

**EU DIAGNOSTIC MANUAL FOR
CLASSICAL SWINE FEVER (CSF) DIAGNOSIS:
TECHNICAL PART
(SECOND DRAFT MARCH 2002)**

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1. Suitable cell lines

Porcine kidney cell line PK15

The PK 15 cell line is suitable for all test systems for CSF diagnosis e.g. virus isolation, virus replication, neutralisation test.

The cell line can be obtained from the CRL. It was established there by single cell cloning.

The cells are supplied free of mycoplasma, BVD virus, other pestiviruses and BVD antibodies.

Cell culture medium

- For the preparation of most culture dishes and replication of the stock cells routinely EMEM with 5% FCS is used.
- For virus isolation after inoculation with diagnostic material or virus multiplication of virus containing cell culture supernatant EMEM with **10% FCS** is the ideal medium.
- For cultivation in a 96 well culture dish EDulb-medium with 5% FCS is used.
- After contact with any kind of diagnostic sample which might be contaminated with bacteria antibiotics and antimycotics should be added (Penicillin/Streptomycin, Fungizone or others) to the medium.
- The fetal calf serum is carefully checked for BVD virus by virus isolation and for BVD antibodies by neutralisation test before use. It is not sufficient to rely on the manufactures certification only.

Traditional Cultivation of PK15 cells

For multiplication PK15 cells are kept in 75 cm² (250 ml) plastic culture flasks with 20 ml cell culture medium. Two passages weekly are ideal, but one passage weekly is sufficient. The routine splitting is 1 : 5.

- Remove medium and wash with PBS/Versene.
- Incubate 10 min at 37°C with PBS/V.
- Add 0,1 ml of Trypsin-solution 1%.
- Incubate 10-20 min at 37°C until cells are removed from the dish.
- Add medium with FCS to stop trypsin reaction.
- Centrifuge cell suspension and discard supernatant.
- Resuspend pellet in 10 ml medium or other appropriate volume.

Seeding of different culture dishes:

A confluent monolayer of a 75 cm² culture flask contains an average of 8,5 x 10⁶ cells.

If the cell pellet of one flask, obtained by trypsination is resuspended with 10 ml medium, the following amounts are seeded in the different cell culture vessels:

Dish	10 ml cell suspension	ml medium for vessel	Total cells / vessel (average)	Cells / ml (average)
75 cm ² flask	2ml	20-25	1.6 x 10 ⁶	8.5 x 10 ⁴
25 cm ² flask	0.5 ml	5-7	4.2 10 ⁵	8.5 x 10 ⁴
Culture tube	0.1 ml	1	8.5 x 10 ⁴	8.5 x 10 ⁴
24 well plate	2 ml / dish	24	1.6 x 10 ⁶	8.5 x 10 ⁴
96 well plate	2 ml / dish	5-7	1.6 x 10 ⁶	3 x 10 ⁵

- Regular screening for Mycoplasma should be performed. The frequency has to be fixed individually. In case of increased doubtful results or reduced cell growth, the cell culture should be examined for Mycoplasma.

Cultivation without centrifugation:

To avoid cell damage by centrifugation and omit time consuming steps this method is also useful.

- Remove medium from a 250 ml culture flask
- Wash monolayer with 5 ml of ATV solution for 30 seconds.
- Remove ATV and replace with 2 ml fresh ATV
- Incubate at 37°C for 15 min until cells are detached
- Fill up with 8 ml of culture medium containing 5% FCS to get a total volume of 10 ml.

Cells are seeded in the different dishes as described above.

Sheep fetal thymoid (SFT-R) cell line:

For isolation and cultivation of BDV and 'non-CSF pestiviruses', a sheep fetal thymoid cell line (SFT-R/CCLV Rie043) is available. The main use is the differential neutralisation test against BD virus. The cell line is available at the cell collection of The Federal Research institute for Virus Diseases of animals, Island of Riems, Germany.

Cell culture medium

- For the preparation of all culture dishes and replication of the stock cells routinely EDulb-medium with 5% FCS is used.
- After contact with any kind of diagnostic sample which might be contaminated with bacteria antibiotics and antimycotics should be added (Penicillin/Streptomycin, Fungizone or others) is added to the medium.
- The fetal calf serum is carefully checked for BVD virus and BVD antibodies before use. It is not sufficient to rely on the manufactures certification only.

Cultivation of SFT-R cells

For multiplication SFT-R cells are kept in 250 ml plastic culture flasks with 20 ml cell culture medium. One passage weekly is sufficient, two passages weekly are possible if necessary. The routine splitting is 1 : 3.

- Remove medium from a 250 ml culture flask
- Wash monolayer with 5 ml of ATV solution for 30 seconds.
- Remove ATV and replace with 2 ml fresh ATV
- Incubate at 37°C for 15 min until cells are detached
- Fill up with 8 ml of culture medium containing 5% FCS to get a total volume of 9 ml.
- Routine splitting 1 : 3
- Seed cell with a density of 1.5×10^5 / ml in 96 well dish
- Seed cells with a density of 9×10^4 / ml in cell culture tubes or 24 well dish
- Regular screening for Mycoplasma should be performed. The frequency has to be fixed individually. In case of increased doubtful results or reduced cell growth, the cell culture should be examined for Mycoplasma.

Other cell lines:

Several other porcine cell lines are in use for CSF diagnosis. Their suitability for CSF diagnosis is under investigation. A Swine Testis Endothelial (STE) cell line does grow with horse serum and is therefore less vulnerable to BVD contamination by fetal calf serum.

For BVDV cultivation several susceptible bovine cell lines are suitable e.g. MDBK, bovine turbinate cells. Primary cells (porcine or bovine) in general have the disadvantage of being less homogenous within one batch and between batches of different laboratories. Nevertheless the sensitivity of these cell might be higher than of permanent cells.

Reagents:

PBSV:

NaCl	8,0 g/l
KCl	0,2 g/l
Na ₂ HPO ₄ x 12 H ₂ O	2,37 g/l
KH ₂ PO ₄	0,2 g/l
Versene	0,2 g/l

ATV:

NaCl	8,0 g/l
KCl	0,4 g/l
Dextrose	1,0 g/l
Na HCO ₃	0,58 g/l
Versene	0,2 g/l
Trypsin	0,5 g/l

Antibiotics if considered necessary

CSF VIRUS ISOLATION

Principle of test

Organ preparations or leucocytes are incubated on CSFV susceptible cells to allow the attachment and replication of the virus. Since growth of the virus does not cause a cytopathic effect, its presence must be demonstrated by an immunostaining method. Cells are fixed and the viral antigen is detected with a peroxidase or fluorescein labelled CSFV-specific antibody. Immunostaining may be carried out after one or two virus passages.

Cell culture system for CSFV isolation

Cell cultures of PK15 cells which are 24 hours old and are 50 - 80% confluent should be used for inoculation with test samples.

Organ preparations

Suitable organ samples are described in the Diagnostic Manual Chapter V/A and B e.g tonsil, spleen, kidney, lymphatic tissue or ileum.

Samples should be kept chilled and processed as soon as possible.

1. Organ samples of 1 cm³ are homogenized in a mortar in 9 ml cell culture medium containing antibiotic solution to produce a 10% organ preparation. Smaller samples sizes can be homogenized with less medium in order not to dilute the virus too much. Sterilised sand can be added to facilitate homogenisation. Also homogenising machines (e.g. Ribolyser) can be used. The advantage is that the tissue is homogenised in a closed system, but high speeds will heat the sample thus may affecting the virus.
2. The preparation is left at room temperature for one hour.
3. Centrifugation for 15 min at 2500 g.
4. The supernatant is used for inoculation of cell cultures. A 1:10 and 1:100 dilution can be processed in parallel, in case of a cytotoxic effect. Sterilfiltration can be performed, if considered necessary.

Pooled organ samples for screening:

Tonsils of five individual pigs can be pooled and homogenised together in 20 ml culture medium and processed as described above. In case of a positive result, the samples have to be retested individually.

Leukocyte preparation

Samples of EDTA anticoagulated blood:

1. 0,5 ml of Dextran solution is added to 10 ml EDTA blood and left at room temperature for one hour. If the sample is smaller also 0,5 ml Dextran should be used.
2. The supernatant containing the leukocytes is collected and centrifuged at 500 g.
3. The pellet is resuspended in 5 ml PBSM, centrifuged and washed again in the same way.
4. Finally the pellet is resuspended in 2 ml PBSM for further use or storage.
5. 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.

