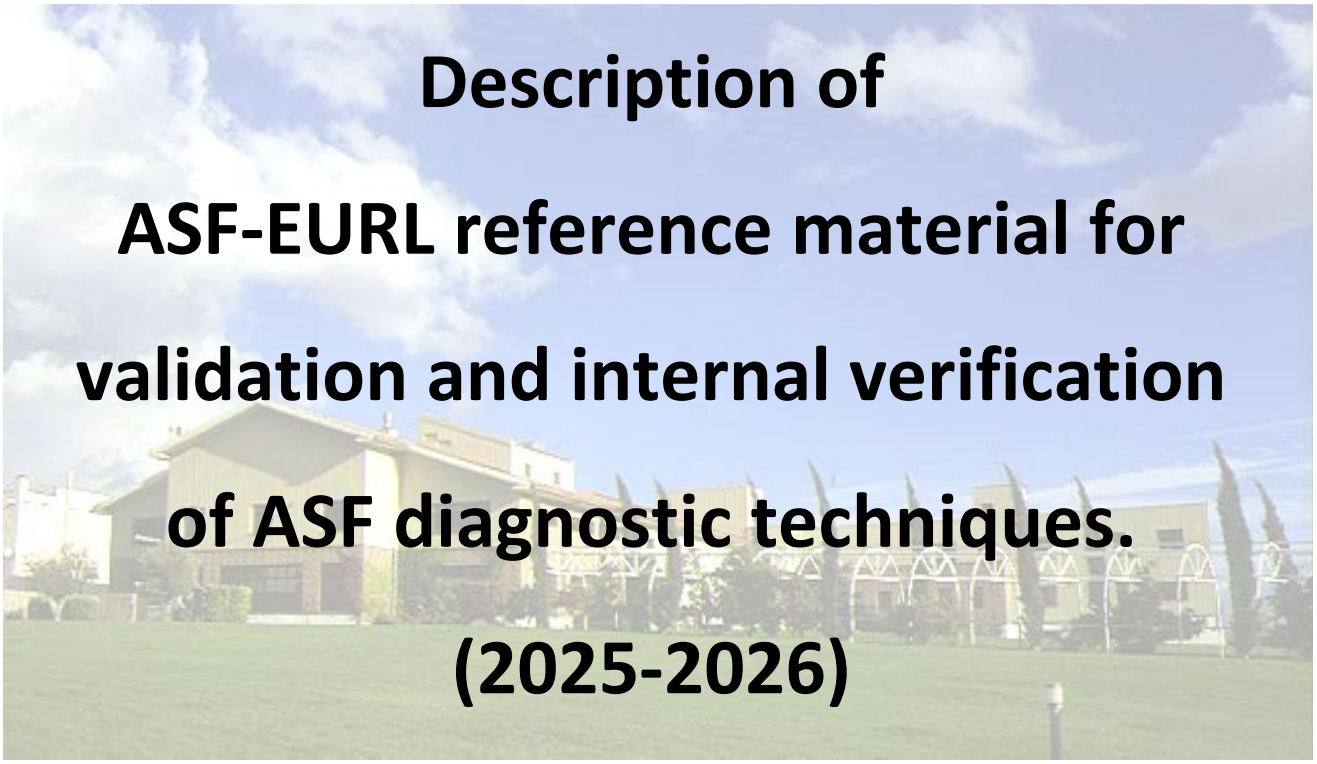
EU Reference Laboratory for ASF
Animal Health Research Centre
(CISA), INIA
Ctra Algete-El Casar s/n
28130, Valdeolmos, Spain



Description of ASF-EURL reference material for validation and internal verification of ASF diagnostic techniques. (2025-2026)

EUROPEAN UNION REFERENCE LABORATORY FOR AFRICAN SWINE FEVER (EURL-ASF)

Centro de Investigación en Sanidad Animal CISA-INIA, CSIC
Valdeolmos 28130, Madrid, Spain.
Tlf; +34916202300

Contacts: : Carmina Gallardo (gallardo@inia.csic.es) and Raquel Nieto (nieto.raquel@inia.csic.es)

1. SCOPE.

In order to assist the National Reference Laboratories (NRLs) within the European Union (EU) in the implementation, validation, and internal verification of official and alternative diagnostic methods for African swine fever (ASF), the EU Reference Laboratory (EURL) for ASF, CISA-INIA (Madrid, Spain), has prepared a panel of inactivated ASF reference materials to be provided to the NRLs upon request.

