Addressing African Swine Fever

Protocols and Guidelines for Laboratory Diagnosis

May 2024

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The World Organisation for Animal Health (WOAH), the Food and Agriculture Organization of the United Nations (FAO) and other partners have been actively working in countries affected or at risk of incursion by African swine fever (ASF). This manual is an updated and expanded version of guidance first published in 2020 following the emergence of ASF in China, other Asian countries, and countries of the Pacific and Caribbean regions.

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www.woah.org/en/disease/african-swine-fever/

Required citation: WOAH (2024). *Addressing African swine fever: Protocols and Guidelines for Laboratory Diagnosis*. Paris, 38 pp., DOI [https://doi.org/10.20506/asf.3475]. Licence: CC BY-SA 3.0 IGO.

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Acknowledgements

We wish to thank the following people for their contributions to this publication:

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Introduction

The World Organisation for Animal Health (WOAH), the Food and Agriculture Organization of the United Nations (FAO) and other partners have been actively working in countries affected or at risk of incursion by African swine fever (ASF). **This manual is an updated and expanded version of guidance first published in 2020 following the emergence of ASF in China, other Asian countries, and countries of the Pacific and Caribbean regions**. Since the emergence of the virulent p72 genotype II strain of ASF virus (ASFV) in China in 2018, several ASFV variants have been identified (Zhang *et al.*, 2020; Sun *et al.*, 2021a). Some of these variants have a non-haemadsorption (HAD) phenotype and have been associated with lower virulence disease forms. Different combinations of point mutations, insertions, or deletions have been found in the genomes of these variants. Notably, there have been reports of a variant containing deletions in the multigene family (MGF) 360 and 505 genes and in the *EP402R* gene (encoding CD2v). These deletions have been widely applied in vaccine development strategies. In 2021, the emergence of genotype I ASFV was reported in China and isolates have been associated with chronic ASF in domestic pigs (Sun *et al.*, 2021b). More recently, detection of recombinant ASFVs comprising genome fragments of genotypes I and II in pigs have been reported in China (Zhao *et al.*, 2023). Currently, the epidemiology of different ASFV genotypes and variants is not well understood and there is limited knowledge of the prevalence of these variant forms of ASF.

For the purposes of the WOAH *Terrestrial Code*, ASF is defined as an infection of suids with ASFV. Early detection of ASF infection in the context of co-circulation of ASF viruses causing different clinical forms of disease (e.g. acute, subacute, chronic and subclinical) requires both virological and serological diagnostic testing for sensitive and accurate laboratory diagnosis.

Recently, significant progress has been made in the development of live attenuated vaccines (LAV), including LAV containing single or multiple gene deletions (Borca *et al.*, 2020; Chen *et al.*, 2020; O'Donnell *et al.*, 2015). In Vietnam, the LAV ASFV-ΔI177L and ASFV-ΔMGF, originally developed by the United States Department of Agriculture (USDA) based on genotype II ASFV, were licensed to local vaccine manufacturers in 2022 and 2023 (FAO, 2023). These vaccines have recently been approved for nationwide use in Vietnam following extensive field trials to assess safety. They have also been evaluated for use in the Dominican Republic and the Philippines (FAO, 2023).

Clinical Signs of ASF

Infection of pigs with ASFV can produce a range of clinical signs associated with the different forms of ASF, which have been identified as peracute, acute, subacute and chronic (FAO, 2017; WOAH, 2022). Virulent strains such as the current pandemic strain can produce peracute or acute ASF, while moderately virulent strains lead to acute and subacute disease, and low virulence ASFV infections have been linked to milder clinical signs, characterised by chronic or subclinical forms of ASF. The range of clinical signs associated with each form of ASF is shown in Table 1. It is important to note that for each form of ASF, the full range of clinical signs may not be observed in infected animals. This is particularly true of early-stage disease, when clinical signs

may be subtle and appear non-specific (e.g. moderate fever, recumbency and mild reddening of the skin). Unlike classical swine fever and foot and mouth disease, ASF has low contagiousness and therefore spreads slowly when introduced into a pig herd, but it has a severe disease course and a high impact.

Table 1. Forms of ASF and associated clinical signs

Peracute	Acute	Subacute	Chronic
• High fever • Anorexia • Recumbency • Sudden death without signs	• High fever • Anorexia • Recumbency, depression • Reddening of the skin • Haemorrhages in the skin, internal organs • Vomiting • Constipation or diarrhoea • Dyspnoea • Nasal or ocular discharge (bloody) • Abortion • Incoordination • Central nervous system signs	• Similar to acute ASF • Intermittent fever • More pronounced haemorrhagic signs • Swollen joints • Oedema	• Low or intermittent fever • Dyspnoea • Swollen joints • Oedema • Reddened areas of skin, papules progressing to necrosis • Anorexia and weight loss • Recumbency
Mortality			
Up to 100%	Up to 100%	30-70%	Sporadic, less than 30%

Emergence of Lower Virulence ASFV

Recently, chronic ASF disease was reported in China, caused by a genotype I virus with high levels of genomic identity to previously characterised isolates from Portugal (Sun *et al.*, 2021). Clinical signs observed on affected farms included paralytic signs, weight loss, intermittent fever, skin ulcers, arthritis and sporadic deaths. Experimentally infected pigs inoculated with the genotype I virus isolated in China showed similar signs, i.e. intermittent fever, swollen joints and the development of papules on the skin and necrotic lesions. Bleeding from the nose and reddening of the skin (hyperaemia) were also observed. Notably, this genotype I virus was demonstrated to have a non-haemadsorbing (HAD) phenotype and mutations in the gene encoding the viral haemagglutinin CD2v (*EP402R*), which is characteristic of non-HAD and lower virulence isolates (Sun *et al.*, 2021). In Europe, there is evidence that genotype II ASFV has evolved into strains causing chronic and subacute disease or subclinical infections in domestic pigs and wild boar, caused by viruses with moderately virulent/HAD and attenuated/non-HAD phenotypes, respectively (Gallardo *et al.*, 2018; Zani *et al.*, 2018). Pigs infected with these lower virulence viruses typically survive long enough to seroconvert to the infecting virus.

This manual has been developed to address the detection of virulent and variant forms of ASFV and the future use of authorised LAVs with defined mutations. It has been developed in consultation with the WOAH ASF Reference Laboratory Network and is based on WOAH recommendations for laboratory diagnosis of ASF.

1. Molecular Diagnosis by PCR

Polymerase chain reaction (PCR) is a highly sensitive and specific method for the detection of ASFV genomic material and it can be used for a wide range of purposes, including confirmation of clinical cases and confirmation of freedom from infection before movement. Several PCR assays have been reported for the detection of ASFV (Table 2). Of these, WOAH recommends two real-time PCR assays for ASFV molecular diagnostics, namely those developed by King *et al.* and by Fernández-Pinero *et al.* (King *et al.*, 2003; Fernández-Pinero *et al.*, 2013; WOAH, 2019). In response to the emergence of lower virulence isolates of ASFV in China and with the development and use of modified LAVs, real-time PCR assays to differentiate wildtype virus from variants or LAV viruses have also recently been reported. This manual describes five validated PCR methods that are used for different purposes:

- **King Assay**, a WOAH-recommended assay targeting the *B646L* gene, encoding the ASFV protein p72 (King *et al.*, 2003).
  Purpose: Detection of all ASFV p72 genotypes.

- **CAHEC Triplex Assay** developed by the Chinese Animal Health and Epidemiology Center (CAHEC).
  Purpose: Differential detection of ASFV genotype II variants with deletions in the *MGF360/505* and *EP402R* genes, which have been associated with subclinical and chronic ASF.

- **ASFV I177L Assay**, based on the method of Velazquez-Salinas *et al.* (2021).
  Purpose: Differential detection of genotype II LAV-derived viruses containing deletions in the *I177L* gene.

