	NATIONAL CENTRE FOR ANIMAL HEALTH **LABORATORY SERVICES UNIT**	**Second edition**
	STANDARD OPERATING PROCEDURE	**Version 2018.1**
	MOLECULAR BIOLOGY	

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TITLE: Real Time RT PCR for AI

PREPARED BY: Molecular section

REVISED BY: Molecular section

APPROVED BY: Head, LSU

DATE: June 12, 2018

1. INTRODUCTION

Avian Influenza (AI) viruses are members of the virus family Orthomyxoviridae. Influenza viruses in this family are designated type A, B or C depending on the antigenic properties of the matrix protein and the nucleoprotein. AI viruses belong to type A. The genome consists of eight single-stranded, negative sense RNA molecules, which encode ten proteins: PB2, PB1, PA, HA, NP, NA, M1, M2, NS1 and NS2. Sixteen serologically distinct Haemagglutinin (H1-H16) and 9 Neuraminidase (N1-N9) subtypes of type A influenza virus exist at present, and representatives of each have been isolated from avian species. To date, all highly pathogenic AI viruses that produce acute clinical disease in avian species have been associated only with H5 or H7 subtypes. Not all H5 and H7 subtypes, however, are virulent in poultry. Pathogenicity of AI viruses is associated with trypsin cleavage of haemagglutinin into two subunits (H1 & H2). Highly pathogenic strains of AI (HPAI) viruses contain several basic amino acid residues at the cleavage site.

Type A in human, birds and some other mammals (swine, equine) can cause disease. Influenza type A has subtypes based on HA and NA proteins. Sixteen HA subtypes (H1 - H16) of which most can be found in avian, 9 NA subtypes, all are found in avian.

2. TEST PRINCIPLES

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 miWXUHDIWHU3K5,WDOORVGHWHFWLRQIWKHDFFXPXODW product without re-opening the reaction tubes after the PCR run.

3. APPLICATION

Detection of type A, H5 and N1specific genes of AIV by real time RT-PCR

4. OBJECTIVE

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

5. APPARATUS/TEST KIT/REAGENTS

5.1 Real-time PCR machine: QuantStudio-5
5.2 MINI spin, Eppendorf, AG-22331, USA
5.3 Mini spin, Spinwin
5.4 Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
5.5 Bio-Safety Cabinet, Class II, Esco

6. REAGENTS

6.1 One step real-time RT-PCR Kit (for example: QuantiTect® Probe RT PCR Kit Cat No. 204443 (QIAGEN) storage at -20°C)
6.2 RNeasy Lipid Tissue Mini Kit (Qaigen USA)
6.3 Primers

Influenza type A

6.4 Forward primer IVA D161M □- AGATGAGYCTTCTAACCGAGGTCG-□
6.5 Reverse primer IVA D162M1 □- TGCAAAAACATCYTCAAGTCTCTG-□
6.6 Reverse primer IVA D162M2 □- TGCAAACACATCYTCAAGTCTCTG-□
6.7 Reverse primer IVA D162M3 □- TGCAAAGACATCYTCAAGTCTCTG-□
6.8 Reverse primer IVA D162M4 □- TGCAAATACATCYTCAAGTCTCTG-□
6.9 Probe: IVA MA □-FAM TCAGGCCCCCTCAAAGCCGA-TAMRA-□

Influenza A, subtype H5

6.10 Forward primer IVA D204f □- ATGGCTCCTCGGRAACCC -□
6.11 Forward primer IVA D148 H5 □- AAA CAG AGA GGA AAT AAG TGG AGT AAA ATT-□
6.12 Reverse primer IVA D205r □- TTYTCCACTATGTAAGACCATTCCG-□
6.13 Reverse primer IVA D149 H5 □- AAA GAT AGA CCA GCT ACC ATG ATT GC-□
6.14 Probe: [illegible] - FAM- TCAACAGTGGCGAGTTCCCTAGCA-TAMRA
6.15 Probe: [illegible] - FAM □ATG TGT GAC GAA TTC MT-MGBNFQ-□

Influenza A, subtype N1

6.16 Forward primer AI_N1 1316F □- GYG GGA GCA GCA TAT CYTT-□
6.17 Reverse primer AI_N1 1379R □- CCG TCT GGC CAA GAC CAA-□
6.18 [illegible] -pdU-G-pdU-GG-pdU-G-pdU-AAAYAG-pdU-GA-pdC-A-pdC-BHQ-1-□
6.19 Sample: Swabs from Cloaca, Tracheal or Tissue
6.20 Positive Control: known Influenza, H5 and N1 positive sample
6.21 Negative control: nuclease free water

7. PROCEDURE

Extraction of RNA (Template RNA)

7.1 Prepare RNA Buffer- (Add 310 µL AVE buffer to Carrier RNA-red cap). Store at -20°C

7.2 Prepare sample buffer- 560 µL AVL buffer with 5.6 µL RNA-AVE buffer (for one sample).
7.3 Add 140 µL of sample to sample buffer
7.4 Mix well and Spin down briefly.
7.5 Incubate for 10 minutes at room temperature
7.6 Add Ethanol (96%-100%) - 560 µL
7.7 Spin down for 1min at 14,000 RPM
7.8 Transfer 630 µL to mini spin column
7.9 Spin down for 1min at 14,000 RPM
7.10 Add wash Buffer (1) - 500 µL
7.11 Spin down for 1min at 14,000 RPM
7.12 Add wash Buffer(2) - 500 µL
7.13 Spin down for 3 mins at 14,000 RPM
7.14 Add AVE buffer - 60 µL (incubate for 1 minute at room temperature).
7.15 Spin down for 1min at 14,000 RPM
7.16 Store at -80°C until used

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Influenza Type A Real-Time TaqMan PCR MasterMix Sheets

Operator: **Date:**

SANs:

AgPath Reagents (Reagent of choice)

Sl no	Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
1	Nuclease-Free Water	4.5	90
2	2X RT-PCR Buffer (Ambion P/N AM1005) Kit lot No:	12.5	250
3	25X RT-PCR Enzyme MIX	1	20
4	FAM-TAMARA PP MIX (Type A PPMIX) Microstores No:	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

SuperScript III Reagent (Backup Reagent)

Sl no	Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
1	Nuclease-Free Water	4.5	90
2	2X Reaction MIX (Invitrogen Cat No. 11731) Kit lot No:	12.5	250
3	SuperScript III/Platinum *Taq* MIX	0.5	10
4	ROX Reference Dye (freshly prepared to 1:10)	0.5	10
	ROX Ref No.		
5	FAM-TAMARA PP MIX (Type A PPMIX) Microstores No:	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

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Influenza H5 Real-Time TaqMan PCR MasterMix Sheets

Operator: **Date:**
SANs:

AgPath Reagents (Reagent of choice)

Sl no	Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
1	Nuclease-Free Water	0.1	2
2	2X RT-PCR Buffer (Ambion P/N AM1005) Kit lot No:	12.5	250
3	25X RT-PCR Enzyme MIX	1	20
4	FAM-TAMARA PP MIX (H5 Primer mix) (H5 Probe mix) Microstores No:	3.4 3	68 60
	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)

Sl no	Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
1	Nuclease-Free Water	1.6	32
2	2X Reaction MIX (Invitrogen Cat No. 11731) Kit lot No:	12.5	250
3	SuperScript III/Platinum *Taq* MIX	0.5	10
4	ROX Reference Dye (freshly prepared to 1:10)	0.5	10
	ROX Ref No.		
5	FAM-TAMARA PP MIX (H5 Primer mix) (H5 Probe mix)	1.9 3	38 60

	Microstores No:		
	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

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Influenza N1 Real-Time TaqMan PCR MasterMix Sheets

Operator: **Date:**
SANs:

AgPath Reagents (Reagent of choice)

Sl no	Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
1	Nuclease-Free Water	3	60
2	2X RT-PCR Buffer (Ambion P/N AM1005) Kit lot No:	12.5	250
3	25X RT-PCR Enzyme MIX	1	20
4	FAM- BHQ 1 PP MIX (N1 PPMIX) Microstores No:	3.5	70
	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)

Sl no	Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
1	Nuclease-Free Water	3	60

2	2X Reaction MIX (Invitrogen Cat No. 11731) Kit lot No:	12.5	250
3	SuperScript III/Platinum *Taq* MIX	0.5	10
4	ROX Reference Dye (freshly prepared to 1:10)	0.5	10
	ROX Ref No.		
5	FAM- BHQ 1 PP MIX (N1 PPMIX) Microstores No:	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

7.17 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

7.18 Set up the reporter dye and quencher as in the worksheet

8. RESULT INTERPRETATION and REPORTING

8.1 Qualitative analysis

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

8.1.2 Ct value Result

8.1.3 > 45 Negative

8.1.4 □40 Positive

8.1.5 40-45 Intermediate

8.2 Quantitative analysis

8.2.1 Assess the Ct value when amplification curve of Standard 1, 2, 3, 4 passes threshold line.

8.2.2 Calculate quantitative value to compare with Ct value of unknown samples and curve of Standard 1, 2, 3, 4

8.2.3 Test validation

8.3 Each Ct value standard should be as follows.

8.4 Standard 1 > Standard 2 > Standard 3 > Standard 4

8.5 R-value of Standard curve should be 0.900~0.999.

8.6 The Standard result should be all positive.

9. WASTE DISPOSAL

9.1 All the wastes should be discarded after being autoclaved

10. RISK ASSESSMENT

10.1 The material to be tested for the presence of highly pathogenic avian influenza (H5N1) viral RNA is potentially contaminated with viable H5N1 or other agents. Work should be conducted in an appropriate containment environment. Good laboratory practices at the P2 level should be used at minimum.

