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virotype® CSFV 2.0 RT-PCR Kit

Validation Report

For the detection of RNA from the *Classical Swine Fever Virus* (CSFV)

INDICAL BIOSCIENCE

Contents

1 Introduction

1.1 Intended use

The virotype CSFV 2.0 RT-PCR Kit is intended for the detection of RNA from *Classical Swine Fever Virus* (CSFV) in blood, tissue, swabs, and samples in stabilizing transport media from pigs and wild boar.

The kit is approved by the Friedrich-Loeffler-Institut and licensed in accordance with § 11 (2) of the German Animal Health Act (FLI-C 113) for use in Germany for veterinary diagnostic procedures.

For veterinary use only.

1.2 General information

The virotype CSFV 2.0 RT-PCR Kit is a highly sensitive and specific solution for the detection of RNA from *Classical Swine Fever Virus* (CSFV) in samples from pigs and wild boar. Classical Swine Fever (CSF) is economically one of the most important viral infectious diseases of swine. CSF is widespread in domestic pig and wild boar populations. CSF is an internationally notifiable animal disease. The causative agent, *Classical Swine Fever Virus*, is a single-stranded RNA virus and a member of the genus *Pestivirus* which belongs to the *Flaviviridae* family like *Bovine Viral Diarrhea Virus* (BVDV) in cattle and *Border Disease Virus* (BDV) in sheep.

The high sensitivity of the virotype CSFV 2.0 RT-PCR Kit allows the early detection of the pathogen in individual or pooled blood samples, in individual or pooled tissue samples, as well as from swabs, and samples in stabilizing transport media from pigs and wild boar. The virotype CSFV 2.0 RT-PCR Kit detects all known CSFV-strains of various genotypes. In rare cases the test may detect CSF vaccine virus. Positive results of animals from areas where CSFV vaccination is performed should therefore be verified.

1.3 Description of the test principle

Polymerase chain reaction (PCR) is based on the amplification of specific regions of the pathogen genome. In real-time PCR, the amplified product is identified using fluorescent dyes. These are usually linked to oligonucleotide probes that bind specifically to the amplified product. Monitoring the fluorescence intensities during the PCR run (i.e., in real-time) allows detection of the accumulating product without the need to re-open the reaction tubes afterward.

The virotype CSFV 2.0 RT-PCR Kit contains all the necessary reagents for the detection of CSFV RNA, including a Positive and Negative Control. With this kit, both reverse transcription and PCR are performed in one reaction tube, reducing the risk of contamination.

An Internal Control excludes the possibility of false-negative results. The kit uses two specific primer/probe combinations:

- FAM™ fluorescence for RNA of CSFV
- JOE™ fluorescence for the endogenous Internal Control (EC; β-actin present within the sample)

A Positive Control serves to verify the functionality of the reaction mix for the amplification of the CSFV RNA target.

1.4 Kit contents

virotype CSFV 2.0 RT-PCR Kit	**(96)**
Cat. no.	**VT281825**
Number of reactions	**96**
Master Mix (tube with orange cap), includes primers, probes and enzymes	2 x 980 µl
Positive Control (tube with red cap)	1 x 150 µl
Negative Control (tube with blue cap)	1 x 150 µl
Handbook	1

1.5 Storage

The components of the virotype CSFV 2.0 RT-PCR Kit should be stored at -30°C to -15°C and are stable until the expiration date stated on the label. Avoid repeated thawing and freezing (> 3x), as this may reduce assay sensitivity. Freeze the components in aliquots if they will only be used intermittently.

1.6 Equipment and reagents to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- Pipets
- Nuclease-free, aerosol-resistant pipet tips with filters
- Sterile 1.5 ml Eppendorf® tubes
- Nuclease-free (RNase/DNase-free) consumables. Special care should be taken to avoid nuclease contamination of all reagents and consumables used to set up PCR for sensitive identification of viral nucleic acids
- Cooling device or ice
- Benchtop centrifuge with rotor for 1.5 ml tubes
- Real-time cycler with appropriate fluorescent channels
- Appropriate software for chosen real-time cycler
- Appropriate strip tubes and caps or 96-well optical microplate with optical sealing film or cover for chosen real-time cycler

1.7 RNA extraction

The virotype CSFV RT-PCR Kit can be used for the detection of CSFV RNA from blood, tissue, swabs, and samples in stabilizing transport media from pigs and wild boar.

Due to the high sensitivity of the test individual or pooled samples can be tested. For domestic pigs, up to 20 individual serum, plasma, or EDTA-blood samples or up to 10 tissue samples can be used. Furthermore, for wild boar, pools can consist of up to 10 serum, plasma, EDTA-blood, or tissue samples. Also swab samples and samples in stabilizing transport media from pigs and wild boar can be tested.

Note: For use in Germany the specifications described in the „*Amtliche Methodensammlung*" apply.

Prior to real-time PCR, viral RNA must be extracted from the starting material. INDICAL offers a range of validated kits for the extraction of RNA from animal samples.

Extraction based on magnetic beads:

- IndiMag® Pathogen Kit* (SP947457)
- IndiMag Pathogen Kit* w/o plastics (SP947257)
- IndiMag Pathogen IM2 Cartridge (SP957654C608)
- IndiMag Pathogen IM48 Cartridge (SP947654P608, SP947654P224)
- IndiMag Pathogen KF96 Cartridge (SP947855P196, SP947855P496)

Extraction based on spin columns:

- IndiSpin® Pathogen Kit* (SP54104, SP54106)
- IndiSpin QIAcube® HT Pathogen Kit (SP54161)

* suitable for simultaneous extraction of CSFV RNA und ASFV DNA

Note: When using difficult sample material, it is recommended to use INDICAL's „Pretreatment T4 (phenol extraction)".

If real-time RT-PCR is not performed immediately after extraction, store the RNA at -20°C or at -80°C for longer storage.

For further information on automated and manual extraction of CSFV RNA from different sample types, refer to the respective handbook or contact INDICAL Support at support@indical.com.

1.8 Important notes

General precautions

The user should always pay attention to the following:

- Use nuclease-free pipet tips with filters.
- Store and extract positive materials (specimens, positive controls and amplicons) separately from all other reagents and add them to the reaction mix in a spatially separated facility.
- Thaw all components on ice before starting the assay.
- When thawed, mix the components by inverting and centrifuge briefly.
- Do not use components of the test kit past the expiration date.
- Keep samples and controls on ice or in a cooling block during the setup of reactions.

Negative control

At least one negative control reaction should be included in each PCR run, containing all the components of the reaction except for the pathogen template. This enables assessment of contamination in the reaction.

Positive control

When performing PCR on unknown samples, it is recommended to include a positive control reaction in the PCR run, containing a sample that is known to include the targeted viral RNA. A positive control serves to prove the functionality of the pathogen assay, for example, the correct setup of the reaction mix. Use 5 µl of the Positive Control provided with the virotype CSFV 2.0 RT-PCR Kit to test for successful amplification of the target.

Extraction and amplification control

For increased process safety and convenience, one extraction and amplification control assay is included in the test kit.

An endogenous internal control (EC) detects the β-actin gene present within the sample.
This allows extraction and amplification to be monitored.

2 Procedure

2.1 Important points before starting

- Please read „Important notes“ before starting.
- Include at least one positive control (Positive Control) and one negative control (Negative Control) per PCR run.
- Before beginning the procedure, read through the protocol and ensure that you are familiar with the operation of the chosen real-time PCR cycler.
- RNA is unstable. Perform the protocol without interruption.

2.2 Things to do before starting

- Thaw all reagents on ice and protect from light.
- Maintain reagents on ice during PCR setup.
- Maintain reagents on ice or in a cooling block during PCR setup.

2.3 Test procedure

1. Before use, mix the Master Mix by inverting 5 times or until mixed thoroughly, then centrifuge briefly to collect the fluids.
2. Pipet 20 µl of the Master Mix into each reaction tube. Then add 5 µl of the sample RNA (Table 1).

   Include positive and negative control reactions.

   Positive Control: Use 5 µl of the positive control (Positive Control) instead of sample RNA.

   Negative Control: Use 5 µl of the negative control (Negative Control) instead of sample RNA.

Table 1. Preparation of reaction mix

Component	Volume
Master Mix	20 µl
Sample	5 µl
Total volume	**25 µl**

3. Close the reaction tubes or seal the plate and invert 5 times or until mixed thoroughly. Then centrifuge briefly to collect the fluids.

4. Set the filters for the reporter dyes in the software of your thermal cycler according to Table 2.

Table 2. Filter settings for the reporter

Pathogen/ Internal Control	Reporter
CSFV	FAM
Endogenous Internal Control	HEX/ JOE™[1]
Passive reference[2]	Texas Red/ ROX™

[1] Use the option appropriate for your thermal cycler.

[2] Internal reference for use with Applied Biosystems® ABI PRISM® Sequence Detection Systems

5. Run the real-time PCR protocol according to Table 3.

Table 3. Real-time RT-PCR protocol for CSFV 2.0.

Step	Temperature	Time	Number of cycles
Reverse Transcription	50°C	10 min	1
Initial Activation	95°C	2 min	1
2-step cycling			
Denaturation	95°C	5 s	40
Annealing/Extension*	60°C	30 s	

* Fluorescence data collection, approximate run time 68 min (Bio-Rad™ CFX96)

Note: The above protocol can also be used in combination with the virotype ASFV 2.0 PCR Kit.

3 Data interpretation

Interpretation of results

For the assay to be valid, the Positive Control must give a signal in the FAM and HEX/ JOE channels with a C_T < 35. The Negative Control must give no signal.

The following results are possible if working with unknown samples. The possible sample results are also summarized in Table 4.

The sample is positive for CSFV, and the assay is valid, if the following criteria are met:

- The sample yields a signal in the FAM channel (regardless of any signal in the HEX/ JOE channel).
- The Positive Control yields a signal in the FAM and HEX/ JOE channel.
- The Negative Control does not yield a signal in the FAM and HEX/ JOE channel.

Note that very high concentrations of CSFV RNA in the sample may lead to a reduced signal or no signal for the endogenous Internal Control (EC; HEX/ JOE channel) due to competition with the internal control.

The sample is negative for CSFV, and the assay is valid, if the following criteria are met:

- The sample **does not yield** any signal in the FAM channel.
- The sample yields a signal in only the HEX/ JOE channel.
- The Positive Control yields a signal in the FAM and HEX/ JOE channels.
- The Negative Control does not yield a signal in the FAM and HEX/ JOE channels.

The sample results are inconclusive, and the assay is invalid, if the following occurs:

- The sample yields no signal in the FAM and HEX/ JOE channels.

If no signal is detected in the FAM (CSFV) and the HEX/ JOE (endogenous Internal Control, EC) channel, the result is inconclusive. The absence of a signal for the housekeeping gene indicates strong PCR inhibition and/or other malfunctions, e.g., during extraction.

To check for inhibition, we recommend 1:5 dilution of the sample RNA in nuclease free water, to repeat the RNA extraction, or repeat the whole test procedure starting with new sample material.

Check that there is a fluorescence signal in the FAM channel for the positive control reaction (Positive Control). Absence of a signal for the Positive Control indicates an error, which could be due to incorrect setup of the reaction mix or incorrect cycling conditions.

Table 4. Results interpretation table*

Sample result	**FAM (CSFV)**	**HEX/JOE (EC)**
CSFV positive	X	X
CSFV strong positive	X	
CSFV negative		X
Inconclusive		

* Interpretation of sample results can be determined provided positive and negative control reactions are performed. The positive control must yield a signal in the FAM and HEX/ JOE channels. The negative control must yield no signal in any channel. For a complete explanation of possible sample results, please refer to “Data interpretation”.

4 Characteristics of the test

4.1 Analytical sensitivity

4.1.1 Analytical sensitivity using the Bio-Rad CFX96 instrument

The high analytical sensitivity of the virotype CSFV 2.0 RT-PCR Kit was verified by a titration series of *Classical Swine Fever Virus* (CSFV) *in vitro* RNA [10^6 – 1 copies/well], performed in triplicates of relevant dilutions using the virotype CSFV 2.0 RT-PCR protocol on the Bio-Rad CFX96™ (Bio-Rad Laboratories, Inc., Hercules, USA) instrument.

