Manual of Diagnostic Tests for Detection of CSF

Contents

1 Purpose

The purpose of this manual is to support and harmonize CSF diagnosis. It shall be understood as a guideline. In general, all methods and protocols have to be validated in the respective laboratory and the guidelines according to ISO/IEC 17025 must be fulfilled. Apart from the methods mentioned, any fully validated test protocol might be used.

This collection of test methodsaims at tests performed on non-vaccinated pigs. It has to be stressed that vaccinated animals (attenuated vaccine strains and marker vaccines) can also give a positive reaction in the below mentioned tests.

2 Suitable cell lines for cell culture based diagnostic assays

2.1 Porcine cell lines for CSF diagnosis:

Porcine kidney cell line PK15

The PK15 cell line is suitable for all test systems for CSF diagnosis e.g. virus isolation, virus propagation, and virus neutralization tests. The cell line was established by single cell cloning. The cells should be tested for absence of mycoplasma and known pestiviruses with diagnostic relevance.

The PK15 cells can be contaminated with porcine circovirus-1 (PCV-1); it is recommended to use PCV1-free cells.

Other porcine cell lines

Several other porcine cell lines can be used for CSF diagnosis, e.g. the porcine kidney cell line Swine Kidney (SK-6) and a Swine Testis Endothelial cell line [STE (e.g. RIE 255)]. As the latter cell line (STE) does also grow with horse serum it is less vulnerable to BVD contamination by fetal calf serum. Both SK6 and STE cell lines propagate some virus strains that do not grow well on PK15 cells.

2.2 Cell lines for differential diagnosis against Border disease virus (BDV) and Bovine viral diarrhea virus (BVDV)

In general, the suitability of particular cell lines for individual virus strains should be evaluated in each laboratory. The cells should be tested for absence of mycoplasma and known pestiviruses with diagnostic relevance.

For isolation and cultivation of Border disease virus (BDV), a sheep fetal thymoid cell line (SFT-R/CCLV Rie043) can be used. The main use is the differential virus neutralization test against BDV.

For Bovine viral diarrhea virus (BVDV) cultivation, several susceptible bovine cell lines are suitable e.g. Madin Darby bovine kidney (MDBK), embryonic bovine trachea (EBTr) and bovine turbinate cells. At the EURL for CSF (European Reference Laboratory for Classical Swine Fever) MDBK cells are used for BVDV diagnostic tests at the moment; a corresponding protocol for seeding MDBK cells into different culture dishes is given below.

Primary cells (porcine or bovine) can also be used for pestivirus diagnosis, and the sensitivity of tests with primary cell lines can be higher compared to tests carried out with permanent cells. However, primary cells have the disadvantage of being less homogenous within and between batches and between different laboratories.

2.3 Cell culture medium

For the preparation of cell stocks, Eagle's Minimum Essential Medium (EMEM), containing an appropriate proportion of fetal calf serum (FCS) depending on the applied cell line can be used; for PK15 cells 7,5% FCS and for SK6, STE, SFT-R, and MDBK cells 10% FCS is recommended. For diagnostic tests as well as for virus multiplication and cell cultivation, EMEM with 10% FCS should be used for all above listed cell lines. The use of antibiotics (e.g. Penicillin/Streptomycin, Gentamycine) is recommended to avoid possible contaminations with bacteria particularly if the medium is used in presence of diagnostic samples, or in culture systems at greater risk of contamination such as macro or micro titre plates. In case a fungal contamination is suspected or probable, the addition of antimycotics can be useful (e.g. Amphotericin B).
Each batch of FCS must be carefully checked for the presence of pestiviruses (e.g. by virus isolation and/or PCR) and related antibodies (e.g. by virus neutralization test) before use in diagnostic assays. It is not sufficient to rely on the manufacture's certification only. Although it does not influence the outcome of serological tests it is still under discussion among the laboratories, if presence of viral genome should be tolerated or not.

2.4 Cultivation

For multiplication, cells can be cultivated at 37°C in plastic cell culture flasks in suitable dimensions; depending on the individual purpose T25- (25 cm^2) or T75- (75 cm^2) flasks with corresponding amounts of cell culture media (app. 7 ml for T25-/ 20 ml for T75-flasks) are used.
The frequence of cell passaging depends on the individual cell line, e.g. PK15 cells are ideally passaged twice per week, but one passage per week is often sufficient if the medium is replaced after app. 4 days; for SFT-R cells one passage per week plus an additional replacement of medium is sufficient, but also two passages per week can be performed if necessary.
For determination of splitting ratios, frequent evaluations of the cell monolayer by light microscopy and results of regular cell counts should be taken into account; for PK15 cells a splitting ratio of 1:5 to 1:10 and for SFT-R cells a splitting ratio of 1:3 is ususally appropriate.
Regular screening for mycoplasma should be carried out and is particularly recommended in case of increased occurance of doubtful results or reduced cell growth.
Different protocols can be applied to trypsinize and seed cells. The most widely used protocols are listed below; quantity specifications refer to a cultivation procedure in T75 flasks.

Protocol for cell cultivation (T75 flask)

- The cell culture medium is removed from the cell culture flask.
- The cell monolayer is washed once or twice with approximately 5 ml of Alsever's-trypsin versene-solution (ATV).
- The ATV is removed and replaced with 1-2 ml fresh ATV so that the cell layer is covered completely.
- The cell culture flask is incubated at 37°C until cells are detached (app. 10 – 25 min).

- Cell culture medium with FCS is added to a total volume of 10 ml (adjust total volume according to the required splitting ratios).
- Cells are seeded into the different dishes as described below.

Protocol for cell counting

- Upon trypsinization the obtained cell suspension is removed and centrifuged at approximately 200 g for 10 min at room temperature.
- The supernatant is discarded and the cell pellet is resuspended with an appropriate amount of cell culture medium (preferably serum-free) or with PBS. The total volume of culture medium/ PBS for resuspension depends on the cell density and should ensure proper determination of cell number.
- Cells are counted either manually by using counting chambers, or automatically by using cell counting machines.
- Finally, the cell pellet is resuspended in the appropriate culture medium volume according to the results of cell counting.

2.5 Seeding cells in different sized cell culture vessels (for example)

PK15 cells

A confluent cell monolayer of a 75 cm^2 cell culture flask contains an average of 8.5 x 10^6 cells. If the cell pellet of one 75 cm^2 flask is resuspended in 10 ml culture medium, it is suggested to use the following amounts for the different cell culture vessels:

type of cell culture vessel	volume cell suspension	volume medium / vessel	number of cells / vessel	number of cells / ml
75 cm^2 flask	2 ml	20-25 ml	1.7 x 10^6	6.8 x 10^4
25 cm^2 flask	0.66 ml	5-7 ml	5.6 x 10^5	8.0 x 10^4
culture tube	0.1 ml	1 ml	8.5 x 10^4	8.5 x 10^4
24 well plate	2 ml / dish	24 ml (1 ml/ well)	1.7 x 10^6	7.1 x 10^4
96 well plate	2 ml / dish	5 ml (0.05 ml/ well)	1.7 x 10^6	3.4 x 10^5

SFT-R cells
A confluent cell monolayer of a 75 cm^2 culture flask contains an average of 9 x 10^6 cells.
If the cell pellet of one flask is resuspended in 9 ml medium, it is suggested to use the following amounts for the different cell culture vessels:

type of cell culture dish	volume cell suspension	volume medium per vessel	number of cells / vessel	number of cells / ml
75 cm^2 flask	3 ml	20-25 ml	3 x 10^6	1.2 x 10^5
25 cm^2 flask	1 ml	5-7 ml	1 x 10^6	1.4 x 10^5
Culture tube	0.12 ml	1 ml	1.2 x 10^5	1.2 x 10^5
24 well plate	3 ml / dish	24 ml (1 ml/ well)	3 x 10^6	1.25 x 10^5
96 well plate	3 ml / dish	5 ml (0.05 ml/ well)	3 x 10^6	6 x 10^5

2.6 Reagents

Alsever's-trypsin versen - solution, pH 7.2

NaCl	8.5 g
KCl	0.4 g
dextrose	1.0 g
NaHCO3	0.58 g
trypsin (1:250)	0.5 g
EDTA	0.2 g

Ad 1 L ddH2O
Filter through a 0.22 μm filter
Store at -20°± 4°C

Antibiotics can be added if considered necessary but are not recommended for routine use in cell cultivation and propagation in closed cell culture flasks (without CO_2) as the addition of antibiotics might mask possible bacterial contaminations.

3 CSF Virology

3.1 Cell culture based CSFV detection: Virus isolation

3.1.1 Principle of virus isolation

Virus isolation technique is used for the detection of infectious Classical swine fever virus (CSFV) in laboratory diagnosis and is best suited for the investigation if samples from small numbers of animals rather than mass surveillance.

This test can be carried out with different sample materials: organ suspensions, anticoagulated whole blood samples after freeze-thaw cycles, leukocyte preparations from fresh anticoagulated whole blood samples, sera, plasma, swab samples and semen. CSFV susceptible cell lines are inoculated with the respective sample material to allow the attachment, entry and replication of the virus either over one or two passages. While virus isolation over one passage is most suitable for screening purposes an improved sensitivity can be achieved by performing the test over two passages (or even a third passage if a slow-growing virus is suspected). Since growth of CSFV does not cause any cytopathic effect, its presence must be demonstrated by an immunostaining method. For this purpose cells are fixed and the presence of intra-cellular CSF antigen (located in the cytoplasma) is detected either with a peroxidase- or a fluorochrome-labelled specific antibody (Chapter 3.2).

3.1.2 Cell culture systems for CSFV isolation

Susceptible cell cultures for CSFV isolation include PK15, SK-6, or STE cells. Further porcine cell lines might be susceptible to CSFV infection *in vitro* but should be at least equally sensitive as PK15; the application of more than one porcine cell line can be beneficial. All applied cell lines must be free from pestiviruses.

Susceptible cells can either be used as pre-formed monolayer or as cell suspension for simultaneous infection. The simultaneous inoculation might be slightly more sensitive, but only fresh and non-cytotoxic samples can be processed this way due to the fact that a removal of the test material including washing of cells upon inoculation is not possible. In addition, simultaneous infection is less suitable for whole blood samples as the anticoagulant may interfere with the adhesion of cells on to the surface of cell culture flask/ plate. Therefore, the application of a pre-formed monolayer is advantageous. To this means, susceptible cells are seeded prior to inoculation with test samples until a confluence of 50 – 80% is reached (1-2 days old cell cultures are commoly used). For samples of bad quality (e.g. from wild boar), cultures with 100% confluence can be beneficial. This approach reduces sensitivity but offers higher robustness.

3.1.3 Organ preparation

Suitable organ samples are tonsil, spleen, kidney, lymph nodes (e.g. retro-pharyngeal, parotid, mandibular or mesenteric lymph nodes) or ileum. In case of autolysed carcasses, an entire long bone or the sternum is the specimen of choice. Samples should be kept cool and processed as soon as possible. For sample processing, different protocols are available depending on the laboratory equipment. Commonly used methods are listed below.

Automated protocols (for example)

Automatic homogenizers utilizing for example ceramic or steel beads can be used to mince the organ material. The advantage is that the tissue is homogenized in a closed system, but high speeds can heat the sample and thus possibly affect the virus. Therefore, cooling of samples (e.g. at 4°C) during the homogenization procedure is recommended.

- Tubes are filled with an appropriate amount (0.5 - 1 ml) of a suitable buffer or cell culture medium [e.g. Earl's with lactalbumine (ELA) medium, Hanks' balanced salts solution (BSS) or Hanks' minimal essential medium (MEM)]. The usage of reaction tubes with a safe-lock system is recommended in order to avoid leakage of potentially infectious fluids during homogenization procedure.
- Organ samples are cut into pieces with a scalpel and an amount of about 10 – 30 mg is put into each tube containing an appropriate amount of preferably serum-free cell culture medium with an increased amount (e.g. 10-fold amount) of antibiotics (e.g. Penicilline/Streptomycin or Glutamine based antibiotic/ antimycotic mixture; recipes see below).
- Tubes are closed and put into the homogenizer; speed, duration and further settings are chosen according to manufacturer's recommendations and internal validations.
- Subsequently, the degree of homogenization is macroscopically assessed and if necessary, the run is repeated.
- Thereafter, the organ suspension is incubated with the cell culture medium containing the high content of antibiotics for app. 1 h at room temperature, so that the added antibiotic compound can take its effect.
- Centrifugation for 15 min at 2500 - 3000 g.
- The supernatant is used for inoculation of cell cultures (see virus isolation procedure below).

