RealPCR* ASFV DNA Test

Validation data report: PCR and complete method

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Test with Confidence

IDEXX

I. Disease Information

African swine fever virus (ASFV) is the causative agent of hemorrhagic African swine fever. ASFV is a highly contagious agent infecting warthogs, bushpigs, and *Ornithodoros* soft ticks. The condition often presents with subclinical symptoms that can persistently infect the animal. A reportable disease to the World Health Organization for Animal Health (OIE), ASFV has a high morbidity and mortality rate in domestic swine. Controlling outbreaks of ASFV relies on early detection of the virus.

II. Glossary of terms

The following definitions have been taken from French standard NF U47-310 entitled, “Méthodes d’analyse en santé animale - Contrôle de réactifs biologiques pour les techniques immunologiques utilisées dans le domaine de la santé animale” [Animal health analysis methods – Biological reagents control for the immunological techniques used in animal health] issued December 2001, updated September 2013 (XP U47-310).

Exclusivity—The exclusivity is the capacity of the assay to detect an analyte or genomic sequence that is unique to a targeted organism and excludes all other known organisms that are potentially cross-reactive.

Inclusivity—The inclusivity is the capacity of an assay to detect several strains or serovars of a species, several species of a genus, or a similar grouping of closely related organisms or antibodies.

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

Reproducibility (interlaboratory)—Degree of agreement between analyses repeated from the same sample in different laboratories, with the same reagent, and following the same technical protocol.

Robustness—Degree of agreement between analyses repeated from the same sample, with the same reagent, in the same laboratory, and under the operating condition limits set forth in the technical protocol.

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

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

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

Stability—Assessment of finished product stability for the period defined by the manufacturer, under recommended storage conditions.

III. Test information

A. Product name

RealPCR* ASFV DNA Test

B. Product description

The IDEXX RealPCR* system is a modular format in which disease-specific target mixes are paired with a standardized DNA or RNA master mix and a single pooled positive control. Reagents are individually packaged and sold separately to allow for flexible reagent handling.

The RealPCR ASFV DNA Mix (ASFV DNA Mix) contains primers and probes for the detection of ASFV DNA when amplified with RealPCR* DNA Master Mix (DNA MMx). The internal control for the test is based on the detection of a genomic DNA sequence in the sample that is conserved in swine. This genomic target is referred to as the internal sample control (ISC). Primers and probe for detection of the internal sample control are included in the ASFV DNA Mix.

IV. Materials and methods

A. Materials required but not provided

- Commercial DNA extraction kit
- Optional—centrifuge with a rotor and adapters for multiwell plates
- Micro-centrifuge for 2 mL microtubes capable of reaching 1,500–3,000 x g
- Appropriate personal protective equipment (e.g., gloves, lab coat)
- Nuclease-free, aerosol-resistant pipette tips
- Sterile microtubes for preparation of PCR mix
- Pipettes (5–1,000 µL); dedicated pipettes for preparation of PCR mix
- 96- or 384-well format PCR plates and optical adhesive film/plate covers
- Real-time PCR instrument (Applied Biosystems* 7500 Fast System (Standard and Fast Mode), Applied Biosystems* ViiA* 7, Applied Biosystems* QuantStudio 5, Agilent Mx3000P*, Agilent Mx3005P*, Agilent AriaMx*, Bio-Rad* CFX96 Touch*, Roche LightCycler* 480, QIAGEN* Rotor-Gene* Q [72-well rotor only], Bio Molecular Systems Mic qPCR Cycler, or equivalent).

B. Test components

Table 1. RealPCR ASFV DNA Test component information

Material	Form (volume)	Storage conditions	Cap color	Description
RealPCR ASFV DNA Mix	Dried (1 mL)	-25ºC to 8°C† -25ºC to -15°C‡	Green	Contains primers and probe for the detection of ASFV DNA, as well as primers and probe for the detection of the internal sample control (ISC).
RealPCR DNA Master Mix	Liquid (1 mL)	-25ºC to -15°C	Purple	Concentrated master mix that includes hot-start polymerase, ROX*, and nucleotides.
RealPCR* Positive Control	Dried (0.5 mL)	-25ºC to 8°C† -25ºC to -15°C‡	Blue	The PC contains all RealPCR and internal control targets (including the target for ASFV) and is intended for use with all RealPCR target mixes. Also includes a unique IDEXX Signature Sequence that can be used in combination with an additional target mix to monitor for positive control contamination of work areas.
RealPCR* PCR Grade Water	Liquid (2 x 1 mL)	-25ºC to 8°C	Clear	Qualified for reverse-transcription PCR (RT-PCR) use. It is used for reconstitution of the target mix and the positive control. It is also used as the PCR negative control for each test run.

† Storage at receipt
‡ Storage after reconstitution

C. Sample types

The RealPCR ASFV DNA Test has been validated for use in serum, plasma, EDTA blood, oral fluids, tissue (including lung, bone marrow, tonsils, kidney, spleen, and lymph nodes) and swab samples from swine, including wild boars. Samples can be tested in pools of up to 20 samples.

D. DNA extraction

DNA was extracted using one of the following commercial extraction methods:

- RealPCR DNA/RNA Magnetic Bead Kit (IDEXX)
- RealPCR DNA/RNA Spin Column Kit (IDEXX)
- MagMAX* 96 Viral RNA Isolation (Thermo Fisher Scientific)
- QIAamp* DNA Mini Kit (QIAGEN*)
- High Pure PCR Template Preparation Kit (Roche)

E. PCR instruments and settings

Data contained in this report was generated using PCR instruments approved for use with the RealPCR reagents.

Table 2 describes the reporter and quencher settings for the ASFV, internal control, and passive reference channels. Table 3 outlines the standard cycling program used for all RealPCR* DNA and RNA tests.

Table 2. Settings for reporter and quencher

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

Table 3. RealPCR standard DNA/RNA cycling program

Step	Temperature	Time	Cycles
Reverse transcription (RT)	50°C	15 min	1
Denaturation	95°C	1 min	1
Amplification†	95°C 60°C	15 sec 30 sec	45

† Ensure the instrument is set to record fluorescence following the 60°C amplification step.
Note: For DNA targets, the cycling protocol can be run without the RNA RT step. However, it is recommended that the RT step is used.

F. Test procedure, validity, and interpretation of results

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

RealPCR ASFV DNA Test procedure

1. Mix 10 µL ASFV DNA Mix and 10 µL DNA MMx per reaction to be tested.
2. Pipette 20 µL of the PCR mix into the required wells of the multiwell plate.
3. Add 5 µL of sample DNA to each well. The final reaction volume is 25 µL.
4. Include the RealPCR* Positive Control (5 µL) and PCR negative control (5 µL RealPCR PCR Grade Water) for each test run.
5. Cover the plate and briefly spin the plate, if necessary, to settle contents and remove air bubbles.
6. Set up thermal cycler with RealPCR standard DNA/RNA cycling program.
7. Insert plate and start the run.

Table 4. Test validity criteria

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

† Cycle threshold

Table 5. Interpretation of results

Sample result	FAM amplification	HEX (VIC) amplification	Sample validity
ASFV DNA detected	Yes	Yes/no	A positive Ct value and characteristic amplification curve in comparison to the PCR negative control. An internal control amplification curve in the HEX (VIC) channel is expected; some strong ASFV positive DNA samples may result in a negative internal control result.†
ASFV DNA not detected	No	Yes	Amplification curve in the HEX (VIC) internal control channel.
Invalid‡	No	No	Absence of amplification curve in the FAM and HEX (VIC) channel indicates an invalid result.‡

† The target mix is optimized for the detection of ASFV DNA; a strong positive DNA sample may out compete the detection of the internal control.

