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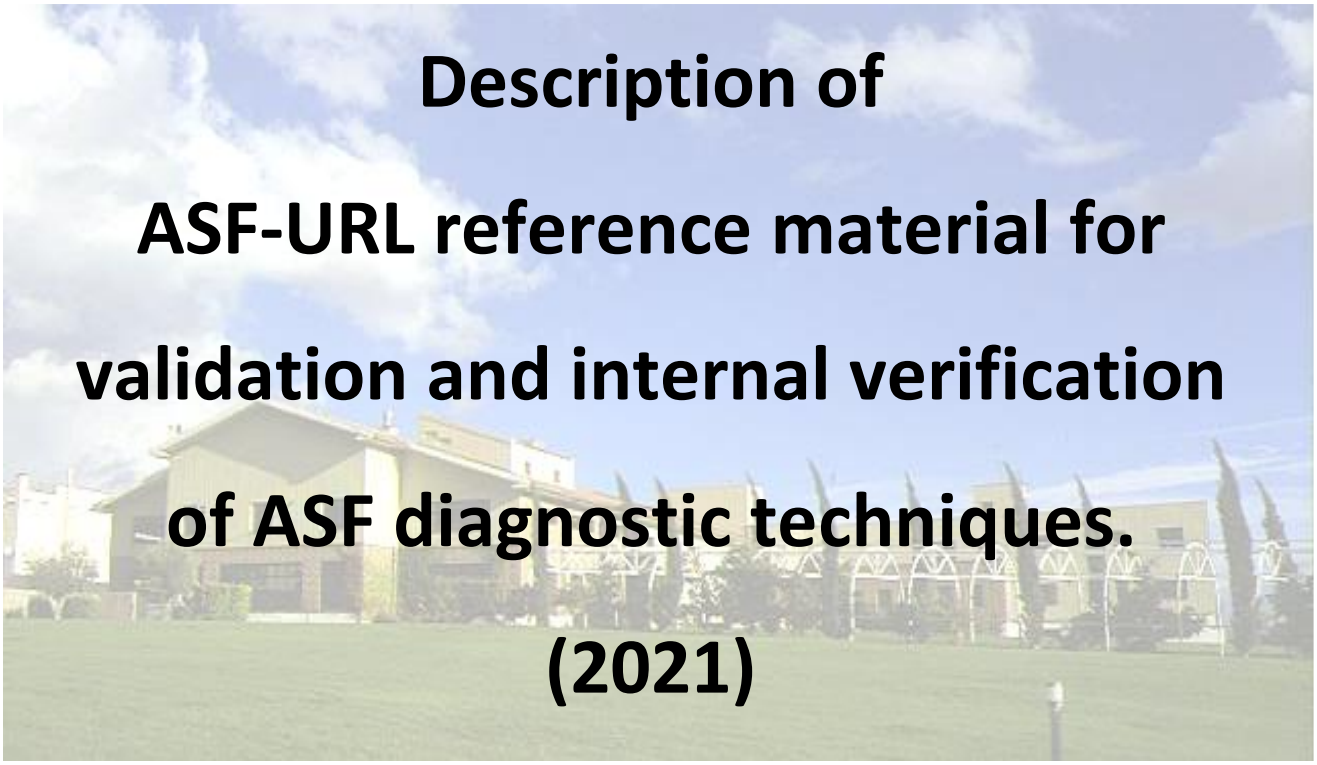

Description of ASF-URL reference material for validation and internal verification of ASF diagnostic techniques. (2021)

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

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1. SCOPE.

In order to assist the National Reference Laboratories (NRLs) within the European Union (EU) on the implementation, validation and internal verification of official and alternative ASF diagnostic methods, the EU Reference laboratory (EU-RL) for African swine fever (ASF) CISA-INIA, (Madrid, Spain) has prepared a **panel of inactivated ASF reference material** to provide to the NRLs previous request.

The **panel of ASF reference material** comprises,

- **Ten URL-ASF reference serum samples** for the evaluation, validation and internal verification of ASF antibody detection techniques**.**

- **Sixteen URL-ASF reference samples** for the evaluation, validation and internal verification of DNA extraction methods.

- **Twenty one URL-ASF reference DNAs** for the evaluation, validation and internal verification of DNA amplification by PCR.

To support NRLs without level 3 biosafety conditions, all samples has been **inactivated** by **heat treatment at 56°C for 70 minutes** followed by **lyophilisation.** The efficacy of virus inactivation has been tested using OIE-prescribed virus isolation technique in three consecutive passages according is described in the Chapter 3.8.1 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2019 Edition).