In addition, alternative automatic techniques are commcercially available and suitable upon adequate validation procedure, e.g. laboratory homogenization of samples by using the Stomacher.

Traditional protocol with mortar and pestle

Organ samples of a maximum of 1 cm^3 (corresponding to app. 1 g) are homogenized in a mortar with the 9-fold amount of cell culture medium containing antibiotics solution (e.g. Penicilline/Streptomycin or Glutamine based antibiotic/ antimycotic mixture; recipes see below) and the mixture is grinded with a pestle to produce a 10% organ preparation. As described above, increased amount of antibiotics should be added to the culture medium (about 10-fold higher than the commonly applied amount). Sterilised sand can be added prior to grinding in order to facilitate homogenisation.

- The organ preparation is incubated at room temperature for 1 h, so that the added antibiotic compound can take its effect.
- Centrifugation for 15 min at appr. 1200 - 1700 g.
- The supernatant is used for inoculation of cell cultures (see virus isolation procedure below).

Prior to the inoculation of cells, for both the automated and the traditional protocol, sterile filtration of the supernatant using syringe filters (0.45 µm followed by 0.22 µm) can be performed, if this is considered necessary with regard to the condition of the sample material.

With samples of lower quality it can be beneficial to perform testing of an additional dilution of the obtained material (e.g. final dilution of 1:100, see below).

3.1.4 **Leukocyte preparation**

Leukocyte preparations can be obtained from EDTA or heparin anticoagulated blood samples; preferably a minimum volume of 10 ml is used.

Dextran method

The sample of choice is EDTA anticoagulated whole blood.

- 0.5 - 1 ml of a 5% Dextran solution is added to the original sample tube containing 5-10 ml EDTA blood. After carefully swiveling, the sample tube is left at room temperature for one to three hours (alternatively 30 minutes at 37°C) until the leukocytes are visibly separated as upper white phase.
- The upper white phase containing the leukocytes is collected and centrifuged at appr. 450 g for 10 min at 4°C.
- The pellet is resuspended in 3-5 ml PBS, centrifuged and the supernatant is discarded. This washing step is repeated once more.
- Finally the pellet is resuspended in 2 ml PBS or serum-free cell culture medium for further use or storage at -20°C to -70°C.
- In case the leukocyte suspension is used directly for inoculation of susceptible cells, the leukocyte preparation can be subjected to a freeze and thaw cycle for lysing the cells and thus increasing the amount of free viral particles during inoculation procedure.

Alternatively, leukocytes may by prepared by density gradient centrifugation. Several appropriate substances are commercially available (e.g. Ficoll- or Pancoll-Paque) and can be applied according to the manufacturers instruction upon internal validation.

Ammoniacal lysis

The sample of choice is Heparin anticoagulated blood.

- Erythrocytes are lysed by adding 20 ml of NH_4Cl solution (0.84%) to 10 ml of Heparin blood sample (2:1) and left at room temperature for 15-30 min. In smaller sample sizes the volume of NH_4Cl is adapted accordingly (2:1).
- The solution is centrifuged at 1000 g for 10 min.
- The pellet is resuspended in 5 ml PBS, centrifuged and the supernatant is discarded. This washing step is repeated once more.
- Finally the pellet is resuspended in 2 ml PBS for further use or storage.
- In case the leukocyte suspension is inoculated simultaneously with susceptible cells it should be frozen at –20°C shortly to lyse the leukocytes and avoid cytotoxic effects.

3.1.5 **Whole blood samples**

EDTA or heparin anticoagulated blood is subjected to a freeze and thaw cycle in order to destroy the leukocyte membrane. The whole blood samples are frozen at least at -20°C and then thawed quickly, e.g. in a waterbath at 37°C in order to lyse the cells. Subsequently, the blood lysate is inoculated directly on the cell culture as described below.

3.1.6 **Test procedure – virus isolation**

Virus isolation over one passage (screening)

- For inoculation of a pre-formed monolayer, 100-300 µl test material (organ suspension, leukocyte preparation,blood lysate, plasma, or serum) are inoculated on a 50- 80% confluent cell culture in multi dish plates (e.g. 24-well-plates or flat-bottomed microtiter plates) or other suitable cell culture systems (e.g. Leighton tubes with cover slips) so that the whole cell layer is covered with a thin film of sample material. For diagnostic samples, which might be cytotoxic, the additional application of dilution steps in an appropriate medium (1:10 and possibly 1:100 dilution) is recommended. In case no dilution steps have been carried out, all diagnostic samples should be tested in duplicates.The cell cultures are incubated at 37°C for 1 - 2 hours to allow viral attachment.
- The cell cultures are initially washed with PBS or another appropriate washing solution (e.g. Hanks' BSS, Hanks' MEM) and subsequently covered with fresh culture medium. The frequency of washing depends on the sample type and if it is diluted or not.
- Alternatively, the cell culture dish can be filled up directly with culture medium, if a cytotoxic effect is unlikely.
- The cell cultures are incubated for preferably 72 hours (at least 2 days, maximum 5 days) at 37°C in a CO_2 incubator. Cell cultures should be checked for possible cytopathic effects regularly during incubation period by light microscopy.
- Positive and negative controls are compulsory in each test and must be processed in the same way as the sample material. As a positive control CSFV reference strains might be used, as negative control cell culture medium can be applied.
- Cells are fixed and stained as described in the Chapter 3.2.

In case of simultaneous inoculation, nine parts of cell suspension and one part of test material are mixed and inoculated in 1.0 – 1.5 ml aliquots in Leighton tubes with cover-slips or other appropriate cell culture flaks or plates. Thereafter, cell cultures are processed as described for the inoculation of a pre-formed cell monolayer.

Virus isolation over two passages

- For virus isolation over two passages, the first virus passage should be carried out in a cell culture system which is suitable for a freeze-thawing cycle (cell culture flaks or tubes).
- The procedure of the first passage is the same as described above. After an incubation period of 72 h, cell culture tubes are frozen at minimum - 70°C for a at least one hour (freeze and thaw cycle).
- The culture tubes are then thawed rapidly and centrifuged for 10 min at appr. 800 g.

- For the second passage, 200 µl of the supernatant collected after centrifugation are incubated for 1-2 hours on a well of a multi dish plate or a cell culture tube with 50 – 80% confluence as described above.
- The cell culture plates or tubes can either be washed as described above or directly refilled with culture medium if a cytotoxic effect is unlikely. The cell cultures are incubated for another 72 hours (minimum 2 days; maximum 4 days) at 37°C in a CO_2 incubator.
- Positive and negative controls must also be included and processed in the same way as described above.
- Cells are fixed and stained as described in Chapter 3.2.

A second passage in a culture tube with a subsequent third passage in a cell culture plate can be performed.

3.1.7 **Reagents – virus isolation**

Dextran solution 5%

Dextran sulfate-Na, MW 500.000	5 g
1.5% EDTA-Solution	ad 100 ml

Dissolve at 45°C and autoclave before use.

PBS (Phosphate Buffered Saline without calcium and magnesium)

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4 x 12 H_2O	2.37 g
KH_2PO_4	0.2 g
Aqua dest.	ad 1000 ml
pH	7.0 – 7.2

Antibiotic solutions

Penicilline/Streptomycin

Na-Benzylpenicilline	10^7 I.U.
Streptomycinsulfate	10 g
PBS	ad 100 ml

1 ml of this solution is added to 1 l culture medium for routine use or 10 ml in 1 l medium for incubation of organ samples. Upon reconstitution, aliquotation and storage at -20°C is recommended.
In case of highly contaminated sample material an additional application of an antimycotic solution can be useful; e.g. Fungizone:
Dissolve the fungizone stock solution fungizone (250 µg/ml) 1:100 in cell culture medium.

Glutamine based antibiotic/ antimycotic mixture

Streptomycinsulfate	11.25 g dissolved in 390 ml Aqua dest.
Na-Benzylpenicilline	9 x 10^6 I.U. dissolved in 90 ml Aqua dest. L-
Glutamine	26.28 g dissolved in 420 ml Aqua dest.
	mix and filter the three solutions.
Fungizone	9 x 10^5 I.U. dissolved in 100 ml Aqua dest.

Add to the above filtrate and store at –20°C in 10 ml aliquotes until use. Application: 10 ml in 1 l medium.

3.2 CSFV detection methods in cell cultures

In general, CSFV does not induce a cytopathic effect. Consequently, the CSFV infected cells have to be immunologically labelled in order to detect the virus antigen in the cytoplasma of the cells. Direct and indirect staining methods as well as mono- and polyclonal antibodies are available. The working dilution of the conjugates should combine a maximum of signal with a minimum of background staining and has to be evaluated in each laboratory.

3.2.1 Cell Fixation

The choice of the fixative will depend on whether the cultures are grown on glass or plastic surfaces. Plastic surfaces are most commonly applied nowadays, allowing the use of different fixation methods, either physical (by heat treatment) or chemical; an exemplary list of fixation strategies for plastic surfaces is given below. For cultures grown on glass surfaces, e.g. fixation in 100% acetone (analytical grade) for 5 min is appropriate.

Heat fixation

- The culture medium is removed and the cell layer is washed once with 1/3 PBS/H_2O.
- The wash solution is removed thoroughly from the culture dish, preferably by using a vacuum pump and subsequent tapping on paper towels to expel all remaining liquid.
- The dry plates are placed in a laboratory air circulating oven at 70-80 °C for fixation. An incubation period of 2-3 hours is usually sufficient for most cell types, however extended time periods are required for certain cell lines (e.g. SFTR-cells need about 5 hours for fixation). After fixation the plates are left at room temperature for cooling.

Alternative protocols

Many traditional protocols using chemical fixatives are available. Each fixation procedure should be validated thoroughly in order to ensure an adequate fixation. For example, chemical fixation and permeabilization can be achieved by using one of the chemical reagents/ compounds listed below:

- Acetone
- Methanol
- Acetone-methanol combined (e.g. 1:1 solution)
- Ethanol
- Ethanol-glacial acetic acid combined (ethanol, 95%; and glacial acetic acid, 5%)
- Ethanol-methanol combinated (e.g. 1:1 solution)
- Formalin (subsequent permeabilization necessary; e.g. with Triton X)
- Paraformaldehyde (subsequent permeabilization necessary; e.g. with Triton X or methanol)

In case the fixed plates are not processed immediately storage in a dry environment is possible until immune labelling. For short-term storage (< 1 week) fixed plates can be left at room temperature, for long-term storage (> 1 week), fixed plates can be stored at -20°C in a sealed plastic bag.

3.2.2 Immune labelling with peroxidase staining

For final detection of CSFV infection in cells, CSFV- or pestivirus-specific monoclonal antibodies or polyclonal serum directed against a pestivirus antigen can be used. The immune staining can be performed directly using a peroxidase-labelled antibody or polyclonal serum, as well as indirectly by using a peroxidase labelled secondary antibody. The reaction of substrate (chromogen-substrate solution AEC (3-Amino-9-ethylcarbazole)/ dimethyl formamide with H_2O_2) leads to a reddish-brown precipitation in the cytoplasm of infected cells.

Direct labelling

Principle
The immune staining is performed directly with a peroxidase-linked CSFV- or pestivirus-specific monoclonal antibody (mAb) or polyclonal serum directed against a pestivirus antigen (conjugate).

Protocol

- The plates are rinsed once with PBS-Tween for moistening the fixed cell monolayer.
- The working dilution of a pestivirus conjugate in PBS-Tween is added to each well so that the cell layer is fully covered, for a 24 well dish 200 µl/ well, for a 48 well dish 150 µl/ well, and for a 96 well dish 50 µl/well are sufficient.
- The plates are incubated for 1 hour at 37 °C in a moist chamber.
- Thereafter, the pestivirus conjugate is removed from the plates which are subsequently washed three times with PBS-Tween and once with Aqua dest.
- Chromogen-substrate solution of equal amounts as stated above for the pestivirus conjugate solution is added to each well and incubated for 10-25 minutes at room temperature. Substrate

solution must be removed from the cell layer when the cytoplasma of infected cells is clearly stained while the cellular nucleus is not yet stained.
- The chromogen-substrate solution is discarded and plates are washed once with Aqua dest.
- The cavities are filled with Aqua dest. for final evaluation by light microscopy (inversion light microscope).
- A cavity is considered as pestivirus positive when the cytoplasma of at least one cell is stained reddish-brown while its nucleus is not stained.

