Food and Agriculture Organization of the United Nations

Addressing African swine fever

Laboratory protocols and algorithms

INTRODUCTION

The Food and Agriculture Organization of the United Nations (FAO) and the World Organisation for Animal Health (OIE) including other partners have been working in countries affected or at risk of incursion by African swine fever (ASF). This document was generated as guidance in response to the emergence of ASF in China, Southeast Asia, and the Pacific.

FAO has provided support for laboratory diagnosis of ASF following OIE recommendations, specifically using Polymerase Chain Reaction (PCR) in detecting ASF virus. PCR is a highly sensitive and specific method for the molecular detecting ASF virus for a wide range of purposes, including confirmation of clinical cases and confirmation of freedom from infection before movement. The Australian Centre for Disease Preparedness (ACDP, formerly the Australian Animal Health Laboratories) has developed a diagnostic algorithm based on OIE recommendations and in consultation with the Association of Southeast Asian Nations (ASEAN) regional animal health laboratory network.

This document describes a validated real time reverse transcription-polymerase chain reaction (RT-PCR) protocol (the 'King assay'), which targets the *B646L* gene, encoding the ASF virus structural protein p72. This assay has been produced in kit form by the ACDP and provided to various veterinary diagnostic laboratories in Southeast Asia by the FAO and OIE. This document also provides links to other reference documents. FAO has provided three categories of guidance for the laboratory testing of pig samples for the presence of ASF virus:

1. Overview of primers and probes
2. PCR protocols
3. Surveillance laboratory flow chart

1. Overview of primers and probes

Table 1. Primers and probes for the detection of ASF VIRUS in real-time PCR assays

ASF Assay	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Probe (5' → 3')	Dye	Quencher
King[1]	CTGCTCATGGTATCAATCTTATCGA	GATACCACAAGATCRGCCGT	CCACGGGAGGAATACCAACCCAGTG	FAM	TAMRA
UPL	CCCAGGRGATAAAATGACTG	CACTRGTTCCCTCCACCGATA	GGCCAGGA[2]	FAM	Dark quencher
USDA	CCTCGGCGAGCGCTTTATCAC	GGAAACTCATTCACCAAATCCTT	CGATGCAAGCTTTAT	FAM	MGB
McKillen	GTTGTTATGGAACGCGAAG	CGCTCCTAGCTGGAAAGAAAA	CTGAAAGTCCTCCGAGT	FAM	Eclipse Dark
Tignon	TGCTCATGGTATCAATCTTATCG	CCACTGGGTTGGTATTCCTC	TTCCATCAAAGTTCTGCAGCTCTT	FAM	TAMRA
Haines[3]	GATGATGATTACCTTYGCTTTGAA	TCTCTTGCTCTRGATACRTTAATATGA	CCACGGGAGGAATACCAACCCAGTG	Cy5	DDQII
Aguero[1,3,4]	AGTTATGGGAAACCCGACCC	CCCTGAATCGGAGCATCCT	NA	NA	NA

[1] Recommended tests by the OIE
[2] UPL#162 probe; Roche cat. No. 04694490001. If the UPL#162 probe is not available, it can be substituted by the following standard probe: 5'-(FAM)-TCCTGGCCRACCAAGTGCTT-(BHQ)-3' (OIE, 2019)
[3] Assay can be duplexed for Classical swine fever virus detection
[4] Conventional PCR

2. PCR protocol

The protocol described below is a recommended real-time RT-PCR procedure for the detection of ASF virus (King assay). The 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 may 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.

2.1 Purpose

This assay can be used to detect viral deoxyribonucleic acid (DNA) of all ASF virus genotypes, including the genotype II lineage currently circulating in Africa, Europe, Asia and the Pacific.

- DNA isolated from various sample types (e.g. blood, tissues and swabs, pork) can be used.
- The King assay has been validated using European and African ASF virus 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.

2.2 DNA extraction

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

i. DNA extraction from clinical specimens

Relevant clinical specimens include whole blood in anticoagulant (EDTA for PCR), serum and tissues, including spleen, lymph nodes, bone marrow, lung, tonsil and kidney. Whole blood can be used undiluted or diluted 1:10 for samples expected to contain high viral loads that may be inhibitory. Tissues should be homogenised (10 percent w/v) using standard protocols.

ii. DNA extraction from pork products

This section describes a procedure for processing pork products to extract DNA for ASF virus PCR testing. This method can be used on various products such as, but not limited to dried pork, jerky, biltong, salami, ham, uncooked pork, sausages and dumplings.

