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virotype® ASFV 2.0 PCR Kit
Validation Report

For detection of DNA from *African Swine Fever Virus* (ASFV)

INDICAL BIOSCIENCE

Contents

1 Introduction

1.1. Intended use

The virotype ASFV 2.0 PCR Kit is intended for the detection of DNA from *African Swine Fever Virus* (ASFV) in serum, plasma, EDTA-blood, tissue, and swab samples from pigs and wild boar.

For veterinary use only.

1.2. General information

The virotype ASFV 2.0 PCR Kit is a highly sensitive and specific solution for the detection of DNA from *African Swine Fever Virus* (ASFV) in samples from pigs and wild boar.

African Swine Fever (ASF) is one of the most important infectious viral diseases of swine of all ages and causes a wide range of clinical signs characterized by a high rate of morbidity and mortality. The disease is notifiable to the World Organization for Animal Health (OIE).

The causative agent is a double-stranded DNA virus belonging to the family *Asfarviridae*, genus *Asfivirus*. ASF virus can be transmitted by vectors (soft ticks of the genus *Ornithodoros*) therefore classified as *Arbovirus* (arthropod-borne virus).

The high sensitivity of the virotype ASFV 2.0 PCR Kit allows early detection of the pathogen in individual as well as in pooled samples of serum, plasma, EDTA-blood, tissue, and swab material from pigs and wild boar.

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 ASFV 2.0 PCR Kit contains all of the necessary reagents for the detection of ASFV DNA, including a Positive and Negative Control.

The kit contains two internal controls. The endogenous control (EC) detects a housekeeping gene present within the sample and the exogenous control (IC-DNA) permits tests for successful extraction and amplification by adding it to the DNA purification procedure.

Both internal control systems exclude the possibility of false-negative results.

The kit uses three specific primer/probe combinations:

- FAM™ fluorescence for DNA of ASFV
- HEX™ fluorescence for the endogenous Internal Control (β-actin present within the sample)
- Cy®5 fluorescence for the exogenous Internal Control (IC-DNA extracted during DNA purification)

1.4. Kit contents

virotype ASFV 2.0 PCR Kit Catalog no. / Number of reactions	**VT281925 / 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
IC-DNA (tube with transparent cap)	1 x 200 µl
Handbook	1

1.5. Storage

The components of the virotype ASFV 2.0 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 (>2x), 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. DNA extraction

The virotype ASFV 2.0 PCR Kit can be used for the detection of ASFV DNA from serum, plasma, EDTA-blood, tissue, and swab samples from pigs and wild boars.

Due to the high sensitivity of the test individual or pooled samples can be tested. Pools of up to 20 individual serum, plasma, EDTA-blood, or tissue samples can be used, provided that the sample quality is good. It is recommended to test dead wildlife samples on an individual basis.

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

Prior to real-time PCR, viral DNA must be extracted from the starting material. The exogenous internal control DNA (IC-DNA) must be added to the lysis buffer prior to the extraction procedure. In most cases, 2 µl IC-DNA per sample is suitable.

INDICAL offers a range of validated kits for the extraction of DNA from animal samples.

Extraction based on magnetic beads:

- IndiMag® Pathogen Kit[1] (SP947457; formerly MagAttract 96 cador® Pathogen Kit)
- IndiMag Pathogen Kit w/o plastics (SP947257; formerly MagAttract 96 cador Pathogen Kit w/o Plastics)

Extraction based on spin columns:

- IndiSpin® Pathogen Kit[1] (SP54104, SP54106; formerly QIAamp® cador Pathogen Mini Kit)
- IndiSpin QIAcube® HT Pathogen Kit (SP54161; formerly cador Pathogen 96 QIAcube HT Kit) – not suitable for blood samples

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

If real-time PCR is not performed immediately after extraction, store the DNA at -20°C or at -70°C for longer storage.

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

[1] Suitable for simultaneous extraction of ASFV DNA und CSFV RNA

1.8. Important notes

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 perform a positive control reaction in the PCR run, containing a sample that is known to include the targeted viral DNA. A positive control serves to prove the functionality of the pathogen assay, e.g., the correct setup of the reaction mix. Use 5 µl of the Positive Control provided with the virotype ASFV 2.0 PCR Kit to test for successful amplification of the target.

Extraction and amplification control

For increased process safety and convenience, two extraction and amplification control assays are included in the test kit.

An endogenous Internal Control (EC) detects a housekeeping gene present within the sample, whereas the exogenous Internal Control detects IC-DNA, which must be added to the lysis buffer prior to extraction. The use of both control systems allows extraction and amplification to be monitored, as well as the sample quality.

It is strongly recommended to add the IC-DNA to the lysis buffer prior to extraction. This allows to monitor extraction and amplification also in samples that would show at least partial inhibition due to the sample quality.

2 Procedure

2.1. Important points before starting

- Please read „Important notes“ before starting.
- It is strongly recommended to use the IC-DNA provided with the kit to monitor extraction and amplification, as well as any partial inhibition. Please add the respective volume to the lysis buffer prior to extraction.
- 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.
- 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.
- Before use, spin the reagents briefly.

2.3. Test procedure

1. Pipet 20 µl of the Master Mix into each reaction tube. Then add 5 µl of the sample DNA (Table 1).
   Include positive and negative control reactions.
   Positive control: Use 5 µl of the positive control (Positive Control) instead of sample DNA.
   Negative control: Use 5 µl of the negative control (Negative Control) instead of sample DNA.

Table 1. Preparation of reaction mix

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

2. Close the reaction tubes with the corresponding caps.
3. Set the filters for the reporter dyes in the software of the thermal cycler according to Table 2.

Table 2. Filter settings for the reporter

Pathogen/ Internal Control	Reporter
ASFV	FAM
Endogenous Internal Control (EC)	HEX/ JOE™[1]
Exogenous Internal Control (IC-DNA)	Cy5
Passive reference[2]	ROX™

[1] Use the option appropriate for your thermal cycler.
[2] Internal reference for use with Applied Biosystems® ABI PRISM® Sequence Detection Systems

4. Run the real-time PCR protocol according to Table 3 if running only the virotype ASFV 2.0 PCR Kit.

Table 3. Real-time PCR protocol for ASFV 2.0

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

* Fluorescence data collection.

5. Run the real-time RT-PCR protocol according to Table 4 if running the virotype CSFV assay simultaneously.

Table 4. Real-time RT-PCR protocol for simultaneous amplification of ASFV 2.0 and CSFV[1]

Step	Temperature	Time	Number of cycles
Reverse Transcription	45°C	10 min	1
Initial Activation	95°C	10 min	1
3-step cycling			
Denaturation	95°C	15 s	40
Annealing*	57°C	30 s	40
Extension	72°C	35 s	40

[1] Valid for viotype CSFV RT-PCR Kit only.
* Fluorescence data collection. Approximate run time 118 min (Rotor-Gene Q).

3 Data interpretation

Interpretation of results

For the assay to be valid the Positive Control must give a signal in the FAM, HEX and Cy5 channels with a CT[2] < 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 5 on page 8.

The sample is positive for ASFV, 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 and/ or Cy5 channel).
- The Positive Control yields a signal in all channels (FAM, HEX and Cy5).
- The Negative Control does not yield a signal in the FAM, HEX and Cy5 channel.

Note that very high concentrations of ASFV DNA in the sample may lead to a reduced or no signal of internal controls due to competition.

The sample is negative for ASFV, 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 channel if no IC-DNA was used or in the HEX and Cy5 channel if IC-DNA was used.
- The Positive Control yields a signal in all channels (FAM, HEX and Cy5).
- The Negative Control does not yield a signal in the FAM, HEX and Cy5 channel.

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

- The sample yields no signal in the FAM, HEX and Cy5 channel.

If no signal is detected in the FAM (ASFV), the HEX (endogenous Internal Control, EC) and Cy5 (exogenous Internal Control, IC-DNA) channel, the result is inconclusive. The absence of a signal for the housekeeping gene and the IC-DNA indicates strong PCR inhibition and/or other malfunctions, e.g. during extraction.

To check for inhibition, we recommend 1:5 dilution of the sample DNA in nuclease free water, to repeat the DNA 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.

[2] Threshold cycle (C_T) — cycle at which the amplification plot crosses the threshold, i.e., there is the first clearly detectable increase in fluorescence

Additional information given by the endogenous and exogenous Internal Control systems:

The lack of Cy5 fluorescence signal can be caused by insufficient sample extraction, competition with a strong positive ASFV signal, PCR inhibition or will occur in cases where the IC-DNA had not been added. Higher C_T-values in the Cy5 channel of a sample compared to the majority of samples may indicate partial inhibition in the sample.

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

The lack of HEX fluorescence signal in presence of a signal in the Cy5 channel indicates poor sample quality and/ or sample amount.

Table 5. Results interpretation table*

Sample result	Reporter			Interpretation
	FAM (ASFV)	**HEX (EC)**	**Cy5 (IC-DNA)**	
ASFV positive	X	X	X	Valid
ASFV positive	X	X		Valid (extraction without IC-DNA)
ASFV strong positive	X	(X)	(X)	Valid (no EC and/ or IC-DNA signal due to competition)
ASFV negative		X	X	Valid
Poor sample quality			X	Successful extraction (no EC-signal due to poor sample quality or amount; recommendation to test a new sample; also applies to non-animal samples
Partial PCR inhibition		X	(X)	No or weak C_T value for IC-DNA when added to the extraction (recommendation to test 1:5 dilution of the sample)

* 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, HEX and Cy5 channel. 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 of virotype ASFV 2.0 PCR Kit

The high analytical sensitivity of the virotype ASFV 2.0 PCR Kit was verified by a titration series of ASFV in-vitro DNA [10^6 – 10^{-1} copies/well], performed in triplicates of ten-fold dilutions using the ASFV 2.0 PCR protocol (Tables 6 and 7).

