MSV should be produced at a level at which it can be tested. Manufacturers may produce virus at a higher level than can be neutralized and dilute it to a neutralizable titer before bottling and testing the MSV. If during MSV qualification the virus can not be tested because of a non neutralizable titer the manufacturer may have the option to produce a new, uniquely identified MSV, by dilution from the previous MSV to the highest titre that can be neutralised. In such cases sterility, mycoplasma, and extraneous agent testing will be required on the new MSV. Previous efficacy, safety, reversion to virulence, etc. qualification should be applicable to the new MSV after evaluation by the competent authority.
2.1.4.3. Procedure.

2.1.4.3.1. General tests to detect presence of extraneous viruses.

2.1.4.3.1.1. Observation of cell cultures.

All cell cultures are maintained for at least 21 days, during which at least 2 subcultures of the cells are made at 7 days intervals, unless the cells do not survive for this length of time, when the subculture shall be made on the latest day possible. At least Sufficient monolayers of suitable cells with a surface area suitable for the tests prescribed below of at least 20 cm² each shall be used prepared for the final subculture.

They are examined as follows:

- All cultures shall be observed at least every second working day
- At the end of the last subculture
  - monolayers are examined for cytopathic effect in accordance with the method described in section 3.1.
  - monolayers are examined for haemadsorption in accordance with the method described in section 3.1

2.1.4.3.2. Tests to detect presence of specific viruses.

For viruses, which are unlikely to be detected by the general tests described above, specific test methods must be applied unless there is justification for not testing as detailed in the introductory remarks to Annex 1. These viruses are indicated under the heading “Specific tests” in the relevant tables.

This shall be done in accordance with section 3.2..
2.2. TEST FOR THE PRESENCE OF EXTRANEOUS VIRUSES IN CELL SEEDS

SCHEMATIC OUTLINE OF TEST FOR EXTRANEOUS VIRUSES.

MASTER CELL SEED.

MASTER CELL SEED SAMPLE

- MCS CELLS
- PRIMARY CELLS of source species or, when justified, cells that are sensitive to a suitable range of viruses that may be present in the source species
- PRIMARY CELLS of source species
- CELL LINE susceptible to viruses of target animal species and to viruses that may be present in substances of animal origin used during establishment of the seed except where the substances of animal origin have been subjected to a suitable validated inactivation procedure and shown to be free from viruses (with the exception of Pestiviruses)

(7 days)

- SUBCULTURE 1
- SUBCULTURE 2

(7 days)

- GENERAL TESTS
- CYTOPATHIC EFFECT
- HAEMADSORPTION

(7 days)

- CELL PASSAGE 1
- CELL PASSAGE 2

(7 days)

- CELL PASSAGE 1
- CELL PASSAGE 2
- CELL PASSAGE 2
SPECIFIC TESTS

IMMUNO_STAINING OR IMMUNOCHEMICAL TESTS

e.g. FA - TEST AND/OR ELISA TEST
2.2.1. General.

The cell seed is tested to the extent possible for the presence of extraneous agents. Tests should be carried out for possible contaminating viruses that may originate from:

- the source species of the cells.
- those animal species to which the cell seed may have been exposed in the preparation of the Master Cell Seed.

...and also...

- for the presence of possible contaminating viruses to which the target animal species is susceptible to infection.

The extent of testing for specific viruses that may have been introduced by exposure of the MCS to materials of animal origin will depend on the available documentation on the origin, history and treatment of these materials. Tests for viruses that may be present in substances of animal origin used in the establishment of the seed are not necessary when these substances have been subjected to a suitable validated inactivation procedure or tested and shown to be free from viruses.

2.2.2. Samples

The size of the sample shall depend on the number of cells present in the cell seed material but shall contain at least \(1 \times 10^5\) cells or sufficient cells to produce a monolayer of at least 70 cm

The size of the sample taken in the case of suspension cultures of non anchorage dependent cells shall be at least \(1 \times 10^5\) cells or at least 1.0 ml of the suspension culture, whichever sample contains the largest number of cells.

