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Validation of the ASF diagnostic kit INgene q PPA Sondas Detection for detection of African Swine Fever (ASFV) by real-time PCR (qPCR), developed by INGENASA (Eurofins)

VALIDATION REPORT

PERFORMED BY THE

Centro de Investigación en Sanidad Animal (CISA-INIA)

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1. OBJECTIVE.

The purpose of the study performed at INIA -CISA as European Union Reference Laboratory for ASF (EURL-ASF) has been to validate **the performance of the INgene q PPA Sondas Detection for the detection of African Swine Fever (ASFV) by real-time PCR (qPCR) in clinical field samples.**

INgene q PPA Sondas Detection is a real-time PCR kit suited for the detection of nucleic acid of ASFV in biological samples in a simple way, with high levels of sensitivity and specificity. This kit makes use of a UPL® probe labelled with FAM. It also includes another probe, labelled in VIC, as internal control of the reaction, allowing detection of false negatives due to an inhibition of the PCR. In addition, the master mix contains a passive reference fluorochrome, ROX, allowing the normalization of the signal, avoiding possible quantification errors deriving from pipetting.

2. PROCEDURE

2.1. Samples included in the validation study.

2.1.1. Domestic pig field samples from genotype II ASFV-infected areas within European countries.

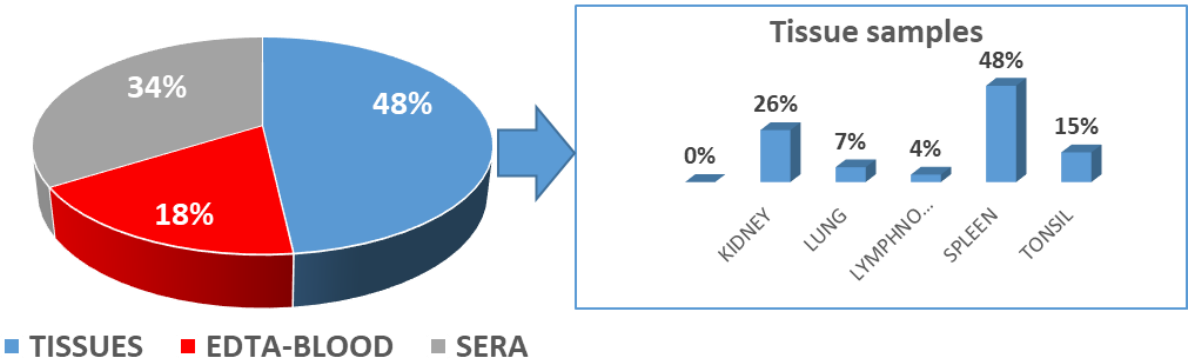
A panel of **56 field samples** collected from **domestic pigs** in the outbreaks occurred during 2020 and 2021 in Europe were included in the validation study (**table 1**). All animals were previously classified as positives combining both virus and antibody detection tests at the EURL-ASF.

Table 1 → *Description of the 57 domestic pig field samples tested with the INGENASA real time PCR kit for validation purpose.*

COUNTRY	Nº SAMPLES TESTED	Nº animals tested
BULGARIA	9	9
GREECE	2	2
LATVIA	6	6
LITHUANIA	2	2
POLAND	35	35
ROMANIA	2	2
TOTAL	**56**	**56**

The samples tested included 27 (48%) tissues, 10 (18%) blood and 19 (34%) serum samples. The tissues tested comprised 7 kidney (26%), 2 lung (7%), 1 lymph node (4%), 13 spleen (48%) and 4 tonsils (15%) (**figure 1)**

Figure 1 → *Description of the domestic pig samples tested with the INGENASA real time PCR kit for validation purpose.*



