Siedlce, dnia 4 listopada 2019 r.

INSPEKCJA WETERYNARYJNA
MAZOWIECKI WOJEWÓDZKI
LEKARZ WETERYNARII
Paweł Jakubczak

WYKONAWCY

- wszyscy -

Nasz znak: WIW-AD.272.78.2019 – Pakiet 2
Dot. sprawy nr: pismo z dnia:

Wyjaśnienie treści specyfikacji istotnych warunków zamówienia

Działając na podstawie art. 38 ust. 2 ustawy z dnia 29 stycznia 2004 r. Prawo zamówień publicznych (Dz. U. z 2018 r., poz. 1986 z późniejszymi zmianami) Wojewódzki Inspektorat Weterynarii z siedzibą w Siedlcach przesyła odpowiedzi na zadane pytania Wykonawcy dotyczące treści specyfikacji istotnych warunków zamówienia w postępowaniu o udzielenie zamówienia publicznego nr sprawy **WIW-AD.272.78.2019** na **dostawę zestawów diagnostycznych do wykrywania wirusa ASF dla Zakładu Higieny Weterynaryjnej w Warszawie** – Pakiet 2: Dostawa zestawów diagnostycznych do wykrywania DNA wirusa afrykańskiego pomoru świń (ASF) u świń i dzików:

Pytanie do pakietu 2:

Pytanie nr 1: Czy Zamawiający dopuści zestaw diagnostyczny do wykrywania i izolacji DNA wirusa afrykańskiego pomoru świń (ASFv) u świń i dzików posiadający kontrolę egzogenną? Oferowany test z kontrolą egzogenną posiada walidację EURL-ASF (European Union Reference Laboratory for ASF) i jest umieszczony w aktualnym „Wykazie testów do diagnostyki in vitro", prowadzonym przez Głównego Lekarza Weterynarii oraz został zgłoszony do rejestracji przez Światową Organizacji ds. Zdrowia Zwierząt (OIE) w kierunku badań nad ASF – dokumenty w załączniku.

Odpowiedź nr 1: Zamawiający nie dopuszcza zestawów diagnostycznych do wykrywania i izolacji DNA wirusa afrykańskiego pomoru świń (ASFv) u świń i

dzików posiadający kontrolę egzogenną. Wymagania zostały określone w SIWZ w sposób jednoznaczny.

Pytania do umowy:

Pytanie nr 2: Czy Zamawiający wyrazi zgodę na naliczanie kar od wartości NETTO niezrealizowanej dostawy?

Odpowiedź nr 2: Zamawiający nie wyraża zgody.

Pytanie nr 3: Czy Zamawiający wyrazi zgodę na zmniejszenie procenta naliczanej kary do max. 5% wartości NETTO niezrealizowanej umowy.

Odpowiedź nr 3: Zamawiający nie wyraża zgody.

Pytanie nr 4: W przypadku braku zgody na powyższe prosimy o okazanie kalkulacji przyszłej, hipotetycznej szkody, jaką ma ponieść zamawiający w związku z niewykonaniem lub nienależytym wykonaniem umowy - zgodnie z przepisami.

Odpowiedź nr 4: Zamawiający nie jest w stanie przedłożyć kalkulacji.

Pytanie nr 5: Czy Zamawiający wyrazi zgodę na zwiększenie cen jednostkowych brutto, a co za tym idzie wartości brutto umowy, w przypadku ustawowej zmiany stawki VAT?

Odpowiedź nr 5: Zamawiający dopuszcza możliwość zmiany umowy w zakresie zmiany obowiązującej stawki podatku VAT w przypadku ustawowej zmiany stawki podatku VAT.

Pytanie nr 6: Czy Zamawiający dopuści zmianę stawki VAT dla produktu w przypadku uzasadnionej przez producenta zmiany klasyfikacji wyrobu i możliwości zastosowania uprzywilejowanej stawki VAT, zgodnie z zapisami Ustawy o VAT?

Odpowiedź nr 6: Zamawiający dopuszcza możliwość zmiany umowy w zakresie zmiany obowiązującej stawki podatku VAT w przypadku ustawowej zmiany stawki podatku VAT.

Pytanie nr 7: Czy Zamawiający dopuści zmianę stawki VAT w przypadku uzasadnionej przez producenta zmiany klasyfikacji wyrobu i braku możliwości dalszego stosowania uprzywilejowanej stawki VAT, zgodnie z zapisami Ustawy o VAT, z jednoczesnym podwyższeniem ceny jednostkowej brutto?

Odpowiedź nr 7: Zamawiający dopuszcza możliwość zmiany umowy w zakresie zmiany obowiązującej stawki podatku VAT w przypadku ustawowej zmiany stawki podatku VAT.

