ViroReal® Kit
Bluetongue Virus (BTV)

Manual

For use with the

- **ABI PRISM® 7500 (Fast)**
- **Mx3005P®**
- **LightCycler® 480**

For veterinary use only

REF DVEV02211, DVEV02213 Σ **100**

REF DVEV02251, DVEV02253 Σ **50**

ingenetix GmbH
Arsenalstraße 11
1030 Vienna, Austria
T +43(0)1 36 198 0 198
F +43(0)1 36 198 0 199
office@ingenetix.com
www.ingenetix.com

Index

1. Product description 3
2. Pathogen information 3
3. Principle of real-time PCR 3
4. General Precautions 4
5. Contents of the Kit 5
5.1. ViroReal® Kit Bluetongue Virus (BTV) order no. DVEV02211 or DVEV02251 5
5.2. ViroReal® Kit Bluetongue Virus (BTV) order no. DVEV02213 or DVEV02253 5
6. Additionally required materials and devices 5
7. Preparation of real-time PCR 6
7.1. Internal RNA positive control (RNA IPC) 6
7.2. Positive Control 6
7.3. Pipetting scheme 6
7.4. Programming of the temperature profile 7
8. Interpretation of PCR-data 7
8.1. Signal in FAM channel 7
8.2. No signal in FAM channel but signal of the RNA IPC 7
8.3. No signal in FAM channel and no signal with the RNA IPC 7
9. Troubleshooting 8
9.1. No BTV specific signal with positive control 8
9.2. No signal with the RNA IPC and no BTV specific signal with the sample 8
9.3. BTV specific signal with the negative control 8
9.4. BTV specific signal with the negative control of RNA-extraction (optional) 8
10. Specifications and performance evaluation 9
10.1. Analytical sensitivity and linearity 9
10.2. Analytical specificity 9
10.3. Kit performance 10

Explanation of symbols

LOT	Batch code		Use by
REF	Catalogue number		Manufactured by
Σ	Contains sufficient for <n> tests		Store at
	Corrosion, GHS05		Exclamation mark, GHS07

1. Product description

ViroReal® Kit Bluetongue Virus (BTV) is a real-time PCR kit for the detection of bluetongue virus (BTV) RNA using one-step reverse transcription real-time PCR. This test was developed for the ABI PRISM® 7500 (Fast) instrument (Thermo Fisher Scientific), LightCycler® 480 (Roche) and for Mx3005P® (Agilent), but is also suitable for other real-time PCR instruments. This test allows the rapid and sensitive detection of BTV RNA purified from tissues (e.g. spleen, lymph nodes, etc.) and blood (e.g. with the QIAamp Viral RNA Mini Kit, Qiagen).

ViroReal® Kit Bluetongue Virus (BTV) detects segment 10 (NS3) of BTV. A probe-specific amplification-curve at 530 nm (FAM channel) indicates the amplification of BTV specific RNA.
An internal RNA positive control system for detection in VIC/HEX channel (order no. DVEV02211 or DVEV02251) or in Cy5 channel (order no. DVEV02213 or DVEV02253) allows control of RNA extraction and excludes false-negative interpretation of results due to inhibition of reverse transcription real-time PCR (see 8. Interpretation of PCR-data).
When using PCR-platforms not validated by ingenetix, an evaluation of the multiplex-PCR is recommended. Please be aware that some PCR-platforms have to be calibrated with the corresponding dye before performing multiplex-PCR.

BactoReal®, MycoReal, ParoReal and ViroReal® Kits are optimized to run under the same thermal cycling conditions. RNA and DNA material can be analysed in one run.

2. Pathogen information

Bluetongue virus (BTV) is a segmented, linear, double-stranded RNA virus (dsRNA), belonging to the *Reoviridae* family and is divided into 28 serotypes. The virus is transmitted by biting midges and infects wild and domestic ruminants such as sheep and less frequently goats, deer and cattle. There is no appropriate treatment of acute disease, which is associated with high morbidity and mortality in some sheep breeds. Prophylactic immunisation with attenuated and inactivated vaccines is available. However, they do not cover all virulent BTV serotypes.

References:
Purse BV, Brown HE, Harrup L, Mertens PP, Rogers DJ. 2008. Invasion of bluetongue and other orbivirus infections into Europe: the role of biological and climatic processes. Rev Sci Tech. 27(2):427-42.