2.1.2. Wild boar field samples from genotype II ASFV-infected areas within the Eastern European countries

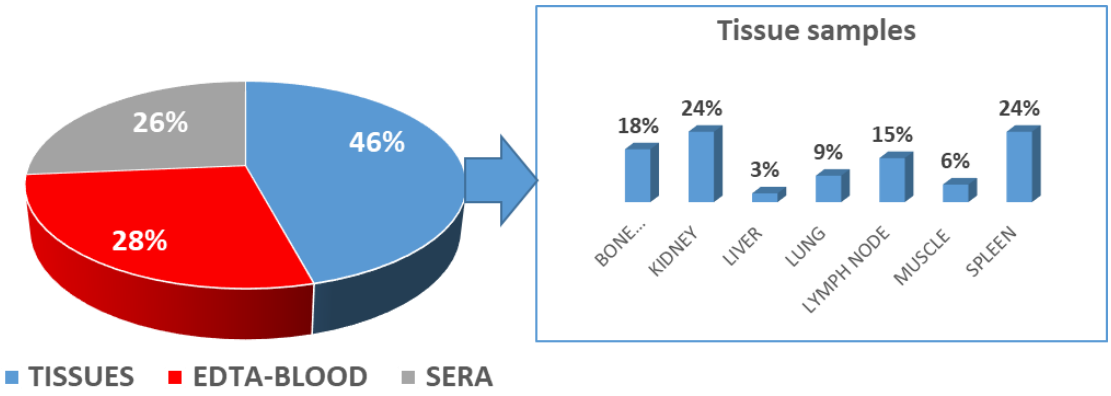
Seventy two field samples (72) collected from wild boar in the affected countries from Europe were tested to assure the diagnostic sensitivity of the INgene q PPA Sondas Detection real time PCR kit (**table 2**).

Table 2 → *Description of the 72 European wild boar (EWB) field samples tested with the INgene q PPA Sondas Detection real time PCR kit for validation purpose.*

COUNTRY	Nº ANIMALS	Nº SAMPLES
LATVIA	29	29
LITHUANIA	15	15
POLAND	26	26
ROMANIA	1	1
SERBIA	1	1
TOTAL	**72**	**72**

Specifically, in the case of wild boar 33 (46%) tissue samples, 20 (28%) blood and 19 (26%) sera were analyzed. The tissues tested mainly included spleen (*n* =8, 24%), kidney (*n* = 8, 24%), bone marrow (*n* = 6, 18%) followed by lymph nodes (n=5, 15%), lungs (*n* =3, 9%), 2 muscle (6%) and 1 liver (3%), **(figure 2).**

Figure 2→ *Description of the wild boar samples tested with the the INGENASA real time PCR kit for validation purpose.*



2.1.3. Negative SAMPLES

A panel of 94 negative blood samples were tested to determine the diagnostic specificity. Samples were taken for negative ASFV domestic pigs.

2.2. Evaluation of the INgene q PPA Sondas Detection to detect the ASFV genome on pooled blood samples.

To determine the most suitable combination ratio for EDTA blood samples to detect ASFV using INgene q PPA Sondas Detection, a panel of 16 experimental PCR-positive blood samples obtained from domestic pigs (table 3) were individually tested and in pools. Pooled blood samples were made by mixing equal volumes of the ASFV-positive sample with 2 (pool of 3), 4 (pool of 5), 9 (pool of 10) and 19 (pool of 20) ASF-negative samples. The negative samples were collected at day 0 from the domestic pigs included in the experimental infections. Samples were tested by the indirect immunoperoxidase test (IPT) [SOP/CISA/ASF/IPT/1] and the UPL real time PCR [SOP/CISA/ASF/PCR/3] to confirm the negative condition for ASF.

Table 3→ *pig experimental blood samples used from the pooling study.*

ASFV STRAIN	ORIGIN	VIRULENCE DESIGNATION	CLINICAL FORM	Nº BLOOD TESTED	Days post infection
Arm07	Armenia 2007	*vir*	acute	2	3-14
Est16/WB-Viru8	Estonia 2016	*mod*	subacute	3	13
Est16/WB-Viru8	Estonia 2016	*mod*	subacute	1	56-69
Est15/WB-Tartu 14	Estonia 2015	*mod*	acute	1	7
Est15/WB-Tartu 14	Estonia 2015	*mod*	chronic	1	14
Est15/WB-Valga6	Estonia 2015	*mod*	acute	1	10
Est15/WB-Valga6	Estonia 2015	*mod*	chronic	2	21-73
LV17/WB-RIE1	Latvia 2017	*att*	chronic	5	29-83

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

To avoid any bias in the pooling experiments and to guarantee representation of a series of samples ranging from strong to weak positives, the 19 experimental blood samples were selected on the basis of their cycle threshold (Ct) value previously obtained with the UPL real time PCR (Fernández *et* al., 2013) [SOP/CISA/ASF/PCR/3] as gold standard. Therefore, real time PCR results were divided into five classes (15<Ct≤20, 20≤Ct<25, 25≤Ct<30, 30≤Ct<35, 35≤Ct<40). Around 2 to 5 samples were selected per class (table 4).