Indirect labelling

Principle
The immune staining is performed indirectly by using a pestivirus specific polyclonal antiserum or prefereably a mAb directed against a pestivirus antigen; this first binding is then detected by a peroxidase-linked secondary monoclonal antibody (conjugate).

Protocol
- The plates are rinsed once with PBS-Tween for moistening the fixed cell monolayer.
- The working dilution of a monoclonal hybridoma supernatant or a pestivirus specific antiserum in PBS-Tween is added to each well so that the cell layer is fully covered, for a 24 well dish 200 µl/ well, for a 48 well dish 150 µl/ well, and for a 96 well dish 50µl/well are sufficient.
- The plates are incubatedfor 1 hour at 37 °C in a moist chamber.
- Thereafter, the supernatant is removed from the plates which are subsequently washed three times with PBS-Tween.
- The working dilution of a commercial antispecies peroxidase conjugate in PBS-Tween (secondary antibody) is added to each well and plates are incubated according to manufacturer's instructions, in general 1 hour at 37°C, in a moist chamber.
- The antispecies peroxidase conjugate is removed and plates are washed three times with PBS-Tween and once with Aqua dest.
- Chromogen-substrate solution is added to each well in suitable amounts (50 µl/ well in a 96 well dish; 150 µl/ well in a 48 well dish; 200 µl/ well in a 24 well dish) and stained for 10-25 minutes at room temperature until the cytoplasma of the infected cells is strongly stained while the cellular nucleus is not yet stained.
- The chromogen-substrate solution is discarded and washed with Aqua dest.
- The wells are filled with Aqua dest. for final evaluation by light microscopy (inversion light microscope).
- A cavity is considered as pestivirus positive when the cytoplasm of at least one cell is stained reddish-brown while its nucleus is not stained.

Incubation times may vary among different conjugates (manufacturer's instructions). Blocking reagents might be used to block the peroxidase reaction of the secondary antibody and reduce background staining, e.g. 4% horse serum can be added. Manufacturer's instructions should be taken into account.

3.2.3 Immune labelling with fluorescence staining

As an alternative to the peroxidase staining a fluorochrome labelled (e.g. FITC, Cy3, Alexa Fluor) conjugate can be applied to generate a specific fluorescence signal in the cytoplasmn of CSFV infected cells while the nucleus remains undyed. The fluorescence signal can be detected by fluorescence microscopy.
Again, direct and indirect labelling methods can be distinguished depending on whether the primary CSFV- or pestivirus-directed antibody is directly labelled with a fluorochrome or a second fluorochrome labelled antispecies antibody is applied.
The respective methods are carried out according to the protocols provided for the peroxidase staining (see above) with the only difference that a fluorochrome is used for final CSFV detection instead of the chromogen-substrate solution (AEC/ dimethyl formamide with H_2O_2).

The fluorochromes are applied according to manufacturer's instructions, nevertheless these must be adjusted (if necessary) and verified by laboratory validation prior to use.

After immune labelling with fluorescence staining, a mounting buffer can be applied in order to prolong the fluorescence signal and thereby, the time span for fluorescence microscopic analysis.

In case Leighton tubes have been used for virus isolation and staining have been carried out with a direct or an indirect anti-CSFV conjugate, cells are washed three times in PBS for 5 minutes each. After washing, the cover-slip cultures are rinsed once in Aqua dest. and covered with the mounting buffer; subsequently the cover-slips can be examined for fluorescent foci.

3.2.4 Reagents

CSFV specific/ Pestivirus specific/ antispecies antibodies/ conjugates
Working dilutions are generally provided by the manufacturer but should be verified by internal laboratory validation procedures prior to use.

For peroxidase staining

PBS-Tween
1 l PBS containing ~0.01% $Tween_{20}$ (2-3 drops)

1/3 PBS
PBS: H_2O = 1:3

Antispecies antibodies
Commercially available, e.g. rabbit anti-mouse peroxidase, RAMPO

Sodium Acetate-Buffer (pH 5.0; 0.05 M)

Sodium Acetate-Trihydrate	6.804 g
Aqua dest.	1000 ml

The pH can be adjusted using acetic acid.

Chromogen-substrate solution

3-Amino-9-Ethylcarbazole (AEC)*	20 mg
Dimethylformamide	3 ml
Sodium Acetate-Buffer	ad 50 ml
H_2O_2 (3%)	0.4 ml

A stock solution can be prepared using AEC and Dimethylformamide. However, the chromogen-substrate solution must always be prepared freshly from the stock solution prior to use!
*Warning instructions for hazardous substances have to be taken into account.

For fluorescence staining

Mounting buffer

PBS (pH 7.4 - 7.6, physiological saline buffered with 0.01 M phosphate)

NaCl	8.78 g/l
Na_2HPO_4 x $12H_2O$	3.58 g/l

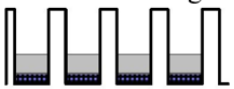
(pH adjusted with 1M KH_2HPO_4)
20% glycerine (buffered glycerine)

Alternatively, a commercial non-fading mountant can be used.

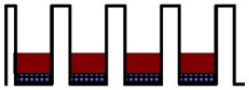
Immune labelling

Peroxidase staining

Direct labelling

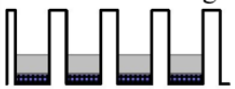


Rinse wells once with 1/3 PBS, add 50 µl, 150 µl, or 200 µl/well of a peroxidase-linked pestivirus directed monocloncal antibody (mAb) (conjugate) and incubate for 1 h at 37 °C

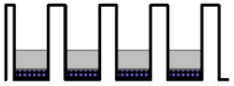


Wash plate 3 x with PBS-Tween and 1x with Aqua dest.; add 50 µl, 150 µl, or 200 µl/well chromogen-substrate solution, incubate 10-25 minutes at room temperature (RT)

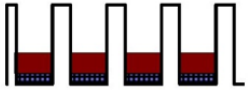
Indirect labelling



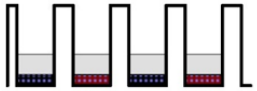
Rinse wells once with 1/3 PBS, add 50 µl, 150 µl, or 200 µl/well of a pestivirus directed mAb/ pestivirus specific antiserum and incubate for 1 h at 37 °C



Rinse wells 3x with PBS-Tween, add 50 µl, 150 µl, or 200 µl/well of a peroxidase-linked secondary mAb (conjugate) and incubate for 1 h at 37 °C



Wash plate 3 x with PBS-Tween and 1x with Aqua dest.; add 50 µl, 150 µl, or 200 µl/well chromogen-substrate solution, incubate 10-25 minutes at room temperature (RT)



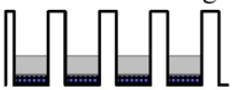
Discard the chromogen-substrate solution, wash and add Aqua dest.; then read by light microscopy

Fluorescence staining

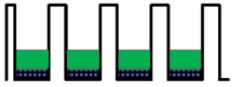
Direct labelling

Rinse wells once with 1/3 PBS, add 50 µl, 150 µl, or 200 µl/well of a fluorochrome-labelled pestivirus directed mAb (conjugate) and incubate for 1 h at 37 °C

Indirect labelling



Rinse wells once with 1/3 PBS, add 50 µl, 150 µl, or 200 µl/well of a pestivirus directed mAb/ pestivirus specific antiserum and incubate for 1 h at 37 °C



Rinse wells 3x with PBS-Tween, add 50 µl, 150 µl, or 200 µl/well of a fluorochrome-labelled secondary mAb (conjugate) and incubate for 1 h at 37 °C

Discard the fluorochrome-conjugate, wash and add Aqua dest.; then read by fluorescence microscopy

3.3 Cell culture based virological differential diagnosis

Further characterization of isolated viruses is commonly achieved by sequencing and subsequent genetic typing according to the methods described in 3.5.2. Before molecular biology diagnostic techniques were broady established, CSFV specific mAbs and mAbs specific for other pestiviruses (BDV and BVDV) were used to differentiate between pestiviruses, and it is still possible to apply specific mAbs for this purpose.

Lists of available antibodies (for example)

Monoclonal antibodies available from EURL for CSF:

Supplier	Specificity, Availability and Name			
	Pestivirus	CSFV	BVDV	BDV
EURL, Hannover	Yes, mAb BVD/C 16 (NS3-specific)	Yes, mAb HC/C 34 (E2-specific); HC/TC 169 (E^{rns}-specific)	Yes, mAb BVD/CA 3; BVD/CA 34 (both E2-specific); BVD/C 46 (E^{rns}-specific)	No

Monoclonal antibodies available from other sources:

Supplier	Specificity, Availability and Name			
	Pestivirus	CSFV	BVDV	BDV
APHA Scientific, UK	Yes, mAb WB103/105	Yes, mAb WH211; WH303	Yes, mAb WB162 (BVDV-1); WS538 (BVDV-2)	Yes, mAb WS371
Bio-X Diagnostics, BE	No	Yes	Yes	No
c.c.pro GmbH, DE	Yes	Yes	Yes	Yes
Noack Group	Yes	Yes	No	No
Thermo Fisher Scientific (PrioCON)	Yes	Yes	No	No

Commercial availabilities change frequently. Please observe the market. This list was sorted alphabetically and shall not be understood as recommendation of certain products.

3.4 CSFV antigen detection

3.4.1 Antigen ELISA

Commercially available antigen capture enzyme-linked immunsorbent assays (ELISAs) can be carried out with serum samples, plasma, blood, and organ suspensions. It is recommended to analyse samples from animals with clinical signs or pathological lesions of the disease.
Most antigen ELISAs detect CSF viral glycoprotein E^{rns} but also other viral proteins can be used. Although this is an easy to handle, rapid method that yields a result after a few hours, can be used fully automated, and does not require special tissue culture facilities, its performance is hampered by a rather low sensitivity and specificity due to possible cross-reactions with other pestiviruses. Therefore, the antigen capture ELISA is not intended for testing of individual animals. Each laboratory is in charge of licensing the different batches of commercial CSF antigen ELISA kits after thorough validation.

3.4.2 Fluorescent antibody test (FAT)

3.4.2.1 Principle of test

Thin cryostat sections of organ material can be used for the detection of intracellular antigen of CSFV. For this purpose, the cryostat sections are affixed to a microscope slide and dyed either directly by using an anti-CSFV antibody conjugated to a fluorescence marker e.g. fluorescein isothiocyanate (FITC) or indirectly with a secondary fluorescent conjugated anti-species antibody. The sections are finally examined by fluorescence microscopy.
Suitable organ samples are e.g. tonsil, spleen, kidney, ileum, lymphnodes, smear of bone marrow.

3.4.2.2 Test procedure of indirect FAT

- Organ samples of app. 1 x 1 x 0.5 cm are treated with a tissue freezing medium buffer or are shock frozen in liquid nitrogen or pre-cooled (app. -70°C) n-Heptan.
- Cryostat sections of maximum 5 µm thickness are cut, affixed to a microscope slides and air-dried at room temperature for 20-30 minutes.
- Fixation is carried out in pre-cooled acetone (analytic grade) at –20°C for 10 – 20 minutes. Fixed cryostat sections can be stored at – 20°C for several months.
- The cryostat section is washed briefly with either a washing buffer or PBS.
- All cryostat sections (sample, negative and positive controls) are blocked with 5% bovine serum albumin (BSA) in PBS for 30 minutes and washed again.
- The sections are incubated with the primary monoclonal antibody at the suitable working dilution in washing buffer/ PBS according to manufacturer's instructions for app. 60 minutes.
- The sections are washed briefly 3 times.
- The FITC-conjugated secondary antibody is added at suitable working dilutions according to manufacturer's instructions for app. 60 minutes. Evans Blue can be added to the working dilution as contrast dye (3 parts conjugate working dilution + 1 part 0.005% Evans blue in PBS).
- The washing step is repeated. Subsequently, the sections are immersed in distilled water for a minimum of 5 minutes and air-dried.

- The slightly moist sections are covered with a PBS-glycerol-mix (1 part PBS + 9 parts glycerol) or an equivalent fluorescence maintaining buffer (mounting buffer) and examined by fluorecence microscopy

3.4.2.3 Controls

Negative and positive control sections from non-infected and infected animals respectively must be included into each test run. The control sections can be prepared in advance and stored after acetone fixation for 2-3 years at -70 °C.