10.2 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.

11. TROUBLESHOOTING

11.1 Remove the possibility of contamination of nucleic acid and PCR product when performing PCR.

11.2 It is recommended to work at the clean bench designated for PCR

11.3 Use sterilized filter tip.

11.4 Do not create any bubbles in the tube

11.5 Avoid detection solution being exposed to strong light

11.6 Do not use expired kits

11.7 Read the result as AIV by following clinical symptoms and autopsy even if the kits show the positive result. You are required to ask for testing at the OIE reference laboratory or when the results are in doubt

11.8 The detection limit of the kit is 10 copies. It may not detect less than 10 copies.

11.9 The kit should be stored at -20(. Under these conditions reagents are stable through the expiration date printed on the label.

11.10 Do not read result when Ct value is less than 5

11.11 AIV Real-Time PCR Kit can be detect AIV RNA with high sensitivity, but it may sometimes show false negative result due to RNA mis-extraction, operating error from unskilled worker, denaturation of the kit, and any other unknown reason.

12. REFERENCES

12.1 AHHL SOP QA; modified Spackman et al 2002 AHHL SOP

12.2 Heine H, Trinidad L, Selleck P (2005) Australian Biosecurity CRC Technical Report - Influenza virus type A and subtype H5-specific real-time reverse transcription (RRT)-PCR for detection of Asian H5N1 isolates.

12.3 Lee MS, Chang PC, Shien JH, Cheng MC, Shieh HK (2001) Identification and subtyping of avian influenza viruses by reverse-transcription PCR. *J. Virol. Meth* 97:13-22

12.4 Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, Lohman K, Daum LT, Suarez DL (2002). Development of a real-time RT PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J. Clin. Microbiol* 40(9):3256-60

TITLE: Real Time RT-PCR for NDV

PREPARED BY: Molecular section

REVISED BY: Molecular section

APPROVED BY: Head, LSU

DATE: June 12, 2018

1. INTRODUCTION
Newcastle disease is an infection of domestic poultry and many other bird species with virulent Newcastle disease virus. NDV synonymous with avian paramyxovirus serotype 1 (PMV-1), is an RNA virus and the most important of the 9 known PMV serotypes as a pathogen for poultry. NDV can be isolated from oropharyngeal or cloacal swabs or tissues from infected birds by inoculation of the allantoic cavity of 9- to 11-day-old embryonated chicken eggs. Infection is confirmed by recovery of a hemagglutinating virus that is inhibited with NDV antiserum or by detection of NDV RNA by reverse transcriptase PCR.

2. TEST PRINCIPLES
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. The fluorometer is designed to detect the amplified product without re-opening the reaction tubes after the PCR run.

3. APPLICATION
Detection of M genes of NDV by real time RT-PCR

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

5. APPARATUS
5.1 QuantStudio-5
5.2 MINI spin, Eppendorf, AG-22331, USA
5.3 Mini spin, Spinwin
5.4 Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
5.5 Bio-Safety Cabinet, Class II, Esco

6. REAGENTS
6.1 Forward primer NDV M 4100 - AGT GAT GTG CTC GGA CCT TC-

6.2 Reverse primer NDVM 4220 □- CCT GAG GAG AGG CAT TTG CTA-□
6.3 Probe 1900□ - FAM TTC TCT AGC AGT GGG ACA GCC TGC BHQ-□
6.4 ODEHOLWKDOXRUHVFHQWUHSRUWHUGH[(FAM)
6.5 ODEHOLWKWKHTXHQFKHU-1
6.6 Sample: Swabs from Cloaca, Tracheal or Tissue
6.7 Positive Control: known NDV positive sample
6.8 Negative control: sterile water

7. PROCEDURE

Extraction of RNA (Template RNA)

7.1 Prepare RNA Buffer- (Add 310 µL AVE buffer to Carrier RNA-red cap). Store at -20°C
7.2 Prepare sample buffer- 560 µL AVL buffer with 5.6 µL RNA-AVE buffer (for one sample).
7.3 Add 140 µL of sample to sample buffer
7.4 Mix well and Spin down briefly.
7.5 Incubate for 10 minutes at room temperature
7.6 Add Ethanol (96%-100%) - 560 µL
7.7 Spin down for 1min at 14,000 RPM
7.8 Transfer 630 µL to mini spin column
7.9 Spin down for 1min at 14,000 RPM
7.10 Add wash Buffer (1) - 500 µL
7.11 Spin down for 1min at 14,000 RPM
7.12 Add wash Buffer(2) - 500 µL
7.13 Spin down for 3 mins at 14,000 RPM
7.14 Add AVE buffer - 60 µL (incubate for 1 minute at room temperature).
7.15 Spin down for 1min at 14,000 RPM
7.16 Store at -80°C until used

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NDV Real-Time TaqMan PCR MasterMix Sheets

Operator: **Date:**

SANs:

AgPath Reagents (Reagent of choice)

Sl no	Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
1	Nuclease-Free Water	5	100
2	2X RT-PCR Buffer (Ambion P/N AM1005) Kit lot No:	12.5	250
3	25X RT-PCR Enzyme MIX	1	20
4	FAM-BHQ-1 PP MIX (NDV M gene)	1.5	30

	Microstores No:		
	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)

Sl no	Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
1	Nuclease-Free Water	5	100
2	2X Reaction MIX (Invitrogen Cat No. 11731) Kit lot No:	12.5	250
3	SuperScript III/Platinum *Taq* MIX	0.5	10
4	ROX Reference Dye (freshly prepared to 1:10)	0.5	10
	ROX Ref No.		
4	FAM--BHQ-1 PP MIX (NDV M gene) Microstores No:	1.5	30
	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

7.17 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

7.18 Set up the reporter dye as FAM for NDV M gene

8. RESULT INTERPRETATION and REPORTING

8.1 Qualitative analysis

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

8.1.2 Ct value Result

8.1.3 > 45 Negative

8.1.4 □40 Positive

8.1.5 40-45 Intermediate

8.2 Quantitative analysis

8.2.1 Assess the Ct value when amplification curve of Standard 1, 2, 3, 4 passes threshold line.
8.2.2 Calculate quantitative value to compare with Ct value of unknown samples and curve of Standard 1, 2, 3, 4
8.2.3 Test validation
8.2.4 Each Ct value standard should be as follows.
8.2.5 Standard 1 > Standard 2 > Standard 3 > Standard 4
8.2.6 R-value of Standard curve should be 0.900~0.999.
8.2.7 The Standard result should be all positive.

9. WASTE DISPOSAL

9.1 All the wastes should be discarded after being autoclaved

10. RISK ASSESSMENT

10.1 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.
10.2 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.

11. TROUBLESHOOTING

11.1 Remove the possibility of contamination of nucleic acid and PCR product when performing PCR.
11.2 It is recommended to work at the clean bench designated for PCR
11.3 Use sterilized filter tip.
11.4 Do not create any bubbles in the tube
11.5 Avoid detection solution being exposed to strong light
11.6 Do not use expired kits
11.7 Read the result as New Castle Disease by following clinical symptoms and autopsy even if the kits show the positive result. You are required to ask for testing at the OIE reference laboratory or when the results are in doubt
11.8 The detection limit of the kit is 10 copies. It may not detect less than 10 copies.
11.9 The kit should be stored at -20(. Under these conditions reagents are stable through the expiration date printed on the label.
11.10 Do not read result when Ct value is less than 5
11.11 NDV Real-Time PCR Kit can be detect ND virus RNA with high sensitivity, but it may sometimes show false negative result due to RNA mis-extraction, operating error from unskilled worker, denaturation of the kit, and any other unknown reason.

12. REFERENCES

12.1 CSIRO AAHL, Quality Assurance manual

12.2 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.

12.3 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.