Table 4→ *nº of pig experimental blood samples per Ct class.*

Ct class	Nº samples per class	Days post infection
15<Ct≤20	2	7-10
20≤Ct<25	2	4-7
25≤Ct<30	2	13-21
30≤Ct<35	5	14-62
35≤Ct<40	5	29-86

2.3. TEST PROCEDURES.

2.3.1. **DNA extraction procedure**; DNA was extracted from organ homogenates, EDTA-blood and 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]**.** The DNA was extracted from 200 µl of each tissue homogenate, blood, and swab samples using the High Pure PCR Template Preparation Kit. DNAs were stored <-10 until use.

2.3.2. **OIE-Real time PCR procedure (UPL method) developed by Fernandez *et* al., 2013 (OIE 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 **OIE-real time PCR (Taqman probe ASFV-VP72P1)** developed by Fernandez *et al*., 2013 and included in the OIE Manual as reference test (OIE 2021) [SOP/CISA/ASF/PCR/3].

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

Reference	Component	Description
EURL-E+ batch 34	**Positive control for the extraction (EURL)**	1:10.000 dilution of the ASFV reference strain E75 (genotype I) diluted in a negative sera.
NEC (E-)	**Negative control for the extraction**	Distilled water
EURL-R+ batch 30	**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 34	Detected in FAM channel	Ct value within the range of 32±4.
NEC (E-)	Non detected	Ct ≥40.
EURL-R+ batch 30	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, in order 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.

2.3.3. INgene q PPA Sondas Detection: 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 **INGENASA kit** according the protocol described by the manufacturers**.**

The following controls were included in the amplification steps:

Reference	Component	Description
EURL-R+ batch 30	**Positive control for the amplification (EURL)**	Ct value within the range of 32±4.
PAC-ASF A1	**Positive control for the amplification included in the kit**	Synthetic DNA provided, containing specific target of ASFV
NEC (E-)	**Negative control for the extraction**	Distilled water
NAC (R-)	**Negative control for the amplification**	Distilled water

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

CONTROLS	FAM	VIC
EURL-R+ batch 30	C_T 32±4	Not relevant
PAC-ASF A1	C_T 32±4*	Not relevant

NEC (E-)	No signal (Ct≥45)	Not relevant
NAC (R-)	No signal (Ct≥45)	Not relevant

**batch Nº Lot 250222: Ct =29.38*

Interpretation of the results:

A positive result during amplification implies a typical fluorescence curve with a Ct value <45. Normally, values are comprised within a range of 30 <Ct < 45

Cases	Channel FAM	Channel VIC	Results
A	+	+	Positive
B	+	-	Positive
C	-	+	Negative
D*	-	-	Null/inhibited

*Repeat the analysis diluting DNA 1/40 to avoid inhibitors, before considering the sample as negative.

2.4. DATA ANALYSIS.

The OIE real time PCR developed by Fernández-Pinero *et* al., 2013 was used as "reference method for diagnostic sensitivity calculation. The ***concordance between each test*** was the overall percentage agreement between the results of the two assays calculated using two-by-two contingency tables. Kappa Coefficient (κ) statistics were used to evaluate the significance of the level of concordance between results beyond that expected by chance, with κ values of 0.81–1.00 representing almost perfect agreement, values of 0.61–0.80 substantial agreement, values of 0.41–0.60 good agreement, values of 0.21–0.40 moderate agreement, values of 0.01–0.20 slight agreement, and values of 0.00 no agreement.

2.5. 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 seven 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 the **11 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 **11 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.

Of the 128 samples analyzed, 8 (6.25%) were considered invalid due to the negative Ct value obtained in the FAM and VIC channels. Following the manufacturer's instructions, the DNA samples were analyzed in a second series of PCR at a dilution of 1:40, six of them giving a validated result. In the two samples that were not validated, a new extraction was performed with similar results. These samples, both wild boar, were removed from the final validation, having a final number of samples tested of 126.