3. Principle of real-time PCR

When detecting pathogens by reverse transcription real-time PCR, a specific RNA sequence of the pathogen genome is transcribed into cDNA and amplified. The generated PCR-product is detected by an oligonucleotide-probe labelled with a fluorescent dye. This technology allows for a sequence-specific detection of PCR amplificates.

4. General Precautions

- Always include a negative control per PCR-run (Nuclease-free water instead of sample).
- Optional: for valid interpretation of results, a negative control should be included during RNA-extraction (for example extraction of water instead of sample material), in order to exclude false-positive results due to contamination with BTV RNA during extraction.
- Be careful when handling the positive control.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated workspace.
- Periodically decontaminate benches and devices.
- Use sterile pipette tips with filters.
- Thaw all components thoroughly at room temperature before starting an assay. When thawed, mix the components and centrifuge briefly.
- Always keep the RNA Reaction Mix on ice.
- Use the RNA immediately after extraction and store at -20°C to -80°C as soon as possible.
- **Caution:** the Positive Control and the RNA IPC Target are stored in RNA stabilizer that contains Guanidinium thiocyanate/Triton X-100 (see MSDS, www.ingenetix.com).

5. Contents of the Kit

5.1. ViroReal® Kit Bluetongue Virus (BTV) order no. DVEV02211 or DVEV02251

Labelling	Content	Amount		Storage
		DVEV02211	DVEV02251	
BTV Assay Mix (green cap)	Primer and probe (FAM) for BTV detection	2 x 50 µl	1 x 50 µl	-15 °C to -25 °C
RNA IPC-1 Assay Mix (yellow cap)	Primer and probe (VIC/HEX) for RNA IPC detection	2 x 50 µl	1 x 50 µl	-15 °C to -25 °C
RNA IPC Target (orange cap)	RNA internal positive control	1 x 100 µl	1 x 100 µl	-15 °C to -25 °C
BTV Positive Control (red cap)	RNA positive control (approx. 1,500,000 target copies/µl)	1 x 15 µl	1 x 15 µl	-15 °C to -25 °C
RNA Reaction Mix (white cap)	Amplification mix for one-step RT real-time PCR	2 x 250 µl	1 x 250 µl	-15 °C to -25 °C
Nuclease-free water (blue cap)	Nuclease-free water	2 x 1000 µl	1 x 1000 µl	-15 °C to -25 °C

5.2. ViroReal® Kit Bluetongue Virus (BTV) order no. DVEV02213 or DVEV02253

Labelling	Content	Amount		Storage
		DVEV02213	DVEV02253	
BTV Assay Mix (green cap)	Primer and probe (FAM) for BTV detection	2 x 50 µl	1 x 50 µl	-15 °C to -25 °C
RNA IPC-3 Assay Mix (yellow cap)	Primer and probe (Cy5) for RNA IPC detection	2 x 50 µl	1 x 50 µl	-15 °C to -25 °C
RNA IPC Target (orange cap)	RNA internal positive control	1 x 100 µl	1 x 100 µl	-15 °C to -25 °C
BTV Positive Control (red cap)	RNA positive control (approx. 1,500,000 target copies/µl)	1 x 15 µl	1 x 15 µl	-15 °C to -25 °C
RNA Reaction Mix (white cap)	Amplification mix for one-step RT real-time PCR	2 x 250 µl	1 x 250 µl	-15 °C to -25 °C
Nuclease-free water (blue cap)	Nuclease-free water	2 x 1000 µl	1 x 1000 µl	-15 °C to -25 °C

The components of ViroReal® Kit Bluetongue Virus (BTV) are stable until the expiry date stated on the label. Repeated thawing and freezing should be avoided. Please protect kit components from light.

6. Additionally required materials and devices

- Reagents and devices for RNA-extraction
- Nuclease-free water for dilution of RNA IPC Target and positive control
- Disposable powder-free gloves
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Desktop centrifuge with rotor for 2 ml reaction tubes
- Real-time PCR instrument which is able to detect and differentiate fluorescence in FAM and VIC/HEX or Cy5 channel
- Appropriate 96 well reaction plates or reaction tubes with corresponding (optical) closing material

7. Preparation of real-time PCR

Please make sure that at least one negative control (water, blue cap), as well as one positive control (red cap) and one extraction negative control (optional, recommended) are included per PCR run.
Ingenetix highly recommends performing PCR analyses in duplicates, which increases the probability of detection of the pathogen and facilitates interpretation of results.

