Evaluation of the ASFV African swine fever virus (ASF) qPCR Diagnostic Kit TARGETEX BIOSCIENCES. ASSESSMENT REPORT

PERFORMED BY THE

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Content

1. OBJECTIVE.

The study conducted at CISA-INIA/CSIC as the European Union Reference Laboratory (EURL) for African swine fever (EURL-ASF) aimed to assess the performance of the ASF Virus qPCR Diagnostic Kit developed by TARGETEX (named in this study TARGETEX ASFV qPCR kit), using **experimental porcine samples**. The multiplex TARGETEX ASFV qPCR kit is designed for the detection of African Swine Fever (ASF) virus DNA by quantitative PCR. In addition to the ASF virus-specific FAM™-labelled probe, the assay is capable of detecting endogenous controls (e.g., swine, boar or warthog DNA, probe labelled with HEX™) as well as exogenous controls (probe labelled with Cy5™).

2. PROCEDURE

2.1. Samples included in the evaluation study

2.1.1. ASFV REFERENCE DNAs.

To assure the detection range of the real time PCR kit, an initial evaluation was carried out by the analysis of a collection of **22 ASFV reference DNAs belonging to 20 of the 24 reported p72 genotypes** as is described in Table 1.

Table 1→ *Description of ASFV reference DNAs.*

ASFV ISOLATE	GENOTYPE	COUNTRY	HOST	OUTBREAK DATE
E70	I	Spain	Domestic pig	1970
Maur08/1	II	Mauritius	Domestic pig	2008
Ukr12/Zapo	II	Ukraine	Domestic pig	2012
RSA2008/1	III	South Africa	Domestic pig	2008
RSA/W/1/99	IV	South Africa	Warthog	1999
Moz64	V	Mozambique	Domestic pig	1964
SPEC/265	VI	Mozambique	Domestic pig	1994
RSA/03/7	VII	South Africa	Warthog	2003
MwLil20/1	VIII	Malawi	Tick	1990
Ken06.Bus	IX	Kenya	Domestic pig	2006
BUR90/1	X	Burundi	Domestic pig	1990
KAB6/2	XI	Zambia	Tick	1983
MZI92/1	XII	Malawi	Domestic pig	1992
SUM14/11	XIII	Zambia	Tick	1983
NYA1/2	XIV	Zambia	Tick	1988
TAN/2008/1	XV	Tanzania	Domestic pig	2008
TAN2003/2	XVI	Tanzania	Domestic pig	2003
NAM/P/1/95	XVIII	Namibia	Domestic pig	1995
SPEC125	XIX	South Africa	Domestic pig	1987
24823	XX	South Africa	Domestic pig	1975
RSA2008/2	XXII	South Africa	Domestic pig	2008
ET13/1505	XXIII	Ethiopia	Domestic pig	2013

2.1.2. EURL-ASF REFERENCE SAMPLES (ASF-Ref-2)

A panel of **13 EURL-ASF REFERENCE SAMPLES (ASF-Ref-2) prepared by the EURL-ASF for the evaluation, validation and internal verification of DNA extraction methods** were included in the evaluation study. The panel of samples consists in 13 ASF lyophilised reference samples including tissues and serum samples collected from different epidemiological situations (Table 2).

Table 2→ *origin of samples included in the URL-ASF reference samples for ASFV DNA extraction methods.*

ID SAMPLE	ASFV ISOLATE	GENOTYPE	ORIGIN OF SAMPLES	
			DPI/DPE*	DESCRIPTION
SAMPLE 34	POL18/WB CASE1794	II	24	Homogenate lung obtained from one pig (C5) kept in contact with pig's experimentally inoculated intramuscular route with the Polish ASFV POL18/WB CASE1794 isolate (10 HAU/ml).
SAMPLE 35	ET13/1505	XXIII	23	Homogenated heart obtained from one pig (C17) kept in contact with pig's experimentally inoculated intramuscular route with the Ethiopia ASFV ET13/1505 (10 HAU/ml).
SAMPLE 36	LV17/WB/RIE14/TUKUMA5	II	9	Serum obtained from one pig (C2) experimentally inoculated intramuscular route with the Latvian ASFV LV17/WB/RIE14/TUKUMA (10 HAU/ml).
SAMPLE 37	Lv19/WB/ Brocenu6	II	20	Homogenate liver from one pig (C15) kept in contact with pig's experimentally inoculated intramuscular route with the Latvia ASFV Lv19/WB/ Brocenu6 (10 HAU/ml).
SAMPLE 39	LV17/WB/RIE14/TUKUMA5	II	9	Dilution **1/50 i**n negative SERUM of serum obtained from one pig (C2) experimentally inoculated intramuscular route with the Latvian ASFV LV17/WB/RIE14/TUKUMA isolate (10 HAU/ml).
SAMPLE 40	NH/P68	I	128	Homogenate LUNG obtained from one pig (C14) inoculated intramuscular route with the Portuguese ASFV NH/P68
SAMPLE 42	Nig08/LaOk	I	8	Homogenate LUNG obtained from one pig (C4) inoculated intramuscular route with the Nigerian ASFV Nig08/LaOK
SAMPLE 43	Ken06.Bus	IX	9	Serum obtained from one pig (C1) experimentally inoculated intramuscular route with the Ken06/Bus (10 HAU/ml).
SAMPLE 44	E75	I	15	Serum obtained from one pig (C1) experimentally inoculated intramuscular route with the E75 100 DOSES (10 HAU/ml).
SAMPLE 45	Nig08	I	9	Dilution **1/20 i**n negative tissue of sample 42
SAMPLE 46	POL18/WB CASE1794	II	36	Homogenate spleen obtained from one pig (C1) kept in contact with pig's experimentally inoculated intramuscular route with the Polish ASFV POL18/WB CASE1794 isolate (10 HAU/ml).
SAMPLE 47	Est15/WB-Valga6	II	25	Serum obtained at 25 dpe from one pig (Cc6) kept in contact with pig's experimentally inoculated intramuscular route with the Est15/WB-Valga6 isolate (10 HAU/ml).
SAMPLE 48	Lv19/WB/ Brocenu6	II	17	Serum obtained at 17 dpi from one pig (C13) inoculated intramuscular route with the Lv19/WB/ Brocenu6 isolate (10 HAU/ml).