Other commercial preparations may be used according to the manufacturers instruction e.g. Ficoll-Paque.

Samples of Heparin anticoagulated blood:

1. 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).
2. The solution is centrifuged at 1000 g.
3. The pellet is resuspended in 5 ml PBSM, centrifuged and washed again in the same way.
4. Finally the pellet is resuspended in 2 ml PBSM for further use or storage.
5. 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.

Plasma samples:

10 ml of EDTA or heparin anticoagulated blood are subjected to a freeze and thaw cycle in order to destroy the leukocytes. After thawing the plasma is inoculated directly on the cell culture as described below.

Test procedure**A. Screening**

1. 200-300 μl of the organ or leukocyte preparation is inoculated on a 50-80% confluent cell culture in multi dish plates or Leighton tube with cover slips, enough to cover the monolayer. Duplicate cultures of each sample should always be prepared.
2. The cell cultures are incubated at 37°C for 1 - 2 hours.

3. The cell cultures are washed once with PBSM and overlaid with fresh medium.
Alternatively the cell culture dish can be filled up directly, if and a cytotoxic effect is unlikely.
4. The cell cultures are incubated up to 72 hours at 37°C in a CO₂ incubator.
5. Simultaneous inoculation of cells and sample is possible, if the sample is fresh and a cytotoxic effect is unlikely.
6. Positive and negative controls must also be processed in the same way.
7. Cells are fixed and stained as described in Chapter 'Immun Labelling'.
A portion of the cultures might be fixed and stained earlier.

B. Virus isolation over two passages

1. 200-300 µl of the organ or leukocyte preparation is inoculated on a cell culture tube.
Always duplicate cultures of each sample should be prepared.
2. The cell cultures are incubated at 37°C for 1 - 2 hours.
3. The cells are washed twice with PBSM and are further cultivated for at least 72 hours at 37°C. EMEM with 10 % FCS is the ideal medium for virus growth.
8. Simultaneous inoculation of cells and sample is possible, if the sample is fresh and a cytotoxic effect is unlikely.
4. The cell culture tubes are frozen at -80°C for at least one hour (freeze and thaw cycle).
5. The culture tube is thawed and centrifuged at 2500 g.
6. 200-300 µl of the supernatant is incubated for 1-2 hours on a well of a multi dish plate or Leighton tubes as described above.
7. The well is filled up with culture medium and incubated 72 hours.
8. Positive and negative controls must also be processed in the same way.
9. Cells are fixed and stained as described in Chapter Immun Labelling.

In case a slow growing isolate is suspected, a second passage in a culture tube can be done, leading to a third passage in a culture dish.

Material:

Dextran solution 5%:

Dextranulphat-Na, MW 500.000	5g
1,5 % EDTA-Solution	ad 100 ml
dissolve at 45°C and autoclave before use	

PBSM (Phosphate buffer without Calcium and Magnesium including Dextrose and Phenolred,

pH 7,3

NaCl 8,0 g

KCl 0,2 g

Na₂HPO₄ 2,37 g

Dextrose 1,0 g

Phenolred 0,016 g

Aqua bidest. ad 1000 ml

Antibiotic solutions:

Penicilline/Streptomycin

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

This solution is used 1 ml in 1 liter medium for routine use or 10 ml in 1 liter medium for incubation of organ samples.

Glutamine based antibiotic cocktail:

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.
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Add to the above filtrate and store at -20°C in 10 ml aliquotes until use.

Application: 10 ml in 1 liter medium

Immune Labelling for the detection of CSF virus in cell cultures

In general CSF does not induce a cytopathic effect. Consequently the CSF infected cells have to be immunologically labelled stained in order to detect the virus antigen in the cytoplasm 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.

Cell Fixation

The choice of fixative will depend on whether the cultures are grown on glass or plastic surfaces.

1. For cultures grown on glass surfaces, fixation in 100% acetone for 5 min is appropriate.
2. In case plastic surfaces are used different fixation methods are available:
 - Remove culture medium very thoroughly and incubate the plates at 70-80 °C for 2-3 hours or
 - add 100µl/well of acetone/methanol (1:1) solution and leave for 10 minutes at room temperature or
 - fix in 20% acetone for 10 minutes. Drain plates thoroughly then dry under a bench lamp for 4 hours at 25-30°C.

Fixed plates may be stored before staining for several days in dry environment and longer periods at – 20°C in a sealed bag.

Other fixation procedures bare the risk of interfering with the penetration of the monoclonal antibody.

Immune labelling with peroxidase staining

Direct labelling

1. Rinse the plates once in PBS-Tween.
 2. Add to each well working dilution of a pestivirus conjugate in PBS Tween. In a 24 well dish 200 µl/well and in a 96 well dish 50 µl/well of conjugate are suitable amounts.
 3. Incubate for 1 hour at 37 °C in a moist chamber.
 4. Wash the plates 3 times with PBS-Tween and one time with Aqua dest.
 5. Add to each well 200 or 50µl of chromogen-substrate solution and stain for 15-30 minutes at room temperature.
1. Discard the chromogen-substrate solution and wash with 1/3 PBS/H₂O.

2. Fill the wells with Aqua dest. and read the test by low-power microscopy. The cytoplasm of infected cells is stained dark red.

Indirect labelling:

3. Add to each well working dilution of a pestivirus specific antiserum or suitable monoclonal hybridoma supernatant and incubate for 1 hour at 37 °C.
4. In a 24 well dish 200 µl and in a 96 well dish 50 µl of conjugate are suitable amounts.
5. Wash the plates 3 times with PBS-Tween and once with Aqua dest.
6. Add to each well working dilution of an commercial antispecies peroxidase conjugate in PBS Tween and incubate for 1 hour at 37 °C.
7. Wash the plates 3 times with PBS-Tween and one time with Aqua dest.
8. Perform incubation with second antibody, if required by manufacturer.
9. Add to each well 200 or 50µl of chromogen-substrate solution and stain for 15-30 minutes at room temperature.
10. Discard the chromogen-substrate solution and wash with 1/3 PBS/H₂O.
11. Fill the wells with Aqua dest. and read the test by low-power microscopy. The cytoplasm of infected cells is stained dark red.

Incubation times may vary according to differences between conjugates.

Immune labelling with FITC staining

1. Rinse the cover slips, plates or chamber slides once with washing buffer.
2. If necessary, the working dilution in washing buffer of the FITC conjugated antibody can be filtered in a millipore filter to remove FITC crystals.
3. Overlay the fixed cells with FITC conjugate in a suitable dilution (in washing buffer) and incubate for 30-60 min at 37 °C in a moist chamber. The cells should be completely covered with conjugate. Some conjugates are not compatible with CO₂ incubators.
4. Rinse in washing buffer 3 times for 5 minutes.
5. Rinse once in distilled water.
6. Place a drop of mounting buffer onto the cells. If necessary use a cover slip to cover the cells
7. Investigate for cytoplasm fluorescence by UV microscopy.

In case labelling is done indirectly one incubation step with a FITC labelled antispecies antibody has to be included.

Material:

To avoid non-specific binding 0.1% CSF antibody negative pig serum can be added to conjugate dilutions.

For peroxidase staining:

PBS-Tween:

1 litre PBS containing ~0,01% Tween20 2-3 drops)

1/3 PBS:

PBS : H₂O = 1 : 3

Antispecies antibodies:

commercially available

Chromogen-substrate solution:

3-Amino-9-Ethylcarbazol (AEC)	20	mg
Dimethylformamide	3	ml
Sodium Acetate-Buffer	ad 50	ml
H ₂ O ₂ (3 %)	0,4	ml

Sodium Acetate-Buffer (pH 5,0; 0,05 M):

Sodium Acetate-Trihydrate	6,804	g
Aqua bidest.	1000	ml

For FITC staining:

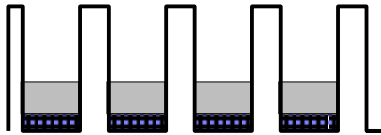
washing buffer: Phosphate buffered saline (pH 7.4 - 7.6):
(Physiological saline buffered with 0.01M phosphate)
NaCl 8.78 g/l
Na₂HPO₄ x 12H₂O 3.58 g/l
(pH adjusted with 1M KH₂HPO₄)

mounting buffer

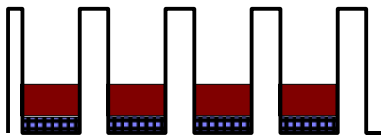
washing buffer with 20% glycerine (buffered glycerine)

Or commercial NON-FADING mountant.