This extraction method has been optimised using the High-Volume Extraction method of the MagMAX™ Viral RNA Isolation kit protocol (ThermoFisher Scientific, undated) but can be adapted for other commercial extraction kits.

- In a class II biosafety cabinet, dissect the product into three equal portions. Using 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 original sample pool) into a sterile 15 ml tube containing 5 ml PBS and antibiotics for processing. Store the remainder of chopped product in a separate 5 ml tube at 4 ^{o}C for subsequent re-sampling or confirmatory testing. For long term storage freeze at -80 ^{o}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: 6m/s, 45sec for 1 cycle using a MP Biomedical FastPrep homogenizer.

- Centrifuge tubes at 1500 *g* for 2 min in a benchtop centrifuge and transfer 200 µl[5] of clarified homogenate into a separate microtube (avoid taking the fatty top layer of sample).
- Perform nucleic acid extraction according to manufacturer's instructions.

Table 2. Difference in proportion of sample and buffer used for high-volume extraction

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

2.3 PCR protocol

iii. Kits and controls

- The assay described uses the AgPath-ID One-Step RT-PCR Kit. The inclusion of a reverse transcription (RT) step does not affect the assay performance. Alternative PCR or RT-PCR kits are expected to work as well, but procedures will likely need to be adjusted to accommodate different settings required by other kits.
- Run controls: a 'no template' control (NTC), as well as strong and weak positive ASF virus DNA controls (PC).
- After preparation of the mastermix (see Table 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.

iv. Preparation of primer/probe mix

King PCR assay (300 nM primers and 250 nM probe)

Table 3. Primer/Probe preparation mix

Primer	Sequence (5' → 3')	Volume per reaction (µl)
ASF VIRUS Fwd (18 µM)	CTGCTCATGGTATCAATCTTATCGA	0.42
ASF VIRUS Rev (18 µM)	GATACCACAAGATCRGCCGT	0.42
ASF VIRUS Probe (5 µM)	FAM- CCACGGGAGGAATACCAACCCAGTG-TAMRA	1.25

[5] This volume is used for the High-Volume Extraction method of the MagMAX™ Viral RNA Isolation kit protocol. **Error! Reference source not found.** shows the difference in proportion of sample and buffer used for high-volume extraction compared to normal extraction methods for the MagMAX™ viral RNA Isolation kit. Similar ratios may be used for other commercial extraction kits.

vi. Preparation of mastermix

Table 4. Preparation of mastermix

Reaction Component	**Volume (µl)**
Nuclease-Free Water	4.41
2X RT-PCR Buffer	12.5
25X Enzyme Mix	1.0
ASF Primer Probe Mix	2.09
Total volume mastermix	20.0/ reaction tube or well
Addition of template	**Volume (µl)**
Sample RNA	5.0
NTC	
PC	
Total volume of reaction mixture	**25.0 µl**

vii. Programming the PCR cycler

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

Table 5. Cycling parameter settings

Setting	**Temperature (degree Celsius)**	**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[6]	60	45 sec	

viii. Analysis

- Positive result: Ct less than 40
- Negative result: Ct greater than 45
- Indeterminate result: Ct between >40 and 45
- If 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 ASF virus DNA. The run is invalid.

[6] Fluorescence is recorded in the FAM-channel during annealing

2.4 Example test worksheet

African swine fever real time-PCR test worksheet

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	4.41	
	2X RT-PCR Buffer (Ambion P/N AM1005)[1] Kit Lot #: ……	12.5	
	25X RT PCR Enzyme Mix	1	
	ASF Primer Probe Mix: Aliquot no. ……..	2.09	
	Total volume	**20.0**	
	Template DNA (Test sample, PC, or NTC)	5.0	
	Total volume per reaction	**25.0**	

