Validation of the ASF diagnostic kit Bosphore® ASFV Detection Kit v2 for detection of African Swine Fever (ASFV) by real-time PCR (qPCR), developed by Anatolia Geneworks

VALIDATION REPORT (modified version V2)

PERFORMED BY THE

Centro de Investigación en Sanidad Animal (CISA-INIA)

European Union reference laboratory for ASF (EURL-ASF)

INIA-CISA. Valdeolmos, Madrid, SPAIN
Ctra. Algete a El Casar, s.n.
Valdeolmos, 28130 Madrid
Tel +34 916202300
Fax +34916202247

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Location: Moldova
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1. OBJECTIVE.

The study conducted at CISA-INIA/CSIC as the European Union Reference Laboratory for African swine fever (EURL-ASF) aimed to assess **the performance of the Bosphore® ASFV Detection Kit v2 for the detection of African Swine Fever Virus (ASFV) in clinical field samples**. This Real-Time PCR assay, developed by Anatolia Geneworks, targets the major capsid protein p72 gene of ASFV. Manufacturers state that it's apt for analyzing diverse swine samples like tissues, swabs, serum, and whole blood. Utilizing fluorescence detection via FAM and HEX filters enables the precise amplification of ASFV DNA, with FAM indicating its presence. To guarantee result reliability and detect probable issues such as DNA extraction complications or PCR inhibition, an internal control based on swine endogenous nucleic acid (GAPDH) detection is included. Its presence is monitored through the HEX channel.

2. PROCEDURE

2.1. Samples included in the validation study.

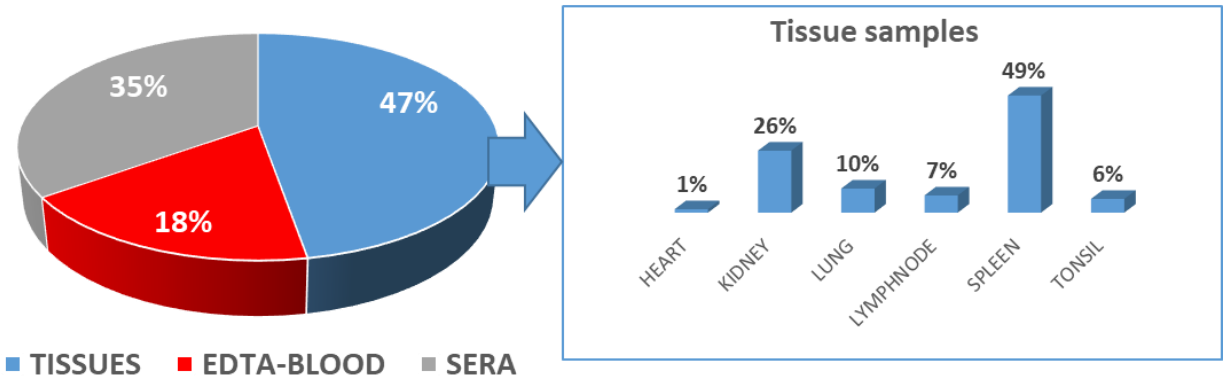
The validation study encompassed **five hundred and fifty two (552) samples** from different sources as showed in Table 1.

Table 1→ *Detailed description of field samples included in the validation study* using the *Bosphore® ASFV Detection Kit v2.*

HOST	Affected ASF European countries	Free ASF European countries	TOTAL
Domestic pig	146	92	238
Wild boar	314	0	314
TOTAL	**460**	**92**	**552**