TITLE: Real Time RT-PCR for CSFV

PREPARED BY: Molecular section

REVISED BY: Molecular section

APPROVED BY: Head, LSU

DATE: June 12, 2018

1. INTRODUCTION

ODVVLFDOVLHHYHU6)DOVRNRDVKRJFKROHUDLVDKLJKOFRQWDJLRVDGRIWH
fatal GLVHDVHRIGRPHVWLFSLJVDQGZLOGERUDUKHDXVDWLYHDJHQW6)9LV
DPHPEHURIWKHIDPLO\ *Flaviviridae*
(+) ss RNA of about 12.3 kb in length, which contains untranslated regions at 5DQGHQGV
and encodes a single polyprotein that is both co- and post-translationally processed to yield four structural (C, EO, E1 and E2) and 7-8 non-structural (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A and non-structural protein 5B [NS5B]) viral proteins (2, 3).

2. TEST PRINCIPLES

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 mi[WXUHDIWHU3K5□WDOORZVGHWHFWLRQRIWKHDFFXPXOD
product without re-opening the reaction tubes after the PCR run.

3. APPLICATION

Detection of CSFV by real time RT-PCR

4. OBJECTIVE

This document is used to support diagnosis of CSF in pigs using real time RT-PCR test

5. APPARATUS

5.1 QuantStudio-5
5.2 MINI spin, Eppendorf, AG-22331, USA
5.3 Mini spin, Spinwin
5.4 Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
5.5 Bio-Safety Cabinet, Class □II, Esco

6. REAGENTS

6.1 Forward primer 6)9□ - CCCTGGGTGGTCTAAG -□

6.2 Reverse primer CSFV 5- CATGCCCTCGTCCAC- □
6.3 Probe CSFV 5□FAM-CCTGAGTACAGGACAGTCGTCAGTAGTT-TAMRA
6.4 ODEHOZLWKDXRUHVFHQWUHSRUWLQJIluorescent (FAM)
6.5 ODEHOZLWKWKHTXHQFKHU-Carboxy-tetramethyl-rhodamine (TAMRA)
6.6 Sample: Triturate from tonsils (throat and caecal)
6.7 Positive Control: known CSF positive sample
6.8 Negative control: sterile water

7. PROCEDURE

Extraction of RNA (Template RNA)

7.1 Prepare RNA Buffer- (Add 310 µL AVE buffer to Carrier RNA-red cap). Store at -20°C
7.2 Prepare sample buffer- 560 µL AVL buffer with 5.6 µL RNA-AVE buffer (for one sample).
7.3 Add 140 µL of sample to sample buffer
7.4 Mix well and Spin down briefly.
7.5 Incubate for 10 minutes at room temperature
7.6 Add Ethanol (96%-100%) - 560 µL
7.7 Spin down for 1min at 14,000 RPM
7.8 Transfer 630 µL to mini spin column
7.9 Spin down for 1min at 14,000 RPM
7.10 Add wash Buffer (1) - 500 µL
7.11 Spin down for 1min at 14,000 RPM
7.12 Add wash Buffer(2) - 500 µL
7.13 Spin down for 3 mins at 14,000 RPM
7.14 Add AVE buffer - 60 µL (incubate for 1 minute at room temperature).
7.15 Spin down for 1min at 14,000 RPM
7.16 Store at -80°C until used

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SERBITHANG: THIMPHU

CSFV Real-Time TaqMan PCR MasterMix Sheets

Operator: **Date:**
SANs:

AgPath Reagents (Reagent of choice)

Sl no	Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
1	Nuclease-Free Water	2.75	55
2	2X RT-PCR Buffer (Ambion P/N AM1005) Kit lot No:	12.5	250

3	25X RT-PCR Enzyme MIX	1	20
4	FAM-TAMARA PP MIX (CSFV Risatti PPMIX) Microstores No:	3.75	75
	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)

Sl no	**Reaction component**	**Volume per reaction (µL)**	**Volume for 20 Rxs (µL)**
1	Nuclease-Free Water	2.75	55
2	2X Reaction MIX (Invitrogen Cat No. 11731) Kit lot No:	12.5	250
3	SuperScript III/Platinum *Taq* MIX	0.5	10
4	ROX Reference Dye (freshly prepared to 1:10)	0.5	10
	ROX Ref No.		
5	FAM-TAMARA PP MIX (CSFV Risatti PPMIX) Microstores No:	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

7.17 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

7.18 Set up the reporter dye as FAM and quencher TAMRA for CSFV

8. RESULT INTERPRETATION and REPORTING

8.1 Qualitative analysis

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

8.1.2 Ct value Result

8.1.3 > 40 Negative

8.1.4 □37 Positive
8.1.5 37-40 Intermediate

8.2 Quantitative analysis
8.2.1 Assess the Ct value when amplification curve of Standard 1, 2, 3, 4 passes threshold line.
8.2.2 Calculate quantitative value to compare with Ct value of unknown samples and curve of Standard 1, 2, 3, 4
8.2.3 Test validation
8.2.4 Each Ct value standard should be as follows.
8.2.5 Standard 1 > Standard 2 > Standard 3 > Standard 4
8.2.6 R-value of Standard curve should be 0.900~0.999.
8.2.7 The Standard result should be all positive.

9. WASTE DISPOSAL
9.1 All the wastes should be discarded after being autoclaved

10. RISK ASSESSMENT
10.1 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.
10.2 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.

11. TROUBLESHOOTING
11.1 Remove the possibility of contamination of nucleic acid and PCR product when performing PCR.
11.2 It is recommended to work at the clean bench designated for PCR
11.3 Use sterilized filter tip.
11.4 Do not create any bubbles in the tube
11.5 Avoid detection solution being exposed to strong light
11.6 Do not use expired kits
11.7 Read the result as CSF by following clinical symptoms and autopsy even if the kits show the positive result. You are required to ask for testing at the OIE reference laboratory or when the results are in doubt
11.8 The detection limit of the kit is 10 copies. It may not detect less than 10 copies.
11.9 The kit should be stored at -20(□. Under these conditions reagents are stable through the expiration date printed on the label.
11.10 Do not read result when Ct value is less than 5
11.11 CSFV Real-Time PCR Kit can be detect CSF virus RNA with high sensitivity, but it may sometimes show false negative result due to RNA mis-extraction, operating error from unskilled worker, denaturation of the kit, and any other unknown reason.

12. REFERENCES

12.1 Risatti et al. (2003) J Clin Microbiol 41: 500-5
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12.3 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
12.4 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.
12.5 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.

TITLE: Real Time RT-PCR for FMDV

PREPARED BY: Molecular section

REVISED BY: Molecular section

APPROVED BY: Head, LSU

DATE: June 12, 2018

1. INTRODUCTION

FMD is a highly contagious viral disease that affects all cloven-footed animals including cattle, yak, sheep, goat, pig and other wild ruminants. The disease is endemic in many parts of the world, particularly in developing countries of Asia, Africa, the Middle East, and some parts of Europe. In FMD-endemic countries, FMD can have serious economic losses through reduced production in terms of milk, meat and draught power and deaths, while in FMD-free countries, the outbreak will impact economic in terms of trade restrictions, costs of disease control and eradication. Foot and mouth disease is caused by the FMD virus (FMDV) which is a non-enveloped RNA virus belonging to the *Aphthovirus* genus of the family *Picornaviridae*. The FMDV has seven clinically indistinguishable serotypes: O, A, C, Asia 1, SAT 1, SAT 2, SAT 3. These serotypes do not induce cross protection against each other and therefore vaccination against one serotype will not provide protection against other serotypes. As FMD is clinically indistinguishable from other vesicular diseases, the disease needs to be confirmed using laboratory tests. The laboratory tests currently used for diagnosis of FMD in Bhutan include lateral flow immunochromatography (rapid NSP antibody test), virus detection tests (PCR, indirect sandwich ELISA), ELISAs to detect FMDV non-structural proteins (NSP) and structural proteins (SP) in sera. One of the highly sensitive tests is the molecular detection of FMD virus.

2. TEST PRINCIPLES

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 IURPWKHIOXRURSKRUHVLQDUHDFWLRQPLWXUHDIWHU3&5WDOORZVGHWHFWLRQRIWKHD product without re-opening the reaction tubes after the PCR run.