4.1.1. Analytical sensitivity using the Biorad CFX96

Table 6. Analytical sensitivity. Individual and mean values of amplificates in triplicates (FAM signal) and amplification plot, Biorad CFX96

Type	Copy number	C_T (FAM)	C_T mean	SD	Result
Standard	10^6	19.25			+
Standard	10^6	19.49	19.37	0.12	+
Standard	10^6	19.36			+
Standard	10^5	22.64			+
Standard	10^5	22.62	22.57	0.10	+
Standard	10^5	22.45			+
Standard	10^4	26.09			+
Standard	10^4	26.05	26.10	0.05	+
Standard	10^4	26.16			+
Standard	10^3	29.30			+
Standard	10^3	29.75	29.49	0.24	+
Standard	10^3	29.40			+
Standard	10^2	33.11			+
Standard	10^2	32.40	32.73	0.36	+
Standard	10^2	32.68			+
Standard	10	35.95			+
Standard	10	36.39	36.24	0.26	+
Standard	10	36.40			+
Standard	5	38.01			+
Standard	5	38.82	38.37	0.41	+
Standard	5	38.29			+
Standard	1	No C_T			-
Standard	1	No C_T	-	-	-
Standard	1	No C_T			-

Figure 1: Analytical sensitivity. Individual values of amplificates in triplicates. Titration of ASFV *in vitro* DNA, Biorad CFX96.

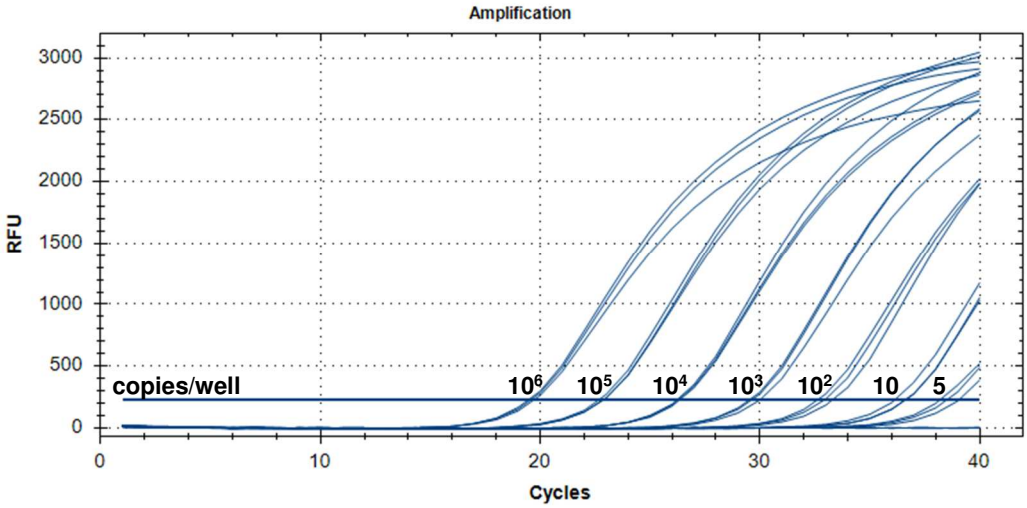
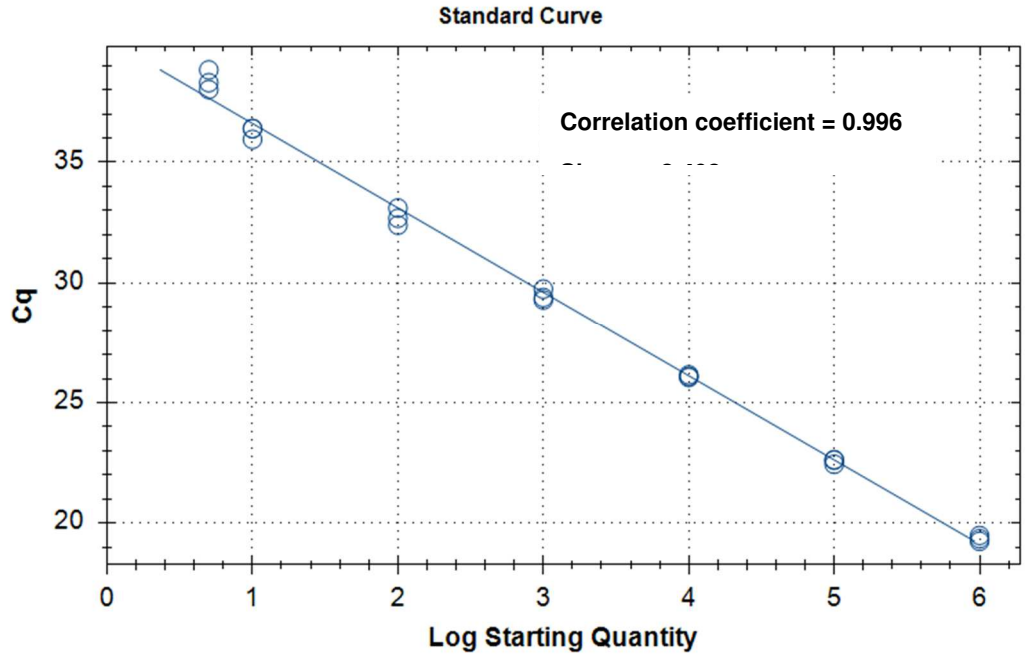


Figure 2: Titration of ASFV *in vitro* DNA using the virotype ASFV 2.0 PCR Kit. Standard curve of obtained C_T values is shown. The Test was performed on the Biorad CFX96 instrument.



Results (ASFV detection using the BioRad CFX96)

The virotype ASFV 2.0 PCR Kit allows the detection of up to five ASFV genome copies per sample (Table 6 and Figure 1). There is a high correlation between DNA copy number and amplification results. A correlation coefficient of 0.996 with an efficiency of 93.3% for the *in vitro* DNA was calculated when using the virotype ASFV 2.0 PCR Kit on the Biorad CFX96 instrument (Figure 2).

4.1.2. Analtyical sensitivity using the Stratagene Mx3005P

Table 7. Analytical sensitivity. Individual and mean values of amplificates in triplicates (FAM signal) and amplification plot, Agilent Stratagene Mx3005P

Type	Copy number	C_T (FAM)	C_T mean	SD	Result
Standard	10^6	18.51			+
Standard	10^6	18.67	18.61	0.09	+
Standard	10^6	18.64			+
Standard	10^5	21.75			+
Standard	10^5	21.93	21.88	0.12	+
Standard	10^5	21.97			+
Standard	10^4	25.13			+
Standard	10^4	25.45	25.30	0.16	+
Standard	10^4	25.33			+
Standard	10^3	28.48			+
Standard	10^3	28.44	28.52	0.11	+
Standard	10^3	28.65			+
Standard	10^2	32.13			+
Standard	10^2	32.01	32.10	0.08	+
Standard	10^2	32.16			+
Standard	10	35.05			+
Standard	10	35.17	35.02	0.17	+
Standard	10	34.83			+
Standard	5	34.92			+
Standard	5	34.29	34.70	0.36	+
Standard	5	34.90			+
Standard	1	No C_T			-
Standard	1	No C_T	-	-	-
Standard	1	No C_T			-

Figure 3: Analytical sensitivity. Individual values of amplificates in triplicates. Titration of ASFV *in vitro* DNA, Agilent Stratagene Mx3005P.

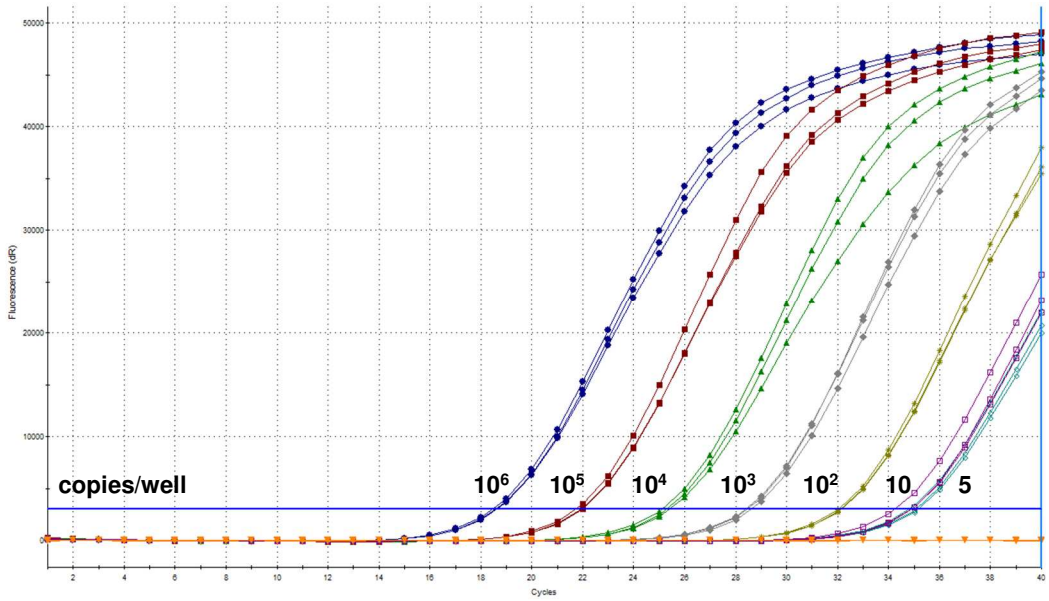
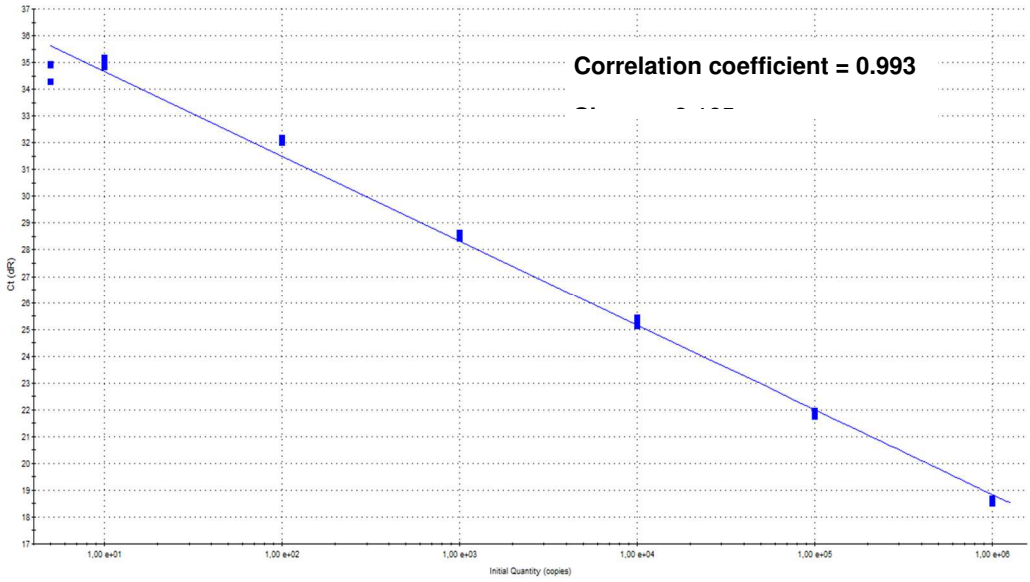


Figure 4: Titration of ASFV *in vitro* DNA using the virotype ASFV 2.0 PCR Kit. Standard curve of obtained C_T values is shown. The Test was performed on the Agilent Stratagene Mx3005P instrument.