**Dpi (days post infection). Dpe (days post exposure)*

2.1.3. Experimental EDTA-BLOOD samples

Analytical sensitivity was initially assessed using **186 experimental EDTA-blood samples collected from domestic pigs infected with different ASFV isolates at the day of necropsy**. These isolates covered a range of virulence levels and represented diverse genotypes, including I, II, IX, X, and XXIII. The pigs displayed varying clinical presentations of ASF, from acute to subclinical infections, as detailed in Table 3.

Table 3→ *pig experimental blood samples used for the determination of the analytical sensitivity.*

ASFV STRAIN	P72 GENOTYPE	ORIGIN	CLINICAL FORM	VIRULENCE DESIGNATION	DAYS POST INFECTION (DPI)	Nº BLOOD TESTED
Arm07	II	Armenia 2007	acute	*vir*	5-8	24
BEL18/WB-LUX1	II	Belgium 2018	acute	*vir*	10-14	2
E75	I	Spain 1975	acute	*vir*	7-15	7
Est15/WB-Tartu14	II	Estonia 2015	acute	*mod vir*	8-13	2
Est15/WB-Tartu14	II	Estonia 2015	chronic	*mod vir*	206	2
Est15/WB-Valga6	II	Estonia 2015	subacute	*mod vir*	25	2
Est15/WB-Valga6	II	Estonia 2015	chronic	*mod vir*	204	2
Est16/WB-VIRU8	II	Estonia 2016	acute	*mod vir*	10-15	2
Est16/WB-VIRU8	II	Estonia 2016	subacute	*mod vir*	72-76	2
Est20/WB/VIRU12	II	Estonia 2020	acute	*vir*	11-19	4
ET13/1505	XXIII	Ethiopia 2013	acute	*mod vir*	10	1
ET13/1505	XXIII	Ethiopia 2013	subacute	*mod vir*	23-93	2
IT 22489,4_2312/RC/2023	II	Italy 2023	Hyperacute	*vir*	4	2
Ken05/Tk1	X	Kenya 2005	subacute	*mod vir*	70	1
Ken05/Tk1	X	Kenya 2005	acute	*mod vir*	7-15	4
Ken06/Bus	IX	Kenya 2006	acute	*vir*	12	1
Lt14/1490	II	Lithuania 2014	acute	*vir*	7-21	13
Lt22/WB/Tau11	II	Lithuania 2022	acute	*vir*	9-16	5
LV17/WB/RIE1	II	Latvia 2017	chronic	*Att*	10-126	35
LV17/WB/RIE1	II	Latvia 2017	subclinical	*Att*	71-105	7
LV17/WB/RIE14/TUKUMA5	II	Latvia 2017	acute	*mod vir*	9	1
LV17/WB/RIE14/TUKUMA5	II	Latvia 2017	chronic	*mod vir*	121	1
Lv17/WB/Zieme3	II	Latvia 2017	acute	*vir*	9-16	3
Lv19/WB/ Brocenu6	II	Latvia 2019	subacute	*mod vir*	17	1
Lv19/WB/ Brocenu6	II	Latvia 2019	chronic	*mod vir*	28-30	2
Lv19/WB/ Brocenu6	II	Latvia 2019	subclinical	*mod vir*	44	1
Lv19/WB/DOBEL9	II	Latvia 2019	acute	*vir*	13-22	3
Lv19/WB/DOBEL9	II	Latvia 2019	subacute	*vir*	17	1
Mu82	I	Spain 1982	subclinical	*att*	105-135	6
NHP/68	I	Lisbon 1968	chronic	*att*	26-125	25
Ourt88/3	I	Portugal 1988	chronic	*att*	22-108	3
Ourt88/3	I	Portugal 1988	subclinical	*att*	97-98	2
Pol16/DP-OUT21	II	Poland 2016	acute	*vir*	13-20	4
POL18/WB CASE1794	II	Poland 2018	subclinical	*att*	64	1
POL18/WB CASE1865	II	Poland 2018	subacute	*vir*	18	1
POL18/WB CASE1865	II	Poland 2018	subclinical	*att*	64-71	5
POL18/WB CASE1865	II	Poland 2018	chronic	*att*	24-66	3
Ukr12/Zapo	II	Ukraine 2012	acute	*vir*	7-12	3
Total						**186**

Att.= attenuated; vir.= virulent, mod. vir.= moderate virulent

In order to assess the ability of the TARGETEX ASFV qPCR kit to detect the ASFV genome throughout the course of infection, **45 experimental EDTA-blood samples** were systematically analyzed using the TARGETEX ASFV qPCR kit. These samples were collected under the following conditions:

a. **Seven (7) blood samples** collected from **0 up to 21 days** from one domestic pigs kept in direct contact with the virulent Latvia genotype II-ASFV isolate (Lv19/WB/DOBEL9). The pig (#C3) developed an acute form of ASF and was slaughtered at 22 days after the exposure.
b. **Twenty (19) blood samples** collected from **0 up to 64 days post inoculation** (dpi) from one animal inoculated with the attenuated Polish genotype II-ASFV isolate (POL18/WB/CASE1794). The pig, named #C4, developed a **chronic form of ASF** and was slaughtered at **64 dpi.**
c. **Nineteen (19) blood samples** collected from **0 up to 93 dpe** from one domestic pig (#CC18) kept in direct contact with pigs infected with the moderate virulence Ethiopia genotype XXIII-ASFV isolate (ET13/1505). The domestic pig developed a subacute form of ASF.

2.1.4. Experimental TISSUE samples.

A panel of **32 tissues** from domestic pigs experimentally infected with ASFV isolates belonging to genotype II and genotype XXIII were included in this study as described below;

a. **15 tissue samples** from the pig named #CC1 exposed to the attenuated Polish genotype II- ASFV isolate (POL18/WB CASE1794). The pig developed a **chronic form of ASF** and was slaughtered at 36 days after the initial exposure**.**

b. **17 tissue samples** from the pig named #C17 exposed to the moderate virulence genotype XXIII-Ethiopia ASFV isolate (ET13/1505). The pig developed a **subacute form of ASF** and was slaughtered at 23 days after the initial exposure.

