RealPCR* CSFV RNA Test

Validation data report

Test **with Confidence**

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I. Disease information

Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF), a highly contagious hemorrhagic disease affecting wild and domestic pigs. CSF is characterized by high morbidity and mortality rates, causes serious economic losses to the pig industry, and is a World Organization for Animal Health (OIE) listed disease.

Pigs infected with CSFV may shed a high amount of virus before showing clinical signs of the disease. If animals survive an acute or subacute infection, they can become chronically infected and excrete the virus intermittently or continuously until death. In pregnant sows, CSFV can cross the placenta and infect fetuses, causing abortions, fetal mummifications, and stillbirths. In midgestation infections (~50–70 days of pregnancy), weak or persistently viremic piglets can be born. These persistently infected piglets can shed high levels of virus for several months.

II. Glossary of terms

The following definitions have been taken from the Glossary of Terms section of the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (World Organization for Animal Health, 2012) and may be used to describe the assay's performance characteristics in this validation report.

Repeatability—Level of agreement between replicates of a sample both within and between runs of the same test method in a given laboratory.

Reproducibility—Ability of a test method to provide consistent results when applied to aliquots of the same sample tested by the same method in different laboratories.

Sensitivity (analytical)—Synonymous with "Limit of Detection," smallest detectable amount of analyte that can be measured with a defined certainty; analyte may include antibodies, antigens, nucleic acids, or live organisms.

Sensitivity (diagnostic)—Proportion of known infected reference animals that test positive in the assay; infected animals that test negative are considered to have false negative results.

Specificity (analytical)—Degree to which the assay distinguishes between the target analyte and other components in the sample matrix; the higher the analytical specificity, the lower the level of false positives.

Specificity (diagnostic)—Proportion of known uninfected reference animals that test negative in the assay; uninfected reference animals that test positive are considered to have false-positive results.

III. Test information

A. Test name

RealPCR* CSFV RNA Test

B. Part number(s), number of units

Part number 99-56022, 100 reactions

C. Product description

The RealPCR* platform is a modular format in which disease-specific target mixes are paired with standardized RNA or DNA master mixes and a single pooled positive control. Reagents are individually packaged and sold separately to allow for flexible reagent handling. All target mixes are qualified with the RealPCR standard reagents. Quality control testing ensures product performance that meets standards for sensitivity and specificity.

The RealPCR* CSFV RNA Mix (CSFV RNA Mix) contains primers and probes for the detection of CSFV RNA when amplified with RealPCR* RNA Master Mix (RNA MMx). The assay is a single-tube reverse transcriptase and polymerase reaction. The internal control for the test is based on the detection of an endogenous swine RNA sequence present in the host sample and is referred to as the internal sample control (ISC) in this protocol. Detection of endogenous RNA in swine samples controls for sample addition, extraction, and amplification. Primers and probe for detection of the internal sample control are included in the CSFV RNA Mix. An optional internal positive control, the RealPCR* Internal Positive Control (IPC ≥v1.1), is also available and should be used when endogenous host RNA is at low levels or unlikely to be present after extraction (such as environmental samples). The IPC contains a synthetic version of the swine ISC RNA target and is therefore compatible with the CSFV RNA Mix. Refer to the RealPCR Internal Positive Control (99-56330) product insert for guidance.

D. Sample types

The RealPCR CSFV RNA Test has been validated for the detection of classical swine fever virus RNA extracted from swine blood (EDTA), serum, plasma, oral fluids, and tissue including spleen, kidney, lymph node, and tonsil and swab samples. Tissue samples can be tested in pools of up to 10 samples and blood (EDTA), plasma, and serum can be tested in pools of up to 20 samples. Oral fluids can be tested as a composite sample taken from pens of up to 30 pigs. Pools containing a single weak sample (for example, cycle threshold [Ct] >32) may yield a negative result due to the dilution effect of pooling.

IV. Materials and methods

A. Materials required but not provided:

- Commercial RNA extraction kit
- (Optional) Centrifuge with a rotor and adapters for multiwell plates
- Micro-centrifuge for 2 mL microtubes capable of reaching 1500–3000 × g
- Appropriate personal protective equipment (e.g., gloves, lab coat)
- Nuclease-free, aerosol-resistant pipette tips
- Sterile microtubes for preparation of PCR mix
- Pipettes (5–1,000 µL); dedicated pipettes for preparation of PCR mix
- 96- or 384-well format PCR plates and optical adhesive film/plate covers
- Real-time PCR instrument (Applied Biosystems* 7500, Applied Biosystems* ViiA* 7, Applied Biosystems QuantStudio* 5, Agilent Mx3000P*, Agilent Mx3005P*, Agilent AriaMx*, Bio-Rad CFX96 Touch*, Bio Molecular Systems Mic qPCR Cycler, QIAGEN* Rotor-Gene* (72-well rotor only), Roche LightCycler* 480, or equivalent).

B. Test components

Table 1. RealPCR CSFV RNA Test component information

Material	Form (volume)	Storage conditions	Cap color	Description
RealPCR CSFV RNA Mix	Dried (1 mL)	-25°C to 8°C[1] -25°C to -15°C[2]	Yellow	Contains primers and probes for the detection of CSFV, and primers and probe for the detection of the internal sample control (ISC).
RealPCR RNA Master Mix	Liquid (1mL)	-25°C to -15°C	Black	Concentrated master mix (2.5X) that includes reverse transcriptase and hot-start polymerase for use with RNA target mixes in the IDEXX RealPCR system.
Real PCR Positive Control (PC)		-25°C to 8°C[1] -25°C to -15°C[2]	Blue	The PC contains all RealPCR and internal control targets (including the target for CSFV); for use with all RealPCR target mixes.
RealPCR* PCR grade water	Liquid (2 x 1 mL)	-25°C to 8°C	Clear	Qualified for reverse transcription PCR (RT-PCR) use. It is used for the reconstitution of the target mix and positive control and as the PCR negative control for each test run.