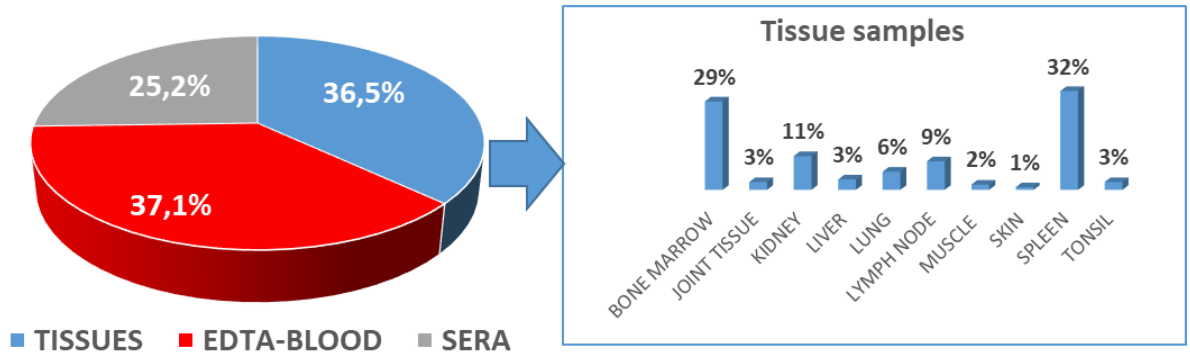
Specifically, for **domestic pigs in European countries affected by genotype II ASFV**, a total of **146 field samples were tested**, obtained from 142 animals involved in outbreaks spanning 2020 to 2022. These samples included 69 (47%) tissue samples, 26 (18%) EDTA-blood samples, and 51 (35%) serum samples. Among the tissues tested, the distribution was as follows: 1 heart sample (1%), 18 kidney samples (26%), 7 lung samples (10%), 5 lymph node samples (7%), 34 spleen samples (49%), and 4 tonsil samples (6%) (Figure 1).

Figure 1 → *Description of the domestic pig samples tested with the Bosphore® ASFV Detection Kit v2 for validation purpose.*



A total of **314 field samples were examined, sourced from 306 wild boars** collected in European countries affected by genotype II ASFV between 2018 and 2022. The sample types comprised 116 (36.5%) tissue samples, 118 (37.1%) blood samples, and 80 (25.2%) sera samples. Among the tissue samples, spleen predominated (n=38, 32%), followed by bone marrow (n=34, 29%), kidney (n=13, 11%), lymph nodes (n=11, 9%), lungs (n=7, 6%), liver (n=4, 3%), with smaller numbers for tonsils, joint tissue, muscle, and skin (Figure 2).

Figure 2 → *Description of the wild boar samples tested with the Bosphore® ASFV Detection Kit v2 for validation purpose.*



Additionally, a set **of 92 blood samples from domestic pigs in Spain** served as **negative samples** for diagnostic specificity assessment.

2.2. TEST PROCEDURES.

2.2.1. DNA extraction procedure: DNA was extracted from organ homogenates, EDTA-blood or sera 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. The DNA was extracted from 200 µl of each tissue homogenate, blood or serum samples using the High Pure PCR Template Preparation kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). DNAs were stored <-10 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 genomic DNA was performed in 96-well plate MX3005P equipment's (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 38	**Positive control for the extraction (EURL)**	1:10.000 dilution of the ASFV reference strain E75 (genotype I) diluted in a negative sera.
NCS (E-)	**Negative control for the extraction**	Distilled water
EURL-R+ batch 33	**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 38	Detected in FAM channel	Ct value within the range of 32±4.
NCS (E-)	Non detected	Ct ≥40.
EURL-R+ batch 33	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. Bosphore® ASFV Detection Kit v2: The amplification of the ASFV genomic DNA was performed in 96-well plate MX3005P equipment's (Stratagene, Agilent Technologies Inc., Santa Clara, CA, USA) using the **Bosphore® ASFV Detection Kit v2** according to the protocol described by the manufacturers.

The following controls were included in the amplification steps:

Reference	Component	Description
Positive control (PC+)	**Synthetic ASFV DNA included in the kit**	Synthetic DNA provided, containing specific target of ASFV
NCA (R-)	**Negative control for the reaction included in the kit**	Distilled water

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

CONTROLS	FAM	HEX
EURL-R+ batch 33	C_T 32±4	C_T ≤35
Positive control (PC+)	C_T ≤ 30	C_T ≤35
NCA (R-)	No signal	No signal

Interpretation of the results:

- **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 and signal/not signal in HEX channel.
  - The PC+ yields a signal in the FAM and HEX channels with C_T ≤ 30 and C_T ≤35 respectively.
  - The NCA(R-) does not yield a signal in the FAM and HEX channels.
- **The sample is negative for ASFV**, and the assay is valid, if the following criteria are met:
  - The sample yields a signal in the HEX (Ct≤35) channel but not in the FAM channel.

- The PC+ yields a signal in the FAM and HEX channels with $C_T \leq 30$ and $C_T \leq 35$ respectively.
- The NCA(R-) does not yield a signal in the FAM and HEX channels.

- **The sample results are inconclusive,** and the assay is invalid, if the following occurs:
  - The sample yields no signal in the FAM and in HEX channels.