3.4.2.4 Interpretation

In case FITC has been used as fluorescence marker, the CSFV antigen is visualized by fluorescence microscopy as cytoplasmatic, mostly homogenous, light green (brilliant) fluorescence while the cell nuclei show no fluorescence. Non-cytoplasmatic as well as yellow-green/ yellow-granular cell-associated colourings must be considered as unspecific. The evaluation of the cryostat sections always has to be carried out in direct comparison to the positive and negative controls. The application of Evans Blue for counterstaining leads to an overall reddish-brown colour and masks minor unspecific fluorescence signals. For correct interpretation of fluorescence signals well-trained and experienced personnel is crucial.

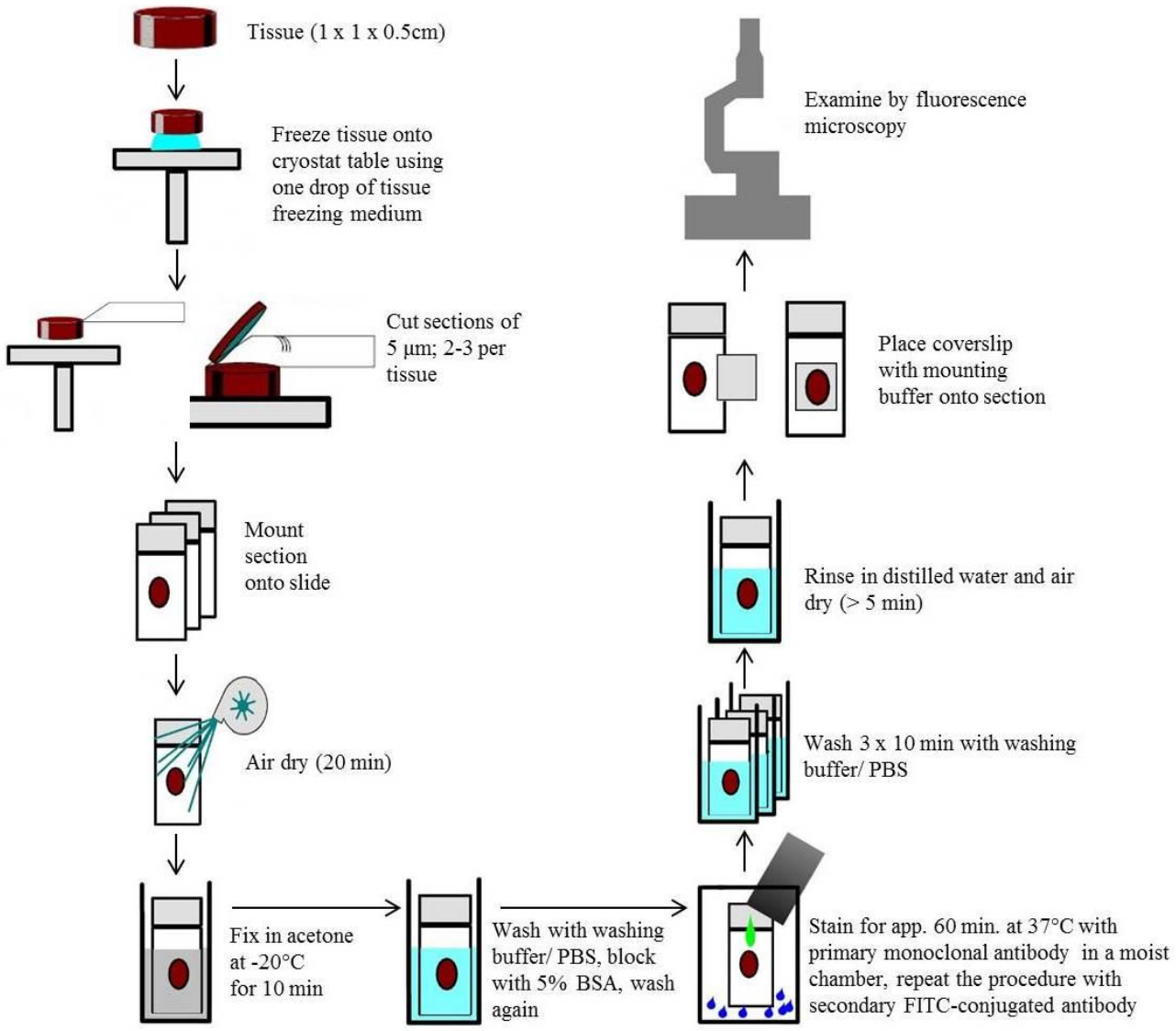
Different FITC-conjugates are commercially available. The working dilution of the conjugate should combine a maximum of signal with a minimum of background staining and has to be evaluated in every laboratory prior to use.

The use of polyclonal CSF specific conjugates in direct FAT ensure that no minor variant viruses will be missed; however they can have the disadvantage of showing cross-reactivity with other pestiviruses or they might even contain marked antibodies against other viruses, as a consequence false positive results can occur. Accordingly, positive results based on tests for which polyclonal antibodies had been used require a confirmation with monoclonal antibodies that can distinguish between CSFV and other pestiviruses, especially BVDV and BDV (see above). For that reason, the application of an indirect FAT combined with a monoclonal anti-CSFV antibody is recommended.

Any sample showing specific cytoplasmic reaction shall be considered positive for pestivirus. Each CSFV infected organ displays a typical fluorescence pattern; fluorescence signals are particularly strong in tonsil sections in the epithelial lining of crypts, in kidney sections in the proximal and distal tubules of the renal cortex and collecting ducts in the medulla and in ileum sections in the epithelial cells of the Lieberkühn glands. Fluorescence signals in spleen sections are more diffuse with concentrations of lymphoid cells in the periarterial lymphoid sheath (PALS).

A negative FAT result does not completely rule out a CSFV infection. In some cases during the terminal stage of the disease a positive reaction can be masked by antibodies which are already induced by the infected animal. On the other hand, caution should be exercised when interpreting results from a vaccination area as pigs vaccinated with modified live virus strains may yield a positive FAT result for two weeks after vaccination due to replication of vaccine virus mainly in the regional lymph nodes and in the crypt epithelium of the tonsils [1]. Depending on the type of vaccine, it may be possible to carry out differential staining with vaccine-specific monoclonal antibodies.

Fluorescent Antibody Test – FAT (indirect)



3.4.2.5 Reagents

Washing buffer

PBS (pH 7.4 - 7.6, physiological saline buffered with 0.01 M phosphate)

NaCl	8.78 g/l
Na_2HPO_4 x $12H_2O$	3.58 g/l

(pH adjusted with 1M KH_2HPO_4)

Evans Blue

Stock solution	1:100 in Aqua dest.
For use dilute	1:2000 in PBS

Mounting buffer

Washing buffer with 20% glycerine (buffered glycerine) or a commercial non-fading mountant.

3.5 CSF Molecular biology

3.5.1 Diagnostic RT-PCR

3.5.1.1 Introduction

Since the introduction of PCR and common availability of adequate laboratory equipment in the laboratories, substantial technological progress has been achieved during the last years. Nowadays, reverse transcriptase (RT)-PCR has become an internationally accepted highly sensitive and accurate standard technology which is routinely applied for the detection and quantification of CSFV genome [2]. The PCR shows clear advantages with regard to a higher sensitivity than other virological detection methods like antigen-capture ELISAs or virus isolation [3, 4]. In comparison to the traditional virus isolation, results are obtained much faster by RT-PCR and a higher number of samples can be processed with less effort and expenditure of time. Moreover, PCR might be the method of choice, if the sample material is autolysed and virus isolation is not possible due to cytotoxicity. Further advantages include a larger time span in which CSFV genome is detectable compared to viable virus, as well as the possibility of automation and pooling of samples to a certain extent. Nevertheless, virus isolation is still essential as a test with regard to the detection of viable virus and the possibility of subsequent agent characterization. A wide spectrum of sample matrices were shown to be suitable for CSFV genome detection by RT-PCR, e.g. anticoagulated whole blood, sera, tissues, swabs (oral, nasal, fecal), or cell culture (supernatants).

3.5.1.2 RNA-Extraction

The RNA extraction represents a critical point in the success of RT-PCR. The first step is lysing the cells by adding a strong denaturant to the sample (such as GITC, LiCl, SDS, phenol) for inactivation of enzymes (including the RNases). In order to optimize the performance of commercially available kits, samples and buffers are pretreated in dependence on their nature (cell culture supernatant, serum, tissue, blood). Therefore, extraction kits should be chosen according to the sample material used to receive optimal yields and a high quality of nucleic acids.

Three technologies are widely in use for nucleic acid isolation: the liquid based, the silica or glass based, and the magnetic bead based methods. For all of them, commercial kits are available which should be applied as described by the manufacturer. In case other techniques or in house protocols are used, they need to be thoroughly validated before they are applied. The liquid-based methods are further developments of the initial nucleic acid isolation procedure using guanidinium thiocyanate/acid phenol:chloroform [5, 6]. Ready-to-use reagents are commercially available under different brand names (TRIZOL, TRI Reagent, Stratagene RNA Isolation Kit, etc.), allowing single-step disruption/ separation procedures.
Silica- or glass-based matrices or filters selectively absorb nucleic acids in the presence of chaotropic salts [7], which immediately inactivate the RNases. After washing off the remaining components of the lysate using a high-salt buffer, bound RNA is eluted with water or another elution buffer supplied by the manufacturer. In many commercially available kits, the glass or silicon filter or fleece is housed in spin columns or in 96-well plates. Solutions are driven through the filter by centrifugation or under vacuum. Some kits also include a proteinase K or a DNase incubation for removal of proteins and DNA, respectively. Silica-based kits can be used in nucleic acid extraction robots and require no hazardous reagents.
Up to now, automated extraction systems that enable high throughput analyses have been implemented widely in routine laboratory diagnosis. Extraction of nucleic acids by most bead-based assays allows simultaneous purification of RNA and DNA, which might be an advantage, if testing for different pathogens is intended (e.g. combined CSFV and ASFV testing).
The correct storage of extracted RNA is crucial for the success of subsequent nucleic acid detection by PCR. For immediate application in PCR the RNA should be kept cool until usage; for storage of RNA freezing conditions are necessary, e.g. at -20°C for short-term and -70°C for long-term storage. RNA samples may be stored in a special RNA-safe buffer as described previously [8] in order to minimize degradation by freezing and thawing.

Independently from the manufacturer's specifications, each RNA extraction kit should be validated in each laboratory prior to use in order to ensure the suitability for the respective sample type. Furthermore, the PCR performance can differ in dependence on the applied RNA extraction method. Therefore, the PCR test performance needs to be re-evaluated when changing the RNA extraction method by using RNAs obtained by the established and by the novel extraction method.

3.5.1.3 Reverse transcription - Polymerase chain reaction (RT-PCR)

Upon extraction, nucleic acids are subjected to RT-PCR. To date, real-time PCR techniques (qPCR) are routinely applied and show clear advantages compared to conventional gel-based PCR methods, e.g. a higher sensitivity and specificity as well as a lower risk of contamination and expenditure of time. Moreover, qPCR methods allow an automation and hence a substantially higher sample throughput. In addition, an accurate CSFV genome quantification is facilitated and different targets can be detected simultaneously in multiplex approaches by using probe-based systems.

Conventional gel-based RT-PCR with electrophoresis in agarose gel

Traditionally, gel-based RT-PCR assays had been applied for the qualitative detection of CSFV/ pestivirus genomes and are still in use in some laboratories or for certain purposes to date.
After performance of RT-PCR, the reaction mixture containing the amplicons is subjected to electrophoresis in agarose gels. Double-stranded DNA is visualised under ultraviolet (UV) light after incubation with a fluorescent intercalating dye, mostly by using ethidium bromide, but also several alternatives are commercially available by now and, according to the manufacturer's information, offer advantages in terms of toxicity and stability. By including a DNA marker, the amplicon size can be estimated and thus, eventual unspecific amplicons and primer-dimers may be identified.
Various pestivirus- or CSFV-specific conventional RT-PCR protocols have been published, e.g. a well-proven pestivirus detection assay [9] in which a 288 bp-fragment of the 5' non-translated region (5`-NTR) is amplified. Furthermore, several CSFV-specific RT-PCR protocols are available, either targeting the E2 glycoprotein gene [10], the NS5B non-structural protein gene [11], or the 5'-NTR region of the CSFV genome [12]. The latter is a multiplex-PCR using two sets of primers whereas one of the primer sets was designed for the amplification of a 257 bp-fragment of the African swine fever virus (ASFV) genome. The mentioned RT-PCR targeting the E2 glycoprotein [10] is a nested PCR, amplifying fragments with a length of 308 bp and 172 bp. Nowadays, conventional RT-PCR protocols for pestivirus or CSFV genome detection have been widely replaced by real-time RT-PCR assays (qRT-PCR). Nevertheless, conventional gel-based RT-PCR is still indispensable for the verification of correct amplification prior to sequencing of PCR products and thus for genetic typing (see Chapter 3.5.2) which can support epidemiological tracing of isolates causing new outbreaks [13-15].