Results / Conclusion

The virotype CSFV 2.0 RT-PCR Kit is able to detect up to ten CSFV virus copies per sample (Table 5, Figure 1 and Figure 2). There is a high correlation between RNA copy number and amplification results. A correlation coefficient of 0.985 with an efficiency of 108.1 % for the *in vitro* RNA was calculated when using the virotype CSFV 2.0 RT-PCR Kit on the Bio-Rad CFX96 instrument (Figure 2).

Table 5. Individual and mean C_T values of **CSFV** (FAM) *in vitro* RNA titration series in triplicates. The test was performed on the Bio-Rad CFX96 instrument using the virotype CSFV 2.0 real-time RT-PCR protocol.

Type	Copy number	C_T (FAM)	C_T mean	SD	Result
Standard	10^6	22.51	22.55	0.05	+
Standard	10^6	22.60			+
Standard	10^6	22.55			+
Standard	10^5	25.87	25.70	0.19	+
Standard	10^5	25.73			+
Standard	10^5	25.49			+
Standard	10^4	28.89	28.75	0.32	+
Standard	10^4	28.39			+
Standard	10^4	28.97			+
Standard	10^3	31.76	31.77	0.07	+
Standard	10^3	31.84			+
Standard	10^3	31.70			+
Standard	100	35.10	34.78	0.36	+
Standard	100	34.39			+
Standard	100	34.85			+
Standard	50	36.62	35.83	0.85	+
Standard	50	34.93			+
Standard	50	35.95			+
Standard	25	38.41	37.08	1.16	+
Standard	25	36.54			+
Standard	25	36.30			+
Standard	10	39.83	39.83	-	+
Standard	10	-			-
Standard	10	-			-
Standard	1	-	-	-	-
Standard	1	-			-
Standard	1	-			-

SD = standard deviation, - = no C_T

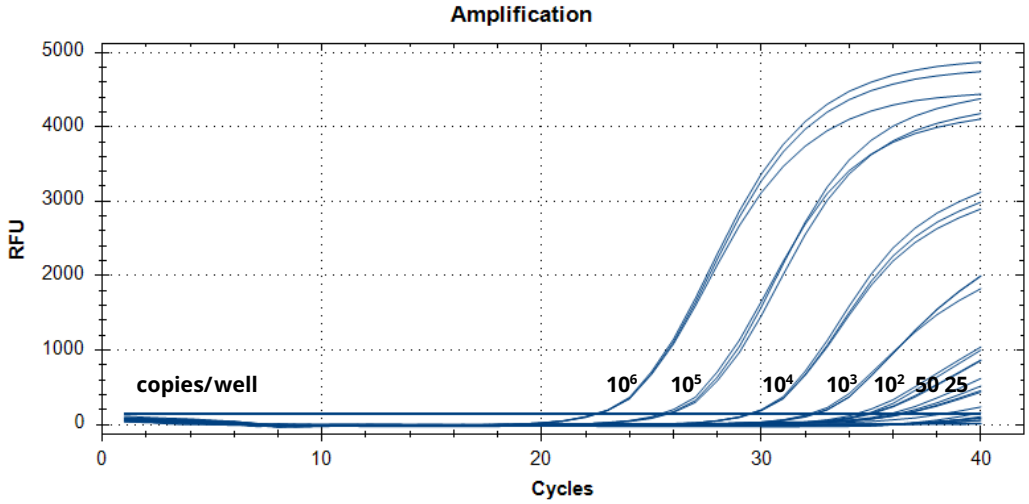

Figure 1. Individual values of a titration series of **CSFV** (FAM) *in vitro* RNA in triplicates. The test was performed on the Bio-Rad CFX96 instrument using the virotype CSFV 2.0 real-time RT-PCR protocol.

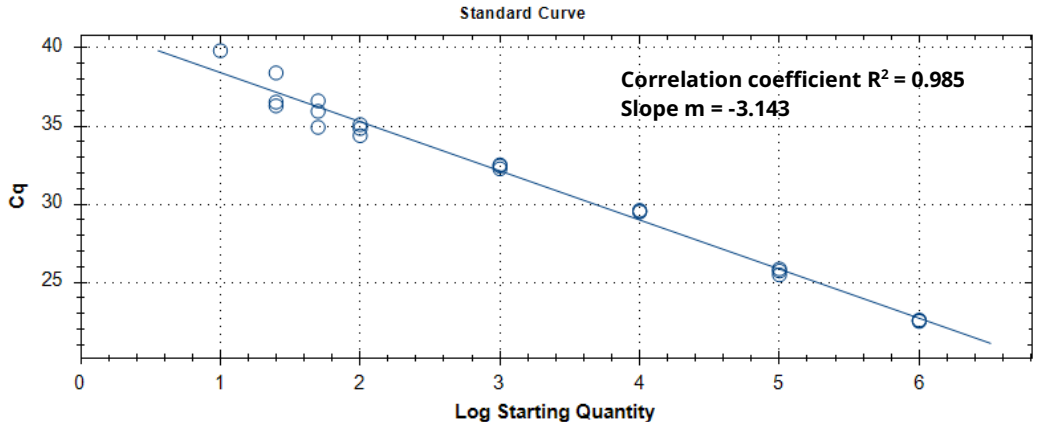

Figure 2. Standard curve of obtained C_T values for a titration series of **CSFV** (FAM) *in vitro* RNA. The test was performed on the Bio-Rad CFX96 instrument using the virotype CSFV 2.0 real-time RT-PCR protocol.

4.1.2 Analytical sensitivity – Limit of detection

The limit of detection (LOD) for the target sequence of the *Classical Swine Fever Virus* was determined by testing individual titration series of *in vitro* RNA of this sequence in octuplicates. The limit of detection with 95 % confidence interval ($LOD_{95\,\%}$: mean number of copies yielding a probability of detection of 0.95) was determined using the web tool https://quodata.de/content/validation-qualitative-pcr-methods-single-laboratory.

Results / Conclusion

Results are summarized in Table 6 and Figure 3. Using the virotype CSFV 2.0 RT-PCR Kit, a high correlation between RNA copy number and the amount of amplified product was demonstrated for the CSFV targeted sequence.

The $LOD_{95\%}$ is 32.9 copies/reaction with a 95 % confidence interval of [18.632, 58.582] (Figure 3).

Table 6. Limit of detection for CSFV *in vitro* RNA tested in octuplicates on the Bio-Rad CFX96 instrument using the virotype CSFV 2.0 real-time RT-PCR protocol.

Copies/test	Total number of replicates	Number of replicates positive	Number of replicates negative
1000,000	8	8	0
100,000	8	8	0
10,000	8	8	0
1,000	8	8	0
100	8	8	0
50	8	8	0
25	8	8	0
10	8	3	5
1	8	1	7
0.1	8	0	8

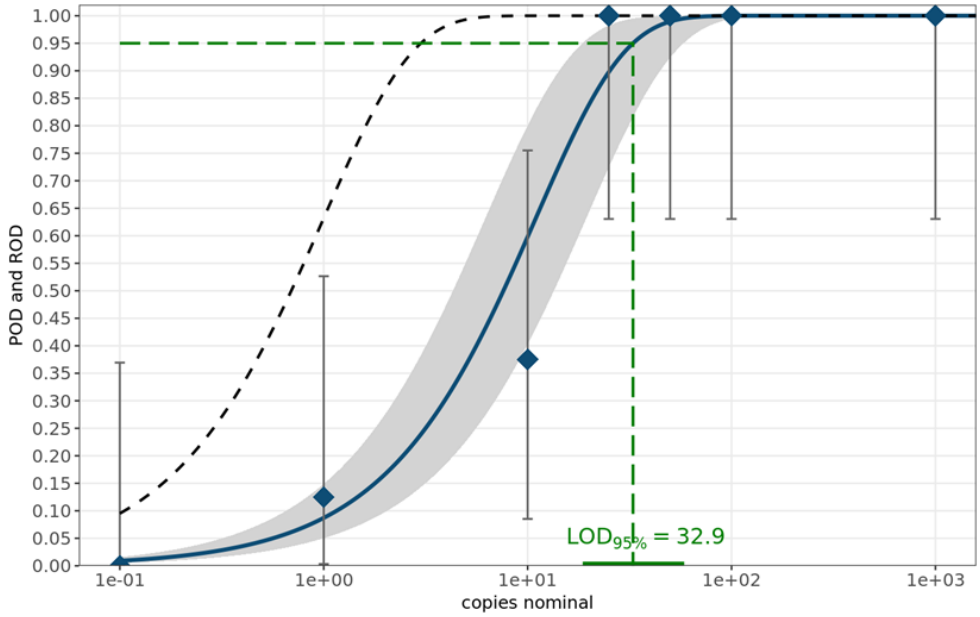

Figure 3. POD (probability of detection) curve and $LOD_{95\,\%}$ for CSFV. The blue diamonds characterize the laboratory-specific rates of detection. The blue curve denotes the mean POD curve along with the corresponding 95 % confidence range highlighted as the grey band. The POD curve under ideal conditions is displayed as the black dashed curve.

4.1.3 Testing of pooled lysates

Pools were generated by mixing nucleic acids from CSFV positive blood, tonsil and swab samples from domestic pigs and wild boars with nucleic acids extracted from a CSFV negative blood sample using the IndiMag Pathogen Kit (INDICAL Bioscience GmbH) according to manufacturer's instructions. Samples were tested with the virotype CSFV 2.0 RT-PCR Kit on the Bio-Rad CFX96 instrument.

Results / Conclusion

The C_T values of the CSFV (FAM) signals of individual and pooled samples are shown in Table 7. CSFV RNA can be reliably detected with the virotype CSFV 2.0 RT-PCR Kit in pools of 5, 10 and 20 of different sample matrices.

Table 7. Testing of individual and pool samples using the virotype CSFV 2.0 RT-PCR Kit on the Bio-Rad CFX96 instrument using the virotype CSFV 2.0 RT-PCR protocol.

		C_T (CSFV; FAM)			
Sample	**Material**	**Individual sample**	**Pool of 5**	**Pool of 10**	**Pool of 20**
1	Blood dp	25.26	26.97	28.64	29.57
2	Blood dp	29.56	31.44	32.64	33.63
3	Blood dp	34.67	35.83	37.68	38.75
4	Plasma dp	21.17	22.29	24.13	25.11
5	Plasma dp	28.21	29.88	31.85	32.98
6	Plasma dp	35.73	36.03	38.01	39.32
7	Tonsil dp	27.67	31.76	32.69	33.09
8	Swab dp	27.31	29.21	31.51	32.09
9	Blood wb	29.51	33.26	34.51	35.33
10	Blood wb	26.64	27.66	29.59	30.14
11	Blood wb	32.57	33.94	35.11	36.98
12	Blood wb	22.77	23.61	26.27	27.40

dp = domestic pig; wb = wild boar

4.2 Specificity

4.2.1 Comparative analysis of the virotype CSFV 2.0 RT-PCR Kit and other commercially available RT-PCR Kits using reference samples (in-house testing)

CSFV positive RNA samples from cell culture, wild boars and pigs and dilutions thereof were provided by PIWet (Polish National Reference Laboratory for CSFV / PAŃSTWOWY INSTYTUT WETERYNARYJNY), the European Reference Laboratory for CSFV (TiHo Hannover) as well as the German National Reference Laboratory for CSFV (Friedrich-Loeffler-Institut, Riems, Germany) (Table 8). The RNA samples were tested using the virotype CSFV 2.0 RT-PCR Kit on the Bio-Rad CFX96 instrument.

Results / Conclusion

Results obtained with the virotype CSFV 2.0 RT-PCR Kit show better or equal sensitivity compared to some competitor RT-PCR kits, especially kits A and C (Table 9).

