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virotype® BTV pan/8 2.0 RT-PCR Kit

Validation Report

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For the detection of RNA from the *Bluetongue Virus* (BTV) *and the European serotype BTV-8*

INDICAL BIOSCIENCE

Contents

1 Introduction

1.1 Intended use

The virotype BTV pan/8 2.0 RT-PCR Kit is intended for the detection of *Bluetongue Virus* (BTV) RNA and differentiation of European serotype BTV-8 specifically, in ruminant whole blood and blood pools (preferred with anticoagulants, for example EDTA-blood) and tissue samples (spleen, lymph nodes) from cattle, sheep and goats.

The kit is approved by the Friedrich-Loeffler-Institut and licensed in accordance with § 11 (2) of the German Animal Health Act (FLI-C 114) for use in Germany for veterinary diagnostic procedures.

For veterinary use only.

1.2 General information

Bluetongue is an infectious, non-contagious disease of ruminants. The agent is the *Bluetongue Virus* (BTV), a double-stranded RNA virus of the genus *Orbivirus* of the family *Reoviridae* which includes 36 known serotypes including atypical BTV. BTV is widely distributed around the world. Sheep, cattle and goats are mainly affected by the disease. Clear clinical signs are usually seen only in sheep. In severe cases the tongue may show intense hyperemia and becomes cyanotic (Bluetongue).

BTV serotype 8 (BTV-8) is of epidemiological importance in Central Europe and cause of recent major Bluetongue Disease outbreaks. The virus is transmitted by certain midges of the genus *Culicoides*. Furthermore, the virus can be spread by contaminated needles and surgery equipment.

1.3 Description of the test principle

Polymerase chain reaction (PCR) is based on the amplification of specific regions of the pathogen genome. In real-time PCR, the amplified product is identified using fluorescent dyes. These are usually linked to oligonucleotide probes that bind specifically to the amplified product. Monitoring the fluorescence intensities during the PCR run (i.e., in real time) allows detection of the accumulating product without the need to re-open the reaction tubes afterward.

The virotype BTV pan/8 2.0 RT-PCR Kit contains all of the necessary reagents for the detection of BTV RNA, including a Positive and Negative Control. With this kit, both, reverse transcription and PCR are performed in one reaction tube, reducing the risk of contamination.

The virotype BTV pan/8 2.0 RT-PCR Kit uses three specific primer/probe combinations:

- FAM™ fluorescence for RNA of all known BTV serotypes (pan BTV)
- Cy®5 fluorescence for RNA of European serotype 8 (BTV-8)
- JOE™ fluorescence for the endogenous Internal Control (EC; β-actin present within the sample)

A Positive Control contains BTV-8 RNA and allows the control of the denaturation step since the successful denaturation of the viral double-stranded RNA is a prerequisite for amplification.

1.4 Kit contents

virotype BTV pan/8 2.0 RT-PCR Kit	**(100)**
Cat. no.	**VT280465**
Number of reactions	**100**
Master Mix (tube with orange cap), includes enzymes, primers, and probes	1 x 800 µl
Positive Control (tube with red cap)	1 x 150 µl
Negative Control (tube with blue cap)	1 x 150 µl
Handbook	1

1.5 Storage

The components of the virotype BTV pan/8 2.0 RT-PCR Kit should be stored at -30°C to -15°C and are stable until the expiration date stated on the label. Avoid repeated thawing and freezing (> 3x), as this may reduce assay sensitivity. Freeze the components in aliquots if they will only be used intermittently.

1.6 Equipment and reagents to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- Pipets
- Nuclease-free, aerosol-resistant pipet tips with filters
- Sterile 1.5 ml Eppendorf® tubes
- Nuclease-free (RNase/DNase-free) consumables. Special care should be taken to avoid nuclease contamination of all reagents and consumables used to set up PCR for sensitive identification of viral nucleic acids
- Cooling device or ice
- Benchtop centrifuge with rotor for 1.5 ml tubes
- Real-time cycler with appropriate fluorescent channels
- Appropriate software for chosen real-time cycler
- Appropriate strip tubes and caps or 96-well optical microplate with optical sealing film or cover for chosen real-time cycler

1.7 RNA extraction

The virotype BTV pan/8 2.0 RT-PCR Kit can be used for the detection of BTV RNA from ruminant whole blood (preferred with anticoagulants, e.g., EDTA-blood) and tissue samples (spleen, lymph nodes) from cattle, sheep and goats.

Due to the high sensitivity of the test, pools of up to 10 individual blood samples may be analyzed. However, the optimum pool size depends on the regional prevalence for BTV.

Note: For use in Germany the specifications described in the „Amtliche Methodensammlung“ apply.

Prior to real-time PCR, viral RNA must be extracted from the starting material.

INDICAL offers a range of validated kits for the extraction of RNA from animal samples.

Extraction based on magnetic beads:

- IndiMag® Pathogen Kit (SP947457)
- IndiMag Pathogen Kit w/o plastics (SP947257)
- IndiMag Pathogen IM48 Cartridge (SP947654P608, SP947654P224)
- IndiMag Pathogen KF96 Cartridge (SP947855P196)

Extraction based on spin columns:

- IndiSpin® Pathogen Kit* (SP54104, SP54106)
- IndiSpin QIAcube® HT Pathogen Kit (SP54161)

If real-time RT-PCR is not performed immediately after extraction, store the RNA at -20°C or at -80°C for longer storage.

For further information on automated and manual extraction of BTV RNA from different sample types, refer to the respective handbook or contact INDICAL Support at support@indical.com.

1.8 Important notes

General precautions

The user should always pay attention to the following:

- Use nuclease-free pipet tips with filters.
- Store and extract positive materials (specimens, positive controls and amplicons) separately from all other reagents and add them to the reaction mix in a spatially separated facility.
- Thaw all components on ice before starting the assay.
- When thawed, mix the components by inverting and centrifuge briefly.
- Do not use components of the test kit past the expiration date.
- Keep samples and controls on ice or in a cooling block during the setup of reactions.

Negative control

At least one negative control reaction should be included in each PCR run, containing all the components of the reaction except for the pathogen template. This enables assessment of contamination in the reaction.

Positive control

When performing PCR on unknown samples, it is recommended to include a positive control reaction in the PCR run, containing a sample that is known to include the targeted viral RNA. A positive control serves to prove the functionality of the pathogen assay, e.g., the correct setup of the reaction mix. Use 5 µl of the Positive Control provided with the virotype BTV pan/8 2.0 RT-PCR Kit to test for successful amplification of the target.

Extraction and amplification control

For increased process safety and convenience, one extraction and amplification control assay is included in the test kit.

An endogenous Internal Control (EC) detects the β-actin gene present within the sample. This allows extraction and amplification to be monitored.

2 Procedure

2.1 Important points before starting

- Please read „Important notes“ before starting.
- Include at least one positive control (Positive Control) and one negative control (Negative Control) per PCR run.
- Before beginning the procedure, read through the protocol and ensure that you are familiar with the operation of the chosen real-time PCR cycler.
- RNA is unstable. Perform the protocol without interruption.

2.2 Things to do before starting

- Thaw all reagents on ice and protect from light.
- Before use, spin the reagents briefly.
- Maintain reagents on ice or in a cooling block during PCR setup.

2.3 Test procedure

1. Pipet 5 µl of RNA samples, Positive Control, and Negative Control into individual reaction tubes. Cover the reaction tubes (e.g., with PCR sealing foil).

   Include positive and negative control reactions.

   Positive Control: Use 5 µl of the positive control (Positive Control) instead of sample RNA.

   Negative Control: Use 5 µl of the negative control (Negative Control) instead of sample RNA.
2. Denature the samples and Controls for 5 min at 98°C in a 96-well plate standard cycler with a heated lid.
3. Immediately cool down on ice water or liquid nitrogen for at least 20 s. Then store on ice or in a cooling device.
4. Before use, mix the Master Mix by inverting 5 times or until mixed thoroughly, then centrifuge briefly to collect the fluids.

5. Pipet 8 µl of the Master Mix into each reaction tube. Thus, the final volume of a test is 13 µl (Table 1).

Table 1. Preparation of reaction mix

Component	Volume
Master Mix	8 µl
Sample	5 µl
Total volume	**13 µl**

6. Close the reaction tubes with the corresponding caps.
7. Set the filters for the reporter dyes in the software of your thermal cycler according to Table 2.

Table 2. Filter settings for the reporter

Pathogen/ Internal Control	Reporter
Pan BTV	FAM
BTV-8	Cy5
Endogenous Internal Control	HEX/ JOE™[1]
Passive reference	Texas Red/ ROX™[2]

[1] Use the option appropriate for your thermal cycler.
[2] Internal reference for use with Applied Biosystems® ABI PRISM® Sequence Detection Systems

8. Run the real-time PCR protocol according to Table 3.

Table 3. Real-time RT-PCR protocol for BTV pan/8 2.0.

Step	Temperature	Time	Number of cycles
Reverse Transcription	50°C	10 min	1
Initial Activation	95°C	2 min	1
2-step cycling			
Denaturation	95°C	5 s	40
Annealing/Extension*	60°C	30 s	

***** Fluorescence data collection, approximate run time 68 min (CFX96, Bio-Rad™)

3 Data interpretation

Interpretation of results

For the assay to be valid the Positive Control must give a signal in the FAM, the Cy5, and HEX/JOE channels with a C_T[1] < 35. If no FAM and no Cy5 signals of the Positive Controls are measured the denaturation and cooling steps were insufficient and the testing should be repeated. The Negative Control must give no signal.

The following results are possible if working with unknown samples. The possible sample results are also summarized in Table 4.

The sample is positive for pan BTV and BTV-8, and the assay is valid, if the following criteria are met:

- The sample yields a signal in the FAM, Cy5 and HEX/JOE channel.
- The Positive Control yields a signal in all channels.
- The Negative Control yields no signal in any of the channels.

Note that very high concentrations of BTV RNA or presence of inhibitors in the sample may lead to a reduced HEX/JOE signal or no HEX/JOE signal due to competition with the Internal Control.

The sample is positive for pan BTV and negative for BTV-8, and the assay is valid, if the following criteria are met:

- The sample yields a signal in the FAM and HEX/JOE channel, but not in the Cy5 channel.
- The Positive Control yields a signal in all channels.
- The Negative Control yields no signal in any of the channels.

Note that very high concentrations of BTV RNA or presence of inhibitors in the sample may lead to a reduced HEX/JOE signal or no HEX/JOE signal due to competition with the Internal Control.

[1] Threshold cycle (C_T) — cycle at which the amplification plot crosses the threshold, i.e., there is the first clearly detectable increase in fluorescence

The sample is negative for both, pan BTV and BTV-8, and the assay is valid, if the following criteria are met:

- The sample yields a signal only in the HEX/JOE channel.
- The Positive Control yields a signal in all channels.
- The Negative Control yields no signal in any of the channels.

A positive HEX/JOE signal rules out the possibility of PCR inhibition and/ or incorrect RNA extraction as the Internal Control is amplified.

The sample results are inconclusive, and the assay is invalid, if the following occurs:

- sample yields no signal in any of the fluorescence channels.

If no signal is detected in the FAM (pan BTV), Cy5 (BTV-8) and the HEX/JOE (endogenous Internal Control, EC) channels, the result is inconclusive. The absence of a signal for the housekeeping gene indicates strong PCR inhibition and/or other malfunctions, e.g., during extraction.

To check for inhibition, we recommend 1:5 dilution of the sample RNA in nuclease-free water, to repeat the RNA extraction procedure, or repeat the whole test procedure starting with new sample material.

Check that there is a fluorescence signal in all the channels for the positive control reaction (Positive Control). Absence of a signal for the Positive Control indicates an error, which could be due to incorrect RNA denaturation or incorrect cycling conditions.

Table 4. Results interpretation table*

Sample result	FAM (pan BTV)	Cy5 (BTV-8)	HEX (EC)
pan BTV positive	X		(X)
pan BTV and BTV-8 positive	X	X	(X)
BTV negative			X
Inconclusive			

***** Interpretation of sample results can be determined provided positive and negative control reactions are performed. The Positive Control must yield a signal in the FAM, Cy5 and HEX/JOE channels. The Negative Control must yield no signal in any channel. For a complete explanation of possible sample results please refer to “Data analysis and interpretation”

4 Characteristics of the test

4.1 Analytical sensitivity

The high analytical sensitivity of the virotype BTV pan/8 2.0 RT-PCR Kit was verified by a titration series of pan BTV and BTV-8 *in vitro* RNA [10^6 – 1 copies/well], performed in triplicates of relevant dilutions using the virotype BTV pan/8 2.0 RT-PCR protocol on the Bio-Rad CFX96, the Thermo Fisher Scientific QuantStudio® 5, the Agilent Technologies Aria Mx, and the Applied Biosystems™ 7500 Fast instrument.

4.1.1 Analytical sensitivity of pan BTV and BTV-8 systems using the Bio-Rad CFX96 instrument

Results / Conclusion

The virotype BTV pan/8 2.0 RT-RT-PCR Kit can detect up to one pan BTV / BTV-8 genome copy per sample (Table 5, Table 6, Figure 1 - Figure 4). There is a high correlation between RNA copy number and amplification results. A correlation coefficient of 0.9990 with an efficiency of 99.6 % for the pan BTV *in vitro* RNA and a correlation coefficient of 0.9970 with an efficiency of 104.7 % for the BTV-8 *in vitro* RNA was calculated when using the virotype BTV pan/8 2.0 RT-PCR Kit on the Bio-Rad CFX96 instrument (Figure 2, Figure 4).

Table 5. Individual and mean C_T values of **pan BTV** (FAM) *in vitro* RNA titration series in triplicates. The test was performed on the Bio-Rad CFX96 instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

Type	Copy number	C_T (FAM)	C_T mean	SD	Result
Standard	10^6	18.98			+
Standard	10^6	19.02	19.03	0.05	+
Standard	10^6	19.09			+
Standard	10^5	21.98			+
Standard	10^5	22.19	22.10	0.11	+
Standard	10^5	22.14			+
Standard	10^4	25.47			+
Standard	10^4	25.43	25.50	0.09	+
Standard	10^4	25.60			+
Standard	10^3	28.76			+
Standard	10^3	28.75	28.76	0.02	+
Standard	10^3	28.78			+
Standard	100	32.20			+
Standard	100	32.04	32.13	0.08	+
Standard	100	32.14			+
Standard	10	35.91			+
Standard	10	35.91	35.68	0.40	+
Standard	10	35.21			+
Standard	5	35.50			+
Standard	5	35.52	35.53	0.03	+
Standard	5	35.56			+
Standard	1	-			-
Standard	1	38.49	38.49	-	+
Standard	1	-			-

SD = standard deviation, - = no C_T

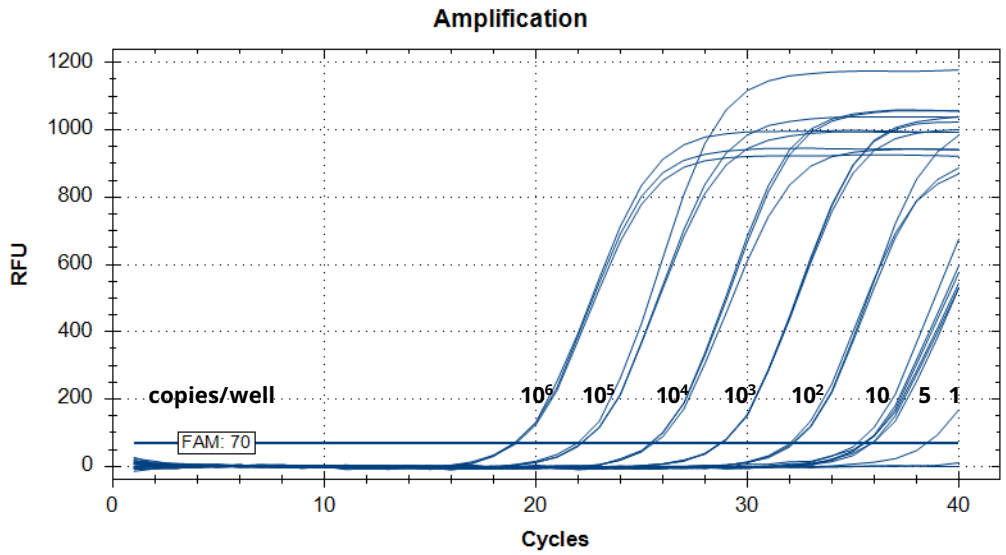

Figure 1. Individual values of a titration series of **pan BTV** (FAM) *in vitro* RNA in triplicates. The test was performed on the Bio-Rad CFX96 instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