The ASF reference material panel comprises:

- **ASF-Ref-1: Ten EURL-ASF reference serum samples** for the evaluation, validation, and internal verification of ASF antibody detection techniques.
- **ASF-Ref-2: Sixteen EURL-ASF reference samples** for the evaluation, validation, and internal verification of DNA extraction methods.
- **ASF-Ref-3: Twenty-five EURL-ASF reference DNA samples** for the evaluation, validation, and internal verification of DNA amplification by PCR.

To support NRLs without biosafety level 3 (BSL-3) facilities, **all samples were inactivated by heat treatment at 56°C for 70 minutes followed by lyophilisation**. The efficacy of virus inactivation was confirmed by the WOAH-prescribed virus isolation test through three consecutive passages, as described in Chapter 3.9.1 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2025 Edition).

2. ASF-Ref-1: ASF reference material for the evaluation, validation and internal verification of ASF antibody detection techniques.

2.1. DESCRIPTION→ a panel of ten lyophilised ASF reference serum samples was selected for the evaluation, validation, and internal verification of ASF antibody detection techniques at the NRL level. The ten inactivated and lyophilised serum samples were obtained from domestic pigs experimentally infected under biosafety level 3 (BSL-3) animal facilities at CISA-INIA. The origin and description of the sera are provided in **Table 1**.

Table 1: *origin of serum samples included in the EURL-ASF reference samples for ASFV antibody detection methods.*

ID Sample	Clinical form	Virulence ASFV	ASFV isolate	Genotype	DPI/DPE*	Description
S33	Subacute	Moderate virulent	Ken05/Tk1	X	D70	Serum obtained from a domestic pig naturally infected with the Kenyan ASFV isolate Ken05/Tk1 (10 HAU/ml). The sample was collected 70 dpe and diluted 1:8 in negative serum.
S34	Chronic	Attenuated + virulent	NH/P68 + Arm07	I	D126	Serum from a domestic pig intramuscularly (i.m.) inoculated with the Portuguese ASFV isolate NH/P68 (10^7 $TCID_{50}$/ml) and challenged at 30 dpi with the virulent ASFV isolate Arm07 (10 HAU). Collected 126 days after the first inoculation.
S35	Subacute	Virulent	Lv19/WB/DOBEL9	II	D22	Serum from a domestic pig i.m. inoculated with the ASFV isolate Lv19/WB/DOBEL9 (10 HAU). Collected 22 dpi.
S36	Acute	Virulent	Est16/WB/VIRU8	II	D16	Serum from a domestic pig i.m. inoculated with the ASFV isolate Est16/WB-VIRU8 (10 HAU). Collected 16 dpi.
S37	Subclinical	Attenuated	LV17/WB/Rie14/ Tukuma5	II	D119	Serum from a domestic pig naturally infected (by contact) with the ASFV isolate LV17/WB/Rie14/Tukuma5. Collected 119 dpe.
S38	Subacute	Moderate virulent	ET13/1505	XXIII	D93	Serum from a domestic pig naturally infected (by contact) with the ASFV isolate ET13/1505 (10 HAU). Collected 93 dpe.
S39	Chronic	Attenuated	POL18/WB/ Case1794	II	D64	Serum from a domestic pig i.m. inoculated with the ASFV isolate POL18/WB/Case1794 (100 $TCID_{50}$). Collected 64 dpi.
S40	Subclinical	Attenuated	Lv19/WB/ Brocenu6	II	D44	Serum from a domestic pig naturally infected (by contact) with the ASFV isolate Lv19/WB/Brocenu6 (10 HAU). Collected 44 dpe.
S41	Chronic	Attenuated	POL18/WB/ Case1794	II	D64	Derived from sample S39, diluted 1:1500 in negative serum.
S42	**Serum from a naïve (ASF-negative) domestic pig.**					