- **ASFV MGF360-12L Assay**, based on the method of Velazquez-Salinas *et al.* (2001).
  Purpose: Differential detection of genotype II LAV-derived viruses containing deletions in the *MGF360-12L* gene.

- **ACDP ASFV Genotype I Assay**, based on a method developed at the Australian Centre for Disease Preparedness (ACDP).
  Purpose: Differential detection of genotype I viruses associated with lower virulence and chronic disease.

Table 2. Primers and probes for the detection of ASFV in real-time PCR assays

ASF assay	Gene target	(5' → 3') Forward primer	Reverse Primer	Probe	Dye*	Quencher*	Reference
King[†]	*B646L*	CTGCTCATGGTATCA-ATCTTATCGA	GATACCACAAGATCR-GCCGT	CCACGGGAGGAATAC-CAACCCAGTG	FAM	TAMRA	King *et al.*, 2003
Fernández-Pinero[†]	*B646L*	CCCAGGRGATAAAAT-GACTG	CACTRGTTCCCTCCAC-CGATA	CCTGGCCRACCAAGT-GCTT^	FAM	BHQ	Fernández-Pinero *et al.*, 2013; WOAH, 2019
Zsak	*B646L*	CCTCGGCGAGCGCTT-TATCAC	GGAAACTCATTCAC-CAAATCCTT	CGATGCAAGCTTTAT	FAM	MGB	Zsak *et al.*, 2005
McKillen	*9GL (B119L)*	GTTGTTATGGAACG-CGAAG	CGCTCCTAGCTGGAAA-GAAAA	CTGAAAGTCCTCCGAGT	FAM	Eclipse Dark	McKillen *et al.*, 2007
Tignon	*B646L*	TGCTCATGGTATCA-ATCTTATCG	CCACTGGGTTGGTA-TTCCTC	TTCCATCAAAGTTCTG-CAGCTCTT	FAM	TAMRA	Tignon *et al.*, 2011
Haines#	*B646L*	GATGATGATTACCTTY-GCTTTGAA	TCTCTTGCTCTRGATA-CRTTAATATGA	CCACGGGAGGAATAC-CAACCCAGTG	Cy5	DDQII	Haines *et al.*, 2013
Aguero[†]#±	*B646L*	AGTTATGGGAAACCC-GACCC	CCCTGAATCGGAG-CATCCT	NA	NA	NA	Aguero *et al.*, 2003
CAHEC triplex	*EP402R*	AGAAGAACAATGTCA-GCATGATGAC	CGACTGTAAGGCTTAG-GAAGTAATGG	CCACTTCCATACAT-GAACCATCTCCCAGA	VIC	BHQ1	Unpublished
	MGF 360-14L	AGAAGACGGGGTTCG-GATACAG	GCAAATCCTGAATATG-GGCTTATACG	CTCCCAGTTCCGCACA-CAGCCGC	CY5	BHQ2	
	B646L	AAGTTTCGGTACGCA-TTCTTTGT	TATGACTGGGACAAC-CAAACACC	ACAAGCGTGTAAACGG-CGCCCTC	FAM	BHQ1	
Shenzen Customs triplex	*EP402R*	GAAGAACAATGTCAG-CATGATGA	ACTGTAAGGCTTAG-GAAGTAATGGTT	ACCACTTCCATACAT-GAACCATCTCCCA	VIC	BHQ1	Lin *et al.*, 2020
	MGF 360-14L	GGGTTCGGATACAG-GCGTTA	CGTGTTCCTGCCGTG-TATCTAA	CCTCCCAGTTCCGCA-CACAGCC	Cy5	BHQ2	
	B646L	AACGCGTTCGCTT-TTCG	CATCGTGGTGGTTATT-GTTGGT	ACGTGTCCATAAAACG-CAGGTGACCC	FAM	BHQ1	
Yang triplex	*EP402R*	CCACCACCTGAATC-TAATGAAGAAG	CTGATAACGACTGTAA-GGCTTAGG	ACAATGTCAGCATGAT-GACACCACTTCC	VIC	BHQ1	Yang *et al.*, 2022
	MGF 360-14L	TTGGGGCGCAA-ATCCTGAAT	GCGTTAAGCCTCCCA-GTTC	ACACAGCCGCTTTAGA-TACACGGCA	Cy5	BHQ2	
	B646L	CTACCTGGAACATCTC-CGATCA	CTTATCTCTGCGTGGT-GAGT	CTCATCAACACCGAGA-TTGGCACAAG	FAM	BHQ1	
Velazquez-Salinas I177L	*I177L*	GAACTGGAAAAAACT-TTAACGGC	CCATTACCGGCAAGC-TAGG	ACGGATCCCCCTTCG-CATTTGA	FAM	MGB-NFQ	Velazquez-Salinas *et al.*, 2021
Velazquez-Salinas MGF360-12L	*MGF360-12L*	CATACCCTTCCCC-TAAAGCTG	CTACTGCTATGTCCT-GGGC	ACCCTCTTCGAAAA-CATCAGCCCC	FAM	MGB-NFQ	Velazquez-Salinas *et al.*, 2021
ACDP ASFV Genotype I	*505-3R*	TCCCTGGGCCACTTA-ATCATT	TGAAACGTCTCGCAG-GAAATG	AATGCAGTCCTGTGGA-CAAGACCCCAC	FAM	BHQ1	Unpublished
VNUA-p54	*E183L*	CAAGTGTAGGCAAGC-CAGTC	GCCATGACTAGTCT-GTCCGT	ACGGGCAGACCGG-CAACAAA	FAM	TAMRA	Trinh *et al.*, 2021

*Fluorophore dyes and quenchers can be substituted to be compatible with the PCR instrument that will be used for testing

[†]Test recommended by WOAH

^The original probe for this assay (UPL#162) is no longer commercially available and can be replaced by this standard probe sequence (WOAH, 2019)

#Assay can be duplexed for classical swine fever virus detection

±Conventional PCR

1.1. PCR Protocol: King Assay

The protocol described below is a WOAH-recommended real-time PCR procedure for the detection of ASFV which targets the *B646L* gene, encoding the ASFV structural protein p72 (King *et al.*, 2003). The reverse transcription (RT) step is not required for amplification of ASF viral DNA; however, it is included in this protocol so that only a single enzyme/buffer system and standard thermocycling conditions can be employed in the laboratory for both DNA and RNA virus targets. The RT step does not affect the assay performance. Standard PCR mixes and cycling conditions without RT can also be used.

This assay has been produced in kit form by the ACDP and has been provided to various veterinary diagnostic laboratories in Southeast Asia by FAO and WOAH.

1.1.1. Purpose

This assay can be used to detect viral DNA of all ASFV genotypes, including the genotype II lineage currently circulating in Africa, Europe, Asia and the Pacific, and genotype I and genotype II recombinants reported in China (Zhao *et al.*, 2022).

- DNA isolated from various sample types can be used (Section 1.1.2.).
- The King assay has been validated using European and African ASFV isolates belonging to different genotypes, including genotype II (King *et al.*, 2003; Gallardo *et al.*, 2015).
- The assay cannot be used to determine genotype. Additional conventional PCR testing and sequencing for p72 genotyping is required using specific primers.
- No cross-reactions with other porcine viruses have been detected.

1.1.2. Samples

Recommended samples are whole blood in EDTA anti-coagulant, serum and tissue (including spleen, lymph node, liver, tonsil, heart, lung, kidney and bone marrow). Blood swabs (dried or in transport medium) or dried blood spots on filter paper can also be useful samples. Blood collected in heparin-based anticoagulant can also be used for PCR testing, but should be diluted (e.g. 1:10) prior to extraction to remove the inhibitory effect of heparin.