Results (ASFV detection using the Stratagene Mx3005P)

The virotype ASFV 2.0 PCR Kit is able to detect up to five ASFV genome copies per sample (Table 7 and Figure 3). There is a high correlation between DNA copy number and amplification results. A correlation coefficient of 0.993 with an efficiency of 107.0% for the *in vitro* DNA was calculated when using the virotype ASFV 2.0 PCR Kit on the Agilent Stratagene Mx3005P instrument (Figure 4).

4.1.3. Analytical sensitivity – limit of detection

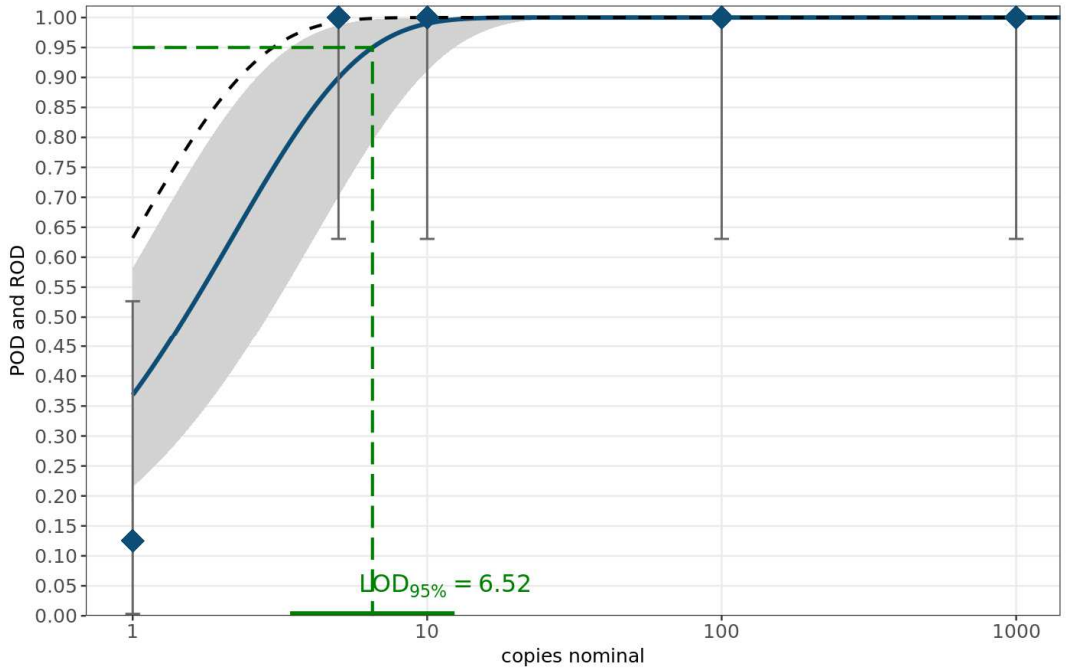
The limit of detection for the target sequence of the *African Swine Fever Virus* was determined by testing individual titration series of *in vitro* DNA of this sequence in 8 replicates. Results are summarized in Table 8. The limit of detection with 95 % confidence interval (LOD95 %: mean number of copies yielding a probability of detection of 0.95) was determined using a web tool (https://quodata.de/content/validation-qualitative-pcr-methods-single-laboratory).

Data are shown in Figure 5.

Table 8. Analytical sensitivity. Limit of detection for ASFV *in vitro* DNA tested in 8 replicates. Biorad CFX96.

Copies/test	Total number of replicates	Number of replicates positive	Number of replicates negative
1000000	8	8	0
100000	8	8	0
10000	8	8	0
1000	8	8	0
100	8	8	0
10	8	8	0
5	8	8	0
1	8	1	7

Figure 5. POD (probability of detection) curve and LOD95 % for ASFV. The blue diamonds characterize the laboratory-specific RODs (rate 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.



Using a different method to determine the limit of detection, five copies of *in vitro* ASFV DNA were tested 50 times in using the Biorad CFX96 thermocycler. Data is shown in Table 9.

Table 9. Analytical sensitivity. Limit of detection for ASFV *in vitro* DNA tested in 50 replicates. Biorad CFX96.

Type	Copy number	C_T (FAM)	C_T mean	SD	Result
Standard	5	38.01	37.12	1.25	+
Standard	5	38.82	37.12	1.25	+
Standard	5	38.29	37.12	1.25	+
Standard	5	37.55	37.12	1.25	+
Standard	5	37.50	37.12	1.25	+
Standard	5	35.33	37.12	1.25	+
Standard	5	38.34	37.12	1.25	+
Standard	5	38.28	37.12	1.25	+
Standard	5	No C_T	37.12	1.25	-
Standard	5	37.46	37.12	1.25	+
Standard	5	35.35	37.12	1.25	+
Standard	5	35.73	37.12	1.25	+
Standard	5	37.43	37.12	1.25	+
Standard	5	36.09	37.12	1.25	+
Standard	5	39.73	37.12	1.25	+
Standard	5	No C_T	37.12	1.25	-
Standard	5	35.19	37.12	1.25	+
Standard	5	38.54	37.12	1.25	+
Standard	5	37.16	37.12	1.25	+
Standard	5	36.28	37.12	1.25	+
Standard	5	34.51	37.12	1.25	+
Standard	5	39.04	37.12	1.25	+
Standard	5	36.12	37.12	1.25	+
Standard	5	37.60	37.12	1.25	+
Standard	5	38.61	37.12	1.25	+
Standard	5	37.25	37.12	1.25	+
Standard	5	36.26	37.12	1.25	+
Standard	5	37.19	37.12	1.25	+
Standard	5	37.20	37.12	1.25	+
Standard	5	38.54	37.12	1.25	+
Standard	5	35.79	37.12	1.25	+
Standard	5	38.12	37.12	1.25	+
Standard	5	37.85	37.12	1.25	+
Standard	5	37.41	37.12	1.25	+

Standard	5	37.20	37.12	1.25	+
Standard	5	37.14	37.12	1.25	+
Standard	5	37.56	37.12	1.25	+
Standard	5	37.32	37.12	1.25	+
Standard	5	37.29	37.12	1.25	+
Standard	5	35.67	37.12	1.25	+
Standard	5	38.20	37.12	1.25	+
Standard	5	33.65	37.12	1.25	+
Standard	5	36.39	37.12	1.25	+
Standard	5	35.27	37.12	1.25	+
Standard	5	37.16	37.12	1.25	+
Standard	5	37.50	37.12	1.25	+
Standard	5	38.49	37.12	1.25	+
Standard	5	36.69	37.12	1.25	+
Standard	5	37.15	37.12	1.25	+
Standard	5	36.46	37.12	1.25	+

Results/Conclusions

Using the virotype ASFV 2.0 PCR Kit, a high correlation between DNA copy number and the amount of amplified product was demonstrated for the ASFV targeted sequence.

The $LOD_{95\%}$ is 6.519 copies with a 95% confidence interval of [3.424, 12.391].

When testing five copies of *in vitro* ASFV DNA in replicates of 50, 48/50 (96.00%) could reproducibly be detected.

4.2. Analytical specificity

4.2.1. Comparative analysis of the virotype ASFV 2.0 and other commercially available kits using field samples (in-house testing)

Eight ASFV positive DNA samples from wild boars and pigs were provided by INIA (EU Reference laboratory for ASFV), PIWET (Polish National Reference Laboratory for ASFV) and BIOR (Estonian National Reference Laboratory for ASFV). The DNA samples were tested using the virotype ASFV 2.0 PCR Kit on the Biorad CFX96 instrument. Results are shown in Table 10.

Table 10. Comparative analysis of the virotype ASFV 2.0 PCR Kit and other commercially available kits.

Sample	Species	Material	Origin	Geno-type	C_T (ASFV)				
					vt ASFV 2.0	Kit A	Kit B	Kit C	Kit D
Ken06.Bus	pig	spleen	INIA	9	22.17	23.25	23.16	22.10	23.91
Ken05/Tk1	pig	lung	INIA	10	22.73	22.39	24.05	22.09	23.89
Z/18/10107(1)	wild boar	bone-marrow	PIWET	2	23.65	22.71	25.33	23.04	24.80
LV24	wild boar	tissue	BIOR	2	27.08	27.81	28.24	26.51	27.79
Z/18/09903(10)	wild boar	lung	PIWET	2	28.27	27.88	29.72	27.64	29.64
Ken06.Bus	pig	spleen	INIA	9	30.55	33.16	31.66	30.94	32.87
LV22	wild boar	blood	BIOR	2	35.08	36.87	36.70	34.46	No C_T
NH/P68	pig	spleen	INIA	1	35.45	No C_T	35.99	35.12	No C_T
Negative Control					No C_T	No C_T	No C_T	No C_T	No C_T
Positive Control					28.87	30.05	30.55	30.06	25.80

Results/Conclusions (ASFV)

Results obtained with the virotype ASFV 2.0 PCR Kit show better or equal sensitivity compared to other available PCR kits. (Table 10).

4.2.2. Discrimination of pathogen for differential diagnosis

Cross-reactivity was tested with samples positive for *Classical Swine Fever Virus* (CSFV), *Porcine Reproductive and Respiratory Syndrome Virus* (PRRSV), *Swine Influenza Virus* (SIV) and *Porcine Circovirus 2* (PCV 2). The samples were kindly provided by the FLI and Veterinary State Diagnostic Laboratories (Table 11).

Table 11. Cross-reactivity of the virotype ASFV 2.0 PCR Kit to other swine-related pathogens. Biorad CFX96.