2.2. TEST PROCEDURES.

2.2.1. DNA extraction procedure.

DNA was extracted from organ homogenates and EDTA-blood using the **High Pure PCR Template Preparation kit** (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany: Ref. 11796828001 (ROCHE)] according the EURL standard operating procedure (SOP) [SOP-ASF-DNA-EXTRACTION-1_REV52021.pdf]. Briefly, 10% (w/v) clarified homogenized tissue suspensions were prepared in phosphate-buffered saline (PBS) following the EURL-SOP [SOP/CISA/ASF/SAMPLES/1]. Supernatants were filter with MINISART filters 0.45µm and then treated with 0.1 % of gentamicin sulphate 50mg/ml (BioWhittaker) during 1h at 4±3ºC prior to use for virus detection; a 10% (w/v) clarified homogenized tissue were prepared in phosphate-buffered saline (PBS). The DNA was extracted from 200 µl of each tissue homogenate or blood samples using the High Pure PCR Template Preparation Kit. DNAs were stored <-10 until use. Extracted DNAs were stored at ≤ –10 °C until use.

2.2.2. WOAH-Real time PCR procedure (UPL method) developed by Fernandez et al., 2013 (WOAH 2021)

The amplification of the ASFV amplification was performed in 96-well plate MX3005P equipment (Stratagene, Agilent Technologies Inc., Santa Clara, CA, USA) using the **WOAH-real time PCR (Taqman probe ASFV-VP72P1)** developed by Fernandez *et al.*, 2013 and included in the WOAH Manual as reference test (WOAH 2021) [SOP/CISA/ASF/PCR/3].

The following controls were included in both extraction and amplification steps:

Reference	Component	Description
EURL-E+ Batch 40	**Positive control for the extraction (EURL)**	1:10.000 dilution of the ASFV reference strain E75 (genotype I) diluted in a negative sera.
NCE (E-)	**Negative control for the extraction**	Distilled water
EURL-R+ batch 38	**Positive control for the amplification (EURL)**	ASFV positive DNA extracted for the EURL
NAC (R-)	**Negative control for the amplification**	Distilled water

Assay validation: results were considered as validated if met the following criteria:

Control	Expected result	Acceptability criteria
EURL-E+ batch 40	Detected in FAM channel	Ct value within the range of 32±4.
NCE (E-)	Non detected	Ct ≥40.
EURL-R+ batch 38	Detected in FAM channel	Ct value within the range of 32±4.
NAC (R-)	Non detected	Ct ≥40.

Interpretation of the results:

- → Samples giving a **Ct value ≤ 35** are considered as **POSITIVE SAMPLES**.
- → Samples giving a **Ct value Ct ≥40** are considered as **NEGATIVE SAMPLES**.
- → Samples giving **35 ≤ Ct value < 40** are considered as **WEAK SAMPLES** if a sigmoidal plot is observed. In this case and to confirm the results, the extracted DNA from the weak sample must be tested by duplicated in a second PCR run. Sample will be considered as positive in case of the Ct value <40 in, at least, one duplicate.
- → Samples showing a Ct value >38 were considered as negative if the amplification plot had a linear shape.

2.2.3. ASF qPCR Diagnostic Kit TARGETEX BIOSCIENCES.

The amplification of the ASFV DNA was performed in 96-well plate AriaMX equipment (Stratagene, Agilent Technologies Inc., Santa Clara, CA, USA) using the TARGETEX ASFV qPCR kit according to the protocol described by the manufacturers. The kit includes both **positive and negative controls, each producing an expected Ct value of approximately 28 during amplification for ASFV and endogenous target**. Additionally, it contains an **exogenous control designed to monitor the efficiency and reliability of the DNA extraction process**. The exogenous extraction control was directly added at a 10^5-fold dilution to the extracted DNA sample (Post-extraction addition) to verify the PCR reaction itself.

Reagent	Description	Volume (µL)
Reaction Mix (100 reactions)	Master Mix includes primers and probes	1500
Positive ASF Control (PC)	ASF-specific target DNA	50
Negative ASF Control (NC)	Endogenous target DNA	50
Pos. Extraction Control (EC)	Exogenous target DNA	50

The thermal cycle used is described below:

	STEP	TEMPERATURE (°C)	DURATION
1	INITIAL DENATURATION	95	3 min.
2	DENATURATION	95	5 sec
3	ANNEAL/EXTENSION	60	30 sec

Repeat steps #2-3 for 40 cycles. The fluorescence is read at the end of the extension phase.

Assay validation: results were considered as validated if met criteria established in the following table.

Control Type	Target	Channel	Expected Ct Value
Positive ASF Control (PC)	Verifies ASFV-specific amplification	FAM	≤ 30
Negative ASF Control (NC)	Verifies endogenous target amplification	HEX	≤ 30
Pos. Extraction Control (EC)	Monitors extraction and PCR efficiency	Cy5	25–35*
No Template Control (NTC)	Excludes reagent contamination	–	no amplification

* Expected Ct value varies depending on the DNA extraction method, sample type, etc.

Interpretation of the results:

- **Positive (FAM +):** ASFV genome detected, regardless of control status. If IC (HEX) or EC (Cy5) fail, repeat extraction/qPCR is recommended to verify sample quality or EC concentration.
- **Negative (FAM – / IC + / EC +):** ASFV not detected.

- **Negative with IC or EC failure:** Result inconclusive; repeat extraction/qPCR required.
- **Invalid (all –):** No amplification of target or controls; repeat full extraction and qPCR workflow.

Result	ASF Virus (FAM)	Endogenous control (HEX)	Exogenous control (Cy5)	Conclusion
Positive	+	+	+	ASFV detected.
Positive (IC failure)	+	–	+	ASFV detected; but endogenous control failed – insufficient sample quality, repeat extraction/qPCR protocol.
Positive (EC failure)	+	+	–	ASFV detected; but exogenous control failed – repeat extraction with higher EC concentration.
Positive (IC and EC failure)	+	–	–	ASFV detected but both endogenous and exogenous control failed – repeat extraction with higher EC concentration.
Negative	–	+	+	ASFV not detected.
Negative (IC failure)	–	–	+	Endogenous control failed – insufficient sample quality, repeat extraction/qPCR protocol.
Negative (EC failure)	–	+	–	Exogenous control failed – repeat extraction with higher EC concentration.
Invalid	–	–	–	No amplification of target or controls – repeat full extraction and qPCR workflow.