**DPI= days post inoculation; DPE= days post infection*

2.2. REFERENCE RESULTS → The samples were analysed after the lyophilisation process in three independent aliquots using the serological tests routinely employed at EURL for ASF specific antibody detection comprising;

- **EURL indirect ELISA (EURL-ELISA)** included as WOAH prescribed serological technique in the Chapter 3.9.1 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2025 Edition. Briefly this ELISA is performed using semi purified virus (E70) produced in Monkey stable (MS) cells as coated antigen and protein-A labelled to HRPO as indicator. [SOP/CISA/ASF/ELISA/1].
- **EURL Immunoblotting (EURL-IB)** included as WOAH confirmatory serological technique in the Chapter 3.9.1 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2025 Edition, using, as well, semi purified virus as antigen [SOP/CISA/ASF/IB/1].
- **EURL Immunoperoxidase technique (EURL-IPT)** included as WOAH confirmatory serological technique in the Chapter 3.9.1 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2025 Edition, using E70-MS infected cells [SOP/CISA/ASF/IPT/1].
- **INGENASA K3 ELISA** commercial kit Ingezim PPA Compac (11.PPA k3) based on the use of the ASFV protein p72/73 as antigen.

The results obtained at EURL by ASF antibody detection are showed in Table 2.

ID	EURL- ELISA		INGENASA K3		EURL-IB	EURL-IPT		*ASF antibody diagnostic conclusion*
SAMPLE	*O.D.*	*RESULT*	*O.D.*	*RESULT*	*RESULT*	*TITER*	*RESULT*	
S33	**0.312**	**DOUBT**	**0.772**	**POSITIVE**	**WEAK**	**1:2560**	**POSITIVE**	**POSITIVE**
S34	**1.275**	**POSITIVE**	**0.080**	**POSITIVE**	**POSITIVE**	**1:655360**	**POSITIVE**	**POSITIVE**
S35	**0.333**	**DOUBT**	**0.825**	**POSITIVE**	**POSITIVE**	**1:20480**	**POSITIVE**	**POSITIVE**
S36	**0.616**	**DOUBT**	1.34	NEGATIVE	**WEAK**	**1:2560**	**POSITIVE**	**WEAK**
S37	**1.235**	**POSITIVE**	**0.08**	**POSITIVE**	**POSITIVE**	**1:655360**	**POSITIVE**	**POSITIVE**
S38	**1.057**	**POSITIVE**	**0.08**	**POSITIVE**	**POSITIVE**	**1:40960**	**POSITIVE**	**POSITIVE**
S39	**1.2850**	**POSITIVE**	**0.098**	**POSITIVE**	**POSITIVE**	**1:163840**	**POSITIVE**	**POSITIVE**
S40	**0.401**	**DOUBT**	**0.43**	**POSITIVE**	**POSITIVE**	**1:10240**	**POSITIVE**	**POSITIVE**
S41	0.271	NEGATIVE	**0.74**	**POSITIVE**	NEGATIVE	**1:80**	**POSITIVE**	**WEAK**
S42	0.145	NEGATIVE	1.65	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE

O.D. optical density

2.3. PRESENTATION→ lyophilized in 1ml vials.

2.4. STORAGE CONDITIONS:

- Before reconstitution: stored at 4 ±-3ºC.
  Expiry date: 2 years.
- After reconstitution: Reconstitute each vial with 1 ml of distilled water. Once rehydrated, store at ≤ −10 °C.
  Expiry date: 18 months.