2.2.3. Substrates

The substrates used for testing the presence of extraneous agents shall consist of at least one or more different types of sensitive cells provided that the following conditions are met:

- primary cells of the source species, or, when justified, cells that are sensitive to a suitable range of viruses that may be present in the source species provided they are more sensitive than available non-primary cells, except in the case of testing of well-known established cell lines of simian origin. In the latter case the test may be done on another established cell line of simian origin.

- cells sensitive to viruses pathogenic for the target species cells and to viruses that may be present in substances of animal origin used during establishment of the cell seed (except where the substance(s) of animal origin have been subjected to a suitable validated inactivation procedure or tested and shown to be free from viruses).

2. cells sensitive to pestiviruses. The sensitivity of these cells shall be demonstrated by the use of positive controls prepared with a non-cytopathic strain of Bovine Viral Diarrhoea virus.
2.2.4. Test method.

The test to determine the presence of extraneous agents in cells consists of three different stages:

- the test on subcultures of the MCS.
- the test on passaged cultures of the MCS.

2.2.4.1. The test on the subcultures of the MCS

2.2.4.1.1. Preparation of the cell cultures.

The Master Cell Seed is used to establish monolayers in suitable culture vessels. The monolayers to be used in the test shall cover an area of at least 70 cm$^2$ and shall be prepared and maintained using medium and additives that support good growth. The monolayers shall be confluent for not more than 70% at the moment of inoculation.

2.2.4.1.2. Procedure.

At least 2 subcultures are made of the monolayers with an interval of 7 days or as frequently as may be necessary for suitable growth. The cell-cultures are maintained for at least 21 days during which period the cultures are examined regularly for cytopathic effect.

At least 7 days after the initiation of the last subculture, the monolayers are examined:

- for cytopathic effect in accordance with the method described in section 2.6.1.
- for haemadsorption in accordance with the method described in section 2.6.2.
- for the presence of specific extraneous agents in accordance with the method described in section 2.7

2.2.4.2. The test on the passaged cultures of the MCS.

2.2.4.2.1. Preparation of the cell cultures.

The Master Cell Seed is used to establish monolayers with a surface of at least 140 cm$^2$. For anchorage-independent cells, cultures containing at least $1 \times 10^7$ cells are used. In the case of non-anchorage dependent cells suitable alternative methods have to be established.

2.2.4.2.2. Procedure.

2.2.4.2.2.1. Cell passage. Inoculum

The cells of the monolayers of each cell type used and supernatant are harvested and the...

...The cells and 50% of the supernatant are three times subjected to freeze-thawing three times and... The freeze-thawed mixtures are subsequently mixed with the remaining supernatant. Each of two monolayers of each required cell type (see section 2.2.3) with a surface of at least 70 cm$^2$ each is inoculated with 1.0 ml of the resulting material.

2.2.4.2.2.2 Cell passage
After incubation for at least 7 days the cells are collected, and the cells and 50% of the supernatant are subjected to freeze-thawing three times and subsequently mixed with the remaining supernatant and treated in the same manner. The material is inoculated into new monolayers of the same cell type and incubated for a period of 7 days. A second subculture is made in the same manner and at the end of the period of 21 days the monolayers are examined:

- for cytopathic effect in accordance with the method described in section 2.6.1.
- for haemadsorption in accordance with the method described in section 2.6.2.
- for the presence of specific extraneous agents in accordance with the method described in section 2.7.
2.3. TEST FOR THE PRESENCE OF EXTRANEOUS VIRUSES IN BOVINE SERUM

SCHEMATIC OUTLINE OF TEST

BOVINE SERUM SAMPLE PRESENT IN CELL CULTURE MEDIUM.