3. APPLICATION

Detection of matrix gene of FMD virus

4. OBJECTIVE

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

5. APPARATUS

5.1 QuantStudio-5
5.2 MINI spin, Eppendorf, AG-22331, USA
5.3 Mini spin, Spinwin
5.4 Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
5.5 Bio-Safety Cabinet, Class II, Esco

6. REAGENTS

Pirbright TaqMan assay for detection of all types IRES conserved sequence

6.1 Forward primer: FMDV SA-IR-219-246F: 5'-CACYTYAAGRTGACAYTGRTACTGGTAC-3'
6.2 Reverse primer : FMDV SA-IR-315-293R: 5'-CAGATYCCRAGTGWCICITGTTA-3'
6.3 Probe: FMDV SA-IR-292-3 -FAM-CCTCGGGGTACCTGAAGGGCATCC-TAMRA-3

Tetracore TaqMan assay for detection of all types RNA polymerase (3D)

6.4 Forward primer: FMDV Tet 6769 F: 5'-ACTGGGTTTTACAAACCTGTGA-3'
6.5 Reverse primer: FMDV Tet 6875 R: 5'-GCGAGTCCTGCCACGGA-3'
6.6 Probe: FMDV Tet 6820-P: -FAM-TCCTTTGCACGCCGTGGGAC-TAMRA-
6.7 Sample: Swabs/epithelial tissue/vesicular fluid
6.8 Positive Control: known FMD positive sample
6.9 Negative control: sterile water

7. PROCEDURE

Extraction of RNA (Template RNA)

7.1 Prepare RNA Buffer- (Add 310 µL AVE buffer to Carrier RNA-red cap). Store at -20°C
7.2 Prepare sample buffer- 560 µL AVL buffer with 5.6 µL RNA-AVE buffer (for one sample).
7.3 Add 140 µL of sample to sample buffer
7.4 Mix well and Spin down briefly.
7.5 Incubate for 10 minutes at room temperature
7.6 Add Ethanol (96%-100%) - 560 µL
7.7 Spin down for 1min at 14,000 RPM
7.8 Transfer 630 µL to mini spin column
7.9 Spin down for 1min at 14,000 RPM
7.10 Add wash Buffer (1) - 500 µL
7.11 Spin down for 1min at 14,000 RPM
7.12 Add wash Buffer(2) - 500 µL
7.13 Spin down for 3 mins at 14,000 RPM
7.14 Add AVE buffer - 60 µL (incubate for 1 minute at room temperature).
7.15 Spin down for 1min at 14,000 RPM
7.16 Store at -80°C until used

NATIONAL VETERINARY LABORATORY
NATIONAL CENTRE FOR ANIMAL HELATH
SERBITHANG: BHUTAN

NDV Real-Time TaqMan PCR MasterMix Sheets

Operator: **Date:**

Accession No:

AgPath Reagents (Reagent of choice)

Sl no	Reaction component	**Volume per reaction (µL)**	**Volume for 20 Rxs (µL)**
1	Nuclease-Free Water	2.75	55
2	2X RT-PCR Buffer (Ambion P/N AM1005)	12.5	250
	Kit lot No:		
3	25X RT-PCR Enzyme MIX	1	20
4	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

SuperScript III Reagent (Backup Reagent)			
Sl no	**Reaction component**	**Volume per reaction (µL)**	**Volume for 20 Rxs (µL)**
1	Nuclease-Free Water	2.75	55
2	2X Reaction MIX (Invitrogen Cat No. 11731)	12.5	250
	Kit lot No:		
3	SuperScript III/Platinum *Taq* MIX	0.5	10
4	ROX Reference Dye (freshly prepared to 1:10)	0.5	10
	ROX Ref No.		
5	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**	

SuperScript Thermal Cycling Parameter
1X 50°C 15 min, 95°C 2 min
45X 95°C 15 sec, 60°C 30 sec

7.17 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
7.18 Set up the reporter dye as FAM for FMDV

8. RESULT INTERPRETATION and REPORTING

8.1 Qualitative analysis
- 8.1.1 Ct (Threshold cycle) value of each sample can be read as follows
- 8.1.2 Ct value Result
- 8.1.3 > 40 Negative
- 8.1.4 □37 Positive
- 8.1.5 37-40 Intermediate

8.2 Quantitative analysis
- 8.2.1 Assess the Ct value when amplification curve of Standard 1, 2, 3, 4 passes threshold line.
- 8.2.2 Calculate quantitative value to compare with Ct value of unknown samples and curve of Standard 1, 2, 3, 4
- 8.2.3 Test validation

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

9. WASTE DISPOSAL

9.1 All the wastes should be discarded after being autoclaved

10. RISK ASSESSMENT

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

10.2 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.

11. TROUBLESHOOTING

11.1 Remove the possibility of contamination of nucleic acid and PCR product when performing PCR.

11.2 It is recommended to work at the clean bench designated for PCR
11.3 Use sterilized filter tip.
11.4 Do not create any bubbles in the tube
11.5 Avoid detection solution being exposed to strong light
11.6 Do not use expired kits
11.7 Read the result as FMD by following clinical symptoms and autopsy even if the kits show the positive result. You are required to ask for testing at the OIE reference laboratory or when the results are in doubt
11.8 The detection limit of the kit is 10 copies. It may not detect less than 10 copies.
11.9 The kit should be stored at -20(□. Under these conditions reagents are stable through the expiration date printed on the label.
11.10 Do not read result when Ct value is less than 5
11.11 FMDV Real-Time PCR Kit can be detect FMD virus RNA with high sensitivity, but it may sometimes show false negative result due to RNA mis-extraction, operating error from unskilled worker, denaturation of the kit, and any other unknown reason.

12. REFERENCES

12.1 CSIRO AAHL, Qualiry assurance manual.
12.2 Boyle, D. B., Taylor, T. and Cardoso, M. (2004). Implementation in Australia of molecular diagnostic techniques for the rapid detection of foot and mouth disease virus. Australian Veterinary Journal, 82, 421-425.
12.3 Callahan, J. D., Brown, F., Osorio, F. A., Sur, J. H., Kramer, E., Long, G. W., Lubroth, J., Ellis, S. J., Shoulars, K. S., Gaffney, K. L., Rock, D. L. and Nelson, W. M. (2002). Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of footand-mouth disease virus. Journal of the American Veterinary Medical Association, 220, 1636-1642.
12.4 Scott M. Reid., Juliet P. Dukes, Katja Ebert, Nigel P. Ferris and Donald P. King. Diagnosis of FMD by RT-PCR: prospects for mobile and portable assays. Institute for Animal Health, Pirbright Laboratory, Ash Road, Woking, Surrey, GU24 0NF, UK

TITLE: Real Time RT-PCR for PPMV-1

PREPARED BY: Molecular section

REVISED BY: Molecular section

APPROVED BY: Head, LSU

DATE: June 12, 2018

1. INTRODUCTION

Avian Paramyxovirus type 1 in pigeons (PPMV-1) is a viral infection that is present in most countries that can spread rapidly and cause high rates of pigeon illness and death. Paramyxovirus strains are generally capable of affecting other avian species including poultry. But so far, there has not been any detection of natural infection of poultry. Avian paramyxovirus type 1 (PPMV-1) is a very serious disease that can kill up to 100% of pigeons in some lofts. Some of the signs of PPMV1infection include lethargy, vomiting or regurgitation, green diarrhoea, twisting of the neck, circling, head flicking, laboured breathing and runny eyes and beak. Sick birds can die within three days. The infection spreads easily between birds and there is no specific treatment. Infected birds may shed the virus in their faeces and other discharges, contaminating the environment (including feed, water, equipment and human clothing) and allowing transmission to other birds. Avian paramyxovirus 1 can survive for several weeks in the environment, especially in cool weather. The spread of PPMV1is typically due to the movement of birds, but it can be carried in eggs or on equipment used with pigeons, as well as on people and their clothing. The clinical signs and gross pathological changes, if any, are not specific to avian paramyxovirus. Many other bird diseases can cause similar signs. Confirmation of PPMV-1 requires laboratory analysis especially the molecular test such as PCR.

2. TEST PRINCIPLES

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 IURPWKHIOXRURSKRUHVLQDUHDFWLRQPLWXUHDIWHU3&5WDOORZGHWHFWLRQRIWKHD product without re-opening the reaction tubes after the PCR run.

