NATIONAL CENTRE FOR ANIMAL HEALTH
NATIONAL VETERINARY LABORATORY
STANDARD OPERATING PROCEDURE

SOP No: NCAH/LAB/MOLE 01
Title: SoP on Sample Collection for Molecular Diagnosis
Version No: 1, Total Pages: 5
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Revision Summary: Added bone marrow sampling for ASFV
Supersedes Version No:2024.01
Prepared by: Molecular Section, LSU,NCAH
Reviewed by: Tshering Choden, Sonam Tshering, Kelzang Lhamo
Approved by:
Application/Distribution: NCAH, RLDC (kanglung)

1. Scope

This procedure can be applied in any kind of sample collection for diagnosis of animal diseases using PCR technology.

2. Objective

This document outlines the methods for sample collection, storage and transportation of the samples from the affected animals to the laboratory for molecular detection of the pathogens.

3. Equipment and Consumables

General materials

- Labels and permanent markers
- Data collection forms, pens, clipboards
- Sharps bin for needle and scalpel disposal
- Autoclavable disposal bags
- Forceps
- Swabs
- Sterile container with PBS/VTM
- Disinfectant (2% Virkon/bleach)

Personal Protective Equipment

- Dedicated clothing (coveralls)
- Rubber boot
- Boot cover
- Gloves
- Facemasks
- Safety glasses for eye protection
- Hand disinfectants
- Boot disinfectant

Materials for sample transport

- Primary containers/sterile tubes/vials (leakproof and clearly labelled)
- Absorbent
- Cool box /Styrofoam box filled with cooling materials (ice, frozen water bottles, or cold packs)

4. Sampling

4.1 Sampling materials for live animals

- Materials for restraining animals
- Cotton wool and disinfectant to clean sampling site
- Sterile vacutainers (10 ml) without anticoagulant (red stoppers) for serum collection
- Sterile vacutainers (10 ml) with EDTA (purple stoppers) for whole-blood collection
- Vacutainer holders and vacutainer needles or 10-20 ml syringe
- Swab
- Injectable local anesthetics, disposable biopsy punches or scalpels and suture material if full-thickness skin samples are to be collected from live animals

4.2 Materials for post-mortem sampling

- Sample racks or cryo-boxes for cryo-vial
- Sterile cryovials of appropriate size for organ collection (can be prefilled with medium for sample preservation if the cold chain is not optimal)
- Knives, knife sharpeners, shears, scalpels and blades, forceps and scissors
- Containers with disinfectant for disinfecting knives, scissors, etc. between organs and between animals, to avoid cross-contamination
- Securely sealable plastic pots filled with 10% neutral buffered formalin (1:10 organ volume: formalin volume ratio)
- Appropriate materials for carcass disposal

5. Procedure

Sampling from Live animals

- Swabs from nodular fluids/discharges from nasal, mouth and ocular sites and preserve in VTM or PBS

Skin Nodular Lesions- Skin scrapings/ Scabs

- Collect skin biopsy from skin nodules or scabs (2-4 numbers) preferably from the upper body surface using sterile forceps or swabs. Place it in a sterile container with viral transport medium (VTM) or sterile phosphate buffer saline (PBS) and store at refrigerated temperature (4°C) and ship immediately in a cool box with ice. If the shipping period is >48 hrs., store in -80°C
- Use local ring block-anesthesia if you surgically collect full-thickness samples from skin lesions – disposable biopsy punches 16 to 17 mm in diameter can be used.

Whole Blood/Serum

- Collect a minimum of 5 ml of blood from the jugular or tail vein (coccygeal vein) in sterile vacutainers (10 ml) with EDTA (purple stoppers) and store at refrigerated

temperature (4°C) until shipping in ice. For serum, collect blood in vacutainer tubes without anticoagulant, stand to separate serum and store at 4°C

Sampling from Dead animals/Meat Samples

- In dead animals, samples should include lesions, lung lesions (including normal tissue), lymph nodes - mediastinal lymph nodes and other organs with nodular lesions.

Bone Marrow Sample Collection

- Select a long bone (commonly femur, humerus, or rib) from the carcass
- Clean the surface of the bone with disinfectant to avoid contamination
- Break or cut the bone using a sterile bone cutter or knife
- Expose the marrow cavity
- Using a sterile swab or spoon, collect the red bone marrow from inside the bon
- Place the marrow into a sterile screw-cap tube (with VTM if available)
- Label the sample clearly (animal ID, location, date). Keep the sample refrigerated at 4°C and transport it to the laboratory as soon as possible.

6. Shipment of samples

Sample Information

- Information and case history should always accompany the samples to the laboratory and should be placed in a plastic envelope on the outside of the shipping container. The sample submission form (See table 1) should be filled and submitted to the receiving laboratory along with the samples.

The recommended procedure for packing samples are as follows:

- Put the samples in a primary container with screw caps and wrap with paraffin film or adhesive tape individually to prevent leakage of fluid. The wrapping of primary containers should be carried out in clean surroundings. Put the primary container into a watertight, spill proof secondary container with absorbent cotton wool sufficient to absorb the entire contents of the primary container (in cases of leakage)
- Place the secondary container in an outer container. This should be a polystyrene foam box covered with a hard box or other appropriate containers (E.g. cool box).
- It is recommended that a freezer box/ice packs is put outside the secondary packaging to ensure that all materials are kept cool and not frozen during shipment. These packs should be pre-frozen at – 20 degrees centigrade before packaging.

Transportation of specimens

- The specimens should be forwarded to the laboratory by the fastest method available. If they can reach the laboratory within 48 hours, samples should be sent refrigerated

Safety

- When samples are taken from live animals, care should be taken to minimize distress to the animal and avoid injury to the animal handlers and sample collector
- All the materials used for sampling should either be autoclaved or safely disposed Disinfect the sample collection site and change needles, scalpels and gloves.

Waste Disposal

- All the waste generated during sample collection should be disposed appropriately based on the zoonotic and non-zoonotic nature of the cases.

References

- *Collection, submission and storage of diagnostic specimens. Chapter 1.1.2. WOAH The Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 2023. WOAH*

SOP No: NCAH/LAB/MOLE 02
Title: SoP on DNA Extraction for Molecular analysis
Version No: 2, Total Pages: 2
Issue Month/Effective Date: May/2026
Revision Summary:
Supersedes Version No:2024.01
Prepared by: Molecular Section, LSU,NCAH
Reviewed by: Tshering Choden, Sonam Tshering, Kelzang Lhamo
Approved by:
Application/Distribution: NCAH, RLDC (kanglung)

1. Scope

The scope of DNA extraction is to isolate nucleic acids from biological samples using validated laboratory methods. It ensures removal of impurities and preservation of nucleic acid integrity for downstream molecular applications.

2. Objective

The objective is to obtain high-quality DNA suitable for accurate analysis and reliable results. It supports diagnostic, surveillance, and research activities by providing reproducible nucleic acid material. Ultimately, the analysis contributes to improved animal health, laboratory capacity, and evidence-based policy decisions.

3. Procedure

i. *Processing of Animal Origin Samples—Homogenization*

a. Cut approximately 1 g of meat/ sausage/organs/bone marrow and mince/homogenize well with PBS in a mortar and pestle.
b. Centrifuge the suspension at 3000× g for 5 min
c. Use the supernatant for further testing.
d. Store at 4 °C until DNA extraction (within 24 h)

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

a. Take 200ul of the supernatant in a UV-crosslinked 1.5mL tube.
b. Add 180 ml Buffer ATL and 20ml Proteinase K and vortex
c. Place in the 55 ^{o}C incubator for 3 hours or overnight
d. Remove from incubator, vortex, add 200ml Buffer AL and vortex
e. Place in a heat block at 70 ^{o}C for 10 minutes
f. Add 200ml 98% Ethanol and transfer the entire volume onto the spin column.
g. Centrifuge at 8000 rpm for 1 minute; discard flow-through
h. Add 500ml Buffer AW1 and centrifuge at 8000 rpm for 1 minute; discard flow-through
i. Add 500ml Buffer AW2 and centrifuge at 13000 rpm for 3 minutes; discard flow through
j. Place spin column on UV-crosslinked 1.5mL tube, add 200ml buffer AE. Let it sit for 1 minute, then centrifuge at 8000 rpm for 1 minute. Repeat and then combine flow-through for a total volume of 400ml
k. Store the extracted DNA at 4^{o}C for immediate use, otherwise at -80^{o}C for long term

Note: The RNA extraction kit from Qiagen can be used for DNA extraction as well

SOP No: NCAH/LAB/MOLE 03
Title: SoP on detection of PCV2 by real time polymerase chain reaction
Version No: 2, Total Pages:5
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Revision Summary:
Supersedes Version No:2024.01
Prepared by: Molecular Section, LSU,NCAH
Reviewed by: Tshering Choden, Sonam Tshering, Kelzang Lhamo
Approved by:
Application/Distribution: NCAH, RLDC (kanglung)

1. Scope

This procedure can be applied in any kind of porcine clinical sample such as EDTA-blood, serum and tissue homogenates and in cell culture supernatants. It is particularly useful for identifying PCV2 DNA in porcine tissues that are unsuitable for virus isolation or antigen detection, because they have undergone putrefaction, or when there is good reason to believe that virus may have been inactivated before samples are received in the laboratory.