Alternative sample types that can be used include oro-nasal swabs, deep throat swabs, and oral fluids from rope chew collections; however, viral loads may be lower in these samples than in blood and tissues. Oral fluids offer a convenient, non-invasive means of sampling at a herd or pen level and can be used for surveillance purposes (Grau *et al.*, 2015; Goonewardene *et al.*, 2022; Havas *et al.*, 2022). For dead pigs, the preferred collection sites are lymph nodes, spleen, bone marrow and lungs. Superficial inguinal lymph nodes are quick and easy to collect and can be used for testing dead pigs with minimum environmental contamination (Goonewardene *et al.*, 2022). Meat juice or tissue exudates can also be used (Onyilagha *et al.*, 2021; Okwasiimire *et al.*, 2023).

Samples should be kept as cold as possible, without freezing, during transit. Serum, tissue samples, and oral fluid should be stored frozen (-20° C or below) if processing and shipping is going to be delayed for more than 24 hours. Whole blood and blood swabs in medium should be stored at 4°C. Dried blood swabs can be stored at ambient temperature in low-gas-permeable plastic bags containing desiccant. Where the maintenance of cold chain is not possible, there are commercially available products that can assist in the stabilisation of samples for room temperature transport.

1.1.3. DNA Extraction

There are several commercially available nucleic acid extraction kits that are suitable for the extraction and purification of ASF viral DNA.

1.1.3.1. DNA Extraction from Clinical Specimens

Whole blood, undiluted or diluted 1:10, can be used for samples expected to contain high viral loads that may be inhibitory. Tissues should be homogenised (10% w/v) using standard protocols. For extraction of DNA from other sample types, refer to the product manual of the DNA extraction kit used.

1.1.3.2. DNA Extraction from Pork Products

This section describes a procedure for processing pork products to extract DNA for ASFV PCR testing. This method can be used on various products, such as dried pork, jerky, biltong, salami, ham, uncooked pork, sausages and dumplings. Feed additives such as spray-dried blood and plasma may also be tested by this method.

This extraction method has been optimised using the High Volume Extraction method of the MagMAX™ Viral RNA Isolation kit protocol. Optimised methods using other commercial extraction kits, including column-based kits, may also be suitable.

- In a class II biosafety cabinet, dissect the product into three equal portions. Using a sterile scalpel or scissors, excise or cut a ~0.5 g cube of tissue from each portion and chop finely using a scalpel blade and tweezers within a petri dish.

- Pool the chopped product and add ~0.5 g (i.e. one third of the original sample pool) into a sterile 15 ml tube containing 5 ml of phosphate-buffered saline and antibiotics for processing. Store the remainder of chopped product in a separate 5 ml tube at 4°C for subsequent re-sampling or confirmatory testing. For long-term storage, freeze at -80°C.

- Homogenise the sample with homogeniser beads or shards using a suitable homogeniser fitted with a 15 ml tube adaptor or with mortar and pestle. *Example settings: 6 m/s, 45 sec for 1 cycle using an MP Biomedical FastPrep homogenizer.*

- Centrifuge tubes at 1500 *g* for 2 min in a benchtop centrifuge and transfer 200 µl* of clarified homogenate into a separate microtube (avoid taking the fatty top layer of sample).

- Perform nucleic acid extraction according to the manufacturer’s instructions.

*This volume can be used for the High Volume Extraction method of the MagMAX™ Viral RNA Isolation kit protocol as well as the MagMax™ Pathogen RNA/DNA kit. The table below shows the difference in the proportion of sample and buffer used for high-volume extraction compared to normal extraction methods (using the MagMAX™ viral RNA Isolation kit). Similar ratios may be used for other commercial extraction kits.

	Sample mixture (µl) (Sample + lysis buffer)	Elution volume (µl)
Normal extraction	180 (50+130)	90
High-volume extraction	725 (200+525)	90

1.1.4. PCR Protocol

1.1.4.1. Kits and Controls

- The assay uses the AgPath-ID One-Step RT-PCR Kit. The inclusion of an RT step does not affect the assay performance. Alternative PCR or RT-PCR kits are expected to work as well, but are recommended to be verified prior to routine use.
- Run controls: a ‘no template’ control (NTC), as well as strong and weak positive ASFV DNA controls (PC).
- After preparation of the mastermix (Section 1.1.4.3.), 10 µl will be used for each reaction and 5 µl template DNA is to be added for a total volume of 15 µl. The volume can be adjusted to accommodate other detection equipment.

1.1.4.2. Preparation of Primer/Probe Mix

Primer	Sequence (5’ →3’)*	Volume per reaction (µl)	Final conc. (nM)
ASFV Fwd (15 µM)	CTGCTCATGGTATCAATCTTATCGA	0.3	300
ASFV Rev (15 µM)	GATACCACAAGATCRGCCGT	0.3	300
ASFV Probe (12.5 µM)	FAM- CCACGGGAGGAATACCAACCCAGTG-TAMRA	0.3	250

*The choice of fluorophores and quenchers will depend on local availability, costs, and the instrumentation used by individual laboratories.

1.1.4.3. Preparation of Mastermix

Reaction component	Volume (µl)
Nuclease-free water	1.0
2X RT-PCR buffer	7.5
25X enzyme mix	0.6
ASFV primer probe mix	0.9
Total volume mastermix	**10.0/ reaction tube or well**
Addition of template	**Volume (µl)**
Sample RNA	5.0
NTC	
PC	
Total volume of reaction mixture	**15.0 (µl)**

1.1.4.4. Programming the PCR Cycler

Standard RT-PCR amplification conditions are used. Cycling parameters can be adjusted to accommodate different kits and machines.

Setting	Temperature (°C)	Time	Cycle
Reverse transcription	45	10 min	1 cycle
Inactivation RT/ activation of Taq	95	10 min	
Denaturation	95	15 sec	45 cycles
Annealing and elongation*	60	45 sec	

*Fluorescence is recorded in the FAM-channel during annealing.

1.1.4.5. Analysis

- Positive result: Ct < 40 AND the appearance of specific amplification curves
- Negative result: Ct > 45
- Indeterminate result: Ct between ≥ 40 and 45
- If an indeterminate result is obtained, repeat the test to confirm.
- Failure to detect the positive control means the run is invalid.
- If 'no template' or negative controls are 'positive', cross-contamination may have occurred during reaction set-up or reagents may have been contaminated with ASFV DNA. The run is invalid.

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1.1.4.6. Example Test Worksheet

AFRICAN SWINE FEVER REAL-TIME PCR TEST WORKSHEET: KING ASSAY

Real-time PCR run #:	Date:
PCR operator:	Time:
Extraction date:	Extraction operator:
Extraction kit used:	Extraction kit batch #:
Remarks:	

When added, tick box	Mastermix	Volume per reaction (µl)	Volume for ..... reactions (µl)
	Nuclease-Free Water	1.0	
	2X RT-PCR Buffer (Ambion P/N AM1005) Kit Lot #:	7.5	
	25X RT PCR Enzyme Mix	0.6	
	ASFV Primer Probe Mix: Aliquot no	0.9	
	Total volume	**10.0**	
	Template DNA (Test sample, PC, or NTC)	5.0	
	Total volume per reaction	**15.0**	

AgPath thermocycling conditions:	1X 45°C 10 min, 95°C 10 min 45X 95°C 15 sec, 60°C 45 sec

96-well plate: SAMPLE IDs

POSITIVE CONTROL

Description (e.g. Strong/Weak positive)	Batch #	Expected result (e.g. Ct = 29-32)	Actual result

NEGATIVE CONTROLS

Description (e.g. NTC)	Batch #	Expected result	Actual result
		Negative	
		Negative	

1.2. PCR Protocol: CAHEC Triplex Assay

The protocol described below was developed by the Chinese Animal Health and Epidemiology Center for the multiplex detection of *MGF360/505, EP402R* (CD2v) and *B646L* (p72) genes of ASFV belonging to genotype II.