[1] Storage at receipt
[2] Storage after reconstitution

C. RNA extraction

RNA was extracted from different sample types using one of the following commercial extraction methods:

- RealPCR* DNA/RNA Spin Column Kit (IDEXX)
- RealPCR* DNA/RNA Magnetic Bead Kit (IDEXX)
- NucleoSpin* RNA Kit (Machery-Nagel)
- QIAamp* Viral RNA Mini Kit (Qiagen)

D. PCR instruments and settings

All data contained in this report was generated using PCR instruments approved for use with RealPCR reagents. Table 2 describes the reporter and quencher settings for the CSFV, ISC, and passive reference channels. Table 3 outlines the standard cycling program used for all RealPCR RNA and RNA tests.

Table 2. Settings for reporter and quencher

	Reporter	Quencher
CSFV	FAM*	BHQ* (none)
Internal control	HEX* (VIC)	BHQ* (none)
Passive reference	ROX*	N/A

Table 3. RealPCR standard DNA/RNA cycling program

Step	Temperature	Time	Cycles
Reverse transcription	50°C	15 min	1
Denaturation	95°C	1 min	1
Amplification‡	95°C 60°C	15 sec 30 sec	45

‡Ensure the instrument is set to record fluorescence following the 60°C amplification step.

E. Test protocol, validity, and interpretation of results

The RealPCR CSFV RNA Test procedure is detailed below. The test validity criteria are shown in table 4 and sample interpretation/validity is described in table 5.

RealPCR CSFV RNA Test procedure:

1. To prepare the PCR mix, add 10 µL CSFV RNA Mix and 10 µL RNA MMx for each reaction.
2. Pipette 20 µL of the PCR mix into the required wells of the multiwell plate.
3. Add 5 µL of sample RNA to each well. The final reaction volume is 25 µL.
4. Include the RealPCR positive control (5 µL) and PCR negative control (5 µL PCR grade water) for each test run.
5. Cover the plate and briefly spin the plate, if necessary, to settle contents and remove air bubbles.
6. Set up the thermal cycler with the RealPCR standard DNA/RNA cycling program.

Table 4. Test validity criteria

	FAM Ct‡ value	HEX (VIC) Ct value
Positive control	<38	<38
PCR negative control	No signal	No signal

‡Ct = Cycle threshold

Table 5. Interpretation of results

Sample result	FAM amplification	HEX (VIC) amplification	Sample validity
CSFV RNA detected[‡]	Yes	Yes/No	A positive Ct value and characteristic amplification curve in comparison to the PCR negative control. An ISC amplification curve in the HEX (VIC) channel is expected; however, some strong CSFV positive samples may result in a negative internal control result.
CSFV RNA not detected	No	Yes	Amplification curve in the HEX (VIC) ISC channel only.
Invalid[§]	No	No	Absence of an amplification curve in the FAM and HEX (VIC) channels indicates an invalid result.

[‡]The target mix is optimized for the detection of CSFV RNA; a strong positive RNA sample may out compete the detection of the internal control.

[§]An invalid sample can be an indication of failed sample addition, extraction, and/ or PCR. It is recommended that the RNA be diluted fivefold into PCR grade water and retested. Include the undiluted RNA as a sample. If the test is still not valid, a new extraction is recommended.

V. RT-PCR characterization

Characterization of RT-PCR focuses only on detection of the target RNA and analysis of the result and is exclusive of biological sample types or processing methods.

Inclusivity, exclusivity, analytical sensitivity, efficiency, repeatability, reagent stability, and robustness are included in the RT-PCR characterization.

VI. Inclusivity

In silico analysis for inclusivity

Purpose: To confirm sequence homology between the RealPCR CSFV RNA Test design and sequences annotated as classical swine fever virus in the National Center for Biotechnology Information (NCBI) sequence database.

Procedure: To demonstrate design specificity, a BLASTn (Basic Local Alignment Search Tool) search was performed using the RealPCR CSFV amplicon sequence. The search was designed to include only results annotated as classical swine fever. This analysis was intended to demonstrate identity between all database classical swine fever virus sequences and the RealPCR CSFV RNA Test design.

Results/conclusions: A total of 140 hits annotated as classical swine fever virus were returned as matches to the complete RealPCR CSFV RNA Test design amplicon with 100% identity to the CSFV test design.

A. Experimental inclusivity, part 1

Purpose: To evaluate the inclusivity of the RealPCR CSFV Mix by testing a CSFV panel comprised of different genotypes/subtypes.

Procedure: A panel of 13 CSFV strains was supplied as extracted nucleic acid by the Friedrich-Loeffler-Institut (FLI), Riems, Germany. CSFV-positive status had been previously confirmed through testing by the EPIZONE international consortium. All samples were supplied at a concentration of 2,000 copies/µL and were tested in duplicate wells using the standard RealPCR CSFV RNA Test reagents and protocol.

Results/conclusions: Experimental inclusivity results are shown in table 6 below. All CSFV strains tested positive on the RealPCR CSFV RNA Test.

Table 6. RealPCR CSFV RNA Test inclusivity (FLI panel)

Sample	Species	Strain name	Subtype/ genotype	RealPCR CSFV result (mean Ct)	Result
Pesti-1	CSFV	C strain	1.1	25.6	Positive
Pesti-2	CSFV	Eystrup91	1.1	26.4	Positive
Pesti-3	CSFV	Alfort187	1.1	26.5	Positive
Pesti-4	CSFV	Koslov1128	1.2	26.3	Positive
Pesti-5	CSFV	Brescia	1.2	27.3	Positive
Pesti-6	CSFV	Schweiz II	2.1	26.9	Positive
Pesti-7	CSFV	Pader	2.1	25.8	Positive
Pesti-8	CSFV	Bergen	2.2	26.8	Positive
Pesti-9	CSFV	D4886/82/Ro	2.2	26.2	Positive
Pesti-10	CSFV	Uelzen	2.3	26.4	Positive
Pesti-11	CSFV	Spante	2.3	25.1	Positive
Pesti-12	CSFV	Congenital Tremor	3.1	28.0	Positive
Pesti-13	CSFV	Kanagawa	3.4	25.0	Positive

B. Experimental inclusivity, part 2

Purpose: To evaluate the inclusivity of the RealPCR CSFV Mix by testing a panel of CSFV isolates comprised of genotypes from different regions.

Procedure: A panel of 50 CSFV isolates, representing a variety of genotypes, was tested by Dr. Alexander Postel's laboratory at the Institute for Virology, University of Veterinary Medicine Hannover, Foundation (Hannover, Germany). CSFV positive status was confirmed using the laboratory's accredited method (Hoffmann, et al., 2006). Each sample was extracted and tested on the RealPCR CSFV RNA Test using the standard protocol.