‡ An invalid sample can be an indication of failed sample addition, extraction and/or PCR. It is recommended that the DNA be diluted five-fold into PCR grade water and retested; include the undiluted DNA as a sample. If the test is still not valid a new extraction is recommended.

V. PCR characterization

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

Exclusivity, inclusivity, analytical sensitivity, efficiency, repeatability, reproducibility, stability, and robustness are included in the PCR characterization.

A. Exclusivity-*In silico* analysis

Purpose: To demonstrate, through *in silico* analysis, the exclusion of reaction of the RealPCR ASFV DNA Test to pathogens other than ASFV.

Procedure: To demonstrate design specificity, the PCR amplicon sequence as well as the primer and probe sequences used in the ASFV test and internal sample control were searched for in the National Center for Biotechnology Information (NCBI) nr/nt nucleotide database. Program selection of "blastn" and a word size of 16 was used; all other settings remained as default. Sequences associated with ASFV were excluded from the results. This analysis was intended to return orthologous sequences that could potentially be amplified with the RealPCR ASFV reagents.

Results/conclusions: *In silico* analysis showed a short 19-base region of similarity to *Escherichia coli* and several serovars of *Salmonella enterica*. The section did not cover the entire RealPCR ASFV design region and, given the limited region of similarity, nonspecific detection of these organisms is not possible. This analysis supports the specificity of the RealPCR ASFV DNA Test to detect ASFV exclusively.

B. Exclusivity- Experimental testing

Purpose: To evaluate the specificity of the PCR by testing a panel of organisms that are similar to ASFV with common ecological niches or clinical symptoms or that are genetically close.

Procedure: The determination of the analytical specificity for the RealPCR ASFV DNA Test was performed using defined bacterial and viral strains, specifically those identified as common swine pathogens.

Results/conclusions: Experimental testing for exclusivity of the RealPCR ASFV DNA Test is shown in table 6. No amplification with the RealPCR ASFV DNA Test design was observed for any of the pathogens tested. These results are consistent with the *in silico* exclusivity analysis and show that the RealPCR ASFV DNA Test detects ASFV exclusively.

Table 6. Exclusivity of the RealPCR ASFV DNA Test

Organism/pathogen	Serovar/strain	Sample type	RealPCR ASFV Ct	RealPCR ASFV (dRn)
Salmonella	(4,5,12:i:-)	Culture	No Ct	0.00
Salmonella	Typhimurium	Culture	No Ct	0.00
Salmonella	Derby	Culture	No Ct	0.00
Salmonella	Dublin ATCC 39184	Culture	No Ct	-0.01
Salmonella	Choleraesuis var. Kunzendorf	Culture	No Ct	0.00
Salmonella	London	Culture	No Ct	0.00
E. coli	ATCC 10798 (K-12)	Culture	No Ct	0.00
E. coli	ATCC 11775 (NCTC 9001)	Culture	No Ct	0.00
E. coli	ATCC 25922 N62/Ec25 (Seattle 1946)	Culture	No Ct	0.00
E. coli	ATCC 8739 N63/Ec8 (Crooks)	Culture	No Ct	0.00
Clostridium perfringens	Strain S 107	Culture	No Ct	0.00
Campylobacter jejuni	Subsp. jejuni	Culture	No Ct	0.00
Porcine epidemic diarrhea virus (PEDV)	N/A	Oral fluid	No Ct	0.00
Porcine deltacoronavirus (PDCoV)	N/A	Oral fluid	No Ct	0.01
PDCoV	N/A	Isolate	No Ct	0.00
Transmissible gastroenteritis virus (TGEV)	N/A	Fecal swab	No Ct	0.00
EU PRRS (type 1)	N/A	Serum	No Ct	-0.03
NA PRRS (type 2)	N/A	Serum	No Ct	-0.04
Mycoplasma hyopneumoniae	N/A	Lung lavage	No Ct	0.01

C. Inclusivity- *In silico* analysis

Purpose: To demonstrate ASFV specificity through *in silico* analysis of primer and probe sequence homology to a highly conserved region of the ASFV genome.

Procedure: The primer and probe sequences were submitted for BLAST* analysis to the NCBI nr/nt database, limiting the search set to ASFV (taxid 10497), optimized for highly similar sequences.

Results/conclusions: A total of 624 hits were returned as matches to the RealPCR ASFV DNA Test design. 99.7% of the returns have a perfect match to the primer and probe sequences. Results of the analysis demonstrate that the RealPCR ASFV DNA Test is highly inclusive of ASFV sequences.

D. Inclusivity- Experimental testing

Purpose: To evaluate the inclusivity of the RealPCR ASFV DNA Test using 22 of the known 23 ASFV reference p72 genotype isolates.[2]

Procedure: ASFV reference isolates were tested at the Instituto Nacional de Innovación Agraria-Centro de Investigacion en Sanidad Animal (INIA-CISA) in Madrid, Spain. Testing of the RealPCR ASFV DNA Test was done in parallel with two other ASFV reference tests, OIE real-time developed by King et al., 2003[1] and the UPL real-time PCR developed by Fernández-Pinero et al., 2013[3].

A second study at the INIA laboratory tested experimental blood samples from the European Union Reference Laboratories (EURL) sample bank. This sample set included a variety of clinical forms of ASFV generated from isolates of different virulence representing genotypes (I, II, IX, and X). The RealPCR ASFV DNA Test was evaluated in parallel with two other ASFV reference tests, the OIE and UPL real-time PCR assays.

An isolate for p72 ASFV genotype XVIII was not available for testing in this study. See results section for an *in silico* analysis of this genotype and the predicted ability of the ASFV DNA Test to detect it.

Results/conclusions: Results for the ASFV reference isolates (n = 22) are shown in table 7. Ct values obtained using the RealPCR ASFV DNA Test are similar to the those obtained using the UPL and OIE real-time PCR reference tests. The RealPCR ASFV DNA Test detected all 22 genotypes, as did the UPL reference test. One genotype was negative on the OIE real-time PCR test.

Results for the ASFV experimental blood samples are shown in table 8. The RealPCR ASFV DNA Test detected the additional ASFV strains as did the UPL reference test. The OIE reference test missed one of the blood samples; Ct values were similar between the three ASFV PCR tests.

In silico analysis of the untested ASFV p27 genotype XVIII demonstrated 100% alignment to the primers and probe used in the RealPCR ASFV DNA Test. Based on this analysis, the RealPCR ASFV DNA Test will detect the untested ASFV genotype as positive.

Table 7. ASFV reference isolates

ASFV isolate	Genotype	Country	Host	Outbreak date	RealPCR ASFV Ct	UPL-PCR Ct value	OIE-PCR Ct value
E75	I	Spain	Domestic pig	1975	19.40	19.44	17.89
Maur08/1	II	Mauritius	Domestic pig	2008	24.21	24.23	23.36
Ukr12/Zapo	II	Ukraine	Domestic pig	2012	18.83	18.01	17.54
RSA2008/1	III	South Africa	Domestic pig	2008	27.22	27.60	27.18
RSA/W/1/99	IV	South Africa	Warthog	1999	23.11	23.17	22.95
Moz64	V	Mozambique	Domestic pig	1964	18.90	18.51	17.58
SPEC/265	VI	Mozambique	Domestic pig	1994	24.07	23.55	23.73
RSA/03/7	VII	South Africa	Warthog	2003	26.43	25.39	25.64
MwLil20/1	VIII	Malawi	Tick	1990	19.20	21.78	18.83
Ken06.Bus	IX	Kenya	Domestic pig	2006	25.63	26.08	25.04
BUR90/1	X	Burundi	Domestic pig	1990	23.92	23.98	23.55
KAB6/2	XI	Zambia	Tick	1983	25.10	27.16	25.22
MZI92/1	XII	Malawi	Domestic pig	1992	24.83	25.74	25.16
SUM14/11	XIII	Zambia	Tick	1983	27.80	28.08	28.49
NYA1/2	XIV	Zambia	Tick	1988	25.57	25.71	25.82
TAN/2008/1	XV	Tanzania	Domestic pig	2008	23.79	24.22	23.02
TAN2003/2	XVI	Tanzania	Domestic pig	2003	23.56	24.01	24.28
NAM/P/1/95	XVII	Namibia	Domestic pig	1995	23.83	25.06	23.81