CELL (LINE) susceptible to bovine viruses

CELL (LINE) susceptible to Pestiviruses

SUBCULTURE 1

SUBCULTURE 2

SUBCULTURE 3

SUBCULTURE 4

GENERAL TESTS

CYTOPATHIC EFFECT

HAEMADSORPTION

SPECIFIC TESTS

IMMUNO_STAINING OR IMMUNOCHEMICAL TESTS
  e.g. FA - TEST and ELISA TEST

COMPARATIVE TITRATION OF BVD VIRUS
2.3. TEST FOR THE PRESENCE OF EXTRANEOUS VIRUSES IN BOVINE SERUM.

2.3.1. General.

Bovine serum may be used in many stages of the manufacture of vaccines for veterinary use and is considered as a material with a potential relatively high risk in respect of acting as a source of contamination for veterinary vaccines, notably with Bovine Viral Diarrhoea Virus and is therefore subject to specific control tests. It shall be tested for the presence of extraneous viruses. Special care shall be taken in demonstrating the absence of BVDV because the presence of BVDV may be masked by the presence of antibodies against BVDV.

The material is tested by applying tests of a general nature that may be expected to detect a broad selection of agents, complemented by specific tests to detect individual agents for which there is a particular risk of occurrence. These tests shall be capable of detecting viruses inducing viraemia and transplacental infection.

2.3.2. Sample.

The size of the sample used for testing shall be justified.

Explanatory remark:

- The explanatory remark is not part of the text of the guideline.
- The subject of the sample size was not yet resolved during the previous meeting of the WG and will have to be decided upon during the meeting in Strasbourg in September 2003.

2.3.3. Substrates

The substrate shall consist of bovine cells demonstrated to be sensitive to pestiviruses including non-cytopathic BVD virus and the other bovine viruses.

2.3.4 Test method.

2.3.4.1 Preparation of the substrate cells.

Cell cultures of the appropriate sensitivity to be used in the test shall cover an area of at least 70 cm².

Control cells cultivated with a serum, known to be free of antibodies against BVDV shall be included.

2.3.4.2. Inoculum.

The test sample shall be incorporated into growth medium at a concentration of not less than 5%. Not more than 25 ml of this medium shall be used per 70 cm² of cell culture. The total
cell surface area is dependent on the serum sample size required for testing by the competent authority.

Explanatory remark:

The explanatory remark is not part of the text of the guideline.

The subject of the sample size was not yet resolved during the previous meeting of the WG and will have to be decided upon during the meeting in Strasbourg in September 2003.

2.3.4.3. Procedure.

2.3.4.3.1. General tests to detect presence of extraneous viruses.

Observation of cell cultures.

All cell cultures are maintained for at least 21 days, during which at least 2 subcultures are made at 7 days intervals. If the cells do not survive for this length of time, then the subcultures shall be made on the latest day possible.

In the case of the cell cultures used to detect the presence of BVD virus at least 4 subcultures shall be made.

Explanatory remark:

The explanatory remark is not part of the text of the guideline.

The U.S. to determine if data still exists for the US conversion from the BVDV interference test to the BVDV FA test presently codified in the 9 CFR and to supply that information prior to the next consultation.

They are examined as follows:

- All cultures shall be observed at least every second working day
- At the end of the last subculture
- monolayers are examined for cytopathic effect in accordance with the method described in section 3.6.1.
- monolayers are examined for haemadsorption in accordance with the method described in section 3.6.2.

2.3.2.3.3.2. Specific tests to detect presence of extraneous viruses.

For viruses, which are unlikely to be detected by the general tests described above, specific test methods must be applied. These viruses are indicated under the heading “Specific tests” in the relevant tables.

2.3.2.3.3.2.1. Preparation of the substrate cells.
The monolayers of cells of the appropriate sensitivity to be used in the test shall cover an area of at least 70 cm$^2$.

The monolayers are maintained in culture for a total of at least 21 days. At least two subcultures are made at 7-day intervals. If the cells do not survive for this length of time, then the subcultures are made on the latest day possible.