Results/conclusions: Experimental inclusivity results are shown in table 7 below. All CSFV strains tested positive on the RealPCR CSFV RNA Test.

Table 7. RealPCR CSFV RNA Test inclusivity (University of Hannover isolates)

#	Genotype	Isolate name	Country	Year	Species (host)	RealPCR CSFV Ct	Result
1	1.1	Vi127/94	Germany	1994	Domestic pig	14.6	Positive
2	1.1	Romania I 01	Romania	2001	Domestic pig	18.5	Positive
3	1.1	94-14901/ 02-94	Costa Rica	1994	Domestic pig	25.1	Positive
4	1.1	664/Ru	Russia	1995	Domestic pig	19.7	Positive
5	1.1	97- 31719/ #4	Mexico	1997	Domestic pig	15.7	Positive
6	1.1	22/97	Brazil	1997	Domestic pig	23.7	Positive
7	1.1	CAP	France	1978	Domestic pig	16.7	Positive
8	1.1	31240/97	Slovakia	1997	Domestic pig	17.3	Positive
9	1.1	Fukuoka/72	Japan	1972	Domestic pig	13.4	Positive
10	1.2	3-Jul	Poland	1993	Domestic pig	19.3	Positive
11	1.2	TVM-1 vac	Czech Republic	-	Domestic pig	18.2	Positive
12	1.2	3795/96	Czech Republic	1996	Wild boar	18.0	Positive
13	1.2	Baker A	United States	-	Domestic pig	19.2	Positive
14	1.3	VRI 4167	Malaysia (MY)	1986	Domestic pig	16.1	Positive
15	1.3	HC/ #4409	Guatemala	-	Domestic pig	18.2	Positive
16	1.3	HCV 31	Honduras (HN)	1992	Domestic pig	19.3	Positive
17	1.4	39/Margarita	Cuba	-	Domestic pig	17.7	Positive
18	1.4	PR, VP32/10	Cuba	2010	Domestic pig	19.8	Positive
19	2.1	V1240/97	Germany	1997	Domestic pig	18.4	Positive
20	2.1	SP 10549/13	Austria	1993	Wild boar	18.3	Positive
21	2.1	18/Cr.	Croatia	1997	Domestic pig	20.2	Positive
22	2.1	2000/8	UK	2000	Domestic pig	18.8	Positive
23	2.1	no. 3, Farm 1	South Africa	2005	Domestic pig	17.1	Positive
24	2.1	907/1	Germany	1989	Domestic pig	19.8	Positive
25	2.1	A-2	Lithuania	2009	Domestic pig	20.0	Positive
26	2.2	Sch 180	Germany	1989	Domestic pig	15.5	Positive
27	2.2	SP 1790/90	Austria	1990	Domestic pig	17.8	Positive
28	2.2	1295/94	Czech Republic	1994	Domestic pig	17.3	Positive
29	2.2	Parma98	Italy	1998	Domestic pig	17.5	Positive
30	2.2	5502/B/5502 B	Czech Republic	1995	Domestic pig	17.9	Positive
31	2.2	VA 531	Italy	2000	Wild boar	17.6	Positive
32	2.2	P29/03/88	Singapore	1988	Domestic pig	17.8	Positive
33	2.2	Nep28/Makwanpur	Nepal	2011	Domestic pig	19.0	Positive
34	2.2	Oct-26	Vietnam	2010	Domestic pig	15.5	Positive
35	2.3	SP373Han82	Germany	1982	Domestic pig	16.9	Positive
36	2.3	Switzerland IV/93	Switzerland	1993	Domestic pig	17.8	Positive
37	2.3	24/93	Poland	1993	Domestic pig	17.9	Positive
38	2.3	5325/96	Czech Republic	1996	Wild boar	18.2	Positive

#	Genotype	Isolate name	Country	Year	Species (host)	RealPCR CSFV Ct	Result
39	2.3	100/06	Croatia	2006	Domestic pig	17.5	Positive
40	2.3	906/2000	Slovakia	2000	Wild boar	18.8	Positive
41	2.3	620.01	Macedonia	2000	Domestic pig	18.0	Positive
42	2.3	Castellon Vinaroz)	Spain	2001	Domestic pig	18.7	Positive
43	2.3	Segarcea/2004	Romania	2004	Domestic pig	16.2	Positive
44	2.3	Alfort/Tuebingen	Germany	-	Domestic pig	18.4	Positive
45	2.3	M7 19928/60	Hungary	2007	Wild boar	17.7	Positive
46	2.3	V1987	Germany	2000	Wild boar	19.4	Positive
47	2.3	92946/4	Latvia	2013	Wild boar	19.1	Positive
48	2.3	26108	Serbia	2010	Domestic pig	18.8	Positive
49	3.4	Okinawa/86	Japan	1986	Domestic pig	18.0	Positive
50	3.4	38/KS/93/TWN	Taiwan	1993	Domestic pig	17.9	Positive

VII. Exclusivity

In silico analysis for exclusivity

Purpose: To determine sequence orthology between the RealPCR CSFV RNA Test design and sequences annotated as non-CSFV sequences.

Procedure: The procedure followed for exclusivity analysis was identical to the procedure for analyzing inclusivity; however, sequences annotated as classical swine fever virus were excluded from the results.

Results/conclusions: Four matches were returned in the exclusivity analysis with 98.3% identity to the RealPCR CSFV RNA Test design amplicon and were associated with CSFV sequences that may have been misannotated. The remaining matches maintained less than 90% identity to the RealPCR CSFV RNA Test design amplicon with a minimum of five mismatches over the complete amplicon. These results demonstrate the RealPCR CSFV RNA Test design will not detect sequences unrelated to the CSF virus.

A. Experimental exclusivity, part 1

Purpose: To evaluate the specificity of the RealPCR CSFV Mix by testing a panel of related pestiviruses.

Procedure: A panel of 22 non-CSFV pestiviruses, including border disease virus (BDV) and bovine viral disease virus (BVDV, Type 1 and Type 2), were supplied as extracted nucleic acid by the Friedrich-Loeffler-Institute (FLI), Riems, Germany. Positive status had been previously confirmed through testing by the EPIZONE international consortium. All samples were supplied at a concentration of 2,000 copies/µL and were tested in duplicate wells using the standard RealPCR CSFV RNA Test reagents and protocol.