Table 8. Samples for comparative testing of the virotype CSFV 2.0 RT-PCR Kit and other commercially available RT-PCR Kits

Sample	CSFV status	Origin	Material	Strain (genotype)	Dilution
1	positive	PIWet	Cell culture	Alfort 187 (gt 1.1)	1:10
2	positive	PIWet	Cell culture	Brescia (gt 1.1)	1:10
3	positive	EURL	Pig blood	CSF385 (gt 2.3)	1:100
4	negative	INDICAL	Wild boar blood	-	-
5	positive	EURL	Pig serum	CSF1060 (gt 2.2)	1:10
6	positive	EURL	Pig plasma	CSF309 (gt 3.4)	1:10
7	NC	INDICAL		Negative Control (virotype CSFV 2.0 RT-PCR Kit, alpha test)	
8	PC	INDICAL		Positive Control (virotype CSFV 2.0 RT-PCR Kit, alpha test)	
9 - 16		FLI (FLI-1)	Pig blood	unknown	10^{-1} - 10^{-6}
17 - 24		FLI (FLI-2)	Pig tonsil	Israel (gt 2.1)	10^{-1} - 10^{-5}

NC = Negative Control, PC = Positive Control, PIWet = PAŃSTWOWY INSTYTUT WETERYNARYJNY, EURL = European Reference Laboratory, FLI = Friedrich-Loeffler-Institut, gt = genotype

Table 9. Comparative analysis of the virotype CSFV 2.0 RT-PCR Kit and competitor kits for CSFV (FAM signal).

Sample	Material	Status	C_T (CSFV)				
			virotype CSFV 2.0 RT-PCR Kit	**Kit A**	**Kit B**	**Kit C**	**Kit D**
#1	Cell culture	pos	30.26	31.54	30.05	33.14	31.21
#2	Cell culture	pos	27.09	27.20	27.45	30.36	28.17
#3	Blood	pos	32.77	-	35.92	-	36.47
#4	Blood	neg	-	-	-	-	-
#5	Blood	pos	29.77	29.99	30.97	32.58	30.99
#6	Blood	pos	30.10	30.23	31.74	33.40	31.18
#7	NC	neg	-	-	-	-	-
#8	PC	pos	29.47	31.21	-	-	-
#9	Blood	FLI 1 (10^{-1})	20.38	20.80	21.72	25.23	22.65
#10		FLI 1 (10^{-2})	23.50	23.75	25.22	29.01	26.75
#11		FLI 1 (10^{-3})	26.42	27.15	28.94	32.18	30.12
#12		FLI 1 (10^{-4})	29.51	30.39	31.83	35.68	32.80
#13		FLI 1 (10^{-5})	33.26	35.26	35.28	39.70	37.29
#14		FLI 1 ($10^{-5.5}$)	34.51	35.97	35.61	-	36.33
#15		FLI 1 ($10^{-5.8}$)	35.33	-	36.34	-	36.39
#16		FLI 1 (10^{-6})	36.72	-	37.58	-	38.23
#17	Tonsil	FLI 2 (10^{-1})	24.74	26.31	26.72	28.75	28.52
#18		FLI 2 (10^{-2})	27.67	30.14	29.72	32.56	31.44
#19		FLI 2 (10^{-3})	31.76	34.47	33.22	38.39	35.42
#20		FLI 2 ($10^{-3.5}$)	32.69	35.56	33.79	36.38	34.96
#21		FLI 2 ($10^{-3.8}$)	33.09	-	34.87	37.73	37.39
#22		FLI 2 (10^{-4})	34.74	-	36.57	38.72	37.82
#23		FLI 2 ($10^{-4.5}$)	36.15	-	36.85	-	38.37
#24		FLI 2 (10^{-5})	-	-	-	-	-

4.2.2 Inclusivity: Detection of CSFV and other genetically related *pestiviruses*

Cell culture RNA samples from the Epizone panel of different relevant CSFV genotypes (Friedrich-Loeffler-Insitut) were tested with the virotype CSFV 2.0 RT-PCR Kit on the Bio-Rad CFX96 instrument using the virotype CSFV 2.0 RT-PCR protocol.

Results/Conclusion

All available CSFV genotypes were correctly detected when using the virotype CSFV 2.0 RT-PCR Kit (Table 10).

Table 10. Testing CSFV cell culture samples of the Epizone panel with the virotype CSFV 2.0 RT-PCR on the Bio-Rad CFX96 instrument using the virotype CSFV 2.0 real-time RT-PCR protocol.

	Sample	**Dilution tested**	**virotype CSFV 2.0 RT-PCR Kit C_T (CSFV)**
***Classical Swine Fever Virus* (CSFV)**	CSFV-C strain_gt1.1	1:10	33.38
	CSFV-Eystrup91_gt1.1	1:100	38.52
	CSFV-Alfort187_gt1.1	1:100	38.52
	CSFV-Koslov1128_gt1.2	1:100	37.73
	CSFV-Brescia_gt1.2	1:10	33.37
	CSFV-Schweiz II_gt2.1	1:100	37.80
	CSFV-Pader_gt2.1	1:100	39.62
	CSFV-Bergen_gt2.2	1:10	33.62
	CSFV-D4886/82/Ro_gt2.2	-	31.31
	CSFV-Uelzen_gt2.3	-	29.69
	CSFV-Spante_gt2.3	1:10	31.81
	CSFV-Congenital Tremor_gt3.1	-	32.01
	CSFV-Kanagawa_gt3.4	-	31.15
	CSFV-CSF 1027	1:100	24.10
	CSFV-CSF 1024	1:100	28.08
	CSFV-CSF 0867	1:100	24.49
	CSFV-CSF 0866	1:100	28.34
	CSFV-CSF 0864	1:100	23.66

CSFV-CSF1015	1:100	24.09
CSFV-CSF 0852	1:100	27.62
CSFV-CSF 0854	1:100	23.69
CSFV-CSF 848	1:100	30.23
CSFV-CSF 847	1:100	25.22
CSFV-CSF 0840	1:100	26.49
CSFV-CSF 0838	1:100	29.81
CSFV-CSF 0822	1:100	25.01

- = no C_T, gt = genotype

4.2.3 Exclusivity: Discrimination of other genetically related *pestiviruses*

Pestivirus RNA samples from the Epizone panel (Friedrich-Loeffler-Insitute) were tested with the virotype CSFV 2.0 RT-PCR Kit on the Bio-Rad CFX96 instrument using the virotype CSFV 2.0 RT-PCR protocol.

Results/Conclusion

No cross-reactivity to other relevant *pestiviruses* was observed when using the virotype CSFV 2.0 RT-PCR Kit (Table 11).

Table 11. Testing cell culture samples of the Epizone panel with the virotype CSFV 2.0 RT-PCR on the Bio-Rad CFX96 instrument using the virotype CSFV 2.0 real-time RT-PCR protocol.

	Sample	**Dilution tested**	**virotype CSFV 2.0 RT-PCR Kit C_T (CSFV)**
***Border Disease Virus* (BDV)**	BDV-Moredun_gt1	-	-
	BDV-Rudolph_gt2	-	-
	BDV-Gifhorn_gt3	-	-
	BDV-Isard_gt4	-	-
	BDV-2 ST 1507	-	-
	BDV-1 SF6/87	-	-
	BDV-1 137/4	-	-
***Bovine Viral Diarrhea Virus* (BVDV)**	BVDV-1-WUS 5708	-	-
	BVDV-1-BO806-17	-	-
	BVDV-1-Arnsby 1599	-	-
	BVDV-1b-Grub	-	-
	BVDV-1-17R507	-	-
	BVDV-1-NADL_gt1a	-	-
	BVDV-1-Paplitz_gt1b	-	-
	BVDV-1-PI809_gt1d	-	-
	BVDV-1-NC3807-1251/1_gt1e	-	-
	BVDV-1-Egbert_gt1f	-	-
	BVDV-1-BO806-17_gt1g	-	-

	BVDV-1-BO807-3_gt1h	-	-
	BVDV-1-NC3807-8757_gt1x	-	-
	BVDV-2-Bure	-	-
	BVDV-2-10/01	-	-
	BVDV-2-P01600	-	-
	BVDV-2-Vepfe	-	-
	BVDV-2-8644_gt2a G	-	-
	BVDV-2-Bure_gt2a US	-	-
	BVDV-2-Walter_gt2b	-	-
	BVDV-2-PO1600_gt2c	-	-
	BVDV-Bayonce	-	-
Pestivirus	Hobi gt atypical	-	-
	Giraffe H138_gtatypical	-	-

- = no C_T, gt = genotype

4.2.4 Exclusivity: Discrimination of pathogens for differential diagnosis

Cross-reactivity was tested with samples positive for *African Swine Fever Virus* (ASFV), *Porcine Circovirus-2* (PCV-2), *Swine Influenza Virus* (SIV) und *Porcine Reproductive and Respiratory Syndrome Virus* (PRRSV). The samples were kindly provided by the Friedrich-Loeffler-Institut and other State Veterinary Laboratories.

Results/Conclusion

No cross-reactivity to other relevant porcine viral pathogens was detected using the virotype CSFV 2.0 RT-PCR Kit (Table 12).

Table 12. Cross-reactivity of the virotype CSFV 2.0 RT-PCR Kit to other swine-related pathogens on the Bio-Rad CFX96 instrument using the virotype CSFV 2.0 real-time RT-PCR protocol.

	Sample	**Sample material**	**virotype CSFV 2.0 RT-PCR Kit**	**Reference assay***
			C_T (CSFV)	**C_T (Pathogen)**
***African Swine Fever Virus* (CSFV)**	Arm07 (gt II)	Lymph node	-	23.77
	Sardinia-ws12-4	Blood	-	17.08
	Kenia05-hs07-7	Blood	-	25.82
***Porcine Circrovirus-2* (PCV-2)**	PCV-2_1	Serum	-	29.73
	PCV-2_2	Serum	-	30.76
	PCV-2_3	Serum	-	38.87
	PCV-2_4	Serum	-	24.63
	PCV-2_5	Serum	-	29.54
***Swine Influenza Virus* (SIV)**	SIV-01	Blood	-	22.81
	SIV-02	Blood	-	26.62
	SIV-03	Blood	-	29.77

***Porcine Reproductive and Respiratory Syndrome Virus* (PRRSV)**	Intervet 10-3	Culture	-	22.38
	Stendal V852-10-3	Culture	-	22.34
	USA 18-18-10-4	Culture	-	26.09
	PRRSV-22	Serum	-	31.59
	PRRSV-23	Serum	-	24.42
	PRRSV-24	Serum	-	25.33

* Reference assay were as follows: virotype ASFV 2.0 PCR Kit (ASFV samples), virotype PCV2/PCV3 Reagent (PCV-2 samples), virotype Influenza A RT-PCR Kit (SIV samples), virotype PRRSV RT-PCR Kit (PRRSV samples)

4.3 Diagnostic sensitivity, specificity and efficiency

4.3.1 Definition diagnostic sensitivity

Percentage of positive samples in the new test of a population of true positive samples. True positive samples giving negative results in the new test are termed false negative.

Calculation: [true positives / (true positives + false negatives)]*100

4.3.2 Definition diagnostic specificity

Percentage of negative samples in the new test of a population of true negative samples. True negative samples giving positive results in the new test are termed false positive.

Calculation: [true negatives / (false positives + true negatives)]*100

4.3.3 Definition diagnostic efficiency

Diagnostic efficiency refers to the amount of agreement between the results from the new test and those from the reference test. It is expressed as a proportion of correctly identified samples among all samples.

Calculation: [(true positives + true negatives) / (true positives + true negatives + false positives + false negatives)]*100

4.3.4 Validation of the virotype CSFV 2.0 RT-PCR Kit

For validation of the virotype CSFV 2.0 RT-PCR Kit, *n* = 472 samples [blood/ blood swab (*n* = 156), tissue (*n* = 107), plasma (*n* = 85), serum (*n* = 55) and cell culture (*n* = 69)] were tested. Tissue samples comprised of undefined tissues samples (*n* = 50), tonsils (*n* = 38), pancreas (*n* = 12) and lymph nodes (*n* = 7).