In the case of the cell cultures used to detect the presence of BVD virus at least 4 subcultures have to be made.

2.3.2.3.2.2. Examination for specified viruses.

This shall be done in accordance with section 2.5.3.

NOTE:

For serum intended for use in growing and maintaining cells for extraneous agent testing it shall be demonstrated that the level of antibodies against BVDV does not interfere with the detection of a low level of BVDV e.g. by use of a positive control.

The following procedures may be used for that purpose:

A. Comparative Titration Test for BVD virus.

This test shall be done after the serum has been subjected to an inactivation treatment.

Appropriate sensitive cells which have been grown with at least 3 subcultures, in the presence of the serum under test, are used. These cells are used to titrate a reference strain of BVD virus.

A similar titration shall be done with identical cells grown in the presence of a previously qualified reference serum.

The result of the titrations shall demonstrate that the serum does not have an apparent inhibitory effect on the multiplication of BVD virus (e.g. within 0.7 log$_{10}$ of the reference BVDV virus grown with the qualified reference serum).

Data should be supplied (if available) from each region for the next consultation (Washington) to determine if 0.7 log is the appropriate number for the inhibitory threshold.

B. Test to detect antibodies against BVDV.

The samples are tested using a validated technique to detect BVDV antibodies. The results of the test shall demonstrate that the serum is either free of BVDV antibodies or that the level of these antibodies does not interfere with the detection of a low titre of BVD virus e.g. by validated spiking techniques.

The validation performed should clearly demonstrate what the limit of detection (LOD) of the testing method is in the presence of any given titre of antibodies and how this compares with the LOD when specific antibodies are not present. Ideally the titre of virus used for spiking should be equivalent to the lower limit of detection of the validated assay for BVD virus meaning that the LOD is not affected by the titre of antibodies present.
Where this is not possible the inactivation procedure shall have a sufficient safety margin to inactivate not only the titre of virus detected but also the additional titre "masked" by the presence of antibody.
2.4. TEST FOR THE PRESENCE OF EXTRANEOUS VIRUSES IN OTHER MATERIALS OF ANIMAL ORIGIN NOT STERILISED BY ACCEPTABLE PROCESSES.

SCHEMATIC OUTLINE OF TEST

SAMPLE OF MATERIAL PRESENT IN CELL CULTURE MEDIUM.

CELLS (LINE) susceptible to viruses that may be present in each of the materials used for the preparation of the substance

CELLS (LINE) susceptible to Pestiviruses

(7 days)

SUBCULTURE

(7 days)

SUBCULTURE

(7 days)

SUBCULTURE

GENERAL TESTS

CYTOPATHIC EFFECT

HAEMADSORPTION

SPECIFIC TESTS

IMMUNO_STAINING OR IMMUNOCHEMICAL TESTS

e.g. FA - TEST and ELISA TEST
2.4.1. General.

Material of animal origin shall be sterilised by acceptable methods as e.g. described in the EP 5.1.1 and 5.2.5. If this is not the case than it shall be tested by applying tests of a general nature that may be expected to detect a broad selection of agents, complemented by specific tests to detect individual agents for which there is a particular risk of occurrence. These agents - only of viral nature - are listed in Annex 2.

2.4.2. Samples.

The sample size shall be justified on the basis of likelihood of contamination (origin and history) degree of purification applied, degree of concentration and availability.

The material, when prepared for testing, shall be dissolved or suspended in a defined medium, that is identical or at least related to the medium in which the substance will be used in the production process, provided this medium is compatible with the test system.

Any solids are dissolved or suspended in a suitable medium in such a way as to create a solution or suspension containing at least 30 per cent w/v of the substance to be examined.

If the substance is not soluble or where cytotoxic reactions occur, a lower concentration may be used when justified. In case of very scarce ingredients a minimum sample size may be used.