		Reference Assay		virotype ASFV 2.0 PCR Kit		
Sample	**Sample material**	**C_T (Pathogen)**	**C_T (Control)**	**C_T (ASFV)**	**C_T (EC)**	**C_T (IC)**
***Classical Swine Fever Virus* (CSFV)**						
Alfort187	culture	32.93	No C_T	No C_T	No C_T	No C_T
Koslov1128	culture	31.18	No C_T	No C_T	No C_T	No C_T
Brescia	culture	32.94	No C_T	No C_T	No C_T	No C_T
Schweiz II	culture	32.91	No C_T	No C_T	No C_T	No C_T
D4886/82/Ro	culture	32.25	No C_T	No C_T	No C_T	No C_T
Spante	culture	31.16	No C_T	No C_T	No C_T	No C_T
Congenital Tremor	culture	28.82	No C_T	No C_T	No C_T	No C_T
Kanagawa	culture	29.71	No C_T	No C_T	No C_T	No C_T
CSF 1027	tissue	19.82	31.46	No C_T	31.06	No C_T
CSF 1024	tissue	19.56	32.75	No C_T	31.91	No C_T
CSF 0867	tissue	19.09	32.36	No C_T	31.87	No C_T
CSF 0866	tissue	23.08	31.46	No C_T	34.03	No C_T
CSF 0840	tissue	22.14	31.92	No C_T	31.88	No C_T
CSF 0822	tissue	20.48	31.94	No C_T	32.12	No C_T
Alfort 187	culture	23.42	No C_T	No C_T	No C_T	No C_T
Norden	culture	22.64	No C_T	No C_T	No C_T	No C_T
HCV 14	culture	22.73	No C_T	No C_T	No C_T	No C_T
Rovac	culture	23.49	No C_T	No C_T	No C_T	No C_T
Brescia	culture	21.44	No C_T	No C_T	No C_T	No C_T
HCV 17	culture	28.20	No C_T	No C_T	No C_T	No C_T
548	culture	32.29	No C_T	No C_T	No C_T	No C_T
385	culture	33.79	No C_T	No C_T	No C_T	No C_T
CSF 1060	culture	29.26	No C_T	No C_T	No C_T	No C_T
***Porcine Circovirus 2* (PCV 2)**						
PCV-2_1	serum	29.73	31.82	No C_T	31.64	No C_T
PCV-2_2	serum	30.76	28.70	No C_T	25.24	No C_T
PCV-2_3	serum	38.87	31.10	No C_T	29.38	No C_T
PCV-2_4	serum	24.63	31.10	No C_T	31.47	No C_T
PCV-2_5	serum	29.54	30.09	No C_T	27.45	No C_T

***Swine Influenza Virus* (SIV)**						
SIV-01 (1:1000)	blood	22.81	26.89	No C_T	29.91	No C_T
SIV-01 (1:1000)	blood	23.74	27.08	No C_T	29.60	No C_T
SIV-03 (1:1000)	blood	26.95	26.86	No C_T	30.11	No C_T
SIV-03 (1:1000)	blood	26.62	27.05	No C_T	30.21	No C_T
***Porcine Reproductive and Respiratory Syndrome Virus* (PRRSV)**						
Intervet	culture	15.41	No C_T	No C_T	No C_T	No C_T
Intervet 10-1	culture	18.74	No C_T	No C_T	No C_T	No C_T
Intervet 10-2	culture	22.38	No C_T	No C_T	No C_T	No C_T
Intervet 10-3	culture	25.53	No C_T	No C_T	No C_T	No C_T
Intervet 10-4	culture	28.97	No C_T	No C_T	No C_T	No C_T
Stendal V852-10-1	culture	19.34	30.97	No C_T	30.66	No C_T
Stendal V852-10-2	culture	22.55	34.05	No C_T	33.85	No C_T
Stendal V852-10-3	culture	26.23	No C_T	No C_T	No C_T	No C_T
USA 18-10-3	culture	22.34	No C_T	No C_T	No C_T	No C_T
USA 18-10-4	culture	26.09	No C_T	No C_T	No C_T	No C_T
USA 18-10-5	culture	29.56	No C_T	No C_T	No C_T	No C_T
PRRSV-22	serum	31.59	32.73	No C_T	31.13	No C_T
PRRSV-23	serum	24.42	31.71	No C_T	28.52	No C_T
PRRSV-24	serum	25.33	29.80	No C_T	31.90	No C_T
PRRSV-25	serum	28.38	30.42	No C_T	29.23	No C_T
PRRSV-26	serum	30.55	32.58	No C_T	31.65	No C_T
PRRSV-27	serum	32.38	31.53	No C_T	31.88	No C_T
PRRSV-28	serum	29.32	31.69	No C_T	30.25	No C_T
PRRSV-29	serum	28.68	32.05	No C_T	32.52	No C_T
PRRSV-34	serum	35.38	32.21	No C_T	30.79	No C_T
PRRSV-42	serum	25.76	32.07	No C_T	31.05	No C_T
PRRSV-43	serum	30.28	29.52	No C_T	26.23	No C_T
PRRSV-45	serum	33.65	31.04	No C_T	26.23	No C_T
PRRSV-48	serum	29.85	30.90	No C_T	31.56	No C_T
PRRSV-49	serum	30.03	30.55	No C_T	31.24	No C_T
PRRSV-50	serum	32.44	32.24	No C_T	30.33	No C_T
PRRSV-57	serum	33.11	32.47	No C_T	32.40	No C_T

* Reference assays were as follows: virotype CSFV RT-PCR Kit, virotype PCV2/PCV3 Reagent, virotype Influenza A RT-PCR Kit, virotype PRRSV RT-PCR Kit

Results

No cross-reactivity to other relevant porcine viral pathogens was detected using the virotype ASFV 2.0 PCR Kit.

4.3. Diagnostic sensitivity and specificity

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)

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)

4.3.3. Validation of the virotype ASFV 2.0 PCR Kit

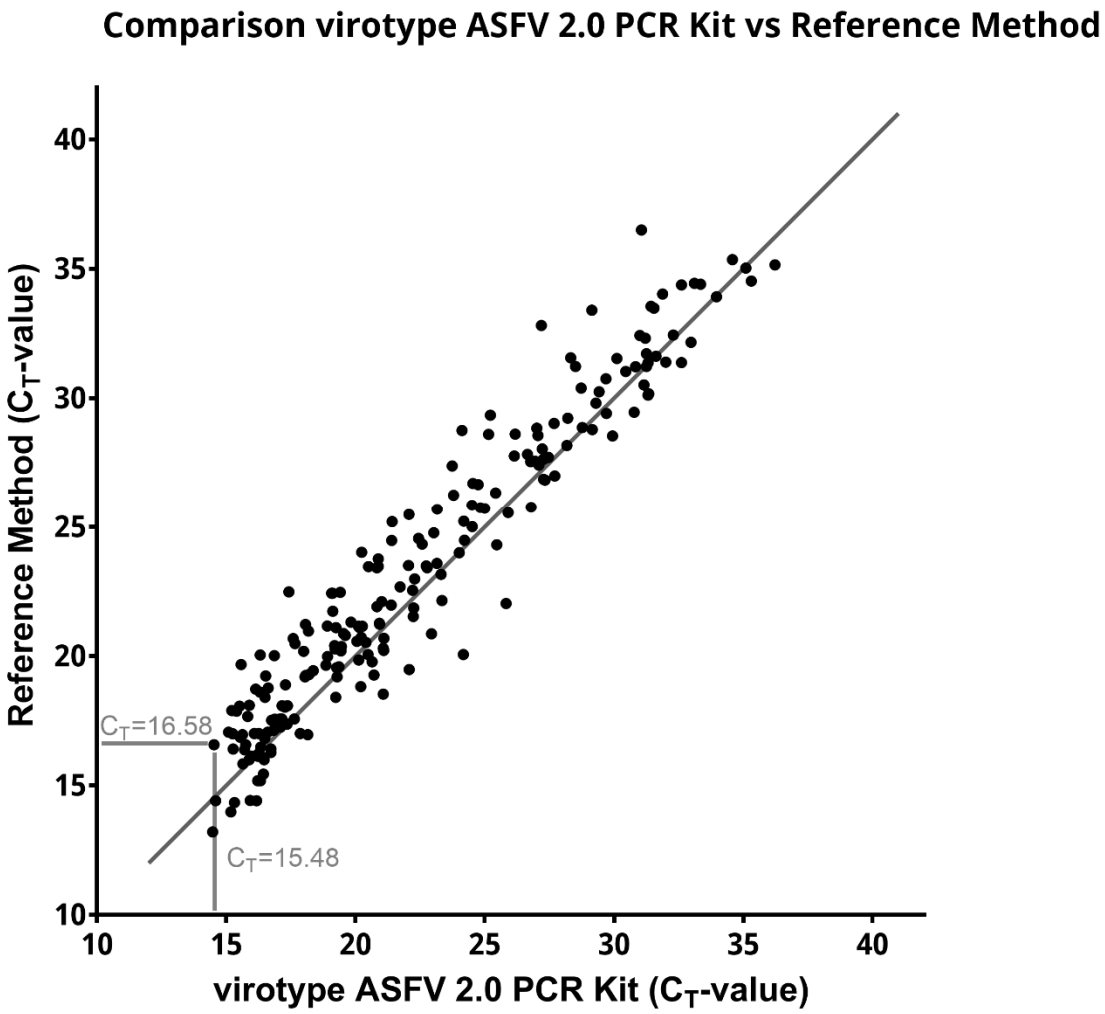
For validation of the virotype ASFV 2.0 PCR Kit, 468 samples (blood, serum, tissue, fecal and oropharyngeal swabs) were tested. Reference samples (245 ASFV-positive, 11 ASFV-negative) were kindly provided by INIA (EU Reference laboratory for ASFV), the FLI (German National Reference Laboratory for ASFV), PIWET (Polish National Reference Laboratory for ASFV) and BIOR (Estonian National Reference Laboratory for ASFV). The ASFV-positive samples comprised of seven different genotypes (I, II, V, VIII, IX, X and XIII). Further 223 ASFV-negative samples were provided by the FLI and Veterinary State Diagnostic Laboratories.