2. Objective

The purpose of this procedure is to rapidly detect the specific presence of Porcine circovirus 2 Virus (PCV2) DNA in porcine clinical material by the real-time polymerase chain reaction (PCR) technique using King et. al 2003, procedure.

3. Principle

Polymerase chain reaction (PCR) is a molecular genetic technique which allows the specific detection of PCV2 DNA by enzyme-based amplification of a short viral genome fragment defined by a specific primer set. Under controlled conditions, multiple copies of DNA are generated by the action of the DNA polymerase enzyme that add complementary deoxynucleotides (dNTPs) to a piece of DNA known as the "template". Real-time PCR is an advanced amplification method, which allows the automated detection of the amplified product, reducing the risk of carry-over contamination with increased specificity and in most cases, even sensitivity. The PCR method requires a first step of viral DNA extraction from the original material to be analysed, which will be the template for the PCR. In real-time PCR, the appearance of amplified products is monitored continuously, in special equipment, with the incorporation in the reaction mix of a fluorescent dye that will give a fluorescence signal in a proportional way to the amplicon accumulation. By determination of fluorescence signal intensity in each amplification cycle, a sigmoid-shaped curve, that represents the amplicon appearance along the PCR, will be obtained. The described PCV2 real-time PCR method uses a primer set and a specific TaqMan probe directed to a highly conserved region of the viral genome, VP72, which ensures the detection of a wide range of PCV2 isolates, belonging to all the 24 known virus genotypes. The primers amplify a DNA fragment of 250 bp, from nucleotide position 2041 to 2290 of the complete VP72 gene sequence of the reference strain BA71V (GenBank accession no. ASU18466). TaqMan probe employed for amplified product detection is labelled with a reporter at 5' end [6-carboxy-fluorescein (FAM)] and a quencher at 3' end [6-carboxy-tetramethyl-rhodamine (TAMRA)]. PCR is a rapid method, which can be performed in less than four hours, and highly sensitive, allowing the viral detection even before the appearance of clinical symptoms.

4. Equipment and Consumables

- QuantStudio-5/real time PCR machine
- MINI spin / micro -centrifuge for Eppendorf tubes
- Heating block/water bath
- Freezers -20^{o}C

- Freezer -80^{o}C
- Fridge 2-8^{o}C
- Vortex
- Bio-Safety Cabinet, Class –II
- 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 filters of 1-10, 2-20, 20-200 and 100- 1000 µl,
- Microcentrifuge tubes of volumes 0.2, 0.5, 1.5, and 2 ml, sterile.
- Ethanol 100%, Merck
- Primer probe sequence for porcine circovirus Type 2
- Forward primer 1 PCV2 5'- CGG ATATTGTAKTCCTGGTCGTA-3'
- Reverse primer 2 PCV2 5- CCTGTCCTAGATTCCCCTATTGATT- 3'
- Probe PCV2 FAM-5' CTAGGCCTACGTGGTCTACATTTC-3' -TAMRA
- AgPath-ID, One-Step RT-PCR Reagents, Catalogue number: 4387391
- Distilled H2O, sterile, PCR grade.
- Positive control; Known diluted PCV sample
- Negative controls: Nuclease free water
- Latex or nitrile gloves
- Biohazard bag

5. Procedure

5.1 *Refer NCAH/ LSU/MOLE-02 for extraction of viral DNA*

5.2 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.

Master mix reagents	1x volume (reaction 25ul)
Nuclease-Free Water	4.3
2X RT-PCR Buffer (Ambion P/N AM1005)	12.5
Primer Forward	0.8
Primer reverse	0.8
Probe	0.5
25X RT-PCR Enzyme MIX	1
Rox	0.1
Total Volume	20
Template DNA	5
FinalVolume	25

5.3 Add 20 µl of the PCR reaction mix to the required number of 0.2 ml optical PCR tubes including the positive controls and the negative controls, adding at least one additional sample to minimize pipetting mistakes

5.4 Sample addition

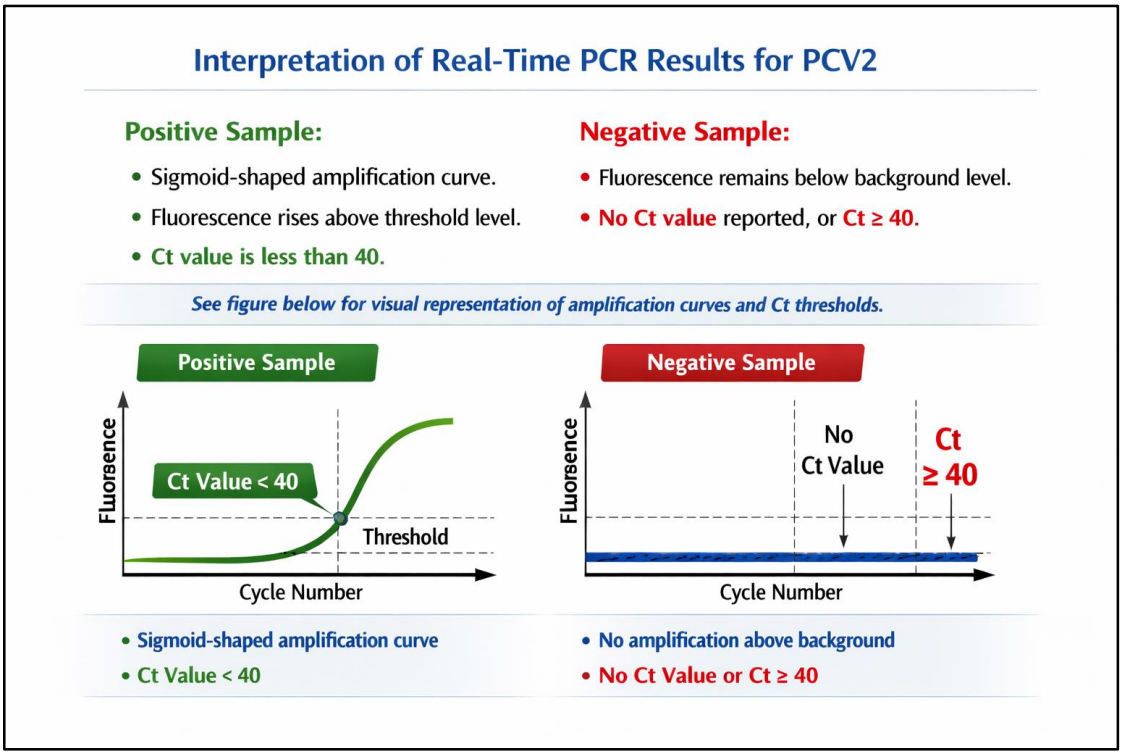
- Add 5µl of DNA template to each PCR tube. Include positive control (5 µl of PCV2 DNA) and negative control (5 µl of nuclease free water)
- After addition of the template, close the reaction tube and spin down the PCR mix.
- Place all tubes in an automated real-time thermocycler.
- Run the incubation program detailed below

5.5 PCR cycle condition

- 1X 45°C 10 min, 95°C 10 min
- 45X 95°C 15 sec, 60°C 45 sec
- Program the fluorescence collection in FAM channel and quencher as TAMRA

6. 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 (see figure). Therefore, a negative sample will show a Ct value ≥40.



7. Quality Control

- Test positive and negative controls every time while testing the samples.
- Use the same positive control and record the CT of the control at every run.

8. Waste Disposal

All the waste generated is disposed of in a waste container and autoclaved.