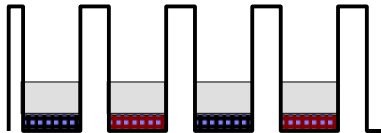
Immune labelling using peroxidase



Rinse wells once with 1/3
50 or 200 μl /well of conjugate and
incubate for 1h at 37 °C



Wash plate 3 x with PBS-Tween
and 1 x with distilled H₂O,
add 50 or 200 μl /well chromogen-
incubate 15-30 minutes at RT



Discard chromogen-substrate,
wash and add distilled H₂O,
read by low power microscopy

Differential Diagnosis of virus isolates

In the case where a pestivirus is isolated during virus isolation, it has to be characterised by using monoclonal antibodies specific for CSF, BD and BVD. CSF specificity is crucial.

List of available antibodies useful for characterisation of pestivirus isolates:

	Specificity			
	Availability and Name			
Supplier	Pestivirus	CSF	BVD	BD
CRL, Hannover	Yes, mAB C16	Yes, mAB HC34	Yes, mAB CA3, CA34	No
Bommeli, CH	Yes, PO-conj.	Yes	Yes	No
ID-Lelystad, NL	Yes, PO-conj.	Yes	No	No
VLA, UK	Yes, W54 Pool	Yes, WH303	Yes, WB	Yes, WS

The CRL can supply the monoclonal Antibodies (mAB) in small quantities in form of hybridoma supernatant.

Commercial availabilities are frequently changing. Please observe the market. This list is not a recommendation of certain products.

References:

Hess, R. G., Coulibaly, C. O., Greiser-Wilke, I., Moennig, V., and Liess, B., 1988. Identification of hog cholera viral isolates by use of monoclonal antibodies to pestiviruses. *Vet.Microbiol.* 16, 4. 315-321.

Moennig, V., Bolin, S. R., Coulibaly, C. O., Gourley, N. E., Liess, B., Mateo, A., Peters, W., and Greiser-Wilke, I., 1987. [Studies of the antigen structure of pestiviruses using monoclonal antibodies] Untersuchungen zur Antigenstruktur von Pestiviren mit Hilfe monoklonaler Antikörper. *Dtsch.Tierarztl.Wochenschr.* 94, 10. 572-576.

Paton, D. J., Sands, J. J., Lowings, J. P., Smith, J. E., Ibata, G., and Edwards, S., 1995. A proposed division of the pestivirus genus using monoclonal antibodies, supported by cross-neutralisation assays and genetic sequencing. *Vet.Res.* 26, 2. 92-109.

Peters, W., Greiser-Wilke, I., Moennig, V., and Liess, B., 1986. Preliminary serological characterization of bovine viral diarrhoea virus strains using monoclonal antibodies. *Vet.Microbiol.* 12, 3. 195-200.

Zhou, Y., Moennig, V., Coulibaly, C. O., Dahle, J., and Liess, B., 1989. Differentiation of hog cholera and bovine virus diarrhoea viruses in pigs using monoclonal antibodies. *Zentralbl.Veterinarmed.B* 36, 1. 76-80.

Fluorescent antibody test on tissue sections

Principle of test

Classical swine fever virus (CSFV) antigen is demonstrated in thin cryostat sections of organ material. The cryostat sections are mounted on a microscope slide and stained directly with anti-CSF immunoglobulin conjugated to fluorescein isothiocyanate (FITC-conjugate). The sections are examined for fluorescence by UV microscopy.

Suitable organ samples are described in the Diagnostic Manual Chapter V/A and B e.g. tonsil, spleen, kidney or lymphnodes.

Test procedure of direct FAT

1. Cut out a piece of tissue of app. 1 x 1 x 0,5 cm and mount it with a cryo-embedding compound or distilled water on a cryostat mounting block (chuck). Tonsils have to be embedded to allow a cross-section.
2. Freeze the piece of organ onto the cryostat chuck. The freezing temperature of the cryostat should be $-15-20^{\circ}\text{C}$. Ideal is shock-freezing of the tissue in n-Heptan cooled with liquid N_2 .
3. Cut sections of maximum 5 μm thickness and mount them onto microscope slides which have been previously cleaned with alcohol. Prepare several slides with sections from the same tissue.
4. Dry the mounted sections at room temperature for 20 minutes.
5. Fix the mounted sections for 10 minutes in acetone (analytic grade) at -20°C .
6. Immerse the section briefly in washing buffer, remove excess fluid with tissue paper and place them on a frame in a humid incubation chamber.
7. Remove a fixed control positive section from the deep freeze (-70°C) and process in parallel.
8. Dispense the FITC-conjugate at the suitable working dilution in washing buffer onto the entire section, close the moist chamber and incubate in the dark for 30 minutes at 37°C .
9. Check, that the conjugate solutions have not evaporated and dried the tissue.
10. Wash the sections 3 x 10 minutes at room temperature with washing buffer;
11. Immerse the section briefly in distilled water.
12. If necessary, counterstain in Evans blue for 30 seconds.
13. Carefully remove excess fluid with tissue paper and place a cover slip with mounting buffer onto the section .

14. Remove excess mounting fluid with tissue paper and examine the sections for fluorescence by UV microscopy.

Controls

Negative and positive control sections must be included in each series of organ samples to be examined. The control sections can be prepared in advance and stored after acetone fixation for 2-3 years at -70 °C.

Interpretation

Any sample showing specific cytoplasmic reaction (brilliant green fluorescence) shall be considered positive for pestivirus.

A negative FAT result does not necessarily rule out CSF in all cases. In some cases during the terminal stage of the disease a positive reaction can be masked by neutralizing antibodies which are already induced by the organism. When suspicion of CSF continues further samples should be examined. In subacute and chronic cases, the ileum is frequently positive and occasionally may be the only tissue to display fluorescence.

Pigs vaccinated with modified live virus strains may yield a positive FAT result for two weeks after vaccination. Strains of modified live virus vaccine multiply mainly in the regional lymph nodes and in the crypt epithelium of the tonsils. Depending on the type of vaccine it may be possible to carry out differential staining with vaccine-specific monoclonal antibodies.

Non-specific staining does also occur, which could lead to false positive results. The experience of the reader is crucial for the interpretation of test results.

Pigs infected with bovine viral diarrhoea (BVD) or border disease (BD) viruses can give false positive results when a FITC-conjugate prepared from a polyclonal antibody specific for pestiviruses has been used. For this reason it is recommended that, especially in CSFV-free areas, duplicate samples should be examined from FAT positive cases using monoclonal antibodies which can distinguish CSF virus from BVD or BD viruses. Alternatively, confirmatory diagnosis should await virus isolation in cell culture with subsequent typing by monoclonal antibodies.

FITC-conjugate

The quality of the FITC-conjugate determines the quality of the reaction. It is recommended that primary diagnosis is carried out with FITC-conjugates prepared from a polyclonal

antibody to CSF. This will not distinguish between the antigens of different pestiviruses, but does provide assurance that minor variant viruses will not be missed. FITC-conjugates should be prepared from hyperimmune serum prepared in specific pathogen free pigs. The serum should be free from any antibody which could affect the specificity or quality of the specific CSF reaction. In case of a positive result, virus isolation should be carried out or staining should be repeated with a CSF specific antibody.

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.

List of available FITC conjugates:

	Polyclonal pestivirus FITC conjugate
CRL, Hannover	FITC-conj.
ID-Lelystad	FITC-conj.
Mevak	FITC-conj.
Bio X Diagnostics	FITC-conj. mAb

The CRL can supply the FITC conjugate in small quantities.

Commercial availabilities are frequently changing. Please observe the market. This list is not a recommendation of certain products.

References:

Hyera, J. M., Dahle, J., Liess, B., Moennig, V., and Frey, H. R., 1987. [Production of high titrating antisera against BVD virus from swine and its use for direct immunofluorescence and immunoperoxidase technics]. Dtsch.Tierarztl.Wochenschr. 94, 10. 576-580.