2.3. DATA ANALYSIS.

The **concordance between the TARGETEX ASFV qPCR kit and the WOAH real-time PCR (gold standard)** was assessed by calculating the overall percentage agreement using two-by-two contingency tables. In addition, the Kappa coefficient (κ) statistic was used to evaluate the level of agreement beyond that expected by chance. According to the interpretation criteria, κ values of 0.81–1.00 indicate almost perfect agreement, 0.61–0.80 substantial agreement, 0.41–0.60 good agreement, 0.21–0.40 moderate agreement, 0.01–0.20 slight agreement, and 0.00 no agreement.

Since the samples originated from experimentally infected animals, their infection status (positive or negative) was already known. This ensured a reliable reference framework for evaluating the diagnostic performance of the TARGETEX ASFV qPCR kit against the WOAH real-time PCR.

2.4. INTER and INTRA-ASSAY REPRODUCIBILITY

The ***inter-assay reproducibility*** was initially estimated **on the ASF positive control included in the kit** and on the **EURL-ASF reference positive control (R+)**. The controls were run in eight different PCRs, to monitor assay-to-assay variation. The Ct values means for the positive controls were calculated and then used to calculate the overall mean, standard deviation (SD), and Coefficient of Variability % (CV). **The average of the % CV was reported as the inter-assay CV.** In addition the **13 EURL ASF-reference samples** were analyzed in two different PCR runs in different equipments. The CV was calculated following the same schedule explained above. **The average of the % CV was reported as the inter-assay CV**.

The ***intra-assay reproducibility*** were additionally assessed with CVs from the **13 EURL ASF-reference samples tested by duplicate in two different equipments.** The Ct values means were calculated and then used to calculate the standard deviation (SD), and % CV. Over all % CV = SD of Ct means ÷ mean of Ct x 100. **The average of the individual CVs was reported as the intra or intra-assay CV.**

3. RESULTS

3.1. Validation criteria and interpretation of the results.

All reactions were validated according to the criteria described in the Methodology section, since all controls met the required values.

Out of the 298 samples initially tested, **18 samples were deemed invalid.** Specifically, four samples showed negative Ct values in all three channels, while the remaining invalid cases were due to negative values obtained in the FAM channel (used for ASFV detection) and the Cy5 channel (used to monitor extraction and PCR efficiency). All invalid cases corresponded to blood samples. Following the manufacturer's instructions, these DNA samples underwent a second extraction and PCR run, tested at 1:5 dilution. As a result, **all previously invalid samples yielded valid results when tested at a 1:5 dilution** (Table 6).

Table 6→ *Comparative results of WOAH real-time PCR and TARGETEX ASFV qPCR kit in samples initially classified as non-validated*

Nº	ID SAMPLE	WOAH real-time PCR		TARGETEX ASFV qPCR kit 1st run				TARGETEX ASFV qPCR kit 2nd run			
		Ct	RES	FAM	HEX	CY5	RES	FAM	HEX	CY5	RES
1	64	34.10	POS	40	11.3	40	NV	31.5	13.89	19.48	POS
2	66	34.24	POS	40	13.34	40	NV	33.74	13.94	19.64	POS
3	71	17.86	POS	40	12.24	40	NV	15.6	15.75	19.75	POS
4	85	21.43	POS	40	15.98	40	NV	19.5	18.3	19.73	POS
5	91	30.60	POS	40	40	40	NV	26.31	18.73	19.9	POS
6	93	35.78	WEAK[1]	40	40	40	NV	34.6	21.73	23.94	POS
7	117	33.69	POS	40	15.34	40	NV	31.77	18.67	19.99	POS
8	119	39.23	WEAK	40	15.03	40	NV	33.27	16.98	20.38	POS
9	120	33.92	POS	40	14.57	40	NV	32.03	17.5	22.79	POS
10	138	37.62	WEAK	40	14.32	40	NV	40	16.09	18.71	FN
11	142	34.55	POS	40	13.65	40	NV	30.72	15.9	19.33	POS
12	145	31.47	POS	40	14.99	40	NV	33.9	17.23	19.66	POS
13	147	35.64	WEAK	40	15.2	40	NV	26.35	17.88	19.66	POS
14	155	34.52	POS	40	14.24	40	NV	32.8	17.53	19.56	POS
15	4	40	NEG	40	40	40	NV	40	17.57	26.2	NEG
16	10	34.31	POS	40	16.42	40	NV	36.06	17.57	25.34	POS
17	37	40	NEG	40	40	40	NV	40	19.92	26.37	NEG
18	95	40	NEG	40	16.88	40	NV	40	18.33	25.50	NEG

[1] *WEAK Ct ≥35; NV = not validated; FN = false negative*

It should also be noted that the exogenous control (EC) was added at a 10^{-4} dilution after the extraction step, instead of the 10^{-5} dilution recommended by the manufacturer. Despite this modification, all samples were successfully detected, and all controls fulfilled the required validation criteria.

3.2. Determination of the analytical sensitivity of the TARGETEX ASFV qPCR kit testing experimental samples.

3.2.1. Evaluation of the suitability of the TARGETEX ASFV qPCR kit for the detection of the circulating ASFV genotypes

Twenty two ASFV reference DNAs out of the 24 reported p72 ASFV genotypes were analyzed in parallel by the two PCR assays. **The TARGETEX ASFV qPCR kit was able to detect the 21 genotypes tested** with **average of difference in the Ct values of -1.632** when comparing to those obtained using the WOAH real-time PCR (as is shown in Table 7).