3.2. Diagnostic sensitivity and specificity.

One hundred and twenty six (126) field samples collected from domestic pigs (56 samples) and wild boar (70 samples) were selected to verify the performance of the test to detect the ASFV genome on real clinical samples. Samples were tested in parallel using the OIE real time PCR (UPL-PCR) as gold standard, and the INgene q PPA Sondas Detection real time PCR kit. By the reference method all samples gave a positive results while by the INGENASA kit, **two samples were negatives, resulting in a diagnostic sensitivity of 98.41%** [94.5%- 98.7% CI95%]

The “discrepant results” were obtained in two wild boar that presented weak Ct values (Ct>35) when tested with the UPL real-time PCR and were negative with the INGENASA kit. These samples tested positive for antibodies, confirming that animals were infected by the ASFV and survived to the infection.

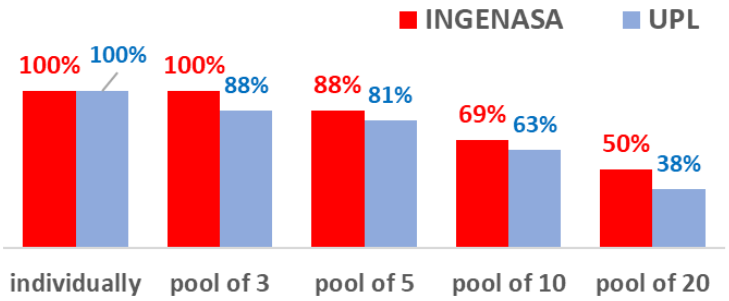
Out of the 94 negative bloods tested only one gave a false positive result with the INGENASA kit giving a diagnostic specificity of **98.9%** [94.2%- 99.8% CI95%]. The false positive sample was tested by duplicate giving the same result.

The (κ) value of 0.97 [95% CI] **indicates ALMOST PERFECT AGREEMENT among the UPL reference method and the INgene q PPA Sondas Detection real time PCR kit.**

3.3. Evaluation of the INgene q PPA Sondas Detection real time PCR kit to detect the ASFV genome in pooled EDTA-blood samples.

In agreement with the results obtained using the UPL real time PCR as gold standard, the **16 (100%) blood samples individually tested** gave a positive result using the **INgene q PPA Sondas Detection real time PCR kit** regardless the Ct range-value. When samples were tested with the INgene q PPA Sondas Detection real time PCR kit in 1:3 dilution (pool of 3) the number of positive was 16 (100%), decreasing to 14 (87.5%), 11 (68.7%) and 8 (50%) using the pool of 5, 10 and 20, respectively. The **UPL real time PCR** showed and slight less sensitivity when samples were tested in pools (figure 3).

Figure 3→ *Percentage of positive blood samples obtained with the **INgene q PPA Sondas Detection real time PCR kit** or with the **UPL real time PCR** when individually tested or in pools of 3, 5, 10 and 20.*



Therefore, it was almost a **perfect agreement** with κ values ranged from 0.81 to 1.00 among the results obtained when **samples were individually tested** and those obtained when testing in **pools of 3 and 5**. The κ value of 0.69 showed a substantial agreement when samples were tested in 1:10 and only good agreement (value of 0.5) when samples were tested in 1:20 dilutions (table 5).

Table 5→ *effecting of pooling blood samples using the INGENASA real time PCR.*

	INGENASA REAL TIME PCR		
	Nº	*%*	*κ value [CI 95%]*
Individual	16	100%	1 [1-00-1.00]
Pools 1/3	16	100%	1 [1-00-1.00]
Pools 1/5	14	87.5%	0.88 [0.74-0.95]
Pools 1/10	11	68.7%	0.69 [0.60-0.91]
Pools 1/20	8	50%	0.5 [0.48-0.82]

Dividing the data set into the five Ct-classes, there was perfect agreement (κ index =1 [95% CI]) in samples with Ct values lower than 30 using both real time PCRs (table $6_{a, b}$). However the percentage of agreement was reduced when samples with Ct values above 30 were tested. The largest differences were observed in the fifth Ct-class (35≤Ct<40) which resulted in a sensitivity of 80% (INGENASA) and 60% (UPL) when pools of 3 samples were analysed. The number of false negative results gradually increased resulting in a sensitivity less than 70% in samples pooled in 1/5, 1/10 and 1/20 (table 6).