2.5. USE OF MATERIAL → the material is intended for use as quality control in ASF antibody detection techniques, serving as positive, limit, and negative reference sera, as described in **Table 2**. It is recommended to use each reference serum in duplicate per run.

For internal verification controls in ASF antibody detection techniques, it is advised to test each sample in duplicate in three independent runs, using the working dilutions specified in the Standard Operating Procedures (SOPs) routinely applied by the NRLs. When using the standardized EURL-SOPs for ASF antibody detection, the recommended working dilutions are: EURL-ELISA: 1/30, EURL-IB: 1/40 and EURL-IPT: 1/40

3. ASF reference material for the evaluation, validation and internal verification of ASFV genome detection techniques (PCR)

The EURL has prepared two distinct panels of reference materials for the evaluation, validation, and internal verification of PCR-based diagnostic techniques:

i) ASF-Ref-2, for the assessment of DNA extraction methods; and

ii) ASF-Ref-3, for the assessment of DNA amplification by PCR.

Quality control of both panels has been performed in accordance with the SOPs for DNA extraction [SOP/CISA/ASF/DNA EXTRACTION/1] and real time PCR [SOP/CISA/ASF/PCR/2].

3.1. ASF-Ref-2: Panel of reference material for DNA extraction.

3.1.1. DESCRIPTION→ a panel of **sixteen lyophilised ASF reference samples**, collected from experimental infections using different ASFV isolates from different epidemiological situations, was prepared at the EURL for the evaluation, validation, and internal verification of the ASFV DNA extraction methods and subsequent ASFV genome detection techniques at the NRL level. The origin and description of the samples are provided in **Table 3**.

Table 3→ *origin of samples included in the EURL-ASF reference samples for ASFV DNA extraction methods.*

ID Sample	Clinical form	Virulence ASFV	ASFV isolate	Genotype	DPI/DPE*	Description
S33	**Negative spleen homogenate obtained from a naïve (ASF-free) domestic pig.**					
S34	Chronic	Attenuated	POL18/WB/Case1794	II	D24	Lung homogenate obtained at 24 dpe from a domestic pig kept in contact with another pig experimentally inoculated intramuscularly (i.m)with the Polish ASFV isolate POL18/WB/Case1794 (10 HAU/ml).
S35	Subacute	Moderate virulent	ET13/1505	XXIII	D23	Heart homogenate obtained at 23 dpe from a domestic pig kept in contact with another pig experimentally inoculated i.m. with the Ethiopian ASFV isolate ET13/1505 (10 HAU/ml).
S36	Subclinical	Attenuated	LV17/WB/Rie14/Tukuma5	II	D9	Serum obtained at 9 dpi from a domestic pig experimentally inoculated i.m. with the Latvian ASFV isolate LV17/WB/Rie14/Tukuma5 (10 HAU/ml).