- Prepare master mix on ice.
- Thaw RNA Reaction Mix on ice, and invert 2 to 3 times to ensure homogenous solution. Do not let it warm to room temperature.
- Use RNA immediately after extraction and store at -20 to -80°C as soon as possible.

7.1. Internal RNA positive control (RNA IPC)

An internal RNA positive control system containing the RNA IPC assay and the RNA IPC Target excludes false-negative interpretation of results due to inhibition of reverse transcription real-time PCR.
→ Dilute RNA IPC Target freshly 1:500 with nuclease-free water and add to the master mix (use 1 µl/reaction).
→ Alternatively, for control of RNA extraction and PCR inhibition the RNA IPC Target can be added during extraction. Spike 1 µl of undiluted RNA IPC Target into the sample material after the lysis buffer was added. Caution: Do not add the RNA IPC Target directly to the sample material.

7.2. Positive Control

The BTV Positive Control is an *in vitro* synthesized RNA in RNA-stabilizer. It has to be stored at -20°C. Before use it has to be freshly diluted 1:500 with nuclease-free water, which corresponds to approx. 3,000 target copies/µl.
→ As positive control use 1 µl of the freshly 1:500 diluted BTV Positive Control + 9 µl nuclease-free water. Caution: The use of more than 1 µl positive control (diluted 1:500) inhibits the RT-PCR reaction.

7.3. Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	Nuclease-free Water*	2.0 µl
	RNA Reaction Mix	5.0 µl
	BTV Assay Mix	1.0 µl
	RNA IPC Assay Mix	1.0 µl
	RNA IPC Target# (freshly diluted 1:500)	1.0 µl
	Total volume Master Mix	**10.0 µl**
Preparation of PCR	Master Mix	10.0 µl
	RNA-Sample*	10.0 µl
	Total volume	**20.0 µl**

*1-10 µl of the sample can be used. When using an amount < 10 µl of the sample, the amount of H_2O has to be changed accordingly.
#If RNA IPC Target not already added during extraction.

7.4. Programming of the temperature profile

Please find further information on programming the real-time PCR instrument in the respective operator's manual. Please be aware that some PCR-platforms have to be calibrated with the corresponding dye before performing multiplex-PCR.

Select dyes: FAM-TAMRA for detection of BTV
Cy5-NONE (RNA IPC-3 Assay Mix) **or** VIC/HEX-TAMRA (RNA IPC-1 Assay Mix) for detection of RNA IPC

Select reference dye (passive reference): ROX

Sample Volume: 20 µl

Temperature Profile:

Program 1	Program 2	Program 3
Cycles: 1 Analysis: None	Cycles: 1 Analysis: None	Cycles: 45 Analysis: Quantification Acquisition at 60°
50°C 15 min	95°C 20 sec	95°C 5 sec 60°C 1 min

For ABI PRISM® 7500:
Ramp speed: Without "fast cycling" parameter

For LightCycler® 480 instrument:
Detection format: 2 Color Hydrolysis Probe (dyes see above)

Note: These instrument parameters can be used for all BactoReal®, MycoReal, ParoReal and ViroReal® kits on all PCR instruments.

8. Interpretation of PCR-data

For a valid interpretation, the following criteria must be fulfilled:

	Ct/Cp (FAM channel) BTV target	Ct/Cp RNA IPC target	Interpretation
Negative control	Negative	26-29*	Valid
Positive control (freshly diluted 1:500), approx. 3,000 copies, 1 µl/PCR	25-27	26-29*	Valid
Extraction negative control (optional)	Negative	26-29	Valid
Negative sample	Negative	26-29	Valid
Positive sample	Positive	26-29/negative	Valid

*In the case that the RNA IPC target has been added to the master mix

For analysis of PCR data please proceed as follows:
For analysis of PCR results gained with ViroReal® Kit Bluetongue Virus (BTV) please select fluorescence display options FAM channel for the BTV target and VIC/HEX channel (order no. DVEV02211, DVEV02251) or Cy5 channel (order no. DVEV02213, DVEV02253) for the internal RNA positive control target (RNA IPC). Samples with a positive Cp or Ct-value are considered positive. Please also check amplification-curves manually.