Positive reference samples were kindly provided by the Tiermedizinische Hochschule Hannover (TiHo Hannover, EURL for CSFV), the Friedrich-Loeffler-Institut (German NRL for CSFV) and PIWet (Polish NRL for CSFV). The CSFV-positive samples comprised of eight different genotypes (1.1, 1.2, 1.3, 2.1, 2.2, 2.3, 3.1, and 3.4).

Negative reference samples from domestic pigs and wild boars were kindly provided by the Friedrich-Loeffler-Institut and PIWet. Additionally, CSFV-negative blood and blood swab samples were acquired from two German state veterinary laboratories or were collected in-house during different hunting seasons. The latter samples were processed using the IndiMag Pathogen Kit (INDICAL Bioscience) according to the manufacturer's instructions and subsequently tested using the virotype CSFV 2.0 RT-PCR Kit as well as the reference Kit A.

Results/ Conclusion

The summary is shown in Table 13. Individual results are shown in Figure 4 and Table 14.

All positive CSFV samples, even the very weak positives, were detected correctly with the virotype CSFV 2.0 RT-PCR Kit. The virotype CSFV 2.0 RT-PCR Kit demonstrated a diagnostic sensitivity of 100 %, a diagnostic specificity of 100 % and a diagnostic efficiency of 100 %. In this study the virotype CSFV 2.0 RT-PCR Kit demonstrated an overall higher sensitivity (with lower C_T values) compared to in-house reference methods.

Table 13. Diagnostic sensitivity, specificity and efficiency of the virotype CSFV 2.0 RT-PCR Kit.

virotype CSFV 2.0 RT-PCR Kit (CSFV; FAM)		**Comparative data**			
Total	**472**	**Reference positive**	**296**	**Reference negative**	**176**
positive	296	true-positive	296	false-positive	0
negative	176	false-negative	0	true-negative	176

Diagnostic sensitivity:	100 %
Diagnostic specificity:	100 %
Diagnostic efficiency:	100 %

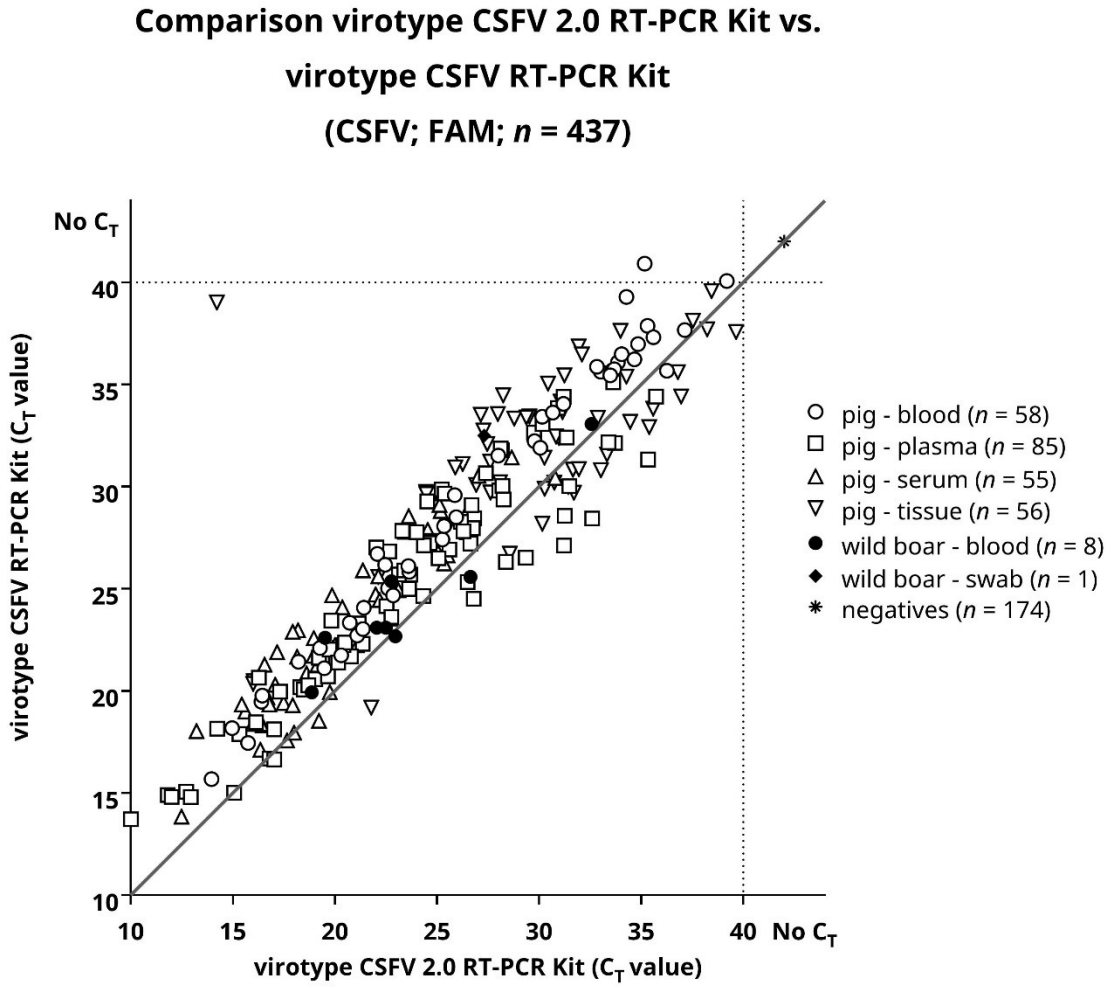

Figure 4. Comparison of C_T values from CSFV-positive and negative samples tested with the virotype CSFV 2.0 RT-PCR Kit compared to the virotype CSFV RT-PCR Kit. All samples situated above the black diagonal line showed lower C_T values with the virotype CSFV 2.0 RT-PCR Kit than tested with the virotype CSFV RT-PCR Kit. Please note that CSFV-positive cell culture samples (*n* = 35) that were tested in a 1:10/ 1:100 dilution (virotype CSFV 2.0 RT-PCR Kit) and values for the virotype CSFV RT-PCR Kit (undiluted samples) were omitted from this graph. CSFV-negative samples are not differentiated by sample type or origin.

Table 14. List of CSFV-positive cell culture samples (*n* = 35) tested with the virotype CSFV RT-PCR Kit during validation.

Sample number	Genotype	CSFV status	virotype CSFV 2.0 RT-PCR Kit	
			C_T (CSFV)	C_T (Internal Control)
132	1.1	pos	33.38	-
133	1.1	pos	38.52	-
134	1.1	pos	38.52	-
192	1.1	pos	28.07	23.22
193	1.1	pos	24.35	21.65
194	1.1	pos	24.69	21.38
135	1.2	pos	37.73	-
136	1.2	pos	33.37	-
195	1.2	pos	24.82	20.65
196	1.2	pos	23.42	20.98
137	2.1	pos	37.80	-
138	2.1	pos	39.62	-
139	2.2	pos	33.62	-
140	2.2	pos	31.31	-
200	2.2	pos	25.99	21.84
141	2.3	pos	29.69	-
142	2.3	pos	31.81	-
197	2.3	pos	25.38	20.59
198	2.3	pos	27.78	21.45
199	2.3	pos	28.48	21.12
143	3.1	pos	32.01	-
144	3.4	pos	31.15	-
163	unknown	pos	24.10	-
164	unknown	pos	28.08	-
165	unknown	pos	24.49	-

166	unknown	pos	28.34	32.16
167	unknown	pos	23.66	38.22
168	unknown	pos	24.09	38.66
169	unknown	pos	27.62	-
170	unknown	pos	23.69	39.28
171	unknown	pos	30.23	30.91
172	unknown	pos	25.22	33.36
173	unknown	pos	26.49	33.80
174	unknown	pos	29.81	33.24
175	unknown	pos	25.01	-

neg = negative, pos = positive, n.d. = not determined, - = no C_T

4.3.5 Testing of different sample types

Altogether n = 148 samples derived from various domestic pig and wild boar sample types (*n* = 1 CSFV-positive blood swab from domestic pig, *n* = 7 CSFV-negative blood swab from wild boar, *n* = 85 CSFV-positive plasma samples as well as *n* = 55 CSFV-positive serum samples from domestic pig) were tested with the virotype CSFV 2.0 RT-PCR Kit on the Bio-Rad CFX96 instrument.

Results / Conclusion

The test results are presented in Table 15. All samples were correctly identified by the virotype CSFV 2.0 RT-PCR Kit. In a direct comparison of samples that have a quantifiable reference result, the virotype CSFV 2.0 RT-PCR kit shows a higher sensitivity (mean ΔC_T = -2.05, not shown).

Table 15. List of blood swab, plasma and serum samples tested with the virotype CSFV 2.0 RT-PCR Kit during validation.

Sample	Species	Geno-type	CSFV status	Reference Assay		virotype CSFV 2.0 RT-PCR Kit	
				C_T (CSFV)	C_T (Control)	C_T (CSFV)	C_T (IC)
Blood swabs (*n* = 8)							
106	wb	n.d.	pos	32.50	n.d.	27.31	19.19
466	wb	n.d.	neg	-	n.d.	-	28.62
467	wb	n.d.	neg	-	n.d.	-	27.17
468	wb	n.d.	neg	-	n.d.	-	28.43
469	wb	n.d.	neg	-	n.d.	-	32.58
470	wb	n.d.	neg	-	n.d.	-	29.73
471	wb	n.d.	neg	-	n.d.	-	25.31
472	wb	n.d.	neg	-	n.d.	-	32.13
Plasma (*n* = 85)							
201	dp	2.1	pos	pos	n.d.	21.26	26.85
202	dp	2.1	pos	pos	n.d.	21.42	26.97
203	dp	2.1	pos	pos	n.d.	24.53	29.88
204	dp	2.1	pos	pos	n.d.	22.24	34.52
205	dp	2.1	pos	pos	n.d.	22.46	34.22
206	dp	2.1	pos	pos	n.d.	25.72	-
207	dp	1.1	pos	16.69	n.d.	16.80	28.41
208	dp	1.1	pos	15.01	n.d.	15.07	30.04
209	dp	3.4	pos	29.81	n.d.	28.05	31.48
210	dp	3.4	pos	27.77	n.d.	26.46	31.62

211	dp	3.4	pos	23.30	n.d.	21.17	31.19
212	dp	3.4	pos	27.21	n.d.	26.64	36.98
213	dp	3.4	pos	21.59	n.d.	19.38	31.20
214	dp	3.4	pos	21.62	n.d.	19.22	31.09
215	dp	3.4	pos	22.02	n.d.	19.74	31.20
216	dp	1.3	pos	30.03	n.d.	28.21	14.85
217	dp	1.3	pos	28.45	n.d.	26.83	30.94
218	dp	1.3	pos	32.65	n.d.	29.75	30.76
219	dp	2.2	pos	27.96	n.d.	26.75	30.27
220	dp	2.2	pos	29.37	n.d.	28.26	28.47
221	dp	2.2	pos	26.92	n.d.	25.61	30.51
222	dp	2.1	pos	32.13	n.d.	33.72	28.58
223	dp	2.1	pos	32.17	n.d.	33.40	28.15
224	dp	2.2	pos	27.35	n.d.	24.73	29.63
225	dp	2.1	pos	34.41	n.d.	35.73	27.38
226	dp	1.3	pos	29.28	n.d.	24.53	32.31
227	dp	1.3	pos	29.86	n.d.	25.24	30.97
228	dp	2.2	pos	25.69	n.d.	23.69	30.11
229	dp	2.2	pos	27.24	n.d.	24.64	28.20
230	dp	2.1	pos	30.02	n.d.	31.48	30.26
231	dp	2.1	pos	28.57	n.d.	31.26	28.09
232	dp	2.2	pos	24.15	n.d.	22.53	29.84
233	dp	2.1	pos	31.32	n.d.	35.34	29.02
234	dp	1.3	pos	24.65	n.d.	24.33	37.67
235	dp	1.3	pos	26.49	n.d.	25.08	38.51
236	dp	1.3	pos	27.13	n.d.	24.36	34.26
237	dp	2.2	pos	23.62	n.d.	22.77	31.94
238	dp	2.2	pos	24.99	n.d.	23.63	30.89
239	dp	2.2	pos	22.30	n.d.	21.36	30.19
240	dp	2.1	pos	26.52	n.d.	29.35	30.45
241	dp	2.1	pos	27.12	n.d.	31.22	30.29
242	dp	2.2	pos	21.69	n.d.	20.79	31.25
243	dp	2.1	pos	28.44	n.d.	32.60	28.89
244	dp	1.3	pos	20.57	n.d.	19.04	28.43
245	dp	1.3	pos	25.70	n.d.	23.13	36.68
246	dp	2.2	pos	20.18	n.d.	18.31	29.97
247	dp	2.2	pos	22.24	n.d.	20.42	30.66
248	dp	2.2	pos	20.71	n.d.	19.66	31.53