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

2.1. DESCRIPTION→ a panel of **ten ASF reference lyophilised experimental serum samples** were selected for the evaluation, validation and internal verification of ASF antibody detection techniques at NRLs level. **The 10 inactivated – lyophilised serum samples** were obtained from domestic pigs experimentally infected at BSL- 3 animal facilities at CISA- INIA. **The origin and description of the sera is described in Table 1.**

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

ID SAMPLE	CLINICAL FORM	VIRULENCE ASFV	ASFV ISOLATE	GENOTYPE	ORIGIN OF SAMPLES	
					DPI/DPE *(days post infection/exposure)*	***DESCRIPTION***
S23	**Naïve pig (ASF negative)**					
S24	**Naïve pig (ASF negative)**					
S25	**SUBACUTE**	**MOD. VIR.**	Est16/WB/Viru8	II	D26	Domestic pig naturally infected with the Estonian ASFV isolate Est16/WB/Viru8. Serum obtained at 26 days after exposure.
S26	**CHRONIC**	**ATT.+ VIR.**	NH/P68+Arm07	I	D52	Domestic pig i.m. inoculated with the Portuguese ASFV isolate NH/P68 i.m. (10^7 $TCDI_{50}$/ml) and challenged at 43 dpi with the ASFV virulent Arm07 (10 HAU) isolate. Serum obtained at 52 days after the first inoculation.
S27	**SUBACUTE**	**MOD. VIR.**	E75	I	D15	Domestic pig i.m. inoculated with the Spanish ASFV isolate $E75_{CV1-4}$ (10^2HAU/ml). Serum obtained at 15 days after the first inoculation.
S28	**SUBACUTE**	**MOD. VIR.**	Ken05/Tk1	X	D70	Domestic pig naturally infected with the Kenyan ASFV isolate Ken05/Tk1 (10HAU/ml). Serum obtained at 70 days after exposure.
S29	**CHRONIC**	**ATT.+ VIR.**	NH/P68+L60+Arm07	I	D72	Domestic pig i.m. inoculated with the Portuguese ASFV isolate NH/P68 i.m. (10^5 $TCDI_{50}$/ml) and challenged at 29 dpi with the ASFV virulent L60 and at 63dpi with the ASFV virulent Arm07 (10 HAU) isolate. Serum obtained at 72 days after the first inoculation.
S30	**ACUTE**	**VIR.**	POL16/DP/OUT21	II	D20	Domestic pig naturally infected with the Polish ASFV isolate POL16/DP/OUT21 (10 HAU/ml). Serum obtained at 20 days after exposure.
S31	**CHRONIC**	**ATT.+ VIR.**	NH/P68+Arm07	I	D126	Domestic pig i.m. inoculated with the Portuguese ASFV isolate NH/P68 i.m. (10^7 $TCDI_{50}$/ml) and challenged at 30 dpi with the ASFV virulent Arm07 (10 HAU) isolate. Serum obtained at 126 days after the first inoculation.
S32	**CHRONIC**	**ATT.+ VIR.**	Es15/WB/Tartu14	II	D206	Domestic pig naturally infected with the Estonian ASFV isolate Est15/WB/Tartu14. Serum obtained at 206 days after exposure.

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

- **URL indirect ELISA (URL-ELISA)** included as OIE prescribed serological technique in the Chapter 3.8.1 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019 Edition. Briefly this ELISA is performed using semi purified virus (E70) produced in MS cells as coated antigen and protein-A labelled to HRPO as indicator. [SOP/CISA/ASF/ELISA/1].
- **URL Immunoblotting (URL-IB)** included as OIE confirmatory serological technique in the Chapter 3.8.1 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019 Edition, using, as well, semi purified virus as antigen [SOP/CISA/ASF/IB/1].

- **URL- Immunoperoxidase technique (URL-IPT)** included as OIE confirmatory serological technique in the Chapter 3.8.1 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019 Edition, using E70-MS infected cells [SOP/CISA/ASF/IPT/11.]
- **INGENASA K3 ELISA** commercial kit Ingezim PPA Compac (11.PPA k3) based on the use of the ASFV protein p73 as antigen.