Pytanie nr 8: Czy Zamawiający wyrazi zgodę na dodanie w projekcie umowy zapisu, że zmiany umowy mogą nastąpić również w przypadku, gdy dotyczą poprawienia błędów i oczywistych omyłek słownych, literowych, liczbowych, numeracji jednostek redakcyjnych lub uzupełnień treści nie powodujących zmiany celu i istoty umowy?

Odpowiedź nr 8: Zamawiający nie wyraża zgody.

Pytanie nr 9: Czy Zamawiający dopuści aneksowanie ze względu na zamianę oferowanego produktu na produkt równoważny w przypadku zmiany produktu lub producenta sprzętu?

Odpowiedź nr 9: Zamawiający nie wyraża zgody.

Pytanie nr 10: Czy Zamawiający dopuści możliwość zaoferowania zamiennika o parametrach nie gorszych od proponowanego w umowie po powiadomieniu Zamawiającego w wypadku wystąpienia przejściowego produktu?

Odpowiedź nr 10: Zamawiający nie wyraża zgody.

Pytanie nr 11: Czy Zamawiający wprowadzi możliwość wstrzymania dostaw w przypadku nierealizowania płatności?

Odpowiedź nr 11: Zamawiający będzie dokonywał płatności zgodnie z zapisami we wzorze umowy stanowiący Załącznik nr 4 do SIWZ.

Pytanie nr 12: Czy Zamawiający dopuści, po każdorazowej konsultacji z Zamawiającym w razie zaistnienia niniejszej sytuacji, możliwość zaoferowania zamiennika produktu w trakcie realizacji umowy, o innej nazwie, kodzie i/lub sposobie opakowania produktu oraz zbliżonych parametrach jakościowych w stosunku do produktu zaoferowanego w danej pozycji oferty w sytuacji, gdy z przyczyn niezależnych od Wykonawcy, jest on niedostępny u producenta? W przypadku innego sposobu pakowania (konfekcji), cena za opakowanie zbiorcze oferowanego zamiennika zostałaby przeliczona w ten sposób, że cena za sztukę lub oznaczenie zamiennika byłaby równa cenie za sztukę lub oznaczenie produktu znajdującego się danej pozycji umowy. Uzasadnienie: Wprowadzenie niniejszego zapisu pozwoli zarówno na zabezpieczenie ciągłości procesu diagnostycznego i uchroni, zarówno Zamawiającego oraz Wykonawcę przed nieoczekiwanymi oraz niezależnymi od nich skutkami wypadków losowych, do których mogą należeć: czasowa awaria linii produkcyjnej u producenta, czasowe wycofanie produktu przez

producenta brak dostępności surowców, niekorzystne zmiany makroekonomiczne czy wpływ klęsk żywiołowych.

Odpowiedź nr 12: Zamawiający nie wyraża zgodny.

Pytanie nr 13: W przypadku konieczności dostarczenia dokumentów w postaci papierowej, czy Zamawiający wyrazi zgodę na przesłanie ich w postaci zbindowanych tomów, gdzie poświadczenie za zgodność będzie widniało tylko na pierwszej stronie tomu dla jego pozostałych stron?

Odpowiedź nr 13: Zamawiający nie dopuszcza możliwości dostarczenia dokumentów w postaci papierowej jako zbindowanych tomów, gdzie poświadczenie za zgodność będzie widniało tylko na pierwszej stronie tomu.

Pytanie nr 14: Czy Zamawiający dopuści możliwość załączenia dokumentacji przetargowej w postaci plików nagranych na płycie CD wraz z oświadczeniem potwierdzającym zgodność kopii na płycie z elektronicznymi pierwowzorami?

Odpowiedź nr 14: Zamawiający nie wyraża zgody.

Pytanie nr 15: Czy Zamawiający po podpisaniu umowy, w trakcie jej realizacji dopuści możliwość dostarczania dokumentacji produktowej do dostaw (specyfikacje produktów) w formie elektronicznej?

Odpowiedź nr 15: Zamawiający dopuszcza każdą formę przekazania informacji z zastrzeżeniem, iż doręczenie musi być skuteczne.

MAZOWIECKI WOJEWÓDZKI LEKARZ WETERYNARII
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MINISTERIO DE
CIENCIA, INNOVACIÓN
Y UNIVERSIDADES

INIA

VALIDATION of the ASF diagnostic kit

LSI VetMAX™ AFRICAN SWINE FEVER VIRUS DETECTION KIT

Taqman Real time PCR detection of African swine fever virus developed by Life Technologies

VALIDATION REPORT

PERFORMED BY THE

Centro de Investigación en Sanidad Animal (CISA-INIA)

European Union reference laboratory for ASF (EURL-ASF)

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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 evaluate **the diagnostic sensitivity of the LSI VetMAX™ African swine fever virus detection (ASFV) KIT, a Taqman Real time PCR for the detection of the ASFV genome IN FIELD SAMPLES.** The assay is a single-well real time PCR in which ASFV and exogenous internal positive control (IPC) targets are amplified and detected using fluorescent TaqMan® probes. The kits includes:

- 3 mix ASFV: master mix for optimized duplex real time PCR amplification of ASFV and IPC targets. It contains primers, Taqman® probes, PCR buffer and enzyme.
- 4a-EPC ASFV: nucleic acid template for p72 target amplification. It serves as an external positive control for real time PCR reaction, and it is used to set the cycle threshold (CT) for evaluating the results.
- 5-IPC ASFV: internal positive control added to each sample and control at the lysis step of the DNA extraction procedure. It serves as a control for the DNA purification process, and it is used to monitor for the presence of PCR inhibitors.