249	dp	2.1	pos	25.33	n.d.	26.50	32.17
250	dp	2.1	pos	26.32	n.d.	28.37	30.93
251	dp	2.2	pos	18.12	n.d.	17.02	31.12
252	dp	2.1	pos	24.51	n.d.	26.80	27.90
253	dp	1.1	pos	16.64	n.d.	17.03	29.15
254	dp	3.4	pos	33.07	n.d.	30.17	29.92
255	dp	1.1	pos	15.06	n.d.	12.72	29.50
256	dp	3.4	pos	31.78	n.d.	28.18	29.82
257	dp	2.3	pos	31.86	n.d.	28.12	30.07
258	dp	1.1	pos	14.90	n.d.	11.82	27.92
259	dp	3.4	pos	29.66	n.d.	25.38	29.38
260	dp	2.3	pos	30.65	n.d.	27.40	29.45
261	dp	3.4	pos	27.77	n.d.	24.01	31.23
262	dp	2.3	pos	29.09	n.d.	26.70	31.20
263	dp	3.4	pos	26.83	n.d.	22.66	32.70
264	dp	2.3	pos	25.90	n.d.	23.40	32.74
265	dp	2.3	pos	22.37	n.d.	20.50	31.59
266	dp	3.4	pos	21.39	n.d.	20.16	30.88
267	dp	2.3	pos	19.97	n.d.	17.31	29.15
268	dp	3.4	pos	23.44	n.d.	19.83	31.51
269	dp	2.3	pos	20.65	n.d.	16.28	27.87
270	dp	2.3	pos	27.02	n.d.	22.03	29.93
271	dp	2.3	pos	27.83	n.d.	23.31	30.41
272	dp	2.3	pos	28.27	n.d.	26.18	30.21
273	dp	2.3	pos	32.40	n.d.	31.35	27.59
274	dp	2.3	pos	20.07	n.d.	18.48	29.67
275	dp	1.1	pos	18.47	n.d.	16.14	27.42
276	dp	1.1	pos	14.80	n.d.	12.95	28.24
277	dp	1.1	pos	14.81	n.d.	12.01	27.91
278	dp	1.1	pos	18.15	n.d.	14.24	28.57
279	dp	2.3	pos	33.85	n.d.	30.92	29.37
280	dp	2.3	pos	35.10	n.d.	33.63	33.10
281	dp	2.3	pos	34.40	n.d.	31.20	32.41
282	dp	1.1	pos	13.72	n.d.	10.01	28.94
283	dp	1.1	pos	20.27	n.d.	18.70	31.66
284	dp	1.1	pos	17.89	n.d.	15.33	29.16
285	dp	2.3	pos	27.81	n.d.	26.30	33.21

Serum (*n* = 55)

286	dp	2.3	pos	24.44	n.d.	21.88	31.03
287	dp	2.3	pos	30.52	n.d.	27.03	36.45
288	dp	2.3	pos	28.80	n.d.	25.18	34.72
289	dp	2.3	pos	27.79	n.d.	23.43	32.69
290	dp	2.3	pos	28.13	n.d.	23.44	33.24
291	dp	2.3	pos	28.69	n.d.	26.42	32.05
292	dp	2.3	pos	31.43	n.d.	28.66	32.65
293	dp	2.3	pos	24.09	n.d.	20.37	31.08
294	dp	2.3	pos	19.41	n.d.	17.49	30.10
295	dp	2.3	pos	21.01	n.d.	19.26	31.59
296	dp	2.3	pos	25.90	n.d.	21.37	39.01
297	dp	2.3	pos	22.96	n.d.	18.20	37.33
298	dp	2.3	pos	22.89	n.d.	17.94	31.42
299	dp	2.3	pos	28.43	n.d.	26.77	30.61
300	dp	2.3	pos	27.88	n.d.	24.55	35.71
301	dp	2.3	pos	28.55	n.d.	23.62	39.51
302	dp	2.3	pos	29.67	n.d.	25.24	34.79
303	dp	2.3	pos	20.85	n.d.	18.62	36.90
304	dp	2.3	pos	18.38	n.d.	15.98	31.10
305	dp	2.3	pos	24.68	n.d.	19.86	32.03
306	dp	2.3	pos	21.67	n.d.	18.15	36.88
307	dp	2.3	pos	18.99	n.d.	15.62	30.12
308	dp	2.3	pos	21.89	n.d.	17.17	30.56
309	dp	1.1	pos	14.87	n.d.	9.63	28.76
310	dp	1.1	pos	20.30	n.d.	17.08	30.37
311	dp	1.1	pos	19.33	n.d.	15.44	28.69
312	dp	2.3	pos	24.72	n.d.	22.00	33.43
313	dp	2.3	pos	22.24	n.d.	20.00	31.83
314	dp	2.3	pos	19.68	n.d.	16.70	30.82
315	dp	2.3	pos	29.10	n.d.	25.12	30.17
316	dp	2.3	pos	22.58	n.d.	18.97	29.52
317	dp	2.3	pos	26.86	n.d.	22.24	30.18
318	dp	2.3	pos	18.03	n.d.	13.23	27.75
319	dp	2.3	pos	21.28	n.d.	16.56	28.77
320	dp	2.3	pos	19.33	n.d.	16.79	28.52
321	dp	2.3	pos	18.53	n.d.	19.22	30.58
322	dp	1.1	pos	18.31	n.d.	16.47	28.63
323	dp	1.1	pos	22.09	n.d.	19.89	22.35

324	dp	1.1	pos	13.84	n.d.	12.49	29.01
325	dp	2.3	pos	24.93	n.d.	23.11	32.23
326	dp	2.3	pos	21.72	n.d.	18.95	26.53
327	dp	2.3	pos	21.27	n.d.	19.15	29.18
328	dp	2.3	pos	22.23	n.d.	21.08	31.25
329	dp	2.3	pos	17.12	n.d.	16.35	31.93
330	dp	2.3	pos	19.30	n.d.	17.94	31.46
331	dp	2.3	pos	19.94	n.d.	19.75	22.10
332	dp	2.3	pos	17.57	n.d.	17.66	29.60
333	dp	2.3	pos	17.96	n.d.	18.00	30.24
334	dp	n.d.	pos	30.42	n.d.	30.79	30.54
335	dp	2.3	pos	18.38	n.d.	16.38	30.95
336	dp	1.1	pos	23.54	n.d.	22.77	31.87
337	dp	2.3	pos	26.63	n.d.	25.52	33.75
338	dp	2.3	pos	24.44	n.d.	22.19	33.25
339	dp	1.1	pos	26.24	n.d.	25.35	30.55
340	dp	2.1	pos	25.61	n.d.	22.13	31.72

dp = domestic pig, wb = wild boar, neg = negative, pos = positive, n.d. = not determined, - = no C_T

4.3.6 Testing of artificial swab samples

Blood samples were artificially created by mixing serum samples from CSFV positive (n = 28, samples 1-28) and CSFV negative (n = 22, samples 29-50) serum samples with CSFV negative domestic pig blood sample. Serum samples 1- 6 and 29 - 31 were included in the ring trial 2014 of the Friedrich-Loeffler-Institut (FLI), Germany. Serum samples 7-11, 14-28 and 32-43 were included in the ring trial trial 2009 of the European Reference Laboratory for CSFV (EURL-CSFV), Germany. Serum samples 12 - 13 and 44-47 were included in the ring trial 2010 of the EURL-CSFV. Serum samples 48 - 50 were included in the ring trial 2012 of the EURL-CSFV. Positive serum samples included CSFV serum samples from genotype 1.3, 2.1, and 2.3.

The artificial blood sample (100 µl) was pipetted on cotton swabs thus generating the swab samples. Swabs were dried and RNA was extracted using the pretreatment for swabs (Pretreatment S1) with the IndiMag Pathogen Kit (INDICAL) on the KingFisher™ Flex instrument (ThermoFisher Scientific™ Inc., Waltham, USA) according to manufacturer's instructions. Samples were tested with the virotype CSFV 2.0 RT-PCR Kit on the Bio-Rad CFX96 instrument.

Results / Conclusion

The C_T values of the CSFV (FAM) signals of swab samples are shown in Table 16. CSFV RNA from swab samples can be reliably detected with the virotype CSFV 2.0 RT-PCR.

Table 16. Testing of artificial swab samples with the virotype CSFV 2.0 RT-PCR Kit on the Bio-Rad CFX96 instrument using the virotype CSFV 2.0 RT-PCR protocol.

Sample number	Genotype	CSFV status	virotype CSFV 2.0 RT-PCR Kit	
			C_T (CSFV)	C_T (Internal Control)
1	unknown	pos	39.99	27.45
2	unknown	pos	33.32	26.69
3	unknown	pos	23.96	28.42
4	unknown	pos	29.72	26.70
5	unknown	pos	26.20	26.67
6	unknown	pos	37.11	27.20
7	1.3	pos	36.55	28.70
8	1.3	pos	32.38	28.36
9	2.1	pos	38.96	28.07
10	2.1	pos	35.07	28.97
11	2.1	pos	37.73	27.93
12	2.1	pos	37.09	26.71
13	2.1	pos	33.35	27.01
14	2.3	pos	30.75	27.58
15	2.3	pos	27.52	27.31
16	2.3	pos	34.03	27.38
17	2.3	pos	37.96	27.66
18	2.3	pos	32.23	27.84
19	2.3	pos	27.66	27.53
20	2.3	pos	35.68	27.91
21	2.3	pos	35.42	27.60
22	2.3	pos	30.99	27.54
23	2.3	pos	27.70	27.74
24	2.3	pos	35.31	28.47

25	2.3	pos	38.30	28.94
26	2.3	pos	30.04	28.73
27	2.3	pos	33.37	28.51
28	unknown	pos	29.46	28.32
29		neg	-	27.76
30		neg	-	31.59
31		neg	-	26.47
32		neg	-	28.01
33		neg	-	28.46
34		neg	-	27.67
35		neg	-	28.97
36		neg	-	28.37
37		neg	-	28.43
38		neg	-	27.67
39		neg	-	27.47
40		neg	-	27.36
41		neg	-	28.62
42		neg	-	28.76
43		neg	-	31.57
44		neg	-	28.06
45		neg	-	28.03
46		neg	-	27.10
47		neg	-	27.62
48		neg	-	30.56
49		neg	-	30.84
50		neg	-	28.76

4.4 Repeatability

The same sample panel comprising five CSFV-positive RNA samples (samples 1-3, 5 and 6), one CSFV-negative sample (sample 3) and the controls (NC, PC) of the test kit was used for assessment of intra-assay variance, inter-assay variance, batch-to-batch variance, stability testing and for comparison of test results obtained with the virotype CSFV 2.0 RT-PCR Kit tested on different real-time PCR thermocyclers (Table 17).

Table 17. Sample panel for assessment of intra-assay variance, inter-assay variance, batch-to-batch variance, stability and for comparison of test results obtained with the virotype CSFV 2.0 RT-PCR Kit on different real-time PCR thermocyclers. Samples 1-3 and 5-6 were diluted in extracted pig blood RNA to obtain signals for the endogenous Internal Control (JOE).

