Validation Report

OneqPCR-realtime™ Bluetongue Virus

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IAS ACCREDITED Management Systems Certification Body MSCB 107 ISO 9001:2015 STAREGISTER

BioinGentech Biotechnology

1. OneqPCR-realtime™ Bluetongue Virus

1.1. General

Bluetongue Virus (BTV) is an arthropod-borne virus that infects domestic and wild ruminants, particularly sheep, cattle, and goats, causing Bluetongue disease, a condition of major veterinary and economic importance. The virus is transmitted mainly by Culicoides spp. midges and is characterized by fever, edema, mucosal lesions, and, in severe cases, high mortality in susceptible breeds. Beyond clinical losses, BTV outbreaks lead to trade restrictions, decreased productivity, and significant economic impact on livestock industries.

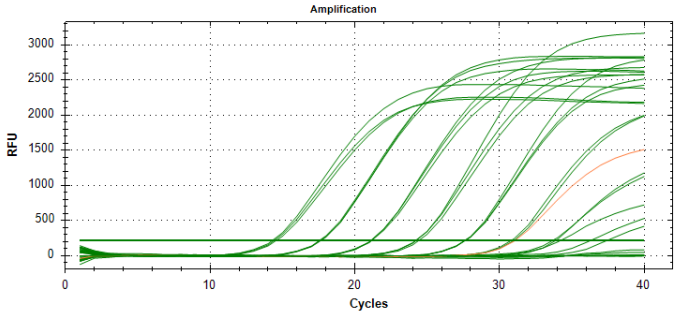
The real-time PCR assay implemented in our laboratory targets segment 10, enabling rapid and sensitive detection of all 29 recognized BTV serotypes. This broad-range molecular approach allows early diagnosis, differentiation from other hemorrhagic or febrile viral diseases of ruminants, and supports surveillance, outbreak control, and epidemiological monitoring. Molecular detection thus represents a critical tool for managing Bluetongue virus circulation and assessing the effectiveness of preventive measures in affected regions.

1.1.1 Method

The OneqPCR-realtime™ Bluetongue Virus kit follows a standardized and systematic approach to ensure its efficiency in detecting viral RNA in avian samples. 1) RNA extraction, 2) reverse transcription, and 3) real-time PCR amplification. During RNA extraction, viral RNA is isolated from the sample using a specific extraction protocol that efficiently recovers viral genomic material. Reverse transcription is then performed to convert the viral RNA into complementary DNA (cDNA), which is subsequently amplified using a specific primer-probe set targeting a highly conserved region of the Bluetongue Virus genome. The OneqPCR-realtime™ Bluetongue Virus kit is compatible with various real-time PCR instruments and is validated for use with multiple avian sample types, such as nasal swabs, tracheal swabs, blood, and respiratory secretions. This assay provides a reliable tool for the detection of Bluetongue Virus, facilitating early diagnosis, surveillance, and control measures in avian populations.

1.1.2 Real-time PCR Data

a) Figure 1. Standard amplification curves using the OneqPCR-realtimeTM Bluetongue Virus on Bio Rad CFX96.



a) Standard amplification curves (10^1-10^9 DNA copies/µL) for the detection of Bluetongue Virus in the FAM channel and positive control.

1.2 Validation data

1.2.1 Specificity

Specificity of the OneqPCR-realtime™ Bluetongue Virus kit has been tested as described (see 2.2.1 Specificity). Results are shown in table 1. The specificity of the real-time PCR system detecting Bluetongue Virus was tested with RNA extracted from several closely related species as well as species commonly detected in poultry. The method is specific for the detection of Bluetongue Virus.

Table 1: Overview of specificity tests using OneqPCR-realtimeTM Bluetongue Virus.

sample	realtime PCR
Bluetongue Virus (BTV-1 to BTV-29)	**POSITIVE**
Epizootic Hemorrhagic Disease Virus (EHDV)	NEGATIVE
Palyam Serogroup Virus	NEGATIVE
Akabane Virus	NEGATIVE
Schmallenberg Virus	NEGATIVE
Bovine Viral Diarrhea Virus (BVDV)	NEGATIVE
Rift Valley Fever Virus (RVFV)	NEGATIVE
Foot-and-Mouth Disease Virus (FMDV)	NEGATIVE
Capripoxvirus (Sheeppox/Goatpox Virus)	NEGATIVE
Bovine Ephemeral Fever Virus (BEFV)	NEGATIVE
Peste des Petits Ruminants Virus (PPRV)	NEGATIVE

1.2.2 Robustness

Temperature stability for OneqPCR-realtime™ Bluetongue Virus has been approved according to specifications (see 2.2.2 Robustness).