Material

washing buffer: Phosphate buffered saline (pH 7.4 - 7.6):
(Physiological saline buffered with 0.01M phosphate)

NaCl	8.78 g/l
Na ₂ HPO ₄ x 12H ₂ O	3.58 g/l

(pH adjusted with 1M KH₂HPO₄)

Evans Blue

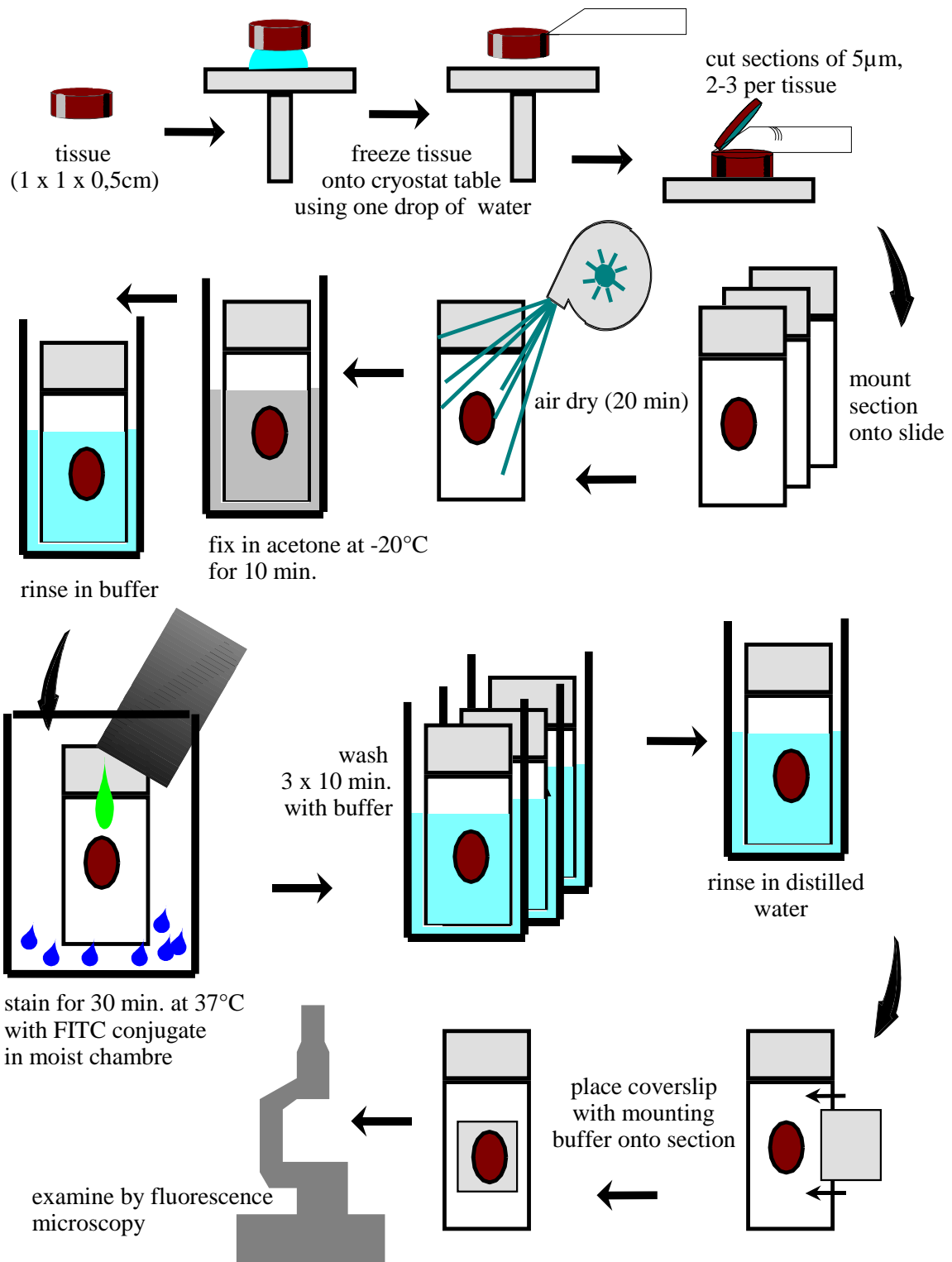
- Stock solution 1:100 in distilled H₂O
- For use dilute 1:2000 in PBS

mounting buffer

washing buffer with 20% glycerine (buffered glycerine)

Or we recommend commercial NON-FADING mountant.

Demonstration of viral antigen in cryostat sections
(Direct Fluorescent Antibody Test - FAT)



CSF antigen ELISA

Each NSFL is in charge for the licensing of different batches of commercial CSF antigen ELISAs. The CRL does not recommend a certain antigen ELISA. At present, CSF antigen ELISAs can be obtained from:

Bommeli, CH

Synbiotics, F

ID-Lelystad, NL

IDEXX, USA and S

Diagnostic RT-PCR

Procedures are described for the detection of CSFV genome in anticoagulated whole blood, serum, tissues or cell cultures.

Many methods can be used for extraction of RNA from clinical and laboratory samples. Two widely used commercially available systems that are fairly simple to use and give satisfactory results are:

- (1) the Total RNA Isolation Reagent (TRIZOL, Gibco Life Technologies)
- (2) the commercial viral RNA Purification Kits (e.g. QIAGEN). However, new kits are being offered frequently. This is not a recommendation of certain commercial products. The market has to be observed by each laboratory.

It has to be pointed out that the protocols described below have to be adapted in each laboratory and validated there individually. Therefore temperatures and incubation times may vary slightly between laboratories.

(1) Use of the Total RNA Isolation Reagent (TRIZOL)

Reagents required:

TRIZOL Reagent (a mono-phasic solution of phenol and guanidine isothiocyanate)
Chloroform (without any additives such as isoamyl alcohol)
Isopropyl alcohol
RNase-free water
Glykogen
Ethanol
yeast-RNA

Preparation of samples

Tissues:

- Homogenise 50-100 mg of tissue in 1 ml of TRIZOL Reagent. The sample volume should not exceed 10% of the volume of TRIZOL Reagent used for homogenisation.

Cells grown in a monolayer:

- Lyse cells directly in a culture dish by adding 1 ml of TRIZOL Reagent to a 3.5 cm diameter dish, and passing the cell lysate several times through a pipette.

Whole anti-coagulated blood or serum:

- Add 200 µl of EDTA blood or serum to 800 µl of TRIZOL Reagent and pipette up and down several times.
- The TRIZOL method does not work with heparinised blood.

Continuation for all kinds of samples:

- Incubate the samples in TRIZOL for 5 minutes at room temperature or 37°C to allow the complete dissociation of nucleoprotein complexes.

- Add 0.2 ml of chloroform per 1 ml of TRIZOL Reagent.
- Shake tubes vigorously by hand for 15 seconds and incubate for a further 3 minutes at room temperature.
- Centrifuge the samples at 12,000 x g for 15 minutes at 2 to 8 °C.
- Transfer the upper, aqueous phase to a fresh tube.
- Precipitate the RNA by mixing with isopropyl alcohol, using 0.5 ml of isopropyl alcohol and 5µl glykogen per 1 ml of TRIZOL Reagent used in the original homogenisation.
- Incubate samples at room temperature for 10 minutes and then centrifuge at 12,000 x g for 10 minutes at 2 to 8 °C.
- The RNA precipitate forms a gel-like pellet on the side and bottom of the tube.
- Remove the supernatant.
- Wash the RNA pellet once with 75% ethanol (in RNase-free water), adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenisation.
- Mix the samples by gently mixing and centrifuge at 7,500 x g for 5 minutes at 2 to 8°C.
- Repeat washing
- At the end of the procedure, briefly dry the RNA pellet (air-dry or vacuum-dry for 5 to 10 minutes).
- Redissolve the RNA in 30 µl RNase-free water on ice (by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55 to 60°C).

(2) Commercial viral RNA Purification kits.

Principle and Procedure

The commercial kits are based on the binding of RNA to a Silica-gel-membrane. A specialised high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the membrane. Biological samples are first lysed and homogenised in the presence of a highly denaturing guanidine isothiocyanate-containing buffer which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is added to a spin column where the total RNA binds to the membrane and contaminants are efficiently washed away.

Different kits are used for different kinds of samples. Important is that the commercial kits have to be validated for each kind of sample, because sample components may interfere with the RNA isolation (Greiser-Wilke and Scheibner, 2000). As an example, at present suitable kits are:

Cells grown in a monolayer

RNeasy Mini Kit (Quiagen)

EDTA and Heparin whole Blood

QIAamp RNA blood mini kit (Quiagen)

Serum	High Pure viral RNA (Roche)
Cell culture supernatant	QIAamp viral RNA kit (Qiagen)

Other available kits may work as well. The market is changing frequently and individual validation is necessary in each laboratory.

In some kits which claim to be for EDTA and heparinised whole blood, the first step is the preparation and washing of the leukocytes. In this case it does of course not matter which anticoagulant is used.

- The instructions of the manufacturers have to be followed.
- Extracted RNA should be held on ice until used in RT-PCR or stored at -70°C.

