STANDARD OPERATING PROCEDURE			
Title:	**Bovine Viral Diarrhoea Virus (BVDV) - Detection of BVDV nucleic acid by RT-PCR**		
Reference:	**Vilcek *et al* (1994)**	*Author:*	**DR N N Barman, MS Elina Khatoon**

1.0 Introduction

1.1 Purpose/Scope Of this SOP

This SOP outlines the method for the detection of BVDV samples. RNA is isolated from samples using either the QIAamp® Viral RNA Mini kit protocol or the conventional method using TRIZOL reagent. Once the RNA has been eluted, store at -18°C or below or keep on ice and proceed to BVDV RNA detection by RT-PCR.

2.0 MATERIALS:

2.1.1	Consumables	Supplier
	0.2ml PCR tubes	GENAXY
	0.2ml Thin Wall Clear PCR 12-Strip Tubes and Flat Strip Caps	GENAXY
	Filter Tips	GENAXY
2.1.2	**Equipment**	**Supplier, Model**
	Class 2 safety microbiological safety cabinet	LABCHEM & LABORTENIK INSTRUMENTS
	Micro-centrifuge	TARSONS
	VERITI 96-well Thermalcycler	APPLIED BIOSYSTEMS
	Micropipette (0.5-10 µl, 10-100 µl, 20-200 µl, 100-1000 µl)	GENAXY (Nichipette)
	7500 Real Time PCR System	Applied Biosystems

3.0 PROCEDURES:

3.1 Collection Of BVDV Suspected Samples

Clinical as well as post mortem samples were collected from suspected samples

3.1.1 Clinical samples:

Samples (semen, liver, spleen)

3.2 Detection Of BVDV Viral Nucleic Acid:

Clinical as well as post mortem samples collected from affected bovines and were processed for detection of viral nucleic acid by RT-PCR. Standardization of RT-PCR was taken from published paper of Vilcek *et al* (1994).

3.3 Extraction of viral RNA:

Extraction of viral RNA was done by QIAamp® Viral RNA Mini kit protocol.

- Pipet 560ul of prepared buffer AVL containing carrier RNA into a 1.5ml microcentrifuge tube.
- Add 140ul plasma, serum, urine, cell-culture supernatant, or cell-free body fluid to the Buffer AVL-carrier RNA in the microcentrifuge tube. Mix by pulse vortexing for 15s.
- Incubate at room temperature (15-25^{o}C) for 10min.
- Briefly centrifuge the tube to remove drops from the inside of the lid.
- Add 560ul of ethanol (96-100%) to the sample, and mix by pulse-vortexing for 15s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.
- Carefully apply 630ul of the solution from previous step to the QIAmp Mini column without wetting the rim. Close the cap, and centrifuge at 6000xg (8000rpm) for 1 min. Place the QIAamp Mini column into a clean 2ml collection tube, and discard the tube containing the filtrate.
- Carefully open the QIAamp Mini column, and repeat previous step.
- Carefully open the QIAamp Mini column, and add 500ul of Buffer AW1.Close the cap, and centrifuge at 6000xg(8000rpm) for 1min. Place the QIAamp Mini column in a clean 2ml collection tube(provided) and discard the tube containing the filtrate.
- Carefully open the QIAamp Mini column, and add 500ul of buffer AW2.Close the cap and centrifuge at full speed (20,000xg; 14000rpm) for 3min.Continue carryover, perform next step.
- Place the QIAamp in a clean 1.5ml microcentrifuge tube. Discard the old collection tube containing filtrate. Carefully open the QIAamp Mini column and add 60ul of Buffer AVE equilibrated to room temperature. Close the cap and incubate at room temperature for 1min. Centrifuge at 8000rpm for1min.