2. PROCEDURE

2.1. Samples included in the validation study.

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

A panel of **90 field samples** collected from 90 domestic pigs in the outbreaks occurred since 2014 in the Eastern European countries were used in this study (**table 1**). All animals were previously classified as positives combining both virus and antibody detection tests at the European Union reference laboratory for ASF (EURL).

Table 1 → *Description of the 90 domestic pig field samples tested for LSI Vet MAX real time PCR kit validation purpose.*

COUNTRY	Nº SAMPLES TESTED
ESTONIA	8
HUNGARY	1
LATVIA	5
LITHUANIA	1
MOLDOVA	10
POLAND	64
ROMANIA	1
TOTAL	**90**

The samples tested included 42 tissues, 1 blood and 5 serum samples. The tissues tested comprised 42 spleen (50%), 29 kidney (35%), 7 lymph nodes (8%), 3 tonsils (4%), 2 lung (2%), and 1 ear (1%).

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

In this study were included **334 field samples** collected from 228 wild boar in the affected countries from Eastern European (**table 2**). Combining both virus and antibody detection tests all samples were classified as positives at the European Union reference laboratory for ASF (EURL).

Table 2 → *Description of the 334 European wild boar (EWB) field samples tested for LSI Vet MAX real time PCR kit validation purpose.*

COUNTRY	Nº SAMPLES TESTED
ESTONIA	24
LATVIA	66
LITHUANIA	152
POLAND	81
CZECH REPUBLIC	11
TOTAL	**334**

Specifically, in the case of wild boar 231 tissues samples, 58 blood and 45 sera were analyzed. The tissues tested mainly included bone marrow (*n* = 101, 44%) followed by spleen (*n* = 65, 28%), kidney (*n* = 29, 13%), lymph nodes (*n* = 11, 5%), muscles (*n* = 10, 4%), tonsils (*n* = 8, 3%), skin (*n* = 3, 1%), 2 liver (1%), 1 lung (0.4%), and 1 tissue without identification.

2.2. TEST PROCEDURES.

2.2.1. Universal probe library (UPL) real time PCR (Fernandez *et* al., 2013): a 10% (w/v) clarified homogenized tissue were prepared in phosphate-buffered saline [PNT/CISA/PPA/MUESTRAS/1]. The DNA was extracted from 200 µl of each tissue homogenate, and blood/serum samples using the High Pure PCR Template Preparation Kit [Ref. 11796828001 (ROCHE)] according the EURL standard operating procedure (SOP) [http://asf-referencelab.info/asf/images/files/PROTOCOLOS-EN/SOP-ASF-DNA-EXTRACTION-1.pdf]. 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 **UPL real time PCR** developed by Fernandez *et al.*, 2013 [PNT/CISA/PPA/PCR/3].

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

Reference	Component	Description
EURL-PEC	**Target positive control EURL-ASF**	1:10.000 dilution of the ASFV reference strain E75 (genotype I) diluted in a virus negative sera.
NEC	**Negative control for the extraction**	Distilled water
EURL-PAC	**Target positive control EURL-ASF**	ASFV positive DNA extracted for the EURL-PEC
NAC	**Negative control for the amplification**	Distilled water

2.2.2. LSI VetMAX™ ASFV real time PCR kit: a 10% (w/v) clarified homogenized tissue were prepared in phosphate-buffered saline [PNT/CISA/PPA/MUESTRAS/1]. The DNA was extracted from 200 µl of each tissue homogenate or blood/serum samples using the High Pure PCR Template Preparation Kit [Ref. 11796828001 (ROCHE)] according the EURL standard operating procedure (SOP) [http://asf-referencelab.info/asf/images/files/PROTOCOLOS-EN/SOP-ASF-DNA-EXTRACTION-1.pdf]. Following the LSI manufactures indications, **5 µl of the internal positive control (IPC ASFV) was added to each sample and controls at the lysis step of the DNA extraction procedure**. 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 **LSI-VetMAX™® ASFV real time PCR kit** according the protocol described by the manufacturers

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

Reference	Component	Description
EURL-PEC	**Target positive control EURL-ASF**	1:10.000 dilution of the ASFV reference strain E75 (genotype I) diluted in a virus negative sera.
NEC	**Negative control for the extraction**	Distilled water
EPC ASFV	**Target positive control LSI**	Nucleic acid template for p72 target amplification. It serves as an external positive control for real time PCR reaction, and it is used to set the cycle threshold (CT) for evaluating the results.
NAC	**Negative control for the amplification**	Distilled water

2.3. DATA ANALYSIS

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.