1.2.1. Purpose

This assay can be used to detect and identify viral DNA of genotype II ASFV variants containing genome deletions in the *MGF360/505* and *EP402R* (CD2v) genes (currently circulating in Asia).

- DNA isolated from various sample types can be used (Section 1.1.2.).
- This assay has been validated using Chinese ASFV isolates belonging to genotype II and candidate LAV viruses developed by CAHEC containing *MGF360/505* and *EP402R* (CD2v) gene deletions.
- The assay cannot be used to determine genotype. Additional conventional PCR testing and sequencing for p72 genotyping is required using specific primers.
- No cross-reactions with other porcine viruses have been detected.

1.2.2. Samples

Refer to Section 1.1.2.

1.2.3. DNA Extraction

Refer to Section 1.1.3.

1.2.4. PCR Protocol

1.2.4.1. Kits and Controls

- The assay uses the AceQ Universal U+ Probe Master Mix V2 (Vazyme Biotech Co., Ltd), or Luna® Universal Probe qPCR Master Mix (New England BioLabs Inc.). Alternative PCR or RT-PCR kits (as described above) are expected to work as well, but are recommended to be verified prior to routine use.
- Run controls: an NTC, as well as strong and weak positive ASFV DNA controls.
- After preparation of the mastermix (Section 1.2.4.3.), 20 µl will be used for each reaction and 5 µl template DNA is to be added for a total volume of 25 µl. The volume can be adjusted to accommodate other detection equipment.

1.2.4.2. Preparation of Primer/Probe Mix

Primer	Sequence (5' →3')*	Genome position of primer sequences^	Volume per reaction (µl)	Final conc. (nM)
CD2v_F (20 µM)	AGAAGAACAATGTCAGCATGATGAC	75103-75127	0.5	400
CD2v_R (20 µM)	CGACTGTAAGGCTTAGGAAGTAATGG	75161-75186	0.5	400
CD2v_P_VIC (10 µM)	VIC-CCACTTCCATACATGAACCATCTCCCAGA-BHQ1	75129-75157	1.0	400
MGF-14L_F (20 µM)	AGAAGACGGGGTTCGGATACAG	33532-33553	0.5	400
MGF-14L_R (20 µM)	GCAAATCCTGAATATGGGCTTATACG	33607-33632	0.5	400
MGF-14L_P_Cy5 (10 µM)	CY5-CTCCCAGTTCCGCACACAGCCGC-BHQ2	33563-33585	0.8	320
p72_F (20 µM)	AAGTTTCGGTACGCATTCTTTGT	105991-106013	0.4	320
p72_R (20 µM)	TATGACTGGGACAACCAAACACC	106072-106094	0.4	320
p72_P_FAM (10 µM)	FAM-ACAAGCGTGTAAACGGCGCCCTC-BHQ1	106045-106067	0.4	160

*The choice of fluorophores and quenchers will depend on local availability, costs, and the instrumentation used by individual laboratories.

^Reference sequence GenBank Acc. No. NC_044959.2.

1.2.4.3. Preparation of Mastermix

Reaction component	Volume (µl)
Nuclease-free water	2.5
2X PCR buffer	12.5
ASFV CD2v primer/probe mix	2
ASFV MGF-14L primer/probe mix	1.8
ASFV p72 primer/probe mix	1.2
Total volume mastermix	20.0/ reaction tube or well
Addition of template	**Volume (µl)**
Sample DNA	5.0
NTC	
PC	
Total volume of reaction mixture	**25.0 µl**

1.2.4.4. Programming the PCR Cycler

Standard RT-PCR amplification conditions are used. Cycling parameters can be adjusted to accommodate different kits and machines.

Setting	Temperature (°C)	Time	Cycle
Pre-incubation	50	2 min	1 cycle
Pre-denaturation	95	5 min	
Denaturation	95	15 sec	45 cycles
Annealing and elongation*	60	60 sec	

*Fluorescence is recorded in the FAM, VIC and Cy5 channels during annealing

1.2.4.5. Analysis

- Positive result: Ct < 35 for CD2v/MGF-14L/p72 AND the appearance of specific amplification curves for each assay
- Negative result: Ct ≥ 40
- Indeterminate result: Ct ≥ 35 and <40
- If an indeterminate result is obtained, repeat the test to confirm.
- Failure to detect the positive control means the run is invalid.
- If negative controls are 'positive', cross-contamination may have occurred during reaction set-up or reagents may have been contaminated with ASFV DNA. The run is invalid.
- Where the p72 assay is positive AND one or both of the MGF and CD2v assays are negative, this indicates that a variant ASFV has been detected.
- Where all assays are positive, this indicates that the parental or pandemic ASFV strain has been detected.

Interpretation	**Test results**		
	P72-FAM	**CD2v-VIC**	**MGF-Cy5**
ASFV pandemic strain positive	+	+	+
ASFV CD2v gene deletion strain positive	+	–	+
ASFV MGF gene deletion strain positive	+	+	–
ASFV CD2v and MGF gene double deletion strain positive	+	–	–
ASFV negative	–	–	–

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1.2.4.6. Example Test Worksheet

AFRICAN SWINE FEVER REAL-TIME PCR TEST WORKSHEET: CAHEC TRIPLEX ASSAY

Real-time PCR run #:	Date:
PCR operator:	Time:
Extraction date:	Extraction operator:
Extraction kit used:	Extraction kit batch #:
Remarks:	

When added, tick box	Mastermix	Volume per reaction (µl)	Volume for ..... reactions (µl)
	Nuclease-free water	2.5	
	2X RT-PCR Buffer **(AceQ #Q513-02 or NEB #M3004)** Kit lot #:	12.5	
	ASFV CD2v primer probe mix: Aliquot no.	2.0	
	ASFV MGF-14L primer probe mix: Aliquot no.	1.8	
	ASFV p72 primer probe mix: Aliquot no.		
	Total volume	**20.0**	
	Template DNA (Test sample, PC, or NTC)	5.0	
	Total volume per reaction	**25.0**	

Thermocycling conditions:	1X 50°C 2 min, 95°C 5 min 45X 95°C 15 sec, 60°C 1 min

96-well plate: SAMPLE IDs

POSITIVE CONTROL

Description (e.g. Strong/Weak positive)	Batch #	Expected result (e.g. Ct = 29–32)	Actual result

NEGATIVE CONTROLS

Description (e.g. NTC)	Batch #	Expected result	Actual result
		Negative	
		Negative	

1.3. PCR Protocol: ASFV I177L Assay

The protocol described below was developed by the USDA Agricultural Research Service at the Plum Island Animal Disease Center. It was developed for the specific detection of the ASFV-ΔI177L LAV candidate developed by the USDA (Velazquez-Salinas *et al.*, 2021). This virus has had the *I177L* gene deleted, resulting in an attenuated virus (Borca *et al.*, 2020).

1.3.1. Purpose

This assay can be used to discriminate between infected pigs and those vaccinated with the ASFV-ΔI177L LAV.

- DNA isolated from various sample types can be used (Section 1.1.2.).
- This assay should be used for further molecular characterisation of samples that have tested positive for p72 detection using either the King assay (Section 1.1.) or the Triplex assay (Section 1.2.).
- This assay has been validated using samples collected from uninfected pigs and from pigs infected with parental ASFV Georgia isolate (ASFV-G) or vaccinated with AFSV-ΔI177L. Analytical sensitivity was shown to be comparable to a validated USDA p72 assay (Zsak *et al.*, 2005). A false negative rate of 0% and a false positive rate of < 1% were found for diagnostic sensitivity and specificity, respectively.
- The assay cannot be used to determine genotype. Additional conventional PCR testing and sequencing for p72 genotyping is required using specific primers.
- No cross-reactions with other porcine viruses have been detected.