Table 7→ *comparative PCR results obtained in the analysis of* ***21 ASFV REFERENCE DNAs***

ASFV	GENOTYPE	WOAH real time PCR Ct value	TARGETEX ASFV qPCR kit Ct value (FAM)	Ct DIFFERENCE
E75	I	21.77	16.71	-5.060
Maur08/1	II	24.23	23.67	-0.560
Ukr12/Zapo	II	18.01	18.44	0.430
RSA2008/1	III	27.6	26.17	-1.430
RSA/W/1/99	IV	23.17	22.3	-0.870
Moz64	V	18.51	16.18	-2.330
SPEC/265	VI	23.55	23.45	-0.100
RSA/03/7	VII	25.39	25.21	-0.180
MwLil20/1	VIII	21.78	17.58	-4.200
Ken06.Bus	IX	26.08	26.33	0.250
BUR90/1	X	23.98	23.6	-0.380
KAB6/2	XI	27.16	24.69	-2.470
MZI92/1	XII	25.74	24.74	-1.000
SUM14/11	XIII	28.08	27.41	-0.670
NYA1/2	XIV	25.71	25.65	-0.060
TAN/2008/1	XV	24.22	26.53	2.310
TAN2003/2	XVI	24.01	23.91	-0.100
NAM/P/1/95	XVIII	25.06	23.5	-1.560
SPEC125	XIX	38.49	32.87	-5.620
24823	XX	36.12	22.95	-13.170
RSA2008/2	XXII	23.9	22.71	-1.190
ET13/1505	XXIII	18.13	20.19	2.060
AVERAGE Ct DIFFERENCE *(TARGETEX – WOAH real time PCR)*				**-1.632**

3.2.2. Analysis of the EURL-ASF REFERENCE SAMPLES (ASF-Ref-2)

When evaluating a panel of 13 EURL-ASF reference samples, there was complete concordance (κ index = 1 [95% CI]) between the WOAH real-time PCR and the TARGETEX ASFV qPCR kit. **The commercial Kit yielded similar Ct (Cycle threshold) values compared to the reference test, with an average disparity of 0.05**, as delineated in Table 8.

Table 8→ *comparative PCR results obtained in the analysis of* ***13 ASF REFERENCE SAMPLES***

ID SAMPLE	WOAH real-time PCR		TARGETEX ASFV qPCR kit					Ct DIFFERENCE
	Ct VALUE	RESULT	Ct VALUE FAM	RESULT	Ct VALUE HEX	Ct VALUE Cy5	RESULT	
SAMPLE 34	27.04	POSITIVE	26.69	POSITIVE	20.4	30.61	VALIDATED	-0.35
SAMPLE 35	30.37	POSITIVE	30.16	POSITIVE	22.02	29.6	VALIDATED	-0.21
SAMPLE 36	19.31	POSITIVE	18.69	POSITIVE	16.15	28.83	VALIDATED	-0.62
SAMPLE 37	21.32	POSITIVE	20.31	POSITIVE	19.17	28.6	VALIDATED	-1.01
SAMPLE 39	23.12	POSITIVE	23.64	POSITIVE	21.09	29.52	VALIDATED	0.52
SAMPLE 40	30.84	POSITIVE	30.32	POSITIVE	18.43	29.35	VALIDATED	-0.52
SAMPLE 42	23.66	POSITIVE	23.36	POSITIVE	20.55	29.35	VALIDATED	-0.3
SAMPLE 43	24.71	POSITIVE	26.03	POSITIVE	20.77	30.34	VALIDATED	1.32
SAMPLE 44	38.54	POSITIVE	38.35	POSITIVE	27.56	20.03	VALIDATED	-0.19
SAMPLE 45	30.46	POSITIVE	32.38	POSITIVE	18.8	31.35	VALIDATED	1.92
SAMPLE 46	32.93	POSITIVE	34.02	POSITIVE	14.73	31.33	VALIDATED	1.09
SAMPLE 47	32.84	POSITIVE	32.42	POSITIVE	33.99	29.66	VALIDATED	-0.42
SAMPLE 48	31.26	POSITIVE	30.69	POSITIVE	24.18	30.19	VALIDATED	-0.57
AVERAGE Ct DIFFERENCE *(TARGETEX – WOAH real time PCR)*								***0.05***

3.2.3. ASFV genome detection in EDTA-BLOOD samples

The **analytical sensitivity** was first assessed using a total of **186 experimental blood samples** obtained from the EURL-ASF sample bank. These samples were collected at different time points post-infection during necropsies of domestic pigs experimentally infected with ASFV isolates representing varying levels of virulence and multiple genotypes, including I, II, IX, X, and XXIII. **All samples tested positive with the WOAH real-time PCR**. In comparison, the **TARGETEX ASFV qPCR kit detected ASFV in 178 samples, resulting in a sensitivity of 96%** when using the WOAH assay as the gold standard. There were **eight samples (4%) which gave a negative result** by the commercial kit resulted as positive when tested using the WOAH real-time PCR (Table 9)**.**

Table 9→ *discrepant results obtained with the TARGETEX ASFV qPCR kit compared to the WOAH real-time PCR.*

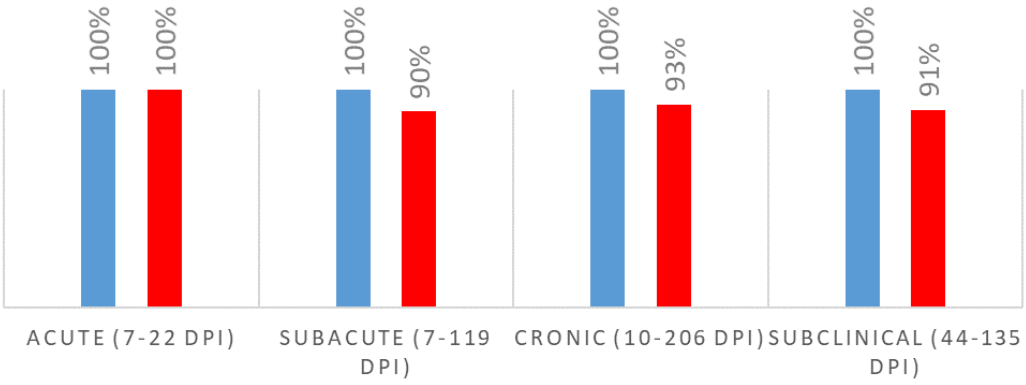
ASFV	CLINICAL FORM	DPI*	WOAH real-time PCR		TARGETEX ASFV qPCR kit			
			Ct	*RES*	*FAM*	*HEX*	*Cy5*	*RES*
Ken05/Tk1	SUBACUTE	64	38.78	WEAK[1]	40	15.97	26.7	FN
OURT88/3	CHRONIC	108	37.91	WEAK	40	13.49	27.34	FN
NH/P68	CHRONIC	65	37.82	WEAK	40	15.54	34.28	FN
NH/P68	CHRONIC	72	38.38	WEAK	40	15.38	25.68	FN
NH/P68	CHRONIC	52	36.79	WEAK	40	11.29	27.07	FN
LV17/WB-RIE1	SUBCLINICAL	101	36.52	WEAK	40	13.53	24.43	FN
LV17/WB-RIE1	SUBCLINICAL	105	38.96	WEAK	40	15.2	30.42	FN
LV17/WB/RIE1	CHRONIC	35	37.62	WEAK	40	16.09	18.71	FN

[1] *WEAK Ct ≥35; *dpi = days post infection; FN = false negative*

These "discrepant samples" were mainly taken at late times post infection (more than one month) from animals with subclinical and chronic forms of the disease that presented Ct values >35 in the WOAH real time PCR (Table 9).

