NATIONAL CENTRE FOR ANIMAL HEALTH,
NATIONAL VETERINARY LABORATORY,
MICROBIOLOGY: VIROLOGY

Copy No.

Proprietary Statement: *This document contains proprietary information on Microbiology Unit National Veterinary Laboratory, NCAH. It is intended solely for the information and use by the authorized staff only. The proprietary information may not be used, edited, reproduced, shared or disclosed to any other parties for any purpose in any form without expressed written permission of the undersigned authority.*

SOP No.	Title	Version No.	Total Pages
NCAH/LAB/MOLE 12	**SOP for detection of Capripox Virus (including LSDV) by real time polymerase chain reaction**	1	6

Issue Date	Effective Date	Review Period
01-11-2020	01-11-2020	

Function	Name	Designation	Signature
Prepared by	Puspa M Sharma	Sr. Laboratory Officer	
Verified by	N.K Thapa	Head, LSU	
Approved by	R.B. Gurung	Program Director, NCAH	

Distribution:			
Location	Molecular section, NCAH		
REVISION SUMMARY			
Version No	**Effective Date**	**Reason for Change**	**Details of the change**
01	01-10-2020	Original release	

A. *Purpose:*

The purpose of this procedure is rapid detection of capripoxvirus (CaPV) genome from clinical samples by the real-time polymerase chain reaction (PCR) technique.

B. *Scope:*

This procedure can be applied in any kind of ruminant clinical samples such as EDTA-blood, serum and tissue homogenates and in cell culture supernatants. It is to support diagnosis of CaPV in Bovine, Caprine and Ovis species using real time RT-PCR test

C. *Test principles*

In RT-PCR, the DNA is used as a template for exponential amplification using PCR. RT-PCR is currently the most sensitive method of RNA/DNA detection available. It can be used for both qualitative analysis and quantitative analysis. In Fluorescent End-Point PCR, the amplified product is detected using fluorescent dyes. These dyes are usually linked to oligonucleotide probes which bind specifically to the amplified product during thermocycling. A multichannel rotor-type fluorometer is specially designed to detect fluorescence emission from the fluorophores in a reaction mixture after PCR. It allows detection of the accumulating product without re-opening the reaction tubes after the PCR run. The method was adapted from Bowden et al., 2008 for detection of all CaPV including Lumpy skin disease virus (Babiuk, et al., 2008). The assay uses a dual labelled fluorogenic (TaqMan®) probe and primers specific to capripoxvirus.

D. *Equipment and Materials:*

1. Equipment:

- QuantStudio-5/real time PCR machine
- MINI spin / micro -centrifuge for Eppendorf tubes
- Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
- Heating block/water bath
- Freezers -20^{o}C
- Freezer -80^{o}C
- Fridge 2-8^{o}C
- Vortex
- Bio-Safety Cabinet, Class –II

2. Materials:

- Single channel pipette 1-10µl.
- Single channel pipette 2-20µl.
- Single channel pipette 20-200µl.
- Single channel pipette 100-1000µl.
- Micropipette tips of 1-200 and 200-1000 µl, sterile.
- Micropipette tips with aerosol resistant filter of 1-10, 2-20, 20-200 and 100- 1000 µl, sterile.
- Microcentrifuge tubes of volumes 0.2, 0.5, 1.5, and 2 ml, sterile.
- DNA extraction kit , Qiagen
- Ethanol 100%, Merck
- AgPath-ID, One-Step RT-PCR Reagents, Catalogue number: 4387391
- Distilled H2O, sterile, PCR grade.
- Positive control; Known diluted ASF sample
- Negative controls: Nuclease free water
- Latex or nitrile gloves
- Biohazard bag

3. Reagents:

- Forward primer CaPV074F1 5'-AAA ACG GTA TAT GGA ATA GAG TTG GAA-3'
- Reverse primer CaPV074R1 5'-AAA TGA AAC CAA TGG ATG GGA TA-3
- Probe: CaPV074P1 5'-FAM-TGG CTC ATA GAT TTC CT-MGB/NFQ-3'
- Positive Control: reference LSDV strain and reference CaPV strain
- Negative control: Nuclease free water

E. Sample:

- Scab, skin nodules, nasal swab, mouth swab, ocular swab, blood in EDTA used for extraction of DNA

F. Procedures:

1. Extraction of DNA (Template DNA) using DNA Extraction – Qiagen DNeasy kit

1. Isolate a suitable piece of tissue and place in a UV-crosslinked 1.5mL tube.
2. Add 180 ml Buffer ATL and 20ml Proteinase K and vortex.
3. Place in the 55 °C incubator for 3 hours or overnight.
4. Remove from incubator, vortex, add 200ml Buffer AL and vortex.
5. Place in heat block at 70 °C for 10 minutes.