9. References

- SOP for PCV2 detection by real time PCR, National Center for Animal health, Laboratory service unit, Sop No. NCAH/LAB/MOLE 16, 2024
- Kwok, S., Higuchi, R. Avoiding false positives with PCR. *Nature* 339, 237–238 (1989). https://doi.org/10.1038/339237a0

SOP No: NCAH/LAB/MOLE 04
Title: SoP on detection of ASFV by real time polymerase chain reaction
Version No: 2, Total Pages:5
Issue Month/Effective Date: May/2026
Revision Summary:
Supersedes Version No:2024.01
Prepared by: Molecular Section, LSU,NCAH
Reviewed by: Tshering Choden, Sonam Tshering, Kelzang Lhamo
Approved by:
Application/Distribution: NCAH, RLDC (kanglung)

1. Scope

This SOP describes the procedure for detecting African swine fever virus from various porcine food products such as meat, sausages, organs, and meat exudates. It is particularly useful for identifying ASFV DNA in imported samples.

2. Objective

To rapidly detect the presence of African swine Fever Virus (ASFV) DNA in food products such as meat, sausages, organs, meat exudates by the real-time polymerase chain reaction (PCR) technique using King et. al 2003, procedure

3. Principle

Polymerase chain reaction (PCR) is a molecular technique which allows the specific detection of ASFV DNA by enzyme-based amplification of a short viral genome fragment defined by a specific primer set. Under controlled conditions, multiple copies of DNA are generated by the action of the DNA polymerase enzyme that adds complementary deoxynucleotides (dNTPs) to a piece of DNA known as the "template". Real-time PCR is an advanced amplification method, which allows the automated detection of the amplified product. The described ASFV real-time PCR method uses a primer set and a specific TaqMan probe directed to a highly conserved region of the viral genome, VP72, which ensures the detection of a wide range of ASFV isolates, belonging to all the 24 known virus genotypes. The primers amplify a DNA fragment of 250 bp, from nucleotide position 2041 to 2290 of the complete VP72 gene sequence of the reference strain BA71V (GenBank accession no. ASU18466). TaqMan probe employed for amplified product detection is labeled with a reporter at 5' end [6-carboxy-fluorescein (FAM)] and a quencher at 3' end [6-carboxy-tetramethyl-rhodamine (TAMRA)]. PCR is a rapid method, which can be performed in less than four hours, and highly sensitive, allowing the viral detection even before the appearance of clinical symptoms

4. Equipment and Consumables

- QuantStudio-5/real time PCR machine
- Micro centrifuge
- Heating block
- Freezers -20^{o}C
- Freezer -80^{o}C
- Fridge 2-8^{o}C
- Vortex
- Bio-Safety Cabinet, Class –II
- Single channel pipette 1-10ml, 2-20ml, 20-200ml, 100-1000ml
- Sterile aerosol resistant micropipette tips of 1-10ml, 2-20ml, 1-200ml and 200-1000 ml.
- Micro centrifuge tubes of volumes 0.2, 0.5, 1.5, and 2 ml, sterile.
- DNA extraction kit, Qiagen

- Ethanol 98%, Merck
- Forward primer ASFV 5'- CTG CTC ATG GTA TCA ATC TTA TCG A -3'
- Reverse primer ASFV 5- GAT ACC ACA AGA TCR GCC GT - 3'
- Probe ASFV 5′ FAM-CCA CGG GAG GAA TAC CAA CCC AGT G-TAMRA
- AgPath-ID, One-Step RT-PCR Reagents, Catalogue number: 4387391/ Invitrogen Platinum II Hot-Start PCR Master Mix
- Nuclease free water, PCR grade.
- Positive control; Known diluted ASF sample
- Negative controls: Nuclease free water
- Latex or nitrile gloves
- Biohazard bag

5. Procedure

5.1 Refer *NCAH/ LSU/MOLE-02 for extraction of viral DNA*

5.2 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.

Master mix reagents	1x volume (reaction 25ul)
Nuclease-Free Water	2.75
2X RT-PCR Buffer (Ambion P/N AM1005)	12.5
FAM-TAMARA PP MIX (ASFV Risatti PPMIX)	3.75
25X RT-PCR Enzyme MIX	1
Template DNA	5
Total Volume	25

Add 20 µl of the PCR reaction mix to the required number of 0.2 ml optical PCR tubes including the positive controls and the negative controls.

5.3 Sample addition

- Add 5µl of sample DNA template to each PCR tube. Close the reaction tube

- Add positive control (5 µl of ASFV DNA) and negative control (5 µl of nuclease free water) in a separate PCR tube. Close the reaction tube
- Spin down the PCR mix and ensure the caps are closed properly
- Place all tubes in an automated real-time thermo cycler.
- Run the incubation program/PCR cycle condition as detailed below

5.4 PCR cycle condition

- 1X 45°C 10 min, 95°C 10 min
- 45X 95°C 15 sec, 60°C 45 sec
- Program the fluorescence collection in FAM channel and quencher as TAMRA

Alternative reagent for DNA amplification

Master mix reagents	1x volume (reaction 25ul)
Nuclease-Free Water	3.7
Invitrogen Platinum II Hot-Start PCR Master Mix (2X)	12.5
ASFV-FAM-TAMARA PPP MIX	3.75
ROX Reference Dye	0.05
Total Volume	20
Template DNA	5
Final Volume	25

6. 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.

7. Quality Control

- Test positive and negative controls every time while testing the samples.

- Use the same positive control and record the CT of the control at every run.

8. Waste Disposal

- All the waste generated is disposed of in a waste container and autoclaved.

9. References

- SOP for ASFV detection by real time PCR, National Center for Animal health, Laboratory service unit, Sop No. NCAH/LSU/MOLE 16, 2024

SOP No: NCAH/LAB/MOLE 05
Title: SoP on detection of LSDV by real time polymerase chain reaction
Version No: 2, Total Pages:5
Issue Month/Effective Date: May/2026
Revision Summary:
Supersedes Version No:2024.01
Prepared by: Molecular Section, LSU,NCAH
Reviewed by: Tshering Choden, Sonam Tshering, Kelzang Lhamo
Approved by:
Application/Distribution: NCAH, RLDC (kanglung)

1. 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.

2. Objective

To rapidly detect the presence of LSDV DNA in food products such as meat, sausages, organs, meat exudates by the real-time polymerase chain reaction (PCR) technique.

3. 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.

4. Equipment and Consumables

- QuantStudio-5/real time PCR machine
- Micro centrifuge
- Heating block
- Freezers -20^{o}C
- Freezer -80^{o}C
- Fridge 2-8^{o}C
- Vortex
- Bio-Safety Cabinet, Class –II
- Single channel pipette 1-10ml, 2-20ml, 20-200ml, 100-1000ml
- Sterile aerosol resistant micropipette tips of 1-10ml, 2-20ml, 1-200ml and 200-1000 ml.
- Micro centrifuge tubes of volumes 0.2, 0.5, 1.5, and 2 ml, sterile.
- DNA extraction kit, Qiagen
- Ethanol 98%, Merck

- AgPath-ID, One-Step RT-PCR Reagents, Catalogue number: 4387391/ Invitrogen Platinum II Hot-Start PCR Master Mix
- Nuclease free water, PCR grade.
- Positive control; Known diluted ASF sample
- Negative controls: Nuclease free water
- Latex or nitrile gloves
- Biohazard bag
- 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
- Scab, skin nodules, nasal swab, mouth swab, ocular swab, blood in EDTA used for extraction of DNA

5. Procedures

5.1 Refer *NCAH/ LSU/MOLE-02 for extraction of viral DNA*

5.2 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.

-

Master mix preparation using qPCR Master Mix-Path ID

Reagent	**µL per reaction**
DNAse/RNAse free water	6.7
2X RT-PCR buffer mix	12.5
Forward primer- 20 µM	0.4
Reverse primer-20 µM	0.4
Probe- 10 µM	0.5
Rox	0.1
Total Volume	20
Template DNA	5
Final Volume	25

(Or)

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 positive controls and the negative controls, adding at least one additional sample to minimize pipetting mistakes.

5.3 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

5.4 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

6. Analysis and interpretation of 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

7. Waste disposal

- All the wastes should be discarded after being autoclaved.

SOP No: NCAH/LAB/MOLE 06
Title: SoP on Extraction of RNA for Molecular Analysis
Version No: 2, Total Pages:4
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Revision Summary:
Supersedes Version No:2024.01
Prepared by: Molecular Section, LSU,NCAH
Reviewed by: Tshering Choden, Sonam Tshering, Kelzang Lhamo
Approved by:
Application/Distribution: NCAH, RLDC (kanglung)

1. Scope

The scope of RNA extraction is to isolate nucleic acids from biological samples using validated laboratory methods. It ensures removal of impurities and preservation of nucleic acid integrity for downstream molecular applications.