In cases where the **Ct values were below 35, a high level of agreement was observed between both assays, with a κ index of 1 (95% CI).** As shown in **Figure 1,** both methods achieved **100% detection in acute infections**, while the TARGETEX ASFV qPCR kit reached 90% in subacute cases, 93% in chronic cases, and 91% in subclinical cases. It is important to highlight that, as indicated in Table 9, all these samples came from animals that, despite the clinical presentation, had survived the infection and developed strong antibody responses with high titers. These particular conditions are typically associated with lower viral loads. Even under these challenging circumstances, the **TARGETEX ASFV qPCR kit demonstrated excellent diagnostic performance**, maintaining detection rates above 90% across all clinical forms and showing a very high level of concordance with the WOAH real-time PCR.

Figure 1 → *Correlation among the percentage of positive samples with regards to the clinical form of the disease obtained with the* ***WOAH-real time PCR*** *and* ***TARGETEX ASFV qPCR kit***

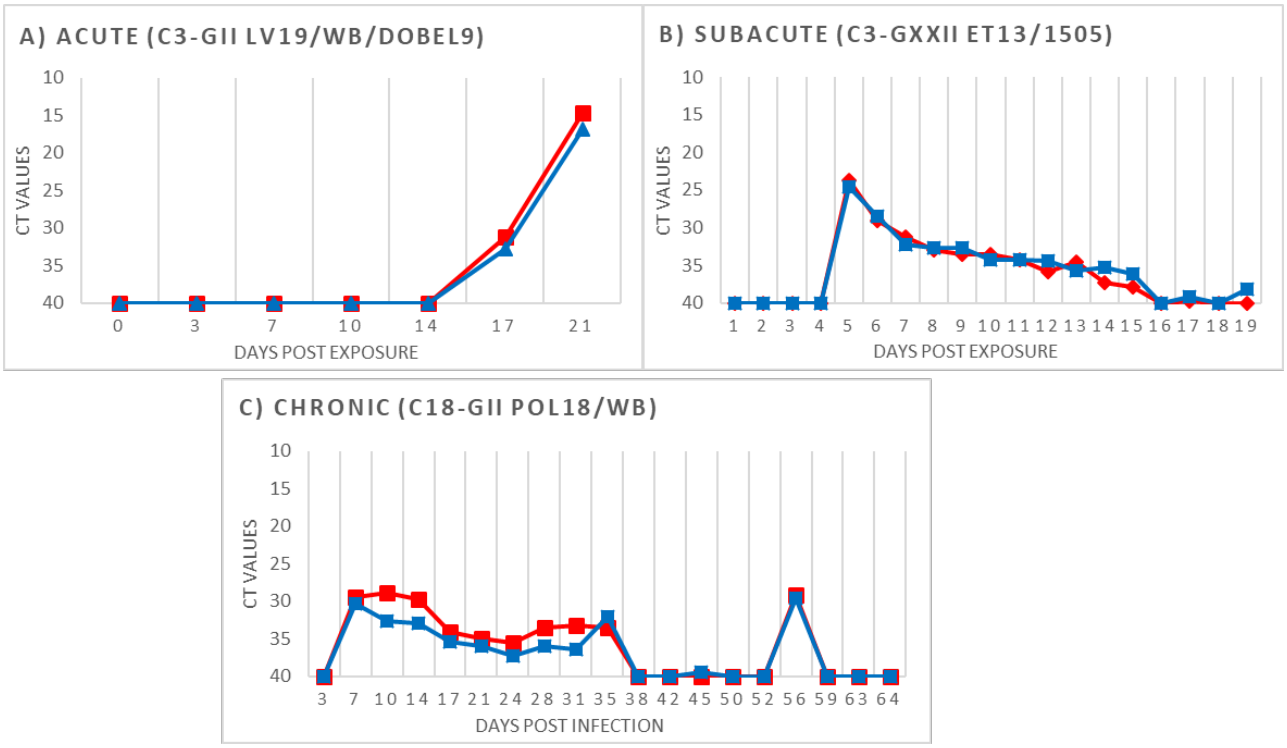


The **performance evaluation of the TARGETEX ASFV qPCR kit for detecting the ASFV genome throughout the course of infection** was carried out using a panel of **45 sequential blood samples collected from experimentally infected animals**. Out of the 45 samples analysed, 25 (55.6%) tested positive with the commercial kit, while 26 (57.8%) tested positive with the reference WOAH real-time PCR. The **overall agreement between the two assays was very high, with a κ index of 0.95** [95% CI: 0.87–1], indicating almost perfect concordance.

As illustrated in **Figure 2**, both methods displayed parallel kinetics of viremia across the different clinical forms of the disease. In acute infections, viremia was detected simultaneously by both assays, showing a progressive decrease in Ct values until animals reached the terminal stage. In subacute infections, both assays also exhibited similar detection dynamics, and only one sample collected at the final stage of infection was negative with the TARGETEX ASFV qPCR kit but positive (Ct = 38.17) with the WOAH real-time PCR. In chronic infections, both assays consistently detected ASFV DNA with fluctuating Ct values,

reflecting persistent but lower viral loads. Overall, these results demonstrate that the commercial kit performs robustly across different infection courses, with only minimal discrepancies observed in samples with very low viral loads.