In rare cases of PCR inhibition due to medication or other PCR inhibitors in the sample, the manufacturers recommend repeating the test of inhibited samples, by freezing and thawing the DNA samples and using them in the PCR after diluting them 1:2 with distilled water.

2.3. DATA ANALYSIS.

Samples were classified as positive or negative for ASF at the European Union Reference Laboratory (EURL) using a combination of virus and antibody detection tests. These tests included the UPL real-time PCR (WOAH-PCR) and the indirect immunoperoxidase test (IPT). Samples were tested in parallel using the both PCR assays and the concordance between the WOAH real-time PCR and the Bosphore® ASFV Detection Kit v2 was assessed. This was done by calculating the overall percentage agreement between the two PCR assays using two-by-two contingency tables. The significance of the concordance level beyond chance was evaluated using the Kappa Coefficient (κ) statistics. The κ values were interpreted as follows: 0.81–1.00 indicated 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 indicated no agreement.

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**. The controls were run in ten different PCRs, respectively, 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). In addition **20 field samples randomly selected** were analyzed by duplicate in two different PCR runs in different equipment's. 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*** was assessed with CVs from the same **20 duplicated field samples using in the inter-assay study.** 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-assay CV.**

3. RESULTS

3.1. Validation criteria and interpretation of the results.

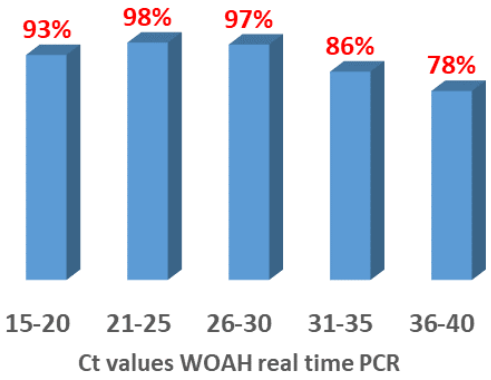
Out of the initial 552 samples tested, **thirty-six were deemed invalid** due to negative Cycle threshold (Ct) values in both the FAM channel (used for ASFV detection) and the HEX channel (used for internal control). This group included nineteen samples from wild boars and seventeen from domestic pigs. The majority of the invalid samples, 27 out of 36 (75%), were blood samples, with or without EDTA, while the remaining nine were tissue samples. Following the manufacturer's recommendations, these DNA samples underwent a second PCR test at a 1:2 dilution. This subsequent test yielded valid results for 34 of these samples, confirming successful detection of the ASFV genome and the internal controls. In the positive and negative controls, all PCR results were validated according to established criteria. Two samples remained invalid and were subsequently excluded from the final analysis. **Therefore, the final datasheet included five hundred and fifty (550) samples.**

3.2. Diagnostic sensitivity.

Diagnostic sensitivity was evaluated using **458 field samples** collected from domestic pigs (146 samples) and wild boars (312 samples) to assess the capacity of detecting the ASFV genome in real clinical scenarios. Initially, all samples were classified as positive for ASF by combining the results from the WOAH real-time PCR (UPL-PCR) and the WOAH-IPT for antibody detection. Among these, **455 samples were confirmed positive for the ASFV genome using the WOAH reference method**. In contrast, the **Bosphore® ASFV Detection Kit v2 identified 425 samples as positive** but also recorded 30 false negatives. This led to a **diagnostic sensitivity of 93.4%,** with a 95% confidence interval ranging from 90.7% to 95.3%.

The decreased sensitivity was mainly attributed to the Ct values obtained from the reference WOAH real-time PCR. In particular, samples with Ct values above 36 displayed the lowest sensitivity at 78%, yielding 12 false negative results. Samples with Ct values ranging from 31 to 35 demonstrated a sensitivity of 86%, with 8 false negatives, when tested using the Bosphore® ASFV Detection Kit v2 (Figure 3).

Figure 3 → *Sensitivity of the Bosphore® ASFV Detection Kit v2 relative to Ct values from positive samples confirmed by the WOAH real-time PCR.*



Moreover, nine samples that tested strongly positive with the WOAH real-time PCR (Ct < 30) were not detected by the Bosphore Detection Kit v2. When these samples were subsequently tested at a 1:10 dilution, seven of them yielded positive results (Table 2).