1.2.3 Correctness

Determination of correctness for qualitative methods must be obtained like described in 2.2.3.a.

1.2.4 Technical equipment verification

Table 2: Overview of tested real-time PCR instrument

Real-time PCR instrument		
Manufacturer	**Model**	**OneqPCR-realtime™ Bluetongue Virus**
Eppendorf	Mastercycler® ep realplex	•
Agilent	Mx3000P QPCR System	•
Agilent	Mx3005P QPCR System	•
Qiagen (Corbett)	RotorGene 3000	•
Qiagen (Corbett)	RotorGene 6000	•
Qiagen	Rotor-Gene Q	•
Sansure (Tianlong)	SLAN® Real-Time PCR	•
Cepheid	SmartCycler II	•
Applied Biosystems	ABI 7300	•
Applied Biosystems	ABI 7500FAST	•
Applied Biosystems	ABI 7900	•
Applied Biosystems	StepOne	•
Applied Biosystems	StepOne Plus	•
Agilent	Mx3005P	•
Bio-Rad	CFX96	•
Bio-Rad	CFX384	•
Bioneer	ExiCycler™ 96	•
Bio-Rad	iQ5	•
Bio-Rad	MyiQ Cycler	•
Illumina	Eco	•
Roche	LightCycler Nano	•
Bio-Rad	CFX Opus 384	•
Bio-Rad	CFX Opus	•
Thermo Fisher Scientific	QuantStudio 3	•
Bioer	QuantGene 9600	•
Thermo Fisher Scientific	QuantStudio 5	•
Roche	LightCycler 2.0	•
Roche	LightCycler 480	•
Bio-Rad	CFX Opus 96	•

1.2.5 Limit of detection (LOD)

The detection limit before enrichment is approximately **100 RNA copies/µL** (depending on the matrix and the vitality of the strain).

LOD may vary depending on matrix, processing grade, RNA-extraction and DNA-content (see 2.2.5 a).

1.2.6 Tested matrices

Table 3 shows different samples analysed for OneqPCR-realtime™ Bluetongue Virus.

Table 3: Overview of different samples analysed using OneqPCR-realtime™ Bluetongue Virus

Sample	
Blood (EDTA) (sheep, cattle, goats)	Tracheal swabs (sheep, cattle)
Spleen tissue (sheep, cattle, goats)	Cloacal swabs (sheep, cattle)
Lymph nodes (sheep, cattle, goats)	Environmental swabs (barns, cages with ruminants)
Lung tissue (sheep, cattle)	Semen (sheep, cattle)
Skin biopsies – ear or coronet (sheep, cattle)	

1.2.7 Ring trials and Proficiency tests

We attend external verification studies and official ring-trials as well as proficiency tests on a regular basis. Please contact us for the current state of external verification, ring trials and proficiency tests.

1.2.8 Ongoing stability studies

Ongoing stability studies are used to verify the shelf life of a OneqPCR-realtime™ Bluetongue Virus. For this purpose one batch of a product is stored over the guaranteed shelf life time plus one month under the conditions stated in the test kit manual (see 2.2.8).

1.2.9 Accelerated stability studies

Accelerated stability testing is carried out at an increased storage temperature within a given storage period (see 2.2.9).

1.2.10 Stability study after opening

The stability of the PCR kit after opening was thoroughly assessed to confirm its continued performance over time. The kit retains its full functionality for up to 2 years once opened, provided it is stored under recommended conditions. This ensures the kit's reliability and consistent performance throughout its validated shelf life (see 2.2.10).