Results/conclusions: Experimental exclusivity results are shown in table 8 below. All the non-CSFV samples gave negative results with the RealPCR CSFV RNA Test.

Table 8. RealPCR CSFV RNA Test exclusivity (FLI Pestivirus Panel)

Sample	Target	Strain name	Subtype/ genotype	RealPCR CSFV result
Pesti-14	BDV	Moredun	1	No Ct
Pesti-15	BDV	Rudolph	2	No Ct
Pesti-16	BDV	Gifhorn	3	No Ct
Pesti-17	BDV	Isard	4	No Ct
Pesti-18	BVDV-1	NADL	1a	No Ct
Pesti-19	BVDV-1	Paplitz	1b	No Ct
Pesti-20	BVDV-1	PI809	1d	No Ct
Pesti-21	BVDV-1	NC3807-1251/1	1e	No Ct
Pesti-22	BVDV-1	Egbert	1f	No Ct

Sample	Target	Strain name	Subtype/ genotype	RealPCR CSFV result
Pesti-23	BVDV-1	BO806-17	1g	No Ct
Pesti-24	BVDV-1	BO807-3	1h	No Ct
Pesti-25	BVDV-1	NC3807-8757	1x	No Ct
Pesti-26	BVDV-2	8644	2a G	No Ct
Pesti-27	BVDV-2	Bure	2a US	No Ct
Pesti-28	BVDV-2	Walter	2b	No Ct
Pesti-29	BVDV-2	PO1600	2c	No Ct
Pesti-30	Pestivirus	Hobi	atypical	No Ct
Pesti-31	Pestivirus	Giraffe H138	atypical	No Ct
Pesti-32	BVDV-1	NCP-2508-FCS	1c	No Ct
Pesti-33	BVDV-1	Böhni	1k	No Ct

B. Experimental exclusivity, part 2

Purpose: To evaluate the specificity of the RealPCR CSFV Mix by testing a panel of pathogens that are genetically related to or found in the same ecological niche as CSFV.

Procedure: Extracted RNA and DNA were used to create a panel of defined bacterial and viral strains, specifically those identified as common swine pathogens. This panel was tested on the RealPCR CSFV RNA Test, following the standard test protocol.

Results/conclusions: Experimental exclusivity results are shown in table 9 below. All the non-CSFV samples gave negative results with the RealPCR CSFV RNA Test.

Table 9. RealPCR CSFV RNA Test Exclusivity (other swine pathogens)

Pathogen	Sample type	RealPCR CSFV result
Porcine reproductive & respiratory syndrome virus (PRRS)	Oral fluids	No Ct
Porcine circovirus type 2 (PCV2)	Lung lavage	No Ct
Porcine epidemic diarrhea virus (PEDV)	Oral fluids	No Ct
Transmissible gastroenteritis virus (TGEV)	Fecal swab	No Ct
Swine influenza A virus (SIV)	Lung lavage	No Ct
Mycoplasma hyopneumoniae (*M. hyo*)	Lung lavage	No Ct
Porcine parvovirus (PPV)	Oral fluids	No Ct
Acholeplasma granularum	Culture	No Ct
Actinobacillus pleuropneumoniae	Culture	No Ct
Mycoplasma flocculare	Culture	No Ct
Mycoplasma hyorhinis	Culture	No Ct
Mycoplasma sualvi	Culture	No Ct
Mycoplasma hyosynoviae	Culture	No Ct
Streptococcus suis	Culture	No Ct
Bordetella bronchiseptica	Culture	No Ct

VIII. Analytical sensitivity

Purpose: To determine the lowest number of target nucleic acids per reaction, generating a positive result in 95% of cases.

Procedure: The limit of detection (LD_{PCR}) was determined using dilutions of synthetic material representing the target sequence for CSFV. For the initial determination of analytical sensitivity, log dilutions in the range of 1 copy to 10,000,000 copies per 25 µL reaction were tested. From this initial evaluation, the LD_{PCR} was estimated at 10 copies per reaction for the CSFV target region. Three sets of tenfold dilutions, in the range of 10 to 1,000 copies per reaction, were then tested for confirmation of LD_{PCR}. Eight replicates of each dilution were tested per session for three sessions by a single operator, for a total of 24 results per dilution.

Results/conclusions: Results for the initial analytical sensitivity determination and the additional testing at the limit of detection are shown in tables 10 and 11, respectively, while figure 1 shows the amplification curves for the initial dilution series.

Initial analytical sensitivity testing showed the limit of detection to be 10 copies per reaction with 3 out of 3 replicates detected as positive. In subsequent testing, CSFV was consistently detected at concentrations of 10 copies per reaction, with positive results in 24 out of 24 wells (100%).

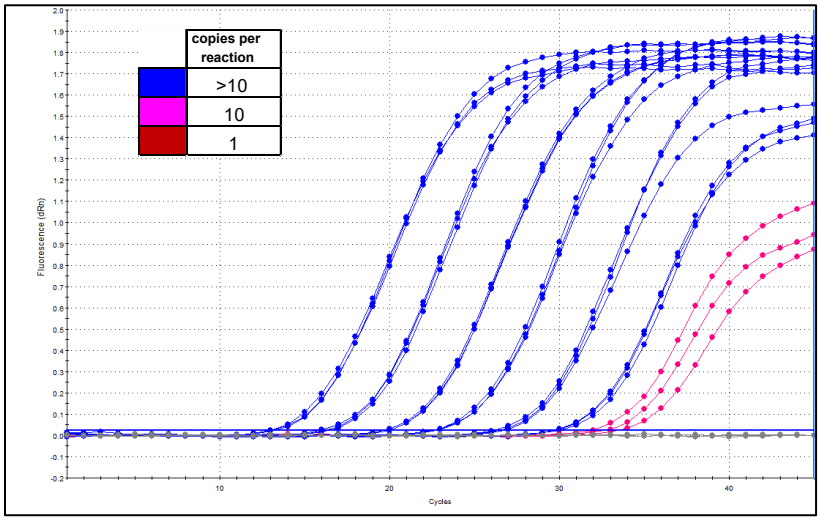


Figure 1. RealPCR CSFV RNA Test amplification curves

Table 10. RealPCR CSFV RNA Test analytical sensitivity

Copies per reaction	Ct-1	Ct-2	Ct-3	Mean Ct	# Positive per 3 replicates
10,000,000	13.6	13.6	13.1	13.4	3
1,000,000	16.6	16.7	16.5	16.6	3
100,000	20.0	19.8	20.0	19.9	3
10,000	23.2	22.9	22.9	23.0	3
1,000	26.1	25.8	26.4	26.1	3
100	29.9	30.2	29.5	29.9	3
10	33.7	33.0	33.0	33.2	3
1	No Ct	No Ct	No Ct	No Ct	0