1.3.2. Samples

Refer to Section 1.1.2.

1.3.3. DNA Extraction

Refer to Section 1.1.3.

1.3.4. PCR Protocol

1.3.4.1. Kits and Controls

- The assay uses the TaqMan™ Universal PCR Master Mix (Applied Biosystems Cat. No. 4305719).

- Alternative PCR or RT-PCR kits (as described above) are expected to work as well, but are recommended to be verified prior to routine use.

- Run controls: an NTC, as well as strong and weak positive ASFV DNA controls.

- After preparation of the mastermix (Section 1.3.4.3.), 20 µl will be used for each reaction and 5 µl template DNA is to be added for a total volume of 25 µl. The volume can be adjusted to accommodate other detection equipment.

1.3.4.2. Preparation of Primer/Probe Mix

Primer	Sequence (5'→3')*	Volume per reaction (µl)	Final conc. (nM)
Forward	GAACTGGAAAAAACTTTAACGGC	0.1	200
Reverse	CCATTACCGGCAAGCTAGG	0.1	200
Probe	FAM-ACGGATCCCCCTTCGCATTTGA-MGB-NFQ	0.25	100

*The choice of fluorophores and quenchers will depend on local availability, costs, and the instrumentation used by individual laboratories.

1.3.4.3. Preparation of Mastermix

Reaction Component	Volume (µl)
Nuclease-free water	7.05
2X PCR buffer	12.50
ASFV I177L primer/probe mix	0.45
Total volume mastermix	**20.0/ reaction tube or well**
Addition of template	**Volume (µl)**
Sample DNA	5.0
NTC	
PC	
Total volume of reaction mixture	**25.0 µl**

1.3.4.4. Programming the PCR Cycler

The following PCR amplification conditions are used. Cycling parameters can be adjusted to accommodate different kits and machines.

Setting	Temperature (°C)	Time	Cycle
Pre-incubation (Uracil N-glycosylase activation)	50	2 min	1 cycle
Pre-incubation (polymerase activation)	95	10 min	1 cycle
Denaturation	95	15 sec	37 cycles
Annealing and elongation*	60	1 min	

*Fluorescence is recorded in the FAM channel during annealing.

1.3.4.5. Analysis

- Positive result: Ct < 37 AND the appearance of a specific amplification curve
- Negative result: Ct ≥ 37
- Failure to detect the positive control means the run is invalid.
- If negative controls are 'positive', cross-contamination may have occurred during reaction set-up or reagents may have been contaminated with ASFV DNA. The run is invalid.
- Samples that test positive for p72 in the King assay or Triplex assay and negative in the ASFV-I177L assay indicate the presence of the ASFV-ΔI177L LAV.
- Samples that test positive in the ASFV I177L assay and negative for MGF and/or CD2v indicate the presence of additional gene deletions.

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1.3.4.6. Example Test Worksheet

AFRICAN SWINE FEVER REAL-TIME PCR TEST WORKSHEET: ASFV I177L ASSAY

Real-time PCR run #:	Date:
PCR operator:	Time:
Extraction date:	Extraction operator:
Extraction kit used:	Extraction kit batch #:
Remarks:	

When added, tick box	Mastermix	Volume per reaction (µl)	Volume for ... reactions (µl)
	Nuclease-free water	7.05	
	2X RT-PCR Buffer **(Applied Biosystems #4305719)** Kit Lot #:	12.50	
	ASFV I177L Primer Probe Mix: Aliquot no.	0.45	
	Total volume	**20.00**	
	Template DNA (Test sample, PC, or NTC)	5.0	
	Total volume per reaction	**25.0**	
Thermocycling conditions:	1X 50°C 2 min 1X 95°C 10 min 37X 95°C 15 sec, 60°C 1 min		

96-well plate: SAMPLE IDs											

POSITIVE CONTROL

Description (e.g. Strong/Weak Pos)	Batch #	Expected result (e.g. Ct = 29-32)	Actual result

NEGATIVE CONTROLS

Description (e.g. NTC)	Batch #	Expected result	Actual result
		Negative	
		Negative	

1.4. PCR Protocol: ASFV MGF360-12L Assay

The protocol described below was developed by the USDA Agricultural Research Service at the Plum Island Animal Disease Center. It was developed for the specific detection of the *MGF360-12L* gene, one of six genes deleted form the ASFV-ΔMGF LAV developed by the USDA (O'Donnell *et al.*, 2015; Velazquez-Salinas *et al.*, 2021).

1.4.1. Purpose

This assay can be used to discriminate between infected pigs and those vaccinated with the ASFV-ΔMGF LAV.

- DNA isolated from various sample types can be used (Section 1.1.2.).
- This assay should be used for further molecular characterisation of samples that have tested positive for p72 detection using either the King assay (Section 1.1.) or the Triplex assay (Section 1.2.).
- This assay has been validated using samples collected from uninfected pigs and from pigs infected with parental ASFV-G virus or vaccinated with AFSV-ΔMGF. Analytical sensitivity was shown to be comparable to a validated USDA p72 assay (Zsak *et al.*, 2005). A false negative rate of 0% and a false positive rate of < 1% were found for diagnostic sensitivity and specificity, respectively.
- The assay cannot be used to determine genotype. Additional conventional PCR testing and sequencing for p72 genotyping is required using specific primers.
- No cross-reactions with other porcine viruses have been detected.

1.4.2. Samples

Refer to Section 1.1.2.

1.4.3. DNA Extraction

Refer to Section 1.1.3.

1.4.4 PCR Protocol

1.4.4.1. Kits and Controls

- The assay described uses the TaqMan™ Universal PCR Master Mix (Applied Biosystems Cat. No. 4305719).

- Alternative PCR or RT-PCR kits (as described above) are expected to work as well, but are recommended to be verified prior to routine use.

- Run controls: an NTC, as well as strong and weak positive ASFV DNA controls.

- After preparation of the mastermix (Section 1.4.4.3.), 20 µl will be used for each reaction and 5 µl template DNA is to be added for a total volume of 25 µl. The volume can be adjusted to accommodate other detection equipment.

1.4.4.2. Preparation of Primer/Probe Mix

Primer	Sequence (5→3')*	Volume per reaction (µl)	Final conc. (nM)
Forward	CATACCCTTCCCCTAAAGCTG	0.1	200
Reverse	CTACTGCTATGTCCTGGGC	0.1	200
Probe	FAM-ACCCTCTTCGAAAACATCAGCCCC-MGB-NFQ	0.25	100

*The choice of fluorophores and quenchers will depend on local availability, costs, and the instrumentation used by individual laboratories.

1.4.4.3. Preparation of Mastermix

Reaction Component	Volume (µl)
Nuclease-free water	7.05
2X PCR buffer	12.5
ASFV MGF360-12L primer/probe mix	0.45
Total volume mastermix	**20.0/ reaction tube or well**
Addition of template	**Volume (µl)**
Sample DNA	5.0
NTC	
PC	
Total volume of reaction mixture	**25.0 µl**

1.4.4.4. Programming the PCR Cycler

- The following PCR amplification conditions are used. Cycling parameters can be adjusted to accommodate different kits and machines.

Setting	Temperature (°C)	Time	Cycle
Pre-incubation (Uracil N-glycosylase activation)	50	2 min	1 cycle
Pre-incubation (polymerase activation)	95	10 min	1 cycle
Denaturation	95	15 sec	40 cycles
Annealing and elongation*	60	1 min	

*Fluorescence is recorded in the FAM channel during annealing.