2. General considerations about verification of OneqPCR-realtime™ Bluetongue Virus methods

2.1 Background of verification

The verification of the OneqPCR-realtime™ Bluetongue Virus methods describe the performance of these tests. BioinGentech defines the verification of the Onestep® methods in an internal Standard Operating Procedure (SOP) based on following performance parameters (excerpt):

Table 1: Performance parameters checked by method verification (+ required, - not required)

No.	Performance parameter	Qualitative PCR	Quantitative PCR
1.	Specificity	+	+
2.	Robustness (chemically, physically)	+	+
3.	Correctness	+	+
4.	Technical equipment verification	+	+
5.	Limit of detection (LOD)	+	+
6.	Limit of quantification (LOQ)	-	+
7.	Precision (intra- and inter-assay)	-	+
8.	Measuring range	-	+
9.	Tested matrices	+	+
10.	Ring trials and Proficiency tests	+	+
11.	Ongoing stability studies	+	+
12.	Accelerated stability studies	+	+
13.	Stability study after opening	+	+

2.2 Information about verification procedure

2.2.1 Specificity

Specificity means the capability of a detection method to detect only the target analyte and discriminate against all other targets. Specificity in real-time PCR is defined by the combination of primers and probes.

For ensuring specificity, DNAs and RNAs of a) closely related species and b) species probably appearing in the main matrices are tested.

2.2.2 Robustness

Temperature stability is the major factor for robustness testing of OneqPCR-realtime™ Bluetongue Virus. Therefore the PCR temperature profile is varied and the correct function of the OneqPCR-realtime™ Bluetongue Virus method is monitored. At least three control samples have to be tested using the kit-specific annealing temperature and with ± 1° C deviating annealing temperature. Maximum allowed variance in obtained Cp values may be ± 1.0 for DNA methods and ± 1.5 for RNA methods.

2.2.3 Correctness

Correctness of qualitative methods is expressed by a percentage of false positive and false negative results for known (negative and positive) samples. Only 1 of 20 samples is allowed to be false positive or false negative.

% false positive = false positives / known negatives x 100

% false negative = false negatives / known positives x 100

Statistical probability for correct (positive and negative) results must be 95 % or higher.

2.2.4 Technical equipment verification

The OneqPCR-realtime™ Bluetongue Virus system has to be tested on a variety of suitable and available real-time PCR cyclers.

2.2.5 Limit of detection (LOD)

The limit of detection (LOD) is defined as the lowest concentration which will be detected with a statistical safety of 50 %. This means 50 % of the results for a repeated measurement of a sample with a concentration at the detection limit will be positive.

The LOD is determined in two ways:

a) Theoretical determination of LOD:

LOD is calculated according to the 95 % confidence level of a standard curve based on a dilution series of different known concentrations.

b) Practical confirmation of theoretical approved values:

The theoretically determined LOD is confirmed in practice by testing relevant concentration levels for detectability.

2.2.6 Tested matrices

A variety of different sample matrices has to be tested.

2.2.7 Ring trials and Proficiency tests

The quality of OneqPCR-realtime™ Bluetongue Virus products are continuously monitored by participation in respective, suitable ring trials and proficiency tests.

2.2.8 Ongoing stability studies

Ongoing stability studies are used to verify the shelf life of a OneqPCR-realtime™ Bluetongue Virus assay. For this purpose one batch of a product is stored over the guaranteed shelf life time plus one month under the conditions stated in the test kit manual. Measurements are performed at the beginning of the storage and after 6, 12, 18 and 24 months. In addition to the negative control and positive control, three control samples (high negative, low positive and moderate positive) have to be tested.

2.2.9 Accelerated stability studies

Accelerated stability testing is carried out at an increased storage temperature within a given storage period as listed in table 2.

Table 2: Test principle and storage conditions for the accelerated stability test.

test principle	storage temperature	storage period
real-time PCR	37°C	7 days

Measurements are performed with one test kit at the beginning of the storage period and with another test kit stored under the conditions stated in table 2. The analysis of the stored kit is carried out immediately after the storage period. In addition to the negative control and positive control, a control sample (moderate positive) is tested, which has not been stored at increased temperature. Ct and fluorescence values are reported.

In order to evaluate the given results, deviations in relation to the initial values are calculated. The deviation must not exceed ± 2.0 cycles and the fluorescence values must not fall below 40 % of the initial fluorescence value.

2.2.10 Stability study after opening

The stability of the PCR kit was evaluated to ensure its performance remains consistent over an extended period once the kit has been opened. Based on the manufacturer's specifications and internal testing, the kit demonstrates a robust stability profile, maintaining its efficacy for up to 2 years following the first use.

To verify this, the stability was assessed under standard storage conditions, which include storage at room temperature protection from prolonged exposure to light or humidity. The results of the stability study showed that all components of the kit, including primers, probes, and enzymes, retain their optimal performance levels, yielding accurate and reliable amplification results over the course of the 2-year period.