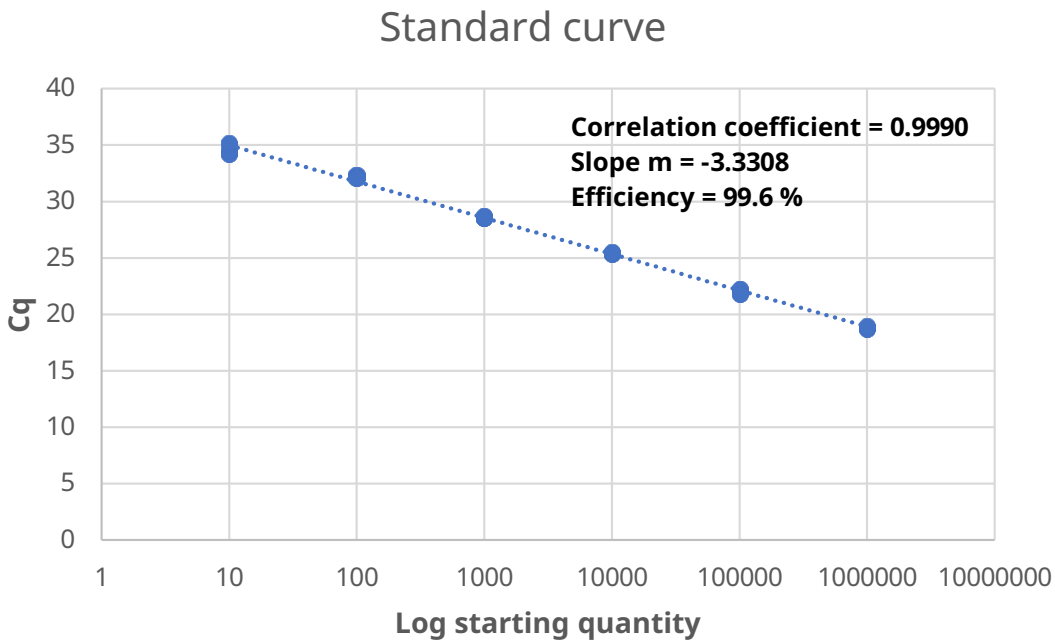

Figure 2. Standard curve of obtained C_T values for a titration series of **pan BTV** (FAM) *in vitro* RNA. The test was performed on the Bio-Rad CFX96 instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

Table 6. Individual and mean C_T values of **BTV-8** (Cy5) *in vitro* RNA titration series in triplicates. The test was performed on the Bio-Rad CFX96 instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

Type	Copy number	C_T (Cy5)	C_T mean	SD	Result
Standard	10^6	18.74			+
Standard	10^6	18.81	18.82	0.09	+
Standard	10^6	18.92			+
Standard	10^5	22.09			+
Standard	10^5	22.24	22.05	0.21	+
Standard	10^5	21.83			+
Standard	10^4	25.32			+
Standard	10^4	25.45	25.41	0.08	+
Standard	10^4	25.46			+
Standard	10^3	28.56			+
Standard	10^3	28.71	28.60	0.10	+
Standard	10^3	28.51			+
Standard	100	32.35			+
Standard	100	32.14	32.19	0.14	+
Standard	100	32.08			+
Standard	10	34.23			+
Standard	10	34.47	34.61	0.46	+
Standard	10	35.12			+
Standard	5	35.72			+
Standard	5	35.64	35.60	0.15	+
Standard	5	35.43			+
Standard	1	-			-
Standard	1	-	37.69	-	-
Standard	1	37.69			+

SD = standard deviation, - = no C_T

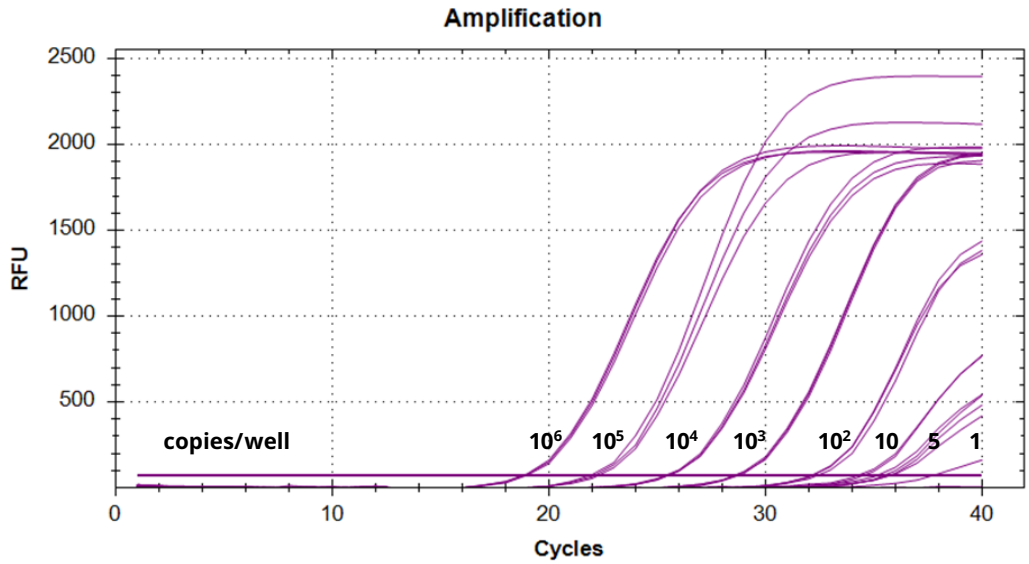

Figure 3. Individual values of a titration series of **BTV-8** (Cy5) *in vitro* RNA in triplicates. The test was performed on the Bio-Rad CFX96 instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

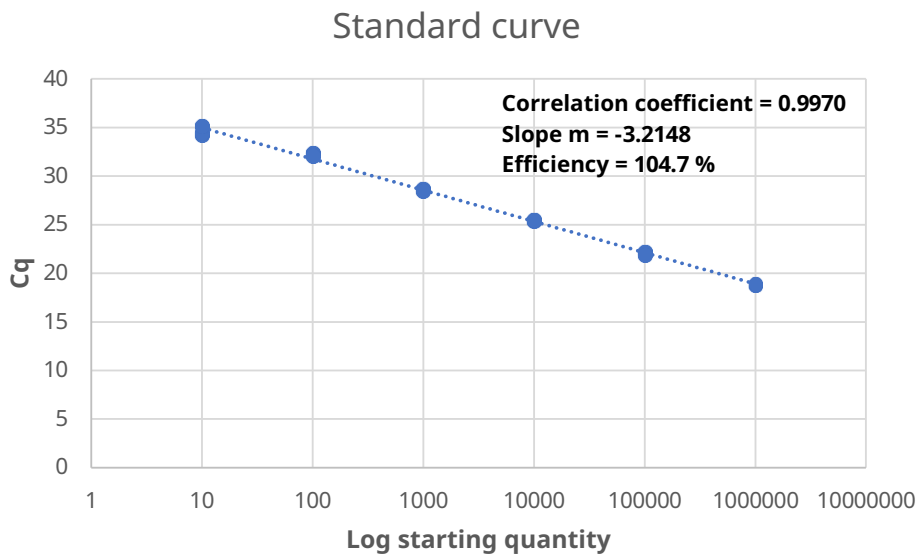

Figure 4. Standard curve of obtained C_T values for a titration series of **BTV-8** (Cy5) *in vitro* RNA. The test was performed on the Bio-Rad CFX96 instrument using the virotype virotype BTV pan/8 2.0 real-time RT-PCR protocol.

4.1.2 Analytical sensitivity of pan BTV and BTV-8 systems using the Thermo Fisher QuantStudio 5 instrument

Results / Conclusion

The virotype BTV pan/8 2.0 RT-PCR Kit can detect up to one BTV genome copies per sample (Table 7, Table 8, Figure 5 - Figure 8). There is a high correlation between RNA copy number and amplification results. A correlation coefficient of 0.9998 with an efficiency of 99.6 % for the pan BTV *in vitro* RNA and a correlation coefficient of 0.9946 with an efficiency of 102.3 % for the BTV-8 *in vitro* RNA was calculated when using the virotype BTV pan/8 2.0 RT-PCR Kit on the Thermo Fisher QuantStudio 5 instrument (Figure 6, Figure 8).

Table 7. Individual and mean C_T values of **pan BTV** (FAM) *in vitro* RNA titration series in triplicates. The test was performed on the Thermo Fisher QuantStudio 5 instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

Type	Copy number	C_T (FAM)	C_T mean	SD	Result
Standard	10^6	18.34			+
Standard	10^6	18.38	18.35	0.03	+
Standard	10^6	18.32			+
Standard	10^5	21.65			+
Standard	10^5	21.61	21.64	0.02	+
Standard	10^5	21.65			+
Standard	10^4	24.79			+
Standard	10^4	25.07	24.99	0.18	+
Standard	10^4	25.12			+
Standard	10^3	28.38			+
Standard	10^3	28.44	28.42	0.04	+
Standard	10^3	28.45			+
Standard	100	31.60			+
Standard	100	31.61	31.61	0.01	+
Standard	100	31.62			+
Standard	10	34.95			+
Standard	10	35.07	35.01	0.06	+
Standard	10	35.00			+
Standard	5	35.35			+
Standard	5	35.19	35.65	0.66	+
Standard	5	36.40			+
Standard	1	37.90			+
Standard	1	36.40	37.15	-	+
Standard	1	-			-

SD = standard deviation, - = no C_T

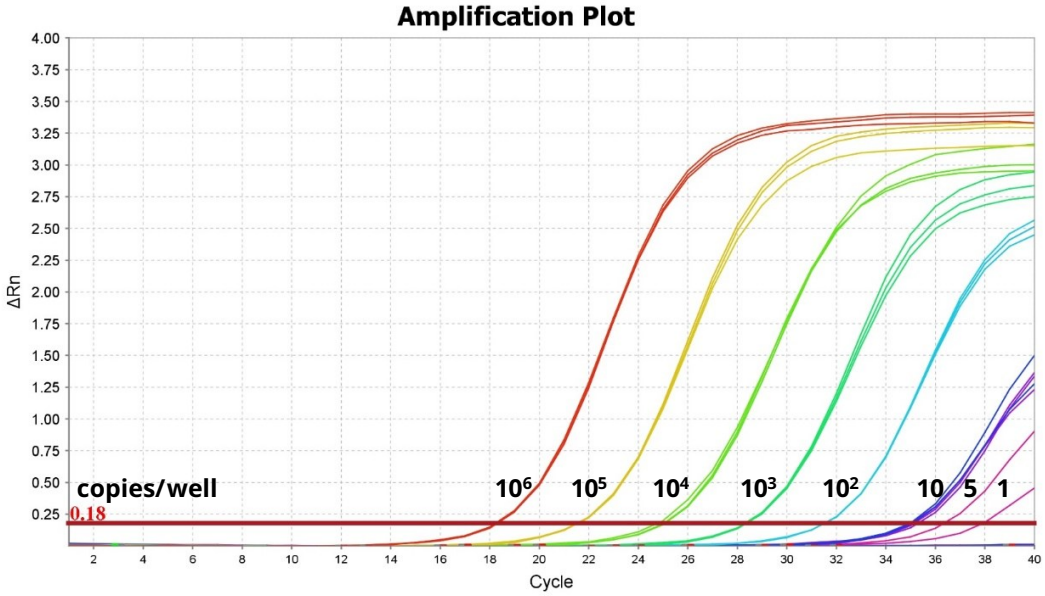

Figure 5. Individual values of a titration series of **pan BTV** (FAM) *in vitro* RNA in triplicates. The test was performed on the Thermo Fisher QuantStudio 5 instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

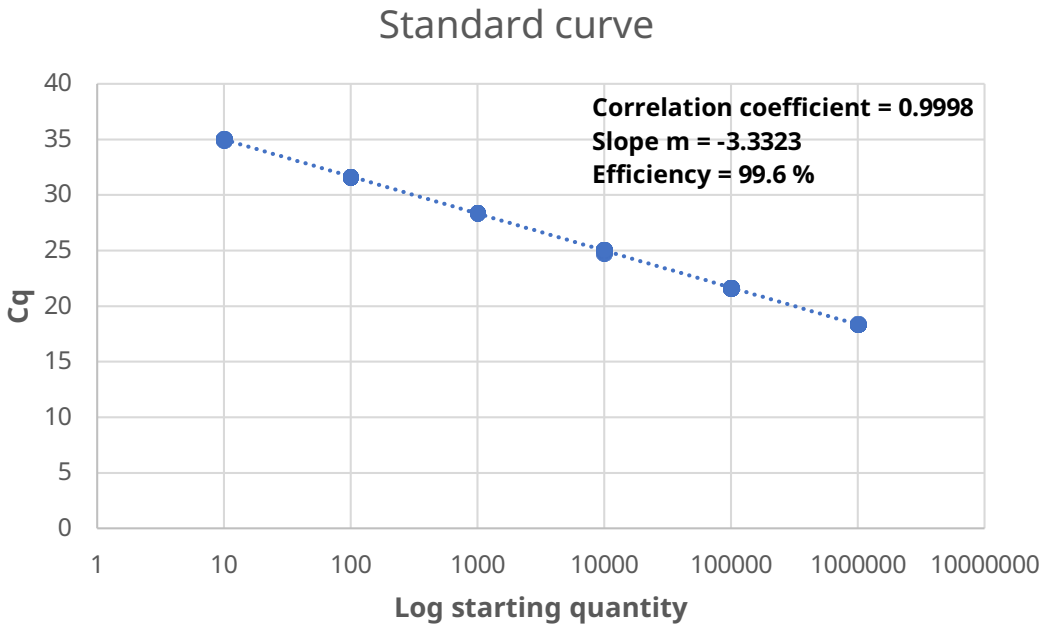

Figure 6. Standard curve of obtained C_T values for a titration series of **pan BTV** (FAM) *in vitro* RNA. The test was performed on the Thermo Fisher QuantStudio 5 instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

Table 8. Individual and mean C_T values of **BTV-8** (Cy5) *in vitro* RNA titration series in triplicates. The test was performed on the Thermo Fisher QuantStudio 5 instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

Type	Copy number	C_T (Cy5)	C_T mean	SD	Result
Standard	10^6	17.90			+
Standard	10^6	17.72	17.77	0.12	+
Standard	10^6	17.68			+
Standard	10^5	20.94			+
Standard	10^5	20.95	20.93	0.04	+
Standard	10^5	20.88			+
Standard	10^4	24.22			+
Standard	10^4	24.28	24.25	0.03	+
Standard	10^4	24.25			+
Standard	10^3	27.43			+
Standard	10^3	27.51	27.46	0.04	+
Standard	10^3	27.46			+
Standard	100	30.83			+
Standard	100	30.63	30.67	0.14	+
Standard	100	30.56			+
Standard	10	35.52			+
Standard	10	33.63	34.16	1.19	+
Standard	10	33.32			+
Standard	5	34.55			+
Standard	5	34.35	34.56	0.21	+
Standard	5	34.77			+
Standard	1	36.44			+
Standard	1	-	36.44	-	-
Standard	1	-			-

SD = standard deviation, - = no C_T

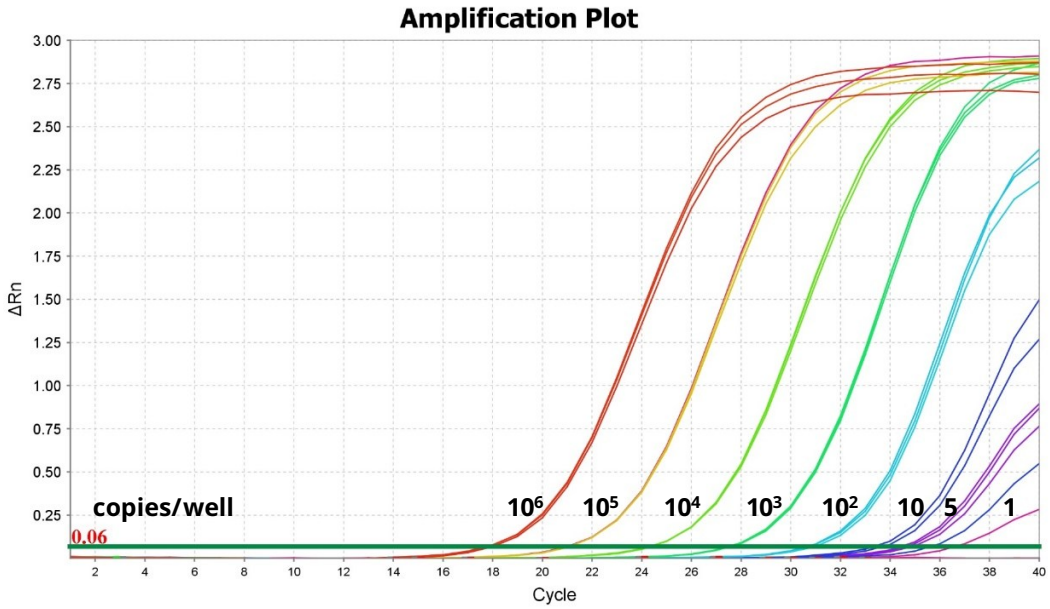

Figure 7. Individual values of a titration series of **BTV-8** (Cy5) *in vitro* RNA in triplicates. The test was performed on the Thermo Fisher QuantStudio 5 instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

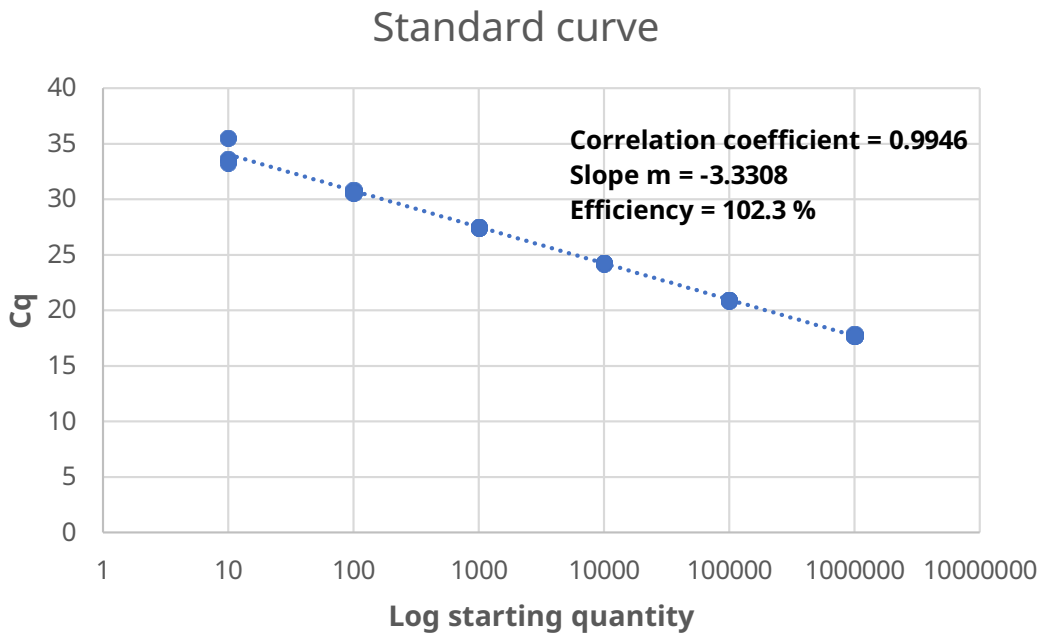

Figure 8. Standard curve of obtained C_T values for a titration series of **BTV-8** (Cy5) *in vitro* RNA. The test was performed on the Thermo Fisher QuantStudio 5 instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

4.1.3 Analytical sensitivity of pan BTV and BTV-8 systems using the Agilent Technologies Aria Mx instrument

Results / Conclusion

The virotype BTV pan/8 2.0 RT-PCR Kit can detect up to one pan BTV genome copy per sample (Table 9, Figure 9, Figure 10) and up to five BTV-8 genome copies per sample (Table 10, Figure 11, Figure 12). There is a high correlation between RNA copy number and amplification results. A correlation coefficient of 0.9937 with an efficiency of 107.3 % for the pan BTV *in vitro* RNA and a correlation coefficient of 0.9994 with an efficiency of 107.3 % for the BTV-8 *in vitro* RNA was calculated when using the virotype BTV pan/8 2.0 RT-PCR Kit on the Agilent Technologies Aria Mx instrument (Figure 10, Figure 12).