Table 2→ *Results obtained in false negative samples tested in 1:10 dilution with the Bosphore Detection Kit v2.*

ID SAMPLE	WOAH REAL TIME		Bosphore Detection Kit v2 Undiluted sample			Bosphore Detection Kit v2 Sample diluted 1:10		
	Ct	*RESULT*	*FAM 1*	*HEX 1*	*RESULT 1*	*FAM 2*	*HEX 2*	*RESULT 2*
1	18.32	**POSITIVE**	No Ct	29.86	NEGATIVE	16.56	28.30	**POSITIVE**
2	19.11	**POSITIVE**	No Ct	26.3	NEGATIVE	18.7	31.3	**POSITIVE**
3	19.23	**POSITIVE**	No Ct	33.59	NEGATIVE	16.45	29.03	**POSITIVE**
4	19.7	**POSITIVE**	No Ct	31.24	NEGATIVE	21.03	22.94	**POSITIVE**
5	22.38	**POSITIVE**	No Ct	30.83	NEGATIVE	19.15	22.03	**POSITIVE**
6	25.18	**POSITIVE**	No Ct	22.72	NEGATIVE	24.2	19.03	**POSITIVE**
7	25.39	**POSITIVE**	No Ct	18.82	NEGATIVE	23.6	18.03	**POSITIVE**
8	28.39	**POSITIVE**	No Ct	21.97	NEGATIVE	No Ct	22.04	NEGATIVE
9	29.74	**POSITIVE**	No Ct	33.93	NEGATIVE	No Ct	29.03	NEGATIVE

Combining the results, the total number of false negative samples detected with the Bosphore® ASFV Detection Kit v2 was 23. This yielded a final diagnostic sensitivity of 94.7%, with a 95% confidence interval ranging from 92.3% to 96.4%.

It should be noted that of the 23 samples that returned a false negative result, only four tested negative for antibodies, suggesting an earlier stage of infection. Therefore, a negative PCR result in samples that are positive for antibodies is not as concerning because it indicates that although the virus might not be detectable, the immune response confirms past or ongoing infection.

3.3. Diagnostic specificity.

In the study, 92 blood samples from domestic pigs collected in an ASF-free country were included to assess the diagnostic specificity of the Bosphore® ASFV Detection Kit v2. All of these samples tested negative for ASFV, thereby confirming the high diagnostic accuracy of the test kit in distinguishing non-infected samples. As a result, the Bosphore Detection Kit v2 demonstrated a **diagnostic specificity of 100%**. This outcome underscores the kit's effectiveness in accurately identifying the absence of the ASFV genome in samples from an environment free from the disease, highlighting its reliability and precision in clinical diagnostics.

3.4. INTER-ASSAY and INTRA-ASSAY REPRODUCIBILITY

Inter-assay reproducibility was initially evaluated using the ASF positive control provided in the kit (PC+) along with the EURL-ASF reference positive controls for both extraction (EURL-E+) and amplification (EURL-R+). These controls underwent analysis in ten separate PCRs to observe variation between assays. The inter-assay coefficient of variation (CV) for the Bosphore® ASFV Detection Kit v2 **ASF positive control (PC+) was determined to be 2.8%.** Similarly, for the EURL controls, **the CV ranged from 1.4% (EURL-E+) to 1.6% (EURL-R+),** demonstrating consistency suitable for routine testing (refer to table 3).

Table 3→ *Coefficient of Variation (CV) per positive controls between ten different PCR runs.*

	DATE	EURL-E+	EURL-R+	PC+
PLATE 1	20/03/2024	31.07	29.18	25.96
PLATE 2	25/03/2024	31.03	29.85	26.22
PLATE 3	01/04/2024	31.35	28.75	26.12
PLATE 4	01/04/2024	32.4	29.98	26.38
PLATE 5	03/04/2024	31.12	29.71	26.43
PLATE 6	04/04/2024	31.15	29.18	26.5
PLATE 7	08/04/2024	31.13	28.67	24.81
PLATE 8	10/04/2024	31.8	28.88	25
PLATE 9	12/04/2024	31.03	28.98	27.24
PLATE 10	12/04/2024	31.08	28.96	25.64
	Average	**31**	**29**	**26**
	SD	**0.446**	**0.469**	**0.724**
	CV	**1.4%**	**1.6%**	**2.8%**

CV = coefficient of variability; SD = standard deviation

The average of the individual CVs reported as the inter-assay CV ranged between 1.4 to 2.8% lower than 5% exhibited a very good stability and inter assay reproducibility.