ID Sample	Clinical form	Virulence ASFV	ASFV isolate	Genotype	DPI/DPE*	Description
S37	Subclinical	Attenuated	Lv19/WB/Brocenu6	II	D20	Liver homogenate obtained at 20 dpi from a domestic pig experimentally inoculated i.m. with the Latvian ASFV isolate Lv19/WB/Brocenu6 (10 HAU).
S38	**Negative spleen homogenate obtained from a naïve (ASF-free) domestic pig.**					
S39	Subclinical	Attenuated	LV17/WB/Rie14/Tukuma5	II	D9	Serum obtained at 9 dpi from a domestic pig experimentally inoculated i.m. with the Latvian ASFV isolate LV17/WB/Rie14/Tukuma5 (10 HAU/ml), diluted 1:32 in negative serum.
S40	Subclinical	Attenuated	NH/P68	I	D128	Lung homogenate obtained at 128 dpi from a domestic pig inoculated i.m. with the Portuguese ASFV isolate NH/P68.
S41	**Negative spleen homogenate obtained from a naïve (ASF-free) domestic pig.**					
S42	Acute	Virulent	Nig08/LaOk1	I	D8	Spleen homogenate obtained at 8 dpi from a domestic pig inoculated i.m. with the Nigerian ASFV isolate Nig08/LaOk1 (10 HAU/ml).
S43	Acute	Virulent	Ken06/Bus	IX	D9	Serum obtained at 9 dpi from a domestic pig inoculated i.m. with the Kenyan ASFV isolate Ken06/Bus (10 HAU/ml).
S44	Subacute	Moderate virulent	E75	I	D15	Serum obtained at 15 dpi from a domestic pig inoculated i.m. with the Spanish ASFV isolate E75 (10 HAU/ml).
S45	Dilution 1:128 in negative tissue of sample S42.					
S46	Chronic	Attenuated	POL18/WB/Case1794	II	D36	Spleen homogenate obtained at 36 dpe from a domestic pig kept in contact with another pig experimentally inoculated i.m. with the Polish ASFV isolate POL18/WB/Case1794 (10 HAU/ml).
S47	Subacute	Moderate virulent	Est15/WB-Valga6	II	D25	Serum obtained at 25 dpe from a domestic pig kept in contact with another pig experimentally inoculated i.m. with the Estonian ASFV isolate Est15/WB-Valga6 (10 HAU/ml).
S48	Subclinical	Attenuated	Lv19/WB/Brocenu6	II	D17	Serum obtained at 17 dpi from a domestic pig inoculated i.m. with the Latvian ASFV isolate Lv19/WB/Brocenu6 (10 HAU/ml).

**DPI= days post inoculation; DPE= days post infection*

3.1.2. EURL REFERENCE RESULTS → DNA was extracted from each of the sixteen inactivated and lyophilised samples using *the High Pure Viral Nucleic Acid Kit (Roche)*, following the manufacturer's instructions and according to the SOP for the extraction of ASFV DNA ([SOP/CISA/ASF/DNA EXTRACTION/1]). Quality control was performed on **two independent extractions using the WOAH-prescribed real-time PCR procedure 2 (UPL-PCR),** as described in Chapter 3.9.1 of the Manual of Diagnostic Tests and

Vaccines for Terrestrial Animals (2025 Edition) ([SOP/CISA/ASF/PCR/3]). The results obtained are presented in **Table 4.**

Table 4→ *PCR results of the EURL reference samples*

ID SAMPLE	Ct 1	Ct 2	Average Ct	PCR CONCLUSION
SAMPLE 33	No ct	No ct	No ct	NEGATIVE
SAMPLE 34	27.62	26.4	27.01	POSITIVE
SAMPLE 35	30.43	29.82	30.12	POSITIVE
SAMPLE 36	19.4	19.01	19.20	POSITIVE
SAMPLE 37	21.64	21.18	21.41	POSITIVE
SAMPLE 38	No ct	No ct	No ct	NEGATIVE
SAMPLE 39	23.55	22.68	23.11	POSITIVE
SAMPLE 40	31.72	31.12	31.42	POSITIVE
SAMPLE 41	No ct	No ct	No ct	NEGATIVE
SAMPLE 42	23.67	23.07	23.37	POSITIVE
SAMPLE 43	24.76	23.89	24.32	POSITIVE
SAMPLE 44	37.51	35.83	36.67	WEAK
SAMPLE 45	32.87	30.63	31.75	POSITIVE
SAMPLE 46	35.02	32.39	33.70	POSITIVE
SAMPLE 47	32.44	31.61	32.02	POSITIVE
SAMPLE 48	31.04	29.87	30.45	POSITIVE

3.1.3. PRESENTATION→ lyophilized in 1ml vials.

3.1.4. STORAGE CONDITIONS:

- Before reconstitution: stored at 4 ±-3ºC.
  Expiry date: 2 years.
- After reconstitution: Reconstitute each vial with 1 ml of distilled water. Once rehydrated, store at ≤ −70 °C.
  Expiry date: 18 months.