Table 9. Individual and mean C_T values of **pan BTV** (FAM) *in vitro* RNA titration series in triplicates. The test was performed on the Agilent Technologies Aria Mx instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

Type	Copy number	C_T (FAM)	C_T mean	SD	Result
Standard	10^6	17.84			+
Standard	10^6	17.97	17.89	0.07	+
Standard	10^6	17.85			+
Standard	10^5	20.94			+
Standard	10^5	21.02	21.02	0.08	+
Standard	10^5	21.09			+
Standard	10^4	24.11			+
Standard	10^4	24.21	24.14	0.06	+
Standard	10^4	24.11			+
Standard	10^3	27.66			+
Standard	10^3	27.34	27.48	0.16	+
Standard	10^3	27.44			+
Standard	100	30.23			+
Standard	100	30.45	30.52	0.34	+
Standard	100	30.89			+
Standard	10	32.51			+
Standard	10	34.03	33.63	0.98	+
Standard	10	34.35			+
Standard	5	35.22			+
Standard	5	35.61	35.14	0.51	+
Standard	5	34.59			+
Standard	1	37.62			+
Standard	1	-	37.05	0.81	-
Standard	1	36.48			+

SD = standard deviation, - = no C_T

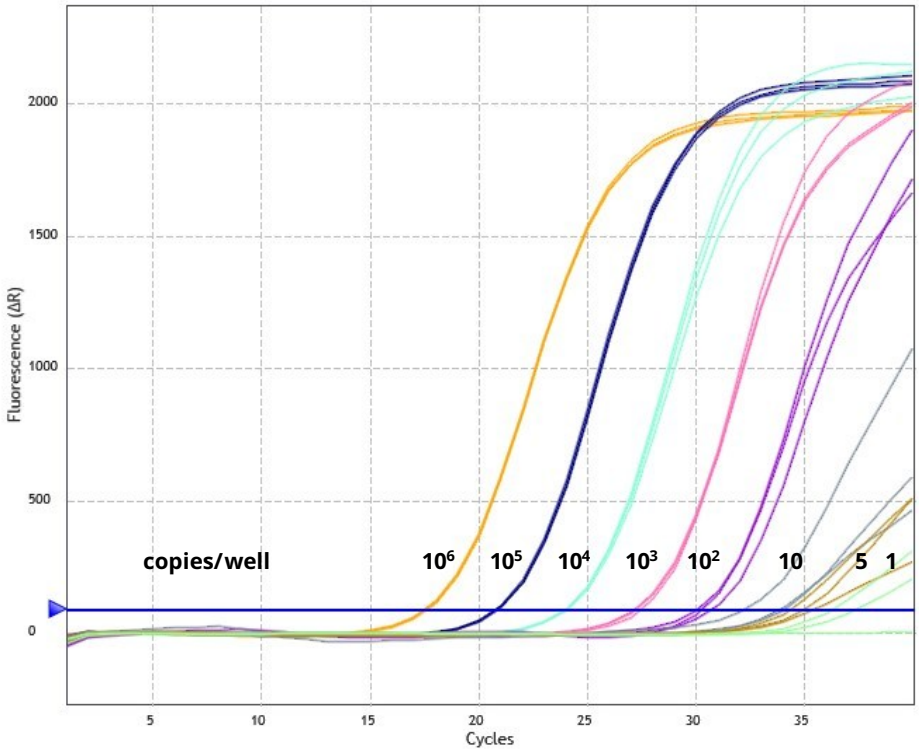

Figure 9. Individual values of a titration series of **pan BTV** (FAM) *in vitro* RNA in triplicates. The test was performed on the Agilent Technologies Aria Mx instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

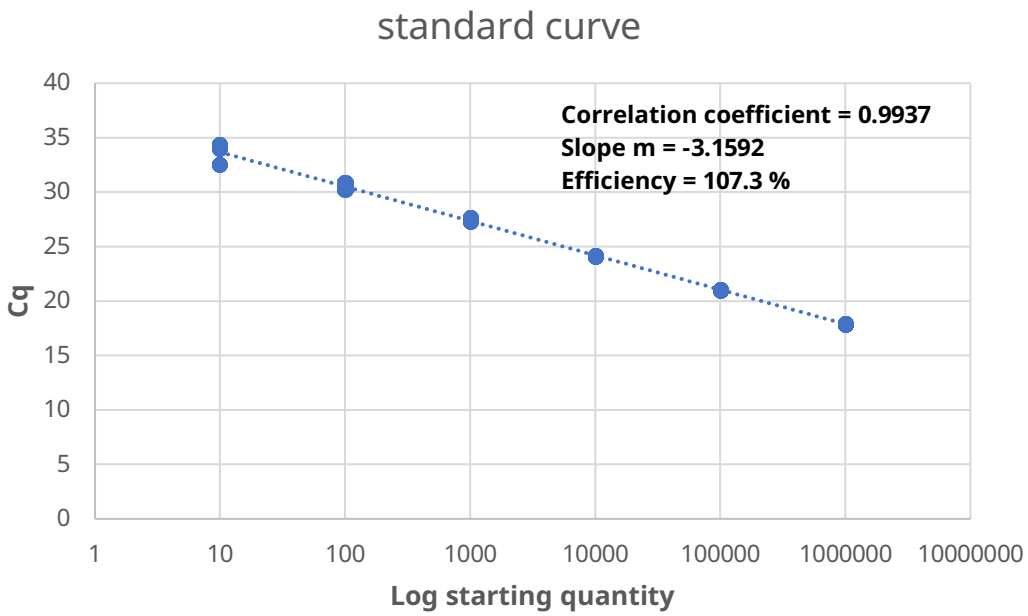

Figure 10. Standard curve of obtained C_T values for a titration series of **pan BTV** (FAM) *in vitro* RNA. The test was performed on the Agilent Technologies Aria Mx instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

Table 10. Individual and mean C_T values of **BTV-8** (Cy5) *in vitro* RNA titration series in triplicates. The test was performed on the Agilent Technologies Aria Mx instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

Type	Copy number	C_T (Cy5)	C_T mean	SD	Result
Standard	10^6	17.60			+
Standard	10^6	17.92	17.70	0.19	+
Standard	10^6	17.59			+
Standard	10^5	20.78			+
Standard	10^5	20.89	20.83	0.06	+
Standard	10^5	20.82			+
Standard	10^4	23.99			+
Standard	10^4	23.99	23.96	0.06	+
Standard	10^4	23.89			+
Standard	10^3	26.92			+
Standard	10^3	27.18	27.04	0.13	+
Standard	10^3	27.01			+
Standard	100	30.09			+
Standard	100	30.27	30.20	0.10	+
Standard	100	30.25			+
Standard	10	33.41			+
Standard	10	33.57	33.57	0.17	+
Standard	10	33.74			+
Standard	5	33.71			+
Standard	5	34.67	34.19	0.48	+
Standard	5	34.20			+
Standard	1	-			-
Standard	1	-	-	-	-
Standard	1	-			-

SD = standard deviation, - = no C_T

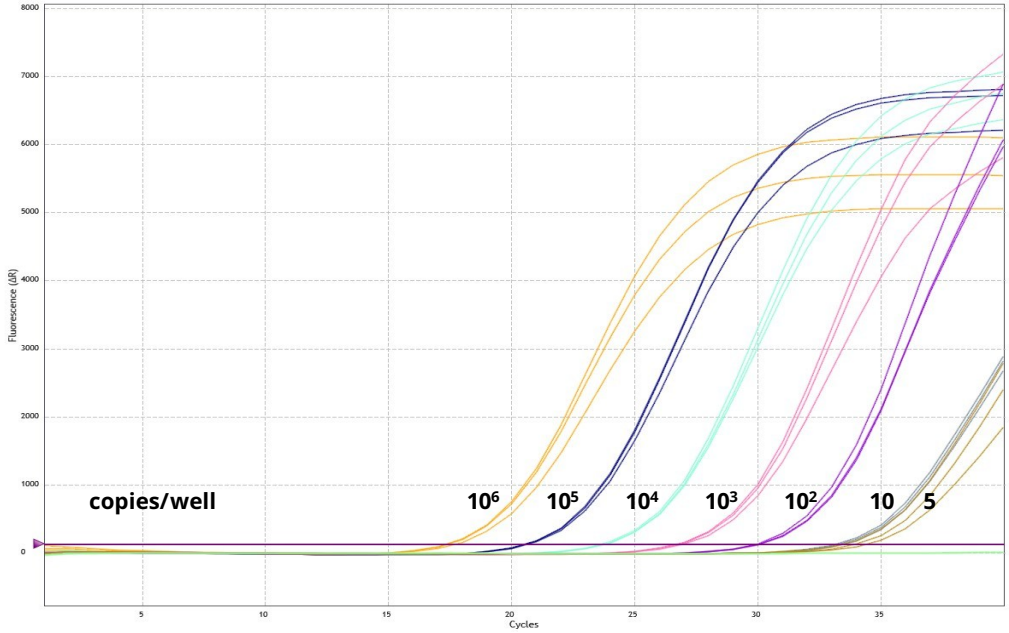

Figure 11. Individual values of a titration series of **BTV-8** (Cy5) *in vitro* RNA in triplicates. The test was performed on the Agilent Technologies Aria Mx instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

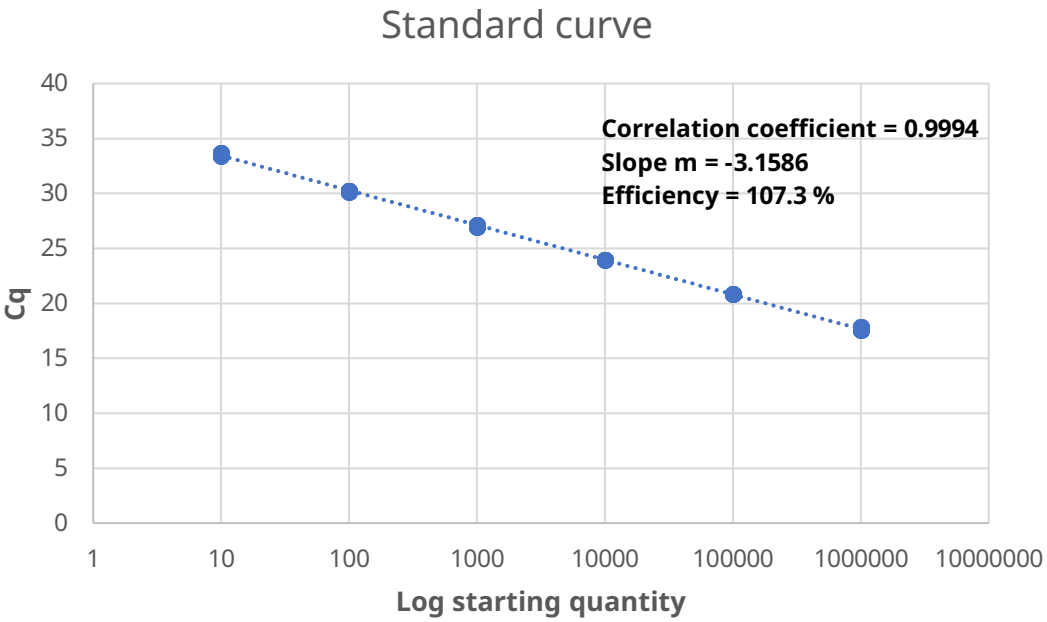

Figure 12. Standard curve of obtained C_T values for a titration series of **BTV-8** (Cy5) *in vitro* RNA. The test was performed on the Agilent Technologies Aria Mx instrument using the virotype virotype BTV pan/8 2.0 real-time RT-PCR protocol.

4.1.4 Analytical sensitivity of pan BTV and BTV-8 systems using the Applied Biosystems™ ABI 7500 Fast instrument

Results / Conclusion

The virotype BTV pan/8 2.0 RT-PCR Kit can detect up to one BTV genome copy per sample (Table 11, Table 12, Figure 13 -Figure 16). There is a high correlation between RNA copy number and amplification results. A correlation coefficient of 0.9992 with an efficiency of 103.2 % for the pan BTV *in vitro* RNA and a correlation coefficient of 0.9983 with an efficiency of 103.7 % for the BTV-8 *in vitro* RNA was calculated when using the virotype BTV pan/8 2.0 RT-PCR Kit on the ABI 7500 Fast instrument (Figure 14, Figure 16).

Table 11. Individual and mean C_T values of **pan BTV** (FAM) *in vitro* RNA titration series in triplicates. The test was performed on the ABI 7500 Fast instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

Type	Copy number	C_T (FAM)	C_T mean	SD	Result
Standard	10^6	18.25			+
Standard	10^6	18.31	18.30	0.04	+
Standard	10^6	18.33			+
Standard	10^5	21.63			+
Standard	10^5	21.54	21.55	0.07	+
Standard	10^5	21.48			+
Standard	10^4	24.78			+
Standard	10^4	24.88	24.81	0.06	+
Standard	10^4	24.78			+
Standard	10^3	28.00			+
Standard	10^3	28.15	28.04	0.10	+
Standard	10^3	27.96			+
Standard	100	31.30			+
Standard	100	31.00	31.24	0.21	+
Standard	100	31.40			+
Standard	10	34.93			+
Standard	10	34.19	34.58	0.37	+
Standard	10	34.62			+
Standard	5	35.03			+
Standard	5	34.73	35.13	0.46	+
Standard	5	35.63			+
Standard	1	37.90			+
Standard	1	37.87	37.58	0.53	+
Standard	1	36.96			+

SD = standard deviation

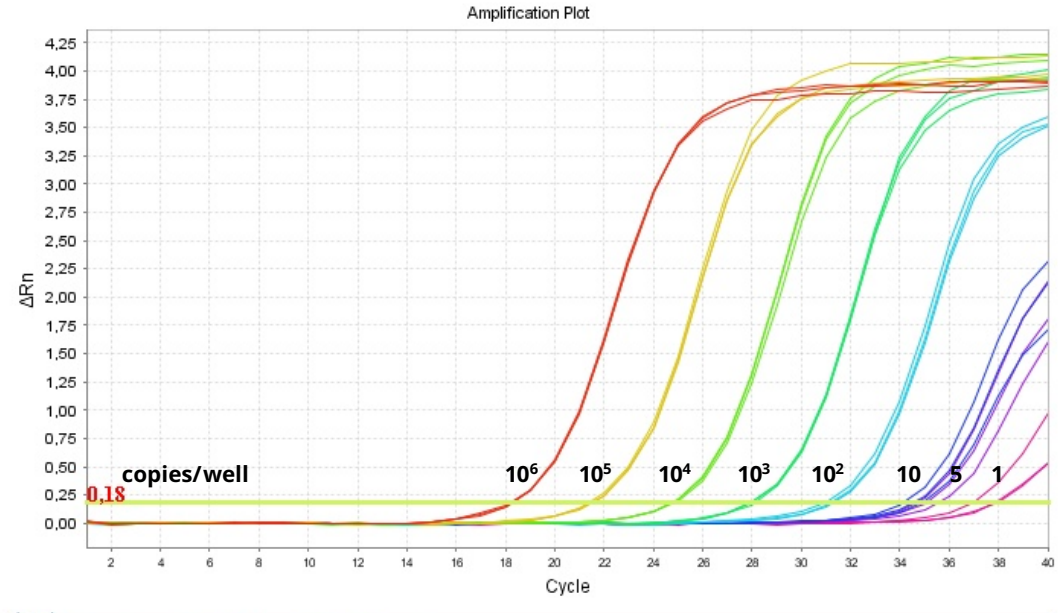

Figure 13. Individual values of a titration series of **pan BTV** (FAM) *in vitro* RNA in triplicates. The test was performed on the ABI 7500 Fast instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