All samples were extracted using the QIAamp Viral RNA Mini Kit (QIAGEN), the IndiSpin Pathogen Kit or the IndiMag Pathogen Kit (INDICAL BIOSCIENCE) or the High Pure Viral Nucleic Acid Kit (Roche) following manufacturer´s instructions and tested with the virotype ASFV 2.0 PCR Kit. Please note that not all institutions added the IC-DNA during sample extraction. The summary is shown in Table 12 and Figure 6.

Table 12. Diagnostic sensitivity, specificity and efficiency of the virotype ASFV 2.0 PCR Kit.

virotype **ASFV 2.0**		**comparative data**			
Total	**468**	**Reference-positive**	**245**	**Reference-negative**	**223**
positive	245	true positive	245	false-positive	0
negative	223	false-negative	0	true-negative	223

Figure 1. Comparison of C_T values from ASFV-positive samples tested with the virotype ASFV 2.0 PCR Kit compared to the Reference method. All samples situated above the black diagonal line showed lower C_T values with the virotype ASFV 2.0 PCR Kit than tested with the Reference method.



Result/Conclusions:

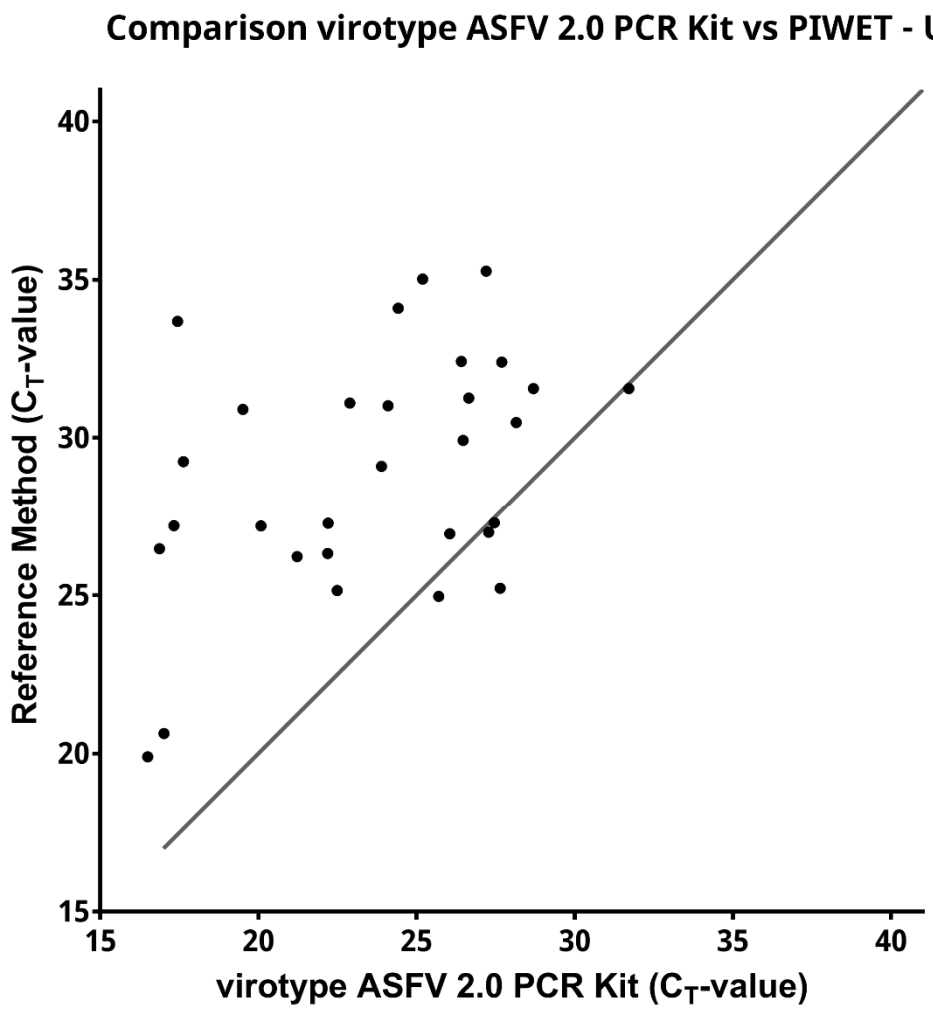
In this study the virotype ASFV 2.0 PCR Kit demonstrated a diagnostic sensitivity of 100.0%, a diagnostic specificity of 100.0% and a diagnostic efficiency of 100.0%.In addition, it also demonstrated an overall higher sensitivity compared to in-house reference methods (Figure 1).

4.4. External test validation

4.4.1. Beta test in comparison to in-house method (PIWET)

30 ASFV positive samples and 30 ASFV negative were tested using the virotype ASFV 2.0 PCR Kit and compared to the in-house method (UPL; Fernandez et al., 2012). Data is shown in Figure 7.

Figure 72. Comparison of C_T values from ASFV-positive samples tested with the virotype ASFV 2.0 PCR Kit compared to the Reference method at PIWET. All samples situated above the black diagonial line showed lower C_T values with the virotype ASFV 2.0 PCR Kit compared to the Reference method.



Results:

The virotype ASFV 2.0 PCR Kit showed a higher sensitivity compared to the in-house method (UPL).

4.5. Testing of pooled samples

Pools were generated by diluting ASFV-positive DNA samples (FLI reference samples) in ASFV-negative pig or wild boar DNA from blood (extracted with the IndiSpin Pathogen Kit following manufacturer´s instructions). ASFV-DNA and DNA of both internal controls could be detected with the virotype ASFV 2.0 PCR Kit in all samples (Table 13).

Table 13. Testing of simulated ASFV pool samples (pool size: 5 to 20) using the virotype ASFV 2.0 PCR Kit

			virotype ASFV 2.0 PCR Kit		
Pool size	**Sample**	**Sample material**	**C_T FAM**	**C_T HEX**	**C_T Cy5**
0	1	wild boar	26.49	27.58	28.71
5			29.14	26.85	28.73
10			30.19	26.59	28.89
20			31.78	26.54	29.09
0	2	pig	29.13	22.34	29.71
5			31.87	21.42	28.79
10			32.40	21.38	29.23
20			33.85	21.15	28.75
0	3	Wild boar	29.42	27.41	29.15
5			32.12	27.57	29.52
10			32.97	27.34	29.60
20			34.36	27.45	29.64
0	4	wildboar	32.21	24.94	28.61
5			34.49	25.99	28.91
10			36.65	26.37	28.68
20			37.04	26.08	28.56
0	5	pig	32.43	21.24	28.70
5			34.67	21.12	28.72
10			36.94	21.34	29.09
20			37.46	21.25	28.18

Results/Conclusion:

Pools consisting of up to 20 samples comprising of at least one strong (C_T<26) or medium (C_T< 32) ASFV-positive sample can be detected (Table 13).

4.6. Reproducibility

4.6.1. Intra-assay variance

ASFV-positive DNA samples (samples 1-4, 6), two ASFV-negative sample (sample 5 and 7) and the controls (Negative Control [NC], Positive Control [PC]) of the test kit were tested in a sevenfold setup in the same PCR run using the ASFV 2.0 PCR Kit (batch Vali05; Table 14, Table 15, Table 16).

Table 14. Intra-assay variance for ASFV (FAM) for the virotype ASFV 2.0 PCR Kit using Biorad CFX96

Intra-assay variance for ASFV (FAM)										
	Reactions (C_T Values)							**Mean**	**SD**	**CV [%]**
Samples	1	2	3	4	5	6	7			
1	28.81	28.65	28.76	28.68	28.59	28.70	28.79	28.71	0.079	0.275
2	25.38	25.77	25.40	25.35	25.20	25.40	25.45	25.42	0.173	0.679
3	34.81	34.10	34.45	34.13	34.42	34.28	34.38	34.37	0.237	0.691
4	24.10	24.08	24.36	24.12	23.97	24.10	24.09	24.12	0.118	0.490
5								-	-	-
6	28.72	28.85	28.62	28.49	28.57	28.66	28.56	28.64	0.119	0.417
7								-	-	-
NC								-	-	-
PC	29.85	30.10	29.66	29.56	29.67	29.82	29.64	29.76	0.181	0.609
MV										**0.53**

CV = Coefficient of variation

Table 15. Intra-assay variance for the endogenous Internal Control (HEX) for the virotype ASFV 2.0 PCR Kit using Biorad CFX96

Intra-assay variance for the endogenous Internal Control (HEX)										
	Reactions (C_T Values)							**Mean**	**SD**	**CV [%]**
Samples	1	2	3	4	5	6	7			
1	30.56	30.76	31.00	30.49	30.62	30.70	30.77	30.70	0.167	0.543
2	26.40	26.96	26.34	26.38	26.25	26.44	26.44	26.46	0.232	0.876
3	29.93	29.93	29.99	29.91	29.73	30.04	30.18	29.96	0.137	0.456
4	26.87	26.89	27.10	26.96	26.88	27.11	26.94	26.97	0.103	0.381
5	24.75	25.09	24.73	25.12	24.82	25.11	25.02	24.95	0.176	0.704
6	33.86	34.50	34.33	34.40	34.58	34.82	34.95	34.49	0.357	1.035
7	26.46	26.35	26.19	26.30	26.31	26.46	26.19	26.32	0.109	0.415
NC								-	-	-
PC	29.17	29.17	29.02	29.34	29.56	29.62	29.78	29.38	0.278	0.946
MV										**0.67**

CV = Coefficient of variation

Table 16. Intra-assay variance for the exogenous Internal Control (Cy5) for the virotype ASFV 2.0 PCR Kit using Biorad CFX96

Intra-assay variance for the exogenous Internal Control (Cy5)										
	Reactions (C_T Values)							**Mean**	**SD**	**CV [%]**
Samples	1	2	3	4	5	6	7			
1	30.71	31.43	31.09	31.21	31.13	30.65	31.29	31.07	0.288	0.928
2	33.17	33.53	33.77	33.66	33.08	33.37	33.69	33.47	0.266	0.794
3	30.64	31.26	30.44	30.77	30.47	30.55	30.40	30.65	0.300	0.977
4	36.65	36.79	36.07	36.83	36.56	36.74	36.35	36.57	0.273	0.747
5	31.31	31.17	31.31	31.90	31.95	31.51	31.24	31.48	0.317	1.006
6								-	-	-
7	28.47	28.47	28.62	28.60	28.47	28.68	28.36	28.52	0.113	0.397
NC								-	-	-
PC	26.10	26.29	26.66	26.45	26.52	26.89	26.01	26.42	0.310	1.175
MV										**0.86**

CV = Coefficient of variation

Results:

The intra-assay variance is in average 0.527% for FAM (ASFV), 0.669% for the endogenous Internal Control (HEX) and 0.861% for the exogenous Internal Control (Cy5).