2.4. INTER and INTRA-ASSAY REPRODUCIBILITY

The ***inter-assay reproducibility*** was estimated **on the ASF positive control included in the kit EPC ASFV** and on the **EURL-ASF reference positive control (EURL-PEC)**. The controls were run in seven and nine 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 **60 field samples with different Ct values** were tested in two different PCR runs. 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 **60 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.**

2.5. Validation criteria and interpretation of the results.

2.5.1. UPL-real time PCR (Fernández-Pinero *et* al., 2013).

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

Control	Expected result	Acceptability criteria
EURL-PEC	Detected in FAM channel	Ct value within the range of 32±4.
NEC	Non detected	Ct ≥40.
EURL-PAC	Detected in FAM channel	Ct value within the range of 32±4.
NAC	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.

2.5.2. LSI VetMAXT^{M} ASFV real time PCR kit:

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

Control reactions	ASFV target (FAM dye)	IPC target (VIC/HEX dye)	Interpretation
Positive control $_{EPC\ ASFV}$	$C_T = 26 \pm 3C_T$ (1)	$C_T < 45$ or $C_T > 45$ (2)	PCR validated
Extraction control $_{NEC}$	$C_T > 45$	$C_T = 30 \pm 3C_T$ (3)	Extraction validated
No-template control $_{NAC}$	$C_T > 45$	$C_T > 45$	PCR reagents validated

(1) Referred to the value indicated in the certificate analysis Batch Lot = ASFV-011

(2) The IPC value of the positive control in not used for test validation

(3) Referred to the value indicated in the certificate analysis Batch Lot = ASFV-011

Interpretation of the results:

ASFV target (FAM dye)	IPC target (VIC/HEX dye)	Interpretation
$C_T < 45$	$C_T < 45$ or $C_T > 45$	ASFV detected
$C_T > 45$	$C_T = C_T$ NEC $\pm 3C_T$	ASFV not detected
$C_T > 45$	C_T is outside this range: C_T NEC $\pm 3C_T$	Not validated

According the manufactures' an invalid sample can be an indication of failed sample addition, extraction and/or PCR. In this case:

- Dilute the DNA of non-validated samples 1:10 in 1x TE buffer (or distilled water).
- Perform real time PCR analysis with 5 µl of this dilution
- The result is validated if:
  - The diluted DNA is positive for ASFV regardless the IPC result
  - The diluted DNA is negative for ASFV with a compliant IPC result
- The result is still not validated if the diluted DNA is negative for ASFV with a non-compliant IPC result. In this case repeat the analysis on a new sample (new extraction).

3. RESULTS

3.1. Validation criteria and interpretation of the results.

The **90 domestic pig samples and the 334 European wild boar samples** obtained from Eastern European affected countries were analyzed in parallel with the UPL real time PCR (Fernández-Pinero *et* al., 2013) and with the commercial LSI VetMAX™ ASFV real time PCR kit. **Each test run was validated according the validation criteria included in the manufacturer's instructions as is described in the section 2.5.**

3.2. Diagnostic sensitivity.

3.2.1. Domestic pig samples from field genotype II ASFV-infected areas within the Eastern European countries

A total of 90 field samples obtained from the domestic pig outbreaks occurred in the Eastern European countries were selected for the validation study. All samples were classified as positives when tested with the UPL-real time PCR. By the LSI VetMAX™ one sample gave a false negative result resulting in the detection of the 99% of the domestic pigs tested. Comparative values are shown in **table 4**.

Table 4→ *comparativ number of positive samples collected from domestic pigs in the Eastern European countries detected by each of the two PCRs assayed.*

COUNTRY	Nº SAMPLES	UPL-real time PCR		LSI VetMAX™	
		Nº POS	% POS	Nº POS	% POS
ESTONIA	8	8	100%	8	100%
HUNGARY	1	1	100%	1	100%
LATVIA	5	5	100%	4	80%
LITHUANIA	1	1	100%	1	100%
MOLDOVA	10	10	100%	10	100%
POLAND	64	64	100%	64	100%
ROMANIA	1	1	100%	1	100%
TOTAL	**90**	**90**	**100%**	**89**	**99%**

The "discrepant sample" was obtained from one animal which presented a weak Ct value (Ct = 35.37) when tested with the UPL real time PCR and resulting negative by antibody detection indicating an early stage of the ASF infection.

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

A second panel of 334 field samples collected from European wild boar in affected areas in Europe where ASFV genotype II is circulating, were included in this study. The LSI VetMAX™ real time PCR kit **was able to detect 89% of the infected or exposed animals against the 93% detected with the UPL-real time PCR** (**table 5**).