Figure 2→ *Comparative viremia results determined by the* ***WOAH real-time PCR*** *and* ***TARGETEX ASFV qPCR kit*** *in blood samples collected from domestic pigs infected developing* ***acute (A), subacute (B) or chronic (C) infections.***



3.2.4. ASFV genome detection in TISSUE samples.

In this study, a panel of 32 tissue samples collected at 23 and 36 dpi from animals infected with genotype II and XXIII ASFVs were examined. Both PCR methods successfully detected the presence of the ASFV genome in all tested tissues, achieving a 100% detection rate (Table 10).

Table 10→ *comparative results obtained in tissue samples*

ASFV / GENOTYPE/ VIRULENCE	ID PIG/ DPI*	TISSUE	WOAH REAL TIME PCR		TARGETEX ASFV Detection Kit			
			Ct	*RES.*	*FAM*	*HEX*	*Cy5*	*RES.*
POL18/WB	C1/D36	LIVER	35.52	WEAK	32.32	14.99	27.1	POSITIVE
CASE1794-		LUNG	32.67	POSITIVE	29.1	17.67	29.83	POSITIVE
GENOTYPE		KIDNEY	35.1	WEAK	31.79	14.91	30.46	POSITIVE
II/ATT-		SPLEEN	32.32	POSITIVE	29.12	15.62	31.25	POSITIVE
CHRONIC		HEART	37.82	WEAK	27.52	17.53	36.43	POSITIVE
		TONSIL	27.03	POSITIVE	24.26	13.79	31.72	POSITIVE
		RENAL LN	27.04	POSITIVE	25.36	12.95	28.33	POSITIVE

		RETROPHARYNGEAL LN	27.79	POSITIVE	24.56	14.08	29.83	POSITIVE
		GASTROHEPATIC LN	31.02	POSITIVE	28.25	14.02	31.14	POSITIVE
		MESENTERIC LN	35.65	WEAK	35.44	13.81	30.28	POSITIVE
		MEDIASTINUM LN	32.71	POSITIVE	30.14	12.82	30.51	POSITIVE
		INGUINAL LN	26.27	POSITIVE	22.72	13.69	39.24	POSITIVE
		SUBMANDIBULAR LN	26.58	POSITIVE	23.84	13.96	29.64	POSITIVE
		SPLENIC LN	33.49	POSITIVE	31.96	15.33	31.61	POSITIVE
		BONE MARROW	34.2	POSITIVE	31.06	13.47	29.49	POSITIVE
ET13/1505-GENOTYPE XXIII/ MOD-VIR SUBACUTE	C17/D23	LIVER	26.36	POSITIVE	25.17	13.93	30.8	POSITIVE
		LUNG	25.56	POSITIVE	23.71	18.26	19.43	POSITIVE
		KIDNEY	28.03	POSITIVE	28.1	12.09	31.68	POSITIVE
		SPLEEN	26.43	POSITIVE	25.4	15.71	32.58	POSITIVE
		HEART	27.45	POSITIVE	24.95	17.68	30.33	POSITIVE
		TONSIL	29.21	POSITIVE	29.17	12.95	30.64	POSITIVE
		RENAL LN	26.16	POSITIVE	24.36	14.51	30.29	POSITIVE
		RETROPHARYNGEAL LN	23.85	POSITIVE	21.76	14	29.2	POSITIVE
		GASTROHEPATIC LN	28.11	POSITIVE	26.96	13.4	35.5	POSITIVE
		MESENTERIC LN	32.46	POSITIVE	31.98	14.09	30.68	POSITIVE
		MEDIASTINUM LN	25.62	POSITIVE	26.04	14.53	29.69	POSITIVE
		INGUINAL LN	26.84	POSITIVE	25.09	13.08	29.95	POSITIVE
		SUBMANDIBULAR LN	24.83	POSITIVE	23.47	13.77	29.09	POSITIVE
		POPLITEAL LN.	29.59	POSITIVE	30.36	17.25	28.33	POSITIVE
		SPLENIC LN.	26.19	POSITIVE	24.9	13.96	29.94	POSITIVE
		BONE MARROW	25.51	POSITIVE	22.84	16.8	28.65	POSITIVE
		DIAPHRAGMATIC MUSCLE	33.09	POSITIVE	30.3	20.77	28.57	POSITIVE

WEAK Ct ≥35; LN = lymph node. DPI = days post-infection.

3.3. OVERALL ANALYSIS OF THE RESULTS.

The results obtained from the analysis of the 13 reference panel samples (EURL-ASF-Ref2), 22 reference panel DNA samples (EURL-ASF-Ref3), 231 experimental EDTA-blood samples, and 32 tissue samples were combined to provide an overall evaluation of the performance of the TARGETEX ASFV qPCR kit for ASF diagnosis. Since all samples originated from animals with a known ASF infection status, the comparative analysis was reliable. Overall, the TARGETEX ASFV qPCR kit correctly identified **90.6%** of infected or exposed animals, while the WOAH real-time PCR detected **93.6%**, resulting in a κ value of **0.79 [95% CI]**, which corresponds to **substantial agreement between the two assays**. Comparative values are summarized in Table 11.

Table 11→ *comparative number of positive samples detected by each of the two PCR assayed.*

ID SAMPLES TESTED		WOAH Real-time PCR		TARGETEX ASFV qPCR kit	
TYPE	Total samples	Nº POS	% POS	Nº POS	% POS
EURL-ASF-Ref DNAs	22	22	100%	22	100%
EURL-ASF-Ref2	13	13	100%	13	100%
BLOODs (necropsy)	186	186	100%	178	95.7%
BLOOD (kinetic)	45	26	58%	25	55.6%
TISSUES	32	32	100%	32	100%
TOTAL	**298**	**279**	**93.6%**	**270**	**90.6%**

3.4. INTER-ASSAY REPRODUCIBILITY

The ***interassay reproducibility*** was initially assessed on the ASF positive control included in the kit (PC) and on the EURL-ASF reference positive control (R+). The controls were run in seven different PCRs, to monitor assay-to-assay variation. **The inter-assay CV for the TARGETEX ASFV qPCR kit positive control (PC) was 3% and for the EURL control 4% considered appropriate in routine testing** (Table 12).