Table 6→ *Correlation among the number and % of positive experimental samples with regards to the Ct ranges using the INGENASA (a) or the UPL (b) real time PCRs*

6A) INGENASA REAL TIME PCR RESULTS						
Ct class	Days post infection	Nº samples per class	Number (%) of positive samples in each category of pools			
			Pools 1/3	*Pools 1/5*	*Pools 1/10*	*Pools 1/20*
15<Ct≤20	7-10	2	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
20≤Ct<25	4-7	2	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
25≤Ct<30	13-21	2	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
30≤Ct<35	14-62	5	5/5 (100%)	5/5 (100%)	**3/5 (60%)**	**2/5 (40%)**
35≤Ct<40	29-86	5	**4/5 (80%)**	**3/5 (60%)**	**1/5 (20%)**	**0/5 (0%)**
6B) UPL REAL TIME PCR RESULTS						
			Pools 1/3	**Pools 1/5**	**Pools 1/10**	**Pools 1/20**
15<Ct≤20	7-10	2	2/2 (100%)	2/2 (100%)	2/2 (100%)	4/4 (100%)
20≤Ct<25	4-7	2	2/2 (100%)	2/2 (100%)	2/2 (100%)	5/5 (100%)
25≤Ct<30	13-21	2	2/2 (100%)	2/2 (100%)	2/2 (100%)	6/6 (100%)
30≤Ct<35	14-62	5	5/5 (100%)	5/5 (100%)	**2/5 (40%)**	**0/5 (0%)**
35≤Ct<40	29-86	5	**3/5 (60%)**	**2/5 (40%)**	**2/5 (40%)**	**0/5 (0%)**

It is important to point out that the domestic pigs which exhibited a Ct-value above 30 gave a positive result on antibody detection using the IPT test, and were collected at late times after the infection, mainly from animals which developed subclinical or chronic forms of the disease.

3.4. INTER-ASSAY REPRODUCIBILITY

The ***interassay reproducibility*** was initially assessed on the ASF positive control included in the kit (PAC-ASF-A1) and on the EURL-ASF reference positive control. The controls were run in 7 different PCRs, respectively, to monitor assay-to-assay variation. **The inter-assay CV for the INGENASA positive control (PAC-ASF A1) was 0.96% and for the EURL control 1.49% considered appropriate in routine testing** (table 7).

Table 7 → *Coefficient of Variation (CV) per positive controls between FOUR different PCR runs.*

	PCR1	PCR2	PCR3	PCR 4	PCR 5	PCR 6	PCR7	MEAN	SD	%CV
PAC-ASFA1	30.95	31.44	31.08	30.55	31.16	30.67	30.99	30.97	0.29	**0.96%**
EURL-PC	29.15	29.59	28.68	28.58	28.79	28.78	29.62	29.02	0.43	**1.49%**

CV = coefficient of variability; SD = standard deviation

In addition **11 field samples randomly selected** 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 8** shows the individual CVs per each sample include in the analysis.

Table 8→ *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	40.0	NEGATIVE	37.1	POSITIVE	38.550	2.051	5.3%
2	KIDNEY	30.0	POSITIVE	29.7	POSITIVE	29.865	0.226	0.8%
3	SERUM	28.4	POSITIVE	28.6	POSITIVE	28.480	0.163	0.6%
4	SPLEEN	33.8	POSITIVE	34.8	POSITIVE	34.308	0.682	2.0%
5	SPLEEN	32.3	POSITIVE	32.4	POSITIVE	32.363	0.039	0.1%
6	SERUM	37.3	POSITIVE	34.3	POSITIVE	35.835	2.135	6.0%
7	BONE MARROW	34.8	POSITIVE	35.0	POSITIVE	34.925	0.163	0.5%
8	SPLEEN	31.4	POSITIVE	31.7	POSITIVE	31.560	0.240	0.8%
9	KIDNEY	35.0	POSITIVE	35.0	POSITIVE	35.003	0.060	0.2%
10	KIDNEY	32.5	POSITIVE	32.9	POSITIVE	32.680	0.269	0.8%
11	KIDNEY	34.1	POSITIVE	33.9	POSITIVE	34.005	0.092	0.3%
AVERAGE %CV								1.6%

CV = coefficient of variability; SD = standard deviation . Eq = Equipment. In blue are showed samples with discrepant results.