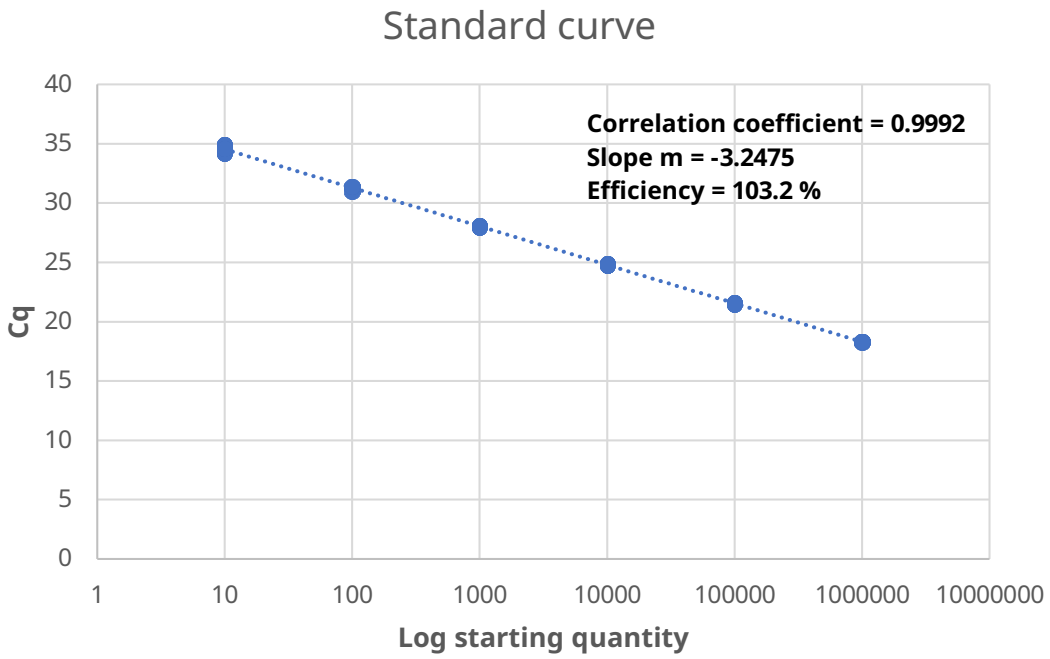

Figure 14. Standard curve of obtained C_T values for a titration series of **pan BTV** (FAM) *in vitro* RNA. The test was performed on the ABI 7500 Fast instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

Table 12. Individual and mean C_T values of **BTV-8** (Cy5) *in vitro* RNA titration series in triplicates. The test was performed on the ABI 7500 Fast instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

Type	Copy number	C_T (Cy5)	C_T mean	SD	Result
Standard	10^6	18.43			+
Standard	10^6	18.57	18.54	0.10	+
Standard	10^6	18.63			+
Standard	10^5	21.88			+
Standard	10^5	21.97	21.89	0.07	+
Standard	10^5	21.82			+
Standard	10^4	25.29			+
Standard	10^4	25.16	25.19	0.09	+
Standard	10^4	25.11			+
Standard	10^3	28.57			+
Standard	10^3	28.48	28.48	0.09	+
Standard	10^3	28.39			+
Standard	100	31.79			+
Standard	100	31.80	31.79	0.01	+
Standard	100	31.77			+
Standard	10	34.85			+
Standard	10	34.02	34.60	0.50	+
Standard	10	34.93			+
Standard	5	35.66			+
Standard	5	35.88	35.50	0.48	+
Standard	5	34.97			+
Standard	1	-			-
Standard	1	-	38.59	-	-
Standard	1	38.59			+

SD = standard deviation, - = no C_T

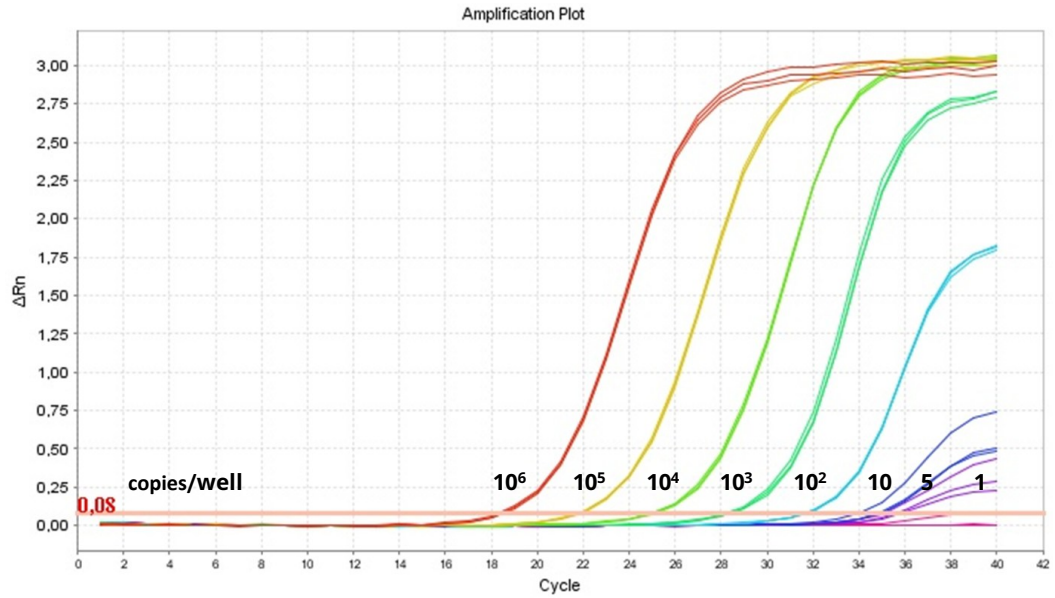

Figure 15. Individual values of a titration series of **BTV-8** (Cy5) *in vitro* RNA in triplicates. The test was performed on the ABI 7500 Fast instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

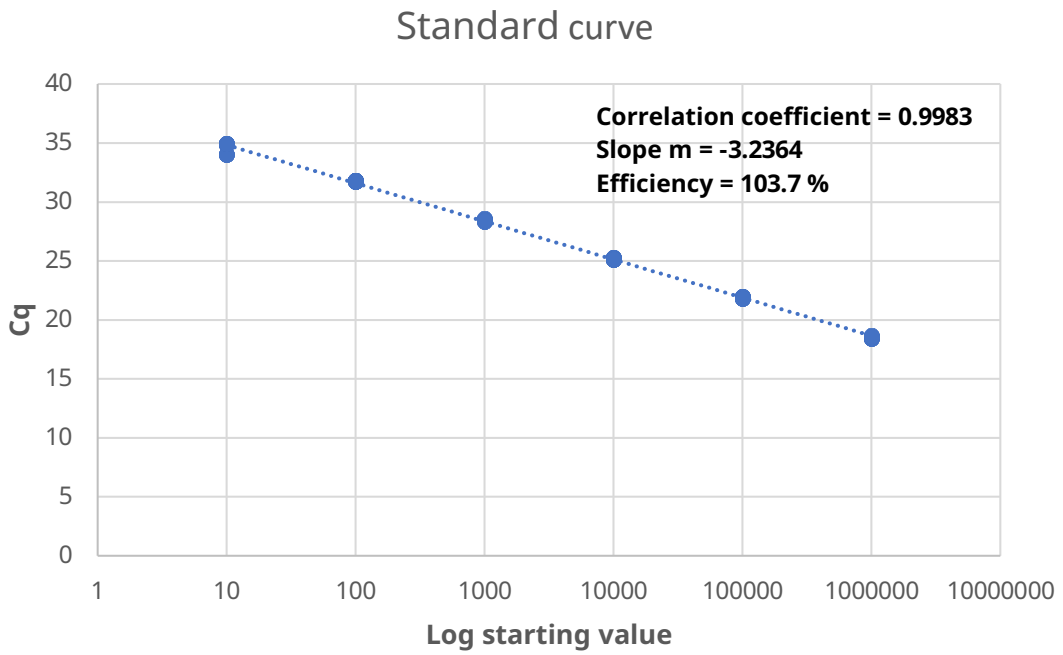

Figure 16. Standard curve of obtained C_T values for a titration series of **BTV-8** (Cy5) *in vitro* RNA. The test was performed on the ABI 7500 Fast instrument using the virotype virotype BTV pan/8 2.0 real-time RT-PCR protocol.

4.1.5 Analytical sensitivity – Limit of detection

The limit of detection (LOD) for the target sequence of the pan BTV and BTV-8 systems was determined by testing individual titration series of *in vitro* RNA of these sequences in octuplicates. The limit of detection with 95 % confidence interval ($LOD_{95\ \%}$: mean number of copies yielding a probability of detection of 0.95) was determined using the web tool https://quodata.de/content/validation-qualitative-pcr-methods-single-laboratory.

Results / Conclusion

Results are summarized in Table 13 and Table 14, as well as Figure 17 - Figure 20. Using the virotype pan/8 2.0 RT-PCR Kit, a high correlation between RNA copy number and the amount of amplified product was demonstrated for the BTV targeted sequences. The $LOD_{95\%}$ for the pan BTV system is 2.9 copies with a 95% confidence interval of [1.307, 6.498] (Figure 17). The $LOD_{95\%}$ for BTV-8 system is 4.6 copies with a 95% confidence interval of [2.272, 9.317] (Figure 19).

Table 13. Limit of detection for **pan BTV** *in vitro* RNA tested in octuplicates on the Agilent Technologies Aria Mx instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

Copies/test	Total number of replicates	Number of replicates positive	Number of replicates negative
10,000	8	8	0
1,000	8	8	0
100	8	8	0
10	8	8	0
5	8	8	0
1	8	5	3

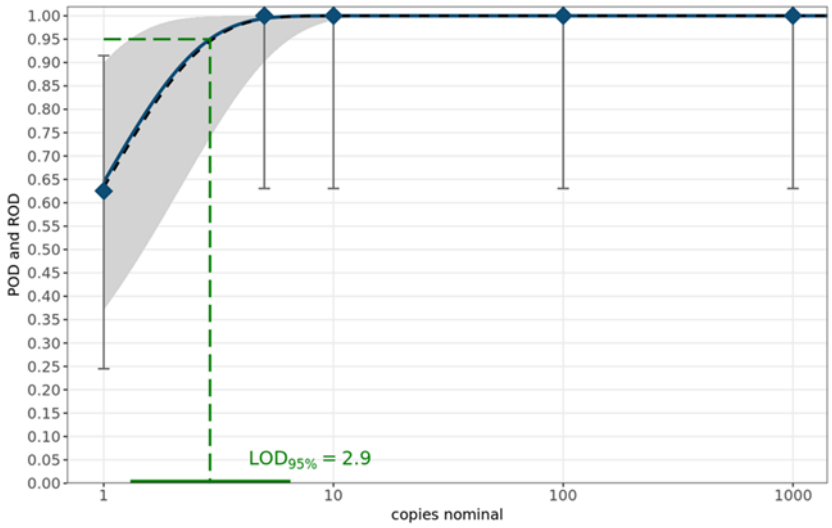

Figure 17. POD (probability of detection) curve and $LOD_{95\ \%}$ for **pan BTV**. The blue diamonds characterize the laboratory-specific rates of detection. The blue curve denotes the mean POD curve along with the corresponding 95 % confidence range highlighted as the grey band. The POD curve under ideal conditions is displayed as the black dashed curve.

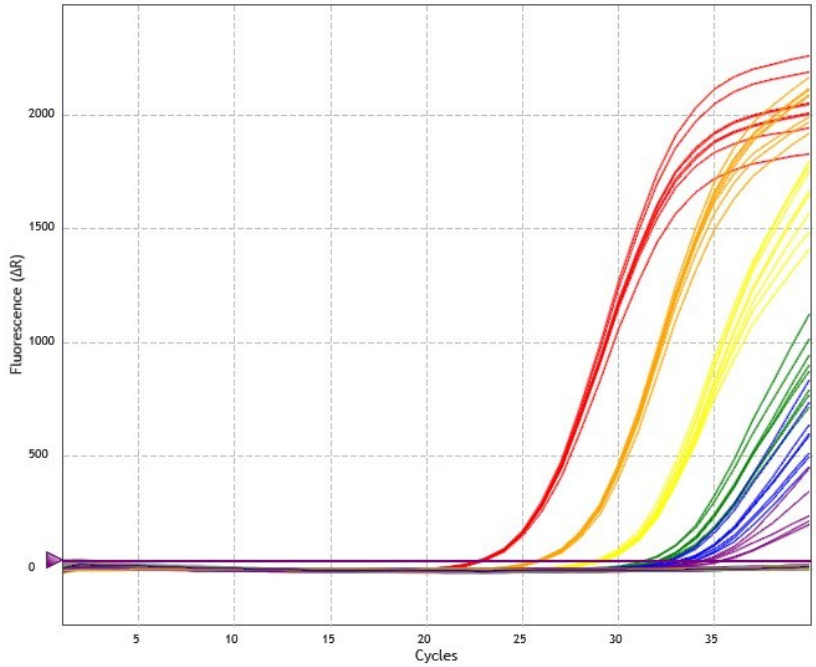

Figure 18. Analytical sensitivity – Limit of detection. Individual values of a titration series of **pan BTV** (FAM) *in vitro* RNA in octuplicates. The test was performed on the Agilent Technologies Aria Mx instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

Table 14. Limit of detection for **BTV-8** *in vitro* RNA tested in octuplicates on the Agilent Technologies Aria Mx instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

Copies/test	Total number of replicates	Number of replicates positive	Number of replicates negative
10,000	8	8	0
1,000	8	8	0
100	8	8	0
10	8	8	0
5	8	8	0
1	8	3	5

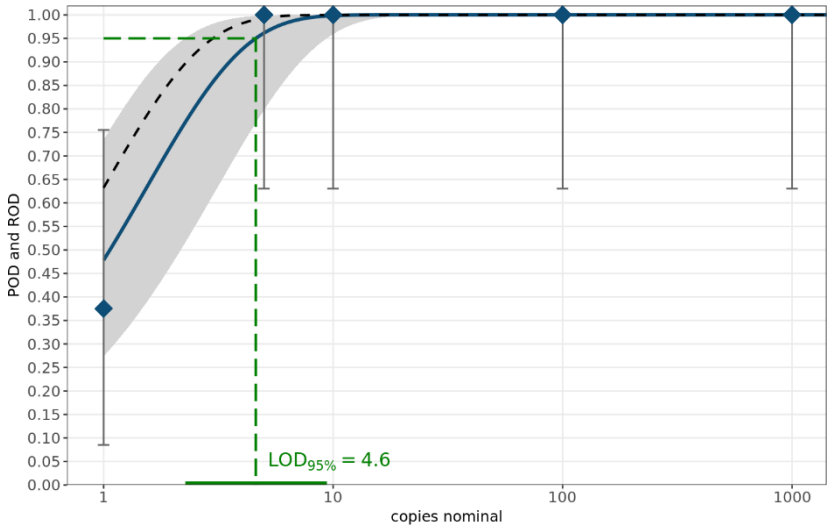

Figure 19. POD (probability of detection) curve and $LOD_{95\,\%}$ for **BTV-8**. The blue diamonds characterize the laboratory-specific rates of detection. The blue curve denotes the mean POD curve along with the corresponding 95 % confidence range highlighted as the grey band. The POD curve under ideal conditions is displayed as the black dashed curve.

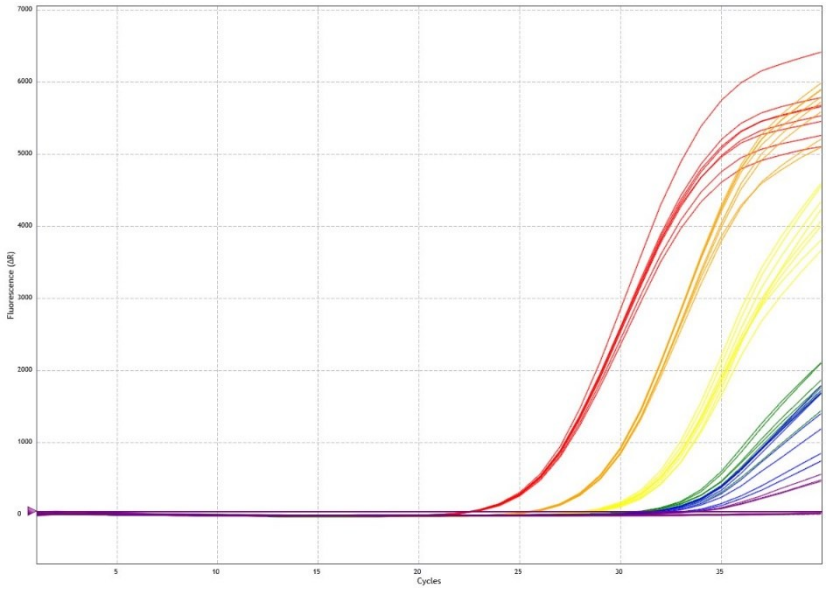

Figure 20. Analytical sensitivity – Limit of detection. Individual values of a titration series of **BTV-8** (Cy5) *in vitro* RNA in octuplicates. The test was performed on the Agilent Technologies Aria Mx instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

4.1.6 Analytical sensitivity of pooled samples

Pools were generated by diluting selected BTV-8 positive samples in BTV negative sheep or cattle blood. Samples were extracted with the IndiMag Pathogen kit (INDICAL) on IndiMag 48s following manufacturer´s instructions. The resulting pools were tested with the virotype BTV pan/8 2.0 RT-PCR Kit on the Agilent Technologies Aria Mx instrument using the virotype BTV pan/8 2.0 RT-PCR protocol.

Results / Conclusion

The C_T values of the pan BTV (FAM), the BTV-8 (Cy5) and the endogenous Internal Control (JOE) are shown in Table 15. Pooled samples up to at least 20 could be detected with the virotype BTV pan/8 2.0 RT-PCR Kit.