Table 12→ *Coefficient of Variation (CV) per positive controls between seven different PCR runs.*

	DATE	R+ EURL	PC
PLATE 1	28/08/2025	30.08	29.12
PLATE 2	28/08/2025	30.67	28.30
PLATE 3	01/09/2025	30.78	29.34
PLATE 4	02/09/2025	27.99	29.35
PLATE 5	04/09/2025	29.84	30.04
PLATE 6	09/09/2025	27.65	30.63
PLATE 7	10/09/2025	29.04	29.14
	Average	**29.44**	**29.42**
	SD	**1.25**	**0.74**
	CV	**4%**	**3%**

CV = coefficient of variability; SD = standard deviation

In addition the inter-assay reproducibility was assessed with CVs obtained testing the 13 EURL-reference samples. These samples **were tested by duplicate in two different runs prepared at different times by different technical staff and using randomly two real time PCR equipment (Stratagene).** The results obtained are showed in Table 14.

Table 14→ *Coefficient of Variation (CV) per sample between two different PCR runs in the analysis of the ASF- Ref2 panel of samples*

ID SAMPLE	PCR RUN (Eq1)		PCR RUN (Eq2)		MEAN	SD	%CV
	C_T1	RESULT	C_T2	RESULT			
SAMPLE 34	26.69	POSITIVE	25.94	POSITIVE	26.32	0.53	2%
SAMPLE 35	30.16	POSITIVE	29.025	POSITIVE	29.59	0.80	3%
SAMPLE 36	18.69	POSITIVE	18.52	POSITIVE	18.61	0.12	1%
SAMPLE 37	20.31	POSITIVE	20.22	POSITIVE	20.27	0.06	0%
SAMPLE 39	23.64	POSITIVE	23.515	POSITIVE	23.58	0.09	0%
SAMPLE 40	30.32	POSITIVE	29.74	POSITIVE	30.03	0.41	1%
SAMPLE 42	23.36	POSITIVE	22.93	POSITIVE	23.15	0.30	1%
SAMPLE 43	26.03	POSITIVE	26.055	POSITIVE	26.04	0.02	0%
SAMPLE 44	38.35	POSITIVE	40	NEGATIVE	39.18	1.17	3%
SAMPLE 45	32.38	POSITIVE	30.24	POSITIVE	31.31	1.51	5%
SAMPLE 46	34.02	POSITIVE	33.98	POSITIVE	34.00	0.03	0%
SAMPLE 47	32.42	POSITIVE	32.435	POSITIVE	32.43	0.01	0%
SAMPLE 48	30.69	POSITIVE	30.31	POSITIVE	30.50	0.27	1%
AVERAGE %CV							**1%**

CV = coefficient of variability; SD = standard deviation .

Eq = Equipment. FP = false positive

The average of the individual CVs reported as the inter-assay CV was 1%. This value, being below 5%, demonstrates very good stability and inter-assay reproducibility. One discrepant result was observed in a weak positive sample, which had an initial Ct value >35.

3.5. INTRA-ASSAY REPRODUCIBILITY

The **intra-assay reproducibility** was evaluated by determining the coefficient of variation (CV) obtained from testing the panel of reference 13 samples (ASF-Ref-2) in duplicate. The results obtained for each of the samples tested in the intra-assay study are presented in Table 15. This assessment helps ensure the reliability and consistency of the testing methodology by examining the variation in results obtained from repeated testing of the same samples within the same assay.

Table 15→ *Coefficient of Variation (CV) in the reference panel of samples tested by duplicate in the same PCR*

ID SAMPLE	PCR RUN				
	C_T1	C_T2	MEAN	SD	%CV
SAMPLE 34	25.99	25.89	25.94	0.0707	0%
SAMPLE 35	29.14	28.91	29.025	0.1626	1%
SAMPLE 36	18.51	18.53	18.52	0.0141	0%
SAMPLE 37	20.05	20.39	20.22	0.2404	1%
SAMPLE 39	23.5	23.53	23.515	0.0212	0%
SAMPLE 40	30.16	29.32	29.74	0.594	2%
SAMPLE 42	23.1	22.76	22.93	0.2404	1%
SAMPLE 43	25.65	26.46	26.055	0.5728	2%
SAMPLE 44	40	40	40	0	0%
SAMPLE 45	31.01	29.47	30.24	1.0889	4%
SAMPLE 46	33.7	34.26	33.98	0.396	1%
SAMPLE 47	32.24	32.63	32.435	0.2758	1%
SAMPLE 48	30.16	30.46	30.31	0.2121	1%
AVERAGE CV%					**1.06%**

CV = coefficient of variability; SD = standard deviation

The average intra-assay CV was 1.06%, indicating high reproducibility of the results.

3.6. CONCLUSIONS.

→ The **commercial TARGETEX ASFV qPCR Detection Kit correctly detected all of the 22 ASFV reference DNAs tested**, representing twenty-two out of the twenty-four reported p72 ASFV genotypes. This demonstrates the kit's capability to effectively detect a wide range of ASFV genotypes.

- → Analysis of 298 samples obtained from experimentally infected animals with ASFV revealed a **κ index of 0.79 [95% CI], indicating substantial agreement between** the TARGETEX ASFV qPCR Detection Kit and the WOAH real-time PCR.
- → Given that all samples were derived from animals with known ASF infection status, the TARGETEX ASFV qPCR Detection Kit correctly identified 90.6% of infected or exposed animals, compared to 93.6% detected with the WOAH real-time PCR. Only **9 samples were found discrepant between the two assays, all of them weak positives with Ct values >35,** obtained from animals in chronic or subclinical stages of infection typically associated with low viral loads.
- → The **inter-assay variability, with values ranging between 1% and 4%,** demonstrated high repeatability across different assay runs, confirming the kit's stability and suitability for routine testing.
- → The **intra-assay variability, with an average CV of 1.06%**, also exhibited high reproducibility in the results obtained, further supporting the robustness of the assay under routine testing conditions.

Overall, the study demonstrates that the TARGETEX ASFV qPCR Detection Kit is effective in detecting a broad range of ASFV genotypes, shows substantial agreement with the WOAH real-time PCR, and offers high repeatability and reproducibility in routine testing. The few discrepancies observed were limited to weak positive samples with Ct values >35 from animals in chronic or subclinical stages of infection, typically associated with low viral loads. These results support the reliability of the TARGETEX ASFV qPCR Detection Kit for ASFV diagnosis in experimental samples. Further validation with field samples is recommended to confirm its performance under diverse epidemiological conditions.

Report performed in Valdeolmos (Madrid) at 12th September 2025

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