The average of the individual CVs reported as the inter assay CV was 1.6% .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 90.9%.** One sample (marked in blue in table 8) initially classified as positive in the first PCR run, resulted negative in the second PCR run.

3.5. INTRA-ASSAY REPRODUCIBILITY

The ***intra-assay reproducibility*** was assessed with CVs obtained testing the same 11 samples used for the inter assay study. The results obtained in each of the samples are showed in **table 9.**

Table 9→ *Coefficient of Variation (CV) in the field samples tested by duplicate in the same PCR run*

ID SAMPLE	PCR RUN (Eq1)					PCR RUN (Eq2)				
	C_T1	C_T2	MEAN	SD	%CV	C_T1	C_T2	MEAN	SD	%CV
1	40	40	40.00	0.000	0.0%	34.2	40	38.55	2.051	5.3%
2	30.45	29.6	29.81	0.301	1.0%	29.75	29.66	29.68	0.032	0.1%
3	28.44	28.29	28.32	0.053	0.2%	27.91	29.28	28.93	0.484	1.7%
4	33.69	33.96	33.89	0.095	0.3%	34.31	35.27	35.03	0.339	1.0%
5	32.17	32.5	32.41	0.117	0.4%	32.27	32.51	32.45	0.085	0.3%
6	36.65	38.04	37.69	0.491	1.3%	34.41	34.24	34.28	0.060	0.2%
7	35.13	34.49	34.65	0.226	0.7%	35.29	34.79	34.91	0.177	0.5%
8	31.14	31.64	31.51	0.177	0.6%	31.52	31.94	31.83	0.148	0.5%
9	35.09	34.83	34.89	0.092	0.3%	35.49	34.6	34.82	0.315	0.9%
10	32.52	32.46	32.47	0.021	0.1%	32.81	32.93	32.90	0.042	0.1%
11	33.6	34.54	34.30	0.332	1.0%	34	33.88	33.91	0.042	0.1%
AVERAGE %CV					0.5%					1.0%

CV = coefficient of variability; SD = standard deviation

The percentage of **agreement among the results obtained was 90.9 %,** with the same discrepant result that in the inter assay (marked in blue in table 9)

The average of the individual CVs reported as the **intra-assay CV was 0.5% and 1.0% exhibited high reproducibility in the results obtained.**

4. CONCLUSIONS.

- From the analysis of **126 POSITIVE field samples obtained from epidemic areas in Europe,** the **diagnostic sensitivity of the INgene q PPA Sondas Detection real time PCR kit was of 98.41%** **[94.5%- 98.7% CI95%]** with only two discrepant results when comparing with the reference method, the OIE- UPL real time PCR.

- From the analysis of **94 blood samples,** the **diagnostic specificity of the INgene q PPA Sondas Detection real time PCR kit was of 98.9%** [94.2%- 99.8% CI95%]. with only one false positive sample.

- **The (κ) value of 0.97%** [95% CI] **indicates PERFECT AGREEMENT among the UPL reference method and the INgene q PPA Sondas Detection real time PCR kit .**

- The **INgene q PPA Sondas Detection real time PCR kit has adequate analytical sensitivity** to provide a reliable diagnosis of ASF for testing individual and pooled samples in **pool sizes of 3 and 5**. **Pools of 10 and 20 resulted in decreased in sensitivity**, mainly in the analysis of samples with low ASFV genome load (Ct>30), obtained in animals with subclinical or chronic infections.

- The **inter-assay variability value** (less than 3%) **exhibited high repeatability in the results obtained and is considered appropriated** in routine testing

- The **intra-assay variability value** (less than 3%) **exhibited high reproducibility in the results obtained and is considered appropriated** in routine testing.

The INgene q PPA Sondas Detection real time PCR kit developed by INGENASA (Eurofins) test has appropriate diagnostic sensitivity and repeatability for performing a confident diagnosis of ASF in samples extracted using the High Pure DNA extraction kit from ROCHE. The commercial kit showed perfect agreement with the OIE reference real time PCR method (UPL – real time)

Report performed in Valdeolmos (Madrid) at 23th May 2022

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