2. Objective

The objective is to obtain high-quality DNA and RNA suitable for accurate analysis and reliable results. It supports diagnostic, surveillance, and research activities by providing reproducible nucleic acid material. Ultimately, extraction underpins molecular testing standards and strengthens laboratory capacity.

3. Equipment and Consumables

- MINI spin / micro -centrifuge for Eppendorf tubes
- Freezers -20^{o}C
- Freezer -80^{o}C
- Fridge 2-8^{o}C
- Vortex
- Bio-Safety Cabinet, Class –II
- 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 filters of 1-10, 2-20, 20-200 and 100- 1000 µl,
- Micro centrifuge tubes of volumes 0.2, 0.5, 1.5, and 2 ml, sterile.
- Ethanol 100%, Merck
- Distilled H2O, sterile, PCR grade.
- Latex or nitrile gloves
- Biohazard bag
- RNA Buffer I
- RNA Buffer II
- RNA-AVE buffer
- AVL buffer
- Elution Buffer
- PBS
- Pestle and Mortar, forceps and blades for tissue sample splicing

4. Procedure

Extraction of RNA (Template RNA)

i. Prepare RNA Buffer- (Add 310 µL AVE buffer to Carrier RNA-red cap). Store at - 20°C
ii. Prepare sample buffer- 560 µL AVL buffer with 5.6 µL RNA-AVE buffer (for one sample).
iii. Add 140 µL of sample to sample buffer
iv. Mix well and spin down briefly.
v. Incubate for 10 minutes at room temperature
vi. Add Ethanol (96%-100%) - 560 µL
vii. Spin down for 1min at 14,000 RPM
viii. Transfer 630 µL to mini spin column
ix. Spin down for 1min at 14,000 RPM
x. Add wash Buffer (1) - 500 µL
xi. Spin down for 1min at 14,000 RPM
xii. Add wash Buffer (2) - 500 µL
xiii. Spin down for 3 mins at 14,000 RPM
xiv. Add AVE buffer - 60 µL (incubate for 1 minute at room temperature).
xv. Spin down for 1min at 14,000 RPM
xvi. Store at -80°C until use.

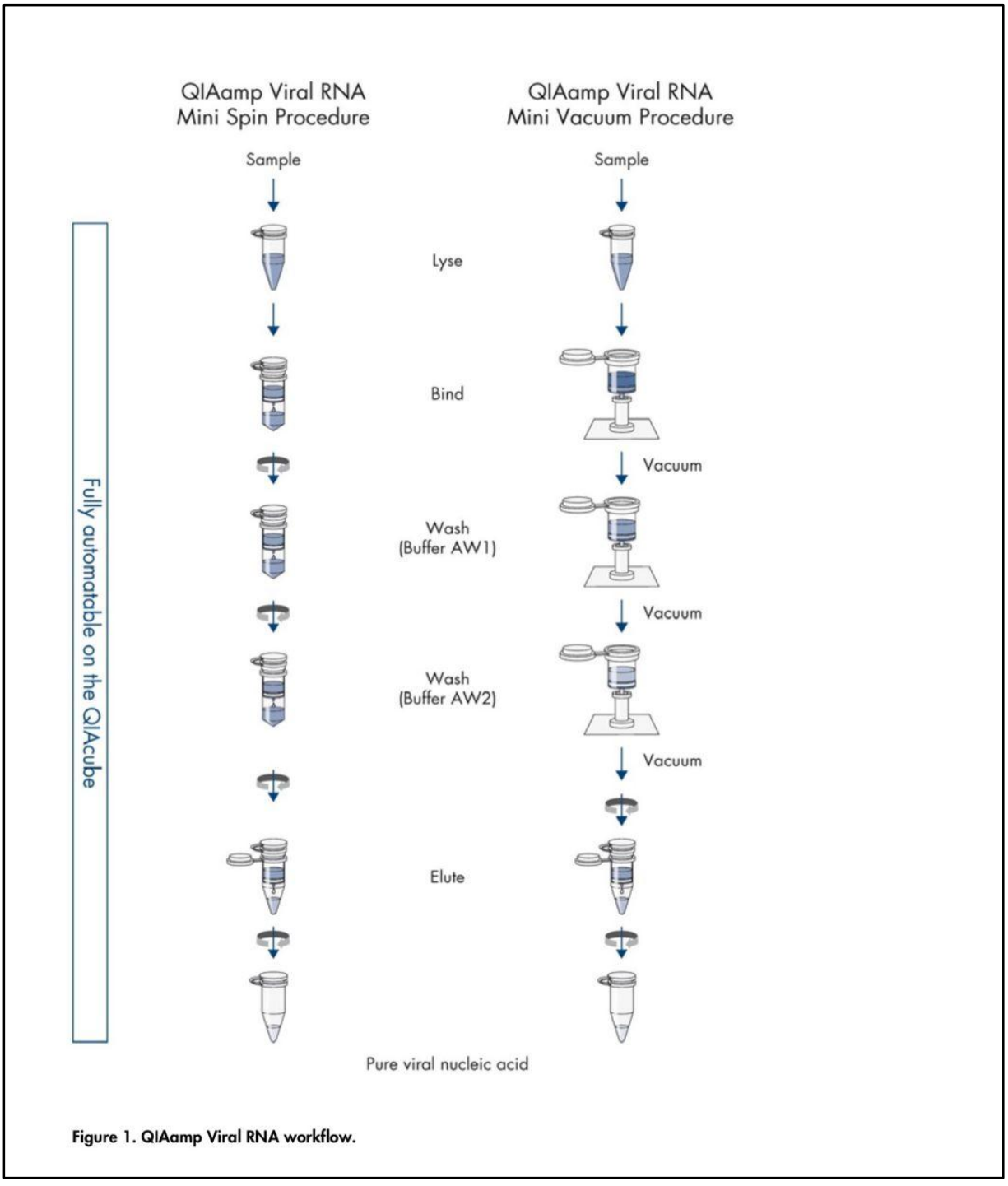

Figure 1. QIAamp Viral RNA workflow.

SOP No: NCAH/LAB/MOLE 07
Title: SoP on detection of FMDV by real time polymerase chain reaction
Version No: 2, Total Pages:5
Issue Month/Effective Date: May/2026
Revision Summary:
Supersedes Version No:2024.01
Prepared by: Molecular Section, LSU,NCAH
Reviewed by: Tshering Choden, Sonam Tshering, Kelzang Lhamo
Approved by:
Application/Distribution: NCAH, RLDC (kanglung)

1.Scope

Detection of matrix gene of FMD virus.

2.Objectives

This document is used to support diagnosis of FMD in cloven hooved animals by RT- PCR test.

3.Principle

In RT-PCR , the RNA template is first converted into a complementary_DNA (cDNA) using a reverse_transcriptase. The cDNA is then used as a template for exponential amplification using PCR. RT-PCR is currently the most sensitive method of RNA detection available. The quantification of mRNA using RT-PCR here is achieved in a one-step reaction. 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

4.Equipment and Consumables

- QuantStudio-5
- MINI spin, Eppendorf, AG-22331, USA
- Mini spin, Spinwin
- Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
- Bio-Safety Cabinet, Class II, Esco
- Pirbright TaqMan assay for detection of all types IRES conserved sequence
- Forward primer: FMDV SA-IR-219-246F: 5'-CACYTYAAGRTGACAYTGRTACTGGTAC-3'
- Reverse primer: FMDV SA-IR-315-293R: 5'-CAGATYCCRAGTGWCICITGTTA-3'
- Probe: FMDV SA-IR-292- 3 FAM-CCTCGGGGTACCTGAAGGGCATCC-TAMRA-3
- Tetracore TaqMan assay for detection of all types RNA polymerase (3D)
- Forward primer FMDV Tet 6769 F: 5'-ACTGGGTTTTACAAACCTGTGA-3'
- Reverse primer: FMDV Tet 6875 R: 5'-GCGAGTCCTGCCACGGA-3'
- Probe: FMDV Tet 6820-P: -FAM-TCCTTTGCACGCCGTGGGAC-TAMRA-
- Sample: Swabs/epithelial tissue/vascular fluid.
- Positive Control: known FMD positive sample
- Negative control: sterile water