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

ID	URL- ELISA		INGENASA K3		URL-IB	URL-IPT		*ASF antibody diagnostic conclusion*
SAMPLE	*O.D.*	*RESULT*	*O.D.*	*RESULT*	*RESULT*	*TITER*	*RESULT*	
S23	0.154	NEGATIVE	1.495	NEGATIVE	NT*	-	NEGATIVE	NEGATIVE
S24	0.119	NEGATIVE	1.509	NEGATIVE	NT	-	NEGATIVE	NEGATIVE
S25	**0.975**	**POSITIVE**	**0.109**	**POSITIVE**	**POSITIVE**	**1:20480**	**POSITIVE**	**POSITIVE**
S26	**1.156**	**POSITIVE**	**0.074**	**POSITIVE**	**POSITIVE**	**1:327780**	**POSITIVE**	**STRONG POSITIVE**
S27	**0.435**	**DOUBT**	1.019	NEGATIVE	**POSITIVE**	**1:2560**	**POSITIVE**	**WEAK**
S28	**0.741**	**POSITIVE**	**0.103**	**POSITIVE**	**WEAK**	**1:5120**	**POSITIVE**	**POSITIVE**
S29	**1.229**	**POSITIVE**	**0.072**	**POSITIVE**	**POSITIVE**	**1:20480**	**POSITIVE**	**POSITIVE**
S30	**0.499**	**POSITIVE**	**0.673**	**POSITIVE**	**POSITIVE**	**1:10240**	**POSITIVE**	**POSITIVE**
S31	**1.337**	**POSITIVE**	**0.063**	**POSITIVE**	**POSITIVE**	**1:163840**	**POSITIVE**	**STRONG POSITIVE**
S32	**0.960**	**POSITIVE**	**0.095**	**POSITIVE**	**POSITIVE**	**1:10240**	**POSITIVE**	**POSITIVE**

NT = no tested

2.3. PRESENTATION→ lyophilized in vials of 1ml.

2.4. STORAGE CONDITIONS:

- Prior to reconstitution: stored at 4 ±-3ºC. **Expiry date: 2 years.**
- Reconstituted: the reference material must be reconstituted with 1ml of distilled water. Once rehydrated store at <-10ºC. **Expiry date: 18 months.**

2.5. USE OF MATERIAL → the material is intended to be used for quality control as positive, limit, and negative reference sera according the description in **Table 2** on ASF antibody detection techniques. It is recommended to use by duplicated per run. For using as internal verification controls in the ASF antibody detection techniques it is recommended to use by duplicated in three different runs at the appropriate working dilution specified in the Standard Operating Procedures (SOP) routinely employed by the NRLs. For the detection of antibodies against ASF using standardized URL-SOPs the appropriate working dilutions are: URL-ELISA 1/30; URL-IB 1/40; URL-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 different panels of reference material** for the evaluation, validation and internal verification of PCR techniques for; i) the DNA extraction methods, and ii) for the DNA amplification by PCR **[SOP/CISA/ASF/DNA EXTRACTION/1]**, **[SOP/CISA/ASF/PCR/1]** and **[SOP/CISA/ASF/PCR/2]**.

3.1. Panel of reference material for DNA extraction.

3.1.1. DESCRIPTION→ a panel of **16 ASF lyophilised reference samples** including experimental and clinical field samples collected from different epidemiological situations, were prepared at URL for the evaluation, validation and internal verification of ASF genome detection techniques at NRLs level. **The origin and description of the samples is described in Table 2**.

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

ID SAMPLE	CLINICAL FORM	VIRULENCE ASFV	ASFV ISOLATE	GENOTYPE	ORIGIN OF SAMPLES	
					DPI (days post infection)	*DESCRIPTION*
SAMPLE 17	**ACUTE**	**VIRULENT**	Ukr12/Zapo	II	D12	Homogenate spleen obtained from one pig kept in contact with pig's experimentally inoculated intramuscular route with the Ukraine ASFV Ukr12/Zapo isolate (10 HAU/ml).
SAMPLE 18	**CHRONIC**	**ATTENUATED**	NH/P68	I	D27	Homogenate spleen obtained from one pig experimentally inoculated intramuscular route with the Portugal ASFV NH/P68 isolate (10^7 TCDI50/ml).
SAMPLE 19	**ACUTE**	**VIRULENT**	L60	I	D7	Homogenate lung obtained from one pig experimentally inoculated intramuscular route with the Portugal ASFV L60 isolate ($3x10^5$ HAU/ml).
SAMPLE 20	**SUBACUTE**	**MODERATE**	Ken05/Tk1	X	D17	Homogenate lung obtained from one pig experimentally inoculated intramuscular route with the Kenya ASFV Ken05/Tk1 isolate (10 HAU/ml).
SAMPLE 21	**ACUTE**	**VIRULENT**	Arm07	II	D9	Homogenate gastro-hepatic lymph node obtained from one pig experimentally inoculated intramuscular route with the Armenia ASFV Arm07 isolate (10 HAU/ml).
SAMPLE 22	**ACUTE**	**VIRULENT**	Ken06.Bus	IX	D17	Homogenate spleen obtained from one pig experimentally inoculated intramuscular route with the Kenya ASFV Ken06.Bus isolate (10 HAU/ml).
SAMPLE 23	1/200 dilution in negative tissue of the **sample 17**					
SAMPLE 24	1/200 dilution in negative tissue of the **sample 22**					
SAMPLE 25	**Negative tissue (lung) obtained from a naive pig (ASF free)**					
SAMPLE 26	**Negative tissue (lung) obtained from a naive pig (ASF free)**					