In addition **20 field samples** were tested in two different runs prepared at different times by different technical staff and using randomly two real time PCR MX3005P equipment's (Stratagene). The **table 4** shows the individual CVs per each sample include in the analysis.

Table 4→ *Coefficient of Variation (CV) per sample between two different PCR runs.*

ID SAMPLE	TYPE OF SAMPLE	PCR RUN (Eq1)		PCR RUN (Eq2)		MEAN	SD	%CV
		C_T1	RESULT	C_T2	RESULT			
1	SERUM	40.00	NEG	38.68	**POSITIVE**	39.34	0.9334	2%
2	SPLEEN	37.33	**POSITIVE**	35.72	**POSITIVE**	36.53	1.1384	3%
3	BLOOD	37.02	**POSITIVE**	34.09	**POSITIVE**	35.56	2.0718	6%
4	SERUM	36.63	**POSITIVE**	37.52	**POSITIVE**	37.08	0.6293	2%
5	TONSIL	35.97	**POSITIVE**	36.26	**POSITIVE**	36.12	0.2051	1%
6	SERUM	35.22	**POSITIVE**	34.96	**POSITIVE**	35.09	0.1838	1%
7	KIDNEY	35.66	**POSITIVE**	34.68	**POSITIVE**	35.17	0.6930	2%
8	KIDNEY	36.61	**POSITIVE**	35.11	**POSITIVE**	35.86	1.0607	3%
9	SPLEEN	37.01	**POSITIVE**	36.15	**POSITIVE**	36.58	0.6081	2%
10	BONE MARROW	36.00	**POSITIVE**	35.51	**POSITIVE**	35.76	0.3465	1%
11	LYMPH NODE	35.09	**POSITIVE**	34.44	**POSITIVE**	34.77	0.4596	1%
12	LYMPH NODE	36.19	**POSITIVE**	37.59	**POSITIVE**	36.89	0.9899	3%
13	TONSIL	38.72	**POSITIVE**	35.84	**POSITIVE**	37.28	2.0365	5%
14	SERUM	32.07	**POSITIVE**	29.01	**POSITIVE**	30.54	2.1637	7%
15	SERUM	29.80	**POSITIVE**	28.70	**POSITIVE**	29.25	0.7778	3%
16	SPLEEN	29.37	**POSITIVE**	28.50	**POSITIVE**	28.94	0.6152	2%
17	BLOOD	29.48	**POSITIVE**	29.38	**POSITIVE**	29.43	0.0707	0%
18	BLOOD EDTA	30.03	**POSITIVE**	29.87	**POSITIVE**	29.95	0.1131	0%
19	SERUM	29.82	**POSITIVE**	29.94	**POSITIVE**	29.88	0.0849	0%
20	SERUM	30.06	**POSITIVE**	29.80	**POSITIVE**	29.93	0.1838	1%
AVERAGE %CV								**2.2%**

CV = coefficient of variability; SD = standard deviation .
Eq = Equipment.

The average of the individual CVs reported as the inter assay CV was 2.2% .The CV% was lower than 3%, therefore exhibiting a very good stability and inter assay reproducibility.

The **percentage of agreement among the results obtained was 95%.** One sample (marked in blue in table 4) initially classified as negative (Ct>35) in the first PCR run, resulted positive in the second PCR run. In cases where the viral load is low, as indicated by a high Ct value, the virus may not be detected in initial tests. However, small variations in sample handling, PCR reagent performance, or the specific section of the sample tested can lead to different results in subsequent tests.

The ***intra-assay reproducibility*** was assessed with CVs obtained testing the same 20 samples used for the inter assay study. The results obtained in each of the samples are showed in **table 5.**

Table 5→ *Coefficient of Variation (CV) in the field samples tested by duplicate in the same PCR run*