2.4.3. Substrates.

It is very important that the substrates are selected by taking into account the origin of the materials of animal origin that were used in the manufacture of the substance to be tested. It is not uncommon that the substance contains materials of animal origin from more than one animal species. This information shall be obtained from the manufacturer of that substance.

The test shall therefore be done with cell cultures, including primary cell cultures of each of the animal species from which material was used in the manufacture of the substance and which cells are sensitive to extraneous viruses that may be present in these materials.

In addition at least one other sensitive cell culture such as VERO cells shall be used.

The monolayers shall have a surface of at least 70 cm²

Explanatory note:

- The explanatory note is for information only and will not be part of the text of the guideline.
- VERO cells are advised because they are well known, are specifically sensitive to Reo viruses and are also recommended in 9CFR.

2.4.4. Test method.

At least 2 subcultures of the inoculated monolayers are made layers with an interval of 7 days. The cell-cultures are maintained for at least 21 days during which period the cultures are examined regularly for cytopathic effect.

At the end of the incubation period the last subculture shall be examined:
- for cytopathic effect in accordance with the method described in section 3.1.
- for haemadsorption in accordance with the method described in section 3.2.
- for the presence of specific extraneous agents in accordance with the method described in section 2.5.3.

2.4.4.1. Preparation of substrate cells.

The monolayers of cells of the appropriate sensitivity to be used in the test shall cover an area of at least 70 cm$^2$.

2.4.4.2. Inoculum.

After discarding the culture medium the cell culture shall be grown in 25 ml medium. In the case the substance is liquid the medium shall contain 3.75 ml of the substance. In other cases the medium shall contain 15% of the substance.

2.4.4.3. Procedure.

At least 2 subcultures are made of the monolayers with an interval of 7 days, or as frequently as may be necessary for suitable growth. The cell-cultures are maintained for at least 21 days during which period the cultures are examined regularly for cytopathic effect.

At least 7 days after the initiation of the last subculture the monolayers are examined:
- for cytopathic effect in accordance with the method described in section 2.6.1.
- for haemadsorption in accordance with the method described in section 2.6.2.
- for the presence of specific extraneous agents in accordance with the method described in section 2.7.
2.5.1. INACTIVATED VACCINES

Normally it will not be necessary to test the intermediate product for extraneous agents, so there is no need to describe test methods.

2.5.2. LIVE VACCINES

**INTERMEDIATE PRODUCT**

**LIVE VACCINE**

1.1 HARVESTED VIRUS POOL SAMPLE

Neutralization of Vaccine Virus with Antiserum

1.1.1.1 SPECIFIC TEST
To detect viruses infective and/or pathogenic to target animal and for potential contaminants during the production process

1.1.2 1.1.3 GENERAL TEST
One or more sensitive cell-lines selected on the basis of their sensitivity for viruses infective and/or pathogenic for target animal species and potential contaminants present in the materials of animal origin.

Subculture
Observation for cytopathic effect and haemadsorption.

* Specific tests may be required by a competent authority.

2.5.2.1. GENERAL TEST

2.5.2.1.1. Samples

The samples shall be taken from the intermediate product. The vaccine virus shall - when necessary - be neutralised with a monoclonal antibody or a polyclonal specific antiserum containing sufficiently high levels of neutralising antibody to the virus present in the material. When a polyclonal specific antiserum is used, it shall be free of antibodies against those viruses for which presence the material is tested as described in the section on testing the Master Seed Virus.

NOTE:

If the vaccine virus cannot be completely neutralised, alternative validated methods to eliminate the vaccine virus may be used when duly justified.

2.5.2.1.2. Substrates.

The substrates used for testing the presence of extraneous agents shall consist of one or more types of cells sensitive to viruses pathogenic for the target species and/or other contaminating viruses.

2.5.2.1.3. Test method

2.5.2.1.3.1. Preparation of the substrate cells.

For initial inoculation, the monolayers of cells of the appropriate sensitivity to be used in the test shall cover an area of at least 70 cm². 