SAMPLE 27	**Negative tissue (tonsil) obtained from a naive pig (ASF free)**					
SAMPLE 28	**ACUTE**	**VIRULENT**	Ukr12/Zapo	II	D7	Serum obtained from one pig experimentally inoculated intramuscular route with the Ukraine ASFV Ukr12/Zapo isolate (10 HAU/ml).
SAMPLE 29	1/50 dilution in negative serum of the **sample 28**					
SAMPLE 30	1/400 dilution in negative serum of the **sample 28**					
SAMPLE 31	**Negative serum obtained from a naive pig (ASF free)**					
SAMPLE 32	**ACUTE**	**VIRULENT**	LT14/1490	II	D17	Homogenate liver obtained from one pig kept in contact with pig's experimentally inoculated intramuscular route with the Lithuania ASFV LT14/1490 isolate (10 HAU/ml).

3.1.2. PRESENTATION→ lyophilized in vials of 1ml.

3.1.3. STORAGE CONDITIONS→

- Prior to reconstitution: stored at 4 ±3ºC. **Expiry date: 2 years.**
- Reconstituted: the reference material must be reconstituted with 1ml of distilled water. Once rehydrated store at <-70ºC. **Expiry date: 18 months.**

3.1.4. USE OF MATERIAL → To reconstitute this material, dissolve the entire contents of the vial **in 1ml of sterile distilled water**, aliquot and keep at <-70ºC until use. Once reconstituted should be treated as PCR positive, limit or negative ASF reference samples according is described in the **Table 3.** For using as internal verification controls in the ASFV genome detection techniques it is recommended to use **by duplicates** at the recommended working dilution specified in the Standard Operating Procedures (SOP) routinely employed by the NRLs for nucleic acid extraction.

3.1.5. URL SAMPLE PREPARATION AND REFERENCE RESULTS → The **DNA was extracted** from each of **16 inactivated – lyophilized samples** using the High Pure Viral Nucleic Acid kit (Roche) following the manufacturer's instructions and three different PCRs routinely employed at URL for ASF diagnostic were set up;

- **OIE conventional PCR** (**OIE-PCR**) included as OIE prescribed PCR technique in the Chapter 2.8.1 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012 Edition. Briefly this PCR is based on the amplification of 257bp within the ASFV p72 protein using the oligo-nucleotide primer set PPA1/PPA2 as it was described by Aguero *et* al., 2003 [SOP/CISA/ASF/PCR/1].
- **Real time PCR (OIE-Real time PCR)** included as OIE prescribed real time-PCR technique in the Chapter 2.8.1 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012 Edition, using the Fast amplification protocol modified by URL. Briefly this PCR is based on the amplification of 250bp within the ASFV p72 protein using the oligo-nucleotide primer set and Taqman probe y King *et* al., 2003 [SOP/CISA/ASF/PCR/2].
- **Real time PCR (UPL-PCR)** using an oligo-nucleotide primers and UPL probe described by Fernandez *et* al., 2012.

The results obtained at URL by PCR in the reference samples are showed in the Table 4;

ID SAMPLE	ASFV genome detection results		
	OIE-Real time PCR	UPL- Real time PCR	*PCR CONCLUSION*
SAMPLE 17	+++	+++	POSITIVE
SAMPLE 18	+	+	POSITIVE
SAMPLE 19	+++	+++	POSITIVE
SAMPLE 20	+++	+++	POSITIVE
SAMPLE 21	+++	+++	POSITIVE
SAMPLE 22	+++	+++	POSITIVE
SAMPLE 23	+	++	POSITIVE
SAMPLE 24	++	++	POSITIVE
SAMPLE 25	No Ct	No Ct	NEGATIVE
SAMPLE 26	No Ct	No Ct	NEGATIVE
SAMPLE 27	No Ct	No Ct	NEGATIVE
SAMPLE 28	+++	+++	POSITIVE
SAMPLE 29	+++	+++	POSITIVE
SAMPLE 30	++	+++	POSITIVE
SAMPLE 31	No Ct	No Ct	NEGATIVE
SAMPLE 32	+	++	POSITIVE