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	

2.5 Algorithm for the detection of ASF virus

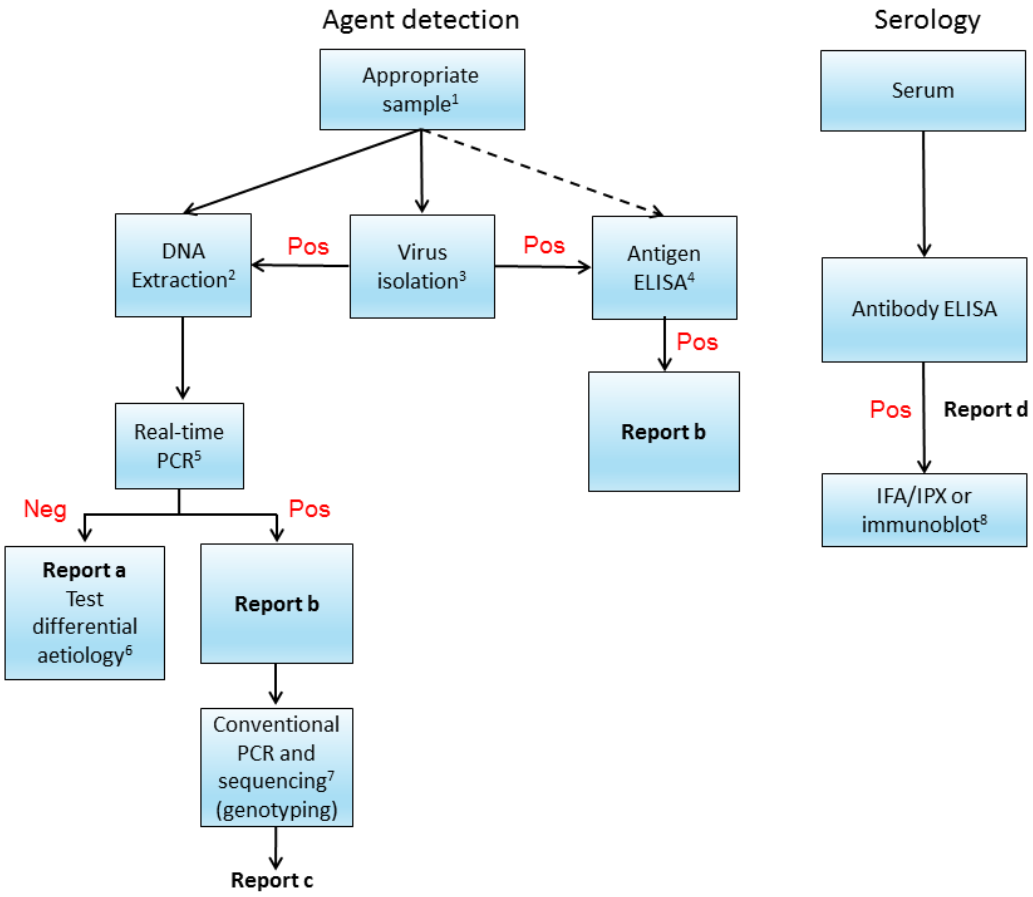

Figure 1. Algorithm for the detection of ASF virus

Notes:

1. Clinical specimens (EDTA blood/serum, spleen, lymph nodes, tonsils, kidneys) or pork products
2. Refer to section 2.2 DNA extraction
3. Virus isolation requires the use of primary porcine cells that may not be available in all laboratories. If no virus isolation, RT-PCR alone is recommended for primary laboratory diagnosis.
4. Antigen ELISA can be used for primary diagnosis if RT-PCR is unavailable.
5. Refer to section 2.3 PCR protocol
6. For differential diagnosis of negative ASF virus diagnostic results, the following diseases should be considered: Classical swine fever, Porcine reproductive and respiratory syndrome virus, Porcine dermatitis and nephropathy syndrome, erysipelas, salmonellosis, pasteurellosis, Aujeszky's disease (pseudorabies), 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 ASF virus 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.

7. Perform genotyping at the start of an outbreak by amplifying and sequencing the *B646L* gene, encoding the ASF VIRUS 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) (Bastos et al., 2003; Gallardo et al., 2009; Gallardo et al., 2014; Nix et al., 2006; and, Rowlands et al., 2008)
8. Perform immunofluorescence antibody (IFA), immunoperoxidase (IPX) or immunoblot tests for confirmation or clarification.

Report a – No ASF present in sample. Differential diagnosis optional.

Report b – Report as ASF detected in sample.

Report c – Report as ASF detected in sample with genotype.

Report d – ASF antibodies detected in sample. Further investigation for active infection in herd required or determination of extent of infection in herd through serological survey.

3. Additional information and references

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