Table 5→ *comparativ number of positive samples collected from European wild boar in Eastern European countries detected by each of the two PCRs assayed.*

COUNTRY	Nº SAMPLES	UPL-real time PCR		LSI VetMAX™	
		Nº POS	*% POS*	*Nº POS*	*% POS*
ESTONIA	24	23	96%	21	88%
LATVIA	66	60	91%	60	91%
LITHUANIA	152	139	91%	135	89%
POLAND	81	77	95%	71	88%
CZECH REPUBLIC	11	11	100%	11	100%
TOTAL	334	310	93%	298	89%

Among the **UPL real time PCR and the LSI VetMAX™ PCR kit** there was **perfect agreement [κ index =1 [95% CI])] in samples with Ct values lower than 30**. Discrepant results were obtained in 32 samples with Ct values ≥30. Twenty two samples that were UPL-PCR positive (Ct values >30) were missed with the LSI VetMAX™ PCR kit, whereas 10 samples which gave a positive result by LSI VetMAX™ resulted negatives when tested using the UPL-real time PCR. **In 20 out of the 32** "discrepant samples" was obtained a positive antibody result which indicates that samples were collected from animals with late infection (**table 6**).

Table 6→ *results obtained using the UPL real time -PCR versus LSI VetMAX™ REAL time PCR in "discrepant" samples.*

SAMPLE	UPL-real time PCR					LSI VetMAX™ REAL time PCR			Antibody RESULT
	Ct1	Ct2	Ct3	AVERAGE	RESULT	FAM	HEX	RESULT	
BONE MARROW	37.12	37.42	35.96	36.83	WEAK[1]	No ct	25.74	FN	NEGATIVE
BLOOD-EDTA	32.63			32.63	POSITIVE	No ct	32.28	FN	POSITIVE
KIDNEY	38.4	37.94	38.99	38.44	WEAK	No ct	25.88	FN	POSITIVE
BLOOD-EDTA	39.16	No ct	39.41	39.28	WEAK	No ct	26.98	FN	NEGATIVE
SERUM	38.69	38.4	37.72	38.27	WEAK	No ct	25.75	FN	POSITIVE
SERUM	38.23	38.28	No ct	38.83	WEAK	No ct	25.72	FN	POSITIVE
SERUM	36.89	35.58	38.3	36.92	WEAK	No ct	26.84	FN	POSITIVE
BONE MARROW	34.68			34.68	POSITIVE	No ct	26.34	FN	NEGATIVE
SKIN	36.49	36.39	35.48	36.12	WEAK	No ct	26.33	FN	NEGATIVE
BLOOD-EDTA	37.37	39.36	39.61	38.78	WEAK	No ct	33.56	FN	NEGATIVE
BLOOD-EDTA	36.59	36.98	35.42	36.33	WEAK	No ct	28.03	FN	NEGATIVE
SPLEEN	37.78	No ct	37.15	38.31	WEAK	No ct	27	FN	WEAK[2]
SERUM	39.94	39.09	No ct	39.67	WEAK	No ct	27.68	FN	NEGATIVE
SERUM	39.05	38.81	39.11	38.99	WEAK	No ct	28.32	FN	POSITIVE
BONE MARROW	34.96				POSITIVE	No ct	27.98	FN	NEGATIVE
BONE MARROW	38.37	38.56	37.44	38.12	WEAK	No ct	27.11	FN	NEGATIVE
BONE MARROW	36.51	37.09	No ct	37.86	WEAK	No ct	28.25	FN	WEAK[2]
SERUM	32.93				POSITIVE	No ct	27.79	FN	POSITIVE
SERUM	38.99	36.98	36.88	37.61	WEAK	No ct	28.71	FN	POSITIVE
BONE	34.43				POSITIVE	No ct	32.23	FN	WEAK[2]
BONE	37.91	36.98	37.84	37.57	WEAK	No ct	29.17	FN	NEGATIVE
MUSCLE	35.81	36.28	37.55	36.54	WEAK	No ct	31.11	FN	NEGATIVE

BLOOD-EDTA	38.97	No ct	No ct	No ct	FN	38.93	27.35	POSITIVE	NEGATIVE
BLOOD-EDTA	No ct				FN	39.57	29.12	POSITIVE	POSITIVE
BLOOD-EDTA	No ct				FN	39.19	28.25	POSITIVE	POSITIVE
TONSIL	No ct				FN	36.47	27.44	POSITIVE	POSITIVE
BLOOD-EDTA	39.03	No ct	No ct	No ct	FN	36.23	26.74	POSITIVE	POSITIVE
BLOOD-EDTA	38.63	No ct	No ct	No ct	FN	38.07	29.26	POSITIVE	POSITIVE
SPLEEN	39.79	No ct	No ct	No ct	FN	38.92	29.06	POSITIVE	POSITIVE
KIDNEY	36.93	No ct	No ct	No ct	FN	34.25	28.6	POSITIVE	POSITIVE
KIDNEY	39.08	No ct	No ct	No ct	FN	34.35	28.42	POSITIVE	POSITIVE
tonsil	39.37	No ct	No ct	No ct	FN	34.12	28.06	POSITIVE	POSITIVE