Table 15. Analysis of the **pan BTV** (FAM), **BTV-8** (Cy5) and the endogenous **Internal Control** (JOE) signals for simulated pool samples with the virotype BTV pan/8 2.0 RT-PCR Kit tested on the Agilent Technologies Aria Mx instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

Pool size	**Sample**	**Species**	**virotype BTV pan/8 2.0 RT-PCR Kit**		
			pan BTV C_T	**BTV-8** C_T	**Internal Control** C_T
0	WIS 167	Sheep	17.67	16.34	20.99
10			21.48	19.98	18.98
20			22.10	20.96	19.26
0	WIS 168	Sheep	16.31	15.06	18.86
10			20.62	19.45	19.81
20			21.97	20.55	19.75
0	CB	Cattle	21.87	20.54	25.06
10			24.97	23.15	26.87
100			29.82	27.14	28.59

4.2 Specificity

4.2.1 Inclusivity

To confirm that the virotype BTV pan/8 2.0 RT-PCR Kit can detect different serotypes of BTV, a panel *n* = 32 reference RNA samples comprising of BTV serotypes 1 through 28, 30, 33, 35, and 36 was tested with the kit on the Agilent Technologies Aria Mx instrument. Samples were kindly provided by the Friedrich-Loeffler-Institut (FLI), Greifswald, Germany.

The reference samples were extracted using the IndiMag® Pathogen Kit (INDICAL BIOSCIENCE) following manufacturer´s instructions and the RNA samples tested with the virotype BTV pan/8 2.0 RT-PCR Kit.

Results / Conclusion

Out of the *n* = 32 reference samples with different BTV serotypes tested, all were detected using the virotype BTV pan/8 RT-PCR Kit (Table 16).

Table 16. Analysis of RNA samples from the FLI reference panel tested with the virotype BTV pan/8 2.0 RT-PCR on the Agilent Technologies Aria Mx instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

BTV serotypes	**virotype BTV pan/8 2.0 RT-PCR Kit**	
	pan BTV C_T	**BTV-8** C_T
1	24.50	-
2	23.21	-
3	21.80	-
4	23.49	-
5	22.51	-
6	23.15	-
7	21.74	-
8	20.82	19.71
9	21.57	-
10	22.36	-
11	22.02	-
12	21.07	-

13	21.31	-
14	21.82	-
15	23.16	-
16	23.11	-
17	22.60	-
18	22.02	-
19	21.81	-
20	21.41	-
21	24.17	-
22	22.79	-
23	23.34	-
24	22.98	-
25	14.48	-
26	17.87	-
27	21.90	-
28	14.89	-
30	14.76	-
33	13.18	-
35	15.93	-
36	16.15	-

- = no C_T

4.2.2 Exclusivity (Discrimination of pathogens for differential diagnosis)

The specificity / exclusivity was tested with nucleic acids from samples positive for other ruminant-related pathogens: *Schmallenberg Virus* (SBV, *n* = 21), *Bovine Viral Diarrhea Virus* (BVDV, *n* = 47), *Epizootic Hemorrhagic Disease Virus* (EHDV, *n* = 15), *Bovine Herpesvirus 1* (BHV1, *n* = 9), and *Mycobacterium avium* subsp. *paratuberculosis* (MAP, *n* = 9). The samples were kindly provided by German Veterinary Laboratories, the FLI (German National Reference Laboratory for BTV) and other reference labs. Specificity testing was performed on the Agilent Technologies Aria Mx or the Thermo Fisher Scientific QuantStudio 5 instrument.

Results / Conclusion

No cross-reactivity to other relevant bovine viral and bacterial pathogens was detected using the virotype BTV pan/8 2.0 RT-PCR Kit (Table 17).

Table 17. Cross-reactivity of the virotype BTV pan/8 2.0 RT-PCR Kit to other ruminant-related pathogens on the Agilent Technologies Aria Mx or the Thermo Fisher Scientific QuantStudio 5 instrument using the virotype BTV pan/8 2.0 real-time RT-PCR protocol.

						virotype BTV pan/8 2.0 RT-PCR Kit		
	Sample / strain (EHDV)	**Species**	**Dil.**	**Material**	**Refe-rence**	**pan BTV**	**BTV-8**	**Int. Control**
					C_T	**C_T**	**C_T**	**C_T**
***Schmallenberg Virus* (SBV)**	SBV #1	Cattle	1:10	Blood	28.01	-	-	23.87
	SBV #2	Cattle	1:10	Blood	25.64	-	-	23.70
	SBV #3	Cattle	1:10	Blood	26.91	-	-	23.38
	SBV #4	Cattle	1:10	Blood	29.59	-	-	23.43
	SBV #5	Cattle	1:10	Blood	31.16	-	-	23.16
	SBV #6	Cattle	1:10	Blood	30.73	-	-	22.83
	SBV #7	Cattle	undil.	Brain	22.20	-	-	21.56
	SBV #8	Cattle	undil.	Brain	30.56	-	-	21.88
	SBV #9	Cattle	undil.	Brain	29.70	-	-	19.78
	SBV #10	Cattle	undil.	Brain	31.92	-	-	22.38
	SBV #10	Cattle	undil.	Brain	27.45	-	-	18.15
	SBV #12	Cattle	undil.	Brain	27.83	-	-	22.19

	SBV #13	Cattle	undil.	Spleen	31.29	-	-	19.24
	SBV #14	Cattle	undil.	Spleen	35.05	-	-	19.99
	SBV #15	Cattle	undil.	Spleen	37.54	-	-	22.23
	SBV #16	Cattle	undil.	Spleen	28.19	-	-	17.10
	SBV #17	Cattle	undil.	Liver	36.55	-	-	19.03
	SBV #18	Cattle	undil.	Liver	37.29	-	-	21.91
	SBV #19	Cattle	undil.	Liver	35.72	-	-	20.51
	SBV #20	Cattle	1:10	Tissue	23.17	-	-	20.61
	SBV #21	Cattle	1:10	Tissue	36.13	-	-	19.26
***Bovine Viral Diarrhea Virus* (BVDV)**	BVDV #1	Cattle	undil.	Blood	19.80	-	-	21.12
	BVDV #2	Cattle	undil.	Blood	21.28	-	-	21.86
	BVDV #3	Cattle	undil.	Blood	21.47	-	-	22.09
	BVDV #4	Cattle	undil.	Blood	37.62	-	-	22.93
	BVDV #5	Cattle	undil.	Blood	34.77	-	-	22.67
	BVDV #6	Cattle	undil.	Blood	20.69	-	-	25.79
	BVDV #7	Cattle	undil.	Blood	20.52	-	-	23.97
	BVDV #8	Cattle	undil.	Blood	20.74	-	-	22.05
	BVDV #9	Cattle	undil.	Blood	22.12	-	-	23.76
	BVDV #10	Cattle	undil.	Blood	20.91	-	-	21.71
	BVDV #10	Cattle	undil.	Blood	20.70	-	-	22.05
	BVDV #12	Cattle	undil.	Blood	21.51	-	-	23.55
	BVDV #13	Cattle	undil.	Blood	18.58	-	-	23.64
	BVDV #14	Cattle	undil.	Blood	20.35	-	-	22.54
	BVDV #15	Cattle	undil.	Blood	21.43	-	-	22.49
	BVDV #16	Cattle	undil.	Blood	19.12	-	-	22.36
	BVDV #17	Cattle	undil.	Blood	21.82	-	-	22.94
	BVDV #18	Cattle	undil.	Blood	20.05	-	-	21.84
	BVDV #19	Cattle	undil.	Blood	20.55	-	-	21.41
	BVDV #20	Cattle	undil.	Blood	20.35	-	-	22.36
	BVDV #21	Cattle	undil.	Blood	20.55	-	-	23.50
	BVDV #22	Cattle	undil.	Blood	20.47	-	-	23.10

	BVDV #23	Cattle	undil.	Blood	23.27	-	-	23.07
	BVDV #24	Cattle	undil.	Blood	20.17	-	-	23.24
	BVDV #25	Cattle	undil.	Blood	19.98	-	-	26.55
	BVDV #26	Cattle	undil.	Blood	21.05	-	-	24.17
	BVDV #27	Cattle	undil.	Blood	20.88	-	-	24.35
	BVDV #28	Cattle	undil.	Blood	22.60	-	-	25.23
	BVDV #29	Cattle	undil.	Blood	26.99	-	-	22.61
	BVDV #30	Cattle	undil.	Blood	21.95	-	-	22.36
	BVDV #31	Cattle	undil.	Blood	20.08	-	-	23.55
	BVDV #32	Cattle	undil.	Blood	37.15	-	-	23.69
	BVDV #33	Cattle	undil.	Blood	18.89	-	-	20.35
	BVDV #34	Cattle	undil.	Blood	30.01	-	-	20.81
	BVDV #35	Cattle	undil.	Blood	19.93	-	-	24.28
	BVDV #36	Cattle	undil.	Blood	18.49	-	-	22.09
	BVDV #37	Cattle	undil.	Blood	19.84	-	-	21.80
	BVDV #38	Cattle	undil.	Blood	19.59	-	-	21.96
	BVDV #39	Cattle	undil.	Blood	18.49	-	-	22.64
	BVDV #40	Cattle	undil.	Blood	20.49	-	-	23.53
	BVDV #41	Cattle	undil.	Liver	24.44	-	-	19.40
	BVDV #42	Cattle	undil.	Liver	27.71	-	-	21.36
	BVDV #43	Cattle	undil.	Liver	27.42	-	-	19.69
	BVDV #44	Cattle	1:50	Tissue	27.12	-	-	24.07
	BVDV #45	Cattle	1:100	Tissue	28.18	-	-	23.67
	BVDV #46	Cattle	1:50	Tissue	26.33	-	-	23.44
	BVDV #47	Cattle	1:100	Tissue	27.47	-	-	23.76
***Epizootic Hemorrhagic Disease Virus* (EHDV)**	FLI-2422-Alb		undil.	Culture	21.76	-	-	30.08
	FLI-2423-3		undil.	Culture	22.98	-	-	28.93
	EHDV-6/CSIR		undil.	Culture	22.89	-	-	-
	EHDV-5/Australia		undil.	Culture	19.10	-	-	-
	EHDV-4/IBAR		undil.	Culture	21.22	-	-	-
	EHDV-3/IBAR		undil.	Culture	21.26	-	-	-

	EHDV-2C/Alberta		undil.	Culture	21.39	-	-	-
	EHDV-2B/CSIR		undil.	Culture	15.27	-	-	-
	EHDV-2A/Ibar		undil.	Culture	19.24	-	-	-
	EHDV-1 /NewJer		undil.	Culture	23.55	-	-	-
	EHDV-1/ISR		undil.	Culture	15.08	-	-	-
	EHDV-6/ISR		undil.	Culture	17.08	-	-	-
	EHDV-7/ISR		undil.	Culture	19.68	-	-	28.00
	EHDV-6		undil.	Culture	21.02	-	-	-
	EHDV-8/CSIR		undil.	Culture	16.99	-	-	-
***Bovine Herpes Virus* (BHV)**	BHV #1	Cattle	undil.	Serum	pos	-	-	25.70
	BHV #2	Cattle	undil.	Serum	pos	-	-	23.79
	BHV #3	Cattle	undil.	Serum	pos	-	-	26.22
	BHV #4	Cattle	undil.	Serum	pos	-	-	27.62
	BHV #5	Cattle	undil.	Serum	pos	-	-	26.92
	BHV #6	Cattle	undil.	Serum	pos	-	-	26.17
	BHV #7	Cattle	undil.	Serum	pos	-	-	25.29
	BHV #8	Cattle	undil.	Serum	pos	-	-	25.33
	BHV #9	Cattle	undil.	Serum	pos	-	-	25.17
***Mycobacterium avium* subsp. *Paratuber-culosis* (MAP)**	MAP #1	Goat	undil.	Lymph node	33.35	-	-	16.61
	MAP #2	Goat	undil.	Lymph node	31.81	-	-	16.47
	MAP #3	Goat	undil.	Colon	32.21	-	-	18.06
	MAP #4	Goat	undil.	Lymph node	34.67	-	-	16.53
	MAP #5	Goat	undil.	Lymph node	32.28	-	-	16.68
	MAP #6	Goat	undil.	Liver	28.03	-	-	19.24
	MAP #7	Goat	undil.	Liver	32.03	-	-	17.53
	MAP #8	Goat	undil.	Spleen	33.19	-	-	17.09
	MAP #9	Goat	undil.	Spleen	33.11	-	-	17.24

Dil. = Dilution, Ref. = Reference, undil = undiluted, n.a. = not applicable, pos = positive, - = no C_T

***** Reference assay were as follows: virotype SBV RT-PCR Kit (SBV samples), virotype BVDV RT-PCR Kit BVDV samples), inhouse PCR (EHDV samples), cattletype BHV1 gE Ab (BHV samples), bactotype MAP PCR Kit (MAP samples)

4.3 Diagnostic sensitivity, specificity and efficiency

4.3.1 Definition diagnostic sensitivity

Percentage of positive samples in the new test of a population of true positive samples. True positive samples giving negative results in the new test are termed false negative.

Calculation: [true positives / (true positives + false negatives)]*100

4.3.2 Definition diagnostic specificity

Percentage of negative samples in the new test of a population of true negative samples. True negative samples giving positive results in the new test are termed false positive.

Calculation: [true negatives / (false positives + true negatives)]*100

4.3.3 Definition diagnostic efficiency

Diagnostic efficiency refers to the amount of agreement between the results from the new test and those from the reference test. It is expressed as a proportion of correctly identified samples among all samples.

Calculation: [(true positives + true negatives) / (true positives + true negatives + false positives + false negatives)]*100

4.3.4 Validation of the virotype BTV pan/8 2.0 RT-PCR Kit

For validation of the virotype BTV pan/8 2.0 RT-PCR Kit, *n* = 348 samples were tested. Samples (*n* = 182 pan BTV-positive of which were *n* = 139 BTV-8-positive, *n* = 166 BTV-negative) were kindly provided by German Veterinary Laboratories, the FLI (German National Reference Laboratory for BTV) and other reference labs.

For validation *n* = 254 cattle, *n* = 16 sheep and *n* = 28 goat samples were tested, of which *n* = 230 were blood, *n* = 9 were serum, and *n* = 59 were tissue samples. In addition, *n* = 50 culture samples were tested.

The latter samples were processed using the IndiMag Pathogen Kit (INDICAL BIOSCIENCE), the IndiSpin® Pathogen Kit (INDICAL BIOSCIENCE), or the QIAamp® Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany), following manufacturer´s instructions and tested with the virotype BTV pan/8 2.0 RT-PCR Kit and the virotype BTV pan/8 RT-PCR Kit as reference.

Results/ Conclusion

The summary is shown in Table 18. Individual results are shown in Figure 21 (pan BTV) and Figure 22 (BTV-8).

All positive BTV samples were detected correctly with the virotype BTV pan/8 2.0 RT-PCR Kit. The virotype BTV pan/8 2.0 RT-PCR Kit demonstrated a diagnostic sensitivity of 100 %, a diagnostic specificity of 100 % and a diagnostic efficiency of 100 %. In this study the virotype BTV pan/8 2.0 RT-PCR Kit demonstrated an overall higher sensitivity (with often lower C_T values) compared to the virotype BTV pan/8 RT-PCR Kit.

Table 18. Diagnostic sensitivity, specificity and efficiency of the virotype BTV pan/8 2.0 RT-PCR Kit.

virotype BTV pan/8 2.0 RT-PCR Kit		**Reference status**			
Total	**348**	**Reference positive**	**182**	**Reference negative**	**166**
BTV-8-positive	139	true BTV-8-positive	139	false BTV-8-positive	0
pan BTV-positive	182	true pan BTV-positive	182	false pan BTV-positive	0
BTV-negative	166	false-negative	0	true-negative	166

Diagnostic sensitivity:	100 %
Diagnostic specificity:	100 %
Diagnostic efficiency:	100 %

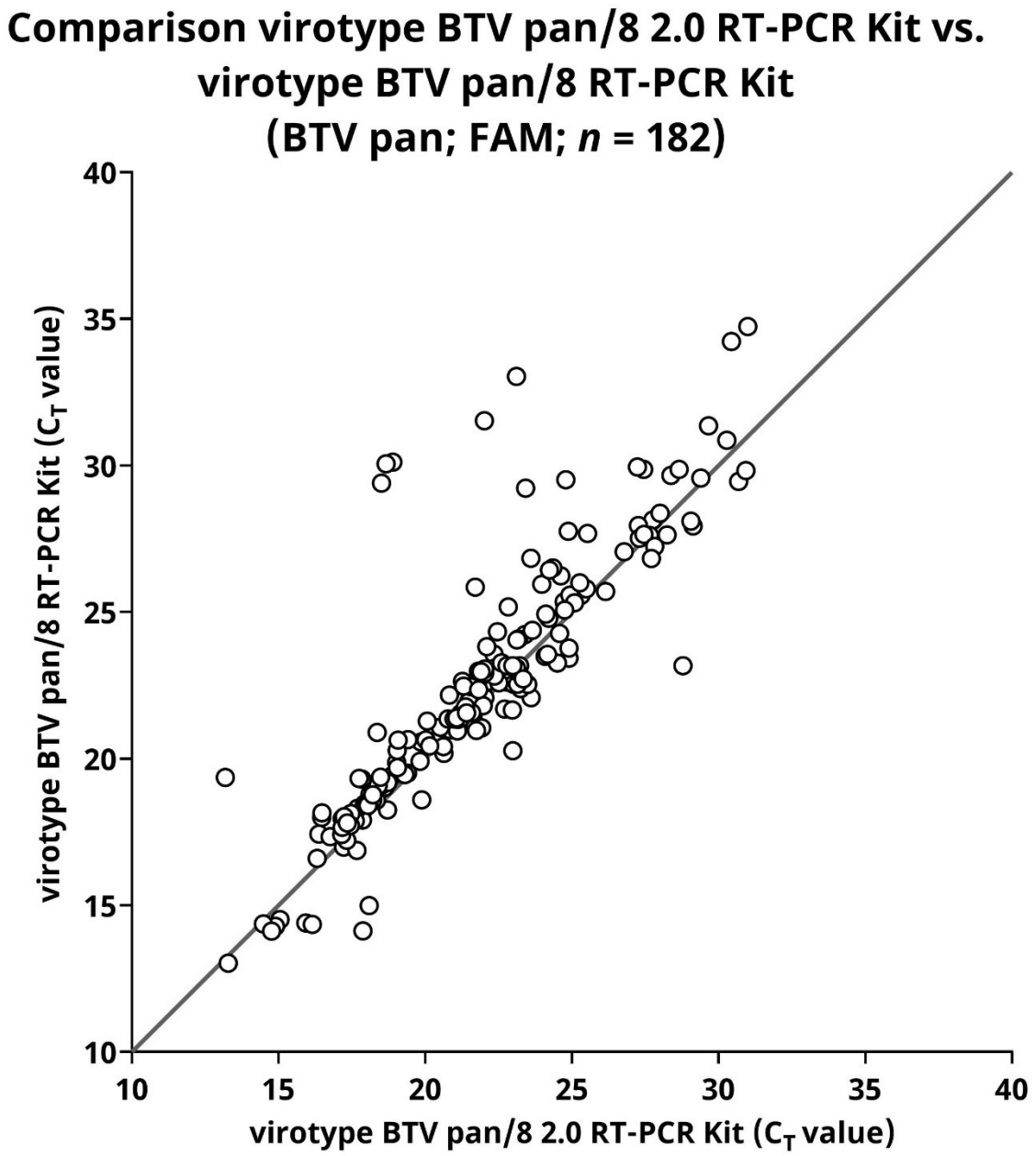

Figure 21. Comparison of FAM C_T values (**pan BTV**) from *n* = 182 pan BTV-positive samples tested with the virotype BTV pan/8 2.0 RT-PCR Kit compared to the virotype BTV pan/8 RT-PCR Kit. All samples situated above the black diagonal line showed lower C_T values with the virotype BTV pan/8 2.0 RT-PCR Kit when compared to the virotype BTV pan/8 RT-PCR Kit.