3. APPLICATION

Detection of PPMV specific genes by real time RT-PCR

4. OBJECTIVE

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

5. APPARATUS

5.1 QuantStudio-5
5.2 MINI spin, Eppendorf, AG-22331, USA
5.3 Mini spin, Spinwin
5.4 Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
5.5 Bio-Safety Cabinet, Class II, Esco

6. REAGENTS

6.1 Forward primer 3309) -TCAGGAGGAAGGAGGCAGAA-
6.2 5HYHUVHSULPHU3095 -CGCAACCCCAAGAGCTACAC-
6.3 3URELH3093 -FAM-TTCATAGGTGCCATTATAG-MGBNFQ-
6.4 Sample: Whole EDTA blood, serum, buffy coat, clarified lung, respiratory tract, spleen and tonsils homogenates. Samples from mummified or aborted litters are also suitable.
6.5 Positive Control: known PPMV-1 positive sample
6.6 Negative control: sterile water

7. PROCEDURE

Extraction of RNA (Template RNA)

7.1 Prepare RNA Buffer- (Add 310 µL AVE buffer to Carrier RNA-red cap). Store at -20°C
7.2 Prepare sample buffer- 560 µL AVL buffer with 5.6 µL RNA-AVE buffer (for one sample).
7.3 Add 140 µL of sample to sample buffer
7.4 Mix well and Spin down briefly.
7.5 Incubate for 10 minutes at room temperature
7.6 Add Ethanol (96%-100%) - 560 µL
7.7 Spin down for 1min at 14,000 RPM
7.8 Transfer 630 µL to mini spin column
7.9 Spin down for 1min at 14,000 RPM
7.10 Add wash Buffer (1) - 500 µL
7.11 Spin down for 1min at 14,000 RPM
7.12 Add wash Buffer(2) - 500 µL
7.13 Spin down for 3 mins at 14,000 RPM
7.14 Add AVE buffer - 60 µL (incubate for 1 minute at room temperature).
7.15 Spin down for 1min at 14,000 RPM
7.16 Store at -80°C until used

NDV Real-Time TaqMan PCR MasterMix Sheets

Operator: **Date:**

Accession No:

AgPath Reagents (Reagent of choice)

Sl no	Reaction component	**Volume per reaction (µL)**	**Volume for 20 Rxs (µL)**
1	Nuclease-Free Water	2.75	55
2	2X RT-PCR Buffer (Ambion P/N AM1005)	12.5	250
	Kit lot No:		
3	25X RT-PCR Enzyme MIX	1	20
4	FAM-TAMARA PP MIX (PPMV-1 PPMIX)	3.75	75
	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)			
Sl no	**Reaction component**	**Volume per reaction (µL)**	**Volume for 20 Rxs (µL)**
1	Nuclease-Free Water	2.75	55
2	2X Reaction MIX (Invitrogen Cat No. 11731)	12.5	250
	Kit lot No:		
3	SuperScript III/Platinum *Taq* MIX	0.5	10
4	ROX Reference Dye (freshly prepared to 1:10)	0.5	10
	ROX Ref No.		
5	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

7.17 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

7.18 Set up the reporter dye as FAM for PPMV-1

8. RESULT INTERPRETATION and REPORTING

8.1 Qualitative analysis

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

8.1.2 Ct value Result

8.1.3 > 40 Negative

8.1.4 □37 Positive

8.1.5 37-40 Intermediate

8.2 Quantitative analysis

8.2.1 Assess the Ct value when amplification curve of Standard 1, 2, 3, 4 passes threshold line.

8.2.2 Calculate quantitative value to compare with Ct value of unknown samples and curve of Standard 1, 2, 3, 4

8.2.3 Test validation

8.3 Each Ct value standard should be as follows.

8.4 Standard 1 > Standard 2 > Standard 3 > Standard 4

8.4.1 R-value of Standard curve should be 0.900~0.999.

8.4.2 The Standard result should be all positive.

9. WASTE DISPOSAL

9.1 All the wastes should be discarded after being autoclaved

10. RISK ASSESSMENT

10.1 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.

10.2 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.

11. TROUBLESHOOTING

11.1 Remove the possibility of contamination of nucleic acid and PCR product when performing PCR.

11.2 It is recommended to work at the clean bench designated for PCR

11.3 Use sterilized filter tip.

11.4 Do not create any bubbles in the tube

11.5 Avoid detection solution being exposed to strong light

11.6 Do not use expired kits

11.7 Read the result as PPVM-1 by following clinical symptoms and autopsy even if the kits show the positive result. You are required to ask for testing at the OIE reference laboratory or when the results are in doubt

11.8 The detection limit of the kit is 10 copies. It may not detect less than 10 copies.

11.9 The kit should be stored at -20(□. Under these conditions reagents are stable through the expiration date printed on the label.

11.10 Do not read result when Ct value is less than 5

11.11 PPMV-1 Real-Time PCR Kit can be detect PPM virus RNA with high sensitivity, but it may sometimes show false negative result due to RNA mis-extraction, operating error from unskilled worker, denaturation of the kit, and any other unknown reason.

12. REFERENCES

12.1 CSIRO, AAHL Quality assurance manual

TITLE: Real Time RT-PCR for PRRS

PREPARED BY: Molecular section

REVISED BY: Molecular section

APPROVED BY: Head, LSU

DATE:

1. INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is a virus that causes a disease of pigs, called porcine reproductive and respiratory syndrome (PRRS), also known as blue-ear pig disease. This economically important, panzootic disease causes reproductive failure in breeding stock and respiratory tract illness in young pigs. Initially referred to as "mystery swine disease" and "mystery reproductive syndrome", it was first reported in 1987 in North America and Central Europe .

2. TEST PRINCIPLES

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 IURP WKH GXRURSKRUHV LQ DUHDFWLRQ LWXUHDIWHU 3&5 WDOORV GHWHFWLRQ WKHD product without re-opening the reaction tubes after the PCR run.

3. APPLICATION

Detection of PRRSV European type (EU) and North American type (NA) by real time PCR

4. OBJECTIVE

This document is used to support diagnosis of PRRS EU/NA in pigs using real time PCR test

5. APPARATUS

5.1 QuantStudio-5
5.2 MINI spin, Eppendorf, AG-22331, USA
5.3 Mini spin, Spinwin
5.4 Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
5.5 Bio-Safety Cabinet, Class II, Esco

6. REAGENTS

PRRS EU

6.1 Forward primer PRRSV EU - GCA CCA CCT CAC CCA GAC -

6.2 Reverse primer PRRSV EU 5- CAG TTC CTG CGC CTT GAT- □
6.3 Probe PRRSV EU 5□FAM-CCT CTG CTT GCA ATC GAT CCA GAC-BHQ1

PRRS-NA

6.4 Forward primer 1 PRRSV 1□ - ATG ATG RGC TGG CAT TCT-□
6.5 Forward primer 2 PRRSV 1□ - ATR ATG RGC TGG CAT TCC-□
6.6 Reverse primer PRRSV NA 5- ACA CGG TCG CCC TAA TTG- □
6.7 Probe PRRSV NA 5□CY5- TGT GGT GAA TGG CAC TGA TTG ACA-BHQ2-3

6.8 Sample: Oral fluids, blood sample / serum samples
6.9 Positive Control: known PRRS positive sample
6.10 Negative control: Nuclease free water

7. PROCEDURE

Extraction of RNA (Template RNA)

7.1 Prepare RNA Buffer- (Add 310 µL AVE buffer to Carrier RNA-red cap). Store at -20°C
7.2 Prepare sample buffer- 560 µL AVL buffer with 5.6 µL RNA-AVE buffer (for one sample).
7.3 Add 140 µL of sample to sample buffer
7.4 Mix well and Spin down briefly.
7.5 Incubate for 10 minutes at room temperature
7.6 Add Ethanol (96%-100%) - 560 µL
7.7 Spin down for 1min at 14,000 RPM
7.8 Transfer 630 µL to mini spin column
7.9 Spin down for 1min at 14,000 RPM
7.10 Add wash Buffer (1) - 500 µL
7.11 Spin down for 1min at 14,000 RPM
7.12 Add wash Buffer(2) - 500 µL
7.13 Spin down for 3 mins at 14,000 RPM
7.14 Add AVE buffer - 60 µL (incubate for 1 minute at room temperature).
7.15 Spin down for 1min at 14,000 RPM
7.16 Store at -80°C until used

Master Mix

NATIONAL VETERINARY LABORATORY
NATIONAL CENTRE FOR ANIMAL HEALTH
SERBITHANG: THIMPHU

PRRS Real-Time TaqMan PCR MasterMix Sheets

Operator: **Date:**

SANs:

AgPath Reagents (Reagent of choice)

Sl no	Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
1	Nuclease-Free Water	3	60
2	2X RT-PCR Buffer (Ambion P/N AM1005) Kit lot No:	12.5	250
3	25X RT-PCR Enzyme MIX	1	20
4	FAM-TAMARA PP MIX (PRRSV EU PPMIX) (PRRSV NA PPMIX) Microstores No:	1.5 2	30 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

SuperScript III Reagent (Backup Reagent)

Sl no	Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
1	Nuclease-Free Water	3	60
2	2X Reaction MIX (Invitrogen Cat No. 11731) Kit lot No:	12.5	250
3	SuperScript III/Platinum *Taq* MIX	0.5	10
4	ROX Reference Dye (freshly prepared to 1:10)	0.5	10
	ROX Ref No.		
4	FAM-TAMARA PP MIX (PRRSV EU PPMIX) (PRRSV NA PPMIX) Microstores No:	1.5 2	30 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

7.17 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

7.18 Set up the reporter dye as FAM and quencher TAMRA for PRRSV

8. RESULT INTERPRETATION and REPORTING

8.1 Qualitative analysis

Ct value Result

8.1.1 Positive : Ct less than 39

8.1.2 Negative : Ct greater than 41

8.1.3 Intermediate: Ct between 39 and 41

8.2 Quantitative analysis

8.3 Assess the Ct value when amplification curve of Standard 1, 2, 3, 4 passes threshold line.

8.4 Calculate quantitative value to compare with Ct value of unknown samples and curve of Standard 1, 2, 3, 4

8.5 Test validation

8.6 Each Ct value standard should be as follows.

8.7 Standard 1 > Standard 2 > Standard 3 > Standard 4

8.8 R-value of Standard curve should be 0.900~0.999.

8.9 The Standard result should be all positive.

9. WASTE DISPOSAL

9.1 All the wastes should be discarded after being autoclaved

10. RISK ASSESSMENT

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

10.2 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.