ASFV isolate	Genotype	Country	Host	Outbreak date	RealPCR ASFV Ct	UPL-PCR Ct value	OIE-PCR Ct value
SPEC125	XIX	South Africa	Domestic pig	1987	36.01	38.49	no ct
24823	XX	South Africa	Domestic pig	1975	33.85	36.12	36.00
SPEC53	XXI	South Africa	Domestic pig	1985	37.13	36.40	38.00
RSA2008/2	XXII	South Africa	Domestic pig	2008	23.78	23.90	24.76
ET13/1505	XXIII	Ethiopia	Domestic pig	2013	17.81	18.13	18.42

Table 8. ASFV experimental blood samples

ASFV strain	Genotype	Country	Clinical form	RealPCR ASFV Ct	UPL-PCR Ct value	OIE-PCR Ct value
Ben97/1	I	Benin 1997	Acute	18.8	19.8	23.2
E70	I	Spain 1970	Acute	19.7	20.9	24.1
L60	I	Lisbon 1960	Acute	18.2	18.5	21.8
Arm07	II	Armenia 2007	Acute	22.3	18.9	21.9
Az08D	II	Azerbaijan 2008	Acute	22.5	21.9	24.1
Lt14/1490	II	Lithuania 2014	Acute	18.9	20.3	28.4
Est15/WB-Valga6	II	Estonia 2016	Subacute	26.4	27.0	25.2
Ken05/Tk1	X	Kenya 2005	Subacute	33.1	32.2	33.7
NHP/68	I	Lisbon 1968	Chronic	23.0	23.0	24.9
646VR85	I	Spain 1960	Subclinical	35.8	35.5	No Ct

E. Analytical sensitivity (LD_{PCR})

Purpose: To determine the smallest number of target nucleic acids per reaction, generating a positive result in 95% of cases when tested on the RealPCR ASFV DNA Test.

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

Results/conclusions: Results for the initial analytical sensitivity determination over a large range of dilutions are shown in table 9 and figure 1. Results for the additional testing at the limit of detection are shown in table 10. The ASFV target was consistently detected at concentrations of 10 copy per reaction, with positive results in 3 out of 3 wells tested initially, and 24/24 wells (100%) in the confirmation tests.

Table 9. ASFV analytical sensitivity

DNA copies	Mean Ct	Replicates
10,000,000	13.8	3/3
1,000,000	17.0	3/3
100,000	20.2	3/3
10,000	23.5	3/3
1,000	26.8	3/3
100	30.0	3/3
10	33.8	3/3

Figure 1. ASFV amplification curves

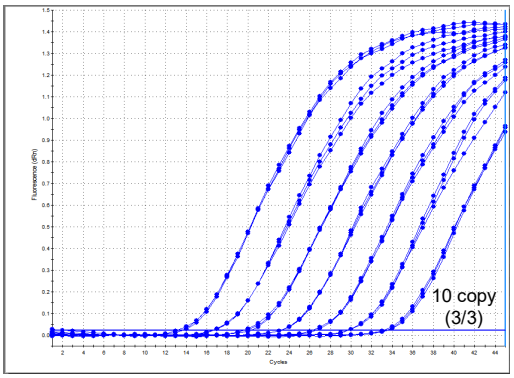


Table 10. RealPCR ASFV DNA Test analytical sensitivity confirmation

		# positive in 8 wells				
DNA copies per 25 µL reaction	Session #1	Session #2	Session #3	Mean Ct value	# positive in 24 tests	% positive results
10	8	8	8	32.1	24/24	100%
100	8	8	8	29.6	24/24	100%
1,000	8	8	8	26.2	24/24	100%

F. Efficiency of PCR

Purpose: To determine the PCR efficiency for the RealPCR ASFV DNA Test.

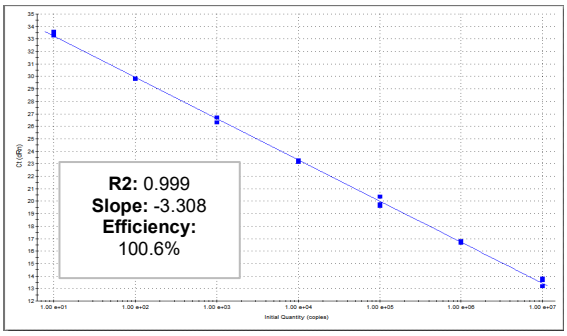
Procedure: Efficiency was evaluated with dilutions of synthetic material representing the target sequence. Results were obtained by testing a series of tenfold dilutions of the stock material, from 10^{-4} (10,000,000 copies/25 µL PCR reaction) through 10^{-10} (10 copies/25 µL PCR reaction). Testing was performed in a single session, testing three replicates of each dilution.

Results/conclusions: Ct results for the DNA dilution series are shown in table 11 below. and the standard curve is shown in figure 2. Efficiency of the test, calculated as $(10^{(-1/slope)-1)} \times 100$, is determined to be 100.6% over a 7-log range.

Table 11. ASFV DNA dilution series

DNA copies	Mean Ct	Replicates
10,000,000	13.8	3/3
1,000,000	17.0	3/3
100,000	20.2	3/3
10,000	23.5	3/3
1,000	26.8	3/3
100	30.0	3/3
10	33.8	3/3

Figure 2. ASFV standard curve



G. Repeatability

Purpose: To demonstrate consistent results between replicates of a sample, both within and between successive runs of the test under constant operator, laboratory, and instrument conditions.

Procedure: Synthetic ASFV DNA target was diluted to high (1,000 copy), medium (mid) (100 copy), and low (10 copy) copies per 25 µL reaction and run with the RealPCR ASFV DNA Test, using standard test reagents and protocol. Samples were tested in triplicate, by the same operator, using the same reagent lots and PCR instrument for each test event. Testing was repeated in three independent events, and the coefficient of variability (%CV) was calculated from nine ASFV (FAM) Ct values for each sample as a measure of repeatability.

Results/conclusions: Results are reported in table 12. For all three target levels tested, the %CV values were less than 2%. This study demonstrates highly repeatable results with the RealPCR ASFV DNA Test.

Table 12. Repeatability ASFV Ct values

		ASFV (FAM) Ct Values				
		Run-1	**Run-2**	**Run-3**	**Mean Ct Value**	**% CV**
Low Level	**Rep-1**	33.77	34.98	33.1	**34.4**	**1.6%**
	Rep-2	33.69	35.44	34.84		
	Rep-3	34.78	34.73	34.47		
Mid Level	**Rep-1**	31.41	31.8	31.03	**31.4**	**1.2%**
	Rep-2	31.5	31.71	31.06		
	Rep-3	31.23	31.79	30.94		
High Level	**Rep-1**	28	28.76	28.48	**28.4**	**1.7%**
	Rep-2	27.26	28.74	28.48		
	Rep-3	28.22	28.8	28.54		

H. Interlaboratory reproducibility/instrument validation

Purpose: To demonstrate consistent results between replicates of a sample under variable conditions of operator, laboratory, and PCR instrument.