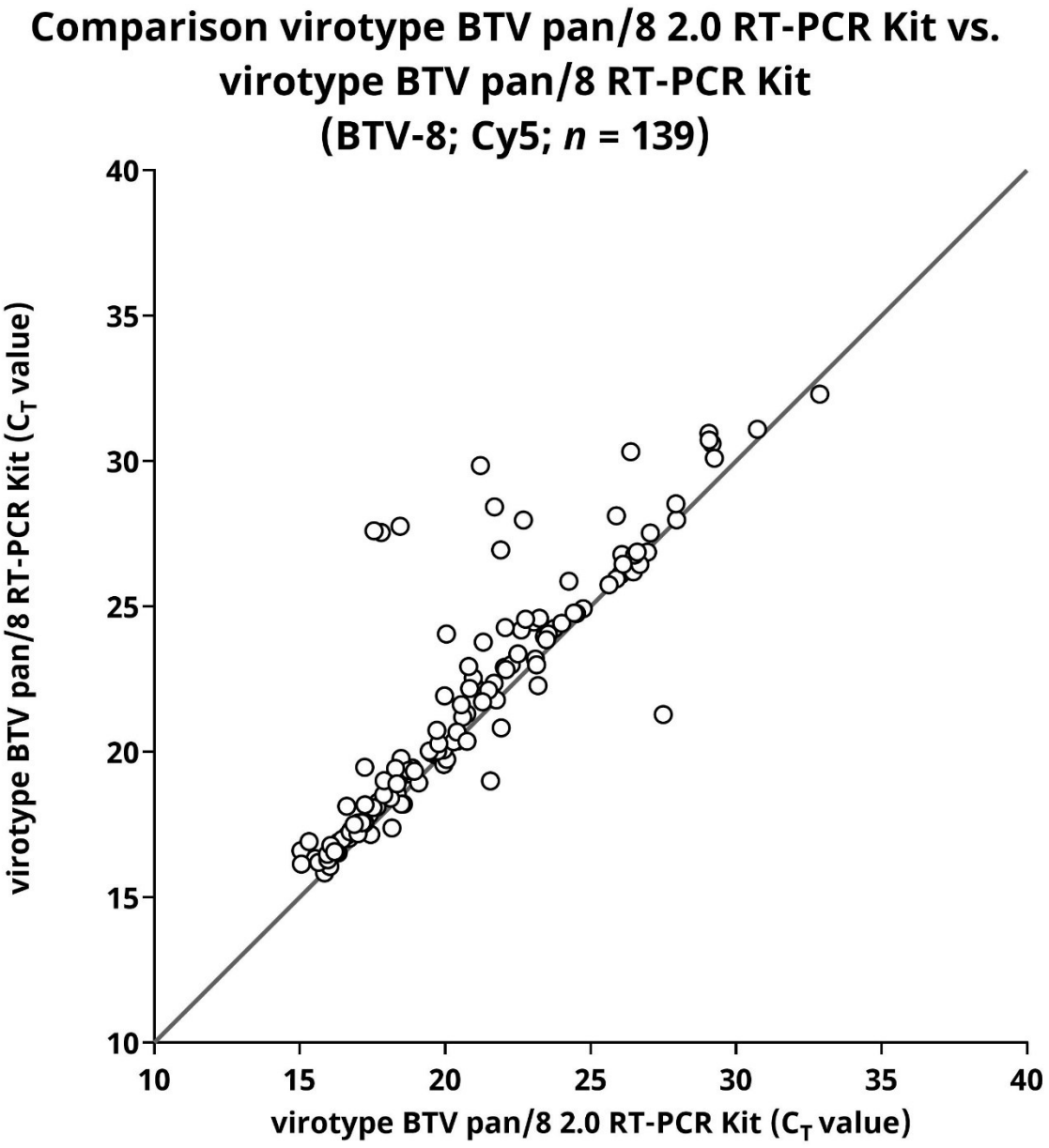

Figure 22. Comparison of Cy5 C_T values (**BTV-8**) from *n* = 139 BTV-8-positive samples tested with the virotype BTV pan/8 2.0 RT-PCR Kit compared to the virotype BTV pan/8 RT-PCR Kit. All samples situated above the black diagonal line showed lower C_T values with the virotype BTV pan/8 2.0 RT-PCR Kit when compared to the virotype BTV pan/8 RT-PCR Kit.

4.4 Repeatability

The same sample panel comprising four BTV-positive RNA samples (samples 1-4), a negative RNA sample from sheep blood (sample 5), and the Positive Control (sample 5) and was used for assessment of intra-assay variance, inter-assay variance, batch-to-batch variance, and stability, testing. BTV-positive samples 1-4 as well as another negative RNA sample from sheep blood (sample 6) was used for robustness testing for the virotype BTV pan/8 2.0 RT-PCR Kit (Table 19).

Table 19. Sample panel for assessment of intra-assay variance, inter-assay variance, batch-to-batch variance, stability, and robustness testing for the virotype BTV pan/8 2.0 RT-PCR Kit.

Sample	BTV status	Species	Description	Dilution
1	positive	Cattle	Extracted nucleic acids from blood of a BTV-8 infected cow	
2	positive	Cattle	sample 1 diluted in RNA from neg cow blood	1:10
3	positive	Cattle	sample 1 diluted in RNA from neg cow blood	1:100
4	positive	Cattle	sample 1 diluted in RNA from neg cow blood	1:1,000
5	negative	Sheep	Extracted nucleic acids from blood	
6	negative	Sheep	Extracted nucleic acids from blood	
PC	PC			

PC = Positive Control

4.4.1 Intra-assay variance

The sample panel listed in Table 19 (sample 1-5, PC) was tested in a sevenfold setup in the same PCR run with the virotype BTV pan/8 2.0 RT-PCR Kit on the Agilent Technologies Aria Mx instrument.

Results / Conclusion

The intra-assay variance is on average 0.75 % for pan BTV (FAM; Table 20), 0.53 % for BTV-8 (Cy5; Table 21), and 0.84 % for the endogenous Internal Control (JOE; Table 22).

These results show an excellent reproducibility in the same RT-PCR run for the virotype BTV pan/8 2.0 RT-PCR Kit.

Table 20. Intra-assay variance for **pan BTV** (FAM) for the virotype BTV pan/8 2.0 RT-PCR Kit using the Agilent Technologies Aria Mx instrument.

Intra-assay variance for pan BTV (FAM)											
Sample	**BTV status**	**Reactions (C_T values)**							**Mean**	**SD**	**CV%**
		1	**2**	**3**	**4**	**5**	**6**	**7**			
1	pos	20.69	20.56	20.56	20.50	20.64	20.47	20.90	20.62	0.146	0.71
2	pos	23.82	23.77	23.76	23.50	23.58	23.78	23.97	23.74	0.156	0.66
3	pos	26.93	26.87	27.32	27.11	27.39	26.92	26.92	27.07	0.213	0.79
4	pos	30.38	30.32	30.44	30.07	30.16	30.18	29.79	30.19	0.220	0.73
5	neg	-	-	-	-	-	-		-	-	-
PC	pos	27.53	27.24	27.97	27.72	27.36	27.48	27.50	27.54	0.240	0.87
Mean											**0.75**

neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Table 21. Intra-assay variance for **BTV-8** (Cy5) for the virotype BTV pan/8 2.0 RT-PCR Kit using the Agilent Technologies Aria Mx instrument.

Intra-assay variance for BTV-8 (Cy5)											
Sample	**BTV status**	**Reactions (C_T values)**							**Mean**	**SD**	**CV%**
		1	**2**	**3**	**4**	**5**	**6**	**7**			
1	pos	19.50	19.42	19.36	19.32	19.40	19.43	19.69	19.45	0.122	0.63
2	pos	22.67	22.50	22.56	22.44	22.51	22.40	22.48	22.51	0.088	0.39
3	pos	25.89	25.54	25.68	25.69	26.00	25.70	25.65	25.74	0.156	0.61
4	pos	29.16	28.60	28.84	28.96	28.78	28.73	28.89	28.85	0.179	0.62
5	neg	-	-	-	-	-	-		-	-	-
PC	pos	26.51	26.29	26.49	26.41	26.25	26.28	26.30	26.36	0.107	0.41
Mean											**0.53**

neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Table 22. Intra-assay variance for the endogenous **Internal Control** (JOE) for the virotype BTV pan/8 2.0 RT-PCR Kit using the Agilent Technologies Aria Mx instrument.

Intra-assay variance for the Internal Control (JOE)											
Sample	**BTV status**	**Reactions (C_T values)**							**Mean**	**SD**	**CV%**
		1	**2**	**3**	**4**	**5**	**6**	**7**			
1	pos	24.30	24.65	24.27	24.23	24.26	24.50	24.71	24.42	0.201	0.82
2	pos	24.56	24.22	24.50	24.15	24.44	24.40	24.54	24.40	0.159	0.65
3	pos	24.65	24.52	24.51	24.39	24.44	24.51	24.50	24.50	0.080	0.33
4	pos	25.33	24.33	24.44	23.89	24.34	24.43	24.83	24.51	0.453	1.85
5	neg	24.61	24.52	24.35	24.56	24.72	24.40	24.50	24.52	0.125	0.51
PC	pos	27.01	26.37	26.47	26.61	26.54	26.30	26.60	26.56	0.230	0.87
Mean											**0.84**

neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation

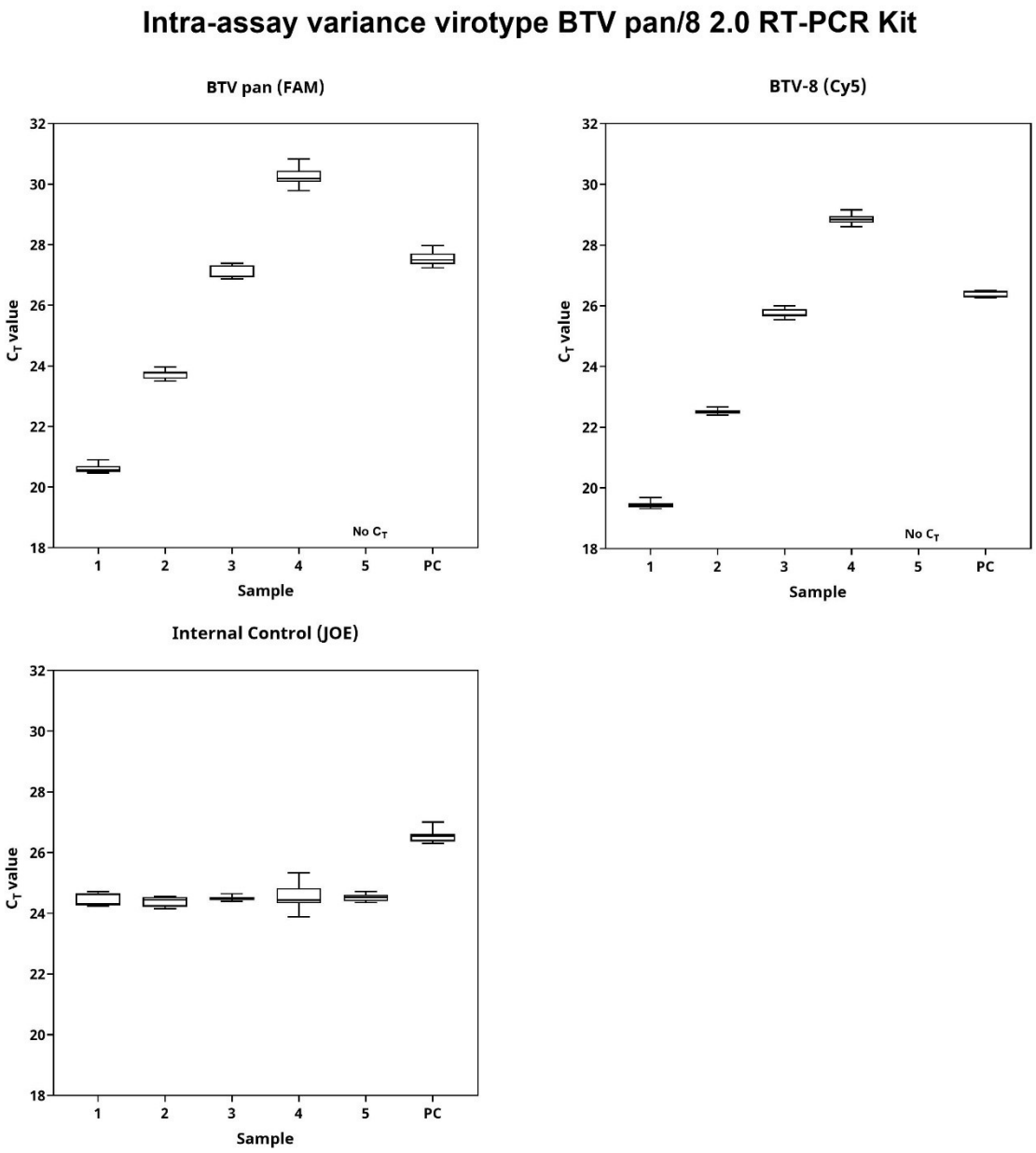

Figure 23. Boxplots of intra-assay variance for pan BTV (FAM), BTV-8 (Cy5), and the endogenous Internal Control (JOE) for the virotype BTV 2.0 pan/8 RT-PCR Kit tested using the Agilent Technologies Aria Mx instrument.

4.4.2 Inter-assay variance

The sample panel listed in Table 19 (sample 1-5, PC) was tested in six different PCR runs using the virotype BTV pan/8 2.0 RT-PCR Kit on the Agilent Technologies Aria Mx instrument.

Results / Conclusion

The inter-assay variance is on average 1.08 % for pan BTV (FAM; Table 23), 0.85 % for BTV-8 (Cy5; Table 24), and 0.96 % for the endogenous Internal Control (JOE; Table 25). These results show an excellent reproducibility in different RT-PCR runs for the virotype BTV pan/8 2.0 RT-PCR Kit.

Table 23. Inter-assay variance for **pan BTV** (FAM) for the virotype BTV pan/8 2.0 RT-PCR Kit using Agilent Technologies Aria Mx instrument.

Inter-assay variance for pan BTV (FAM)										
Sample	**BTV status**	**Reactions (C_T values)**						**Mean**	**SD**	**CV%**
		1	**2**	**3**	**4**	**5**	**6**			
1	pos	21.21	20.69	21.55	21.59	21.11	20.82	21.16	0.368	1.74
2	pos	24.16	23.82	23.90	24.17	24.00	24.23	24.05	0.165	0.69
3	pos	27.24	27.11	27.22	27.24	27.13	27.23	27.2	0.059	0.22
4	pos	30.22	30.32	29.38	30.23	30.55	30.43	30.19	0.415	1.38
5	neg	-	-	-	-	-		-	-	-
PC	pos	27.29	27.24	26.92	27.64	27.52	28.00	27.44	0.372	1.36
Mean										**1.08**

neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Table 24. Inter-assay variance for **BTV-8** (Cy5) for the virotype BTV pan/8 2.0 RT-PCR Kit using the using the Agilent Technologies Aria Mx instrument.

Inter-assay variance for BTV-8 (Cy5)										
Sample	**BTV status**	**Reactions (C_T values)**						**Mean**	**SD**	**CV%**
		1	**2**	**3**	**4**	**5**	**6**			
1	pos	19.94	19.5	20.02	20.02	19.70	19.71	19.82	0.211	1.07
2	pos	22.74	22.67	22.72	22.89	22.58	22.76	22.73	0.103	0.45
3	pos	25.70	25.69	25.68	25.83	25.69	25.88	25.75	0.087	0.34
4	pos	29.00	28.6	28.56	29.52	29.56	29.07	29.05	0.43	1.48
5	neg	-	-	-	-	-		-	-	-
PC	pos	26.42	26.29	26.10	26.56	26.69	26.73	26.47	0.243	0.92
Mean										**0.85**

neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Table 25. Inter-assay variance for endogenous **Internal Control** (JOE) for the virotype BTV pan/8 2.0 RT-PCR Kit using the Agilent Technologies Aria Mx instrument.