11. TROUBLESHOOTING

11.1 Remove the possibility of contamination of nucleic acid and PCR product when performing PCR.

11.2 It is recommended to work at the clean bench designated for PCR

11.3 Use sterilized filter tip.

11.4 Do not create any bubbles in the tube

11.5 Avoid detection solution being exposed to strong light

11.6 Do not use expired kits

11.7 Read the result as PRRS by following clinical symptoms and autopsy even if the kits show the positive result. You are required to ask for testing at the OIE reference laboratory or when the results are in doubt

11.8 The detection limit of the kit is 10 copies. It may not detect less than 10 copies.

11.9 The kit should be stored at -20(□. Under these conditions reagents are stable through the expiration date printed on the label.
11.10 Do not read result when Ct value is less than 5
11.11 PRRSV Real-Time PCR Kit can be detect PRRS virus RNA with high sensitivity, but it may sometimes show false negative result due to RNA mis-extraction, operating error from unskilled worker, denaturation of the kit, and any other unknown reason.

12. REFERENCES

12.1 Kleiboeker et al. (2005), J Vet Diagn Invest 17: 165-170
12.2 CSIRO , AAHL Quality Assurance manual

TITLE: Real Time RT-PCR for ASFV

PREPARED BY: Molecular section

REVISED BY: Molecular section

APPROVED BY: Head, LSU

DATE:

1. INTRODUCTION

African swine fever virus (ASFV) is the causative agent of African swine fever (ASF). ASFV is the only known virus with a double-stranded DNA genome transmitted by arthropods. The virus causes a lethal haemorraghic disease in domestic pigs. Some isolates can cause death of animals as quickly as a week after infection. In all other species, the virus causes no obvious disease. ASFV is endemic to sub-Saharan Africa and exists in the wild through a cycle of infection between ticks and wild pigs, bushpigs, and warthogs.

2. TEST PRINCIPLES

Real time PCR is currently the most sensitive method for DNA/RNA 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 miWXUHDIWHU3K5,WDOORV detection of the accumulating product without re-opening the reaction tubes after the PCR run.

3. APPLICATION

Detection of ASFV by real time PCR

4. OBJECTIVE

This document is used to support diagnosis of ASF in pigs using real time RT-PCR test

5. APPARATUS

5.1 QuantStudio-5
5.2 MINI spin, Eppendorf, AG-22331, USA
5.3 Mini spin, Spinwin
5.4 Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
5.5 Bio-Safety Cabinet, Class II, Esco

6. REAGENTS

6.1 Forward primer A6)9 - CTG CTC ATG GTA TCA ATC TTA TCG A -
6.2 Reverse primer ASFV 5- GAT ACC ACA AGA TCR GCC GT -
6.3 Probe ASFV 5FAM-CCA CGG GAG GAA TAC CAA CCC AGT G-TAMRA

6.4 ODEHOLWKLRXUHVFHQWUHSRUWHUGHV (6-carboxyfluorescent (FAM)
6.5 ODEHOLWKWKHTXHQFKHUG 6-carboxy-tetramethyl-rhodamine (TAMRA)
6.6 Sample: Triturate from tonsils (throat and caecal)
6.7 Positive Control: known ASF positive sample
6.8 Negative control: sterile water

7. PROCEDURE

Extraction of DNA (Template DNA)

7.1 Prepare RNA Buffer- (Add 310 µL AVE buffer to Carrier RNA-red cap). Store at -20°C
7.2 Prepare sample buffer- 560 µL AVL buffer with 5.6 µL RNA-AVE buffer (for one sample).
7.3 Add 140 µL of sample to sample buffer
7.4 Mix well and Spin down briefly.
7.5 Incubate for 10 minutes at room temperature
7.6 Add Ethanol (96%-100%) - 560 µL
7.7 Spin down for 1min at 14,000 RPM
7.8 Transfer 630 µL to mini spin column
7.9 Spin down for 1min at 14,000 RPM
7.10 Add wash Buffer (1) - 500 µL
7.11 Spin down for 1min at 14,000 RPM
7.12 Add wash Buffer(2) - 500 µL
7.13 Spin down for 3 mins at 14,000 RPM
7.14 Add AVE buffer - 60 µL (incubate for 1 minute at room temperature).
7.15 Spin down for 1min at 14,000 RPM
7.16 Store at -80°C until used

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

NATIONAL VETERINARY LABORATORY
NATIONAL CENTRE FOR ANIMAL HEALTH
SERBITHANG: THIMPHU

ASFV Real-Time TaqMan PCR MasterMix Sheets

Operator: **Date:**
SANs:

AgPath Reagents (Reagent of choice)

Sl no	Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
1	Nuclease-Free Water	2.75	55
2	2X RT-PCR Buffer (Ambion P/N AM1005) Kit lot No:	12.5	250
3	25X RT-PCR Enzyme MIX	1	20

4	FAM-TAMARA PP MIX (ASFV Risatti PPMIX) Microstores No:	3.75	75
	Total Volume	20	400
	Template DNA	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)

Sl no	Reaction component	Volume per reaction (µL)	Volume for 20 Rxs (µL)
1	Nuclease-Free Water	2.75	55
2	2X Reaction MIX (Invitrogen Cat No. 11731) Kit lot No:	12.5	250
3	SuperScript III/Platinum *Taq* MIX	0.5	10
4	ROX Reference Dye (freshly prepared to 1:10)	0.5	10
	ROX Ref No.		
5	FAM-TAMARA PP MIX (ASFV Risatti PPMIX) Microstores No:	3.75	75
	Total Volume	20	400
	Template DNA	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

7.17 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

7.18 Set up the reporter dye as FAM and quencher TAMRA for ASFV

8. RESULT INTERPRETATION and REPORTING

8.1 Qualitative analysis
Ct value Result

8.1.1 Positive : Ct less than 39

8.1.2 Negative : Ct greater than 41
8.1.3 Intermediate: Ct between 39 and 41

8.2 Quantitative analysis
8.3 Assess the Ct value when amplification curve of Standard 1, 2, 3, 4 passes threshold line.
8.4 Calculate quantitative value to compare with Ct value of unknown samples and curve of Standard 1, 2, 3, 4
8.5 Test validation
8.6 Each Ct value standard should be as follows.
8.7 Standard 1 > Standard 2 > Standard 3 > Standard 4
8.8 R-value of Standard curve should be 0.900~0.999.
8.9 The Standard result should be all positive.

9. WASTE DISPOSAL

9.1 All the wastes should be discarded after being autoclaved

10. RISK ASSESSMENT

10.1 The material to be tested for the presence of ASFV may be potentially contaminated with viable ASFV or other agents. Work should be conducted in an appropriate containment environment. Good laboratory practices at the P2 level should be used at minimum.
10.2 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.

11. TROUBLESHOOTING

11.1 Remove the possibility of contamination of nucleic acid and PCR product when performing PCR.
11.2 It is recommended to work at the clean bench designated for PCR
11.3 Use sterilized filter tip.
11.4 Do not create any bubbles in the tube
11.5 Avoid detection solution being exposed to strong light
11.6 Do not use expired kits
11.7 Read the result as ASF by following clinical symptoms and autopsy even if the kits show the positive result. You are required to ask for testing at the OIE reference laboratory or when the results are in doubt
11.8 The detection limit of the kit is 10 copies. It may not detect less than 10 copies.
11.9 The kit should be stored at -20(□. Under these conditions reagents are stable through the expiration date printed on the label.
11.10 Do not read result when Ct value is less than 5
11.11 ASFV Real-Time PCR Kit can be detect ASF virus RNA with high sensitivity, but it may sometimes show false negative result due to RNA mis-extraction, operating error from unskilled worker, denaturation of the kit, and any other unknown reason.