6. Add 200ml 100% Ethanol and transfer entire volume onto spin column.
7. Centrifuge at 8000 rpm for 1 minute; discard flow-through.
8. Add 500ml Buffer AW1 and centrifuge at 8000 rpm for 1 minute; discard flow-through.
9. Add 500ml Buffer AW2 and centrifuge at 13000 rpm for 3 minutes; discard flow through.
10. Place spin column on UV-crosslinked 1.5mL tube, add 200ml buffer AE. Let sit for 1 minute, then centrifuge at 8000 rpm for 1 minute. Repeat and then combine flow-throughs for a total volume of 400ml
11. Store the extracted DNA at 4^{o}C for immediate use, otherwise at -80^{o}C for long term

The RNA extraction kit from Qiagen can be used for DNA extraction in cases of non-availability of DNA extraction kit.

G. DNA amplification

1.1 Master mix preparation

In a sterile 1.5 ml Microcentrifuge tube prepare the PCR reaction mixtures described below for the number of samples to be assayed.

-

Table 1: Master mix preparation using qPCR Master Mix-Path ID

Reagent	**Final Conc**	**µL per reaction**
DNAse/RNAse free water		6.7
Forward primer- 20 µM	400 nM	0.4
Reverse primer-20 µM	400 nM	0.4
Probe- 10 µM	250 nM	0.5
2X qPCR Master Mix-Path ID	1X	10
Total volume		18 µL

(Or)

Table 2: Master mis preparation using AmpliTaq Gold DNA Polymerase

Reagent.	Final Conc	µL per reaction
DNAse/RNAse free water		2.7
Forward primer- 20 µM	400 nM	0.4
Reverse primer-20 µM	400 nM	0.4
Probe- 10 µM	250 nM	0.5
2X RT-PCR buffer mix	2X	12.5
AmpliTaq Gold DNA Polymerase	1X	1
ROX reference dye		0.5
Total volume		18 µL

Add 18 µl of the PCR reaction mix to the required number of 0.2 ml optical PCR tubes including the positives controls and the negative controls, adding at least for one additional sample to minimize pipetting mistakes.

Sample addition:

- Add 2µl of DNA template to each PCR tube. Include positive control (2 µl of LSDV/CaPV DNA) and negative control (2 µl of nuclease free water)
- After addition of the template, close the reaction tube and spin down the PCR mix.
- Place all tubes in an automated real-time thermocycler.
- Run the incubation program detailed below

PCR cycle condition

- 1X 95°C 10 min,
- 45X 95°C 15 sec, 60°C 45 sec (Read)
- Program the fluorescence collection in FAM channel and quencher as MGB/NFQ

H. Analysis and interpretation of results

Interpretation of the results:

In a positive sample, a sigmoid-shaped amplification curve will be obtained, indicating the cycles number versus read fluorescence level, where the Ct value will be under 40.

A negative sample will maintain the fluorescence profile under background fluorescence level and the equipment will not report any Ct value. Therefore, a negative sample will show a Ct value ≥40

I. Critical points

Because of PCR is a highly sensitive technique, the most critical point along all the analysis procedure is the considerable risk of carry-over contamination, and the false positive results that could be obtained in this situation. The contamination could be due to the ASFV itself present in the positive analysed samples or in the positive controls included in the DNA extraction procedure; also, it could be due to ASFV DNA obtained after amplification of a previous PCR. It is mandatory that personnel working on PCR follow and carry out some strict work rules in order to minimize the contamination risk associated to PCR technique:

♣ All steps of sample analysis by PCR should be performed in separate locations, using equipment and material specific for each one: sample preparation, DNA extraction, PCR mix preparation, and removal of PCR products.

♣ Personnel must work always with clean nitrile or latex gloves in the PCR laboratory.

♣ Change of gloves whenever personnel go into a different PCR area,

♣ Tubes containing amplified product should never be opened and manipulated in other laboratory distinct to that exclusively assigned to their analysis by electrophoresis, where they will be discarded.

J. Waste disposal

All the wastes should be discarded after being autoclaved.