Inter-assay variance for the endogenous Internal Control (JOE)										
Sample	**BTV status**	**Reactions (C_T values)**						**Mean**	**SD**	**CV%**
		1	**2**	**3**	**4**	**5**	**6**			
1	pos	24.77	24.3	24.83	24.74	24.64	24.64	24.65	0.188	0.76
2	pos	24.51	24.56	24.13	24.41	24.32	24.40	24.39	0.153	0.63
3	pos	24.43	24.39	24.31	24.73	24.50	24.31	24.45	0.157	0.64
4	pos	24.27	24.33	24.56	24.30	24.78	24.58	24.47	0.202	0.83
5	neg	24.26	24.52	24.19	24.41	24.65	24.61	24.44	0.187	0.77
PC	pos	26.13	26.37	26.39	27.29	27.15	27.47	26.80	0.568	2.12
Mean										**0.96**

neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation

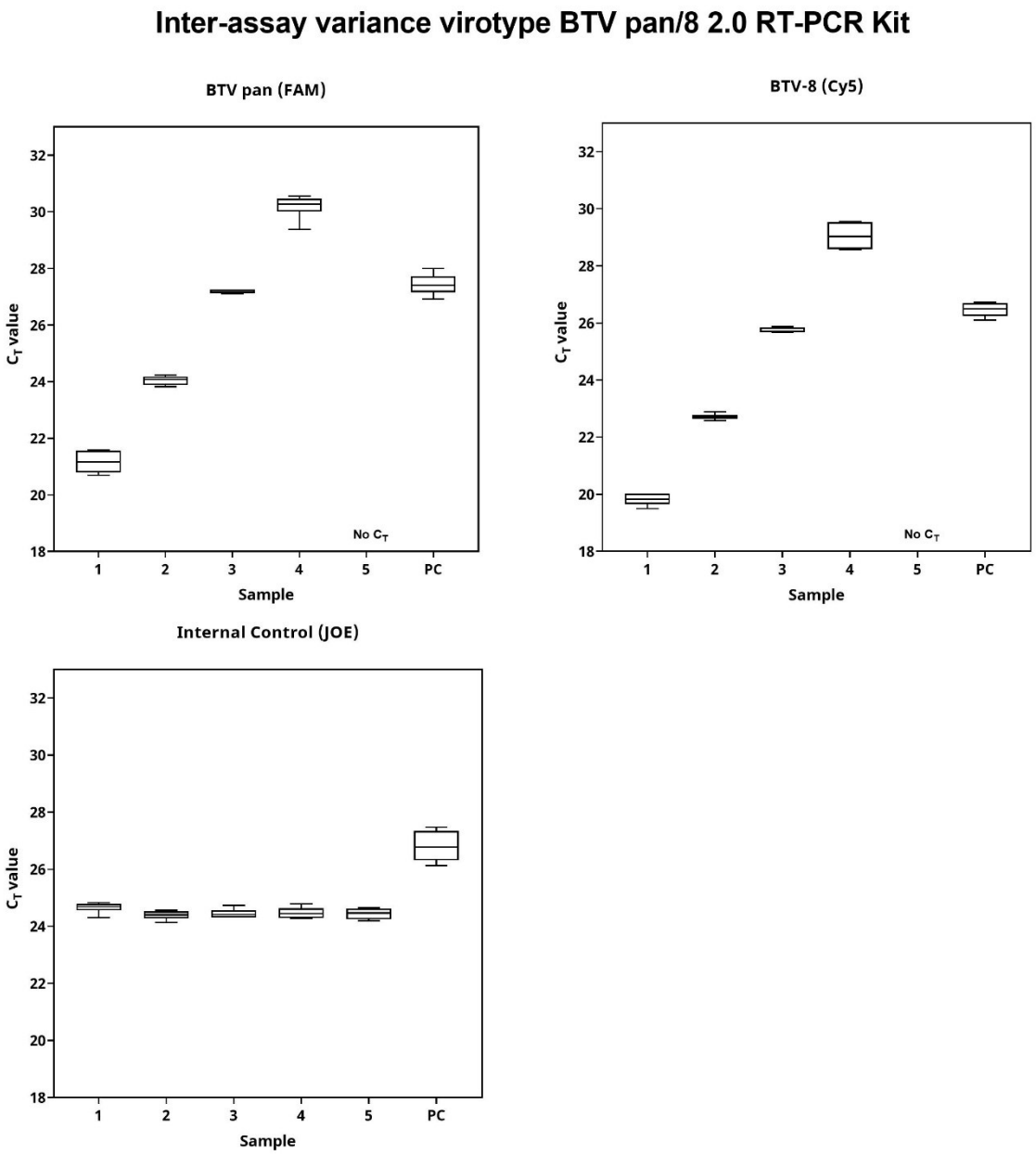

Figure 24. Boxplots of inter-assay variance for pan BTV (FAM), BTV-8 (Cy5), and the endogenous Internal Control (JOE) for the virotype BTV 2.0 pan/8 RT-PCR Kit tested using the Agilent Technologies Aria Mx instrument.

4.4.3 Batch-to-batch comparison

The sample panel listed in Table 19 (sample 1-5, PC) was tested in in the same PCR run using three different batches (1, 2, 3) of the virotype BTV pan/8 2.0 RT-PCR Kit on the Agilent Technologies Aria Mx instrument.

Results / Conclusion

The batch-to-batch performance showed on average variance of 1.36 % for pan BTV (FAM), 0.94 % for BTV-8 (Cy5), 0.78 % for the endogenous Internal Control (JOE).

These results show an excellent reproducibility of different batches for the virotype BTV pan/8 2.0 RT-PCR Kit (Table 26 - Table 28, Figure 25).

Table 26. Batch-to-batch variance for **pan BTV** (FAM) for the virotype BTV pan/8 2.0 RT-PCR Kit using the Agilent Technologies Aria Mx instrument.

Batch-to-batch variance for pan BTV (FAM)							
Sample	**BTV status**	**Batch number (C_T values)**			**Mean**	**SD**	**CV%**
		1	**2**	**3**			
1	pos	21.59	21.61	21.62	21.61	0.015	0.07
2	pos	24.17	24.36	23.81	24.11	0.279	1.16
3	pos	27.81	26.82	27.24	27.29	0.497	1.82
4	pos	29.90	30.23	30.63	30.25	0.366	1.21
5	neg	-	-	-	-	-	-
PC	pos	28.52	27.64	27.13	27.76	0.703	2.53
Mean							**1.36**

neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Table 27. Batch-to-batch variance for **BTV-8** (Cy5) for the virotype BTV pan/8 2.0 RT-PCR Kit using the Agilent Technologies Aria Mx instrument.

Batch-to-batch variance for BTV-8 (Cy5)							
Sample	**BTV status**	**Batch number (C_T values)**			**Mean**	**SD**	**CV%**
		1	**2**	**3**			
1	pos	20.02	19.98	20.02	20.01	0.023	0.12
2	pos	22.89	22.67	22.61	22.72	0.147	0.65
3	pos	26.35	25.67	25.83	25.95	0.356	1.37
4	pos	29.00	29.52	29.43	29.32	0.278	0.95
5	neg	-	-	-	-	-	-
PC	pos	27.10	26.56	26.24	26.63	0.435	1.63
Mean							**0.94**

neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Table 28. Batch-to-batch variance for the endogenous **Internal Control** (JOE) for the virotype BTV pan/8 2.0 RT-PCR Kit using the Agilent Technologies Aria Mx instrument.

Batch-to-batch variance for the endogenous Internal Control (JOE)							
Sample	**BTV status**	**Batch number (C_T values)**			**Mean**	**SD**	**CV%**
		1	**2**	**3**			
1	pos	24.74	24.72	24.81	24.76	0.047	0.19
2	pos	24.41	24.19	24.04	24.21	0.186	0.77
3	pos	24.60	24.60	24.73	24.64	0.075	0.30
4	pos	24.26	24.30	24.40	24.32	0.072	0.230
5	neg	24.41	24.65	24.78	24.61	0.188	0.76
PC	pos	28.33	27.29	27.13	27.58	0.652	2.36
Mean							**0.78**

neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation

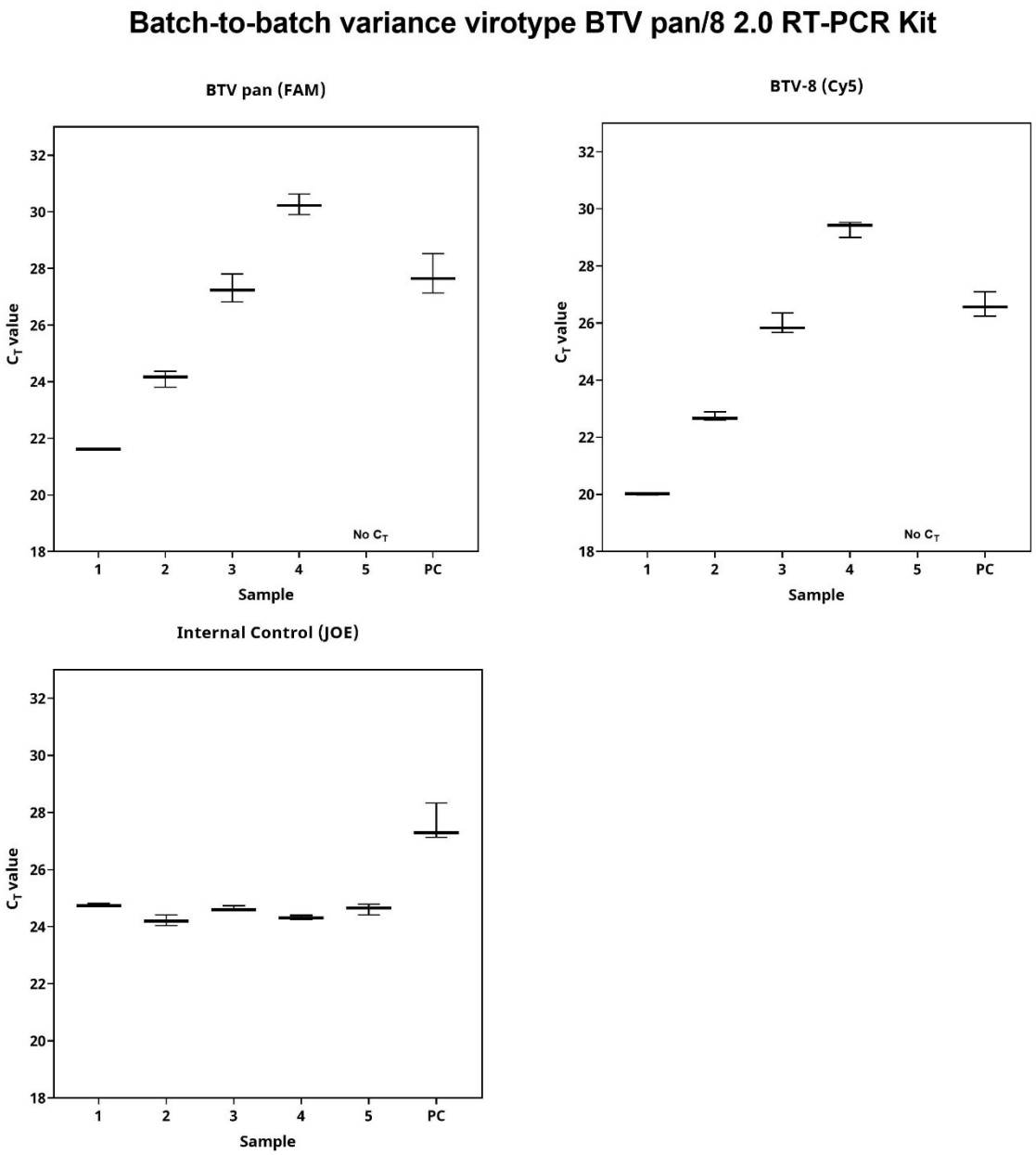

Figure 25. Boxplots of batch-to-batch variance for pan BTV (FAM), BTV-8 (Cy5), and the endogenous Internal Control (JOE) for the virotype BTV 2.0 pan/8 RT-PCR Kit tested using the Agilent Technologies Aria Mx instrument.

4.4.4 Comparison of real-time PCR thermocyclers

The virotype BTV pan/8 2.0 RT-PCR Kit can be used on different standard real-time PCR cyclers. Table 29 gives an overview of selected PCR cyclers and their approximate run times, using the virotype BTV pan/8 2.0 RT-PCR protocol.

Note: The use of the RT-PCR Kit is not limited to the mentioned instruments.

Table 29. Selected overview of real-time thermocyclers and their approximate run times for the virotype BTV pan/8 2.0 RT-PCR protocol.

	Thermocycler			
	Manufacturer	**Model**	**Filters**	**Run time [minutes]**
A	Agilent Technologies, Santa Clara, California, USA	AriaMx	FAM, HEX, Cy5	68
B	Bio-Rad Laboratories, Inc., Hercules, California, USA	CFX96	FAM, HEX, Cy5	67
C	Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA	Applied Biosystems™ 7500 Fast[1]	FAM, JOE, Cy5	52
D	Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA	QuantStudio 5	FAM, VIC/JOE, Cy5	68

[1] Fast Mode setting

A titration series of pan BTV and BTV-8 *in vitro* RNA (10^6 - 1 copies/reaction) were analyzed in the same PCR run using the virotype BTV pan/8 2.0 RT-PCR Kit when determining the variance in FAM (pan BTV) and Cy5 channels (BTV-8). Furthermore, six bovine blood samples were analyzed in the same PCR run using the virotype BTV pan/8 2.0 RT-PCR Kit when determining the variance in the JOE channel (endogenous Internal Control). For the Thermo Fisher ABI 7500 Fast instrument, the Standard Mode with ROX as passive reference dye was performed.

Results / Conclusion

The results are summarized in Table 30 (pan BTV / FAM channel), Table 31 (BTV-8, Cy5 channel), and Table 32 (endogenous Internal Control/ JOE channel). All samples tested on different real-time PCR thermocycler instruments showed comparable results.

Based on this data set, INDICAL can recommend using the virotype BTV pan/8 2.0 RT-PCR Kit on the Bio-Rad CFX96, Agilent Technologies AriaMx, Thermo Fisher ABI 7500 Fast, and Thermo Fisher QuantStudio 5 instruments.

Table 30. Inter-thermocycler variance for **pan BTV** (FAM).

Inter-thermocycler variance for pan BTV (FAM)						
Copy number	**Material**	**BTV status**	**Thermocycler (C_T values)**			
			A	**B**	**C**	**D**
10^6	*in-vitro* RNA	pos	17.89	19.03	18.30	18.35
10^5	*in-vitro* RNA	pos	21.02	22.10	21.55	21.64
10^4	*in-vitro* RNA	pos	24.14	25.50	24.81	24.99
10^3	*in-vitro* RNA	pos	27.48	28.76	28.04	28.42
10^2	*in-vitro* RNA	pos	30.52	32.13	31.24	31.61
10	*in-vitro* RNA	pos	33.63	35.68	34.58	35.01
5	*in-vitro* RNA	pos	35.14	35.53	35.13	35.65
1	*in-vitro* RNA	pos	37.05	38.49	37.58	37.15

pos = positive

Table 31. Inter-thermocycler variance for **BTV-8** (Cy5).

Inter-cycler variance for BTV-8 (Cy5)						
Copy number	**Material**	**BTV status**	**Thermocycler (C_T values)**			
			A	**B**	**C**	**D**
10^6	*in-vitro* RNA	pos	17.70	18.82	18.54	17.77
10^5	*in-vitro* RNA	pos	20.83	22.05	21.89	20.93
10^4	*in-vitro* RNA	pos	23.96	25.41	25.19	24.25
10^3	*in-vitro* RNA	pos	27.04	28.60	28.48	27.46
10^2	*in-vitro* RNA	pos	30.20	32.19	31.79	30.67
10	*in-vitro* RNA	pos	33.57	34.61	34.60	34.16
5	*in-vitro* RNA	pos	34.19	35.60	35.50	34.56
1	*in-vitro* RNA	pos	-	37.69	38.59	36.44

pos = positive, - = no C_T

Table 32. Inter-thermocycler variance for the endogenous **Internal Control** (JOE).

Inter-cycler variance for the endogenous Internal Control (JOE)						
Sample	**Material**	**BTV status**	**Thermocycler (C_T values)**			
			A	**B**	**C**	**D**
S1	Blood	pos	24.97	23.05	24.27	24.97
S2	Blood	pos	22.22	23.34	22.17	22.22
S3	Blood	pos	24.37	25.36	23.40	24.37
S4	Blood	pos	21.51	22.97	21.97	21.51
S5	Blood	pos	22.00	22.61	22.61	22.00
S6	Blood	pos	17.51	19.42	18.43	17.51

pos = positive

4.5 Stability testing

4.5.1 Freeze-thaw-cycles

The sample panel listed in Table 19 (sample 1-5, PC) was tested at the time of production and after ten freeze/thaw cycles. The mean value (Mean), standard deviation (SD) and coefficient of variation (CV) were calculated.

Results / Conclusion

The virotype BTV pan/8 2.0 RT-PCR Kit shows excellent stability after 10 freeze/thaw cycles of the virotype BTV pan/8 2.0 Master Mix. The average variance was 0.70 % for pan BTV (FAM), 0.53 % for BTV-8 (Cy5), and 0.99 % for the endogenous Internal Control (JOE) (Table 33 - Table 35).