Procedure: High, mid, and low levels of a synthetic ASFV target were used for this testing. Samples were tested in duplicate wells for each PCR run. Testing was performed by two operators working in different laboratories. Operator 1 ran the test on six instruments, while Operator 2 used two instrument models. The coefficient of variability (%CV) was calculated from mean ASFV (FAM) Ct values to determine overall variability of the replicates.

Results/conclusions: Results are reported in table 13. For all three target levels tested, the overall %CV values were <5.0%. This study demonstrates highly repeatable results with the RealPCR ASFV DNA Test under variable operating conditions including PCR instrument model.

Table 13. Reproducibility/instrument validation ASFV (FAM) Ct values

		Applied Biosystems QuantStudio 5	Applied Biosystems 7500 FAST	Roche LC480	Qiagen Rotor Gene Q	Agilent Mx3005P	Agilent AriaMx	BioRad CFX96	BMS Mic qPCR cycler		
		Operator 2	Operator 1	Operator 1	Operator 1	Operator 2	Operator 1	Operator 1	Operator 1	Mean Ct	Mean % CV
ASFV Low	Rep 1	35.4	35.9	35.7	34.2	32.6	33.3	34.9	32.3	34.3	4.1%
	Rep 2	36.1	36.3	35.5	32.9	32.7	34.0	33.9	32.9		
ASFV Mid	Rep 1	31.9	32.5	32.5	30.7	29.7	30.7	31.3	30.5	31.2	2.9%
	Rep 2	31.8	32.3	32.2	30.8	29.7	31.1	31.3	31.0		
ASFV High	Rep 1	27.7	29.1	28.8	27.3	26.6	27.7	29.0	27.5	28.0	3.2%
	Rep 2	27.6	29.2	28.9	27.3	26.7	27.8	28.9	27.4		

I. Stability

Purpose: To demonstrate the stability of the RealPCR ASFV DNA Mix when stored at the recommended temperature for an extended time and then tested.

Procedure: Stability testing for the RealPCR ASFV DNA Test was performed with two lots of ASFV target mix. Testing was performed at 1 month, 9 months, and 18–19 months after manufacture date. Three samples containing high, moderate (mid), and low levels of synthetic ASFV target were used for this testing. The low level panel member also contained the synthetic target for the internal sample control.

Results/conclusions: Real-time stability results for the two batches of RealPCR ASFV DNA Mix are shown in tables 14a and 14b. At 18 and at 19 months after manufacture, both batches of ASFV DNA Mix perform within the expected guidelines, and Ct values for the aged mixes are similar to those seen at the time of manufacture for both batches. The data generated for real-time stability of the dried RealPCR ASFV Mix is consistent with other RealPCR target mixes supporting the 18-month expiration assigned to this reagent.

Table 14a. Real-time stability for RealPCR ASFV DNA Mix Lot #1

	Expected Ct Values		Time Post Manufacture					
			1 Month		9 Months		19 Months	
	ASFV Ct	ISC Ct	ASFV Ct	ISC Ct	ASFV Ct	ISC Ct	ASFV Ct	ISC Ct
ASFV Low	**< 38**	**< 34**	32.3	23.7	32.6	24.1	33.2	24.1
ASFV Mid	**< 35**		29.4	--	29.2	--	30.3	--
ASFV High	**< 32**		25.8	--	26.3	--	27.1	--

Table 14b. Real-time stability for RealPCR ASFV DNA Mix Lot #2

	Expected Ct Values		Time Post Manufacture					
			1 Month		9 Months		19 Months	
	ASFV Ct	ISC Ct	ASFV Ct	ISC Ct	ASFV Ct	ISC Ct	ASFV Ct	ISC Ct
ASFV Low	**< 38**	**< 34**	32.2	23.2	32.5	24.6	33.7	23.1
ASFV Mid	**< 35**		30.1	--	30.3	--	30.8	--
ASFV High	**< 32**		26.6	--	26.9	--	27.3	--

J. Robustness

Purpose: To demonstrate robustness of performance for the RealPCR ASFV DNA Test despite small variations in critical parameters of the RT-PCR reaction.

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

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

Testing included an RT step which is standard for the RealPCR cycle protocol. The condition is not shown as it is not the focus of robustness testing for a DNA target.

All conditions were tested with three wells of synthetic DNA representing the target sequence, diluted to 10 copies per 25 µL reaction, the LD_{PCR} determined in Analytical sensitivity (LD_{PCR}). ASFV should be detected in 3/3 wells.

Results/conclusions: Robustness results for the RealPCR ASFV DNA Test are shown in table 15. The 10-copy sample was detected in 3 out of 3 wells for each set of conditions. Additionally, there was one cycle difference in the mean Ct values between the test conditions. This demonstrates acceptable robustness of the test to variation in the test parameters.

Table 15. Robustness of the RealPCR ASFV DNA Test

	Hybridization			ASFV LOW (10-copy sample)			
Conditions	**Time**	**Temp.**	**Sample volume**	**Ct-1**	**Ct-2**	**Ct-3**	**Mean Ct**
Low	27 sec	59°C	4.5 µL	33.9	32.5	32.1	32.8
Standard	30 sec	60°C	5.0 µL	31.9	31.6	31.8	31.8
High	33 sec	61°C	5.5 µL	32.2	32.2	32.8	32.4

VI. Characterization of the complete method

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

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

A. Diagnostic sensitivity/specificity

Purpose: To demonstrate the diagnostic sensitivity and specificity of the RealPCR ASFV DNA Test.

Procedure: Testing was performed at multiple locations using populations of field and experimentally infected samples (n = 439) sourced from the United States (negatives) and areas of the world with characterized ASFV-positive samples. Sample types included in the population were oral fluids, serum, plasma, EDTA blood, and tissue (multiple sources). DNA was extracted for PCR using one of the commercial methods described in Materials and methods. Each sample was tested as a single replicate on the RealPCR ASFV DNA Test, using standard test reagents and protocol. The ASFV status of each sample was confirmed using a real-time PCR reference test or herd status (in cases of negative samples). A total of 230 ASFV-positive and 209 ASFV-negative samples were evaluated in this manner.

Results/conclusions: Results for diagnostic sensitivity and specificity are reported in table 16. Diagnostic sensitivity is presented as the percentage of test positives compared to true positives, and diagnostic specificity as the percentage of test negatives compared to true negatives. Confidence intervals at the 95% level are also included. The RealPCR ASFV DNA Test performed with 98.7% sensitivity and 100% specificity for the 439 samples in the field test population.

Table 16. Diagnostic sensitivity and specificity

		Confirmed ASFV status	
		Pos	**Neg**
RealPCR ASFV DNA Test	**Pos**	227	0
	Neg	3	209
	Total	230	209

		95% confidence limits	
		Low CL	**High CL**
Sensitivity	98.7%	96.01	99.7
Specificity	100%	97.8	100

B. Detection of ASFV DNA in pooled samples

Purpose:

The purpose of this study was to evaluate sample pooling for the RealPCR ASFV DNA Test. IDEXX does not have access to ASFV samples. Therefore, the following evaluation was conducted by Centro de Investigacion en Sanidad Animal, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA-CISA) in Madrid, Spain.

Procedure:

The RealPCR ASFV DNA Test components and product insert were supplied by IDEXX. Test results for individual samples and pools of 20 are presented in the tables below.

INIA-CISA prepared a panel of 59 individual blood samples from pigs experimentally infected with genotype II ASFV isolates currently circulating in Eastern EU affected countries. The samples were from animals that developed different clinical forms of ASF infection. Samples were selected based on a range of early to late Ct values as tested on the INIA-CISA UPL real-time PCR test.[2] In addition, 24 blood samples collected from infected wild boar were included in this study. Samples had been previously diagnosed as positive for ASFV at the European Union Reference Laboratory for ASF (EURL for ASF) (INIA-CISA).