4.6.2. Inter-assay variance

ASFV-positive DNA samples (samples 1-5), one negative sample and the controls of the test kit were tested in a sevenfold setup in the same PCR run using the ASFV 2.0 PCR Kit (batch Vali05; Table 17, Table 18, Table 19).

Table 17. Inter-assay variance for ASFV (FAM) for the virotype ASFV 2.0 PCR Kit using Biorad CFX96

Inter-assay variance for ASFV (FAM)										
	Reactions (C_T Values)							**Mean**	**SD**	**CV [%]**
Samples	1	2	3	4	5	6	7			
1	28.79	28.55	28.21	28.27	28.20	28.90	28.38	28.47	0.285	1.000
2	25.45	25.24	25.85	25.85	25.86	25.85	25.20	25.61	0.308	1.204
3	34.38	33.93	33.50	33.84	34.08	34.64	34.53	34.13	0.410	1.200
4	24.09	24.08	23.70	23.53	23.47	24.02	23.53	23.77	0.279	1.174
5								-	-	-
6	28.56	28.51	28.20	28.28	28.50	29.27	28.48	28.54	0.345	1.207
7								-	-	-
NC								-	-	-
PC	29.64	29.61	29.01	29.28	29.14	29.93	29.24	29.41	0.326	1.108
MV										**1.15**

CV = Coefficient of variation

Table 18. Inter-assay variance for the endogenous Internal Control (HEX) for the virotype ASFV 2.0 PCR Kit using Biorad CFX96

Inter-assay variance for the endogenous Internal Control (HEX)										
	Reactions (C_T Values)							**Mean**	**SD**	**CV [%]**
Samples	1	2	3	4	5	6	7			
1	30.77	30.76	30.98	30.32	30.53	30.60	30.42	30.63	0.229	0.748
2	26.44	26.56	26.56	26.32	26.63	26.80	26.56	26.55	0.150	0.566
3	30.18	29.96	30.27	29.75	30.13	30.33	30.68	30.18	0.295	0.976
4	26.94	26.83	27.03	26.73	27.12	26.75	27.05	26.92	0.154	0.571
5	25.02	24.88	24.88	25.43	25.06	24.30	26.44	25.14	0.662	2.634
6	34.95	34.34	34.07	34.71	34.23	34.57	34.44	34.47	0.298	0.865
7	26.34	26.03	26.11	26.20	25.97	26.22	26.35	26.18	0.146	0.559
NC								-	-	-
PC	29.78	29.37	29.36	29.52	30.04	29.44	29.82	29.62	0.262	0.885
MV										**0.98**

CV = Coefficient of variation

Table 19. Inter-assay variance for the exogenous Internal Control (Cy5) for the virotype ASFV 2.0 PCR Kit using Biorad CFX96

Inter-assay variance for the exogenous Internal Control (Cy5)										
	Reactions (C_T Values)							**Mean**	**SD**	**CV [%]**
Samples	1	2	3	4	5	6	7			
1	31.29	31.08	31.37	31.66	31.41	31.60	31.10	31.36	0.224	0.716
2	33.69	33.45	33.61	33.81	33.19	34.11	34.15	33.72	0.342	1.015
3	30.40	30.35	30.43	31.07	30.63	30.84	31.03	30.68	0.305	0.993
4	36.35	35.93	35.94	35.78	35.68	35.84	35.72	35.89	0.224	0.625
5	31.24	31.11	30.88	31.66	31.36	31.20	31.47	31.27	0.252	0.806
6								-	-	-
7	28.25	28.13	28.61	28.13	28.24	28.59	28.38	28.33	0.200	0.706
NC								-	-	-
PC	26.01	25.69	25.84	25.62	25.92	25.80	26.08	25.85	0.166	0.643
MV										**0.79**

CV = Coefficient of variation

Results:

The inter-assay variance is in average 1.15% for FAM (ASFV), 0.98% for the endogenous Internal Control (HEX) and 0.79% for the exogenous Internal Control (Cy5).

4.6.3. Batch-to-batch comparison

The six fold Positive Control of the kit, the titration series of ASFV in-vitro DNA [10^{7}-10^{-1} copies/well] and six ASFV-positive DNA samples (samples 1-6) and the controls of the test kit were tested in the same PCR run using two different batches of the ASFV 2.0 PCR Kit (batch 1=Vali05; batch 2=Vali06). The mean value (Mean), standard deviation (SD) and coefficient of variation (CV) were calculated. Data is shown in Table 20, Table 21, Table 22.

Table 20. Batch-to-Batch – variance for ASFV (FAM) for the virotype ASFV 2.0 PCR Kit using Biorad CFX96

	Batch-to-batch variance (ASFV; FAM)				
	Batch number (C_T Values)		**Mean**	**SD**	**CV [%]**
Samples	1	2			
PC 1	30.11	29.76	29.93	0.246	0.821
PC 2	30.03	29.70	29.87	0.233	0.781
PC 3	30.11	29.82	29.96	0.203	0.677
PC 4	30.09	29.73	29.91	0.253	0.846
PC 5	29.91	29.83	29.87	0.057	0.192
PC 6	30.04	29.75	29.90	0.199	0.666
standard 1	19.71	19.50	19.60	0.155	0.790
standard 2	22.98	22.86	22.92	0.086	0.377
standard 3	26.45	26.50	26.47	0.029	0.109
standard 4	29.71	29.62	29.66	0.061	0.205
standard 5	32.80	32.23	32.51	0.400	1.230
standard 6	36.50	35.59	36.04	0.645	1.790
standard 7	36.12	36.41	36.26	0.199	0.550
standard 8			-	-	-
1	28.38	28.72	28.55	0.242	0.846
2	25.20	25.38	25.29	0.128	0.505
3	34.53	35.02	34.78	0.344	0.990
4	23.53	23.81	23.67	0.197	0.833
5			-	-	-
6	28.48	28.53	28.50	0.038	0.133
NC			-	-	-
PC	29.24	29.39	29.32	0.104	0.354
MV					**0.67**

CV = Coefficient of variation

Table 21. Batch-to-Batch – variance for the endogenous Internal Control (HEX) for the virotype ASFV 2.0 PCR Kit using Biorad CFX96

	Batch-to-batch variance (EC; HEX)				
	Batch number (C_T Values)		**Mean**	**SD**	**CV [%]**
Samples	1	2			
PC 1	29.55	29.12	29.34	0.308	1.051
PC 2	29.50	29.19	29.35	0.220	0.749
PC 3	29.57	29.61	29.59	0.030	0.102
PC 4	29.76	29.32	29.54	0.307	1.040
PC 5	29.19	29.23	29.21	0.033	0.112
PC 6	29.71	29.42	29.56	0.206	0.695
standard 1			-	-	-
standard 2			-	-	-
standard 3			-	-	-
standard 4			-	-	-
standard 5			-	-	-
standard 6			-	-	-
standard 7			-	-	-
standard 8			-	-	-
1	30.42	30.60	30.51	0.131	0.430
2	26.56	26.80	26.68	0.171	0.642
3	30.68	30.33	30.51	0.246	0.807
4	27.05	26.75	26.90	0.210	0.782
5	26.44	26.30	26.37	0.099	0.375
6	34.44	34.57	34.50	0.089	0.257
NC			-	-	-
PC	29.82	29.44	29.63	0.268	0.906
MV					**0.61**

CV = Coefficient of variation

Table 22. Batch-to-Batch – variance for the exogenous Internal Control (Cy5) for the virotype ASFV 2.0 PCR Kit using Biorad CFX96

Batch-to-batch variance (IC; Cy5)					
	Batch number (C_T Values)		Mean	SD	CV [%]
Samples	1	2			
PC 1	27.42	26.69	27.05	0.518	1.914
PC 2	27.08	26.83	26.95	0.173	0.640
PC 3	27.13	27.36	27.24	0.157	0.577
PC 4	27.40	27.11	27.25	0.203	0.745
PC 5	27.13	26.83	26.98	0.209	0.775
PC 6	27.24	26.73	26.99	0.363	1.343
standard 1			-	-	-
standard 2			-	-	-
standard 3			-	-	-
standard 4			-	-	-
standard 5			-	-	-
standard 6			-	-	-
standard 7			-	-	-
standard 8			-	-	-
1	31.10	31.60	31.35	0.354	1.129
2	34.15	34.11	34.13	0.026	0.076
3	31.03	31.84	31.43	0.569	1.810
4	35.72	35.84	35.78	0.085	0.237
5	31.47	31.20	31.34	0.187	0.598
6			-	-	-
NC			-	-	-
PC	26.08	26.80	26.44	0.506	1.913
MV					**0.98**

CV = Coefficient of variation

Results:

The batch-to-batch performance showed on average variance of 0.67% for FAM (ASFV), 0.61% for the endogenous control (HEX) and 0.98% for the exogenous control (Cy5).

4.6.4. Comparison of real-time PCR thermocyclers

The virotype ASFV 2.0 PCR Kit can be used on any standard real-time PCR cycler. Table 23 gives an overview of selected PCR cyclers and their approximate run times, using the virotype ASFV 2.0 protocol.

Note: the use of the PCR kit is not limited to the mentioned instruments.

Table 23. Selected overview of real-time thermocyclers and their approximate run times for the virotype ASFV 2.0 protocol

Thermocycler (manufacturer)	**Model**	**Dyes**	**Run time** [min]
Agilent	Stratagene Mx3000P	FAM, HEX, Cy5	58
Agilent	AriaMx	FAM, HEX, Cy5	55
BioRad	CFX96	FAM, HEX, Cy5	61
QIAGEN	RGQ 5	Green, Yellow, Red	67-70
ThermoFisher	ABI 7500 Fast [1]	FAM, JOE, Cy5	63
ThermoFisher	QuantStudio 5	FAM, VIC, Cy5	56
Roche	LightCycler 480 [2]	465-510, 533-580, 618-660	55

1 Standard Mode setting

2 no color compensation

A titration series of ASFV *in vitro* DNA (10^6 - 1 copies/well), five ASFV-positive DNA samples (samples 1-4, 6), two ASFV-negative samples (sample 5 and 7), two different positive controls (sample 8, PC) and seven negative controls (Neg 1 -7) of the test kit were analyzed in the same PCR run using the virotype ASFV 2.0 PCR Kit. Tree different real-time PCR thermocycler instruments were used in this direct comparison and all used the standard virotype ASFV 2.0 PCR Kit cycling protocol. For ABI 7500, the Standard Mode with ROX as passive reference dye was performed.