Reverse transcription - polymerase chain reaction (RT-PCR)

It is recommended to perform the diagnostic PCR on two regions of the CSFV genome, 5'NCR and E2. The CSF-specificity of the methods listed below is not really clear to date. Therefore either subsequent genetic typing has to be performed or CSF specific probes (e.g Taqman(McGoldrick et al., 1998)) have to be included. However, even a pestivirus-specific PCR as described below can be helpful in performing a quick diagnosis to rule out CSF.

(1) The E2 gene primers (Paton et al., 2000) are rather CSF specific.

Primers used for amplification of a 652 nt fragment of E2 of CSFV:

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)

(2) The 5'non-coding region (5'NCR) using pan-pestivirus primers (Vilcek et al., 1996)

Primers used for amplification of a 284 nt fragment of 5'NCR of CSFV:

324 Forward 5' ATG CCC T/ATA GTA GGA CTA GCA 3' (100-120)

326 Reverse 5' TCA ACT CCA TGT GCC ATG TAC 3' (383-363)

The primers used for genetic typing in the 5'NCR region (Greiser-Wilke et al., 1998) may also be used for diagnostic PCR. This has the advantage that sequencing can be performed directly. In this case, one primer has to be biotinylated. Figures in parenthesis correspond to nucleotide positions in Alfort-187 (Ruggli et al., 1996).

The following steps are identical for the E2 and the 5'NCR tests except for the differences in the primers used.

Step I

Prepare a 50 µl RT-PCR reaction mix in the bottom of the eppendorf tube. The reagents (Promega) consist of:

- 5 µl 10 x buffer,
- 12 µl MgCl₂ (25 mM),
- 2 µl dNTPs (10 mM),
- 0.25 µl of each primer (20 pmolµl⁻¹),
- 1 µl Triton X-100 (10% stock),
- 0.5 µl Taq polymerase (2.5 U),
- 0.25 µl RNasin (10 U),
- 0.5 µl MMLV reverse transcriptase (100 U).
- Finally add 2 µl of RNA and make up to 50 µl with water.
- Add a mineral oil overlay and then close the tubes for thermocycling.

Step II

Place the tubes in a thermocycling device and subject to the following cycle parameters:

Reverse transcription	42°C for 30 minutes, 95°C for 3 minutes
PCR	20 cycles of 94°C - 1 min, 60°C - 1 min, 72°C - 1 min
A single extension step	72°C for 1 min (5'NCR) or 10 min (E2)

Step III

Detection of PCR products by agarose gel electrophoresis using 2% agarose containing (0.2 µg/ml) and visual inspection for a band of the correct size under UV transillumination.

Alternatively the gel can be stained with ethidium bromide after electrophoresis.

The results can be recorded by taking a polaroid picture of the gel under UV-light.

Quality control

RT-PCR is only diagnostically reliable when carried out in a suitably equipped and organised laboratory operated by properly trained and supervised staff. False positive results due to contamination can be fatal. It is essential to achieve complete separation of so-called 'clean' and 'dirty' procedures.

- Preparation of reagents and master mixes must be carried out in a facility that has no direct or indirect contact with any materials potentially contaminated with pestivirus RNA or DNA.
- A separate facility is needed to prepare samples and extract RNA. This must be set up so as to avoid both extraneous contamination and cross-contamination between test samples.
- An entirely separate facility is needed for analysis of PCR products.
- Strict codes must be enforced to ensure that personnel does not accidentally carry-over materials from one facility to another.
- Inclusion of negative and positive controls in all tests. As guideline approx. 6 samples plus controls can be processed for RNA isolation by one person per day. The following sample controls are required:

Negative controls

- Ideally there should be one negative control for every other sample tested.
- The negative controls should be the same type of sample as that being tested i.e. blood, serum, tissues or culture supernatants.
- The controls are processed in exactly the same way as the real samples and are interspersed evenly throughout the samples.
- As a minimum, one negative control for each batch of samples tested together and one water control for each sample included in the pipetting of the PCR.

Positive controls

- There should be one positive control for every batch of samples tested together.
- The positive controls should be the same type of sample as that being tested (if available) i.e. blood, serum, tissues or culture supernatants.
- The control is processed in exactly the same way as the real samples.
- Ideally, the positive control virus should be of a distinctive type such that the amplicon derived from it can readily be distinguished by sequencing from that likely to be found in a sample.

Interpretation of results

Any test that gives rise to incorrect results for negative or positive controls should be considered invalid. Wherever possible, positive results should be confirmed by repeating the RT-PCR test and by an alternative assay such as virus isolation or serology, where necessary obtaining additional sample materials.

In case of a positive PCR result, the identity of the PCR products should be confirmed by sequencing, see appropriate chapter.

References:

Greiser-Wilke, I. and Scheibner, H., 2000. Template preparation for RT-PCR. SANCO/2589/2000, EU Commission, Brussels, Belgium. 26-28.

Greiser-Wilke, I., Zimmermann, B., Fritzemeier, J., Floegel, G., and Moennig, V., 2000. Structure and presentation of a world wide web database of CSF virus isolates held at the EU reference laboratory. *Vet.Microbiol.* 73, 2-3. 131-136.

Greiser-Wilke, I., Depner, K., Fritzemeier, J., Haas, L., and Moennig, V., 1998. Application of a computer program for genetic typing of classical swine fever virus isolates from Germany. *J.Virol.Methods* 75, 2. 141-150.

McGoldrick, A., Lowings, J. P., Ibata, G., Sands, J. J., Belak, S., and Paton, D. J., 1998. A novel approach to the detection of classical swine fever virus by RT-PCR with a fluorogenic probe (TaqMan). *J. Virol. Methods* 72, 2. 125-135.

Paton, D. J., McGoldrick, A., Greiser-Wilke, I., Parchariyanon, S., Song, J., Liou, P. P., Stadejek, T., Lowings, J. P., Bjorklund, H., and Belak, S., 2000. Genetic typing of classical swine fever virus. *Vet. Microbiol.* 73, 2-3. 137-157.

Ruggli N, Tratschin JD, Mittelholzer C & Hofmann MA (1996) Nucleotide sequence of classical swine fever virus strain Alfort/187 and transcription of infectious RNA from stably cloned full-length cDNA. *J Virol* 70, 3478-3487.

Vilcek, S. and Paton, D. J., 1998. Application of genetic methods to study the relationship between classical swine fever outbreaks. *Res. Vet. Sci.* 65, 1. 89-90.

Vilcek, S., Stadejek, T., Takacsova, I, Strojny, L., and Mojzis, M., 1997. Genetic analysis of classical swine fever virus isolates from a small geographic area. *Dtsch. Tierarztl. Wochenschr.* 104, 1. 9-12.

Vilcek, S., Stadejek, T., Ballagi, Pordany, Lowings, J. P., Paton, D. J., and Belak, S., 1996. Genetic variability of classical swine fever virus. *Virus Res.* 43, 2. 137-147.

Wonnemann, H., Floegel-Niesmann, G., Moennig, V., and Greiser-Wilke, I., 2001. Genetic typing of classical swine fever isolates from Germany. *Dtsch. Tierarztl. Wochenschr.* 108, 6. 252-256.

3. Laboratory test for the detection of CSF antibodies

The neutralization test (NT)

Principle of test

The test is based on the determination of the neutralizing 50% endpoint. Therefore a constant amount of CSF virus of 100 (plus/minus 0.5 log₁₀) virus infectious doses (TCID₅₀) is incubated with diluted serum for one hour at 37 °C. For screening purposes, the sera are initially diluted 1/5. When a full titration is necessary two-fold dilutions of serum starting at 1/2 or 1/5 are prepared. At least two wells of PK-15 cell cultures in a 96-well microplate are inoculated with mixtures of virus and diluted serum at each dilution. The plates are incubated at 37 °C for 2 to 3 days. After this incubation period the cell cultures are fixed and the viral antigen is detected by an immune labelling system. Either the neutralization peroxidase-linked antibody (NPLA) or the neutralization-immunofluorescence (NIF) assays may be used. The results are expressed as the reciprocal of the initial serum dilution at which half the inoculated cell cultures fail to show any specific labeling (no viral replication detectable in the cell culture). A point between two dilution levels is estimated.

The peroxidase system has the advantage that the results can be read with a light microscope or even with the naked eye. For the NIF test a fluorescence microscope is needed.

CSF virus strain for the NT

a) Stock virus

The reference strain of virus for use in the European Union is Alfort/187 (CSF 902). The reference virus can be supplied by the Community Reference Laboratory for CSF, upon request. It can be useful to include additionally a CSF field virus isolates which occurs in the country.