3.1.5. USE OF MATERIAL → To reconstitute the material, dissolve the entire contents of the vial in 1 ml of sterile distilled water, aliquot, and store at ≤ −70 °C until use. Once reconstituted, the material should be handled as PCR-positive, limit, or negative ASF reference samples, as described in **Table 4**.

For use as internal verification controls in ASFV genome detection techniques, it is recommended to test each sample in duplicate at the working dilution specified in, and according to, the SOP(s) routinely applied by the NRLs for nucleic acid extraction and PCR amplification of the target ASFV genome.

3.2. ASF-Ref-3: Panel of DNAs reference material for PCR amplification.

3.2.1. DESCRIPTION→ a panel of **twenty-five ASF reference DNA samples** was prepared at the EURL for the evaluation, validation, and internal verification of ASFV-specific DNA amplification techniques by PCR at the NRL level. The DNA panel comprises reference materials obtained from twenty-five ASFV isolates representing **seven different p72 genotypes (I, II, V, VIII, IX, X, and XXIII),** which together encompass a broad geographical and temporal distribution of ASF outbreaks worldwide. Among these, genotype II is the most extensively represented, including isolates from multiple European countries collected between 2007 and 2023. Within **genotype II, the panel includes nine distinct p72 GII genetic groups** (1, 3, 5, 6, 7, 9, 12, 16, 17, 19, 21, 22, 24, 27, and 28), thus reflecting the genetic diversity and molecular evolution of ASFV circulating in Europe. The remaining genotypes—I, V, VIII, IX, X, and XXIII—are represented by well-characterised isolates from Africa and Europe, including historical strains such as E70 (Spain, 1970; genotype I) and Moz64 (Mozambique, 1964; genotype V), as well as more recent isolates such as Ken11/KisP52 (Kenya, 2011; genotype IX) and Eth13/1505 (Ethiopia, 2013; genotype XXIII).

The origin and detailed description of each reference DNA included in the EURL-ASF PCR panel are provided in **Table 5.**

Table 5→ *origin of reference DNAs included in the EURL-ASF reference samples for ASFV PCR methods.*

ASFV ISOLATE	Country of origin	Host Species	Year of outbreak	P72 genotype	p72 GII genetic group*
E70	Spain	DP	1970	I	-
SS14/DP-Cagliari1	Italy	DP	2014	I	-
BF07 OUAGA 2	Burkina faso	DP	2007	I	-
Arm07	Armenia	DP	2007	II	1
Ukr12/Zapo	Ukraine	DP	2012	II	3
LT14/1490	Lithuania	EWB	2014	II	3
Est15/WB-Tartu14	Estonia	EWB	2015	II	5
Pol21/DP/OUT17	Poland	DP	2021	II	6
Pol21/DP/OUT56	Poland	DP	2021	II	7
Est17/WB/Parnu12	Estonia	EWB	2017	II	9
Lv18/WB/Kepo5	Latvia	EWB	2018	II	12
Lt18/DP/Moletai1	Lithuania	DP	2018	II	16
Lv18/DP/Brocenu1	Latvia	DP	2018	II	17
BOS23/DP/1	Bosnia	DP	2023	II	19
RO19/DP/ARGES1	Romania	DP	2019	II	21
RO19/DP/GALA1	Romania	DP	2019	II	22
RO21/DP14/ARAD	Romania	DP	2021	II	24
Pol21/DP/OUT59	Poland	DP	2021	II	27
Est22/WB/VIRU2212	Estonia	EWB	2022	II	28

ASFV ISOLATE	Country of origin	Host Species	Year of outbreak	P72 genotype	p72 GII genetic group*
Moz64	Mozambique	DP	1964	V	-
MwLil 20/1	Malawi	TK	1983	VIII	-
Ken11/KisP52	Kenya	DP	2011	IX	-
Ken06.Bus	Kenya	DP	2006	IX	-
Ken08Tk.2/1	Kenya	TK	2007	X	-
Eth13/1505	Ethiopia	DP	2013	XXIII	-