1.4.4.5. Analysis

- Positive result: Ct < 40 AND the appearance of a specific amplification curve
- Negative result: Ct ≥ 40
- Failure to detect the positive control means the run is invalid.
- If negative controls are 'positive', cross-contamination may have occurred during reaction set-up or reagents may have been contaminated with ASFV DNA. The run is invalid.
- Samples that test positive for p72 in the King assay or Triplex assay and negative in the ASFV MGF360-12L assay indicate the presence of the ASFV- ΔMGF LAV.
- Samples that test positive in the ASFV MGF360-12L assay and negative for I177L and/or CD2v indicate the presence of additional gene deletions.

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1.4.4.6. Example Test Worksheet

AFRICAN SWINE FEVER REAL-TIME PCR TEST WORKSHEET: ASFV MGF360-12L ASSAY

Real-time PCR run #:	Date:
PCR operator:	Time:
Extraction date:	Extraction operator:
Extraction kit used:	Extraction kit batch #:
Remarks:	

When added, tick box	Mastermix	Volume per reaction (µl)	Volume for ... reactions (µl)
	Nuclease-free water	7.05	
	2X PCR buffer (Applied Biosystems #4305719) Kit Lot #:	12.50	
		0.45	
	ASFV MGF360-12L primer/probe mix: Aliquot no.		
	Total volume	**20.00**	
	Template DNA (Test sample, PC, or NTC)	5.0	
	Total volume per reaction	**25.0**	

Thermocycling conditions:	1X 50°C 2 min 1X 95°C 10 min 37X 95°C 15 sec, 60°C 1 min

96-well plate: SAMPLE IDs											

POSITIVE CONTROL			
Description (e.g. Strong/Weak Pos)	Batch #	Expected result (e.g. Ct = 29-32)	Actual result

NEGATIVE CONTROLS			
Description (e.g. NTC)	Batch #	Expected result	Actual result
		Negative	
		Negative	

1.5. PCR Protocol: ACDP ASFV Genotype I Assay

The protocol described below was developed by the ACDP for the specific detection of ASFV genotype I isolates to address the recent emergence of this type in China. This assay targets the *MGF505-3R* gene of ASFV.

1.5.1. Purpose

This assay can be used to detect and identify viral DNA of ASFV isolates belonging to genotype I.

- DNA isolated from various sample types can be used (Section 1.1.2.).
- This assay should be used for further molecular characterisation of samples that have tested positive for p72 detection using either the King assay (Section 1.1.) or the Triplex assay (Section 1.2.).
- This assay has been validated using a panel of reference isolates belonging to genotypes 1, 2, 7, 9 and 10, as well as diagnostic specimens. No cross-reactions with other genotypes tested were detected and no false positive results were found.
- No cross-reactions with other porcine viruses tested have been detected.

1.5.2. Samples

Refer to Section 1.1.2.

1.5.3. DNA Extraction

Refer to Section 1.1.3.

1.5.4. PCR Protocol

1.5.4.1. Kits and Controls

- The assay uses the AgPath-ID One-Step RT-PCR Kit. The inclusion of an RT step does not affect the assay performance. Alternative PCR or RT-PCR kits are expected to work as well, but are recommended to be verified prior to routine use.
- Run controls: an NTC, as well as strong and weak positive ASFV DNA controls.
- After preparation of the mastermix (Section 1.5.4.3.), 10 µl will be used for each reaction and 5 µl template DNA is to be added for a total volume of 15 µl. The volume can be adjusted to accommodate other detection equipment.

1.5.4.2. Preparation of Primer/Probe Mix

Primer	Sequence (5'→3')*	Volume per reaction (µl)	Final conc. (nM)
ASFV G1 Fwd (15 µM)	TCCCTGGGCCACTTAATCATT	0.3	900
ASFV G1 Rev (15 µM)	TGAAACGTCTCGCAGGAAATG	0.3	900
ASFV G1 Probe (12.5 µM)	FAM-AATGCAGTCCTGTGGACAAGACCCCAC-BHQ1	0.3	250

*The choice of fluorophores and quenchers will depend on local availability, costs, and the instrumentation used by individual laboratories.

1.5.4.3. Preparation of Mastermix

Reaction Component	Volume (µl)
Nuclease-free water	1.0
2X RT-PCR buffer	7.5
25X enzyme mix	0.6
ASFV G1 primer/probe mix	0.9
Total volume mastermix	10.0/ reaction tube or well
Addition of template	Volume (µl)
Sample RNA	5.0
NTC	
PC	
Total volume of reaction mixture	**15.0 µl**

1.5.4.4. Programming the PCR Cycler

Standard RT-PCR amplification conditions are used. Cycling parameters can be adjusted to accommodate different kits and machines.

Setting	Temperature (°C)	Time	Cycle
Reverse transcription	45	10 min	1 cycle
Inactivation RT/ activation of Taq	95	10 min	
Denaturation	95	15 sec	45 cycles
Annealing and elongation*	60	45 sec	

*Fluorescence is recorded in the FAM-channel during annealing.

1.5.4.5. Analysis

- Positive result: Ct < 40 AND the appearance of specific amplification curves
- Negative result: Ct > 45
- Indeterminate result: Ct ≥ 40 and ≤45
- If an indeterminate result is obtained, repeat the test to confirm.
- Failure to detect the positive control means the run is invalid.
- If negative controls are 'positive', cross-contamination may have occurred during reaction set-up or reagents may have been contaminated with ASFV DNA. The run is invalid.

1.5.4.6. Example Test Worksheet

AFRICAN SWINE FEVER REAL-TIME PCR TEST WORKSHEET: ACDP GENOTYPE I ASSAY

Real-time PCR run #:	Date:
PCR operator:	Time:
Extraction date:	Extraction operator:
Extraction kit used:	Extraction kit batch #:
Remarks:	

When added, tick box	Mastermix	Volume per reaction (µl)	Volume for ... reactions (µl)
	Nuclease-free Water	1.0	
	2X RT-PCR Buffer (Ambion P/N AM1005) Kit lot #:	7.5	
	25X RT PCR Enzyme Mix	0.6	
	ASFV Primer Probe Mix: Aliquot no.	0.9	
	Total volume	**10.0**	
	Template DNA (Test sample, PC, or NTC)	5.0	
	Total volume per reaction	**15.0**	

AgPath thermocycling conditions:	1X 45°C 10 min, 95°C 10 min 45X 95°C 15 sec, 60°C 45 sec

96-well plate: SAMPLE IDs

POSITIVE CONTROL

Description (e.g. Strong/Weak Pos)	Batch #	Expected result (e.g. Ct = 29-32)	Actual result

NEGATIVE CONTROLS

Description (e.g. NTC)	Batch #	Expected result	Actual result
		Negative	
		Negative	

2. Serology

Since variant forms of ASFV have been associated with lower virulence and non-lethal, subacute and chronic disease leading to seroconversion in infected animals, the use of serological diagnostic techniques for laboratory diagnosis has become important in regions where these viruses are circulating. Previous recommendations emphasised the importance of PCR as the frontline detection method for China and countries in Southeast Asia and the Indo-Pacific, where the acute form of ASF first appeared; however, the emergence of lower virulence forms warrants the wider use of serodiagnosis for disease investigation and surveillance. This manual provides guidance for the use and application of serological tests in these scenarios.

Antibodies against ASFV appear from 7–10 days post-infection and persist for long periods of time. In surviving pigs, high levels of antibodies are found, which can be present for years and are usually accompanied by intermittent viraemia that may not be detectable by PCR. For outbreaks caused by highly virulent strains of ASFV, serological testing is of lower diagnostic value, since most pigs die before antibody response. However, for disease caused by low or moderately virulent strains, serological testing is a valuable laboratory technique for disease diagnosis and determination of infection status. The presence of antibodies can indicate current or past infection. In countries where approved vaccines are used or where illegal vaccine use is suspected, current serological tests are unable to distinguish infection by wildtype and infection by vaccine strains. In ASF endemic areas, where attenuated and lower virulence viruses circulate, serological testing is valuable for identifying evidence of previous exposure to ASFV in animals experiencing subacute, chronic or subclinical infections, or those that have recovered. Since ASFV infection does not induce a robust neutralising antibody response, neutralisation tests are not recommended for antibody testing.