Pooled blood samples were made by mixing equal volumes for each ASFV-positive sample with 19 volumes of ASF-negative samples. The negative samples were collected at day 0 from domestic pigs used in experimental infections.

Results/conclusions:

Table 17a below summarizes the results for INIA-CISA pooling of experimental and field ASFV-positive samples. Details for each experimental blood sample selected for the pooling study are shown in table 17b. One hundred percent (100%) of the experimental samples with moderate or strong individual Ct values (≤ 32) were positive when tested in pools of 20. As expected, when weak samples (individual Ct values >32) were pooled and tested, only 39% of the pools were detected as positive. Ct values in this range correspond to less than 10 copies of virus per reaction (see Analytical Sensitivity in this report). Therefore, a significant number of samples would be expected to test negative when diluted in pools of 20. Many of the samples with late Ct values were from animals that had chronic infections and/or were exposed to attenuated virus.

Results for pooled wild boar samples collected from the field (table 17c) were similar to the experimental results. Nine out of 10 pooled samples tested positive when the individual Ct value was ≤32. When weak samples (individual Cts >32) were pooled, five out of 13 samples (38%) tested positive.

A 1:20 dilution of a single sample into a pool of 19 negative samples resulted in a theoretical delay in the Ct value of 4.32 cycles. For the 60 samples for which a Ct difference could be calculated (i.e., those samples for which both the individual and the pool of that same sample returned a Ct value), a distribution analysis was performed. The resulting distributions of the individual and pooled Ct values along with quantile values are shown in figure 3.

The differences between the quantile values for individual and pooled samples are shown in table 17d. As shown, pooled Ct values are shifted, on average, 4.5 cycles later than the respective individual sample. Additionally, a regression plot was made using the same 60 samples as above. As seen in figure 4, lines of fit for both individual and pooled Ct values show nearly identical slopes, with a Y-intercept approximately 4.8 cycles apart. These data strongly suggest Ct values are shifting as expected for the pooled dilutions.

The RealPCR ASFV DNA Test detected 98% of sample pools when the starting individual sample had a Ct value less than or equal to 32. When individual samples had low levels of virus (Ct value >32), the test detected 39% of the pooled samples. Distribution analysis and regression plots show that the observed shift in Ct values between individual samples and pooled samples is very close to the expected theoretical shift based on the dilution factor. Collectively, these data support the use of the RealPCR ASFV DNA Test for the detection of ASFV in pools of up to 20 samples. Pools containing a single weak positive sample (for example, Ct >32) may yield negative results due to the dilution effect of pooling.

Table 17a. Pooling summary table

Ct range	Sample type	Total #	# positive	# negative	# positive	# negative
			Individual		Pools of 20	
≤32	Experimental	36	36	0	36	0
	Field	11	11	0	10	1
>32	Experimental	23	23	0	9	14
	Field	13	13	0	5	8

Table 17b. Pooling results for ASF experimental study blood

ID sample	DPI/ DPE	ID animal	ASFV	Virulence	ASF clinical form	RealPCR ASFV DNA Test Ct values	
						Individual	1:20
1	7	IPC15	Est15/WB-TARTU14	Moderate virulence	Acute	15.3	21.3
2	8	IPC13	Est15/WB-VALGA6	Moderate virulence	Acute	17.2	20.8
3	13	PD10	POL16/DP-OUT 21	Virulent	Acute	16.0	20.6
4	7	C17	ARM07	Virulent	Acute	15.8	19.7
5	7	L8	LT14/1490	Virulent	Acute	17.0	21.1
6	10	CPC14	Est15/WB-VALGA6	Moderate virulence	Acute	18.7	24.41
7	8	C23	ARM07	Virulent	Acute	16.8	21.4
8	4	C12	ARM07	Virulent	Acute	20.6	27.9
9	7	IPC7	Est15/WB-TARTU14	Moderate virulence	Acute	19.9	25.1
10	12	U5	Uk12/Zapo	Virulent	Peracute	17.5	24.1
11	20	L14	LT14/1490	Virulent	Acute	23.4	25.8
12	11	U6	Uk12/Zapo	Virulent	Peracute	18.6	25.5
13	16	E2	ES16/WB-VIRU8	Virulent	Subacute	20.9	25.6
14	6	L7	LT14/1490	Virulent	Acute	21.3	26.8
15	21	(CC)L2	LT14/1490	Virulent	Acute	21.9	26.7
16	24	IPC2	Est15/WB-VALGA6	Moderate virulence	Chronic	23.3	28.0
17	21	CPC6	Est15/WB-VALGA6	Moderate virulence	Chronic	22.6	29.2
18	13	E6	ES16/WB-VIRU8	Virulent	Subacute	28.1	30.8
19	37	E2	ES16/WB-VIRU8	Virulent	Subacute	22.8	29.0

ID sample	DPI/ DPE	ID animal	ASFV	Virulence	ASF clinical form	RealPCR ASFV DNA Test Ct values	
						Individual	1:20
20	13	L15	LT14/1490	Virulent	Acute	22.2	27.7
21	14	C20	ARM07	Virulent	Acute	20.1	27.3
22	24	CPC5	Est15/WB-VALGA6	Moderate virulence	Chronic	25.5	29.5
23	44	E2	ES16/WB-VIRU8	Virulent	Subacute	25.4	30.5
24	17	CPC3	Est15/WB-VALGA6	Moderate virulence	Chronic	25.7	34.3
25	21	CPC3	Est15/WB-VALGA6	Moderate virulence	Chronic	27.1	31.4
26	24	CPC3	Est15/WB-VALGA6	Moderate virulence	Chronic	27.1	31.4
27	4	P2	ARM07	Virulent	Acute	27.1	33.9
28	56	E2	ES16/WB-VIRU8	Virulent	Subacute	26.1	34.5
29	28	CPC3	Est15/WB-VALGA6	Moderate virulence	Chronic	26.1	33.7
30	65	E2	ES16/WB-VIRU8	Virulent	Subacute	28.5	33.5
31	91	PW13	LV17/WB-RIE1	Attenuated	Chronic	30.6	34.9
32	106	PW13	LV17/WB-RIE1	Attenuated	Chronic	30.3	36.9
33	3	C17	ARM07	Virulent	Acute	27.5	31.9
34	14	CPC10	Est15/WB-TARTU14	Moderate virulence	Chronic	32.1	36.2
35	29	PW14	LV17/WB-RIE1	Attenuated	Chronic	33.3	36.8
36	3	E5	ES16/WB-VIRU8	Virulent	Acute	32.3	38.9
37	56	E6	ES16/WB-VIRU8	Virulent	Subacute	32.8	No Ct
38	70	PW15	LV17/WB-RIE1	Attenuated	Chronic	37.5	No Ct
39	65	E6	ES16/WB-VIRU8	Virulent	Subacute	33.5	37.2