Batches of stock virus can be produced by inoculating one day old PK-15 cell cultures in tissue culture flasks with the reference virus. The cultures are incubated for 3 to 4 days at 37 °C and thereafter frozen at -80 °C. After thawing, the culture fluids are clarified by centrifugation for 60 minutes at 3000g. The supernatants are dispensed in small (0.5-1 ml) amounts in ampoules which are labeled, packed and stored at -80 °C until use in the NT. Their titre is determined by 10 fold titration as described below.

b) Calculation of infectivity titres

The highest dilution of virus which infected 50% of the cell cultures is regarded as the endpoint of infectivity. This value is estimated or calculated using the method of KÄRBER

(1931). Virus infectivity titres are expressed as tissue culture infectious doses (TCID₅₀) per volume (e.g. 0.1 ml) of virus suspension.

Example of TCID₅₀-Calculation (Method of Kärber)

$$\log \text{TCID}_{50} = L_{1,0} - L_{\text{int}} (S - 0,5)$$

$L_{1,0}$ = 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)

0,5 = Constant factor

virus dilutions	infected wells / total wells	R
10 ⁰	4/4	1,0
10 ⁻¹	4/4	1,0
10 ⁻²	4/4	1,0
10 ⁻³ (= L _{1,0})	4/4	1,0
10 ⁻⁴	1/4	0,25
10 ⁻⁵	0/4	0,0
		S = 1,25

$$\begin{aligned} \log \text{TCID}_{50} &= -3 - 1,0 (1,25 - 0,5) \\ &= -3 - 0,75 \\ &= -3,75 \end{aligned}$$

The virus titer is 10^{3,75} TCID₅₀ /0,1 ml in the original virus suspension or 10^{4,75} TCID₅₀ /ml.

c) Backtitration

A back-titration must be mounted on every occasion that a NT is carried out. It is carried out using the actual virus added to the NT plate, and covers a range of 4 log dilutions (ie 10⁻¹ to 10⁻⁴). The back titration thus acts as one of the internal quality controls. If the back titration is outside the tolerance limits (30-300 TCID₅₀ per well) then the test is invalid and must be repeated.

Calculation of dilution factor for obtaining 10² TCID₅₀/50µl:

As an example consider the stock virus has a titre of 10^{5.7} TCID₅₀/100µl. The virus dilution employed in the VNT should have a titre of 100 (10²) TCID₅₀/50µl.

$10^{5.7}$ TCID₅₀/100µl correspond to $10^{5.4}$ TCID₅₀/50µl

calculation:

$10^{5.7}$ TCID₅₀/100µl is to be divided by 2 (2 is equivalent to $10^{0.3}$) - that means:
 $10^{5.7}$ divided by $10^{0.3}$ - that means: $5.7 - 0.3 = 5.4$ ($10^{5.4}$)

$10^{5.4}$ TCID₅₀/50µl are to be diluted to obtain 10^2 TCID₅₀/50µl

calculation of dilution factor:

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

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

(From 1ml stock virus containing $10^{5.7}$ TCID₅₀/100µl (or $10^{5.4}$ TCID₅₀/50µl) one can make 2600ml of virus solution containing 10^2 TCID₅₀/50µl)

Test procedure

a) Neutralization reaction

1. Load 80µl cell culture medium plus 20µl serum sample in the first row of wells of the microtitre plate to obtain the initial serum dilution of 1/5. Two wells per serum dilution are used. The remaining wells of the plate are loaded with 50µl of medium. Thereafter, 50µl of the 1/5 serum dilution are withdrawn by means of a 12 channel pipette and diluted serially two-fold. (When titration is finished each well contains 50 µl of serum-medium dilution.)
2. Add 50µl/well of test virus suspension containing 100 TCID₅₀/50µl and gently shake the plate. The required dilution of the test virus to obtain 100 TCID₅₀/50µl has to be prepared shortly before use by diluting the virus in growth medium.
3. Place the plates in a moist chamber and incubate in a CO₂ incubator (4-5% CO₂) for 1 hour at 37 °C. (Alternatively the plates can be sealed air tight and incubated for 1 hour at 37 °C)
4. Back titrate the virus dilution and incubate together with the NT plates.
5. Add 50µl/well of growth medium containing approximately 3×10^5 cells/ml and shake gently for 5-10 seconds. The cell suspension should be prepared during the 1 hour incubation period.
6. Place the plates in a moist chamber and incubate in a CO₂ incubator (4-5% CO₂) for 2-3 days at 37 °C. Alternatively the plates can be sealed air tight and incubated for 2-3 days at 37 °C.
7. Discard the growth medium, fix and stain the plates as described in Chapter immune labelling for peroxidase conjugated antibodies.

The neutralisation test can be performed in Leighton tubes, following the same principle and stained with FITC conjugated antibodies.

Controls

Back-titration of test virus must be carried out each time to check if the virus titre was 100 TCID₅₀/50µl. Tolerance limits of 0.5 log either way: 30-300 TCID₅₀/50µl had been recommended in Annex I of EU Directive 80/217. However, this was a pure mathematical figure. Evaluation of neutralisation tests over a longer period and in different laboratories show, that there is no linear correlation between back-titration and antibody titre.

A CSF antibody positive reference serum samples as well as cell controls containing medium and cells have to be included in the test. Reference sera can be supplied by the Community Reference Laboratory for CSF upon request.

The test has to be repeated if the reference sera do not give the expected result and the back titration is out of the limit. The obtained antibody titre of the reference serum should be given priority, when deciding if a neutralisation test is valid or not.

Evaluation / Estimation of ND₅₀

Serum titres are recorded as reciprocal of the highest **initial dilution** of sera (dilution of serum and growth medium without virus suspension) which prevented virus replication in 50% of the wells. Wells are scored as virus positive even if only one cell within the monolayer stains specifically. A point between two dilution levels is estimated. Serum titres are expressed as neutralization dilution 50% (ND₅₀) and can be also calculated using the method of KÄRBER (1931).

Example 1: Only one of the two wells of the serum dilution of 1:10 has infected cells. In this case the neutralization titre would be 10 ND₅₀.

Example 2: All wells up to the dilution of 1/80 are free of viral antigen while the remaining wells with serum dilutions equal and higher than 1/160 are positive. In this case the neutralization titre is estimated to be 120 ND₅₀.

When reporting test results, especially for export tests to other countries, it is important to specify clearly that the titre is expressed as Initial Dilution, and that for Final Dilution (which is the method mostly used in America) the result should be multiplied by 2 (*ie* 1/10 initial, corresponds to 1/20 final). The final dilution system is based on the actual dilution of serum

during the neutralization reaction, *ie* after addition of virus but before adding the cell suspension.

References:

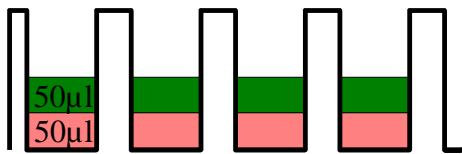
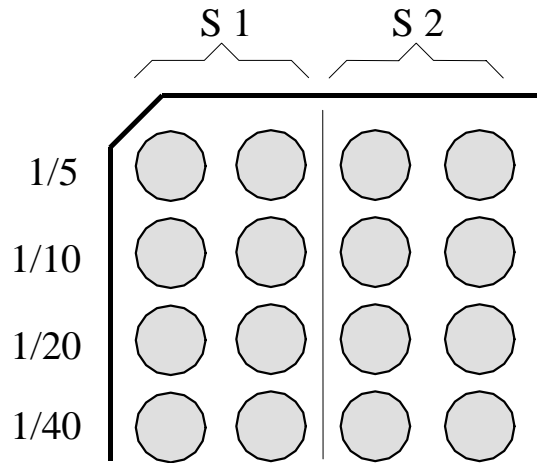
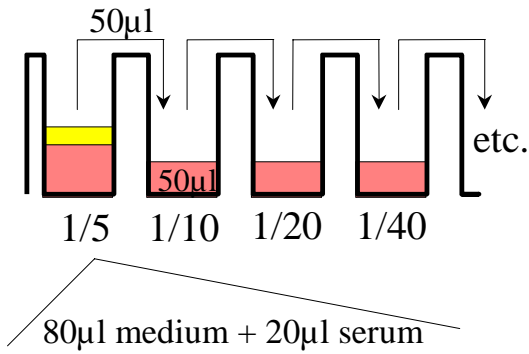
Hyera, J. M., Liess, B., and Frey, H. R., 1987. A direct neutralizing peroxidase-linked antibody assay for detection and titration of antibodies to bovine viral diarrhoea virus. Zentralbl.Veterinarmed.B 34, 3. 227-239.