Real-time RT-PCR (qRT-PCR)
The basic principle of qRT-PCR is the simultaneous detection of fluorescence signals which are directly correlated to the amplification of the target sequence. To this means, fluorescence signals are measured in each cycle in real-time. Thereby, the intensity of fluorescence signals is proportional to the amount of the generated amplicon. This provides a distinct advantage over gel-based PCR detection, where only the end-point of the PCR reaction is evaluated. Fluorescence signals can be produced by use of different techniques, including intercalating fluorescence dyes binding to double-stranded DNA or fluorescence-labelled hybridization probes binding to the corresponding complementary sequence [16-19]. To date, SYBR-Green based assays (an intercalating fluorescence dye) and Taqman probe systems (a fluorescence-labelled hybridization probe) are most commonly used for pestivirus and CSFV genome diagnosis. With regard to the risk of contamination, one-step RT-PCR systems should be preferred towards two-step assays (in which the reverse transcription and cDNA-synthesis are performed in two different tubes, hence bearing a higher risk of contamination.

1. SYBR-Green based qRT-PCR:

The underlying mechanism is the emission of a fluorescence signal of a defined wave length generated through binding of the fluorescent dye SYBR-Green to double-stranded DNA. At the end of the PCR run, the melting point of the product should be determined to distinguish between

specific and unspecific amplification. An advantage of this technique is the detection of the target sequence without the necessity of expensive hybridization probes. In addition, SYBR-Green based assays own a broad reactivity, making them particularly suitable for panpesti-assays. However, assays should be checked for specificity thoroughly prior to use in order to achieve reliable results [20]. The specificity of the reaction needs to be monitored by evaluation of melting temperatures in order to recognize false positive results which might arise due to mis-priming, primer-dimerization, contamination or other reasons [21, 22]. Several SYBR-Green based qRT-PCR assays for the detection of CSFV genome have been successfully applied [23-25]. Similarly, SYBR-Green based protocols for pan-pestivirus genome detection [9, 26, 27] are available.

2. TaqMan-based qRT-PCR:

TaqMan probes are the most common fluorescent-labelled hybridization probes containing a 5'exonuclease-activity. In detail, those are sequence specific oligonucleotides with a fluorescent dye at the 5' base ("reporter") and a fluorescence absorbing molecule at the 3' base ("quencher"). The close proximity of reporter and quencher prevents emission of any fluorescence while the probe is intact. During qPCR reaction the probe binds specifically to the target sequence which is flanked by the primer binding sites. During template amplification (annealing and extension phase) the exonuclease activity of the DNA-polymerase cleaves the probe [28]. This spatial separation between reporter and quencher results in an emission of a fluorescence signal which increases in each cycle in direct proportion to the rate of probe cleavage. The accumulation of PCR products is detected by monitoring the increase of fluorescence signals generated by the reporter dye. TaqMan-based qPCR assays have an increased specificity through the additional specific binding of the oligonucleotide probe molecule to the target sequence and offer the opportunity for multiplexing by use of different fluorescent dyes with distinct emission spectra [21, 22].
Many TaqMan-based qRT-PCR assays for the specific detection of CSFV genome have been published to date. Most of them amplify fragments of the 5'-NTR [29-34], but also protocols targeting alternative genomic areas are available, namely the NS5A [35] or the NS5B coding region [33, 36]. Similarly, TagMan-based qRT-PCR assays for the pan-pestivirus detection have been published using highly conserved portions of the 5'NTR [8, 36]. The method of choice has to be evaluated thoroughly in each laboratory prior to use.

Furthermore, several CSFV genome specific qRT-PCR kits containing all reagents for the test in a single ready-to-use reaction mix are commercially available to date [37].
Apart from the nucleic acid, only the primer pairs, and the polymerase (all reagents supplied by the manufacturer) have to be added, and thus reducing the probability of contamination and of pipetting errors. However, only kits with an official approval from the responsible national licensing authority are to be used for diagnostic purposes.
Apart from the CSFV specific and pan-pestivirus qRT-PCR assays, several other assays for the simultaneous detection and differentiation from further pathogenic agents have been developed. For example, a multiplex test to distinguish CSFV from the differential diagnostic relevant ASFV [38]. Moreover, qRT-PCRs for the differentiation of natural wild-type infection from vaccination (DIVA-principle) were published to reveal veiled infections in the field for early-warning. Those

assays either specifically distinguish between certain vaccine virus strains and natural infection [39, 40], or allow the differentiation of different vaccine strains [41].

Moreover, many recent and ongoing research activities aim to reduce the need of special laboratory equipment in order to transfer the basic principle of PCR to field conditions and by that facilitate a rapid, reliable, and cost-efficient diagnosis in the field. In this context, loop-mediated isothermal amplification methods [42-45] as well as further alternative techniques like penside tests have been developed taking advantage of the enormous technical progress during the last years, e.g. PCR technique modifications in favor of simple field applications [46] or usage of DNA chips [47]. However, those novel methods have not gained general acceptance for application in CSF diagnosis and therefore represent no suitable replacement for the approved qRT-PCR techniques to date. Hence, recent developments should be revised critically and have to be used for research purposes only at the moment.

3.5.1.4 Quality control

A stringent quality control system of (q)RT-PCR is essential. In this regard, mandatory prerequisites for performing (q)RT-PCRs, like a suitable laboratory equipment and organization as well as a properly trained and supervised staff, are designated in order to minimize the risk of laboratory contaminations leading to false positive or false negative results.

For quality control and verification of (q)PCR results the inclusion of several controls is highly recommended. In this context, controls should be used to evaluate both: the success of nucleic acid extraction (extraction controls) and the (q)PCR itself (PCR controls).
For both, RNA extraction and PCR, positive and negative controls should be applied. The RNA extraction control can act as a PCR control at least on a run-level or positive PCR controls should be RNAs containing CSFV genomes of a defined amount; extraction controls should be well characterized sample material (e.g. from experimental *in vitro* or *in vivo* infection) and should be processed in exactly the same way as the test samples in question. With regard to the extraction controls the same sample type as being tested should be used. However, in case this material is not available or various sample types are investigated at the same time heterologous sample material can be used as controls.
The number of negative extraction controls should be in accordance with the total amount of samples. Multiple negative extraction controls evenly distributed in a set of samples can help to identify cross-contamination during pipetting. With respect to the PCR positive control, the containing virus strain should be preferably of a distinct genotype than the one derived from the expected amplicon in order to facilitate a clear differentiation between positive control and target upon sequencing.

In addition, the inclusion of internal controls into each (q)PCR run is strongly recommended e.g. by OIE guidelines. Depending on the assay, different internal controls can be applied:

- One possibility is the simultaneous amplification of at least one ubiquitous internal reference gene (so-called “housekeeping” gene) which were shown not to be altered by CSFV infection (e.g. β-Actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyl transferase (HPRT), 18S ribosomal RNA), providing the advantage that such controls are simple and all steps that are required to

obtain the final PCR measurement are controlled. Some materials with low cell content (plasma or serum from routine surveillance) may show difficulties in the homogeneous detection of such a control.

- A second frequently used approach is spiking of the sample during nucleic acid extraction with a defined amount of a unique or artificial RNA which was produced by cloning and subsequent *in vitro* transcription or generated synthetically. As this artificial RNA is added during RNA extraction it serves as control for nucleic acid isolation and qPCR and is independent from biological variations that might effect reference genes. A general drawback is that those are not extracted from the cells unlike the RNA of interest [48]. The first and the second approach require thorough validation prior to use.
- In case of large numbers of samples, it is recommended to include several negative controls distributed all over the plate.

Only if the controls (positive and negative) provide plausible results, the whole assay can be considered as valid. Depending on the control leading to an invalid test, either the (q)PCR or both [RNA extraction and (q)PCR] must be repeated.
In general, it is highly recommended to have a second (q)PCR method with comparable sensitivity in place targeting a different genome region for confirmation of doubtful and weak positive results (e.g. not allowing subsequent confirmation by sequencing).

3.5.2 Genetic Typing of CSFV isolates

3.5.2.1 Introduction

In general, CSFV strains can be assigned to three distinct genotypes each comprising three to six subtypes [14, 49]. Further discrimination and comparison of genetic differences between virus isolates facilitate to assess the degree of viral relationship, representing the basis for molecular epidemiology of CSFV. Genetic typing has become an important tool for tracing disease spread and outbreak dynamics as the appearance of different subtypes is often linked to particular geographical distributions [14, 15]. The simplest option for genetic typing is to amplify a particular region of the CSFV genome by (q)RT-PCR followed by nucleotide sequencing. This is mandatory to assign newly identified CSFV isolates to a genotype and to describe its phylogenetic relations to other known isolates.
Several CSFV-specific RT-PCR protocols were established to amplify fragments of the viral genome for subsequent nucleotide sequencing and genetic typing. In brief, random primer and dNTPs (deoxynucleotide triphosphates) are used within a first step followed by addition of specific primer and a thermostable DNA polymerase for amplification. Subsequently, the amplified DNA is subjected to agarose gel electrophoresis and finally, PCR products are purified for removal of inhibitors. Those PCR products serve as basic material for the final sequencing reaction. A more detailed description of the sequencing method is provided below.

Various genomic regions have been proposed as a basis for genetic typing including mostly part of the 5'NTR [14, 50, 51], but also different parts of the E2 [14, 50] and NS5B coding regions as well as the entire N^{pro} [51] and E2 coding regions [52]. To enable a standardized and harmonized genetic characterization and procedure for genetic typing, sequences of the 5'NTR, the E2 and

NS5B-coding regions have been included into the CSFV database (CSF-DB) of the EURL for CSF in Hannover (see below for more detailed information) [51, 53-55].
Partial 5´NTR and E2 coding sequences have been routinely used for characterization of CSFV isolates despite of several limitations mainly due to the short sequence lengths of the chosen fragments. In case short-length genome fragments are used for genetic typing of CSFV isolates, it is recommended that at least two different genomic target regions (5'NTR and E2) are analyzed. Phylogenetic analysis based on the genetically variable E2 fragment (190 nt) were shown to provide more reliable results in direct comparison to the conserved partial 5'NTR (150 nt) sequences [49]. In case of incongruent results of 5`NTR and E2 fragment based genetic typing the analysis of the latter usually provides the more reliable information with regard to subgenotype affiliation. Due to the frequent use of the E2 region for typing in recent decades, much sequence information has been made available by now making this strategy advantageous. However, usage of short fragment sequences for genetic typing only allows phylogenetic analysis with restricted resolution and often gives insufficient statistical support when closely related CSFV isolates are analyzed [49, 55].
To overcome this drawback phylogenetic analyses of longer genome fragments, e.g. full-length E2 encoding sequences, can be performed. This region was shown to allow a clear assignment of CSFV isolates to a subgenotype even when closely related isolates are analyzed [49]. Further extension of sequencing did not significantly increase the outcome of phylogenetic analysis. This strategy is therefore recommended by the EURL for CSF as it provides solid and improved results of CSFV genetic typing and molecular epidemiology based on high-resolution phylogenetic analyses.
Besides, further approaches for genetic typing of CSFV isolates have been suggested and are developed continuously. New molecular biological techniques like next-generation sequencing (NGS) which allows a rapid determination of full-length sequences have been developed. However, at the moment only a few institutions can afford this technique for a small number of selected CSFV isolates due to associated costs and efforts.
The standardized and easy-to-use CSF-DB of the EURL for CSF in Hannover offers a platform for the exchange of CSFV genome data within one of the world's largest semi-public virus-specific sequence collections combined with an automated module for a harmonized phylogenetic analysis.
This database now contains partial 5′NTR (150 nt), and E2 (190 nt) coding sequences as well as complete E2 (1119 nt) coding sequences. In addition, a geographic "CSF Maps" tool has been integrated allowing geocoding and subsequent graphical display of outbreak locations [54].
The database is accessible through the homepage of the EURL for CSF (http://viro60.tiho-hannover.de/eg/csf/) [51, 53-55]. Username and password can be obtained upon request by sending an e-mail to: csf.eurl@tiho-hannover.de

The EURL for CSF is in charge of genetic typing in case that virus typing cannot be performed in a national reference laboratory or in any other laboratory. It is recommandend to forward sequence data generated by the diagnostic laboratories, as well as the virus isolates, to the EURL for CSF for integration in the CSF-DB. In return, containing data are available for these laboratories.