**Genetic groups of genotype II ASFVs determined using the multigene approach described by Gallardo et al. (2023).*

DP = domestic pig; EWB = European wild boar; TK = Tick

3.2.2. EURL REFERENCE RESULTS → the reference DNAs were obtained directly from ASFV reference isolates propagated in primary cell cultures (porcine blood monocytes). DNA was extracted using the *High Pure Viral Nucleic Acid Kit (Roche),* following the manufacturer's instructions and according to the SOP for the Extraction of ASFV DNA (SOP/CISA/ASF/DNA EXTRACTION/1). Quality control was performed on **twenty independent extractions using the WOAH-prescribed real-time PCR procedure 2** (UPL-PCR), as described in Chapter 3.9.1 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2025 Edition) (SOP/CISA/ASF/PCR/3). The reference results are shown in **Table 6.**

Table 6→ *Reference results of ASFV reference DNAs*

ASFV ISOLATE	P72 genotype	p72 GII genetic group	Ct value	SD	%CV
E70	I	-	**28.72**	0.30	1%
SS14/DP-Cagliari1	I	-	**31.93**	0.48	2%
BF07 OUAGA 2	I	-	**27.96**	0.47	2%
Arm07	II	1	**27.57**	0.43	2%
LT14/1490	II	3	**24.58**	0.89	4%
Ukr12/Zapo	II	3	**25.44**	0.57	2%
Est15/WB-Tartu14	II	5	**28.63**	0.45	2%
Pol21/DP/OUT17	II	6	**29.57**	0.45	2%
Pol21/DP/OUT56	II	7	**26.58**	0.38	1%
Est17/WB-Parnu12	II	9	**29.25**	0.86	3%
Lv18/WB/Kepo5	II	12	**30.68**	0.82	3%
Lt18/DP-Moletai1	II	16	**25.79**	0.68	3%
Lv18/DP/Brocenu1	II	17	**28.47**	0.45	2%
BOS23/DP/1	II	19	**30.17**	0.72	2%
RO19/DP/ARGES1	II	21	**27.55**	0.74	3%
RO19/DP/GALA1	II	22	**33.21**	0.87	3%
RO21/DP14/ARAD	II	24	**28.38**	0.37	1%
Pol21/DP/OUT59	II	27	**27.10**	0.95	4%
Est22/WB/VIRU2212	II	28	**29.61**	0.81	3%

ASFV ISOLATE	P72 genotype	p72 GII genetic group	Ct value	SD	%CV
Moz64	V	-	**21.36**	0.85	4%
MwLil 20/1	VIII	.	**30.31**	0.91	3%
Ken11/KisP52	IX	.	**28.44**	0.43	2%
Ken06.Bus	IX	.	**29.09**	0.58	2%
Ken08Tk.2/1	X	.	**24.27**	0.51	2%
Eth13/1505	XXIII	.	**25.85**	0.76	3%

SD = standard deviation; CV = coefficient of variation. The values were calculated based on the mean Ct values obtained from twenty aliquots per reference DNA sample.

The extracted DNAs are preserved by the addition of 1/10 volume of 3 M sodium acetate and three volumes of cold absolute ethanol. This material has an expiry date of five years. Accelerated degradation studies have indicated that the material remains stable when stored at temperatures below –70 °C.

3.2.3. PRESENTATION→ dry in 0.5 Eppendorf tubes.

3.2.4. STORAGE CONDITIONS:

- Before reconstitution: stored at –70 °C.
  Expiry date: 5 years.
- After reconstitution: Reconstitute each tube with **100 µl of sterile distilled water**, aliquot and store at temperatures below –10 °C until use.
  Expiry date: 2 years.

3.2.5. USE OF MATERIAL → once reconstituted, it should be handled as ASF reference positive nucleic acid, as described in Table 6. For use as internal verification controls in ASFV genome detection techniques, it is recommended to test in duplicate across three independent PCR runs using the SOPs routinely employed by the NRL.