ID	PCR RUN (Eq1)					PCR RUN (Eq2)				
SAMPLE	C_T1	C_T2	MEAN	SD	%CV	C_T1	C_T2	MEAN	SD	%CV
1	40	40	**40**	0.00	0.0%	40	37.4	**38.68**	1.84	4.8%
2	36.2	38.4	**37.3**	1.56	4.2%	35.4	36	**35.72**	0.42	1.2%
3	36.2	37.9	**37.02**	1.20	3.2%	33.5	34.7	**34.09**	0.85	2.5%
4	36.8	36.5	**36.63**	0.21	0.6%	38.5	36.6	**37.52**	1.34	3.6%
5	36.1	35.8	**35.97**	0.21	0.6%	36.2	36.4	**36.26**	0.14	0.4%
6	35	35.5	**35.22**	0.35	1.0%	35.6	34.3	**34.96**	0.92	2.6%
7	35.3	36	**35.66**	0.49	1.4%	34.4	34.9	**34.68**	0.35	1.0%
8	34.8	38.4	**36.61**	2.55	7.0%	35.2	35	**35.11**	0.14	0.4%
9	37.1	37	**37.01**	0.07	0.2%	36.2	36.1	**36.15**	0.07	0.2%
10	36.3	35.7	**36**	0.42	1.2%	35.1	36	**35.51**	0.64	1.8%
11	34.7	35.5	**35.09**	0.57	1.6%	34.3	34.6	**34.44**	0.21	0.6%
12	36.8	35.5	**36.19**	0.92	2.5%	35.2	40	**37.59**	3.39	9.0%
13	40	37.4	**38.72**	1.84	4.7%	35.7	36	**35.84**	0.21	0.6%
14	33.2	30.9	**32.07**	1.63	5.1%	29	29	**29.01**	0.00	0.0%
15	29.7	29.9	**29.8**	0.14	0.5%	28.6	28.8	**28.7**	0.14	0.5%
16	29.2	29.6	**29.37**	0.28	1.0%	28.3	28.7	**28.5**	0.28	1.0%
17	29.9	29.1	**29.48**	0.57	1.9%	29.1	29.7	**29.38**	0.42	1.4%
18	30.2	29.9	**30.03**	0.21	0.7%	29.9	29.9	**29.87**	0.00	0.0%
19	29.9	29.8	**29.82**	0.07	0.2%	29.8	30.1	**29.94**	0.21	0.7%
AVERAGE %CV					**2.0%**					**1.7%**

CV = coefficient of variability; SD = standard deviation

The percentage of **agreement among the results obtained was 95 %,** with only one discrepant result, the same sample that in the inter assay (marked in blue in table 5)

The average of the individual CVs reported as the **intra-assay CV was 2.0% and 1.7% exhibited high reproducibility in the results obtained.**

4. CONCLUSIONS.

- An analysis of 455 ASFV genome positive field samples from epidemic regions in Europe showed that the **Bosphore® ASFV Detection Kit v2 had a diagnostic sensitivity of 94.7%,** with a 95% confidence interval ranging from 92.3% to 96.4%. This analysis identified 23 false negative results when compared to the reference method, the WOAH UPL real-time PCR.

- Analysis of 92 negative samples from an ASF-free country demonstrated that the **diagnostic specificity of the Bosphore® ASFV Detection Kit v2 is 100%.**

- The **Kappa value (κ) of 0.87 [95% CI]** suggests **almost perfect agreement** between the UPL reference method and the Bosphore® ASFV Detection Kit v2.

- The **inter-assay variability**, being **less than 3%,** indicates high repeatability of the results, making it **suitable for routine testing**.

- The **intra-assay variability**, also **less than 3%,** shows **high reproducibility of the results**, further affirming its suitability for routine testing.

The Bosphore® ASFV Detection Kit v2 has demonstrated suitable diagnostic sensitivity and repeatability for reliable diagnosis of ASF in clinical field samples processed with the High Pure DNA extraction kit from Roche. This commercial kit achieves almost perfect agreement with the WOAH reference real-time PCR method (UPL – real-time PCR).

Report performed in Valdeolmos (Madrid) at 16th May 2024 (modified version V2)

GALLARDO FRONTAURA M.DEL CARMEN - DNI 03867289T

Firmado digitalmente por GALLARDO FRONTAURA M.DEL CARMEN - DNI 03867289T
Fecha: 2024.05.16 10:19:39 +02'00'

Dr. Carmina Gallardo

Laboratory Coordinator ASF-EURL

Approval

ARIAS NEIRA MARIA LUISA - DNI 00683346Q

Firmado digitalmente por ARIAS NEIRA MARIA LUISA - DNI 00683346Q
Fecha: 2024.05.16 10:29:08 +02'00'

Dr. Marisa Arias

Director EURL/ Deputy Director CISA-INIA/CSIC