5. Procedure.

5.1 Refer *SoP on Extraction of RNA for Molecular Analysis, NCAH/LAB/MOLE 06*

5.2 Master mix preparation

- NDV Real Time Taqman PCR master mix sheet, AgPath Reagents (Reagent of choice)

Reaction component	**Volume per reaction (µL)**	**Volume for 20 Rxn (µL)**
Nuclease-Free Water	2.76	55
2X RT-PCR Buffer (Ambion P/N AM1005)	12.5	250
25X RT-PCR Enzyme MIX	1	20
FAM-TAMARA PP MIX (Pirbright PP MIX)	1.4	28
FAM TAMARA PP MIX (Tetracore PP MIX)	2.34	46.8
Total Volume	20	400
Template RNA	5	
Final volume	**25**	

AgPath Thermal Cycling Parameter

- 1X 45°C 10 min, 95°C 10 min
- 45X 95°C 15 sec, 60°C 45 sec

OR

SuperScript III Reagent (Backup Reagent)

Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
Nuclease-Free Water	2.76	55
2X Reaction MIX (Invitrogen Cat No. 11731)	12.5	250
SuperScript III/Platinum *Taq* MIX	0.5	10
ROX Reference Dye	0.5	10
FAM-TAMARA PP MIX (Pirbright PP MIX)	1.4	28
FAM TAMARA PP MIX (Tetracore PP MIX)	2.34	46.8
Total Volume	20	400
Tempate RNA	5	
Final Volume	25	

SuperScript Thermal Cycling Parameter

- 1x50°C 15 min, 95°C 2 min
- 45x95°C 15 sec, 60°C 30 sec

5.3 Set up the reaction samples, internal positive control and Negative control (Nuclease free water) in separate tubes/wells. For quantitative analysis set up reaction samples, standards 1, 2, 3 and 4 and negative control (Nuclease free water) in separate tubes/wells, Set up the reporter dye as FAM for FMDV

6. Result Interpretation and Reporting.

- Ct (Threshold cycle) value of each sample can be read as follows
- Ct value result.
- >40 Negative
- < 37 Positive
- 37-40 Intermediate

7**. Quantitative Analysis**

- Assess the Ct value when the amplification curve of Standard 1, 2, 3, 4 passes the threshold line.
- Calculate quantitative value to compare with Ct value of unknown samples and curve of Standard 1, 2, 3, 4

Test validation, Each Ct value standard should be as follows.

- Standard 1 > Standard 2 > Standard 3 > Standard 4
- R-value of Standard curve should be 0.900~0.999.
- The Standard result should be all positive

8. Waste disposal.

All the waste should be disposed of after being autoclaved.

SOP No: NCAH/LAB/MOLE 08
Title: SoP on detection of AIV by real time polymerase chain reaction (AIV,H5,N1)
Version No: 2, Total Pages:9
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Revision Summary:
Supersedes Version No:2024.01
Prepared by: Molecular Section, LSU,NCAH
Reviewed by: Dr. Tshering Choden, Sonam Tshering, Kelzang Lhamo
Approved by:
Application/Distribution: NCAH, RLDC (kanglung)

1. Scope

Detection of type A, H5 and N1 specific genes of AIV by real time RT-PCR.

2. Objective.

This document is used to support diagnosis of AIV in chicken and other birds using real time RT-PCR test.

3. Principle.

In RT-PCR , the RNA template is first converted into a complementary_DNA (cDNA) using a reverse_transcriptase. The cDNA is then used as a template for exponential amplification using PCR. RT-PCR is currently the most sensitive method of RNA detection available. The quantification of mRNA using RT-PCR here is achieved in a one-step reaction. 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.

4.Equipment and Consumables

- Real-time PCR machine: QuantStudio-5
- MINI spin, Eppendorf, AG-22331, USA
- Mini spin, Spinwin
- Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
- Bio-Safety Cabinet, Class II, Esco
- One step real-time RT-PCR Kit (for example: QuantiTect® Probe RT PCR Kit Cat No. 204443 (QIAGEN) storage at -20°C)
- RNeasy Lipid Tissue Mini Kit (Qaigen USA)
- Primers

Influenza type A

- Forward primer VA D161M - AGATGAGYCTTCTAACCGAGGTCG-
- Reverse primer IVA D162M1 - TGCAAAAACATCYTCAAGTCTCTG-
- Reverse primer IVA D162M2 - TGCAAACACATCYTCAAGTCTCTG-
- Reverse primer IVA D162M3 - TGCAAAGACATCYTCAAGTCTCTG-
- Reverse primer IVA D162M4 - TGCAAATACATCYTCAAGTCTCTG-
- Probe: IVA MA -FAM TCAGGCCCCCTCAAAGCCGA-TAMRA-

Influenza type A,subtype H5.

- Forward primer IVA D204f - ATGGCTCCTCGGRAACCC -
- Forward primer IVA D148 H5 - AAA CAG AGA GGA AAT AAG TGG AGT AAAATT-
- Reverse primer IVA D205r - TTYTCCACTATGTAAGACCATTCCG-
- Reverse primer IVA D149 H5 - AAA GAT AGA CCA GCT ACC ATG ATT GC-
- Probe,:- FAM- TCAACAGTGGCGAGTTCCCTAGCA-TAMRA
- Probe:- FAM ATG TGT GAC GAA TTC MT-MGBNFQ-

Influenza A,subtype N1

- Forward primer AI_N1 1316F 5'- GYGGGAGCAGCATATCYTT-3'
- Reverse primer AI_N1 1379R 5'- CCGTCTGGCCAAGACCAA -3'
- Probe: N1-2(jvm)-p FAM-TGGTCTTGG GACGGTGC-BHQ1
- Sample: Swabs from Cloaca, Tracheal or Tissue
- Positive Control: known Influenza, H5 and N1 positive sample
- Negative control: nuclease free water.

5. Procedure.

5.1 Refer NCAH/LSU/MOLE 06 for the extraction of viral RNA

5.2 Influenza Type A Real-Time TaqMan PCR MasterMix Sheets, AgPath Reagents (Reagent of choice)

Reaction component	Volume per reaction (µL)	Volume for 20 Rxn (µL)
Nuclease-Free Water	4.5	90
2X RT-PCR Buffer (Ambion P/N AM1005)	12.5	250
25X RT-PCR Enzyme MIX	1	20
FAM-TAMARA PP MIX (Type A PPP MIX)	2	40
Total Volume	20	400
Template RNA	5	
Final volume	25	

AgPath Thermal Cycling Parameter

- 1X 45°C 10 min, 95°C 10 min
- 45X 95°C 15 sec, 60°C 45 sec

OR

SuperScript III Reagent (Backup Reagent)

Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
Nuclease-Free Water	4.5	90
2X Reaction MIX (Invitrogen Cat No. 11731)	12.5	250
SuperScript III/Platinum *Taq* MIX	0.5	10
ROX Reference Dye	0.5	10
FAM-TAMARA PP MIX (Type A PPP MIX)	2	40
Total Volume	20	400
Template RNA	5	
Final volume	25	

SuperScript Thermal Cycling Parameter

- 1X 50°C 15 min, 95°C 2 min
- 45X 95°C 15 sec, 60°C 30 sec

Influenza H5 Real-Time TaqMan PCR MasterMix Sheets

- AgPath Reagents (Reagent of Choice)

AgPath Thermal Cycling Parameter

- 1X 45°C 10 min, 95°C 10 min
- 45X 95°C 15 sec, 60°C 45 sec

Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
Nuclease-Free Water	0.1	2
2X RT-PCR Buffer	12.5	250
25X RT-PCR Enzyme MIX	1	20
FAM-TAMARA PP MIX (H5 PPP mix)	3.4	68
(H5 Probe mix)	3	60
Total Volume	20	400
Template RNA	5	
Final volume	25	

SuperScript III Reagent (Backup Reagent)

Reaction component	Volume per reaction (µL)	Volume for 20 Rxn (µL)
Nuclease-Free Water	1.5	32
2X RT-PCR Buffer	12.5	250
Primer Mix (PPP)	1.9	38
Probe mix H5 (FAM-TAMRA)	3	60
25X RT-PCR Enzyme MIX	1	20
Rox	0.1	70
Total Volume	20	400
Template RNA	5	
Final volume	25	

SuperScript Thermal Cycling Parameter

- 1X 50°C 15 min, 95°C 2 min
- 45X 95°C 15 sec, 60°C 30 sec

Influenza N1 Real-Time TaqMan PCR MasterMix Sheets
AgPath Reagents (Reagent of choice)

Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
Nuclease-Free Water	2.9	58
2X RT-PCR Buffer	12.5	250
25X RT-PCR Enzyme MIX	1	20
FAM- BHQ 1 PP MIX (N1 PPMIX)	3.5	70
Rox	0.1	2
Total Volume	20	400
Template RNA	5	
Final volume	25	

AgPath Thermal Cycling Parameter

- 1X 45°C 10 min, 95°C 10 min
- 45X 95°C 15 sec, 60°C 45 sec

SuperScript III Reagent (Backup Reagent)

Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
Nuclease-Free Water	3	60
2X RTPCR buffer	12.5	250
SuperScript III/Platinum *Taq* MIX	0.5	10
ROX Reference Dye (freshly prepared to 1:10)	0.5	10
FAM- BHQ 1 PP MIX (N1 PPMIX)	3.5	70
Total Volume	20	400
Template RNA	5	
Final volume	25	

SuperScript Thermal Cycling Parameter

- 1X 50°C 15 min, 95°C 2 min
- 45X 95°C 15 sec, 60°C 30 sec

5.3 Set up the reaction samples, internal positive control and Negative control (Nuclease free water) in separate tubes/wells. For quantitative analysis set up reaction samples, standards 1, 2, 3 and 4 and negative control (Nuclease free water) in separate tubes/wells. Set up the reporter dye and quencher as in the worksheet.

6. Result Interpretation and Reporting

Ct (Threshold cycle) value of each sample can be read as follows

- Ct value Result
- >45 Negative
- <40 Positive
- 45 Intermediate

7. Quantitative analysis

- Assess the Ct value when the amplification curve of Standard 1, 2, 3, 4 passes the threshold line.
- Calculate quantitative value to compare with Ct value of unknown samples and curve of Standard 1, 2, 3, 4

Test validation

- Each Ct value standard should be as follows.
- Standard 1 > Standard 2 > Standard 3 > Standard 4
- R-value of Standard curve should be 0.900~0.999.
- The Standard result should be all positive.

8. Waste disposal

- All the wastes should be discarded after being autoclaved

SOP No: NCAH/LAB/MOLE 09
Title: SoP on detection of CSFV by real time polymerase chain reaction
Version No: 2, Total Pages:5
Issue Month/Effective Date: May/2026
Revision Summary:
Supersedes Version No:2024.01
Prepared by: Molecular Section, LSU,NCAH
Reviewed by: Tshering Choden, Sonam Tshering, Kelzang Lhamo
Approved by:
Application/Distribution: NCAH, RLDC (kanglung)

1. Scope

The scope of real-time RT-PCR is to detect and quantify CSF viral RNA in pig samples with high sensitivity and specificity. It involves reverse transcription of RNA into cDNA followed by exponential amplification and fluorescent probe-based detection.

2. Objective

The objective is to provide rapid and accurate molecular confirmation of CSF infection. It supports both qualitative and quantitative analysis of viral load for diagnostic and surveillance purposes. Ultimately, the method strengthens laboratory capacity and ensures reliable data for disease control and research.

3. Principle

In RT-PCR, the RNA template is first converted into a complementary DNA (cDNA) using a reverse transcriptase. The cDNA is then used as a template for exponential amplification using PCR. RT-PCR is currently the most sensitive method of RNA detection available. The quantification of mRNA using RT-PCR here is achieved in a one-step reaction. 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.

4. Equipments and Consumables

- QuantStudio-5
- MINI spin, Eppendorf, AG-22331, USA
- Mini spin, Spinwin
- Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
- Bio-Safety Cabinet, Class –II, Esco
- Forward primer: CSFV 5’CCCTGGGTGGTCTAAG-3
- Reverse primer: CSFV 5’ CATGCCCTCGTCCAC-3’
- Probe: CSFV 5’FAM-CCTGAGTACAGGACAGTCGTCAGTAGTT-TAMRA
- 5’ label with fluorescent reporter dye: 6-carboxyfluorescent (FAM)
- 3’label with the quencher 6-carboxy-tetramethyl-rhodamine (TAMRA)
- Sample: Triturate from tonsils (throat and caecal)
- Positive Control: known CSF positive sample
- Negative control: sterile water

5. Procedure

5.1. *Extraction of RNA (Template RNA): refer NCAH/LSU/MOLE/06*

5.2 Process of mastermix (AgPath Reagents (Reagent of choice)

Reaction component	**Volume per reaction (µL)**	**Volume for 20 Rxs (µL)**
Nuclease-Free Water	2.65	55
2X RT-PCR Buffer	12.5	250
25X RT-PCR Enzyme MIX	1	20
FAM-TAMRA PPP Mix (CSFV)	3.75	75
Rox	0.1	2
Total Volume	20	400
Template RNA	5	
Final volume	**25**	

5.3 AgPath Thermal Cycling Parameter

- 1X 45°C 10 min, 95°C 10 min
- 45X 95°C 15 sec, 60°C 45 sec

SuperScript III Reagent (Backup Reagent)

Reaction component	Vol per reaction (µL)	Volume for 20 Rxs (µL)
Nuclease-Free Water	2.75	55
2X Reaction MIX (Invitrogen Cat No. 11731)	12.5	250
SuperScript III/Platinum *Taq* MIX	0.5	10
ROX Reference Dye (freshly prepared to 1:10)	0.5	10
FAM-TAMARA PP MIX (CSFV PPMIX)	3.75	75
Total Volume	20	400
Template RNA	5	
Final volume	**25**	

SuperScript Thermal Cycling Parameter

- 1X 50°C 15 min, 95°C 2 min
- 45X 95°C 15 sec, 60°C 30 sec

Set up the reaction samples, internal positive control and Negative control (Nuclease free water) in separate tubes/wells. For quantitative analysis set up reaction samples, standards 1, 2, 3 and 4 and negative control (Nuclease free water) in separate tubes/wells. Set up the reporter dye as FAM for CSFV.

6. Result interpretation and reporting

Ct (Threshold cycle) value of each sample can be read as follows

Ct value Result

- 40 Negative
- ≤ 40 Positive

Quantitative analysis

- Assess the Ct value when amplification curve of Standard 1, 2, 3, 4 passes threshold line.
- Calculate quantitative value to compare with Ct value of unknown samples and curve of Standard 1, 2, 3, 4

Test validation

- Each Ct value standard should be as follows.
- Standard 1 > Standard 2 > Standard 3 > Standard 4
- R-value of Standard curve should be 0.900~0.999.
- The Standard result should be all positive.

7. Quality Control

The positive control RNA used in the performance of this assay has to be treated to kill any viruses that may have been in samples at the time of production. Accordingly, caution should be exercised in handling all materials associated with this test.

8. Waste Disposal

The material to be tested for the presence of CSFV may be potentially contaminated with viable CSFV or other agents. Work should be conducted in an appropriate containment environment. Good laboratory practices at the P2 level should be used at minimum. All the wastes should be discarded after being autoclaved

9. Reference

- Leifer I, Hoffmann B, Hoper D, Rasmuseen T.B, Blome S, Strebelow G, Horeth-Bontgen D, Staubach C, Beer M. Molecular epidemiology of current classical swine fever virus isolates of wild boar in Germany J. Gen. Virol. 2010;91:2687–2697
- Chakraborty S, Veeregowda B.M. A study report on phylogenetic analysis of classical swine fever virus isolated in different parts of the world. Vet. World. 2012;5(7):437–442.
- Luo T.R, Liao S.H, Wu X.S, Feng L, Yuan Z.X, Li H, Liang J.J, Meng X.M, Zhang H.Y. Phylogenetic analysis of the E2 gene of classical swine fever virus from the Guangxi province of Southern China. Virus Genes. 2011;42(3):347–354.

SOP No: NCAH/LAB/MOLE 10
Title: SoP on detection of PPMV by real time polymerase chain reaction
Version No: 2, Total Pages:5
Issue Month/Effective Date: May/2026
Revision Summary:
Supersedes Version No:2024.01
Prepared by: Molecular Section, LSU,NCAH
Reviewed by: Tshering Choden, Sonam Tshering, Kelzang Lhamo
Approved by:
Application/Distribution: NCAH, RLDC (kanglung)

1. Scope

This document is used to support diagnosis of PPMV-1 disease in pigeon using real time RT-PCR.