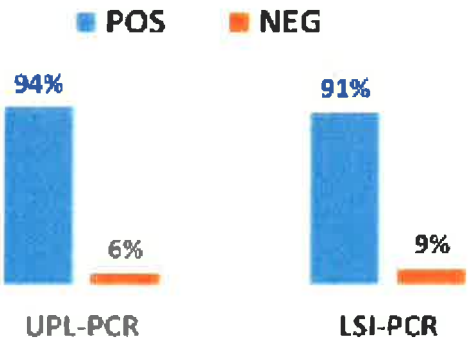
** ASFV antibody result determined using the IPT, as described by EURL (2014).*

1 = CT value≥35; FN = false negative

3.2.3. Overall analysis of the results.

The results obtained in the analysis of the domestic pig and the European wild boar field samples obtained from ASFV genotype II infected animals in the Eastern European countries have been combining to provide an overall approach about the performance of the LSI VetMAX™ real time PCR kit for diagnosing ASF in field conditions. Out of the **424 samples the number of positives** using the UPL-PCR was 400 (94%) and 387 **(91%) using the LSI VetMAX™ real-time PCR** (**figure 1**).

Figure 1 → *Percentage of **positive** and **negative** samples obtained with the two PCRs after the analysis of the **424 field samples tested**.*



Although the UPL-PCR test was able to detect the highest percentage of the infected or exposed animals. the (κ) value of 0.87 % [95% CI] **indicates PERFECT AGREEMENT among the UPL reference method and the LSI VetMAX™ real time PCR kit.**

3.3. INTER-ASSAY REPRODUCIBILITY

The ***interassay reproducibility*** was assessed on the LSI ASF positive control included in the kit (EPC-ASFV) and on the EURL-ASF reference positive control (EURL-PEC). The controls were run in 7 and 9 different PCRs, respectively, to monitor assay-to-assay variation. **The inter-assay CV for**

the LSI VetMAX™ positive control was 2% and for the EURL control was 4% considered appropriate in routine testing (table 7).

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

	PCR1	PCR2	PCR3	PCR 4	PCR 5	PCR 6	PCR 7	PCR 8	PCR 9	MEAN	SD	CV	
EPC-ASFV LSI	28.29	28.19	28	27.27	28.61	28.17	27.22	28.78	27.99	28.11	0.46	0.02	2%
EURL -PEC	28.0	27.3	28.0	27.5	26.9	29.4	30.0	*nt*	*nt*	28.14	1.13	0.04	4%

CV = coefficient of variability; SD = standard deviation; nt = no tested

In addition **60 field samples with different Ct values** (included negative 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 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	PCR 1		PCR 2		MEAN	SD	%CV
	C_T1	RESULT	C_T2	RESULT			
1	22.63	POSITIVE	22.8	POSITIVE	22.7	0.1	0%
2	22.68	POSITIVE	22.5	POSITIVE	22.6	0.2	1%
3	24.8	POSITIVE	24.5	POSITIVE	24.6	0.2	1%
4	26.11	POSITIVE	25.6	POSITIVE	25.8	0.4	2%
5	26.79	POSITIVE	28.4	POSITIVE	27.6	1.1	4%
6	27.77	POSITIVE	27.5	POSITIVE	27.6	0.2	1%
7	27.79	POSITIVE	27.3	POSITIVE	27.5	0.4	1%
8	27.94	POSITIVE	27.7	POSITIVE	27.8	0.2	1%
9	28.08	POSITIVE	28.1	POSITIVE	28.1	0.0	0%
10	28.63	POSITIVE	28.3	POSITIVE	28.5	0.2	1%
11	28.95	POSITIVE	29.3	POSITIVE	29.1	0.2	1%
12	29.36	POSITIVE	28.4	POSITIVE	28.9	0.7	2%
13	29.39	POSITIVE	29.1	POSITIVE	29.2	0.2	1%
14	29.74	POSITIVE	29.6	POSITIVE	29.7	0.1	0%
15	29.78	POSITIVE	29.8	POSITIVE	29.8	0.0	0%
16	29.87	POSITIVE	30.2	POSITIVE	30.0	0.2	1%
17	29.91	POSITIVE	29.3	POSITIVE	29.6	0.4	1%
18	29.91	POSITIVE	29.7	POSITIVE	29.8	0.1	0%
AVERAGE CV% Ct value range < 30: 100% agreement							1%
19	30.1	POSITIVE	30.0	POSITIVE	30.1	0.1	0%
20	30.38	POSITIVE	29.9	POSITIVE	30.1	0.3	1%
21	30.41	POSITIVE	29.7	POSITIVE	30.1	0.5	2%
22	30.55	POSITIVE	30.6	POSITIVE	30.6	0.1	0%
23	30.88	POSITIVE	31.0	POSITIVE	31.0	0.1	0%
24	31.94	POSITIVE	31.4	POSITIVE	31.7	0.4	1%
25	32.06	POSITIVE	32.2	POSITIVE	32.1	0.1	0%
26	33.03	POSITIVE	33.5	POSITIVE	33.3	0.3	1%
27	33.88	POSITIVE	33.1	POSITIVE	33.5	0.5	2%
28	33.97	POSITIVE	34.4	POSITIVE	34.2	0.3	1%
29	34	POSITIVE	33.8	POSITIVE	33.9	0.1	0%
30	34.38	POSITIVE	33.4	POSITIVE	33.9	0.7	2%
AVERAGE CV% 30 > Ct value < 35: 100% agreement							1%
31	35.06	POSITIVE	34.2	POSITIVE	34.6	0.6	2%
32	35.19	POSITIVE	35.8	POSITIVE	35.5	0.4	1%
33	36.26	POSITIVE	36.1	POSITIVE	36.2	0.1	0%
34	37.78	POSITIVE	38.9	POSITIVE	38.4	0.8	2%
35	38.05	POSITIVE	38.8	POSITIVE	38.4	0.5	1%
36	38.82	POSITIVE	36.9	POSITIVE	37.9	1.4	4%
37	39.9	POSITIVE	33.1	POSITIVE	36.5	4.8	13%
38	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
39	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%