2.5.2.1.3.2. Inoculum.
An inoculum of 1.0 ml of the material combined if necessary with appropriate antiserum shall be used to inoculate each surface area of at least 70 cm$^2$.

2.5.2.1.3.3. Procedure.

All cell cultures are maintained for at least 14 days, during which at least 1 subculture of the cells are made at 7 days intervals, unless the cells do not survive for this length of time, when the subculture shall be made on the latest day possible. At least 8 monolayers of suitable cells with a surface area of at least 20 cm$^2$ each shall be used for the final subculture.

They are examined as follows:

- All cultures shall be observed at least every second working day
- At the end of the last subculture
  - monolayers are examined for cytopathic effect in accordance with the method described in section 3.1.
  - monolayers are examined for haemadsorption in accordance with the method described in section 3.1

2.5.2.2. SPECIFIC TEST

Specific tests may be required by a competent authority.

The specific tests shall normally be done as described for Master Seed Virus using Immuno Staining (IS) and Enzymelinked Immuno Assays (EIA)
2.6. TEST FOR THE PRESENCE OF EXTRANEOUS VIRUSES IN FINISHED PRODUCT.

2.6.1. INACTIVATED VACCINES.

Normally it will not be necessary to test the finished product for extraneous agents, so there is no need to describe test methods.

2.6.2. LIVE VACCINES

FINISHED PRODUCT

2.1 VACCINE

Neutralization of Vaccine Virus with Antiserum

2.1.1.1 SPECIFIC TEST*
To detect viruses infective and/or pathogenic to target animal and for potential contaminants during the production process

2.1.2 GENERAL TEST
One or more sensitive cell-lines selected on the basis of their sensitivity for viruses infective and/or pathogenic for target animal species and potential contaminants present in the materials of animal origin.

Subculture

Observation for cytopathic effect and haemadsorption.

* Specific tests may be required by a competent authority.
2.6.2.1. GENERAL TEST

2.6.2.1.1 Samples

The samples shall be taken from the batch of finished product.

The vaccine virus shall, when necessary, be neutralised with a monoclonal antibody or a polyclonal specific antiserum containing sufficiently high levels of neutralising antibody to the virus present in the vaccine.

When a polyclonal specific antiserum is used, it shall be free of antibodies against those viruses for which presence the vaccine is tested as described in the section on testing the Master Seed Virus.

NOTE:

If the vaccine virus cannot be completely neutralised, alternative validated methods to eliminate the vaccine virus may be used when duly justified.

2.6.2.1.2 Substrates.

The substrates used for testing the presence of extraneous agents shall consist of one or more types of cells sensitive to viruses pathogenic for the target species.

2.6.2.1.3 Test method

2.6.2.1.3.1 Preparation of the substrate cells.

For initial inoculation, the monolayers of cells of the appropriate sensitivity to be used in the test shall cover an area of at least 70 cm².

2.6.2.1.3.2 Inoculum.

An inoculum containing 1 dose of vaccine virus, combined if necessary with appropriate antiserum shall be used to inoculate each surface area of at least 70 cm².

2.6.2.1.3.3 Procedure.

All cell cultures are maintained for at least 14 days, during which at least 1 subculture of the cells are made at 7 days intervals, unless the cells do not survive for this length of time, when the subculture shall be made on the latest day possible. At least 8 monolayers of suitable cells with a surface area of at least 20 cm² each shall be used for the final subculture.

They are examined as follows:

- All cultures shall be observed at least every second working day
- At the end of the last subculture
- monolayers are examined for cytopathic effect in accordance with the method described in section 3.1.

- monolayers are examined for haemadsorption in accordance with the method described in section 3.1

2.6.2.2. SPECIFIC TEST

Specific tests may be required by a competent authority. The specific tests shall normally be done as described for Master Seed Virus using Immuno Staining (IS) and Enzymelinked Immuno Assays (EIA)