3.5.2.2 Sequencing method

An overview of the sequencing method with the focus on Sanger sequencing is given below.
The provided information concerning products and companies have to be regarded as examples for the current availability on the market, these are no recommendations. As the market is changing continuously, current developments should always be taken into consideration.

- Total RNA is extracted from clinical samples or from susceptible cell cultures that have been infected with low passaged CSF virus. RNA extraction is performed as described above (see Chapter 3.5.1.2) according to manufacturer's instructions. For sequencing purposes, in particular if the nucleic acid is intended for NGS, RNA extraction should be combined with a DNA digestion step in order to remove host DNA. NGS can either be performed at the laboratory itself, or, in case the technique is not available, the thoroughly isolated RNA is transferred to a specialized laboratory upon quality control of the prepared nucleic acid (estimation of RNA quantity with a special spectrophotometer or advanced ELISA Reader). In case Sanger sequencing is performed, the procedure is continued as described below.
- RT-PCR is performed to amplify the target fragments chosen for genetic typing (5'NTR, E2 and/ or NS5B genes). The detailed performance of RT-PCR including cycling conditions must be adjusted to the applied protocol as well as to the PCR chemistry according to manufacturer's recommendations and internal validation procedure.
- Reverse transcription is carried out by addition of random primers and all four dNTPs. Subsequently, PCR is conducted with specific primer pairs and a thermostable DNA-polymerase (e.g. Taq-polymerase) for DNA amplification.
- The primer sequences which are in use at the EURL for CSF are given below. All required components for reverse transcription and PCR as well as random primer, RNase inhibitors, and dNTP sets are commercially available by different companies.
- The generated CSFV amplicons are subjected to agarose gel electrophoresis. Due to the fact that the separation during electrophoresis is size-dependent, the concentration of agarose gel must conform to the total length of the target fragment. Subsequently, the PCR products are visualized by use of an intercalating dye binding to double-stranded DNA (e.g. ethidium bromide) in combination with UV-light.
- The PCR products are purified in order to remove possible inhibitory substances [e.g. proteins (including enzymes), primer pairs, salts, dNTPs, unincorporated labeled nucleotides and other contaminations]. Several PCR purification kits are commercially available.
- Depending on the guidelines of the assigned sequencing laboratory, the purified DNA is either directly sent to the sequence laboratory together with the corresponding forward and reverse primer upon assessment of DNA quantity in the purified product with a special spectrophotometer, or a sequencing PCR has to be conducted first by the ordering laboratory. In the latter case, forward and reverse primer have to be applied separately within the sequencing PCR reaction by using a commercially available cycle sequencing kit according to the protocol given by the manufacturer. The product of the sequencing PCR has to be cleaned in order to remove residual dNTPs and unincorporated dye terminators by use of commercially available kitsaccording to manufacturer's manual. Afterwards, the product must be denatured by addition of an equal volume of formamide. The denatured sequencing sample is ready for use in an automated sequencer representing a capillary electrophoresis machine for fully

automated determination of DNA sequences of the prepared samples. This final procedure is basing on fluorescence-labelled dNTPs which are separated by capillary electrophoresis.

The quality of the obtained sequence data are evaluated by use of a suitable computer software and after trimming of sequence ends a consensus sequence is generated. The obtained consensus sequence is subsequently compared with the available sequence information provided within specific databases (e.g. CSF-DB of the EURL for CSF; GenBank, NCBI; EMBL). Detailed information concerning sequence data comparison are given below.

Examples for primer sequences which can be used for genetic typing

Primer pairs applied at the EURL for CSF:

Primer sequences for generation of 5'NTR sequences [51] (total fragment length: 420 nucleotides (nt)):

UP- 1	5´-CTA-GCC-ATG-CCC-WYA-GTA-GG- 3´	(94-113)
UP- 2	5´-CAG-CTT-CAR-YGT-TGA-TTG-T- 3´	(495-514)

Primer sequences for generation of partial E2 coding sequences (unpublished; total fragment length: 471 nt):

E2-s1	5´-TTR-AGA-GGD-CAG-GTT-GTG-CAA-G-3´	(2381-2402)
E2-as1	5´-CTA-YKA-CRC-CMG-TCC-ATC-CTA-T-3´	(2831-2852)

Primer sequences for generation of complete E2 gene sequences [49] (total fragment length: 1505 nt):

CSF2250f	5`-TGT-TAG-RCC-RGR-YTG-GTG-GC- 3´	(2224-2243)
CSF3710r	5`-TRG-TYT-TRA-CTG-GRT-TGT-TRG- 3´	(3708-3728)

Numbers in parentheses refer to the genomic sequence of CSFV strain Alfort 187 (GenBank Acc. No. X87939).

The given primer are adjusted to 50 pmol.

In addition, the following primer sequences have been used for genetic typing (not routinely applied at the EURL for CSF):

Supplementary primer for nested PCR targeting an inner part of the 5'NTR fragment [51]:
CSFV/SQ-1 Forward: 5'-AGC TCC CTG GGT GGT CTA -3' (146-163)
CSFV/SQ-2 Reverse: 5'-TGT TTG CTT GTG TTG TAT A -3' (417-399)

Primer sequences for E1 and E2 gene [14] (total fragment length: 670 nt):
Forward 5'-AGR CCA GAC TGG TGG CCN TAY GA-3' (2228-2250)
Reverse 5'-TTY ACC ACT TCT GTT CTC A-3' (2898-2880)

Supplementary primer for nested PCR targeting an inner part of the E2 fragment [14]:
Forward *5'* TCR WCA ACC AAY GAG ATA GGG 3' (2477-2497)
Reverse *5*' CAC AGY CCR AAY CCR AAG TCA TC 3' (2748-2726)

Primer sequences for generation of partial NS5B coding sequences [52] (total fragment length: 449 nt):
S1 Forward 5' GAC ACT AGY GCA GGC AAY AG 3' (11138-11157)
S2 Reverse 5' AGT GGG TTC CAG GAR TAC AT 3' (11586-11567)

3.5.2.3 Sequence data comparison

In the course of recent technological progress, different methods and computer software have been developed which are (freely) available nowadays facilitating a harmonized evaluation of sequence data. The hereinafter stated approaches shall be understood as examples and do not represent recommendations.

For comparative analyses of genomic sequences, alignmet of the virus genomes in question can be carried out by using computer programs, i.e. Pileup and ClustalW of the Multiple Sequence Comparison by Log-Expectation (MUSCLE) tool provided by EMBL-EBI [56].
Nucleotide and deduced amino acid (aa) identities can be calculated with the help of programs like Homologies and MegA-lign [57]. Amino acid sequences can be scanned with the sequence distance calculation tool of the SSE soft ware platform [58].
For choosing the appropriate substitution model to calculate genetic distances different applications are available. For CSFV the Kimura-2 parameter substitution model was widely used in the past [59]. Generated matrices of corrected genetic distances between sequence pairs are the basis for subsequent phylogenetic analysis.
Phylogenetic analyses can be generated by applying different methods, amongst them the neighbor-joining method was frequently used in the past. Different software can be used for analysis, e.g. NEIGHBOR (PHYLIP inference package programs) [60-62], DNASTAR (Lasergene 8.1, DNASTAR, Inc., USA) [63].
Grouping of viruses should be confirmed by at least one additional method, e.g. maximum likelihood [49] or Bayesian analysis.
Statistical analysis of phylogenetic trees should be performed. The robustness of phylogenetic trees can be determined e.g. by bootstrap analysis on 1000 replicates using programs SEQBOOT and CONSENSE from PHYLIP [49]. Bootstrap values in excess of 70% are considered to be significant [59, 60, 62, 64].

The public accessible EURL CSF-DB contains a sequence analysis tool, including the opportunity for generation of phylogenetic trees. The genetic typing module enables phylogenetic analysis of up to ten sequences of choice (partial 5′NTR sequences, partial E2 sequences, and complete E2 coding sequences) including bootstrap analysis for statistical evaluation. For phylogenetic analysis, newly generated sequences and additional sequences from the CSFV-DB can be included, which are integrated in a pre-defined phylogenetic tree of reference sequences and rooted to the sequence of CSFV isolate "Congenital Tremor" (CSF0410) [55]. The analysis of newly generated CSFV sequences in the context of a pre-defined set of reference sequences

increases the comparability among different studies and allows a reliable genotype assignment without detailed background knowledge.

Epidemiological data are useful for the correct interpretation of genetic typing results, especially with regard to new CSF virus isolates, i.e. date of isolation, species (domestic pig or wild boar), geographic origin (country and area).

4 CSF Serology

4.1 Cell culture based CSFV antibody detection: Virus neutralization test (VNT)

4.1.1 Principle of the test

The test is applied for the qualitative and quantitative detection of neutralizing antibodies. It is intended for use on serum samples. In case no serum is available, other sample types like e.g. plasma, meat juice, exsudates of body cavities may be used as an alternative. For complement inactivation and in order to lower the risk of bacterial contaminations it is recommended to incubate the sera at 56°C for 30 minutes prior to use in VNT.
For the detection of the neutralizing activity of antibodies [expressed as neutralizing dose 50% end point (ND_{50})], a constant amount of CSF virus [100 (plus/minus 0.5 log10) tissue culture infectious dose 50 ($TCID_{50}$)] is incubated with equal volumes of diluted serum for one hour at 37 °C. The choice of the initial serum dilution depends on the respective purpose. For a full titration a two-fold serial dilution starting at ½ or 1:5 should be prepared, wheras for screening purpose an initial serum dilution of 1:10 can be applied. Subsequently, susceptible cells (e.g. PK-15) are incubated with the mixtures of virus and diluted serum at each dilution. After an incubation period of 3 to 5 days, the cell cultures are fixed and the viral antigen is detected by immune labelling. The detailed protocol of the test procedure is provided below.

Either the neutralization peroxidase-linked antibody test (NPLA) [65] or the neutralization-immunofluorescence test (NIF/ fluorescent antibody virus neutralization (FAVN)) [66] may be used. The peroxidase system has the advantage that the results can be read with a light microscope and the antibody titre can be crudely assessed even with the naked eye. In addition, the evaluation is possible for a long time after staining. For the NIF test a fluorescence microscope is needed. Repetitive microscopical examination or prolonged storage time may result in fading fluorescence signals.

4.1.2 CSFV strains for VNT

Stock virus

The CSFV reference strain in the European Union is Alfort/187 (CSF 902; genotype 1.1). In addition, the usage of further strains particularly of recently circulating genotypes in the region (if available the corresponding field virus isolates) is clearly recommended as antibody titres can vary considerably depending on the virus genotype used in the assay.

Batches of stock virus can be produced by inoculating one day old susceptible cell cultures in tissue culture flasks with the respective virus isolate. The cultures are incubated for optimally 3 days (but no longer than 4 days) at 37 °C. Thereafter, cell-associated virus is released through a freeze-thaw-cycle at -80 °C and cell components are removed by centrifugation for 10 min at about 778 g. The resulting virus amount can be estimated by 10-fold virus titration which is carried out as end-point dilution method as described below. The newly gained virus-containing supernatants can be aliquoted (0.5 - 1 ml), labelled, packed and stored at -80 °C until use in the VNT.

Calculation of infectivity titres

Before the new virus stock is used in VNT, at least three independend virus titrations should be performed. Later on, when the virus titre has been established, a virus titration of the original stock virus in each VNT can be carried out for constant control of the virus stock´s titre.
A 10-fold serial dilution of the virus stock is prepared with 8 different dilutions by e.g. using 900 µl of cell culture medium to which 100 µl of each virus dilution step is added (see figure B). Subsequently, 100 µl of each dilution are pipetted into 4 wells of a microtiter plate and susceptible cells are added. Plates are incubated for about 72 h at 37°C in a 5% CO_2 humidified atmosphere. After incubation, the cell layer is fixated and virus antigen is detected by immune staining (see Chapter 3.2).
The highest dilution of virus which infects 50% of the cell cultures is regarded as the endpoint of infectivity. This value is estimated or calculated using the method of KAERBER (1931) [67]. Virus infectivity titres are expressed as tissue culture infectious doses ($TCID_{50}$) per volume (e.g. 0.1 ml) of virus suspension.