2. Objective

Detection of PPMV specific genes by real time RT-PCR

3. Principle

In RT-PCR, the RNA template is first converted into a complementary DNA (cDNA) using a reverse transcriptase. The cDNA is then used as a template for exponential amplification using PCR. RT-PCR is currently the most sensitive method of RNA detection available. The quantification of mRNA using RT-PCR here is achieved in a one-step reaction. 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.

4. Equipment and Consumables

- QuantStudio-5
- MINI spin, Eppendorf, AG-22331, USA
- Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
- Bio-Safety Cabinet, Class –II, Esco
- Forward primer: PPMV1-5’ TCAGGAGGAAGGAGGCAGAA-3’
- Reverse primer: -PPMV1- 5’ CGCAACCCCAAGAGCTACAC-3’
- Probe: FAM-TTCATAGGTGCCATTATAG-MGBNFQ 5’
- Sample: Swabs from Cloaca, Tracheal or Tissue
- Positive Control: known PPMV-1 positive sample
- Negative control: sterile water

5. Procedure

5.1 Extraction of RNA (Template RNA): refer NCAH/LSU/MOLE/06

5.2 Master mix process

AgPath Reagents (Reagent of choice)

Reaction component	**Volume per reaction (µL)**	**Volume for 20 Rxn (µL)**
Nuclease-Free Water	2.65	53
2X RT-PCR Buffer (Ambion P/N AM1005)	12.5	250
25X RT-PCR Enzyme MIX	1	20
FAM-TAMARA PP MIX (PPMV-1 PPMIX)	3.75	75
Rox	0.1	2
Total Volume	20	400
Template RNA	5	
Final volume	**25**	

AgPath Thermal Cycling Parameter

- 1X 45°C 10 min, 95°C 10 min
- 45X 95°C 15 sec, 60°C 45 sec

SuperScript III Reagent (Backup Reagent)

Reaction component	**Volume per reaction (µL)**	**Volume for 20 Rxs (µL)**
Nuclease-Free Water	2.75	55
2X Reaction MIX (Invitrogen Cat No. 11731)	12.5	250
SuperScript III/Platinum *Taq* MIX	0.5	10
ROX Reference Dye (freshly prepared to 1:10)	0.5	10
FAM-TAMARA PP MIX (PPMV-1 PPMIX)	3.75	75
Total Volume	20	400
Template RNA	5	
Final volume	**25**	

SuperScript Thermal Cycling Parameter

- 1X 50°C 15 min, 95°C 2 min
- 45X 95°C 15 sec, 60°C 30 sec

5.3 Set up the reaction samples, internal positive control and Negative control (Nuclease free water) in separate tubes/wells. For quantitative analysis set up reaction samples, standards 1, 2, 3

and 4 and negative control (Nuclease free water) in separate tubes/wells. Set up the reporter dye as FAM for PPMV-1.

6. Result interpretation and reporting

a) Qualitative analysis

Ct (Threshold cycle) value of each sample can be read as follows

- Ct value Result
- 45 Negative
- ≤ 40 Positive

b) Quantitative analysis

- Assess the Ct value when amplification curve of Standard 1, 2, 3, 4 passes threshold line.
- Calculate quantitative value to compare with Ct value of unknown samples and curve of Standard 1, 2, 3, 4

c) Test validation

- Each Ct value standard should be as follows.
- Standard 1 > Standard 2 > Standard 3 > Standard 4
- R-value of Standard curve should be 0.900~0.999.
- The Standard result should be all positive.

7. Quality Control

- The material to be tested for the presence of PPMV may be potentially contaminated with viable PPMV or other agents. Work should be conducted in an appropriate containment environment. Good laboratory practices at the P2 level should be used at minimum.
- The positive control RNA used in the performance of this assay has to be treated to kill any viruses that may have been in samples at the time of production. Accordingly, caution should be exercised in handling all materials associated with this test.

8. Waste disposal

- All the wastes should be discarded after being autoclaved

SOP No: NCAH/LSU/MOLE 11
Title: SoP on detection of Porcine Reproductive and Respiratory Syndrome Virus (NA & EU) by real time polymerase chain reaction
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Supersedes Version No: 2020.01
Prepared by: Molecular Section, LSU, NCAH
Reviewed by: Tshering Choden, Sonam Tshering, Kelzang Lhamo
Approved by:
Application/Distribution: NCAH, RLDC (kanglung)

1. Scope

The scope of real-time RT-PCR is to detect and quantify PRSS-NA and PRSS-EU viral RNA in poultry samples with high sensitivity and specificity.

2. Objective

The objective is to provide rapid and accurate molecular confirmation of PRSS infection by detection of NA and EU by real time RT-PCR. It supports both qualitative and quantitative analysis of viral load for diagnostic and surveillance purposes. Ultimately, the method strengthens laboratory capacity and ensures reliable data for disease control and research.

3. Principle

The RNA template is first converted into a complementary DNA (cDNA) using a reverse transcriptase. The cDNA is then used as a template for exponential amplification using PCR. RT-PCR is currently the most sensitive method of RNA detection available. The quantification of mRNA using RT-PCR here is achieved in a one-step reaction. 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.

4. Equipment and Consumables

- QuantStudio-5
- MINI spin, Eppendorf, AG-22331, USA
- Mini spin, Spinwin
- Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
- Bio-Safety Cabinet, Class –II, Esco
- Nuclease-Free Water
- PCR Master Mix (2X)
- ROX Reference Dye
- 25X RT-PCR Enzyme MIX
- PRRS-NA Primer Forward- 5’ ATG ATG RGC TGG CAT TCT 3’
- Primer Reverse- 5’ AGA CCG TCG CCC TAA TTG 3’
- Probe- 5’ TGT GGT GAA TGG CAC TGA TTG ATA 3’
- Sample
- Positive Control: known PRSS positive sample
- Negative control: sterile water
- For PRRS-EU
- Forward primer (Fwd) GCACCACCTCACCCAGAC

- Reverse primer (Rev) CAGTTCCTGCGCCTTGAT
- Probe: FAM-CCTCTGCTTGCAATCGATCCAGAC-BHQ1

5. Procedure

5.1 Extraction of RNA (Template RNA): *refer NCAH/LSU/MOLE/06*

5.2 *Amplification*

Master mix reagents	1x volume (reaction 20ul)
Nuclease-Free Water	4.4
PCR Master Mix (2X)	12.5
PPP mix (PRRS NA) CY5- BHQ2	2
ROX Reference Dye	0.1
25X RT-PCR Enzyme MIX	1
Total Volume	20

Master mix reagents	1x volume (reaction 20ul)
Nuclease-Free Water	4.9
PCR Master Mix (2X)	12.5
PPP mix (PRRS EU) FAM- BHQ1	1.5
ROX Reference Dye	0.1
25X RT-PCR Enzyme MIX	1
Total Volume	20
Template RNA	5
Final Volume	25

- Add 20 µl of the PCR reaction mix to the required number of 0.2 ml optical PCR tubes including the positive controls and the negative controls.

5.3 Sample addition

- Add 5µl of sample DNA template to each PCR tube. Close the reaction tube
- Add positive control (2 µl of PRRS RNA) and negative control (5 µl of nuclease free water) in a separate PCR tube. Close the reaction tube
- Spin down the PCR mix and ensure the caps are closed properly
- Place all tubes in an automated real-time thermo cycler.
- Run the incubation program/PCR cycle condition as detailed below

5.4 PCR cycle condition

- 1X 45°C 10 min, 95°C 10 min
- 45X 95°C 15 sec, 60°C 45 sec
- Program the fluorescence collection in FAM channel and quencher as BHQ1 for PRRS EU and CY5- BHQ2 for PRRS NA.

Set up the reaction samples, internal positive control and Negative control (Nuclease free water) in separate tubes/wells. For quantitative analysis set up reaction samples, standards 1, 2, 3 and 4 and negative control (Nuclease free water) in separate tubes/wells. Set up the reporter dye

6. 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 (see figure). Therefore, a negative sample will show a Ct value ≥40.

7. References

7.1 Kwok, S., Higuchi, R. Avoiding false positives with PCR. *Nature* 339, 237–238 (1989). https://doi.org/10.1038/339237a0

SOP No: NCAH/LSU/MOLE 12
Title: SoP on detection Infectious Bursal Disease (IBD) by Polymerase Chain Reaction
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Issue Month/Effective Date: May/2026
Revision: Summary:
Supersedes Version No: 2026.1
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Application/Distribution: NCAH, RLDC (kanglung)

1. Scope

The scope of real-time RT-PCR is to detect and quantify Infectious Bursal Disease viral (IBDV) RNA in poultry samples with high sensitivity and specificity.