Kärber, G., 2001. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Arch.Exp.Pathol.Pharmakol. 162, 480-483.

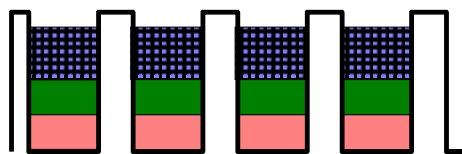
Neutralisation Test

Test Procedure

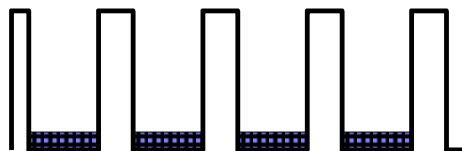
1. Titration of serum in growth medium, starting with 1/5, 2 wells/serum dilution



2. Add 50µl test virus/well, incubate 1h at 37 °C



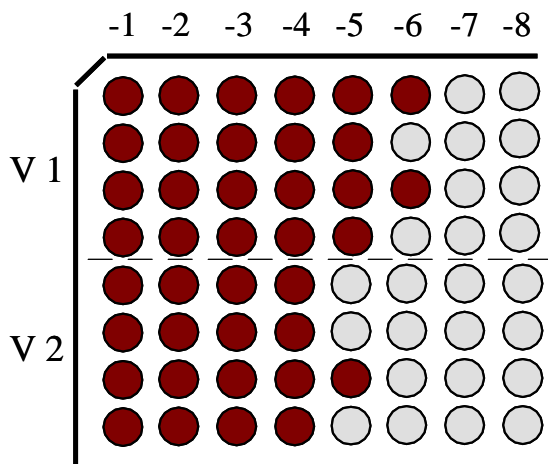
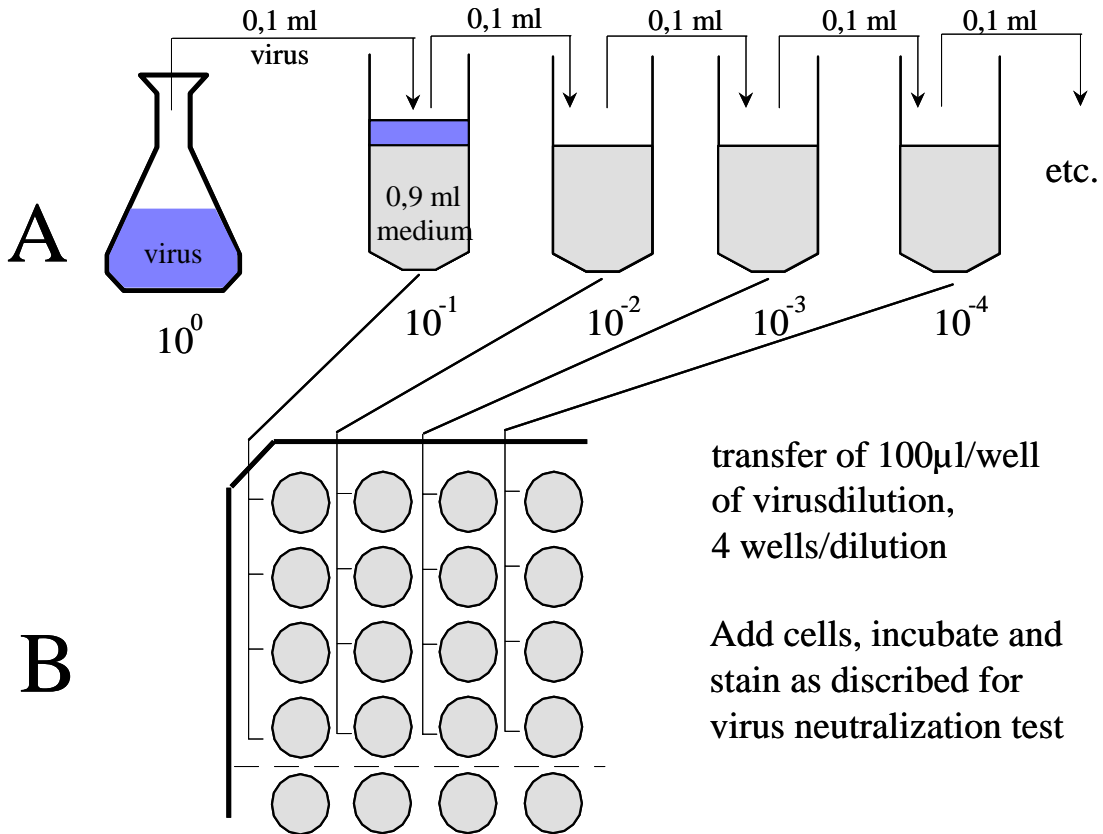
3. Add 50µl cell culture/well, incubate 3-4 days at 37 °C, CO₂



4. Discard medium, fix and stain

Virus titration

log 10 virus dilution



(infected cells are stained dark red)

Virus titre (TCID₅₀):

V 1: 10^6 /0,1 ml

V 2: $10^{4,7}$ /0,1 ml

TCID₅₀: reciprocal of the initial virus dilution at which 50% of the wells showed virus replication. A point between two dilution levels is estimated.

Example of ND₅₀-Calculation (Method of *Kärber*)

$$\log \text{ND}_{50} = L_{1,0} - L_{\text{int}} (S - 0,5)$$

$L_{1,0}$ = 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

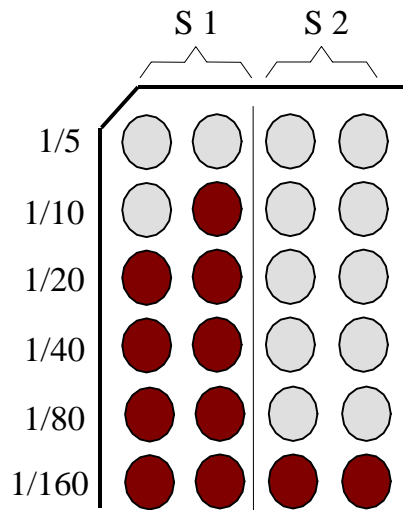
0,5 = Constant factor

serum dilution	infected wells / total wells	R
1 : 40	0/2	1,0
1 : 80	1/2	0,5
1 : 160	2/2	0,0
1 : 320	2/2	0,0
1 : 640	2/2	0,0

$$S = 1,5$$

$$\begin{aligned} \log \text{ND}_{50} &= -1,6 - 0,3 (1,5 - 0,5) \\ &= -1,6 - 0,3 \\ &= -1,9 \end{aligned}$$

$$\text{ND}_{50} = 10^{1,9} = 80$$



(infected cells are stained dark red)

Estimation of neutralization titre (ND₅₀)

S 1: 10

S 2: 120

ND₅₀ reciprocal of the highest initial dilution of the sera which prevented virus replication in 50% of the wells. A point between two dilution levels is estimated.

4. Serological differential diagnosis of CSF

A differential neutralisation test against a BVDV and BDV strain should be carried out simultaneously in order to detect and to interpret cross reaction between CSF and other pestiviruses.

Neutralisation test against BVDV:

The test procedure is the same as described for CSF.

There is at present no recommended permanent cell line for the BVD neutralisation test. Some suitable cell lines are described in Chapter 1. The BVD reference strain is NADL and can be supplied from the CRL.

References:

Mueller, T., Depner, K. R., Burow, J., Ahl, R., Conraths, F. J., and Moennig, V., 1997. Comparison of different BVD virus strains for their use in the differential diagnosis of classical swine fever--an attempt to standardize neutralization tests. Dtsch.Tieraerztl.Wochenschr. 104, 3. 91-96.

Neutralisation test against BDV:

The test procedure is the same as described for CSF.

Fetal sheep thymoid cell line (SFT-R) is the cell line of choice and can be supplied from the cell collection of the Federal Research Institute for virus diseases of animals, Island of Riems, Germany. BD reference strain Moredun, Strain Frijters and 137/4 can be supplied from the CRL.

References:

Oguzoglu, T. C., Floegel-Niesmann, G., Frey, H. R., Moennig, V.: Differential diagnosis of classical swine fever and border disease: seroepidemiological investigation of a pestivirus infection on a mixed sheep and swine farm. In: Dtsch.Tieraerztl.Wochenschr. 108 (2001) 210-213.