Table 11. Analytical sensitivity confirmation for the RealPCR CSFV RNA Test

	# Positive in 8 test wells				
Copies per reaction	Session #1	Session #2	Session #3	# Positive in 24 tests	% Positive results
1,000	8	8	8	24/24	100%
100	8	8	8	24/24	100%
10	8	8	8	24/24	100%

IX. Efficiency of PCR

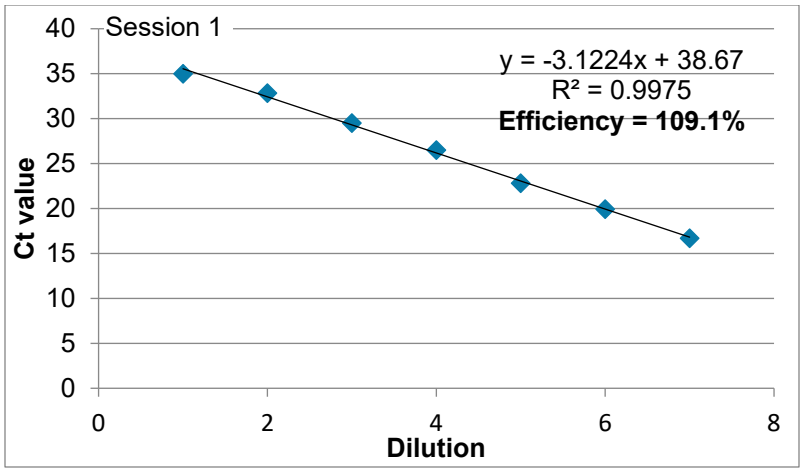
Purpose: To determine the efficiency of the PCR reaction for the RealPCR CSFV RNA Test.

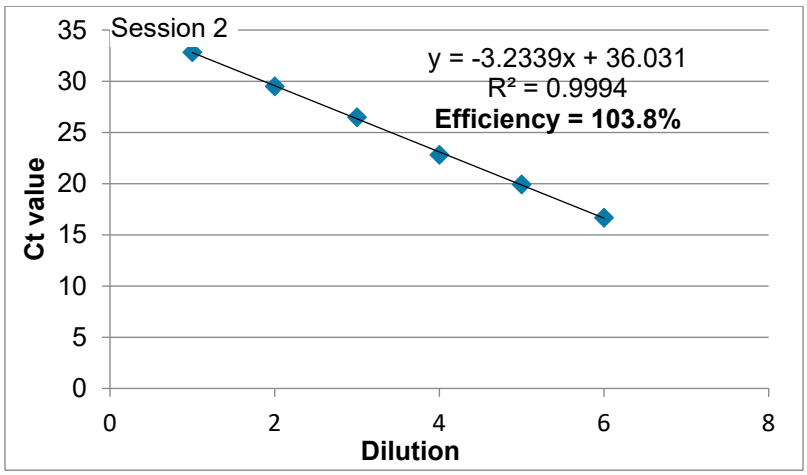
Procedure: Efficiency was evaluated with dilutions of synthetic material representing the target sequence for CSFV. Results were obtained by testing a series of 10-fold dilutions of the stock material, from 1,000,000 copies/25 µL PCR through 10 copies/25 µL PCR reaction. Each dilution was tested in duplicate in three separate test events using the RealPCR CSFV RNA Test.

Results/conclusions: Ct results for the synthetic dilution series are shown in table 12 below, and the standard curves are shown in figure 2. Efficiency of the test, calculated as $(10^{(-1/slope)} - 1) \times 100$, was determined to be 105.7%, which meets acceptable standards of 90%–110%.

Table 12. Mean Ct results for CSFV dilution series

Copies per reaction	Session #1	Session #2	Session #3	Mean Ct
1,000,000	16.7	16.8	16.6	16.7
100,000	20.1	19.8	19.8	19.9
10,000	22.8	22.7	22.9	22.8
1,000	26.6	26.5	26.4	26.5
100	29.6	29.7	29.3	29.5
10	32.7	32.2	33.6	32.8
1	35.1	No Ct	34.8	34.9





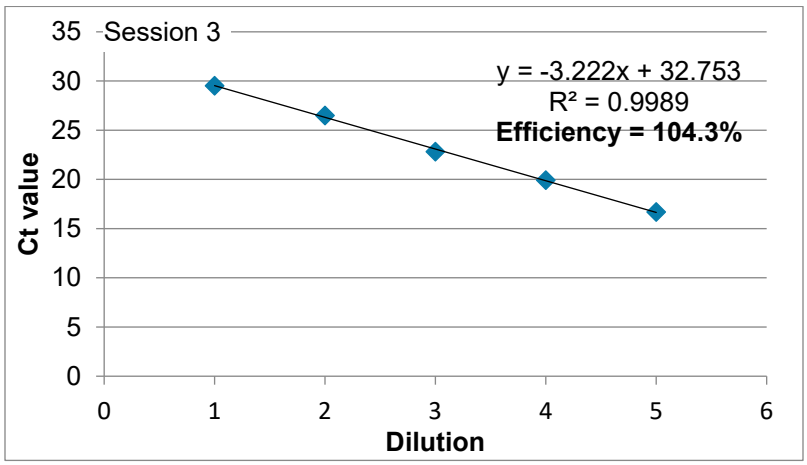


Figure 2. RealPCR CSFV RNA Test standard curves

X. Repeatability

Purpose: To demonstrate consistent results for replicates of a sample between successive runs of the RealPCR CSFV RNA Test under constant laboratory and instrument conditions.