2. Objective

The objective is to provide rapid and accurate molecular confirmation of IBDV infection by real time RT-PCR. It supports both qualitative and quantitative analysis of viral load for diagnostic and surveillance purposes. Ultimately, the method strengthens laboratory capacity and ensures reliable data for disease control and research.

3. Principle

The real-time PCR assay for Infectious Bursal Disease (IBD) is designed to specifically amplify and detect a target region of the IBD viral genome using sequence-specific primers and a fluorescently labeled probe.

4. Equipment and Consumables

- QuantStudio-5
- MINI spin, Eppendorf, AG-22331, USA
- Mini spin, Spinwin
- Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
- Bio-Safety Cabinet, Class –II, Esco
- Forward primer: IBD 5' AGGTCGAGGTCTCTGACCTGAG 3'
- Reverse primer:IBD 5' TGTAGGTTGAGGTCTCTGACCTGAG 3
- Probe: IBD 5'-FAM-CAACCGGACCGGCGT-MGBNFQ-3'
- Sample (RNA template)
- Positive Control: known IBD positive sample
- Negative control: sterile water
- Nuclease-Free Water
- PCR Master Mix (2X)
- ROX Reference Dye
- 25X RT-PCR Enzyme MIX
- QuantStudio-5
- MINI spin, Eppendorf, AG-22331, USA
- Mini spin, Spinwin
- Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
- Bio-Safety Cabinet, Class –II, Esco

5. Procedure

5.1 Extraction of RNA (Template RNA): *refer NCAH/LSU/MOLE/06*
5.2 *Amplification*

Master mix reagents	1x volume (reaction 20ul)
Nuclease-Free Water	4.9
PCR Master Mix (2X)	12.5
FAM- MGBNFQ (IBD PPP mix)	1.5
ROX Reference Dye	0.1
25X RT-PCR Enzyme MIX	1
Total Volume	20
Template RNA	5
Final Volume	25

Add 20 µl of the PCR reaction mix to the required number of 0.2 ml optical PCR tubes including the positive controls and the negative controls.

5.3 Sample addition

- Add 5µl of sample DNA template to each PCR tube. Close the reaction tube
- Add positive control (2 µl IBD RNA) and negative control (5 µl of nuclease free water) in a separate PCR tube. Close the reaction tube
- Spin down the PCR mix and ensure the caps are closed properly
- Place all tubes in an automated real-time thermo cycler.
- Run the incubation program/PCR cycle condition as detailed below

5.4 PCR cycle condition

- 1X 45°C 10 min, 95°C 10 min
- 45X 95°C 15 sec, 60°C 45 sec
- Program the fluorescence collection in FAM channel and quencher as MGBNFQ.

6. 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 (see figure). Therefore, a negative sample will show a Ct value ≥40.

SOP No: NCAH/LSU/MOLE 13
Title: SoP on detection of NDV by real time polymerase chain reaction
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Prepared by: Molecular Section, LSU, NCAH
Reviewed by: Tshering Choden, Sonam Tshering, Kelzang Lhamo
Approved by:
Application/Distribution: NCAH, RLDC (kanglung)

1. **Scope**

The scope
of real-time RT-PCR is to detect and quantify NDV viral RNA in poultry samples

with high sensitivity and specificity.

2. Objective

The objective is to provide rapid and accurate molecular confirmation of NDV infection by detection of M, L or F-genes of NDV by real time RT-PCR. It supports both qualitative and quantitative analysis of viral load for diagnostic and surveillance purposes. Ultimately, the method strengthens laboratory capacity and ensures reliable data for disease control and research.

3. Principle

In RT-PCR, the RNA template is first converted into a complementary DNA (cDNA) using a reverse transcriptase. The cDNA is then used as a template for exponential amplification using PCR. RT-PCR is currently the most sensitive method of RNA detection available. The quantification of mRNA using RT-PCR here is achieved in a one-step reaction. 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.

4. **Consumables and Reagents**

- QuantStudio-5
- MINI spin, Eppendorf, AG-22331, USA
- Mini spin, Spinwin
- Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
- Bio-Safety Cabinet, Class –II, Esco
- Forward primer 5’ ACT GAT GTC CTC GGA CCT TC 3’
- Reverse primer 5’ CCT GAC GAG AGG CAT TTC CTA 3’
- Probe: 5’ TTCTC TAG CAG TGG GAC AGC CTG C 3’
- 5’ label with fluorescent reporter dye : 6-carboxyfluorescent (FAM)
- 3’label with the quencher 6-carboxy-tetramethyl-rhodamine (TAMRA)
- Sample: Swabs from Cloaca, Tracheal or Tissue
- Positive Control: known NDV positive sample
- Negative control: sterile water

5. Procedure

5.1 Extraction of RNA (Template RNA): *refer NCAH/LSU/MOLE/06*
5.2 *Amplification*

Reaction component	Volume per reaction (µL)
Nuclease-Free Water	2.75
2X RT-PCR Buffer (Ambion P/N AM1005)	12.5
25X RT-PCR Enzyme MIX	1
FAM-TAMARA PP MIX (NDV PPP Mix)	3.75
Total Volume	20
Template RNA	5
Final volume	**25**

5.3 Set up the reaction samples, internal positive control and Negative control (Nuclease free water) in separate tubes/wells. For quantitative analysis set up reaction samples, standards 1, 2, 3 and 4 and negative control (Nuclease free water) in separate tubes/wells. Set up the reporter dye as FAM for NDV-1

6. Result interpretation and reporting

a) Qualitative analysis

Ct (Threshold cycle) value of each sample can be read as follows

- Ct value Result
- 45 Negative
- ≤ 40 Positive

b) Quantitative analysis

- Assess the Ct value when amplification curve of Standard 1, 2, 3, 4 passes threshold line.
- Calculate quantitative value to compare with Ct value of unknown samples and curve of Standard 1, 2, 3, 4

Test validation

- Each Ct value standard should be as follows.
- Standard 1 > Standard 2 > Standard 3 > Standard 4
- R-value of Standard curve should be 0.900~0.999
- The Standard result should be all positive.

7. Quality Control

a) The material to be tested for the presence of NDV may be potentially contaminated with viable NDV or other agents. Work should be conducted in an appropriate containment environment. Good laboratory practices at the P2 level should be used at minimum.

b) The positive control RNA used in the performance of this assay has to be treated to kill any viruses that may have been in samples at the time of production. Accordingly, caution should be exercised in handling all materials associated with this test.

8. Waste disposal

a) All the wastes should be discarded after being autoclaved

9. References

- Alexander DJ (1997). Newcastle disease and other avian Paramyxoviridae infections. In: Calnek BW, Barnes HJ, Beard CW, McDougald LR, Saif YM (ed), Disease of poultry, 10th edn. Iowa State University Press, Ames Iowa, pp 541–570.
- Anon (1971). Methods for examining poultry biologics and for identifying and quantifying avian pathogens. Newcastle disease, p. 66. National Academy of Science, Washington, D. C.

ANNEXURE: Trouble shooting in PCR

PCR is a highly sensitive technique, the most critical point along all the analysis procedures 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 agent itself present in the positive analysed samples or in the positive controls included in the DNA extraction procedure; also, it could be due to the agent itself (DNA/RNA) 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. The samples may be prepared in a bio-safety cabinet.
- Personnel must always work with clean nitrile or latex gloves in the PCR laboratory.
- Change of gloves whenever personnel go into a different PCR area,
- Tubes containing amplified products should never be opened and manipulated in other laboratories distinct from those exclusively assigned to their analysis by electrophoresis, where they will be discarded.
- The place should be irradiated with UV light from time to time or when contamination is suspected. UV light can destroy DNA rendering it unsuitable for further amplification. Clean lab benches and equipment periodically with freshly diluted 10% bleach solution.
- Instrument calibration, the Thermal cyclers requires calibration from time to time
- Inhibitors of PCR such as Heparin, porphyrin, SDS, phenol, and proteinase-K are potent inhibitors of PCR. The effect of inhibitors can be reduced or abolished by diluting the sample as it also dilutes the inhibitor. Errors in PCR-There are numerous factors that may affect the efficiency of PCR. The quality of reagents, primers, buffer pH, quality and quantity of DNA and PCR tubes etc. are all important.

References

8.1 Kwok, S., Higuchi, R. Avoiding false positives with PCR. *Nature* 339, 237–238 (1989). https://doi.org/10.1038/339237a0