Example of $TCID_{50}$-calculation (Method of KAERBER)

$\log TCID_{50} = L_{1.0} - L_{int} (S - 0.5)$

$L_{1.0}$ = Logarithm of the highest virus dilution with the reaction rate (R) = 1.0
L_{int} = Logarithm of the dilution interval (int)
S = Sum of reaction rates (R) starting with the last reaction rate with 1.0
0.5 = Constant factor

virus dilutions	logarithm	infected wells / total wells	R
10^0	0	4/4	1.0
10^{-1}	-1	4/4	1.0
10^{-2}	-2	4/4	1.0
10^{-3} (= $L_{1.0}$)	-3	4/4	1.0
10^{-4}	-4	1/4	0.25
10^{-5}	-5	0/4	0.0
			S = 1.25

$\log TCID_{50}$ = -3 - 1.0 (1.25 – 0.5)
= -3 – 0.75
= -3.75

The virus titer is $10^{3.75}$ $TCID_{50}$/0.1 ml, corresponding to $10^{4.75}$ $TCID_{50}$ /ml.

Back-titration

A back-titration is an essential internal quality control and must be included in each VNT in order to determine the actual amount ($TCID_{50}$) of the applied test virus. To this means, a 10-fold virus titration can be performed (as described above) using the actual applied virus suspension (working dilution) which has been added to the VNT plate. The titration of virus working dilution should cover a range of 4 log dilutions [10^0 (undiluted) to 10^{-4}]. Alternatively, back-titration can be performed similar to the processing of serum samples during VNT. To this means, the adjusted virus solution is diluted in log-2-steps starting from 1/10 (180 µl cell culture medium + 20 µl virus solution) until 1:1280 in the same way as the test sera are diluted (see below). Similar to the 10-fold virus titration, 4 adjacent wells are used for each dilution step, containing a total amount of 100 µl of virus dilution at the end of titration procedure. If the titre of the virus working solution determined in back-titration is not within the tolerance limits (30-300 $TCID_{50}$ per well) the test is invalid and must be repeated.

Calculation of dilution factor for obtaining 10^2 $TCID_{50}$/50µl (corresponding to 2000 $TCID_{50}$/ml): As an example it is considered that the stock virus has a titre of $10^{5.7}$ $TCID_{50}$/100µl. The virus solution applied in the VNT should have a titre of 100 (10^2) $TCID_{50}$/50µl.

$10^{5.7}$ $TCID_{50}$/100 µl corresponds to $10^{5.4}$ $TCID_{50}$/50 µl

Calculation

$10^{5.7}$ $TCID_{50}$/100 is to be divided by 2 (2 is equivalent to $10^{0.3}$), that means
$10^{5.7}$ divided by $10^{0.3}$, corresponding to 5.7 - 0.3 = 5.4 ($10^{5.4}$)

$10^{5.4}$ $TCID_{50}$/50 µl is to be diluted to obtain 10^2 $TCID_{50}$/50 µl

Calculation of dilution factor

$10^{5.4}$ is to be divided by 10^2 , that means 5.4 - 2 = 3.4 ($10^{3.4}$)

dilution factor = $1/10^{3.4}$ that means approx. 1/2600

(From 1 ml stock virus containing $10^{5.7}$ $TCID_{50}$/100 µl (or $10^{5.4}$ $TCID_{50}$/50µl) one can make 2600 ml of virus solution containing 10^2 $TCID_{50}$/50 µl).

4.1.3 Test procedure

- The first row of a microtiter plate is filled with 80 µl cell culture medium; and all other rows are filled with 50 µl culture medium.
- 20 µl of each serum sample are added to the first row into at least two adjacent wells and thoroughly mixed with the culture medium to obtain an initial serum dilution of 1/5.
- Subsequently, 50 µl of the **initial 1/5 serum dilution** are transferred into the next row, and again mixed with the culture medium by using a multichannel pipette. This step is repeated until a complete two-fold dilution series is prepared on the microtiter plate (see figure A); the remaining 50 µl from the last row are discarded. Consequently, each well contains 50 µl of serum-medium-dilution when titration is finished.
- The previously prepared virus suspension containing 100 $TCID_{50}$/50 µl is added to each well; 50 µl per cavity are used. The required dilution of the test virus (working dilution) to obtain 100 $TCID_{50}$/50 µl has to be prepared shortly before use by diluting the virus in cell culture medium (see above).
- The plates are placed in a moist chamber in a CO_2 incubator at 5% CO_2 (alternatively plates can be sealed air tight) and incubated at 37°C for 1 to a maximum of 2 hours.
- The virus working dilution is back-titrated (see above) and incubated together with the VNT plates. In addition, a titration of the original virus stock used for the virus working dilution can be performed.
- After incubation of the mixtures of virus and serum dilutions, a suspension of susceptible cells in culture medium (50 to 100 µl/ well) is added to each cavity. The chosen cell density must

ensure that a confluent cell monolayer can develop within 24 hours (see Chapter 2.5). The cell suspension should be prepared during the incubation period.

- The plates are placed in a moist chamber in an incubator at 5% CO_2 (alternatively plates can be sealed air tight) and incubated at 37°C for 3 to 4 days (at maximum 5 days).
- After incubation, cultures are fixed and stained as described in Chapter 3.2

4.1.4 Controls

Back-titration (see above) of the applied test virus solution must be included in each test to ensure correct adjustment of the virus working dilution to 100 $TCID_{50}$/50µl (tolerance limits ±0.5 log, corresponding to 30-300 $TCID_{50}$/50µl). If the virus titres determined in back-titration are not within the defined tolerance limits, the test is invalid and should be repeated. The back-titration serves as virus positive control for the test.
In addition, at least one positive control serum should be included in each VNT. To this means, a CSF antibody positive reference serum sample (with a well characterized antibody titre) is applied, and treated in the same way as the test samples. For a valid VNT, the antibody titer of the reference serum should be within a defined range of the expected value. Reference sera can be monitored over time using internal laboratory control charts.

Furthermore, a negative control (cell control) must be included in each VNT, containing only cells in culture medium. In case any specific labelling is detected in the cell control the VNT is invalid and must be repeated.
Taken together, the test has to be repeated in case of one or more of the following criteria:

- no specific labelling in virus positive control
- specific labelling in cell control
- back-titration not within the defined tolerance limits
- reference serum not within the defined range

4.1.5 Interpretation

Estimation of ND_{50}
CSFV infection of cells is detected by immune staining. A non-infected well is regarded as result of virus neutralizing activities of anti-CSFV antibodies present in the applied test serum.
Serum titres are recorded as reciprocal of the highest initial dilution of sera (dilution of serum and cell culture medium without virus suspension) which prevented virus replication in 50% of the wells. Wells are scored as virus positive as soon as at least one cell in the monolayer demonstrates specific staining. A point between two dilution levels is estimated. Serum titres are expressed as 50% neutralizing dose (ND_{50}) and can be also calculated using the method of KAERBER (1931).

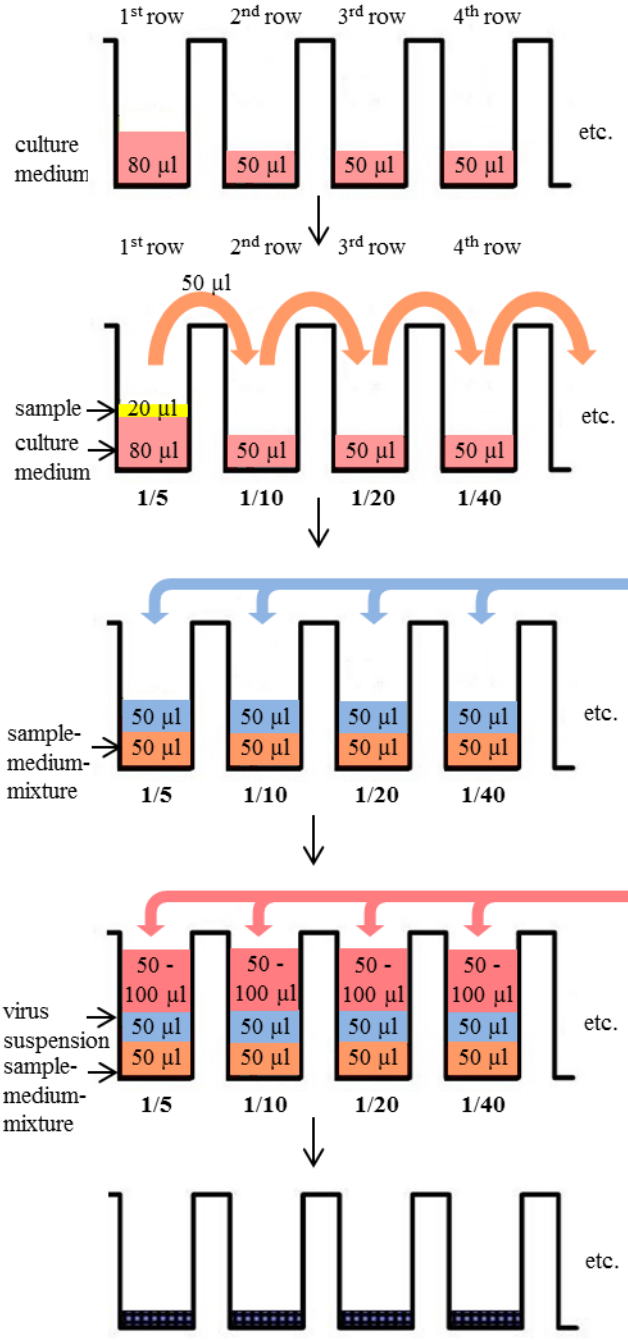
Example 1: Only one of the two wells of the initial serum dilution of 1:5 has infected cells. In this case the neutralization titre would be 5 ND_{50}.
Example 2: All wells up to the dilution of 1/40 are free of viral antigen and one of the two wells in the serum dilution 1:80 has infected cells, all dilutions higher than 1/80 are positive. In this case the neutralization titre would be 80 ND_{50}.
Example 3: All wells up to the dilution of 1/80 are free of viral antigen while the remaining

wells with initial serum dilutions equal and higher than 1/160 are positive. In this case the neutralization titre is estimated to be 120 ND_{50}.

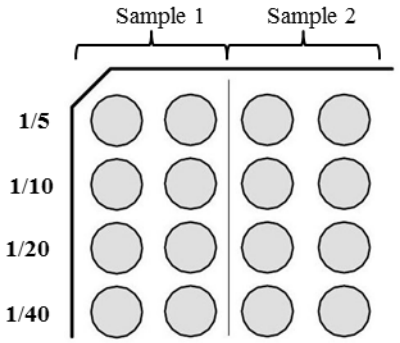
In case of a full neutralization (all wells virus negative) the inclusion of higher dilution steps is necessary for an exact determination of the ND_{50}. For screening purposes, the titre can be given as exceeding the last step, e.g. >640 ND_{50}.

When reporting test results, it is important to specify clearly that the titre is expressed as initial dilution, and that for final dilution (this method is only used in exceptional cases) the result should be multiplied by 2 (e.g. 1/10 initial, corresponds to 1/20 final). The final dilution system is based on the actual dilution of serum during the neutralization reaction, e.g. after addition of virus but before adding the cell suspension.

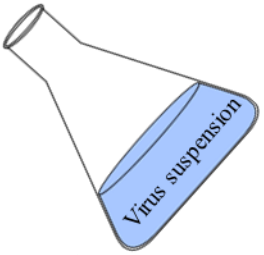
Test procedures VNT



Load the microtiter plate with culture medium:
80 µl into the first row; 50 µl into all other rows.



Add 20 µl sample in two adjacent wells of the first row to prepare the initial dilution, mix thoroughly with culture medium and transfer 50 µl of sample-medium mixture into cavities of the next row. Repeat this procedure until the complete dilution series is finished; discard the remaining 50 µl from the last row.

