rRT-PCR FOR BLUETONGUE DETECTION METHOD (Hofmann *et al.* 2008b)		
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1. PURPOSE

The purpose of this method is to rapidly detect the specific presence of Bluetongue virus RNA by a real time reverse transcription polymerase chain reaction assay (rRT-PCR).

Currently this method is included in the OIE Chapter 3.1.3 of the Manual of Diagnostic Test and Vaccines for the Terrestrial Animals (updated in 2014) as a recommended nucleic acid detection test to detect Bluetongue Virus (BTV).

2. SCOPE

The method is applied to specifically detect BTV RNA in clinical samples from ruminants, such as EDTA-blood or tissue homogenates, and inoculated cell culture.

3. MATERIALS AND EQUPMENTS

Material

Disposable hand gloves

Microcentrifuge tubes (1,5 - 2 ml), PCR clean, RNase free

Centrifuge tubes (12 ml)

96-well PCR plate

Pippete filter tips, sterile (10µl, 100-200µl and 1000µl)

PCR water

Optic sealing film for real time PCR

Reagents (see description in point 4. Reagents)

rRT-PCR Kits

Primers

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Probe

Equipment

Adjustable micropipettes (full range)

Laminar flow cabinet

Ice or Labtop Cooler

Real time Thermal cycler

Refrigerator (-20ºC)

Micro-centrifuge (up to 15000 rpm)

Vortex mixer

4. REAGENTS

Amplification kit

Some examples of amplification kit to be used are given below:

AGPATH-ID ONE-STEP RT-PCR KIT (for fast RRT-PCR)	
Components:	RT-PCR Buffer RT-PCR Enzyme Mix Nuclease-free water

RRT-PCR AgPath-ID™ One-Step RT-PCR (Thermo Fisher Scientific) Kit manual.

QUANTITEC PROBE RT-PCR KIT	
Components:	Quantitec Probe RT-PCR Master Mix Quantitec RT-Mix RNase- free water

RRT-PCR QuantiTect Probe RT-PCR (Qiagen) Kit manual

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Primers and Probe

Primer Forward:	BTV-S10Hf-F	5´- TGG AYA AAG CRA TGT CAA A- 3´
Primer Reverse:	BTV-S10Hf-R	5´- ACR TCA TCA CGA AAC GCT TC- 3´
Probe:	BTV-S10Hf-S	5´-FAM- ARG CTG CAT TCG CAT CGT ACG C-BHQ1-3´

Controls

- Extraction controls.

  -*Extraction Positive Control* (EPC) should contain the target virus

  -*Extraction Negative Control* (ENC): Water or buffer is normally used

- Amplification controls.

  -*Amplification Positive Control (APC):* PCR mix prepared with the same volume and concentration of reagents as the PCR mix being used to test specimens, but with the addition of target nucleic acid

  -*Amplification Negative Control (ANC):* PCR mix being used to test specimens, but with the addition of a volume of sterile nuclease free water or sterile nuclease free buffer instead of extracted template nucleic acid.

5. METHOD

General Aspects

The assay amplifies a RNA fragment of 97 bp of the BTV S10 genome region.

The PCR is carried out in a final volume of 20 µl.

RNA sample is added to each reaction tube containing the primers mix and heat denatured.

One step RT-PCR master mix including the probe is added to reaction tubes after denaturation.

Protocol

- Commercial kits to extract viral nucleic acid are widely available. RNA extraction from blood and tissue samples can be performed following manufacturer´s instructions.
- Thaw all reagents and store in ice, except the enzyme mix.

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- Maintain an RNAse and DNAse free work environment.
- Mix all individual reagents thoroughly and spin down.

a) Protocol if using the Quantitect Probe RT-PCR Kit (Qiagen)

- A test plate layout should be designed and loaded into the real time PCR machine software. Using the layout as a guide 2,5 µl of each primer working stock 10 µM (final concentration 1,25 µM) is added to each well that will contain RNA samples, positive and/or negative controls. The plate is held on ice.

  Note: PCR plates can be replaced with tubes or strips as appropriate.

Reagent	µl/sample	Working concentration	Final reaction mix concentration
Primer BTV- S10Hf-F	2,5	10 µM	1,25 µM
Primer BTV- S10Hf-R	2,5	10 µM	1,25 µM
Sample (RNA)	2		
Total Volume	**7**		

- Heat denaturation at 95°C for 5 minutes, followed by rapid cooling on ice for further 5 minutes.
- An appropriate volume of real time one-step complete RT-PCR mix for the number of samples to be tested is prepared following manufacturer´s instructions. Probe should be included in a final concentration of 0,25 µM (0.1µl of probe working stock 50 µM per sample).
- 13 µl of complete RT-PCR mix is distributed in each well on the PCR plate containing the denatured primers and RNA.