Sample	CSFV status	Origin	Material	Strain (genotype)	Dilution
1	positive	PIWet	Cell culture	Alfort 187 (gt 1.1)	1:10
2	positive	PIWet	Cell culture	Brescia (gt 1.1)	1:10
3	positive	EURL	Pig blood	CSF385 (gt 2.3)	1:100
4	negative	INDICAL	Wild boar blood	-	-
5	positive	EURL	Pig serum	CSF1060 (gt 2.2)	1:10
6	positive	EURL	Pig plasma	CSF309 (gt 3.4)	1:10
NC	NC	INDICAL		Negative Control (virotype CSFV 2.0 RT-PCR Kit, alpha test)	
PC	PC	INDICAL		Positive Control (virotype CSFV 2.0 RT-PCR Kit, alpha test)	

NC = Negative Control, PC = Positive Control, PIWet = PAŃSTWOWY INSTYTUT WETERYNARYJNY, EURL = European Reference Laboratory, FLI = Friedrich-Loeffler-Institut, gt = genotype

4.4.1 Intra-assay variance

The sample panel listed in Table 17 was tested in a sevenfold setup in the same PCR run with the virotype CSFV 2.0 RT-PCR Kit (batch Valid-5) on the Bio-Rad CFX96 instrument using the virotype CSFV 2.0 RT-PCR protocol.

Results / Conclusion

The intra-assay variance is on average 0.72 % for CSFV (FAM) and 0.89 % for the endogenous Internal Control (JOE) (Table 18, Table 19, and Figure 5).

Table 18. Intra-assay variance for **CSFV** (FAM) for the virotype CSFV 2.0 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Intra-assay variance for CSFV (FAM)											
Sample	**CSFV status**	**Reactions (C_T values)**							**Mean**	**SD**	**CV%**
		1	**2**	**3**	**4**	**5**	**6**	**7**			
1	pos	30.52	30.64	31.02	30.75	30.43	30.47	30.72	30.65	0.205	0.669
2	pos	27.06	27.20	27.50	27.21	27.10	27.07	27.24	27.20	0.153	0.563
3	pos	33.78	33.24	33.24	33.10	33.65	33.26	33.36	33.38	0.244	0.731
4	neg	-	-	-	-	-	-	-	-	-	-
5	pos	30.31	30.02	30.27	30.34	30.44	30.37	30.36	30.30	0.134	0.443
6	pos	30.76	30.41	31.03	30.37	30.63	30.85	30.78	30.69	0.237	0.773
NC	neg	-	-	-	-	-	-	-	-	-	-
PC	pos	30.31	29.77	29.74	29.48	30.17	30.27	29.63	29.91	0.333	1.112
Mean											**0.72**

NC = Negative Control, PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Table 19. Intra-assay variance for the **endogenous Internal Control** (JOE) for the virotype CSFV 2.0 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Intra-assay variance for the endogenous Internal Control (JOE)											
Sample	**CSFV status**	**Reactions (C_T values)**							**Mean**	**SD**	**CV%**
		1	**2**	**3**	**4**	**5**	**6**	**7**			
1	pos	22.19	22.06	22.14	22.17	22.46	22.17	22.25	22.20	0.127	0.572
2	pos	21.86	21.94	22.28	22.28	22.00	21.95	22.03	22.05	0.167	0.757
3	pos	22.78	22.76	23.04	22.62	22.58	22.97	22.75	22.79	0.169	0.741
4	neg	20.88	20.59	20.54	20.47	20.34	20.44	19.99	20.47	0.268	1.307
5	pos	22.88	22.56	23.03	23.04	22.82	23.06	22.45	22.83	0.244	1.068
6	pos	22.59	22.70	23.02	22.68	22.87	22.97	23.08	22.85	0.188	0.824
NC	neg	-	-	-	-	-	-	-	-	-	-
PC	pos	24.68	24.67	24.26	24.05	24.45	24.57	24.48	24.45	0.230	0.941
Mean											**0.89**

NC = Negative Control, PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

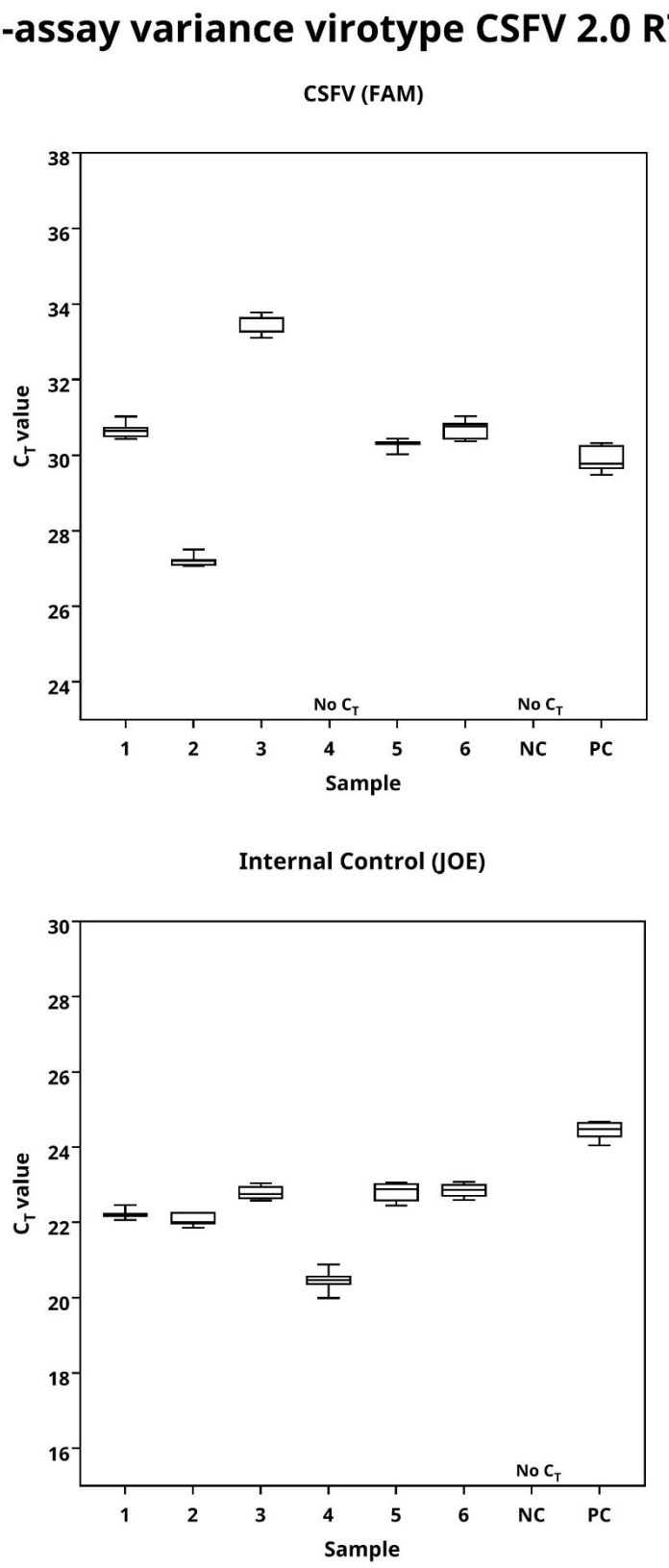

Figure 5. Boxplots of intra-assay variance for **CSFV** (FAM) and the **endogenous Internal Control** (JOE) for the virotype CSFV 2.0 RT-PCR Kit tested using the Bio-Rad CFX96 instrument.

4.4.2 Inter-assay variance

The sample panel listed in Table 17 was tested in seven independent PCR runs using the virotype CSFV 2.0 RT-PCR Kit (batch Valid-5) on the Bio-Rad CFX96 instrument using the virotype CSFV 2.0 RT-PCR protocol.

Results / Conclusion

The inter-assay variance is on average 0.82 % for CSFV (FAM) and 0.92 % for the endogenous Internal Control (JOE) (Table 20, Table 21, and Figure 6).

Table 20. Inter-assay variance for **CSFV** (FAM) for the virotype CSFV 2.0 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Inter-assay variance for CSFV (FAM)											
Sample	**CSFV status**	**RT-PCR runs (C_T values)**							**Mean**	**SD**	**CV%**
		1	**2**	**3**	**4**	**5**	**6**	**7**			
1	pos	30.72	30.59	31.09	30.58	30.63	30.72	30.98	30.76	0.198	0.644
2	pos	27.24	27.05	27.51	27.25	27.34	27.16	27.51	27.29	0.172	0.630
3	pos	33.36	33.41	34.24	33.24	34.03	33.65	34.01	33.71	0.389	1.154
4	neg	-	-	-	-	-	-	-	-	-	-
5	pos	30.36	29.81	30.40	30.15	30.38	30.06	30.48	30.23	0.240	0.794
6	pos	30.78	30.47	30.64	30.42	30.43	30.29	30.85	30.55	0.207	0.677
NC	neg	-	-	-	-	-	-	-	-	-	-
PC	pos	29.63	29.70	29.34	29.62	29.61	29.08	30.03	29.57	0.296	1.002
Mean											**0.82**

NC = Negative Control, PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Table 21. Inter-assay variance for the **endogenous Internal Control** (JOE) for the virotype CSFV 2.0 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Inter-assay variance for the endogenous Internal Control (JOE)											
Sample	**CSFV status**	**RT-PCR runs (C_T values)**							**Mean**	**SD**	**CV%**
		1	**2**	**3**	**4**	**5**	**6**	**7**			
1	pos	22.25	22.36	22.35	21.86	22.21	22.65	22.41	22.30	0.240	1.076
2	pos	22.03	22.09	22.46	22.25	22.67	22.72	22.59	22.40	0.280	1.250
3	pos	22.75	23.10	23.00	23.09	22.89	22.94	23.12	22.98	0.136	0.590
4	neg	19.99	20.15	19.85	20.19	19.98	19.94	20.03	20.02	0.118	0.589
5	pos	22.45	22.28	22.19	22.25	22.91	22.57	22.28	22.42	0.253	1.130
6	pos	23.08	23.30	23.22	22.97	22.71	22.86	23.02	23.02	0.203	0.881
NC	neg	-	-	-	-	-	-	-	-	-	-
PC	pos	24.48	24.21	24.87	24.67	24.74	24.80	24.74	24.64	0.229	0.927
Mean											**0.92**