3.2. Panel of DNAs reference material for PCR amplification.

3.2.1. DESCRIPTION→ a panel of twenty one ASF reference DNAs were prepared at URL for the evaluation, validation and internal verification of specific ASFV DNA amplification by PCR techniques at NRLs level. The DNA samples were obtained from **21 ASFV reference isolates** representatives of **seven different p72 genotypes** as is specified in the **Table 5**.

Table 5, origin of reference DNAs included in the URL-ASF reference samples for ASFV PCR methods.

Isolates	Country of origin	Host Species	Year of outbreak	Town/ Province	P72 genotype	Reference
E70	Spain	Domestic pig	1970	Pontevedra	I	Zsak *et* al 2005
BF07 OUAGA 2	Burkina Faso	Domestic pig	2007	Ouaguodaga	I	Unpublished data INIA-CISA
SS14/WB-Sassari1	Italy	Wild boar	2014	Sassari	I	Unpublished data INIA-CISA
SS14/DP-Cagliari1	Italy	Domestic pig	2014	Cagliari	I	Unpublished data INIA-CISA
Arm07	Armenia	Domestic pig	2007	Dilijan	II	Unpublished data INIA-CISA
Ukr12/Zapo	Ukraine	Domestic pig	2012	Zaporozhye region	II	Gallardo et al 2014
Ukr15/DP-Kieve 1	Ukraine	Domestic pig	2015	Kiev	II	Unpublished data INIA-CISA
LT14/1490	Lithuania	Wild boar	2014	Vilnius	II	Gallardo et al 2014
Pol14/Krus	Poland	Wild boar	2014	Podlaskie	II	Gallardo et al 2014
Lv14/DP/Robez3	Latvia	Domestic pig	2014	Dienvidlatgale	II	Unpublished data INIA-CISA
Est14/WB-Valga-1	Estonia	Wild boar	2014	Valga	II	Unpublished data INIA-CISA
Est15/WB-Tartu14	Estonia	Wild boar	2015	Tartu	II	Unpublished data INIA-CISA
MOL16/DP-CERNO1	Moldova	Domestic pig	2016	Cernoleuca	II	Unpublished data INIA-CISA
MOL16/DP-MOSA1	Moldova	Domestic pig	2016	Mosana	II	Unpublished data INIA-CISA
Moz64	Mozambique	Domestic pig	1964	NK	V	Gallardo et al 2009
MwLil 20/1	Malawi	Tick	1983	Chalaswa	VIII	Complete genome
Ken11/KisP52	Kenya	Domestic pig	2011	Kisumu	IX	Unpublished data INIA-CISA
Ken06.Bus	Kenya	Domestic pig	2006	Busia	IX	Gallardo et al 2009
Ken08Tk.2/1	Kenya	Tick	2007	Kapiti	X	Gallardo et al 2011
UG10/Tk3.2	Uganda	Tick	2010	Mburu	X	Unpublished data INIA-CISA
Eth13/1505	Ethiopia	Domestic pig	2013	Bishoftu	XXIII	Achenbach et al 2016

3.2.2. STORAGE CONDITIONS→ Prior to reconstitution, this material has an **expiry date of five years**. Accelerated degradation studies have indicated that this material is suitably stable when **stored at <-70ºC.**

3.2.3. USE OF MATERIAL→ To reconstitute this material dissolve the entire contents of the vial in **100µl of sterile distilled water**. Aliquot and keep at <-10ºC until use. This material contains no preservative and has an **expired date following the reconstitution of 2 years at appropriated storage conditions.** Once reconstituted should be treated as ASF reference positive nucleic acid according the description in Table 5. For using as internal verification controls in the ASFV genome detection techniques it is recommended to use by duplicated in three different runs at the

recommended concentration specified in the Standard Operating Procedures (SOP) routinely employed by the NRL.

3.2.4. URL SAMPLE PREPARATION → the reference DNAs were obtained directly from ASFV reference isolates grown in primary cell cultures using the High Pure Viral Nucleic Acid kit (Roche) following the manufacturers procedures. Ethanol precipitation was used to concentrate DNA by addition of 1/10 volume of 3M Sodium Acetate and 3 volume of cold absolute ethanol.