Reagent (Quantitect Probe RT-PCR Kit (Qiagen)	µl/sample	Working concentration	Final reaction mix concentration
RT-PCR Master Mix	10	2x	
RT Mix	0,2	100x	
Sonda BTV-S10Hf-S	0,1	50µM	0,25 µM
RNase free water	2,7		
Denatured mix (Sample RNA+primers)	7		
Total Volume	**20**		

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The plate is placed in a real time thermal cycler programmed with the following profile:

Step	Cycle step	Temperature	Time	Nº cycles
Reverse transcription		48°C	25 min	1x
"Hot start"		95°C	10 min	1x
PCR cycle	DNA denaturation	95°C	15 sec	40x
	Primer annealing/extension	56°C*	1 min	

* Fluorescence data are acquired at the end of the 56°C step.

b) Protocol if using the AgPath-ID One step RT-PCR Kit (Thermofisher Scientific)

- A test plate layout should be designed and loaded into the real time PCR machine software. Using the layout as a guide 2.5 µl of each primer working stock 10 µM (final concentration 1.25 µM) is added to each well that will contain RNA samples, positive and/or negative controls of extraction (EPC, ENC) and amplification (AMP, ANC). The plate is held on ice.

  Note: PCR plates can be replaced with tubes or strips as appropriate.

Reagent	µl/sample	Working concentration	Final reaction mix concentration
Primer BTV-S10Hf-F	2,5	10 µM	1,25 µM
Primer BTV-S10Hf-R	2,5	10 µM	1,25 µM
Sample (RNA)	2		
Total Volume	**7**		

- Heat denaturation at 95°C for 5 minutes, followed by rapid cooling on ice for further 5 minutes.
- An appropriate volume of real time one-step complete RT-PCR mix for the number of samples to be tested is prepared following manufacturer´s instructions. Probe should be included in a final concentration of 0.25 µM (0.1µl of probe working stock 50 µM per sample).
- 13 µl of complete RT-PCR mix is distributed in each well on the PCR plate containing the denatured primers and RNA.

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Reagent (AgPATH-ID One Step RT-PCR kit)	µl/sample	Working concentration	Final reaction mix concentration
RT-PCR Buffer	10	2x	
RT-PCR Enzyme	0,8	25x	
Sonda BTV-S10Hf-S	0,1	50µM	0,25 µM
RNase free water	2,1		
Denatured mix (Sample RNA+primers)	7		
Total Volume	**20**		

The plate is placed in a real time thermal cycler programmed with the following profile:

Step	Cycle step	Temperature	Time	Nº cycles
Reverse transcription		48°C	10 min	1x
“Hot start”		95°C	10 min	1x
PCR cycle	DNA denaturation	97 °C	2 sec	40x
	Primer annealing/extension	56°C*	30 sec	

* Fluorescence data are acquired at the end of the 56°C step.

6. ASSAY VALIDATION

<u>Test validation</u>

The PCR assay will be valid if after <u>completion of thermocycling program:</u>

- All positive controls (EPC, APC) should produce amplification curves. Positive control Ct values should be in the range of the previously assigned Ct value ±2. If not, the assay should be repeated.
- All negative controls (ENC, ANC) should not produce any amplification curve and the finding of PCR amplification curves in any negative control means that the assay should be repeated. If only ANC showed amplification, the new PCR test could be performed using the same extracted nucleic acid, otherwise the sample should be retested from the nucleic acid extraction.

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Sample results interpretation

- The sample result is **POSITIVE** when a typical amplification curve is obtained and the Ct value (cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold) is lower or equal to the defined Ct threshold (35) within 40 PCR cycles (Ct≤35).
- The sample result is **INCONCLUSIVE** when a typical amplification curve is obtained and the Ct value is higher to the defined Ct threshold (35) within 40 PCR cycles (Ct>35).
- The sample result is **NEGATIVE** when a horizontal amplification curve is obtained and does not cross the threshold line within 40 PCR cycles (No Ct).

If atypical amplification curves are obtained the sample result is not valid and the assay must be repeated.

7. REFERENCES

OIE. Manual for Diagnostic Tests and Vaccines for Terrestrial Animals. English version in force at date. Chapter 3.1.3.: Bluetongue (Infection with Bluetongue virus).

Hofmann M., Griot C., Chaignat V., PERLER L. & THÜR B. (2008a). Bluetongue disease reaches Switzerland. Schweiz. Arch. Tierheilk.,150, 49–56.

Hofmann M. et al, (2008b). Genetic Characterization of Toggenburg Orbivirus, a New Bluetongue Virus, from Goats, Switzerland. Emerging Infectious Diseases, vol 14, nº 12, December 2008

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