3.4 Reverse transcription: cDNA preparation step:

Complementary DNA was synthesized using following reaction conditions:

Total reaction volume : 13µl		
Reagents/Ingredients	**Volume**	**Supplier**
Total RNA	6.0µl	
Random primer (50 ng/ µl)	1µl	Fermentus
Nuclease free water to make	13µl	Fermentus

The contents were mixed properly and spun briefly and placed in the thermal cycler for incubation on following condition:

70^0C	5 minutes
25^0C	10 minutes
$4\ ^0$C	Hold

The contents were brought to the bottom of the tube by brief spinning. Thereafter, the following reagents were added to the mixture:

Total reaction volume : 7µl		
Reagents/Ingredients	**Volume**	**Supplier**
5X RT buffer	4.0µl	Fermentus
RNAase inhibitor (1U)	1µl	Fermentus
10mM dNTP mix	1µl	Fermentus
M-MuLV RT (200U/ µl)	1µl	Fermentus

The contents were mixed properly and spun briefly and placed in the thermal cycler for incubation on following condition:

Temperature	**Time**
25^0C	5 minutes
42^0C	1hour
70^0C	10 minute
4^0C	Hold

The cDNA thus formed was stored at -20 ^{0}C after proper labeling till further use.

3.5 RT-PCR (GEL BASED):

TABLE 1: DESCRIPTION OF THE PRIMERS USED IN THE PRESENT STUDYTO AMPLIFY BVDV.

Primer designation	Genomic region	Primer sequence	Amplifies	Reference
Primer XI	V-324	5'ATGCCCTATAGTAGGACTAGCA3'	All pesti-viruses	Vilcek *et al* (1994)
Primer XII	V-326	5'TCAACTCCATGTGCCATGTAC 3'		Vilcek *et al* (1994)

3.6 Reaction master mixture:

The targeted gene of the BVDV was amplified using following reagents (Table: 2)

TABLE: 2 DETAILS OF THE PCR REACTION MIXTURE

Total reaction volume : 45 µl		
Reagents/Ingredients	**Volume**	**Supplier**
Water, Nuclease-free	33.5µl	Fermentus
10x Buffer	5µl	Fermentus
25mM MgCl2	3µl	Fermentus
10 mM dNTPs	1µl	Fermentus
Forward primer (10 pmol/µl)	1ul	XXIDT
Reverse primer (10 pmol/µl)	1µl	XXIDT
Taq polymerase	0.5µl	

- Thoroughly mix the master mix and aliquot 45µl into the appropriate number of 0.2ml PCR tubes
- Remove the tubes containing the PCR master mix and place an appropriate number of tubes.
- Add 5µl of the cDNA samples to the 45µl master mix.
- Once cDNA is added, fit caps to all wells, including empty wells if a PCR plate is used. It is important that the caps are fitted firmly and correctly onto the wells before being used on the real time machine.

3.7 Amplification of V-324 & V-326:

- Place the reaction tubes or plate into the Thermal cycler (Veriti 96-well Applied Biosystems).
- Incubate the reactions with the following thermo cycling profile:

TABLE 3: DETAILS OF THE THERMAL CYCLER CONDITION

Number of cycles	Temperature	Time
1 cycle	94^0C	5 minutes
40 cycles	94^0C	1 minute
	56^0C	1 minute
	72^0C	1 minutes
1 cycle	72^0C	10 minutes
	4^0C	Stored until use

The complete run takes approximately 1 hour and 35 minutes and the PCR amplicons thus obtained were stored at -20 ^{0}C, till further use..

3.8 Confirmation of PCR amplicons : Gel Electrophoresis

The confirmation of RT-PCR amplicons was carried out by their sizes in agarose gel. The PCR products were electrophorosed in 1.7% agarose gel containing ethidium bromide in 0.5X tris borate EDTA and visualized on a UV transilluminator as per standard procedures. For size comparision, a 100bp DNA ladder marker was run parallel to the PCR amplicons.

The PCR amplicons of Vilcek primer shows at 288 bp.

3.9 Interpretation:

The amplified product visualized as a single compact fluorescent band of 288bp under UV light and documented by gel documentation system is considered positive for BVDV.