The used samples are of following origin:

Sample 1: pool of two INIA reference samples (1+2) in a 1:10 dilution

Sample 2: pool of two INIA reference samples (4+6) in a 1:100 dilution

Sample 3: pool of two INIA reference samples (7+8) in a 1:10 dilution

Sample 4: pool of two INIA reference samples (12+13) in a 1:10 dilution, resembling weak exogenous internal control (inhibition) after extraction

Sample 5: pool of two ASFV-negative INIA reference samples in a 1:10 dilution

Sample 6: ASFV-positive FLI reference sample in a 1:1000 dilution

Sample 7: Negative Control (Kit component)

Sample 8: Positive Control (Kit component)

INIA pool samples are comprised of ASFV genotypes I (NH/P68; L60), II (Ukr12/Zapo; Arm07), IX (Ken06.Bus) and X (Ken95/Tk1). Sample materials were spleen homogenates, lung homogenates, lymph node and serum. Since they were pooled, sample may contain more than one genotype/sample material.

The FLI DNA-sample was blood from domestic pig infected with genotype II ASFV strain (Arm02) diluted in negative DNA extracted from wildboar sample (with IC-DNA added).

The results are summarized in Table 24 (ASFV pathogen/ FAM channel), Table 25 (endogenous control/ HEX channel), and Table 26 (exogenous control/ Cy5 channel).

The cyclers used in this comparison are the following:

Cycler A: BioRad CFX96

Cycler B: Stratagene Mx3000P

Cycler C: AriaMx

Cycler D: QIAGEN RGQ 5

Cycler E: ABI 7500 (standard mode)

Cycler F: QuantStudio 5

Table 24. Inter-thermocycler variance for the pathogen ASFV (FAM) channel

Inter-cycler variance (ASFV; FAM) – C_T Values						
	Cycler					
Samples	**A**	**B**	**C**	**D**	**E**	**F**
10^6	19.25	18.61	19.51	18.32	19.80	20.11
10^5	22.45	21.88	22.54	21.55	23.32	23.11
10^4	26.05	25.3	26.24	24.99	26.68	26.82
10^3	29.3	28.52	28.74	28.51	30.37	30.60
10^2	32.4	32.1	33.57	32.01	32.61	33.77
10	35.95	35.02	35.93	34.78	36.01	35.93
5	38.98	38.37	38.38	37.75	38.92	38.82
1	-	-	-	-	-	-
Sample 1	28.21	28.71	27.14	27.57	25.83	27.03
Sample 2	25.85	25.42	23.28	24.38	23.34	23.98
Sample 3	31.5	30.37	31.94	30.44	31.21	31.78
Sample 4	23.7	24.12	22.55	23.33	23.12	23.87
Sample 5	-	-	-	-	-	-
Sample 6	28.2	28.64	26.92	26.54	26.93	27.66
Sample 7	-	-	-		-	-
Sample 8	29.01	29.76	30.25	28.97	27.67	28.92
Neg 1	-	-	-	-	-	-
Neg 2	-	-	-	-	-	-
Neg 3	-	-	-	-	-	-
Neg 4	-	-	-	-	-	-
Neg 5	-	-	-	-	-	-
Neg 6	-	-	-	-	-	-
Neg 7	-	-	-	-	-	-
PC	27.99	28.25	27.88	27.90	27.34	27.71

Table 25. Inter-thermocycler variance for the endogenous control (HEX) channel

Inter-cycler variance (endogenous control; HEX) – C_T Values						
	Cycler					
Samples	**A**	**B**	**C**	**D**	**E**	**F**
10^6	-	-	-	-	-	-
10^5	-	-	-	-	-	-
10^4	-	-	-	-	-	-
10^3	-	-	-	-	-	-
10^2	-	-	-	-	-	-
10	-	-	-	-	-	-
5	-	-	-	-	-	-
1	-	-	-	-	-	-
Sample 1	29.32	28.32	28.99	28.05	29.84	29.77
Sample 2	24.32	26.46	24.78	24.89	25.52	26.62
Sample 3	29.75	29.96	28.66	28.30	28.99	29.26
Sample 4	26.73	26.97	25.05	25.32	25.62	26.20
Sample 5	23.43	24.95	23.47	23.14	24.03	25.06
Sample 6	26.20	26.18	28.95	27.71	26.49	26.02
Sample 7	-	-	-	-	-	-
Sample 8	29.52	29.38	29.25	28.67	28.66	29.03
Neg 1	-	-	-	-	-	-
Neg 2	-	-	-	-	-	-
Neg 3	-	-	-	-	-	-
Neg 4	-	-	-	-	-	-
Neg 5	-	-	-	-	-	-
Neg 6	-	-	-	-	-	-
Neg 7	-	-	-	-	-	-
PC	28.00	28.38	27.28	27.17	27.63	28.04

Table 26. Inter-thermocycler variance for the exogenous control (Cy5) channel

Inter-cycler variance (exogenous control; Cy5) – C_T Values						
	Cycler					
Samples	**A**	**B**	**C**	**D**	**E**	**F**
10^6	-	-	-	-	-	-
10^5	-	-	-	-	-	-
10^4	-	-	-	-	-	-
10^3	-	-	-	-	-	-
10^2	-	-	-	-	-	-
10	-	-	-	-	-	-
5	-	-	-	-	-	-
1	-	-	-	-	-	-
Sample 1	29.95	31.36	32.94	30.14	29.94	30.60
Sample 2	31.37	32.72	30.98	30.47	31.91	31.97
Sample 3	29.55	30.68	29.53	28.41	31.49	29.55
Sample 4	36.74	35.89	34.42	34.92	38.08	35.67
Sample 5	31.51	31.27	31.02	30.13	32.36	32.20
Sample 6	28.18	28.33	27.08	27.55	28.40	28.85
Sample 7	-	-	-	-	-	-
Sample 8	24.89	25.85	24.61	24.43	25.54	24.89
Neg 1	-	-	-	-	-	-
Neg 2	-	-	-	-	-	-
Neg 3	-	-	-	-	-	-
Neg 4	-	-	-	-	-	-
Neg 5	-	-	-	-	-	-
Neg 6	-	-	-	-	-	-
Neg 7	-	-	-	-	-	-
PC	27.85	28.98	27.81	27.34	28.38	27.82

Results:

All samples tested on different real-time PCR thermocycler instruments showed comparable results.

No false-positive or false-negative were observed. Differences between all real-time PCR thermocycler instruments were within the normal deviation (SD = 0.13 - 1.5) expected when performing inter-instrument comparison studies. The inter-instrument variability value (less than 4 %) exhibited high repeatability in the results obtained.

Based on this data set, INDICAL can recommend using the virotype ASFV 2.0 PCR Kit on the BioRad CFX96, the Agilent AriaMx, the Agilent Stratagene 3005P, the Rotor-Gene and the ABI 7500 instruments.

4.7. Stability

4.7.1. Stability testing (freeze-thaw-cycles)

Validating the stability of the virotype ASFV 2.0 PCR Kit, the six fold Positive Control of the kit, the titration series of ASFV in-vitro DNA [10^{7}-10^{-1} copies/well] and six ASFV-positive DNA samples (samples 1-6) and the controls of the test kit were tested using one batch (Vali06) at the time of production and after six freeze/thaw cycles (Table 27, Table 28, Table 29).The mean value (Mean), standard deviation (SD) and coefficient of variation (CV) were calculated.

Table 27. Stability testing for ASFV (FAM) of the virotype ASFV 2.0 PCR Kit using Biorad CFX96

	Stability (ASFV; FAM)				
	freeze/thaw cycles (C_T Values)		**Mean**	**SD**	**CV [%]**
Samples	1	6			
PC 1	29.67	29.54	29.61	0.092	0.311
PC 2	29.76	29.56	29.66	0.141	0.476
PC 3	29.56	29.68	29.62	0.088	0.297
PC 4	29.46	29.60	29.53	0.099	0.335
PC 5	29.51	29.43	29.47	0.056	0.189
PC 6	29.56	29.55	29.56	0.008	0.028
standard 1	19.51	19.19	19.35	0.224	1.155
standard 2	22.80	22.39	22.60	0.291	1.286
standard 3	26.13	25.84	25.98	0.204	0.784
standard 4	29.36	29.45	29.40	0.066	0.224
standard 5	32.40	32.26	32.33	0.098	0.302
standard 6	34.87	34.96	34.92	0.060	0.171
standard 7	36.73	35.34	36.03	0.986	2.736
standard 8			-	-	-
1	28.71	28.48	28.59	0.165	0.578
2	25.35	25.30	25.33	0.032	0.128
3	34.68	34.16	34.42	0.365	1.061
4	23.97	24.01	23.99	0.023	0.098
5			-	-	-
6	29.16	29.11	29.13	0.035	0.119
NC			-	-	-
PC	29.75	29.71	29.73	0.026	0.089
MV					**0.55**

CV = Coefficient of variation

Table 28. Stability testing for the endogenous Internal Control (HEX) of the virotype ASFV 2.0 PCR Kit using Biorad CFX96

	Stability (EC; HEX)				
	freeze/thaw cycles (C_T Values)		**Mean**	**SD**	**CV [%]**
Samples	1	6			
PC 1	29.64	29.47	29.55	0.124	0.419
PC 2	29.79	29.47	29.63	0.225	0.761
PC 3	29.41	29.58	29.49	0.118	0.400
PC 4	29.35	29.40	29.38	0.035	0.120
PC 5	29.28	29.62	29.45	0.235	0.799
PC 6	29.53	29.78	29.65	0.178	0.600
standard 1			-	-	-
standard 2			-	-	-
standard 3			-	-	-
standard 4			-	-	-
standard 5			-	-	-
standard 6			-	-	-
standard 7			-	-	-
standard 8			-	-	-
1	30.73	30.57	30.65	0.114	0.372
2	26.52	26.68	26.60	0.111	0.417
3	30.34	30.23	30.29	0.083	0.274
4	27.04	27.18	27.11	0.105	0.388
5	25.63	25.94	25.79	0.220	0.853
6	34.61	34.80	34.71	0.135	0.390
NC			-	-	-
PC	29.21	29.98	29.59	0.539	1.821
MV					**0.59**