8.1. Signal in FAM channel

→ RNA of BTV was amplified. The sample has to be interpreted as positive.
BTV RNA can lead to a reduced or absent fluorescence signal of the RNA IPC.

8.2. No signal in FAM channel but signal of the RNA IPC

→ No BTV RNA is detectable in the sample. The sample has to be interpreted as negative.
The positive signal of the RNA IPC assay excludes a putative PCR inhibition.

8.3. No signal in FAM channel and no signal with the RNA IPC

→ No interpretation statement can be made.
Information about possible sources of error and their solution can be found in 9. Troubleshooting.

9. Troubleshooting

9.1. No BTV specific signal with positive control

- Incorrect programming of the temperature profile of the real-time PCR instrument.
  → Compare the temperature profile with the protocol (see 7. Preparation of real-time PCR).
- Incorrect configuration of the PCR reaction.
  → Check your work steps (see 7. Preparation of real-time PCR) and repeat the PCR, if necessary.
- RNA might be degraded.
  → Prepare a fresh 1:500 dilution of the positive control and repeat the PCR.

9.2. No signal with the RNA IPC and no BTV specific signal with the sample

- The PCR reaction was inhibited. No interpretation can be made.
  → Make sure that you use a recommended method for RNA isolation and stick closely to the manufacturer's instructions.
  → If no operating mistakes during extractions can be retraced, it is recommended to repeat the PCR with lower amounts of RNA-eluate (1/5 or 1/10 of sample volume + the adequate amount of H_2O).
- Incorrect PCR conditions.
  → Check the PCR conditions and repeat the PCR, if necessary.

9.3. BTV specific signal with the negative control

- A contamination occurred during preparation of the PCR.
  → Repeat PCR with new reagents in replicates.
  → Strictly pipette the positive controls at last.
  → Make sure that work space and instruments are decontaminated at regular intervals.

9.4. BTV specific signal with the negative control of RNA-extraction (optional)

- A contamination occurred during extraction.
  → Repeat the extraction and PCR using new reagents.
  → Make sure that work space and instruments are decontaminated at regular intervals.

10. Specifications and performance evaluation

ViroReal® Kit Bluetongue Virus (BTV) was evaluated with the ABI PRISM® 7500 (Fast) instrument. For further validation data please contact ingenetix GmbH.

10.1. Analytical sensitivity and linearity

ViroReal® Kit Bluetongue Virus (BTV) was tested with a 10-fold dilution series of a synthetic RNA representing a fragment of BTV RNA. At least 50 target copies/reaction could be detected.
The assay shows **linearity** over the range of 100 to 1,000,000 target copies/reaction with a slope of -3.3 and a R_2 of > 0.9 as shown in Figure 1.

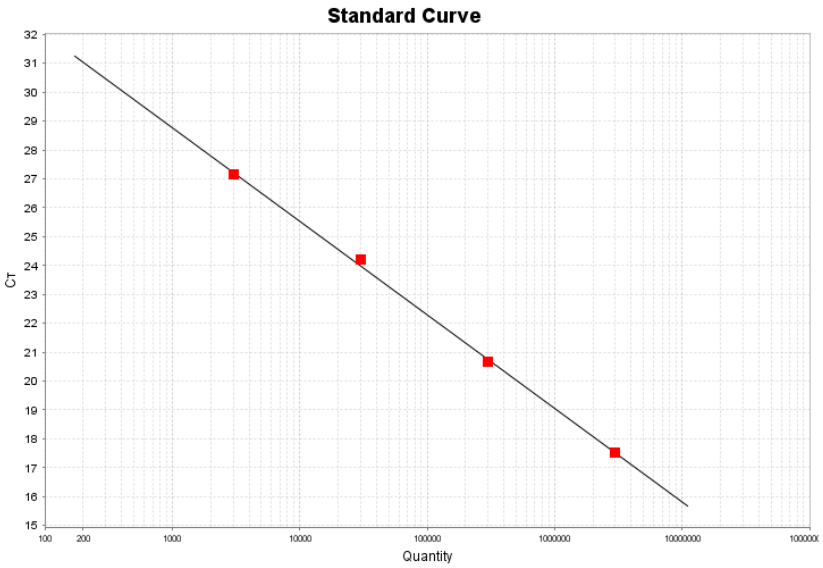

Figure 1 Ten-fold dilution series of a BTV RNA standard plotted against CT

10.2. Analytical specificity

The specificity is ensured by the selection of highly specific primers and probes. The primers and probes were checked for possible homologies to currently published sequences by sequence comparison analyses. This also validated the detection of so far known BTV strains. Sequence comparison ensures the detection of 24 serotypes of BTV.