ID sample	DPI/ DPE	ID animal	ASFV	Virulence	ASF clinical form	RealPCR ASFV DNA Test Ct values	
						Individual	1:20
40	29	PW17	LV17/WB-RIE1	Attenuated	Chronic	35.3	39.1
41	10	PD8	POL16/DP-OUT 21	Virulent	Acute	25.1	29.6
42	72	E6	ES16/WB-VIRU8	Virulent	Subacute	33.7	39.8
43	62	E6	ES16/WB-VIRU8	Virulent	Subacute	34.2	37.88
44	3	E1	ES16/WB-VIRU8	Virulent	Acute	31.0	34.2
45	69	E6	ES16/WB-VIRU8	Virulent	Subacute	32.8	37.0
46	22	PW13	LV17/WB-RIE1	Attenuated	Chronic	35.3	No Ct
47	24	CPC10	Est15/WB-TARTU14	Moderate virulence	Chronic	33.0	37.3
48	112	PW13	LV17/WB-RIE1	Attenuated	Chronic	32.8	No Ct
49	45	CPC5	Est15/WB-VALGA6	Moderate virulence	Chronic	34.6	No Ct
50	86	PW15	LV17/WB-RIE1	Attenuated	Chronic	33.8	No Ct
51	77	PW15	LV17/WB-RIE1	Attenuated	Chronic	37.0	No Ct
52	119	PW13	LV17/WB-RIE1	Attenuated	Chronic	34.4	No Ct
53	31	CPC12	Est15/WB-TARTU14	Moderate virulence	Chronic	36.4	No Ct
54	73	CPC5	Est15/WB-VALGA6	Moderate virulence	Chronic	37.4	No Ct
55	49	PW14	LV17/WB-RIE1	Attenuated	Chronic	37.8	No Ct
56	84	PW15	LV17/WB-RIE1	Attenuated	Chronic	33.2	No Ct
57	35	PW17	LV17/WB-RIE1	Attenuated	Chronic	36.6	No Ct
58	94	PW13	LV17/WB-RIE1	Attenuated	Chronic	28.3	37.9
59	73	CPC3	Est15/WB-VALGA6	Moderate virulence	Chronic	36.6	No Ct

Table 17c. Pooling results for ASF wild boar blood

ID sample	Country	RealPCR ASFV DNA Test Ct values	
		Individual	1:20
1	Lithuania	22.4	25.8
2	Lithuania	24.0	25.0
3	Poland	30.1	37.8
4	Lithuania	29.8	36.1
5	Lithuania	29.7	34.1
6	Lithuania	29.9	32.1
7	Estonia	31.2	34.9
8	Lithuania	30.3	33.0
9	Lithuania	30.2	32.1
10	Estonia	32.4	No Ct
11	Estonia	31.6	No Ct
12	Lithuania	33.4	No Ct
13	Lithuania	32.2	37.5
14	Lithuania	32.6	35.9
15	Estonia	33.1	No Ct
16	Lithuania	33.0	No Ct
17	Lithuania	32.8	35.6
18	Lithuania	33.0	No Ct
19	Lithuania	35.5	35.9
20	Lithuania	35.9	No Ct
21	Estonia	35.0	No Ct
22	Lithuania	33.5	No Ct
23	Lithuania	32.4	36.0
24	Lithuania	20.0	24.2

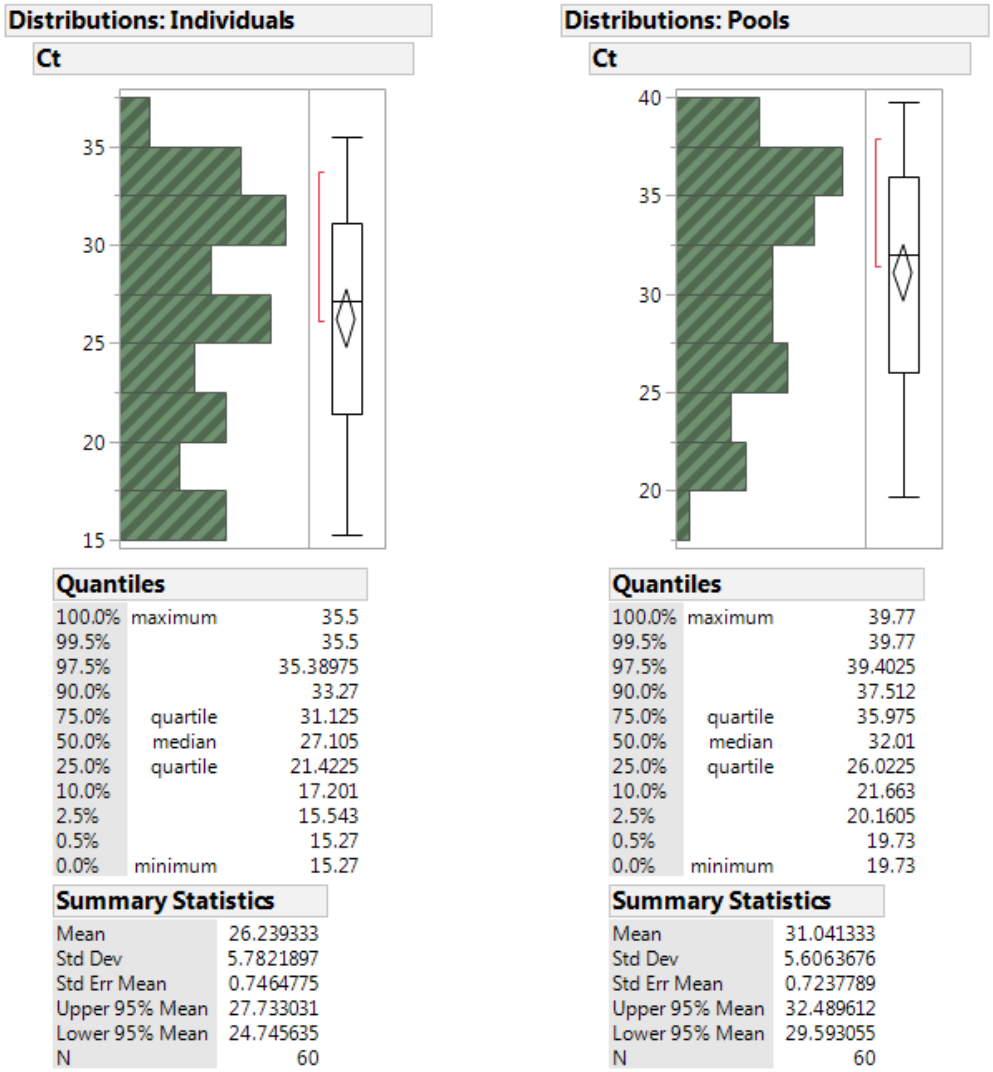
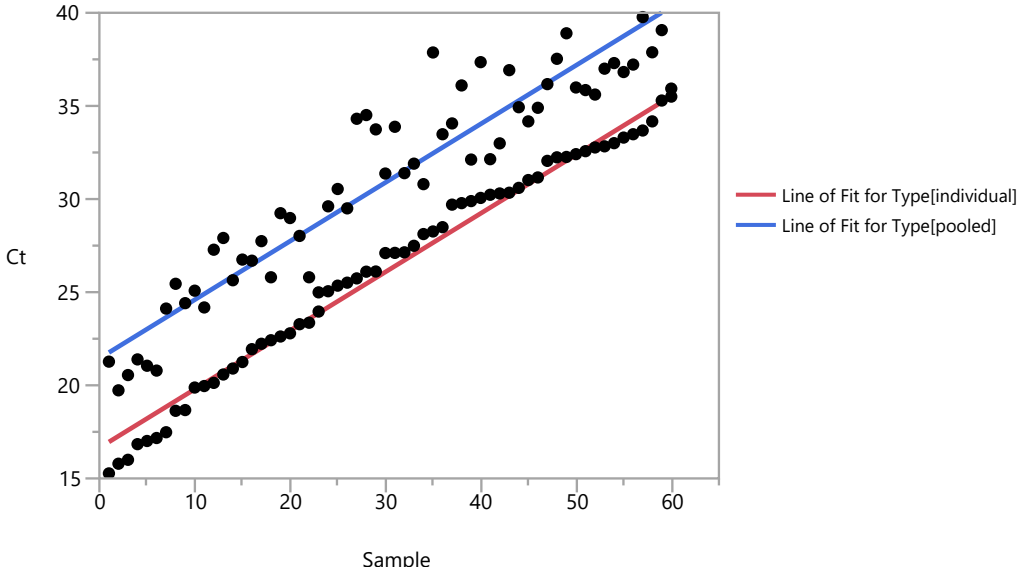