Procedure: Dilutions of synthetic material representing the target sequence for CSFV were prepared to represent low (10), medium (100), and high (1,000) copy numbers per 25 µL reaction. Dilutions were tested in triplicate, in three sessions, with the same reagent lots and PCR instrument for each test event. All testing used the RealPCR CSFV RNA Test standard protocol. The coefficient of variability (%CV) was then calculated from nine CSFV Ct values for each sample as a measure of repeatability.

Results/conclusions: Results for all three target levels are reported in table 13 below. The %CV values were all less than 2%.

Table 13. Repeatability of the RealPCR CSFV RNA Test

		CSFV (FAM) Ct values			Mean		
Panel	**Rep**	**Run-1**	**Run-2**	**Run-3**	**Ct value**	**SD**	**% CV**
Low level	**#1**	32.8	32.7	32.6	32.6	0.407	1.2%
	#2	32.0	32.9	32.2			
	#3	33.2	32.2	33.2			
Mid level	**#1**	30.1	29.4	29.1	29.9	0.457	1.5%
	#2	30.1	30.5	29.6			
	#3	30.5	29.7	29.9			
High level	**#1**	26.9	27.0	26.6	26.7	0.304	1.1%
	#2	27.1	26.4	27.0			
	#3	26.7	26.7	26.1			

XI. PCR instrument evaluation

Purpose: To demonstrate performance of the RealPCR CSFV RNA Test on different PCR instruments.

Procedure: Synthetic material representing the target sequence of CSFV was used for this testing. Dilutions were prepared to represent low (10), medium (100), and high (1,000) copy numbers per 25 µL reaction.

Results/conclusions: Results from all the instruments are reported in table 14. Ct values were consistent across the instruments tested.

Table 14. PCR instrument comparison of RealPCR CSFV RNA Test Ct values

Copies/ reaction	Agilent Mx3000P	Agilent AriaMx	ABI 7500	ABI Quant-Studio 5	Roche Light-Cycler 480	QIAGEN Rotor-Gene Q	Mean	% CV
0	-	-	-	-	-	-	-	-
10	33.8	33.6	37.8	36.5	36.0	34.2	35.3	4.8
100	30.2	30.2	34.2	32.9	33.6	31.2	32.1	5.5
1,000	26.7	27.5	30.9	29.1	30.2	27.9	28.7	5.7

XII. Reagent stability

Purpose: To demonstrate stability of test reagents over the expiration period when stored under the appropriate conditions as indicated on the product labels.

Procedure: Stability of the RealPCR CSFV RNA Mix was evaluated for batches that had been manufactured, dried, and held at the recommended storage temperature of 2°C–8°C. Vials were reconstituted with PCR-grade water at the time of testing. Dilutions of synthetic nucleic acid representing the target sequence were prepared at low (10/reaction), medium (100/reaction), and high (1,000/reaction) copy numbers. Samples were tested in duplicate wells for each PCR run.

Results/conclusions: Real-time stability results for the batches of RealPCR CSFV RNA Mix are shown in table 15 with "time 0" QC release data shown for comparison. Timing for testing varied across the batches. The lots demonstrated acceptable performance for at least 16 months. The batches detected all replicates of the 10-copy sample, and Ct values for the final test date were no more than one Ct later than the time 0 Ct value.

Table 15. Real-time stability of the RealPCR CSFV RNA Test

	Lot #1					Lot # 2				
		Copies per reaction					Copies per reaction			
	Age at test	0	10	100	1,000	Age at test	0	10	100	1,000
Time 0	0	No Ct	35.0	29.6	26.8	0	No Ct	33.8	30.2	26.7
1–3 months	1 month	No Ct	31.8	29.5	26.2	1 month	No Ct	33.8	30.2	26.7
4–8 months	4 months	No Ct	31.8	29.5	26.2					
9–14 months	10 months	No Ct	33.1	30.1	27.0	12 months	No Ct	36.5	33.5	29.5
15–20 months	16 months	No Ct	33.0	29.9	26.4	17 months	No Ct	34.1	30.4	27.0

XIII. Robustness

Purpose: To demonstrate robustness of performance for the RealPCR CSFV RNA Test despite small variations in critical parameters of the PCR reaction.

Procedure: Robustness of the RealPCR CSFV RNA Test was evaluated by performing the test under the following variable conditions:

- Hybridization time (+/-3 sec)
- Hybridization temperature (+/-1°C)
- Sample volume (+/-10%)

All conditions were tested with three wells of synthetic material representing the target sequence, diluted to 10 copies per reaction, which was the detection limit determined in the analytical sensitivity section of this report. CSFV should be detected in 3/3 wells.

Results/conclusions: RealPCR CSFV RNA Test robustness results are shown in table 16. The 10-copy sample was detected in 3/3 wells for each set of conditions. Additionally, there was less than one cycle difference in the mean Ct values between the low and high test conditions. This demonstrates acceptable robustness of the test to variation in the test parameters.

Table 16. Robustness of the RealPCR CSFV RNA Test

	Hybridization		Sample	CSFV LOW (10-copy sample)			
Conditions	**Time**	**Temp.**	**volume**	**Ct-1**	**Ct-2**	**Ct-3**	**Mean Ct**
Low	27 sec	59°C	4.5 µL	32.3	32.2	32.4	32.3
Standard	30 sec	60°C	5.0 µL	32.6	32.2	33.2	32.6
High	33 sec	61°C	5.5 µL	32.7	33.5	32.7	33.0

XIV. Characterization of the complete method

Characterization of the complete method encompasses the entire testing process from biological sample to final result, including sample extraction and PCR.

Sensitivity and specificity, as well as validation of individual sample types, pooled samples, and extraction methods, are included in the characterization of the complete method.

A. Sample types

- Serum—individual and pools of up to 20
- Tissue (spleen, kidney, lymph node, and tonsil)—individual and pools of up to 10
- Plasma
- Blood swabs
- Oral fluids
- Fecal swabs

B. Extraction methods

- RealPCR DNA/RNA Spin Column Kit (IDEXX)
- NucleoSpin RNA Kit (Machery Nagel)
- QIAamp Viral RNA Mini Kit (QIAGEN)

XV. Diagnostic sensitivity/specificity

Purpose: To demonstrate diagnostic sensitivity and specificity of the RealPCR CSFV RNA Test.