NC = Negative Control, PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

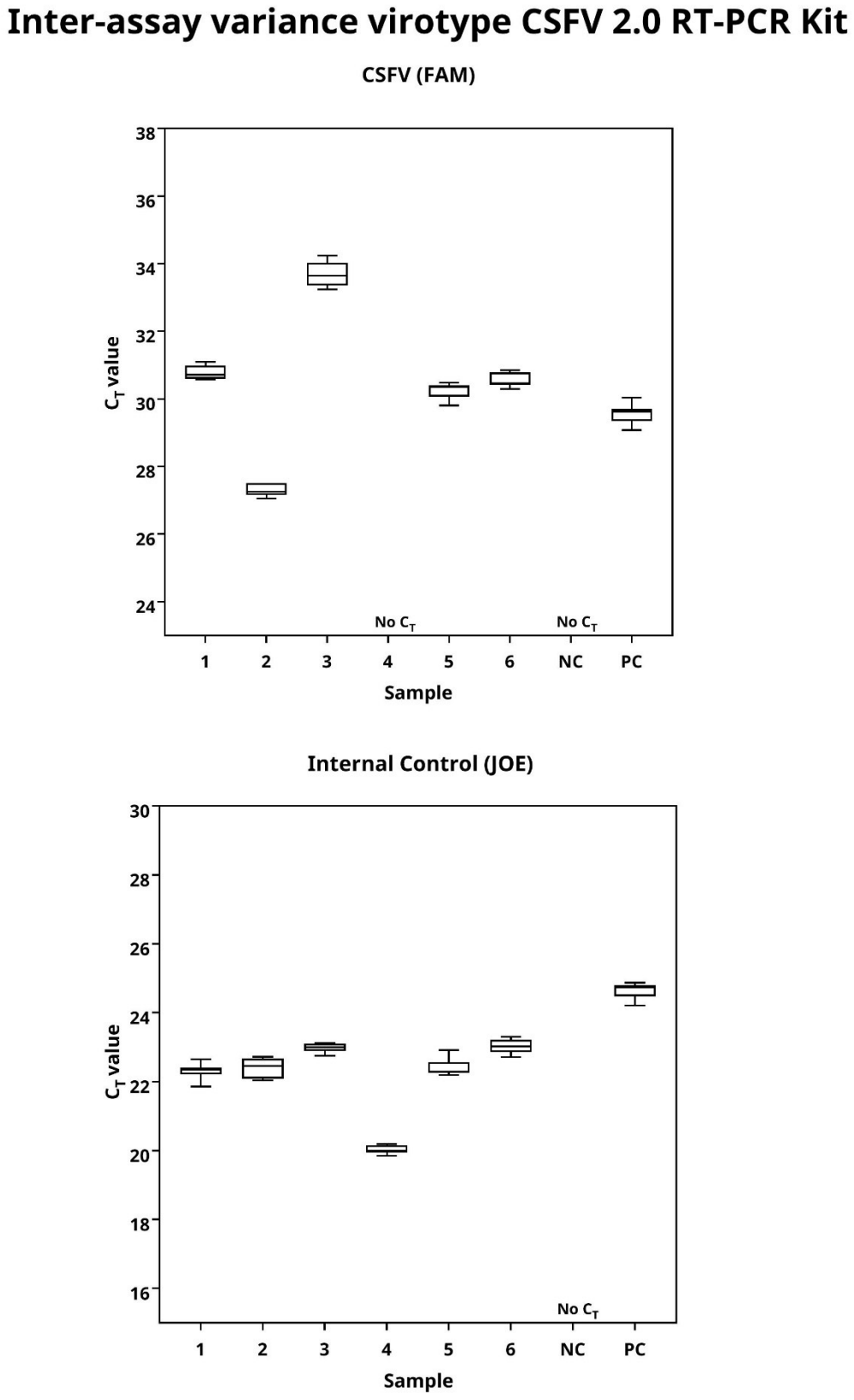

Figure 6. Boxplots of inter-assay variance for **CSFV** (FAM) and the **endogenous Internal Control** (JOE) for the virotype CSFV 2.0 RT-PCR Kit tested using the Bio-Rad CFX96 instrument.

4.4.3 Batch-to-batch comparison

The sample panel listed in Table 17 was tested in the same PCR run using three different batches of the CSFV 2.0 RT-PCR Kit (batch 1 = Valid-4; batch 2 = Valid- 5; batch 3 = Valid-6) on the Bio-Rad CFX96 instrument using the virotype CSFV 2.0 RT-PCR protocol.

Results / Conclusion

The batch-to-batch performance showed on average variance of 0.82 % for CSFV (FAM) and 1.00 % for the endogenous Internal Control (JOE) (Table 22, Table 23, and Figure 7).

Table 22. Batch-to-batch variance for **CSFV** (FAM) for the virotype CSFV 2.0 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Batch-to-batch variance for CSFV (FAM)							
Sample	**CSFV status**	**Batch number (C_T values)**			**Mean**	**SD**	**CV%**
		1	**2**	**3**			
1	pos	30.97	30.95	29.93	30.62	0.595	1.942
2	pos	27.42	27.27	27.41	27.36	0.086	0.314
3	pos	33.60	33.07	33.59	33.42	0.300	0.898
4	neg	-	-	-	-	-	-
5	pos	30.82	30.46	30.73	30.67	0.188	0.613
6	pos	30.91	30.81	30.75	30.82	0.082	0.266
NC	neg	-	-	-	-	-	-
PC	pos	29.58	29.31	29.06	29.32	0.263	0.896
Mean							**0.82**

NC = Negative Control, PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Table 23. Batch-to-batch variance for the **endogenous Internal Control** (JOE) for the virotype CSFV 2.0 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Batch-to-batch variance for the endogenous Internal Control (JOE)							
Sample	**CSFV status**	**Batch number (C_T values)**			**Mean**	**SD**	**CV%**
		1	**2**	**3**			
1	pos	23.40	23.17	22.90	23.15	0.250	1.079
2	pos	22.31	22.43	22.01	22.25	0.215	0.966
3	pos	23.23	23.10	22.33	22.89	0.487	2.126
4	neg	20.85	20.63	20.83	20.77	0.124	0.597
5	pos	23.34	23.49	23.74	23.52	0.198	0.843
6	pos	23.79	23.69	23.56	23.68	0.118	0.496
NC	neg	-	-	-	-	-	-
PC	pos	25.31	25.18	25.63	25.37	0.232	0.914
Mean							**1.00**

NC = Negative Control, PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

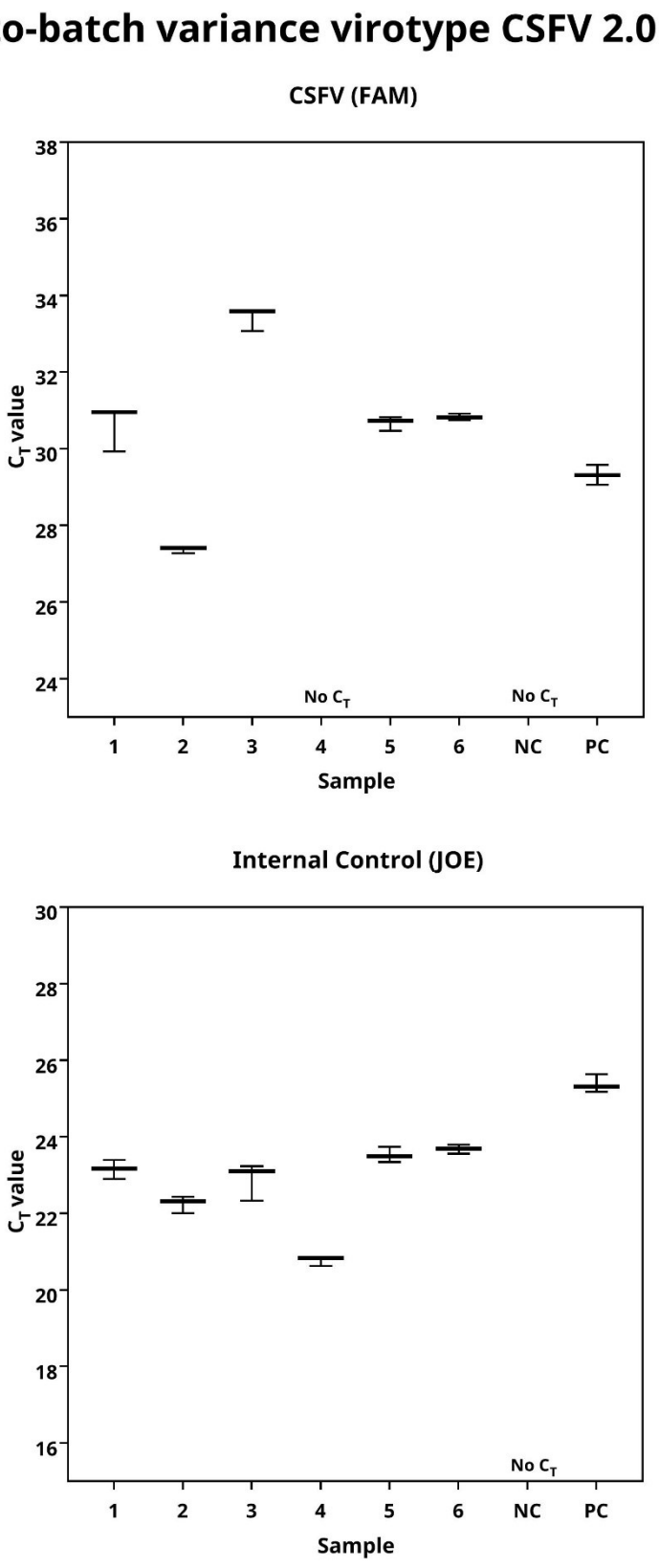

Figure 7. Boxplots of batch-to-batch variance for **CSFV** (FAM) and the **endogenous Internal Control** (JOE) for the virotype CSFV 2.0 RT-PCR Kit tested using the Bio-Rad CFX96 instrument.

4.4.4 Comparison of real-time PCR thermocyclers

The virotype CSFV 2.0 RT-PCR Kit can be used on different standard real-time PCR cycler. Table 24 gives an overview of selected PCR cyclers and their approximate run times, using the virotype CSFV 2.0 RT-PCR protocol.

Note: The use of the RT-PCR Kit is not limited to the mentioned instruments.

Table 24. Selected overview of real-time thermocyclers and their approximate run times for the virotype CSFV 2.0 RT-PCR protocol.

	Thermocycler			
	Manufacturer	**Model**	**Filters**	**Run time [minutes]**
A	Bio-Rad Laboratories, Inc., Hercules, California, USA	CFX96	FAM, HEX	72
B	Agilent Technologies, Santa Clara, California, USA	AriaMx	FAM, HEX	59
C	Agilent Technologies, Santa Clara, California, USA	Stratagene Mx3005P	FAM, HEX	66
D	Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA	Applied Biosystems™ 7500 Fast[1]	FAM, JOE	75
E	Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA	QuantStudio® 5	FAM, VIC	65
F	QIAGEN® GmbH, Hilden, Germany	Rotor-Gene® Q 5	Green, Yellow	85
	Theoretical run time of the virotype CSFV 2.0 RT-PCR Kit:			*36*

[1] Standard Mode setting

The sample panel listed in Table 17 was tested with the virotype CSFV 2.0 RT-PCR Kit (batch Valid-5) on six different real-time PCR thermocycler instruments (named A-F, see Table 24) using the virotype CSFV 2.0 RT-PCR protocol. For the Thermo Fisher ABI 7500 Fast instrument, the Standard Mode with ROX as passive reference dye was performed.

Results / Conclusion

The results are summarized in Table 25 (CSFV pathogen/ FAM channel) and Table 26 (endogenous Internal Control/ JOE channel). All samples tested on different real-time PCR thermocycler instruments showed comparable results.

Based on this data set, INDICAL can recommend using the virotype CSFV 2.0 RT-PCR Kit on the Bio-Rad CFX96, Agilent Technologies AriaMx, Agilent Technologies Stratagene Mx3005P, Thermo Fisher ABI 7500 Fast, Thermo Fisher QuantStudio 5 and QIAGEN Rotor-Gene instruments.

Table 25. Inter-thermocycler variance for the pathogen **CSFV** (FAM).

Inter-thermocycler variance for CSFV (FAM)								
Sample	**Material**	**CSFV status**	**Thermocycler (C_T values)**					
			A	**B**	**C**	**D**	**E**	**F**
1	Cell culture	pos	30.26	30.79	31.41	31.72	31.65	29.10
2	Cell culture	pos	27.09	26.91	27.92	27.15	27.72	25.85
3	Blood	pos	32.77	34.14	34.49	34.05	34.33	33.29
4	Blood	neg	-	-	-	-	-	-
5	Serum	pos	29.77	29.99	30.79	31.97	30.44	28.63
6	Plasma	pos	30.10	30.03	30.62	30.74	30.33	28.79
NC	NC	neg	-	-	-	-	-	-
PC	PC	pos	29.47	28.97	29.50	29.01	28.30	27.93

NC = Negative Control, PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Table 26. Inter-thermocycler variance for the **endogenous Internal Control** (JOE).

Inter-thermocycler variance for the endogenous Internal Control (JOE)								
Sample	**Material**	**CSFV status**	**Thermocycler (C_T values)**					
			A	**B**	**C**	**D**	**E**	**F**
1	Cell culture	pos	22.63	23.26	23.36	23.87	23.46	22.25
2	Cell culture	pos	22.79	22.91	23.13	24.00	23.91	22.60
3	Blood	pos	23.20	22.96	22.66	23.85	23.40	22.19
4	Blood	neg	21.15	19.76	20.15	20.54	19.59	19.36
5	Serum	pos	23.28	23.17	23.86	24.36	24.10	22.79
6	Plasma	pos	23.30	23.00	23.11	24.05	23.62	22.56
NC	NC	neg	-	-	-	-	-	-
PC	PC	pos	25.21	25.27	24.31	25.40	23.01	21.74

NC = Negative Control, PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

4.5 Stability testing

4.5.1 Freeze-thaw-cycles

The sample panel listed in Table 17 was used for validating the stability of the virotype CSFV 2.0 RT-PCR Kit. One kit batch (batch: Valid-6) was tested at the time of production and after six freeze/thaw cycles. The mean value (Mean), standard deviation (SD) and coefficient of variation (CV) were calculated.