Table 33. Stability testing for **pan BTV** (FAM) of the virotype BTV pan/8 2.0 RT-PCR Kit using the Agilent Technologies Aria Mx instrument.

Stability for pan BTV (FAM)						
Sample	**BTV status**	**Freeze-thaw-cycle (C_T values)**		**Mean**	**SD**	**CV%**
		1	**10**			
1	pos	21.21	20.82	21.02	0.276	1.31
2	pos	24.16	24.23	24.20	0.049	0.21
3	pos	27.17	27.23	27.20	0.042	0.16
4	pos	30.44	30.43	30.44	0.007	0.02
5	neg	-	-	-	-	-
PC	pos	27.29	28.00	27.65	0.502	1.82
Mean						**0.70**

PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Table 34. Stability testing for **BTV-8** (Cy5) of the virotype BTV pan/8 2.0 RT-PCR Kit using the Agilent Technologies Aria Mx instrument.

Stability for BTV-8 (Cy5)						
Sample	**BTV status**	**Freeze-thaw-cycle (C_T values)**		**Mean**	**SD**	**CV%**
		1	**10**			
1	pos	19.94	19.71	19.83	0.163	0.82
2	pos	22.74	22.76	22.75	0.014	0.06
3	pos	25.75	25.88	25.82	0.092	0.36
4	pos	28.84	29.07	28.96	0.163	0.56
5	neg	-	-	-	-	-
PC	pos	26.42	26.73	26.58	0.219	0.83
Mean						**0.53**

PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation, - = no C_T

Table 35. Stability testing for the endogenous **Internal Control** (JOE) of the virotype BTV pan/8 2.0 RT-PCR Kit using the Agilent Technologies Aria Mx instrument.

Stability for the endogenous Internal Control (JOE)						
Sample	**BTV status**	**Freeze-thaw-cycle (C_T values)**		**Mean**	**SD**	**CV%**
		1	**10**			
1	pos	24.77	24.64	24.71	0.092	0.37
2	pos	24.51	24.40	24.46	0.078	0.32
3	pos	24.31	24.31	24.31	0.000	0.00
4	pos	24.44	24.58	24.51	0.099	0.40
5	neg	26.13	27.47	26.8	0.948	3.54
PC	pos	18.56	18.22	18.39	0.240	1.31
Mean						**0.99**

PC = Positive Control, neg = negative, pos = positive, SD = standard deviation, CV = coefficient of variation

4.5.2 Heparin inhibition

To test the stability of the virotype BTV pan/8 2.0 RT-PCR Kit, sample inhibition was simulated by treating a BTV-positive sample with an increasing concentration of heparin (0.025 – 1.00 µg/reaction). The sample was tested in duplicate using the virotype BTV pan/8 2.0 RT-PCR Kit.

Results / Conclusion

The inhibition study using heparin showed that the endogenous Internal Control signal will be affected first in case of sample inhibition. The increased C_T value will help to identify partially and fully inhibited samples (Table 36).

Table 36. Stability testing of the virotype BTV pan/8 2.0 RT-PCR Kit for inhibition by heparin.

Heparin [µg / reaction]	Stability (heparin inhibition) virotype BTV pan/8 2.0 RT-PCR		
	pan BTV C_T	BTV-8 C_T	Internal Control C_T
0.00	25.14	24.16	20.19
	24.94	23.66	20.22
0.025	24.79	23.89	19.61
	25.06	23.7	20.09
0.05	24.88	23.62	20.16
	24.78	23.56	20.03
0.25	26.10	24.43	21.85
	26.30	24.54	22.00
0.50	34.65	31.64	-
	32.83	29.45	-
0.75	36.47	35.04	-
	36.98	33.36	-
1.00	-	-	-
	-	-	-

- = no C_T

4.5.3 EDTA inhibition

To test the stability of the virotype BTV pan/8 2.0 RT-PCR Kit, sample inhibition was simulated by treating a positive sample with an increasing concentration of Ethylenediaminetetraacetic acid (0.1 – 0.6 mM EDTA final concentration in reaction mix). The sample was tested in duplicate using the virotype BTV pan/8 2.0 RT-PCR Kit.

Results / Conclusion

The inhibition study using EDTA showed that the endogenous Internal Control signal will be affected first in case of sample inhibition. The increased C_T value will help to identify partially and fully inhibited samples (Table 37).

Table 37. Stability testing of the virotype BTV pan/8 2.0 RT-PCR Kit for inhibition by EDTA.

	Stability (EDTA inhibition)		
	virotype BTV pan/8 2.0 RT-PCR		
EDTA [mM]	**pan BTV**	**BTV-8**	**Internal Control**
	C_T	**C_T**	**C_T**
0.0	24.54	23.43	19.75
	24.52	23.50	19.64
0.1	24.01	23.15	19.28
	23.95	23.09	19.46
0.2	24.04	23.18	19.22
	24.19	23.16	18.96
0.3	23.80	22.77	19.63
	24.63	23.08	20.28
0.4	24.68	23.59	21.4
	24.22	23.03	20.9
0.5	31.39	36.2	-
	31.42	36.91	-
0.6	-	-	-
	-	-	-

- = no C_T

4.6 Robustness

4.6.1 Robustness: Variation of sample volume

To test the robustness of the virotype BTV pan/8 2.0 RT-PCR Kit, the sample volume was varied by 10 % to simulate errors in the reaction mix preparation. Therefore, the sample panel listed in Table 19 (sample 1-4, 6, PC) was tested in triplicates in one PCR run on the Agilent Technologies Aria Mx instrument.

Results / Conclusion

Results are shown in Table 38 - Table 40. The virotype BTV pan/8 2.0 RT-PCR Kit shows excellent robustness for 10 % sample volume variation with an average variance of 1.20 % for pan BTV (FAM), 1.01 % for BTV-8 (Cy5), and 0.86 % for the endogenous Internal Control (JOE).

Table 38. Robustness testing of the virotype BTV pan/8 2.0 RT-PCR Kit (**pan BTV** / FAM) for different sample volumes used in the RT-qPCR. The tests were performed on the Agilent Technologies Aria Mx instrument.

Sample	pan BTV (FAM) C_T			Mean	SD	CV%
	Sample volume					
	4.5 µl	**5.0 µl**	**5.5 µl**			
1	22.48	21.76	21.18	21.49	0.470	2.19
	21.60	21.11	21.31			
	21.80	21.17	21.01			
2	24.19	24.39	24.52	24.29	0.174	0.72
	24.45	24.00	24.16			
	24.25	24.18	24.46			
3	27.25	26.78	26.95	27.08	0.346	1.28
	27.58	27.13	27.26			
	27.24	26.37	27.16			
4	29.99	30.95	30.45	30.54	0.302	0.99
	30.18	30.85	30.58			
	30.62	30.55	30.67			
5	-	-	-	-	-	-
	-	-	-			
	-	-	-			
PC	27.61	28.00	27.69	27.77	0.234	0.84
	27.83	27.52	27.70			
	28.18	27.91	27.46			
Mean						**1.20**

SD = standard deviation; CV = coefficient of variation, - = no C_T

Table 39. Robustness testing of the virotype BTV pan/8 2.0 RT-PCR Kit (**BTV-8** / Cy5) for different sample volumes used in the RT-qPCR. The tests were performed on the Agilent Technologies Aria Mx instrument.

Sample	BTV-8 (Cy5) C_T			Mean	SD	CV%
	Sample volume					
	4.5 µl	**5.0 µl**	**5.5 µl**			
1	20.91	20.16	19.64	20.00	0.415	2.07
	20.07	19.70	19.70			
	20.28	19.79	19.71			
2	22.88	22.95	22.92	22.85	0.147	0.64
	23.02	22.58	22.81			
	22.88	22.64	22.94			
3	26.23	25.76	25.68	25.93	0.247	0.95
	26.30	25.69	25.83			
	26.22	25.81	25.87			
4	29.22	29.27	29.18	29.29	0.196	0.67
	29.28	29.40	28.89			
	29.50	29.56	29.30			
5	-	-	-	-	-	-
	-	-	-			
	-	-	-			
PC	26.55	26.71	26.63	26.69	0.196	0.73
	26.75	26.69	26.72			
	26.91	26.97	26.30			
Mean						**1.01**

SD = standard deviation; CV = coefficient of variation, - = no C_T

Table 40. Robustness testing of the virotype BTV pan/8 2.0 RT-PCR Kit (endogenous **Internal Control** / JOE) for different sample volumes used in the RT-qPCR. The tests were performed on the Agilent Technologies Aria Mx instrument.

Sample	Endogenous Internal Control (JOE) C_T			Mean	SD	CV%
	Sample volume					
	4.5 µl	5.0 µl	5.5 µl			
1	25.26	24.92	24.59	24.86	0.362	1.46
	25.15	24.64	24.56			
	25.48	24.71	24.43			
2	24.68	24.73	24.65	24.60	0.156	0.63
	24.76	24.32	24.43			
	24.48	24.64	24.74			
3	24.80	24.37	24.38	24.57	0.201	0.82
	24.95	24.50	24.53			
	24.51	24.40	24.68			
4	24.40	24.50	24.52	24.61	0.155	0.63
	24.70	24.73	24.48			
	24.85	24.78	24.57			
5	18.39	18.56	18.31	18.44	0.135	0.73
	18.59	18.39	18.38			
	18.27	18.67	18.41			
PC	27.31	27.23	26.91	27.06	0.235	0.87
	27.03	27.15	26.84			
	27.30	27.16	26.61			
Mean						**0.86**

SD = standard deviation; CV = coefficient of variation

4.6.2 Robustness: Variation of annealing time

To test the robustness of the virotype BTV pan/8 2.0 RT-PCR Kit, the annealing time during RT-qPCR reaction was varied by 10 % to simulate cycling errors. Therefore, the sample panel listed in Table 19 (sample 1-4, 6, PC) was tested in triplicates in one PCR run on the Agilent Technologies Aria Mx instrument.

Results / Conclusion

Results are shown in Table 41 - Table 43. The virotype BTV pan/8 2.0 RT-PCR Kit shows excellent robustness for 10 % annealing time variation with an average variance of 1.13 % for pan BTV (FAM), 0.86 % for BTV-8 (Cy5), and 0.80 % for the endogenous Internal Control (JOE).

Table 41. Robustness testing of the virotype BTV pan/8 2.0 RT-PCR Kit (**pan BTV** / FAM) for different annealing times used in the RT-qPCR. The tests were performed on the Agilent Technologies Aria Mx instrument.

Sample	pan BTV (FAM) C_T			Mean	SD	CV%
	Annealing time					
	27 sec	30 sec	33 sec			
1	21.38	21.76	21.59	21.46	0.253	1.18
	21.67	21.11	21.70			
	21.17	21.17	21.55			
2	24.33	24.39	24.39	24.28	0.146	0.60
	24.20	24.00	24.34			
	24.21	24.18	24.48			
3	27.33	26.78	27.32	27.01	0.317	1.18
	27.23	27.13	27.05			
	26.74	26.37	27.10			
4	30.26	30.95	30.63	30.28	0.509	1.68
	30.00	30.85	30.08			
	29.72	30.55	29.46			
5	-	-	-	-	-	-
	-	-	-			
	-	-	-			
PC	27.91	28.00	27.27	27.61	0.285	1.03
	27.52	27.52	27.58			
	27.18	27.91	27.64			
Mean						**1.13**

SD = standard deviation; CV = coefficient of variation, - = no C_T

Table 42. Robustness testing of the virotype BTV pan/8 2.0 RT-PCR Kit (**BTV-8** / Cy5) for different annealing times used in the RT-qPCR. The tests were performed on the Agilent Technologies Aria Mx instrument.

Sample	BTV-8 (Cy5) C_T Annealing time			Mean	SD	CV%
	27 sec	30 sec	33 sec			
1	20.00	20.16	20.15	20.03	0.202	1.01
	20.15	19.70	20.27			
	19.87	19.79	20.20			
2	22.84	22.95	22.98	22.92	0.245	1.07
	22.83	22.58	23.04			
	22.96	22.64	23.42			
3	25.91	25.76	26.21	25.90	0.167	0.65
	25.87	25.69	26.09			
	25.79	25.81	25.98			
4	29.22	29.27	29.71	29.25	0.263	0.90
	29.04	29.40	28.96			
	29.06	29.56	29.01			
5	-	-	-	-	-	-
	-	-	-			
	-	-	-			
PC	26.69	26.71	26.51	26.67	0.178	0.67
	26.44	26.69	26.76			
	26.45	26.97	26.83			
Mean						**0.86**

SD = standard deviation; CV = coefficient of variation, - = no C_T

Table 43. Robustness testing of the virotype BTV pan/8 2.0 RT-PCR Kit (endogenous **Internal Control** / JOE) for different annealing times used in the RT-qPCR. The tests were performed on the Agilent Technologies Aria Mx instrument.

Sample	Endogenous Internal Control (JOE) C_T			Mean	SD	CV%
	Annealing time					
	27 sec	30 sec	33 sec			
1	24.89	24.92	24.88	24.91	0.186	0.75
	25.30	24.64	24.99			
	24.91	24.71	24.98			
2	24.79	24.73	24.58	24.58	0.141	0.57
	24.48	24.32	24.49			
	24.60	24.64	24.63			
3	24.67	24.37	24.44	24.49	0.124	0.50
	24.72	24.50	24.40			
	24.50	24.40	24.43			
4	24.36	24.50	24.67	24.55	0.173	0.70
	24.65	24.73	24.39			
	24.60	24.78	24.30			
5	18.25	18.56	18.10	18.44	0.224	1.22
	18.31	18.39	18.38			
	18.84	18.67	18.44			
PC	27.92	27.23	27.04	27.19	0.287	1.05
	27.16	27.15	27.11			
	26.94	27.16	27.02			
Mean						**0.80**

SD = standard deviation; CV = coefficient of variation

4.6.3 Robustness: Variation of annealing temperature

To test the robustness of the virotype BTV pan/8 2.0 RT-PCR Kit, the annealing temperature during RT-qPCR reaction was varied by 1°C to simulate cycling errors. Therefore, the sample panel listed in Table 19 (sample 1-4, 6, PC) was tested in duplicates in one PCR run on the Agilent Technologies Aria Mx instrument.

Results / Conclusion

Results are shown in Table 44 - Table 46. The virotype BTV pan/8 2.0 RT-PCR Kit shows excellent robustness for 1°C annealing temperature variation with an average variance of 0.95 % for pan BTV (FAM), 0.69 % for BTV-8 (Cy5), and 1.27 % for the endogenous Internal Control (JOE).

Table 44. Robustness testing of the virotype BTV pan/8 2.0 RT-PCR Kit (**pan BTV** / FAM) for different annealing temperatures used in the RT-qPCR. The tests were performed on the Agilent Technologies Aria Mx instrument.

	pan BTV (FAM) C_T					
Sample	**Annealing temperature**			**Mean**	**SD**	**CV%**
	59°C	**60°C**	**61°C**			
1	21.49	21.11	21.20	21.34	0.216	1.01
	21.42	21.17	21.66			
2	24.36	24.00	24.73	24.40	0.274	1.12
	24.49	24.18	24.62			
3	27.43	26.78	27.76	27.25	0.357	1.31
	27.41	27.13	26.96			
4	30.72	30.85	30.66	30.62	0.172	0.56
	30.60	30.55	30.34			
5	-	-	-	-	-	-
	-	-	-			
PC	27.98	27.52	28.00	27.91	0.205	0.73
	27.94	27.91	28.12			
Mean						**0.95**

SD = standard deviation; CV = coefficient of variation, - = no C_T

Table 45. Robustness testing of the virotype BTV pan/8 2.0 RT-PCR Kit (**BTV-8** / Cy5) for different annealing temperatures used in the RT-qPCR. The tests were performed on the Agilent Technologies Aria

Sample	BTV-8 (Cy5) C_T Annealing temperature			Mean	SD	CV%
	59°C	**60°C**	**61°C**			
1	19.89	20.16	19.92	19.99	0.151	0.76
	20.03	19.79	20.16			
2	22.96	22.95	23.15	22.96	0.193	0.84
	22.90	22.64	23.17			
3	26.04	25.76	26.14	25.99	0.169	0.65
	26.17	25.81	26.02			
4	29.16	29.27	29.26	29.31	0.169	0.58
	29.14	29.56	29.47			
5	-	-	-	-	-	-
	-	-	-			
PC	26.51	26.71	26.88	26.77	0.162	0.61
	26.70	26.97	26.83			
Mean						**0.69**

SD = standard deviation; CV = coefficient of variation, - = no C_T

Table 46. Robustness testing of the virotype BTV pan/8 2.0 RT-PCR Kit (**endogenous Internal Control** / JOE) for different annealing temperatures used in the RT-qPCR. The tests were performed on the Agilent Technologies Aria Mx instrument.

Endogenous Internal Control (JOE) C_T						
Sample	**Annealing temperature**			**Mean**	**SD**	**CV%**
	59°C	**60°C**	**61°C**			
1	24.33	24.92	24.79	24.67	0.200	0.81
	24.60	24.71	24.68			
2	24.33	24.73	24.79	24.63	0.161	0.65
	24.60	24.64	24.68			
3	24.53	24.37	24.86	24.53	0.175	0.71
	24.50	24.40	24.52			
4	24.52	24.50	24.62	24.64	0.181	0.73
	24.94	24.78	24.50			
5	18.66	18.56	18.34	18.47	0.195	1.06
	18.40	18.67	18.18			
PC	27.23	27.23	28.95	27.79	1.009	3.63
	26.95	27.16	29.21			
Mean						**1.27**

SD = standard deviation; CV = coefficient of variation