40	No ct	NEGATIVE	39.4	POSITIVE	39.7	0.4	1%
41	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
42	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
43	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
44	No ct	NEGATIVE	38.8	POSITIVE	39.4	0.8	2%
45	No ct	NEGATIVE	No ct	POSITIVE	No ct	0.0	0%
46	No ct	NEGATIVE	38.9	NEGATIVE	39.4	0.8	2%
47	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
48	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
49	No ct	NEGATIVE	39.3	POSITIVE	39.7	0.5	1%
50	No ct	NEGATIVE	No ct	POSITIVE	No ct	0.0	0%
51	No ct	NEGATIVE	No ct	POSITIVE	No ct	0.0	0%
52	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
53	No ct	NEGATIVE	No ct	POSITIVE	No ct	0.0	0%
54	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
55	No ct	NEGATIVE	39.7	POSITIVE	39.9	0.2	1%
56	No ct	NEGATIVE	31.7	POSITIVE	35.9	5.9	16%
57	No ct	NEGATIVE	39.4	POSITIVE	39.7	0.4	1%
58	No ct	NEGATIVE	37.6	POSITIVE	38.8	1.7	4%
59	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
60	No ct	NEGATIVE	38.7	POSITIVE	39.3	1.0	2%
AVERAGE CV% Ct value range > 35: 70% agreement							2%

CV = coefficient of variability; SD = standard deviation

The average of the individual CVs reported as the inter-assay CV was 1% in samples with Ct values lower than 35 and 2% in samples with Ct values > 35. In all cases the CV% was lower than 5%, therefore exhibiting a very good stability and inter assay reproducibility.

It is important to point out that the analysis of the 29 samples with Ct values > 35 resulted in an percentage of agreement among the results obtained of 70% against the 100% found in samples with Ct values lower than 35. Nine samples (marked in grey in table 8) initially classified as negatives in the first PCR run resulted as positives in the second PCR run.

3.4. INTRA-ASSAY REPRODUCIBILITY

The ***intra-assay reproducibility*** was assessed with CVs obtained testing the same 60 samples used for the inter assay study. The results obtained in each of the samples tested within the intra-assay study are showed in **table 9.**

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

ID SAMPLE	Ct1	RESULT	Ct2	RESULT	MEAN		%CV
1	22.44	POSITIVE	22.46	POSITIVE	22.5	0.0	0%
2	22.47	POSITIVE	23.09	POSITIVE	22.8	0.4	2%
3	24.55	POSITIVE	24.42	POSITIVE	24.5	0.1	0%
4	25.3	POSITIVE	25.81	POSITIVE	25.6	0.4	1%
5	27.5	POSITIVE	27.81	POSITIVE	27.7	0.2	1%
6	27.76	POSITIVE	26.83	POSITIVE	27.3	0.7	2%
7	27.82	POSITIVE	28.91	POSITIVE	28.4	0.8	3%
8	27.86	POSITIVE	27.09	POSITIVE	27.5	0.5	2%
9	28.32	POSITIVE	27.91	POSITIVE	28.1	0.3	1%
10	28.39	POSITIVE	28.3	POSITIVE	28.3	0.1	0%