Results / Conclusion

The virotype CSFV 2.0 RT-PCR Kit shows excellent stability with an average variance of 0.82 % for CSFV (FAM) and 0.68 % for the endogenous Internal Control (JOE) (Table 27, Table 28).

Table 27. Stability testing for **CSFV** (FAM) of the virotype CSFV 2.0 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Sample	**CSFV status**	**Stability for CSFV (FAM)** Freeze-thaw-cycle (C_T values)		**Mean**	**SD**	**CV%**
		1	**6**			
1	pos	30.95	30.41	30.68	0.378	1.232
2	pos	27.27	27.87	27.57	0.429	1.554
3	pos	33.07	33.21	33.14	0.095	0.288
4	neg	-	-	-	-	-
5	pos	30.46	30.09	30.27	0.264	0.872
6	pos	30.81	31.18	30.99	0.261	0.843
NC	neg	-	-	-	-	-
PC	pos	29.31	29.26	29.29	0.034	0.116
Mean						**0.82**

NC = Negative Control, PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Table 28. Stability testing for the **endogenous Internal Control** (JOE) of the virotype CSFV 2.0 RT-PCR Kit using the Bio-Rad CFX96 instrument.

Sample	CSFV status	Stability for Internal Control (JOE) Freeze-thaw-cycle (C_T values) 1	6	Mean	SD	CV%
1	pos	23.17	23.01	23.09	0.109	0.471
2	pos	22.43	22.36	22.39	0.050	0.224
3	pos	23.10	22.99	23.04	0.083	0.360
4	neg	20.63	21.05	20.84	0.301	1.444
5	pos	23.49	23.24	23.37	0.174	0.743
6	pos	23.69	23.96	23.82	0.188	0.790
NC	neg	-	-	-	-	-
PC	pos	25.18	25.43	25.31	0.178	0.704
Mean						**0.68**

NC = Negative Control, PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

4.5.2 Real-time stability testing

Real-time storage tests of the virotype CSFV 2.0 RT-PCR Kit (batch F202400001) are carried out at specific time timepoints during the shelf-life of the assay. The storage tests were conducted with CSFV-positive RNA-positive samples as well as the kit controls (Positive Control [PC], Negative Control [NC]). The difference of obtained C_T values (Δ C_T) per sample and fluorescence channel as well as mean (Δ C_T) for all tested samples per fluorescence channel were calculated.

Results/ Conclusion

Results of the storage test of kit batch F202400001 at the end of the shelf life (12 months) compared to test results obtained at batch control directly after kit production are shown in Table 29. C_T values after 12 months of storage showed only minor deviation of -0.77 % (CSFV, FAM channel) on average. The virotype CSFV 2.0 RT-PCR Kit shows excellent stability.

Table 29. Real-time storage test results of the virotype CSFV 2.0 RT-PCR Kit performed after 12 months of storage compared batch control after kit production

Storage test for virotype CSFV 2.0 RT-PCR Kit (batch F202400001)			
Sample	**CSFV (FAM)**		
	0 months	**12 months**	
	C_T	**C_T**	**Δ C_T**
NC	-	-	**-**
PC	28.83	28.11	-0.72
Sample #1	32.35	31.24	-1.11
Sample #2	36.19	35.25	-0.94
Sample #3	39.54	39.25	-0.29
Mean Δ C_T			**-0.77**

NC = Negative Control, PC = Positive Control, - = no C_T

4.5.2 Heparin inhibition

To test the stability of the virotype CSFV 2.0 RT-PCR Kit, sample inhibition was simulated by treating the sample FLI-2 (Israel, genotype 2.1, 1: 100 dilution) with an increasing concentration of heparin (0.17 – 3.40 U/reaction). The sample was tested in duplicate using the virotype CSFV 2.0 RT-PCR Kit (batch: Valid-6) and the Kit D.

Results / Conclusion

Whilst complete inhibition of the sample by heparin was observed at 1.70 U/reaction with the competitor Kit D, the CSFV signal (FAM) was fully inhibited at a higher heparin concentration of 3.40 U/reaction when using the virotype CSFV 2.0 PCR Kit (Table 30). The inhibition study using heparin showed that the virotype CSFV 2.0 RT-PCR Kit shows better resilience to inhibition by heparin than the competitor Kit D.

Table 30. Stability testing of the virotype CSFV 2.0 RT-PCR Kit for inhibition by heparin.

Stability (heparin inhibition)		
Heparin [U/reaction]	**CSFV (FAM) (C_T value)**	
	virotype CSFV 2.0 RT-PCR Kit	**Kit D**
-	31.12	31.32
-	30.44	31.27
0.17	30.44	32.03
0.17	30.43	32.04
0.34	30.30	34.21
0.34	30.50	34.24
1.70	35.04	-
1.70	34.57	-
3.40	-	-
3.40	-	-

- = no C_T

4.5.3 EDTA inhibition

To test the stability of the virotype CSFV 2.0 RT-PCR Kit, sample inhibition was simulated by treating the sample FLI-2 (Israel, genotype 2.1, 1: 100 dilution) with an increasing concentration of Ethylenediaminetetraacetic acid (EDTA; 0.5 – 6.0 mM final concentration in reaction mix). The sample was tested in duplicate using the virotype CSFV 2.0 RT-PCR Kit (batch: Valid-6) and the Kit D.

Results / Conclusion

The inhibition study using EDTA showed that complete sample inhibition (CSFV, FAM) occurs at high concentrations only (> 5.0 mM) when using the virotype CSFV 2.0 RT-PCR Kit (Table 31).

However, using the competitor Kit D, inhibition of the pathogen signal (CSFV, FAM) already occurred at concentration > 2.5 mM EDTA. Thus, the virotype CSFV 2.0 RT-PCR Kit shows better resilience to inhibition by EDTA than the competitor Kit D.

Table 31. Stability testing of the virotype CSFV 2.0 RT-PCR Kit for inhibition by EDTA.

Stability (EDTA inhibition)		
EDTA [mM]	**CSFV (FAM) (C_T value)**	
	virotype CSFV 2.0 RT-PCR Kit	**Kit D**
-	30.08	31.68
-	30.02	31.37
0.5	30.05	n.d.
0.5	30.11	n.d.
1.0	30.17	n.d.
1.0	30.41	n.d.
1.5	30.60	n.d.
1.5	30.51	n.d.
2.0	31.30	35.49
2.0	30.85	-
2.5	31.28	38.48
2.5	30.70	-
3.0	32.11	-
3.0	31.97	-
3.5	32.43	-
3.5	32.84	-
4.0	33.41	-
4.0	33.58	-
5.0	34.18	-
5.0	-	-
6.0	-	-
6.0	-	-

n.d. = not done, - = no C_T

4.6 Robustness

A panel comprising four CSFV-positive samples was used for assessment of the effect of small variations of critical assay parameters (samples A – D).

4.6.1 Robustness: Variation of sample volume

To test the robustness of the virotype CSFV 2.0 RT-PCR Kit, the sample volume was varied by 10 % to simulate errors in the reaction mix preparation. Therefore, the four CSFV-positive samples A – D were tested in triplicates for each condition in one PCR run on the Bio-Rad CFX 96.

Results / Conclusion

Results are shown in Table 32. The virotype CSFV 2.0 RT-PCR Kit shows excellent robustness for 10 % sample volume variation with an average variance of 0.77 % for CSFV (FAM).

Table 32. Robustness testing of the virotype CSFV 2.0 RT-PCR Kit (**CSFV** / FAM) for different sample volumes used in the RT-qPCR. The tests were performed on the Bio-Rad CFX 96 instrument.

Robustness for CSFV (FAM)						
Sample	**Ref. result**	**Sample volume (C_T values)**		**Mean**	**SD**	**CV%**
		4.5 µl	**5.5 µl**			
A	22.91	22.52	22.57	22.62	0.18	0.81
	22.86	22.73	22.43			
	22.50	22.40	22.66			
B	26.15	26.09	25.78	26.05	0.17	0.65
	26.28	26.15	25.86			
	26.21	26.06	25.90			
C	29.25	29.39	29.29	29.25	0.16	0.55
	29.33	29.25	28.86			
	29.21	29.40	29.27			
D	32.44	33.04	32.56	32.52	0.35	1.08
	32.37	32.26	32.56			
	31.99	32.40	33.08			
Mean						**0.77**

SD = standard deviation; CV = coefficient of variation

4.6.2 Robustness: Variation of annealing time

To test the robustness of the virotype CSFV 2.0 RT-PCR Kit, the annealing time during RT-qPCR reaction was varied by 10 % to simulate cycling errors. Therefore, the four CSFV-positive samples A – D were tested in triplicates for each condition in one PCR run on the Bio-Rad CFX 96 instrument.

Results / Conclusion

Results are shown in Table 33. The virotype CSFV 2.0 RT-PCR Kit shows excellent robustness for 10 % annealing time variation with an average variance of 0.75 % for CSFV (FAM).

Table 33. Robustness testing of the virotype CSFV2.0 RT-PCR Kit (**CSFV** / FAM) for different annealing times used in the RT-qPCR. The tests were performed on the Bio-Rad CFX 96 instrument.

		Robustness for CSFV (FAM)				
Sample	**Ref. result**	**Annealing time (C_T values)**		**Mean**	**SD**	**CV%**
		27 sec	**33 sec**			
A	22.91	22.73	22.80	22.63	0.21	0.94
	22.86	22.36	22.42			
	22.50	22.41	22.71			
B	26.15	25.80	26.03	26.01	0.18	0.68
	26.28	25.85	26.01			
	26.21	25.95	25.80			
C	29.25	29.17	29.43	29.28	0.14	0.48
	29.33	29.04	29.42			
	29.21	29.20	29.45			
D	32.44	32.71	32.49	32.34	0.30	0.92
	32.37	31.92	32.69			
	31.99	32.02	32.42			
Mean						**0.75**

SD = standard deviation; CV = coefficient of variation

4.6.3 Robustness: Variation of annealing temperature

To test the robustness of the virotype CSFV 2.0 RT-PCR Kit, the annealing temperature during RT-qPCR reaction was varied by 1°C to simulate cycling errors. Therefore, the four CSFV-positive samples A – D were tested in triplicates for each condition in one PCR run on the Bio-Rad CFX 96 instrument.

Results / Conclusion

Results are shown in Table 34. The virotype CSFV3 2.0 RT-PCR Kit shows excellent robustness for 1°C annealing temperature variation with an average variance of 0.83 % for CSFV (FAM).

Table 34. Robustness testing of the virotype CSFV 2.0 RT-PCR Kit (**CSFV** / FAM) for different annealing temperatures used in the RT-qPCR. The tests were performed on the Bio-Rad CFX 96 instrument.

Robustness for CSFV (FAM)						
Sample	**Ref. result**	**Annealing temperature (C_T values)**		**Mean**	**SD**	**CV%**
		59°C	**61°C**			
A	22.91	22.58	22.63	22.55	0.22	0.99
	22.86	22.45	22.32			
	22.50	22.24	22.46			
B	26.15	26.00	25.81	25.96	0.21	0.80
	26.28	25.79	25.83			
	26.21	25.70	25.88			
C	29.25	28.99	29.16	29.29	0.27	0.92
	29.33	29.09	29.25			
	29.21	29.35	29.94			
D	32.44	32.51	32.69	32.40	0.20	0.62
	32.37	32.52	32.50			
	31.99	32.35	32.24			
Mean						**0.83**

SD = standard deviation; CV = coefficient of variation