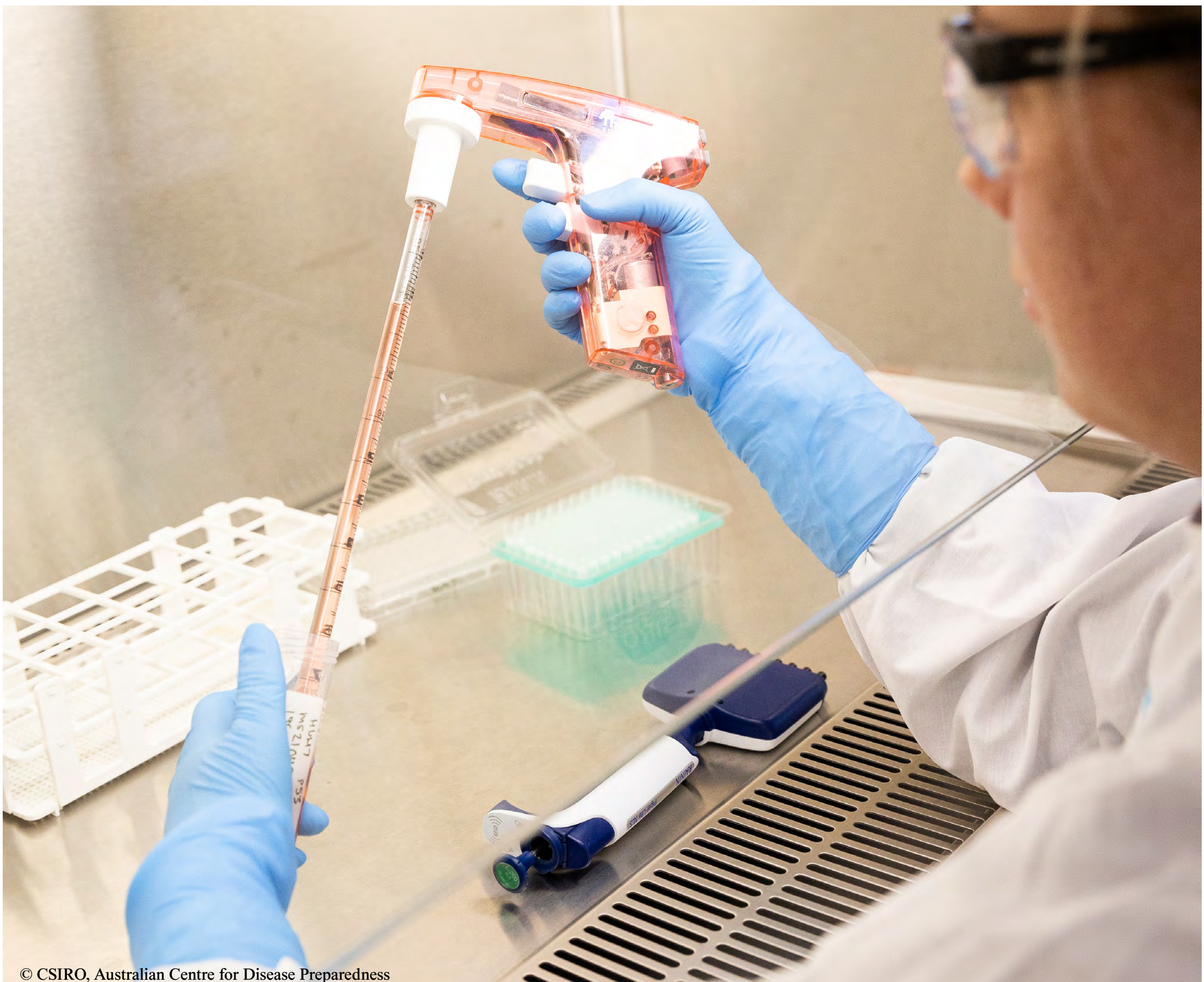
Of the laboratory tools available for ASFV antibody testing, enzyme-linked immunosorbent assay (ELISA) is the quickest and has the highest throughput, making it suitable as a screening test. Several commercial ELISAs as well as in-house ELISA protocols (in competitive or indirect formats) are available to reliably detect ASFV antibodies. ELISAs allow rapid, high-throughput testing and interpretation of results, and they can be adapted for automation where suitable robotic systems are available. ELISA tests can be prone to false-positive results, particularly when hemolysed samples are tested. Therefore, positive and suspicious samples should be subject to confirmatory testing, where possible, using additional methods such as the immunoperoxidase antibody (IPX), immunofluorescent antibody (IFA) or immunoblot (IB) tests. Due to their higher levels of sensitivity, these tests can also detect antibody at earlier stages of infection compared to some ELISAs.

IPX and IFA tests can be established using fixed infected cell monolayers on infected glass slides or in multi-well (e.g. 96-well) format for higher throughput. The IB test follows standard western blot protocols using solubilised infected cell extracts or recombinant virus proteins and can be used as an alternative or back-up test for IFA/IPX tests. However, these tests are more labour-intensive than ELISAs, and require highly skilled staff both to perform the test and to accurately interpret the test results. They also require access to ASFV propagation capability (Biosafety Level 3) or recombinant protein technology (for IB test).

WOAH-recommended protocols are available for an indirect (in-house) ELISA, and for IPX, IFA and IB tests (WOAH, 2019). In addition, point-of-care tests for antibody detection are described by the WOAH ASF Reference Laboratory Network in their overview of diagnostic tests for field application (WOAH, 2022).

2.1. Samples

In addition to serum, various specimen types contain antibodies and can be used for diagnostic testing, including whole blood, plasma, dried blood on filter paper, oral fluids and meat juice or tissue exudate. Oral fluids offer a convenient, non-invasive means of sampling at a herd or pen level and can be used for surveillance purposes (Mur *et al.*, 2013; Giménez-Lirola *et al.*, 2016). Similarly, meat juice or tissue exudate can be readily sampled at abattoirs or markets for detection of contaminated pork entering the food chain. ELISA tests can vary in sensitivity and specificity for testing alternative sample types other than serum (Gallardo *et al.*, 2015; Onyilagha *et al.*, 2021). It is therefore recommended to undertake verification testing beforehand or, in the case of commercially available ELISA kits, to ensure that the test has been validated for the sample intended for testing. The more sensitive formats such as the IPX or IFA tests are more suitable for testing oral fluids, which contain lower levels of antibody compared to serum.