12. REFERENCES

12.1 CSIRO , AAHL, Quality assurance manual.

TITLE: **Conventional PCR for diagnosis of Brucella abortus**

PREPARED BY: **Molecular section**

REVISED BY: **Molecular section**

APPROVED BY: **Dr RB Gurung, Head, LSU**

DATE: **June 12, 2018**

1. INTRODUCTION

Brucella is a small Gram-negative bacterium (0.4-0.8 µm in diameter and 0.4-3.0 µm in length) which is non-flagellated, and non-spore forming. Four species are pathogenic to human: *Brucella abortus*, *Brucella melitensis*, *Brucella suis* and *Brucella canis*. All four species are the essential causative agent for Brucellosis, a disease characterized by undulating fever. Depending on exciter the disease is also called Morbus Bang (*B abortus*) or Malta fever (*B melitensis*). The pathogens are transmitted from infected animals. The infection is caused by contact with infected animals or their excrements as well as by non-pasteurized milk and milk products like fresh cheese from sheep or goat. Main entrances are skin wounds, conjunctiva and digestive tract. The intact pathogens are transported by granulocytes into local lymph nodes, from where they spread haematogenously. All kind of organs can be infected. Diagnosis is based on isolation and identification of the causative agent or by serology. The organisms can usually be readily isolated from vaginal exudates, aborted foetus, blood, milk, or semen of infected males. The most widely used serologic test is an agglutination test by a tube or slide method.

2. PRINCIPLES

Brucella spp detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using special *Brucella* spp primers. In conventional PCR the amplified product is bound to chemical dye, allowed to migrate through agar gel electrophoretically and captured at specific band location. The migrated band is visualized under UV light. Presence of target band base pair amplicon is considered positive result. In contrast, absence of target band is considered as negative result.

3. APPLICATION

This conventional PCR was developed for the detection of *Brucella abortus* from samples such as aborted materials and milk.

4. OBJECTIVE

This document is developed to support diagnosis of *Brucella abortus* infection using aborted materials without having to culture the pathogen.

5. APPARATUS AND EQUIPMENT

5.1 Thermo Cycler, 2720, Thermo fisher Scientific, Singapore
5.2 E-Gel Imager, MNO-25-1071, Life Technologies, Israel
5.3 MINI spin, Eppendorf, AG-22331, USA
5.4 Mixer Reamix, 2789
5.5 Mini spin, Spinwin
5.6 Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
5.7 Bio-Safety Cabinet, Class □II, Esco
5.8 Gel tank, BioRad, USA

6. REAGENTS, SOLUTION AND BUFFER

Master-mix for 5 reactions

6.1	Nuclease free water	50 µl
6.2	DNTP mix	25 µl
6.3	MgCl	12.5 µl
6.4	FW1	3.125 µl
6.5	R1	3.125 µl
6.6	FW2	3.125 µl
6.7	R2	3.125 µl
6.8	DNA polymerase	7.5 µl
6.9	PCR buffer	12.5 µl
6.10	Total	120 µl
6.11	Template	1 µl

7. PROCEDURE

7.1 Sample preparation

7.1.1 Prepare master mix as above
7.2.1 Triturate 1 g of placental cotyledon in distilled water (I BSC Class II)
7.3.1 Take 40 µl of the triturated material or foetal stomach content in a tube and heat at 95°C for 10 min in thermal cycler/heating block
7.4.1 Centrifuge after heating and use 1 µl of supernatant as template

7.2 Preparation of 1.5% of agarose gel

7.2.1 0.75 g of agar in 50 ml of TBE
7.2.2 Dissolve till agarose melt
7.2.3 Do not boil
7.2.4 Let it remain for some time (to lower the temperature so that you are able to handle without causing any injury) and pour it into the tray without comb
7.2.5 After uniformly pouring, place the comb immediately
7.2.6 Transfer/Place the gel in another tank with TBE/TEA buffer
7.2.7 Load the ladder on first well

7.2.8 Load the sample in remaining wells
7.2.9 Run at 100 V and 400 Amp for 32-40 min
7.2.10 Place gel carefully after removing the buffer in a container and flood gel red (5-10 µl) in 50 ml distilled water. Gel red is available as10000 x, so prepare 1x
7.2.11 Leave it for 10-20 min
7.2.12 Rinse with distilled water once and again add distilled water and let it remain undisturbed for 10-20 min
7.2.13 It will de-stain the Gel Red from gel except the strained DNA product, so can remain for longer time.
7.2.14 Discard the distilled water and observe the gel under UV light

L: Ladder (1.5 kb)
P: 450 bp for brucellosis (should be seen for all Brucella)
Primers used are for 450 bp and 1071 bp

Cycling conditions

Temperature (°C)	Time	Stage	Number of cycle
90°C	5 min	Denaturation	1
95°C	30 sec	Annealing	25
55°C	30 sec	Annealing	
72°C	30 sec	Annealing	
72°C	7 min	Final extension	1
4°C		Hold	

7.3 Use the following information while loading on to gel

7.3.1 Gel loading dyes 1 µl
7.3.2 PCR product 5 µl
7.3.3 100 bp DNA ladder 6 µl
7.3.4 The gel is read using E-Gel Imager (MNO-25-1071, Life Technologies, Israel)
7.3.5 Capture the image and store in the PC

8. RESULT INTERPRETATION

Presence of amplicon band (450 bp and 1071 bp) on agarose gel size is considered positive for *B abortus*

9. WASTE DISPOSAL

All the wastes should be discarded after being autoclaved

10. RISK ASSESSMENT

Brucella organism being a zoonotic agent, all possible safety measures should be complied while handling samples until it is completely inactivated. Personal protective such as gloves, eye protection and apron should be donned all time while handling sample.

11. TROUBLESHOOTING

If the cycling conditions are not followed the amplification process may not occur and fail to produce the target size product. There is also a possibility of primer-dimer formation due to sticky ends of primers.

12. REFERENCES

12.1 Debeaumont C., Falconnet P.A., Maurin M. Real-time PCR for detection of Brucella spp., DNA in human serum samples. Eur. J. Clin. Microbiol. Infect Dis. 2005 Dec, 24 (12):842-845.

12.2 DGERRN6DPSOLJTUDVSRUWDWLRDG6WRUDJHRIOLFDO0DWHULDOIRU35 LDJRVWLFVGHHORSHGE)HGHUDO6WDWH,VWRMWLHEHWUDO 5HVHDUFK,VWLWWHRI(SLGHPLRORJRI)HGHUDO6HUYLFHIRU6UHLOODFHR RVPHUV5LJKWV3URWHFWLRDGPDHOO -Being, Moscow, 2008.

12.3 L9URGDUG,7KRPDHWL0DNDD39)UHEULORHO identification and differentiation of *Brucella melitensis, B. abortus, B. suis, B. ovis, B. canis, and B. neotomae* suitable for both conventional and real-time PCR systems. *J Microbiol Methods*. 2008 Oct;75(2):375-8. PMID: 18675856

12.4 L9URGDUG,7KRPDIROE00LVHUH5EULO6 -based real-time PCR assay as a tool for detection of Brucella spp. in wild boars and comparison with bacteria

TITLE: **Conventional PCR for diagnosis of Footrot in sheep**

PREPARED BY: **Molecular section**

REVISED BY: **Molecular section**

APPROVED BY: **Head, LSU**

DATE: **June 12, 2018**

1. INTRODUCTION

Footrot is a mixed bacterial infection of the feet of ruminants in which *Dichelobacter nodosus* is the essential transmitting agent (1, 2). *D. nodosus* is a slow growing anaerobe requiring special media and conditions for growth. Antigenic variation among strains of *D. nodosus* was first observed by Beveridge (1941) and ascribed to surface (K) antigens that were detected using a slide agglutination test (3) The K agglutination reaction, attributable to fimbriae which is the major immunogen (4,5) was used to define a number of serogroups of *D. nodosus* (6,7,8). In the Australian classification system there are 9 serogroups (A-I). Serotypes have been described within most serogroups. Molecular diagnosis of footrot involves serogroup specific PCR.

2. PRINCIPLES

D. nodosus detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome serogroup specific region using special *D. nodosus* primers. In conventional PCR the amplified product is bound to chemical dye, allowed to migrate through agar gel electrophoretically and captured at specific band location. The migrated band is visualized under UV light. Presence of target band base pair amplicon is considered positive result. In contrast, absence of target band is considered as negative result.

3. APPLICATION

This conventional PCR was developed for the detection of *D nodosus* from interdigital swab samples

4. OBJECTIVE

This document is developed to support diagnosis of Footrot in sheep.