Procedure: Testing was performed at the Institute for Virology, University of Veterinary Medicine Hannover, Foundation (Hannover, Germany) and IDEXX Laboratories. The CSFV status was confirmed by the laboratory's accredited method or exposure status. The test population was 543 samples, comprised of fecal swabs, oral swabs, blood, serum, and tissue samples. Of these, 193 were considered positive and 350 were negative for CSFV.

Each sample was processed by one of the commercial extraction methods listed here and tested on the RealPCR CSFV RNA Test using standard test reagents and protocol.

Results/conclusions: Results for diagnostic sensitivity and specificity are reported in table 17. Diagnostic sensitivity is presented as the percentage of test positives relative to true positives, and diagnostic specificity as the percentage of test negatives relative to true negatives. Confidence intervals at the 95% level are also included.

The RealPCR CSFV RNA Test performed with 100% sensitivity and 100% specificity for this population.

Table 17. Overall diagnostic sensitivity and specificity

		Confirmed status		
		Positive	**Negative**	**Totals**
RealPCR CSFV RNA Test	**Positive**	193	0	193
	Negative	0	350	350
	Totals	193	350	543

		95% confidence limits (CL)	
		Low CL	**High CL**
Diagnostic sensitivity	100%	98%	100%
Diagnostic specificity	100%	98%	100%

A. Sample types

Purpose: To demonstrate CSFV detection in multiple sample types.

Procedure: Several sample types were used to evaluate the overall diagnostic sensitivity and specificity of the RealPCR CSFV RNA Test. All samples were tested on the RealPCR CSFV RNA Test using standard test reagents and protocol.

Results/conclusions: Table 18 shows a summary of RealPCR CSFV RNA Test performance, by sample type, for 610 total samples (including those in the diagnostic sensitivity/specificity population and additional extracts). All samples were correctly identified.

Table 18. Number of correctly identified samples, shown by sample type

	CSFV status	
Sample type	**Pos**	**Neg**
Blood	32	49
Serum	47	46
Plasma	0	50
Oral fluid/swab	19	59
Fecal swab	19	35
Tissue type		
Kidney	47	30
Spleen	32	30
Tonsil	32	31
Lymph node	32	20
Total	**260**	**350**

B. Detection of CSFV RNA in blood swabs

Purpose: To demonstrate CSFV RNA detection in blood swabs from domestic swine.

Procedure: In this study, paired samples (serum and blood swab) were collected from a small population of animals. Samples were extracted using one of the approved methods and tested on the RealPCR CSFV RNA Test using standard test reagents and protocol.

Results/conclusions: Table 19 shows a summary of RealPCR CSFV RNA Test performance for the two sample types. CSFV RNA was detected in both serum and blood swab samples when the serum Ct value was < 35. For weaker samples (serum Ct values > 35), blood swabs sometimes tested negative for CSFV RNA. These results suggest very weak CSFV samples may test negative in blood swabs.

Table 19. Detection of CSFV RNA in serum and blood swabs

	RealPCR CSFV RNA Test serum sample		RealPCR CSFV RNA Test blood swab sample	
Animal number	**CSFV Ct result**	**CSFV Result (serum)**	**CSFV Ct result**	**CSFV Result (Blood swab)**
1	36.22	Positive	No Ct	Negative
2	38.25	Positive	37.72	Positive
3	22.52	Positive	20.55	Positive
4	20.97	Positive	19.34	Positive
5	20.70	Positive	19.84	Positive
6	37.97	Positive	No Ct	Negative
7	17.01	Positive	19.13	Positive
8	36.26	Positive	38.12	Positive
9	39.09	Positive	No Ct	Negative
10	39.91	Positive	No Ct	Negative
11	37.72	Positive	No Ct	Negative
12	37.83	Positive	No Ct	Negative
13	38.41	Positive	No Ct	Negative
14	37.01	Positive	No Ct	Negative
15	38.75	Positive	No Ct	Negative

C. Extraction methods

Purpose: To demonstrate CSFV detection using multiple extraction methods.

Procedure: Several extraction methods were used to purify nucleic acid from different CSFV-positive sample types. All samples were tested on the RealPCR CSFV RNA Test using standard test reagents and protocol.

Results/conclusions: Table 20 shows a summary of RealPCR CSFV RNA Test performance, by sample type, for each extraction method. All samples were correctly identified.

Table 20. Extraction method evaluation (CSFV-positive samples)

	QIAamp Viral RNA		MN NucleoSpin		QIAzol RNeasy		RealPCR Spin Column	
Sample type	**Ct mean**	**# Pos**	**Ct mean**	**# Pos**	**Ct mean**	**# Pos**	**Ct mean**	**# Pos**
Blood	18.5	4/4	-	-	22.4	4/4	23.0	4/4
Serum	18.3	4/4	-	-	23.2	4/4	22.3	4/4
Oral fluids	25.2	3/3	-	-	31.9	3/3	25.4	3/3
Fecal swabs	25.8	3/3	-	-	30.7	3/3	28.1	3/3
Kidney	-	-	18.2	4/4	20.7	4/4	20.2	4/4
Lymph node	-	-	13.8	4/4	15.0	4/4	15.9	4/4
Spleen	-	-	16.5	4/4	18.2	4/4	17.6	4/4
Tonsil	-	-	16.4	4/4	16.7	4/4	16.4	4/4

XVI. Detection of CSFV in sample pools

Purpose: To demonstrate the sensitivity of the RealPCR CSFV RNA Test when testing pools of serum or tissue.

Procedure: Testing was performed at the Institute for Virology, University of Veterinary Medicine Hannover, Foundation (Hannover, Germany). Fifteen confirmed CSFV-positive serum samples, and 15 CSFV-positive kidney samples were diluted 1:20 or 1:10, respectively, into CSFV-negative sample matrix (serum or kidney homogenate). Pooled serum samples were extracted using the QIAamp Viral RNA Mini Kit; pooled kidney samples were extracted using the IDEXX RealPCR DNA/RNA Spin Column Kit. Extracted RNA samples were tested with the RealPCR CSFV RNA Test using standard test reagents and protocol.