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3. Algorithm for the Detection of ASF Virus

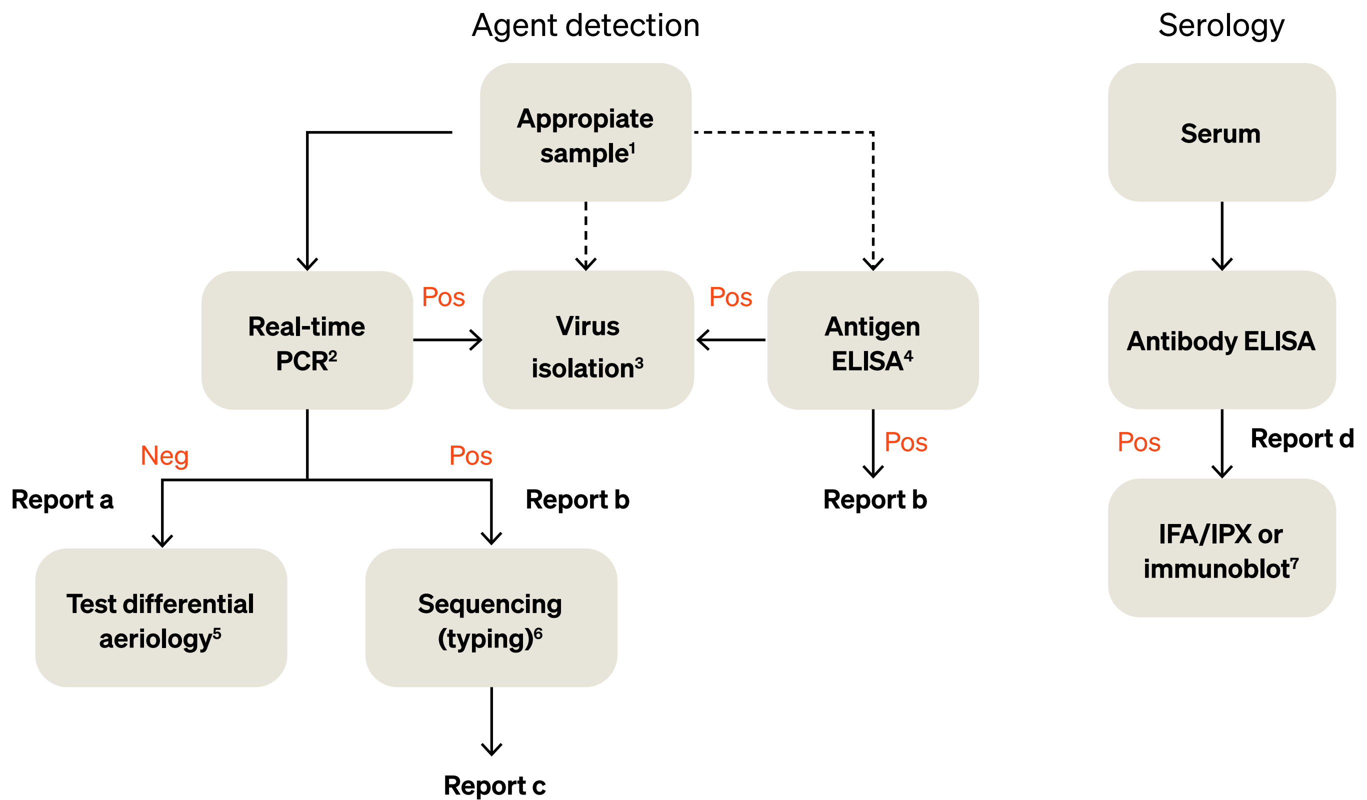

Figure 1. Algorithm for the detection of ASFV

Notes:

1. Clinical specimens (EDTA blood/serum, spleen, lymph nodes, tonsils, kidneys), pork products, meat juice/tissue exudate, oral fluids.

2. Refer to Sections 1.1. to 1.5. The use of a WOAH-recommended or validated PCR test (Table 2) capable of detecting all genotypes and variants of ASFV is recommended for initial testing. Where the circulation of genotype I or variant forms of genotype II associated with chronic, subacute or subclinical ASF is suspected, an additional test is recommended (Section 1.2. and Section 1.5.), as relevant. For testing to distinguish infected from vaccinated pigs, the Triplex PCR, ASFV I177L or ASFV MGF360-12L assays are recommended (Sections 1.2. to 1.4.).

3. Virus isolation requires the use of primary porcine cells that may not be available in all laboratories. Certain continuous cell lines have been reported that have comparable sensitivity to primary porcine cells (e.g. Portugal *et al.*, 2020; Masujin *et al.*, 2021; Rai *et al.*, 2021). Virus isolation is typically performed on PCR-positive samples. Virus replication in cell culture can be detected using one or a combination of the following tests: haemadsorption assay, antigen detection using an IFA or IPX antibody test, or PCR.

4. Antigen ELISA can be used for primary diagnosis if PCR is unavailable. It is also useful, in combination with PCR, for providing additional confidence that a diagnosis is correct (detection by two different methodologies), where sequencing is not readily available. Please note that antigen ELISA is generally less sensitive than PCR.

5. For differential diagnosis of negative ASFV diagnostic results, the following diseases should be considered: classical swine fever, porcine reproductive and respiratory syndrome, porcine dermatitis and nephropathy syndrome, erysipelas, salmonellosis, pasteurellosis, Aujeszky's disease (pseudorabies), and swine enteric coronavirus diseases (e.g. those caused by porcine endemic diarrhoea virus and transmissible gastroenteritis virus). Detection of other pathogens does not exclude an underlying ASFV infection. In addition, other non-infectious causes should be considered, including warfarin poisoning, fungal poisoning (aflatoxicosis, stachybotryotoxicosis), heavy metal poisoning, salt poisoning with water deprivation, and thrombocytopaenia purpura.

6. Perform genotyping at the start of an outbreak by amplifying and sequencing the *B646L* gene, encoding the ASFV p72 protein. Other gene targets may also be used for molecular characterisation, including one or more of the following: *B602L* central variable region, *I73R-I329L* intergenic region, *CP204L* (p30), and *E183L* (p54), as described in Bastos *et al.* (2003), Nix *et al.* (2006), Rowlands *et al.* (2008), and Gallardo *et al.* (2009, 2014). Next-generation sequencing (NGS) can also be used to obtain the sequence information for these gene targets. NGS can also be employed to determine whether an ASFV isolate is a recombinant virus. Conventional PCR targeting different genes belonging to the constituent genotypes making-up the recombinant can also be used, followed by sequencing and analysis. For example, for the genotype I/genotype II recombinant reported in China (Zhao *et al.*, 2023), conventional PCR and sequencing of the *B646L* or *B602L*, belonging to genotype I, and *E183L* or *EP402R* genes, belonging to genotype II, can be used to identify this recombinant.

7. Perform IFA, IPX or IB tests for confirmation or clarification.

   Reporting:
   a. No ASFV present in sample. Differential diagnosis optional.
   b. Report as ASFV detected in sample.
   c. Report as ASFV detected in sample with genotype/types.
   d. ASFV antibodies detected in sample. Further investigation for active infection in the herd is required or the extent of infection in the herd must be determined through serological survey. Assessment of the circulating virus strain causing infection should take into account PCR testing and/or sequencing and typing results. Prior use of vaccine (authorised or not) should also be considered.

4. Diagnostic Approaches for Different Scenarios

The following table provides a summary of different scenarios for ASF disease investigation and shows recommended laboratory diagnostic methods that may be employed in countries where ASF is endemic or where the disease is not present.

When ASFV is detected in a country previously free of ASF, samples should be submitted to a WOAH ASF Reference Laboratory for confirmatory testing and characterisation of the virus.

Purpose	ASF Status	Testing Method						
		Virus detection and identification					Antibody detection	
		ASFV generic PCR	Antigen ELISA*	ASFV variant/ DIVA PCR†	Sequencing/ typing	Virus isolation/ HAD	Antibody ELISA^	IPX/IFA/ IB
Outbreak investigation	Endemic	X	X	X	X	X	X	X
	Free	X	X		X	X	X	X
Active surveillance	Endemic	X	X	X			X	
	Free	X	X				X	X
Passive surveillance	Endemic	X	X	X			X	
	Free	X	X				X	X
Quarantine	Endemic						X#	
		X#	X#				X#	X

*Can be used for primary diagnosis if PCR is unavailable.
†Can be used where circulation of genotype I or variant forms of genotype II is suspected, or when there is a need to distinguish LAV from wildtype virus.
^Positive ELISA results should be confirmed by IPX, IFA or IB tests. Antibody ELISA can be used for outbreak investigations involving low or moderately virulent ASFV strains.
#Test at the beginning and at the end of the quarantine period.
IPX: immunoperoxidase antibody
IFA: immunofluorescent antibody
IB: immunoblot

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5. References

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