Figure 3. Ct distribution for individual and pooled samples

Table 17d. Ct differences for individual and pooled samples

Quantile	Pooled	Individual	ΔCt
100%	39.8	35.5	4.3
99.5%	39.8	35.5	4.3
97.5%	39.4	35.4	4
90%	37.5	33.3	4.2
75%	36.0	31.1	4.9
50%	32.0	27.1	4.9
25%	26.0	21.4	4.6
10%	21.7	17.2	4.5
2.5%	20.2	15.5	4.7
0.5%	19.7	15.3	4.4
0%	19.7	15.3	4.4
		Average	**4.5**

Figure 4. Regression plot for individual and pooled samples



C. Detection of ASFV DNA in blood swab samples

Purpose: The purpose of this study was to evaluate the detection of ASFV DNA in blood swab samples using the RealPCR ASFV DNA Test. The evaluation of ASFV positive samples was conducted in Poland. Negative samples were sourced and tested in the United States.

Procedure: Testing of positive and negative samples was conducted using RealPCR ASFV DNA Test components. Blood swab samples were prepared by immersing a cotton swab in each individual blood sample. Swabs were stored overnight at 2°C–8°C and resuspended in 0.5 mL of phosphate buffered saline before nucleic acid extraction.

Testing of ASFV positive samples:

A panel of 20 ASFV confirmed positive EDTA whole blood samples from domestic pigs and wild boars was selected for the study. Samples were extracted using the QIAamp DNA Mini Kit and tested according to the RealPCR ASFV DNA Test product insert.

Results, generated in two PCR instruments, were compared with the paired whole-blood sample result.

Testing of negative samples:

A set of 30 confirmed ASFV negative samples were extracted using the RealPCR DNA/RNA Magnetic Bead Kit and tested according to the RealPCR ASFV DNA Test product insert.

Results/conclusions: Test results are summarized in tables 18 and 19 for whole blood and blood swabs, respectively. Individual results for the positive samples are presented in table 20. The RealPCR ASFV DNA Test detected ASFV DNA in 20 out of 20 known positive whole-blood samples and in 19 out of 20 ASFV positive blood swab samples (95%). The one blood swab sample that tested negative had a late Ct on the paired whole-blood sample (Ct = 35.9)

All the negative samples tested showed no amplification.

Table 18. ASFV whole blood

		Reference Method	
		Pos	Neg
IDEXX	Pos	20	0
	Neg	0	30

Table 19. ASFV blood swab

		Reference Method	
		Pos	Neg
IDEXX	Pos	19	0
	Neg	1	30

Table 20. ASFV DNA detection in paired whole-blood and blood swab samples.

ID sample	Animal	Paired Whole blood Ct values	Blood swab (QuantStudio 5) Ct values	Blood swab (RotorGene) Ct values
1	Domestic pig	15.9	21.7	20.8
2	Domestic pig	21.9	26.7	26.3
3	Domestic pig	17.0	20.4	19.7
4	Domestic pig	20.9	23.8	23.2
5	Domestic pig	20.2	22.6	21.9
6	Domestic pig	20.0	23.8	23.5
7	Domestic pig	22.7	26.8	26.5
8	Domestic pig	21.2	25.1	24.3
9	Domestic pig	17.6	21.9	21.5
10	Domestic pig	22.3	26.1	25.0
11	Wild boar	24.0	27.2	27.3
12	Wild boar	32.5	37.3	35.8
13	Wild boar	31.3	35.9	37.6
14	Wild boar	25.2	28.9	28.3
15	Wild boar	30.8	35.4	34.8
16	Wild boar	23.3	26.8	26.7
17	Wild boar	35.9	-	-
18	Wild boar	21.9	22.9	22.4
19	Wild boar	22.0	24.7	24.5
20	Wild boar	28.0	30.9	30.7

D. Detection of ASFV DNA in spleen tissue samples

Purpose: To evaluate the detection of ASFV DNA in spleen samples using the RealPCR ASFV DNA Test. This study was conducted by SCIENSANO, the Belgian National Reference Laboratory (Schoder, *et al*., 2020).

Procedure: Three hundred characterized spleen samples, obtained from wild boars during the 2018–2019 ASFV outbreak in Belgium, were used in this study. An in-house PCR test (Tignon, *et al*., 2011) was used as the reference method to establish positive or negative status of the samples.

Panels 1 and 2 each consisted of 50 ASFV negative samples, originating from two different geographical areas, while Panel 3 included 100 ASFV positive samples. Panel 4 was comprised of 100 samples collected from infected areas or areas that recently had the ASFV status changed from free to infected. Although this sample set was considered doubtful, the results were included in the sensitivity/specificity analysis.

Extracted samples were tested according to the RealPCR ASFV DNA Test product insert using a LightCycler* 480 Instrument (Roche) and results were compared with the reference PCR method.

Results/conclusions: Results are summarized in Table 21, below. The RealPCR ASFV DNA Test correctly identified the 100 ASFV negative samples in Panels 1 and 2, and the 100 ASFV positive samples in Panel 3. For Panel 4 (dubious,) the RealPCR ASFV DNA test agreed with the reference method for 90/93 negative and 5/7 weak positive samples. Overall, the sensitivity and specificity were calculated at 98.1% and 98.5%, respectively, compared to the reference method.

References:

1. Schoder ME, Tignon M, Linden A, Vervaeke M, Cay AB. Evaluation of seven commercial African swine fever virus detection kits and three Taq polymerases on 300 well-characterized field samples. *J Virol Methods*. 2020; 280:113874.
2. Tignon M, Gallardo C, Iscaro C, Hutet E, Van der Stede Y, Kolbasov D, De Mia GM, Le Potier MF, Bishop RP, Arias M, Koenen F. Development and inter-laboratory validation study of an improved new real-time PCR assay with internal control for detection and laboratory diagnosis of African swine fever virus. *J Virol Methods.* 2011;178:161–170.

Table 21. ASFV DNA detection in spleen tissue

	Reference Positive		Reference Negative	
	IDX Pos	IDX Neg	IDX Neg	IDX Pos
Panel 1	--	--	50	--
Panel 2	--	--	50	--
Panel 3	100	--	--	--
Panel 4	5	2	90	3
Total	**105**	**2**	**190**	**3**

E. Detection of ASFV DNA in different tissue types

Purpose: To evaluate the detection of ASFV DNA in multiple tissue types using the RealPCR ASFV DNA Test.

Procedure: Testing was performed at multiple locations, using tissue samples from field and experimentally infected domestic pig populations, sourced from the United States (negatives) and areas of the world with characterized ASFV-positive samples. A total of 231 samples were tested in this study.

Tissue types included in the population were bone marrow, kidney, lung, lymph node, muscle, spleen and tonsil. DNA was extracted for PCR using one of the commercial methods described in Materials and methods. Each sample was tested as a single replicate on the RealPCR ASFV DNA Test, using standard test reagents and protocol.

The ASFV status of each sample was confirmed using a real-time PCR reference test or herd status (in cases of negative samples). A total of 142 ASFV-positive and 89 ASFV-negative samples were evaluated in this manner.