11	28.76	POSITIVE	29.45	POSITIVE	29.1	0.5	2%
12	28.96	POSITIVE	29.64	POSITIVE	29.3	0.5	2%
13	29.07	POSITIVE	27.78	POSITIVE	28.4	0.9	3%
14	29.31	POSITIVE	29.19	POSITIVE	29.3	0.1	0%
15	29.6	POSITIVE	29.65	POSITIVE	29.6	0.0	0%
16	29.74	POSITIVE	29.83	POSITIVE	29.8	0.1	0%
17	29.78	POSITIVE	29.68	POSITIVE	29.7	0.1	0%
18	29.82	POSITIVE	29.56	POSITIVE	29.7	0.2	1%
19	29.93	POSITIVE	30.1	POSITIVE	30.0	0.1	0%
AVERAGE CV Ct value range < 30: 100% agreement							**1%**
20	30.11	POSITIVE	29.69	POSITIVE	29.9	0.3	1%
21	30.72	POSITIVE	29.62	POSITIVE	30.2	0.8	3%
22	30.8	POSITIVE	30.49	POSITIVE	30.6	0.2	1%
23	31.14	POSITIVE	30.92	POSITIVE	31.0	0.2	1%
24	31.75	POSITIVE	31.09	POSITIVE	31.4	0.5	1%
25	32.22	POSITIVE	32.2	POSITIVE	32.2	0.0	0%
26	32.33	POSITIVE	31.12	POSITIVE	31.7	0.9	3%
27	33.12	POSITIVE	33.1	POSITIVE	33.1	0.0	0%
28	33.32	POSITIVE	32.93	POSITIVE	33.1	0.3	1%
29	33.35	POSITIVE	33.63	POSITIVE	33.5	0.2	1%
30	33.43	POSITIVE	33.46	POSITIVE	33.4	0.0	0%
31	33.78	POSITIVE	34.54	POSITIVE	34.2	0.5	2%
32	33.89	POSITIVE	33.72	POSITIVE	33.8	0.1	0%
33	34.41	POSITIVE	34.35	POSITIVE	34.4	0.0	0%
AVERAGE CV range 30 > Ct value < 35: 100% agreement							**1%**
34	36.16	POSITIVE	35.45	POSITIVE	35.8	0.5	1%
35	36.38	POSITIVE	35.81	POSITIVE	36.1	0.4	1%
36	36.8	POSITIVE	36.99	POSITIVE	36.9	0.1	0%
37	38.8	POSITIVE	No ct	NEGATIVE	39.4	0.8	2%
38	No ct	NEGATIVE	37.87	POSITIVE	38.9	1.5	4%
39	No ct	NEGATIVE	37.53	POSITIVE	38.8	1.7	5%
40	No ct	NEGATIVE	35.14	POSITIVE	37.6	3.4	9%
41	No ct	NEGATIVE	37.3	POSITIVE	38.7	1.9	5%
42	No ct	NEGATIVE	37.62	POSITIVE	38.8	1.7	4%
43	No ct	NEGATIVE	37.77	POSITIVE	38.9	1.6	4%
44	No ct	NEGATIVE	38.67	POSITIVE	39.3	0.9	2%
45	No ct	NEGATIVE	38.87	POSITIVE	39.4	0.8	2%
46	No ct	NEGATIVE	39.42	POSITIVE	39.7	0.4	1%
47	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
48	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
49	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
50	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
51	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
52	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
53	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
54	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
55	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
56	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
57	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
58	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
59	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
60	No ct	NEGATIVE	No ct	NEGATIVE	No ct	0.0	0%
AVERAGE CV Ct value range > 35: 63% agreement							**2%**

CV = coefficient of variability; SD = standard deviation

The average of the individual CVs reported as the intra-assay CV was 1% in samples with Ct values lower than 35 and 2% in samples with Ct values > 35. In all cases the CV% was lower than 5%, therefore exhibiting a very good stability and inter assay reproducibility.

Similarly to that observed in the inter assay study, in samples with Ct values > 35 the percentage of agreement among the results obtained was 63 % against the 100% found in samples with Ct values lower than 35.

4. CONCLUSIONS.

- From the analysis of **404 field samples obtained from epidemic areas in Eastern Europe,** the (κ) value was 0.87 % [95% CI] **indicating a PERFECT AGREEMENT between the UPL reference method and the LSI VetMAX™ real time PCR kit, therefore showing an appropriated diagnostic sensitivity for ASFV genome detection in field conditions.**

- The **inter-assay variability value** (less than 5%) **exhibited high repeatability in the results obtained and is considered appropriated** in routine testing

- The **intra-assay variability value** (less than 5%) **exhibited high reproducibility in the results obtained and is considered appropriated** in routine testing.

From the final validation of the LSI VetMAX™ ASFV Real time PCR kit throughout the analysis of 424 field samples collected from areas infected with genotype II ASFV in Eastern Europe, we conclude that the test is VALIDATED for performing a confident diagnosis of ASF by the detection of the ASFV genome in field samples extracted using the High Pure DNA extraction kit from ROCHE.

Report performed in Valdeolmos (Madrid) at 6th July 2018

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