CSF antibody ELISA:

Each NSFL is in charge for the licensing procedure of different batches of commercial CSF antibody ELISAs. The CRL does not recommend a certain commercial product. At present CSF antibody ELISAs are available from:

ID-Lelystad, NL

Bommeli, CH

IDEXX, USA

A panel of reference sera with different titres of CSF antibodies and antibodies to other pestiviruses will be supplied by the CRL in April 2002.

6. Genetic Typing of CSF virus isolates

Introduction

Strains of CSFV can be classified into a number of subgroups according to their nucleotide sequence homology (Paton et al., 2000). Further discrimination can allow even small differences between isolates to be determined and compared in order to establish degrees of viral relatedness. This genetic typing can be used to support or refute hypotheses on likely routes of virus spread and this is the basis of molecular epidemiology. Reverse-transcription polymerase chain reaction (RT-PCR) amplification of CSF virus RNA followed by nucleotide sequencing is the simplest option for generating the sequence data to perform these comparisons.

A number of different regions of the CSF virus genome may be targeted for molecular epidemiological studies. However, two particular regions have been extensively studied and provide large sequence data-sets with which new isolates can be compared. These two regions lie within the 5' non coding region (5'NCR) of the genome, and within the E2 major glycoprotein gene (Lowings et al., 1996; Greiser-Wilke et al., 1998). A database of these sequences is accessible through the home page of the CRL, on the world wide web (Greiser-Wilke et al., 2000):

<http://viro08.tiho-hannover.de>

Username and password can be obtained by sending an EMAIL to Irene.Greiser-Wilke@tiho-hannover.de

For fine discrimination between closely related virus isolates, it is recommended that at least two different genomic target regions (5'NCR and E2) are analysed to ensure that small differences in sequence are reliable indicators of genetic relationships. Where discordant results are achieved, a third region can be examined (a part of the NS5B polymerase gene, Bjorklund et al., 1999) and the sequences from all three regions analysed independently and as a single, summated data set (Stadejek et al., 1997).

Sequencing Method

- 1) Extract CSF virus RNA from clinical samples or from PK15 cell cultures that have been infected with low passage CSF virus.
- 2) Perform RT-PCR to amplify targets within the 5'NCR, E2 and/or NS5B genes.

3) Determine the nucleotide sequence of the product or products (150 nucleotides of the 5'NCR, 190 nucleotides of the E2 gene and 409 nt of the NS5B gene) and compare with stored sequence information held in the databases.

The primer sequences and the PCR thermoprofiles for their use are given below:

5'NCR

Primers used for amplification of a 421 nt fragment of CSFV:

CSFV-UP1 Forward: 5' CTA GCC ATG CCC WYA GTA GG 3' (94-113)

CSFV-UP2 Reverse: 5' CAG CTT CAR YGT TGA TTG T 3' (514-496)

PCR thermoprofile: 35 x (95°C/45sec, 50°C/1 min, 72°C/1min), 1x (72°C/5 min).

Primers for sequencing 150 nt of the above:

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)

E2 gene

Primers used for amplification of a 671 nt fragment of E1 and E2 of CSFV:

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)

PCR thermoprofile: 35 x (95°C/45sec, 55°C/1 min, 72°C/1min), 1x (72°C/5 min).

Inner set (for nested PCR - same profile, and for sequencing 190 nt of the above):

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)

NS5B gene

Primers used for amplification of a 449 nt fragment of NS5B of CSFV:

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)

PCR thermoprofile: 35 x (94°C/1 min, 56°C/1 min, 72°C/1min), 1x (72°C/7 min).

The same primers (S1 and S2) used for sequencing 409 nt of the above:

Figures in parenthesis correspond to nucleotide positions in Alfort-187 (Ruggli et al., 1996).

Sequence Data Comparisons

The following method is recommended to ensure comparability of results.

Each virus sequence is stored as text in the Microsoft Notepad program from whence it can be directly imported into the ClustalW program in order to carry out multiple sequence alignments (Thompson et al., 1994). Once aligned, the file is converted into Phylip format and used in the Puzzle4 program to estimate the transition – transversion (Ts-Tv) ratio of each data set (Strimmer and von Haeseler, 1996; 1997). The data sets are next analysed by DNAdist producing distance matrices derived from the number of nucleotide substitutions between the sequences (Felsenstein, 1989). In DNAdist, the maximum likelihood method of evolution is chosen and the estimated Ts/Tv ratio is set to the above-calculated values. The output from DNAdist is then used as the input file in a neighbor-joining tree generating method, in this case Neighbor. Since the topology of the trees becomes more complex as the number of viruses increases, multiple jumbles (x99) should be used and the outgroup should always be specified as the sequence of the Kanagawa virus (CSF0309). This ensures that the trees will have a similar topography each time they are drawn. The treefile generated in the Neighbor program is then used as the input for a tree drawing program, namely; drawtree or drawgram although the trees can be looked at using other programs such as Treeview (Page, 1996).

To assess the statistical reliability of the dendrograms produced, each data set is repeatedly reanalysed using the Seqboot program. As before, the phylogeny for each repeat is calculated using DNAdist and then Neighbor. Both programs are run as described above except that the multiple data sets option (M) was set to 100 to indicate how many replicates of the alignment are in the input file. The treefile from Neighbor is then used as the input file in the Consense program, again designating the outgroup as the Kanagawa sequence. The bootstrap values are then readable in a text format which can be directly correlated to the dendrogram. Values in excess of 70% are considered to be significant (Clewley, 1999).

Interpretation

For the interpretation of the genetic typing of a new CSF virus isolate it is vital to have the epidemiological data available i.e. date of isolation, species (domestic pig or wild boar) place (country and area). These data are necessary for the new virus isolate as well as from those virus isolates the new one is compared with.

References:

Björklund H, Lowings P, Paton D, Stadejek T, Vilcek S, Greiser-Wilke I, Belák S (1999) Phylogenetic comparison and molecular epidemiology of classical swine fever virus. *Virus Genes*, in press.

Clewley JP (1999) A user's guide to producing and interpreting tree diagrams in taxonomy and phylogenetics. Part 2. The multiple alignment of DNA and protein sequences to determine their relationships. *Communicable Disease and Public Health* 1, 132-134.

Felsenstein J. (1989) Phylip: phylogeny inference package (version 3.5c). *Cladistics* 5, 164-166.

Greiser-Wilke I, Depner, K, Fritzemeier J, Haas L, Moennig V (1998) Application of a computer program for genetic typing of classical swine fever virus isolates from Germany. *J Virol Methods* 75, 141-150.

Greiser-Wilke I, Zimmermann B, Fritzmeier J, Floegel G, Moennig V. (2000) Structure and presentation of a database in the World Wide Web of the CSF virus isolates held at the EU reference laboratory. *Veterinary Microbiology* 73.

Lowings JP, Paton DJ, Sands JJ, De Mia GM, Rutili D (1994) Classical swine fever: genetic detection and analysis of differences between isolates. *Journal of General Virology* 75, 3461-3468.

Lowings P, Iyata G, Needham J, Paton D (1996) Classical swine fever diversity and evolution. *Journal of General Virology* 77, 1311-1321.

Page R.D.M. (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* 12, 357-358.

Paton DJ, McGoldrick A, Greiser-Wilke I, Parchariyanon S, Song J-Y, Liou PP, Stadejek T, Lowings JP, Bjorklund H, Belak S (2000) Genetic typing of classical swine fever virus. *Veterinary Microbiology* 73, 132-137

Ruggli N, Tratschin JD, Mittelholzer C & Hofmann MA (1996) Nucleotide sequence of classical swine fever virus strain Alfort/187 and transcription of infectious RNA from stably cloned full-length cDNA. *J Virol* 70, 3478-3487.

Stadejek T, Vilcek S, Lowings JP, Ballagi-Pordany A, Paton DJ & Belák S (1997) Genetic heterogeneity of classical swine fever virus in central Europe. *Virus Research* 52, 195-204.

Stadejek T, Pejsak Z, (2000) Diagnosis of pestivirus infections of pigs by simplified RT-nested PCR. *Medycyna Weterynaryjna* 56(2): 121-124.

Strimmer K. & von Haeseler A. (1996) Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. *Mol. Biol. Evol.* 13, 964-969.

Strimmer K. & von Haeseler A. (1997) Likelihood-mapping: a simple method to visualize phylogenetic content of a sequence alignment *PNAS*. (USA) 94, 6815-6819.

Thompson J.D., Higgins D.G., Gibson T.J. (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673-4680.

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