5. APPARATUS AND EQUIPMENT

5.1 Thermo Cycler, 2720, Thermo fisher Scientific, Singapore
5.2 E-Gel Imager, MNO-25-1071, Life Technologies, Israel
5.3 MINI spin, Eppendorf, AG-22331, USA
5.4 Mixer Reamix, 2789
5.5 Mini spin, Spinwin
5.6 Refrigerated centrifuge, PK-121R, Thermo Electron Corporation
5.7 Bio-Safety Cabinet, Class □II, Esco
5.8 Gel tank, BioRad, USA

6. REAGENTS, SOLUTION AND BUFFER

6.1 10 x PCR buffer
6.2 2mm dNTP mix
6.3 50mm Magnesium chloride
6.4 Taq polymerase
6.5 Sterile DNA free water
6.6 Template DNA

Details of common forward primer and serogroup-specific reverse primers used in PCR assays

Primer name	Nucleotide sequence	Position in *fim A*	Product size
FP		26-46	-
RA		421-441	415bp
RB		286-309	283bp
RC		331-351	325bp
RD		325-345	319bp
RE		367-389	363bp
RF		250-267	241bp
RG		283-305	279bp
RH		412-435	409bp
RI		194-215	189bp

Note: product sizes given are of amplicons produced with forward primer (FP) and the respective serogroup reverse primers.

Master-mix for 5 reactions

Serogroup specific - A, B & C multiplex PCR Worksheet

Footrot PCR No: _____Date: _____ Officer: _____

Serogroup specific A, B, & C multiplex PCR on footrot DNA samples as per key list

Reagents		Concentration		x
10x PCR Buffer _____	#		2.0	
dNTP _____	#	0.2mM	2.0	
MgCl	# _____	3.0mM	1.2	
Forward Primer _____	ID: FP #	0.2uM	2.5	
Multiplex Reverse _____	ID: RA #	0.2uM	1.0	
	ID: RB # _____	0.2uM	1.0	
	ID: RC # _____	0.2uM	1.0	
H2O	# _____		7.1	
Taq Polymerase			0.2	
DNA template			2.0	
Final Volume			20	

Results:

Conclusion:

Serogroup specific D, E, & F multiplex PCR on footrot DNA samples as per key list

Reagents		Concentration		x
10x PCR Buffer _____	#		2.0	
dNTP _____	#	0.2mM	2.0	
MgCl	# _____	3.0mM	1.2	
Forward Primer _____	ID: FP #	0.2uM	2.5	
Multiplex Reverse _____	ID: RD #	0.2uM	1.0	
	ID: RE # _____	0.2uM	1.0	
	ID: RF #	0.2uM	1.0	

_____			
H2O # _____		**7.1**	
Taq Polymerase		**0.2**	
DNA template		**2.0**	
Final Volume		**20**	

Results:

Conclusion:

Serogroup specific G, H, & I multiplex PCR on footrot DNA samples as per key list

Reagents	**Concentration**		**x**
10x PCR Buffer # _____		**2.0**	
dNTP # _____	**0.2mM**	**2.0**	
MgCl # _____	**3.0mM**	**1.2**	
Forward Primer ID: FP # _____	**0.2uM**	**2.5**	
Multiplex Reverse ID: RG # _____	**0.2uM**	**1.0**	
ID: RH # _____	**0.2uM**	**1.0**	
ID: RI # _____	**0.2uM**	**1.0**	
H2O # _____		**7.1**	
Taq Polymerase		**0.2**	
DNA template		**2.0**	
Final Volume		**20**	

Results:

Conclusion:

7. PROCEDURE

PCR amplifications can be performed in a 20µL volume in 0.2mL thin-walled centrifuge tubes. The PCR mixture contains a final concentration of 20mM Tris-HCl, 50mM KCl, 3mM MgCl2 and 200µM each of the four nucleotides. The concentration of each of the primers is 0.25-0.5µM. One unit of Taq polymerase (GIBCO-BRL) is added to the reaction mix. Approximately 50- 100ng of DNA or 1µL of cell lysate is

added as template. The amplification cycles in Peltier thermal cycler PTC 200 (M J Research, Inc. Massachusetts, USA) consists of 94°C for 4 minutes, followed by 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds for 5 cycles, 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds for 25 cycles, and final extension at 72°C for 4 minutes. The PCR products are electrophoresed on 0.8%-3.0% agarose gels, stained with ethidium bromide and visualised under ultraviolet (UV) illumination. The amplification conditions for multiplex PCR are same except for an increased concentration of forward primer (2.5 times) compared to reverse primers.

7.1 Preparation of 1.5% of agarose gel

7.1.1 0.75 gm of agar in 50 ml of TBE
7.2.1 Dissolve till agarose melt
7.3.1 Do not boil
7.4.1 Let it remain for some time (to lower the temperature so that you are able to handle without causing any injury) and pour it into the tray without comb
7.5.1 After uniformly pouring, place the comb immediately
7.6.1 Transfer/Place the gel in another tank with TBE/TEA buffer
7.7.1 Load the ladder on first well
7.8.1 Load the sample in remaining wells
7.9.1 Run at 100 V and 400 Amp for 32-40 min
7.10.1 Place gel carefully after removing the buffer in a container and flood gel red (5-10 µl) in 50 ml distilled water. Gel red is available as10000 x, so prepare 1x
7.11.1 Leave it for 10-20 min
7.12.1 Rinse with distilled water once and again add distilled water and let it remain undisturbed for 10-20 min
7.13.1 It will de-stain the Gel Red from gel except the strained DNA product, so can remain for longer time.
7.14.1 Discard the distilled water and observe the gel under UV light

7.2 Cycling conditions (touch down)

7.2.1 Cycle 1 - 94°C-3 mins, 1 repeat
7.2.2 Cycle 2 - 94°C-30 sec, 60°C-30 sec, 72°C-30 sec, 2 repeats
7.2.3 Cycle 3 - 94°C-30 sec, 58°C-30 sec, 72°C-30 sec, 2 repeats
7.2.4 Cycle 4 - 94°C-30 sec, 55°C-30 sec, 72°C-30 sec, 10 repeats
7.2.5 Cycle 5 - 94°C-30 sec, 52°C-30 sec, 72°C-30 sec, 15 repeats
7.2.6 Cycle 6 - 72°C-4 mins, 1 repeat
7.2.7 Cycle 7 - 4°C, 1 repeat

8. RESULT INTERPRETATION

Presence of serogroup specific amplicon band (in the table) on agarose gel size is considered positive for *D nodosus*

9. WASTE DISPOSAL

All the wastes should be discarded after being autoclaved. If ETB used it should be disposed as per the waste disposal protocol for carcinogen

10. RISK ASSESSMENT

Personal protective such as gloves, eye protection and apron should be donned all time while handling sample.

11. TROUBLESHOOTING

If the cycling conditions are not followed the amplification process may not occur and fail to produce the target size product. There is also a possibility of primer-dimer formation due to sticky ends of primers.

12. REFERENCES

12.1 Beveridge, W. I. B. (1941). Footrot in sheep: a Transmissible disease due to infection with Fusiformis nodosus. Studies on its cause, epidemiology and control. Council for Scientific and Industrial Research, Melbourne, Bulletin No. 140.

12.2 Egerton, J. R., Roberts, D. S. & Parsonson, I. M. (1969). The aetiology and pathogenesis of ovine footrot. A histological study of the bacterial invasion. Journal of Comparative Pathology 79, 207-16.

12.3 Egerton, J. R. (1973). Surface and somatic antigens of Fusiformis nodosus. Journal of Comparative Pathology 83, 151-9.

12.4 Thorley, C. M. & Egerton, J. R. (1981). Comparison of Alum-absorbed or non-alum-absorbed oil emulsion vaccines containing either pilate or non-pilate Bacteriodes nodosus cells in inducing and maintaining resistance of sheep to experimental footrot. Research in Veterinary Science 30, 32-7.

12.5 Egerton, J. R., Cox, P. T., Anderson, B. J., Kristo, C. Norman, M & Mattick, J. S. (1987). Protection of sheep against footrot with a recombinant DNA based Fimbrial vaccine. Veterinary Microbiology 14, 393-409.

12.6 Schmitz, J. A. & Gradin, J. L. (1980). Serotypic and biochemical characterisation of Bacteriodes nodosus isolates from Oregon. Canadian Journal of Comparative Medicine 44, 440-6.

12.7 Claxton, P. D., Ribeiro, L. A. & Egerton, J. R. (1983). Classification of Bacteroides nodosus by agglutination tests. Australian Veterinary Journal 60, 331-4.

12.8 Claxton, P. D. (1986). Serogrouping of Bacteroides nodosus isolates. In Footrot in Ruminants, Proceedings of a Workshop (Stewart, D. J., Peterson, J. E., McKern, N. M. & Emery, D. L. Eds.) pp. 131-4. Melbourne 1985: CSIRO