Results/conclusions: Table 22 shows a summary of RealPCR ASFV DNA Test performance, by sample type compared to the confirmed ASFV status of the sample. Three experimentally infected kidney tissue samples were negative for ASFV using the RealPCR ASFV DNA Test; however, the reference method was also negative for these samples. For the remaining tissue samples there was 100% alignment for the RealPCR ASFV DNA Test to the known ASFV status of each tissue sample tested.

Table 22. ASFV Detection by Tissue Type

	Reference Positive		Reference Negative	
	IDX Pos	IDX Neg	IDX Neg	IDX Pos
Bone Marrow	21	--	14	--
Kidney	24	--	17*	--
Lung	21	--	19	--
Lymph Node	29	--	14	--
Spleen	15	--	14	--
Tonsil	25	--	14	--
Muscle	4	--	--	--

** Includes 3 experimental samples, also negative by the reference method.*

F. RealPCR DNA/RNA Spin Column extraction

Purpose: To evaluate the detection of ASFV DNA using the RealPCR DNA/RNA Spin Column kit on multiple sample types.

Procedure: Multiple sample types of known ASFV status were extracted using the RealPCR DNA/RNA Spin Column kit to evaluate the detection of ASFV DNA. All extracted samples were tested on the RealPCR ASFV DNA Test using standard mix reagents and protocol.

Results/conclusions: Table 23 shows a summary of RealPCR ASFV DNA Test performance, by sample type for the RealPCR DNA/RNA Spin Column extracted samples. Table 24 shows the individual results for the sample extracted using the RealPCR DNA/RNA Spin Column kit. There was 100% alignment for the samples extracted using the RealPCR DNA/RNA Spin Column kit to the known ASFV status of each sample.

Table 23. Sample types extracted by the RealPCR DNA/RNA Spin Column kit

	Reference Positive		Reference Negative	
	IDX Pos	IDX Neg	IDX Neg	IDX Pos
Serum	13	--	14	--
Oral Fluids	--	--	2	--
Nasal swab	4	--	11	--
Anal swab	6	--	--	--
Environmental swab	4	--	4	--

Table 19. ASFV DNA detection in samples extracted with RealPCR DNA/RNA Spin Column kit

Sample ID	Sample Type	ASFV Sample Status	IDEXX RealPCR ASFV			
			ASFV Ct value	ASFV Result	ISC Ct value	ISC Result
1	Serum	Positive	19.46	Positive	23.13	Positive
2	Serum	Positive	24.17	Positive	27.61	Positive
3	Serum	Positive	28.84	Positive	27.84	Positive
4	Serum	Positive	30.68	Positive	30.18	Positive
5	Serum	Positive	24.37	Positive	27.61	Positive
6	Serum	Positive	20.40	Positive	24.99	Positive
7	Serum	Positive	36.69	Positive	28.34	Positive
8	Serum	Positive	36.29	Positive	31.10	Positive
9	Serum	Positive	36.47	Positive	28.86	Positive
10	Serum	Positive	17.69	Positive	23.18	Positive
11	Serum	Positive	35.01	Positive	29.69	Positive
12	Serum	Positive	36.87	Positive	31.97	Positive
13	Serum	Positive	25.43	Positive	27.41	Positive
14	Anal Swab	Positive	29.79	Positive	26.30	Positive
15	Anal Swab	Positive	35.98	Positive	35.35	Positive
16	Anal Swab	Positive	36.06	Positive	36.90	Positive
17	Anal Swab	Positive	26.79	Positive	26.80	Positive
18	Anal Swab	Positive	35.78	Positive	33.47	Positive
19	Anal Swab	Positive	25.88	Positive	23.46	Positive
20	Environmental Swab	Positive	35.88	Positive	33.98	Positive
21	Environmental Swab	Positive	35.87	Positive	31.93	Positive
22	Environmental Swab	Positive	33.72	Positive	34.01	Positive
23	Environmental Swab	Positive	34.66	Positive	29.29	Positive
24	Nasal Swab	Positive	31.51	Positive	30.27	Positive
25	Nasal Swab	Positive	33.23	Positive	37.77	Positive
26	Nasal Swab	Positive	38.81	Positive	28.50	Positive
27	Nasal Swab	Positive	29.99	Positive	26.35	Positive
28	Serum	Negative	No Ct	Negative	27.13	Positive
29	Serum	Negative	No Ct	Negative	27.88	Positive
30	Serum	Negative	No Ct	Negative	26.80	Positive
31	Serum	Negative	No Ct	Negative	29.13	Positive
32	Serum	Negative	No Ct	Negative	28.41	Positive
33	Serum	Negative	No Ct	Negative	32.34	Positive

Sample ID	Sample Type	ASFV Sample Status	IDEXX RealPCR ASFV			
			ASFV Ct value	ASFV Result	ISC Ct value	ISC Result
34	Serum	Negative	No Ct	Negative	31.94	Positive
35	Serum	Negative	No Ct	Negative	30.93	Positive
36	Serum	Negative	No Ct	Negative	31.25	Positive
37	Serum	Negative	No Ct	Negative	30.92	Positive
38	Serum	Negative	No Ct	Negative	32.06	Positive
39	Serum	Negative	No Ct	Negative	28.61	Positive
40	Serum	Negative	No Ct	Negative	32.03	Positive
41	Serum	Negative	No Ct	Negative	29.56	Positive
42	Nasal Swab	Negative	No Ct	Negative	36.05	Positive
43	Nasal Swab	Negative	No Ct	Negative	39.19	Positive
44	Nasal Swab	Negative	No Ct	Negative	38.41	Positive
45	Nasal Swab	Negative	No Ct	Negative	29.03	Positive
46	Nasal Swab	Negative	No Ct	Negative	37.40	Positive
47	Nasal Swab	Negative	No Ct	Negative	29.64	Positive
48	Nasal Swab	Negative	No Ct	Negative	32.25	Positive
49	Nasal Swab	Negative	No Ct	Negative	28.10	Positive
50	Nasal Swab	Negative	No Ct	Negative	28.69	Positive
51	Nasal Swab	Negative	No Ct	Negative	35.11	Positive
52	Nasal Swab	Negative	No Ct	Negative	40.00	Positive
53	Oral Fluids	Negative	No Ct	Negative	30.04	Positive
54	Oral Fluids	Negative	No Ct	Negative	33.80	Positive
55	Environmental Swab	Negative	No Ct	Negative	30.28	Positive

VII. Summary

A. Characteristics of PCR

- Exclusive detection of Africa swine fever virus (ASFV)
- Detection of 23 ASFV genotypes, 22 isolates and 1 predicted through *in silico* analysis
- Analytical sensitivity to ≤10 copies per reaction
- Efficiency of 100.6% for the RT-PCR reaction over 7-log range
- Within-laboratory repeatability %CV of <2%
- Interlaboratory reproducibility %CV of <5%
- Robustness demonstrated for variations in time, temperature, and sample volume for RT-PCR
- Reagent stability demonstrated out to 18 months expiration
- PCR instruments validated: Applied Biosystems* 7500, Applied Biosystems* QuantStudio 5, Agilent Mx3005P*, Agilent AriaMx*, Bio-Rad* CFX96 Touch*, Roche LightCycler* 480, QIAGEN* Rotor-Gene* Q (72-well rotor only), and Bio Molecular Systems Mic qPCR Cycler

B. Characteristics of the complete method

- Diagnostic sensitivity of 98.4% (n = 230)
- Diagnostic specificity of 100% (n = 209)
- Detection of ASFV DNA in pools of up to 20 samples
- Detection of ASFV in serum, plasma, EDTA blood, oral fluids, tissue (including lung, bone marrow, tonsils, kidney, spleen, and lymph nodes) and swab samples from swine, including wild boars.
- Detection of ASFV DNA in samples extracted with RealPCR DNA/RNA Spin Column kit

VIII. References

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