10.3. Kit performance

Performance of ViroReal® Kit Bluetongue Virus (BTV) with an Applied Biosystems® 7500 Fast Real-time PCR System is shown in Figure 2.

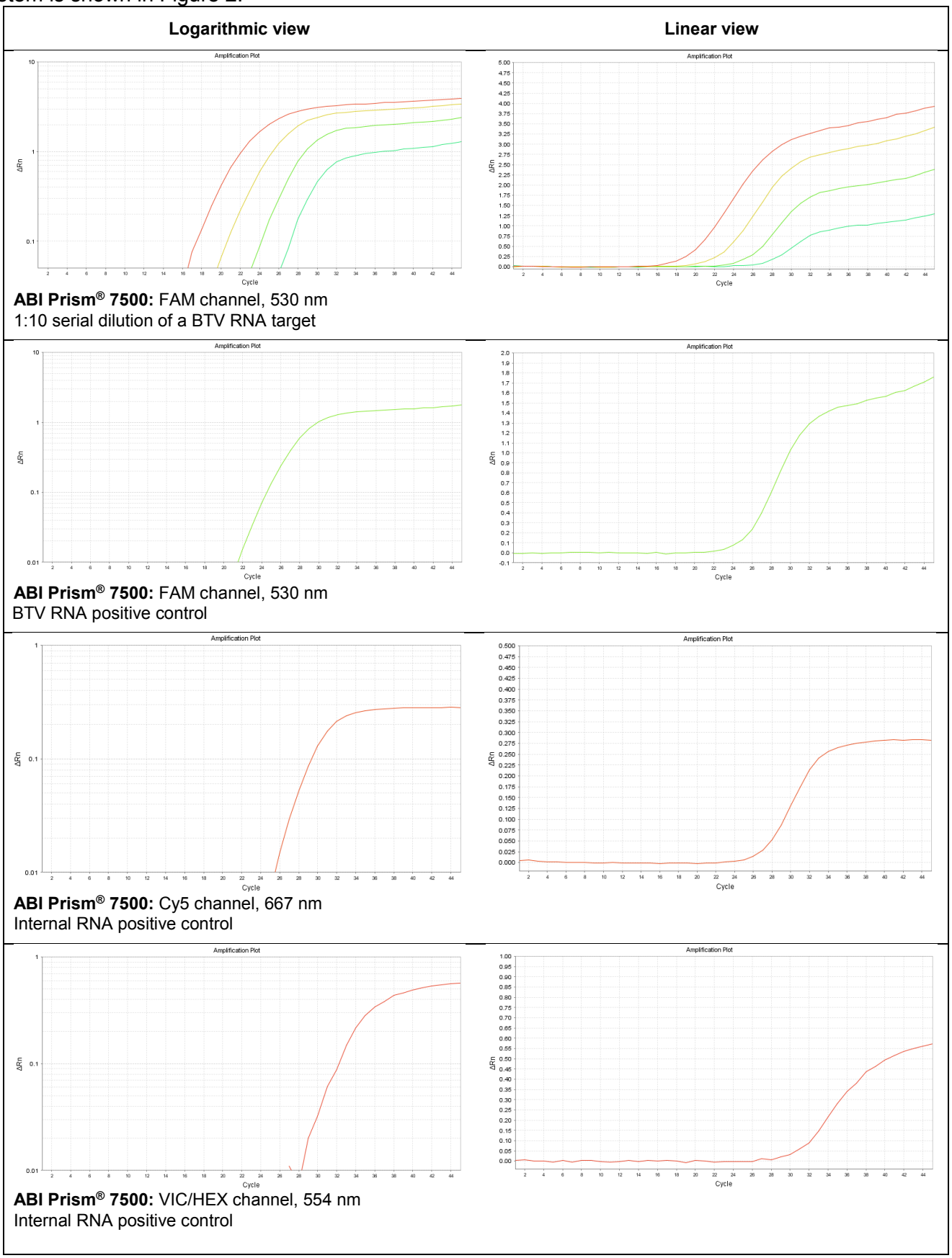

Figure 2 Performance of ViroReal® Kit Bluetongue Virus (BTV)