CV = Coefficient of variation

Table 29. Stability testing for the exogenous Internal Control (Cy5) of the virotype ASFV 2.0 PCR Kit using Biorad CFX96

	Stability (IC; Cy5)				
	freeze/thaw cycles (C_T Values)		**Mean**	**SD**	**CV [%]**
Samples	1	5			
PC 1	26.26	27.01	26.64	0.527	1.980
PC 2	26.23	26.14	26.18	0.061	0.234
PC 3	26.07	25.99	26.03	0.058	0.224
PC 4	25.66	26.15	25.90	0.341	1.318
PC 5	26.29	27.36	26.82	0.754	2.812
PC 6	26.43	26.92	26.67	0.353	1.324
standard 1			-	-	-
standard 2			-	-	-
standard 3			-	-	-
standard 4			-	-	-
standard 5			-	-	-
standard 6			-	-	-
standard 7			-	-	-
standard 8			-	-	-
1	31.16	31.23	31.19	0.051	0.162
2	35.14	34.72	34.93	0.292	0.837
3	31.29	31.47	31.38	0.130	0.414
4	36.21	36.99	36.60	0.548	1.499
5	31.84	32.58	32.21	0.522	1.619
6			-	-	-
NC			-	-	-
PC	25.14	25.81	25.48	0.476	1.870
MV					**1.19**

CV = Coefficient of variation

Results:

The virotype ASFV 2.0 PCR Kit shows excellent stability with an average variance of 0.67% for FAM (ASFV), 0.61% for the endogenous control (HEX) and 0.98% for the exogenous control (Cy5).

4.7.2. Stability testing (heparin inhibition)

To test the stability of of the virotype ASFV 2.0 PCR Kit, sample inhibition was simulated by treating the Positive control and one sample with an increasing concentration of heparin (0.85-17 U/µl). The samples were tested using one batch (Vali06). Results are shown in Table 30.

Table 30. Stability testing of the virotype ASFV 2.0 PCR Kit for inhibition by heparin.

Stability (Heparin inhibition)				
	Heparin (U/µl)	**target (C_T Values)**		
Samples		FAM (ASFV)	EC (HEX)	IC (Cy5)
PC	-	30.03	29.81	26.41
PC	0.85	29.99	29.66	26.48
PC	0.85	29.95	29.98	27.44
PC	1.7	29.87	29.29	26.27
PC	1.7	30.06	29.45	26.12
PC	8.5	38.12		
PC	8.5			
PC	17			
PC	17			
Sample	-	26.08	26.19	29.67
Sample	0.85	26.13	27.18	30.65
Sample	0.85	26.07	27.15	30.48
Sample	1.7	26.18	26.48	32.19
Sample	1.7	26.12	27.03	33.23
Sample	8.5	33.80		
Sample	8.5	35.50		
Sample	17			
Sample	17			

Results:

The inhibition study using heparin showed that when sample inhibition occurred that the internal control signals will be reduced first. Both Internal Control will help to identify partially and fully inhibited samples.

4.7.3. Stability testing (real-time stability)

To evaluate the real time stability, the virotype ASFV 2.0 PCR Kit (batch Vali05) was tested after 8 months storage at -30°C to -15°C using ASFV-positive and
ASFV-negative field samples (INIA), as well as the Negative Control (NC) and Positive Control (PC).

The delta C_T (ΔC_T), standard deviation (SD) and coefficient of variation (CV) were calculated. Results are shown in Table 31, Table 32, and Table 33.**Fehler! Verweisquelle konnte nicht gefunden werden.**

Table 31. Stability testing for ASFV of the virotype ASFV 2.0 PCR Kit after 8 months storage.

			Storage Evaluation				
			FAM (C_T Values)		**ΔC_T**	**SD**	**CV [%]**
Sample	ASFV Status	Material	0 months	8 months			
INIA-17	+	spleen	22.19	22.85	-0.66	0.47	2.07
INIA-18	+	spleen	33.47	33.01	0.46	0.33	0.98
INIA-19	+	lung	21.02	21.44	-0.42	0.30	1.40
INIA-20	+	lung	21.18	20.51	0.67	0.47	2.27
INIA-21	+	lymph	21.65	22.40	-0.75	0.53	2.41
INIA-22	+	spleen	20.28	20.00	0.28	0.20	0.98
INIA-23	+	spleen	29.91	30.72	-0.81	0.57	1.89
INIA-24	+	spleen	28.12	27.98	0.14	0.10	0.35
INIA-28	+	serum	18.09	18.27	-0.18	0.13	0.70
INIA-29	+	serum	21.03	21.30	-0.27	0.19	0.90
INIA-30	+	serum	25.21	25.37	-0.16	0.11	0.45
INIA-32	+	liver	26.73	26.87	-0.14	0.10	0.37
I-1	+	spleen	27.81	27.24	0.57	0.40	1.46
I-2	+	lung	25.38	25.32	0.06	0.04	0.16
I-3	+	spleen	34.81	34.43	0.38	0.27	0.77
I-4	+	serum	24.10	24.47	-0.37	0.26	1.08
INIA-25	-	kidney					
INIA-26	-	lung					
INIA-27	-	tonsil					
INIA-31	-	serum					
I-5	-	blood					
I-6	-	blood					
NC							
PC			29.85	29.83	0.02	0.01	0.05
MV							**1.09**

CV = Coefficient of variation

Table 32. Stability testing for the endogenous Internal Control of the virotype ASFV 2.0 PCR Kit after 8 months storage.

			Storage Evaluation				
			HEX (C_T Values)		**ΔC_T**	**SD**	**CV [%]**
Sample	ASFV Status	Material	0 months	8 months			
INIA-17	+	spleen	25.80	25.81	-0.01	0.01	0.03
INIA-18	+	spleen	29.47	30.04	-0.57	0.40	1.35
INIA-19	+	lung	24.81	25.20	-0.39	0.28	1.10
INIA-20	+	lung	23.53	23.69	-0.16	0.11	0.48
INIA-21	+	lymph	26.46	26.74	-0.28	0.20	0.74
INIA-22	+	spleen	21.98	22.32	-0.34	0.24	1.09
INIA-23	+	spleen	26.34	26.82	-0.48	0.34	1.28
INIA-24	+	spleen	25.46	26.40	-0.94	0.66	2.56
INIA-28	+	serum	22.23	22.25	-0.02	0.01	0.06
INIA-29	+	serum	25.03	25.59	-0.56	0.40	1.56
INIA-30	+	serum	28.15	29.29	-1.14	0.81	2.81
INIA-32	+	liver	22.29	23.29	-1.00	0.71	3.10
I-1	+	spleen	30.56	30.79	-0.23	0.16	0.53
I-2	+	lung	26.40	26.02	0.38	0.27	1.03
I-3	+	spleen	29.93	29.45	0.48	0.34	1.14
I-4	+	serum	26.87	26.72	0.15	0.11	0.41
INIA-25	-	kidney	25.31	25.98	-0.67	0.47	1.85
INIA-26	-	lung	25.94	26.16	-0.22	0.16	0.60
INIA-27	-	tonsil	25.50	25.80	-0.30	0.21	0.83
INIA-31	-	serum	33.52	34.22	-0.70	0.49	1.46
I-5	-	blood	24.75	25.70	-0.95	0.67	2.67
I-6	-	blood	26.46	26.89	-0.43	0.30	1.14
NC							
PC			29.17	29.87	-0.70	0.49	1.67
MV							**1.25**

CV = Coefficient of variation

Table 33. Stability testing for the exogenous Internal Control of the virotype ASFV 2.0 PCR Kit after 8 months storage.

			Storage Evaluation				
			Cy5 (C_T Values)		ΔC_T	SD	CV [%]
Sample	ASFV Status	Material	0 months	8 months			
INIA-17	+	spleen	27.60	26.27	1.33	0.94	3.49
INIA-18	+	spleen	27.84	27.98	-0.14	0.10	0.35
INIA-19	+	lung	31.42	31.18	0.24	0.17	0.54
INIA-20	+	lung	30.83	31.12	-0.29	0.21	0.66
INIA-21	+	lymph	26.99	27.51	-0.52	0.37	1.35
INIA-22	+	spleen	32.21	32.84	-0.63	0.45	1.37
INIA-23	+	spleen	27.72	28.19	-0.47	0.33	1.19
INIA-24	+	spleen	27.33	27.91	-0.58	0.41	1.48
INIA-28	+	serum	37.57	37.89	-0.32	0.23	0.60
INIA-29	+	serum	29.19	28.69	0.50	0.35	1.22
INIA-30	+	serum	26.79	26.97	-0.18	0.13	0.47
INIA-32	+	liver	27.59	28.02	-0.43	0.30	1.09
I-1	+	spleen	30.71	30.14	0.57	0.41	1.33
I-2	+	lung	33.17	33.78	-0.61	0.43	1.28
I-3	+	spleen	30.64	30.72	-0.08	0.06	0.20
I-4	+	serum	36.65	37.23	-0.58	0.41	1.12
INIA-25	-	kidney	27.12	27.59	-0.47	0.33	1.21
INIA-26	-	lung	27.28	27.24	0.04	0.03	0.10
INIA-27	-	tonsil	27.28	27.52	-0.24	0.17	0.62
INIA-31	-	serum	26.81	26.89	-0.08	0.06	0.21
I-5	-	blood	31.31	31.95	-0.64	0.45	1.42
I-7	-	blood	28.47	28.89	-0.42	0.30	1.03
NC							
PC			26.10	26.78	-0.68	0.48	1.83
MV							**1.05**

CV = Coefficient of variation

Results:

After 8 months of storage at -30°C to -15°C the mean C_T value results showed only a minor decrease of 1.1 % for ASFV (FAM), 1.3 % for the endogenous Internal Control (HEX) and 1.1 % for the exogenous Internal Control (Cy5). Therefore, the virotype ASFV PCR Kit demonstrated an excellent real-time stability.