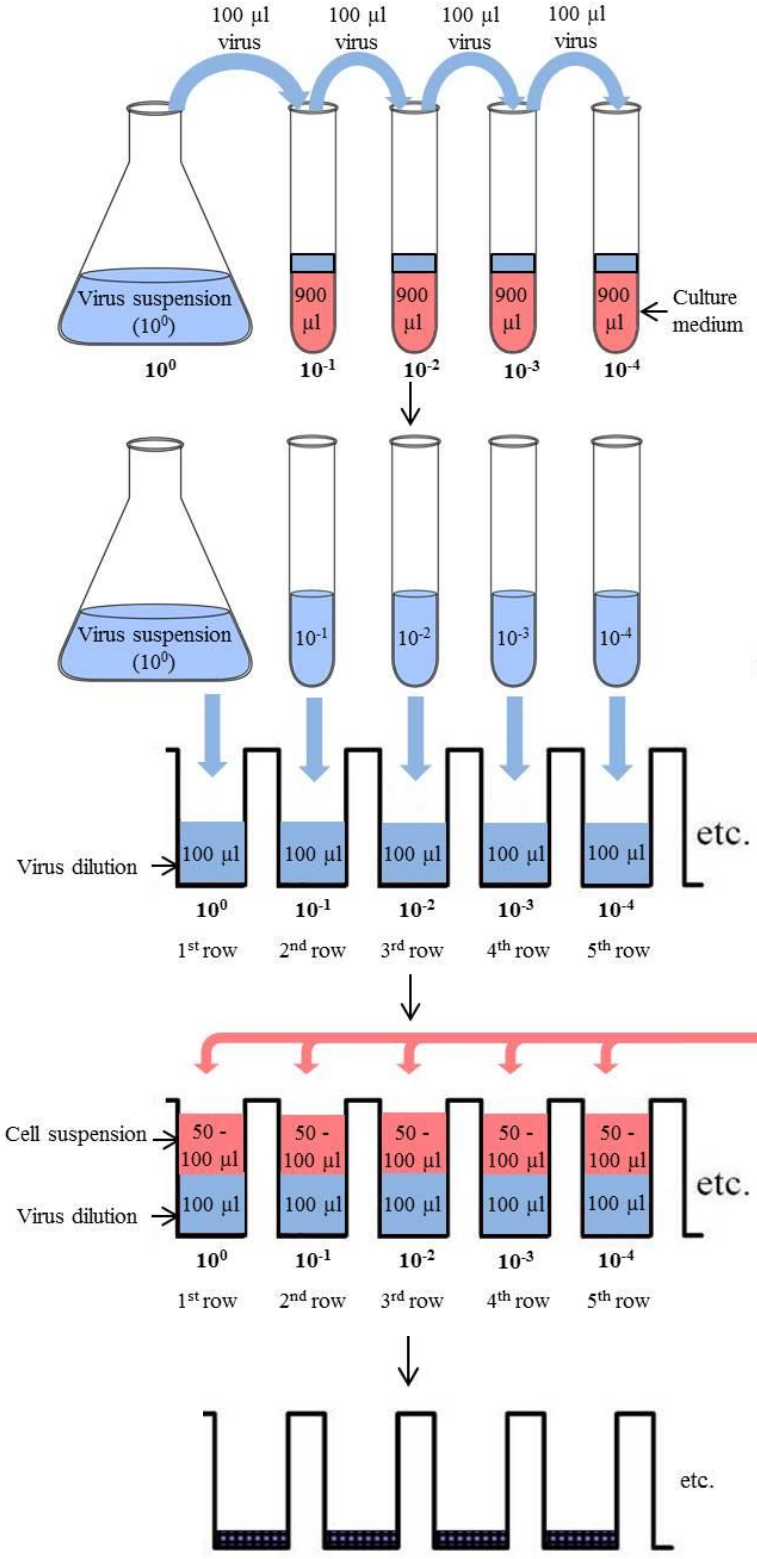


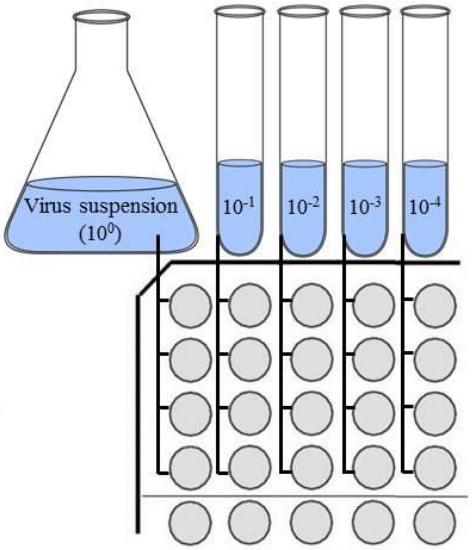
Prepare virus suspension adjusted to 100 $TCID_{50}$/50 µl (= virus working dilution) shortly before use, add 50 µl to each well and incubate for 1 h to maximum 2 h at 37°C. Incubate together with back-titration of virus working dilution figure "Virus (back-) titration").

Prepare cell suspension shortly before use, add 50 µl – 100 µl (depending on cell density) to each well and incubate for 72 to 96 hours (at maximum 120 hours) at 37°C in a CO_2 incubator.

Discard all contents;
fix and stain (see Chapter: 3.2)

Virus titration and back-titration





For preparing a 10-fold virus dilution series add 100 µl of virus supension to test tubes containing 900 µl cell culture medium; for back titration use virus working dilution adjusted to 100 $TCID_{50}$/50 µl, for titration of original virus stock use the original virus. Mix each diltuion step thoroughly.

Add 100 µl of each dilution step to 4 wells of a microtiter plate. For back titration start with the virus working dilution adjusted to 100 $TCID_{50}$/50 µl; for titration of original virus stock it is sufficient to start with the 10^{-1} virus dilution.

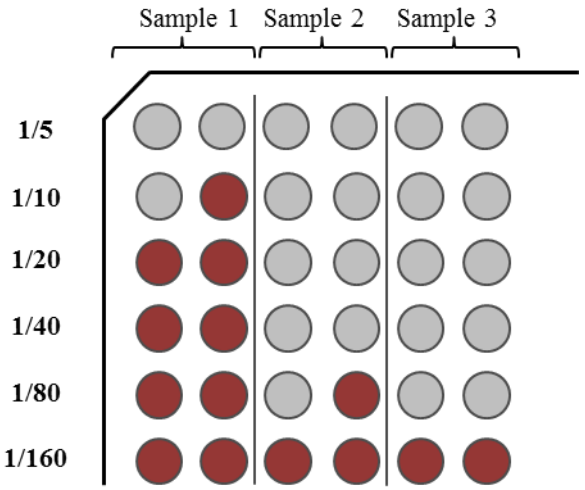
Prepare cell suspension shortly before use, add 50 µl – 100 µl (depending on cell density) to each well and incubate for 72 to 96 hours (at maximum 120 hours) at 37°C in a CO_2 incubator

Discard all contents; fix and stain (see Chapter: 3.2).

Evaluation of results -VNT

Estimation of Neutralization titre (ND_{50}):

ND_{50} = reciprocal of the highest initial serum dilution which prevented virus replication in 50% of the wells.
The ND_{50} can be determined by either estimating a point between two dilutions or by calculating using the method of KAERBER.



Results (ND_{50}) of the displayed example:

Sample 1 = 10 ND_{50}
Sample 2 = 80 ND_{50}
Sample 3 = 120 ND_{50}

Example of ND_{50}-Calculation (method of Kaerber) for sample 2:

$\log ND_{50} = L_{1.0} - L_{int} \times (S - 0.5)$

$L_{1.0}$ = Logarithm of the highest serum dilution with the reaction rate (R) =1.0
L_{int} = Logarithm of the dilution interval (int)
S = Sum of reaction rates (starting with the last reaction rate that is 1.0)
0.5 = Constant factor

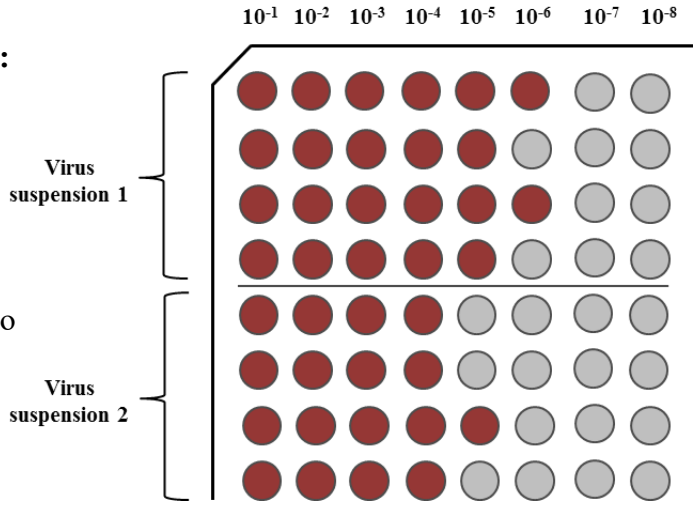
serum dilution	logarithm of serum dilution	infected wells/ total wells	R
1:5	-0.7	0/2	1.0
1:10	-1	0/2	1.0
1:20	-1.3	0/2	1.0
1:40 (= $L_{1.0}$)	-1.6	0/2	1.0
1:80	-1.9	1/2	0.5
1:160	-2.2	2/2	0.0
1:320	-2.5	2/2	0.0
1:640	-2.8	2/2	0.0
			S= 1.5

$\log ND_{50}$ = $-1.6 - 0.3 \times (1.5 - 0.5)$
= $-1.6 - 0.3$
= -1.9
ND_{50} = $10^{1.9} = 80$

Evaluation of results - Virus titration

Estimation of Virus titre ($TCID_{50}$):

$TCID_{50}$ = reciprocal of the initial virus dilution at which 50% of the wells showed virus replication.
The $TCID_{50}$ can be determined by either estimating a point between two dilutions or by calculating using the method of KAERBER.



Results ($TCID_{50}$) of the displayed example:

Virus suspension 1= 10^{6}/ 0.1 ml

Virus suspension 1= $10^{4.75}$/ 0.1 ml

Example of $TCID_{50}$-Calculation (method of Kaerber) for virus suspension 2:

$\log TCID_{50} = L_{1.0} - L_{int} \times (S - 0.5)$

$L_{1.0}$ = Logarithm of the highest virus dilution with the reaction rate (R) =1.0
L_{int} = Logarithm of the dilution interval (int)
S = Sum of reaction rates (starting with the last reaction rate that is 1.0)
0.5 = Constant factor

virus dilution	logarithm of virus dilution	infected wells/ total wells	R
10^{-1}	-1	4/4	1.0
10^{-2}	-2	4/4	1.0
10^{-3}	-3	4/4	1.0
10^{-4} (= $L_{1.0}$)	-4	4/4	1.0
10^{-5}	-5	1/4	0.25
10^{-6}	-6	0/4	0.0
10^{-7}	-7	0/4	0.0
10^{-8}	-8	0/4	0.0
			S= 1.25

$\log TCID_{50} = -4 - 1.0 \times (1.25 - 0.5)$
$= -4 - 0.75$
$= -4.75$
$TCID_{50} = 10^{4.75}$

4.2 Cell culture based serological differential diagnosis

Cross-neutralizing antibodies specific for ruminant pestiviruses (BVDV and BDV) can cause false positive results in CSF VNT. The chosen strain of ruminant pestivirus as well as the interval between infection and sampling time point determine the extent of cross-reactivity [68]. In case of a positive result in VNT with a CSF strain, VNTs against BVDV and BDV should also be carried out in order to facilitate the discrimination of antibodies directed against either CSFV or BVDV/ BDV. The respective test procedures correspond to the VNT procedure for CSF.

4.2.1 VNT against BVDV

Test procedure is the same as described for CSF. Appropriate cell lines are stated in Chapter 2.2, e.g. MDBK cells are suitable for BVDV diagnostic assays and are routinely used at the EURL. The BVDV strain NADL is a reference strain for BVDV and is suitable for the assay [69]. In case there is information available about circulating field strains of the geographic area where the samples had been collected it is strongly recommended to include these strains into the analyses.

4.2.2 Neutralization test against BDV

The test procedure is the same as described for CSF. The test can be carried out with fetal sheep thymoid cells (SFT-R; described in Chapter 2.2). Several BDV reference strains are in routine use, e.g. Moredun [70], Frijters and 137/4. In case there is information available about circulating field strains of the geographic area where the samples had been collected it is strongly recommended to include these strains into analyses.

4.3 CSF antibody ELISA

The antibody ELISA is one of the serological tests of choice for CSF diagnosis and it is recommended to be used as a screening test on a herd basis. This assay can be performed much more rapidly than a VNT.

Each laboratory is in charge of licensing the different batches of commercial CSF antibody ELISAs which are to be applied in the respective country. For quality control of commercial test kit batches it is recommended to use a panel of different sera with certain characteristics (sera taken before 21 days post infection, sera from convalescent pigs and sera from pigs infected with ruminant pestiviruses).

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