Results/conclusions: Results for the individual and pooled serum and kidney samples are shown in tables 21 and 22 respectively. Positive serum samples were detected in all 20 sample pools. Results were similar for the kidney samples, with 15 out of 15 positive results for the 10-sample pools. All the individual samples had early Ct values. It is expected that if a weak sample is pooled (for instance, Ct value >32), the pool may test negative due to some loss of sensitivity caused by the dilution effect of pooling.

Table 21. Detection of CSFV in serum pools using the RealPCR CSFV RNA Test

	Individual samples			Pools of 20		
Number	CSFV Ct	ISC Ct	Result	CSFV Ct	ISC Ct	Result
1	16.8	23.1	Positive	22.0	26.4	Positive
2	12.1	25.4	Positive	16.2	26.7	Positive
3	14.9	29.1	Positive	19.6	26.8	Positive
4	19.2	24.2	Positive	26.1	28.2	Positive
5	18.3	24.0	Positive	23.6	27.1	Positive
6	19.3	24.2	Positive	25.4	27.0	Positive
7	15.9	24.6	Positive	21.0	27.5	Positive
8	17.9	29.1	Positive	25.5	27.3	Positive
9	23.6	24.6	Positive	29.6	27.2	Positive
10	22.6	29.2	Positive	28.0	27.0	Positive
11	11.9	26.1	Positive	16.3	27.3	Positive
12	22.9	27.6	Positive	28.3	27.8	Positive
13	18.6	27.6	Positive	24.0	27.5	Positive
14	16.1	27.9	Positive	21.3	27.2	Positive
15	19.7	25.9	Positive	24.3	27.2	Positive

Table 22. Detection of CSFV in kidney sample pools using the RealPCR CSFV RNA Test

	Individual samples			Pools of 10		
Number	**CSFV Ct**	**ISC Ct**	**Result**	**CSFV Ct**	**ISC Ct**	**Result**
187	15.8	19.5	Positive	20.2	22.5	Positive
188	19.5	13.0	Positive	22.0	16.5	Positive
189	18.4	13.2	Positive	21.3	17.2	Positive
190	23.4	12.5	Positive	27.0	16.6	Positive
191	20.8	12.2	Positive	23.9	17.5	Positive
192	24.9	10.7	Positive	28.1	17.0	Positive
193	19.1	13.5	Positive	23.2	18.2	Positive
194	15.8	17.8	Positive	19.2	20.5	Positive
195	19.7	11.3	Positive	22.8	17.0	Positive
196	19.2	14.6	Positive	22.0	18.4	Positive
197	15.8	14.2	Positive	18.0	19.8	Positive
198	19.0	10.5	Positive	22.4	15.3	Positive
199	16.3	14.9	Positive	19.4	19.4	Positive
200	18.2	19.6	Positive	20.3	20.0	Positive
201	14.8	13.9	Positive	17.6	18.0	Positive

XVII. Limit of detection—complete method (LD_{method})

Purpose: To determine the relative limit of detection of the RealPCR CSFV RNA Test compared to two other real-time PCR CSFV tests.

Procedure: Dilution series were prepared from positive serum, with 10-fold dilutions of moderately strong positive samples diluted into negative serum. Samples were extracted with the QIAamp Viral RNA Mini Kit and then tested with the RealPCR CSFV RNA Test and a published method[1] using standard test reagents and protocol for each method.

Results/conclusions: Table 23 shows the relative limit of detection for the RealPCR CSFV RNA Test for three different serum samples. The final dilution detected with the RealPCR CSFV RNA Test was 1:1,000 to 1:10,000. This was equivalent to or greater than the last dilution detected by the published PCR[1] for each of the samples.

Table 23. Limit of detection for RealPCR CSFV RNA Test in serum samples

Genotype	Sample dilution	RealPCR CSFV	Published PCR
Genotype 2.1	1:10	30	33.2
	1:100	32.1	35.4
	1:1,000	36.2	No Ct
	1:10,000	35.1	39.2
	1:100,000	No Ct	No Ct
Genotype 2.3	1:10	30.2	31.6
	1:100	32.8	34.6
	1:1,000	36.2	No Ct
	1:10,000	No Ct	No Ct
	1:100,000	No Ct	No Ct
Genotype 2.2	1:10	29.3	30.6
	1:100	31.3	33.8
	1:1,000	33.6	No Ct
	1:10,000	No Ct	No Ct
	1:100,000	No Ct	No Ct

Reference:

1. Hoffmann B, Beer M, Schelp C, Schirrmeier H, Depner K. Validation of a real-time RT-PCR assay for sensitive and specific detection of classical swine fever. *J Virol Methods*. 2005;130(1–2):36–44.

XVIII. Conclusions

A. Characteristics of PCR

- Detection of classical swine fever virus
- Exclusive detection of classical swine fever virus
- Analytical sensitivity of 10 copies per reaction
- PCR efficiency of approximately 106%
- Repeatability <2% CV
- Robustness for variations in time, temperature, and sample volume for the PCR reaction
- Reagent stability demonstrated at >15 months
- PCR instruments validated: Applied Biosystems* 7500, Applied Biosystems* ViiA* 7, Applied Biosystems QuantStudio* 5, Agilent Mx3000P*, Agilent Mx3005P*, Agilent AriaMx*, Bio-Rad CFX96 Touch*, Bio Molecular Systems Mic qPCR Cycler, QIAGEN* Rotor-Gene* (72-well rotor only), Roche LightCycler* 480, or equivalent

B. Characteristics of the complete method

- Diagnostic sensitivity of 100% (n = 193)
- Diagnostic specificity of 100% (n = 350)
- Relative limit of detection equivalent to or better than reference CSFV PCR Test for serum samples.
- Detection of CSFV in the following sample types:
    - Blood (EDTA) samples and pools of up to 20 samples
    - Serum samples and pools of up to 20 samples
    - Plasma samples and pools of up to 20 samples
    - Oral fluids (composite sample up to 30 pigs)
    - Swabs (fecal and blood)
    - Tissue samples (spleen, kidney, lymph node, and tonsil) and pools of up to 10 samples
- Sample extraction with the following methods:
    - RealPCR DNA/RNA Spin Column Kit (IDEXX)
    - NucleoSpin RNA Kit (Machery Nagel)
    - QIAamp Viral RNA Mini Kit (Qiagen)

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