European Commission

Joint Research Centre

Wastewater-Based Detection of Viral Pathogens: Preliminary Methodological Framework – Ebola Bundibugyo

Validation of Concentration, Extraction and Quantification Assay

2026

HIGHLIGHTS

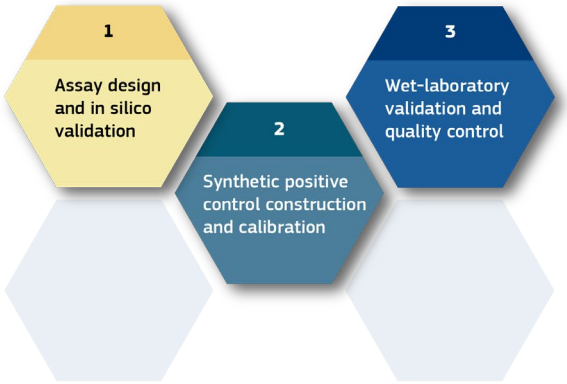
- A validated one-step reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay targeting the nucleoprotein gene enables sensitive and specific detection of **Ebola Bundibugyo** virus (Bdbv-2026) in wastewater samples.
- Validation according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines demonstrated high analytical performance, including robust sensitivity, specificity, repeatability, reproducibility and quantification accuracy.
- This preliminary validation focused exclusively on the RT-qPCR detection phase; subsequent validation encompassing the full concentration and extraction process will be conducted upon the availability of the complete Bdbv-2026 genome.

Introduction

This document supports wastewater-based surveillance (WBS) activities within the EU Super-Site Sentinel System (EU4S) Network, an initiative aimed at strengthening integrated environmental and public health surveillance through advanced monitoring infrastructures. The strategy involves European Health and Digital Executive Agency (HaDEA) and the European Commission (through the Health Emergency Preparedness and Response Authority (HERA) and the Joint Research Centre (JRC)) collecting and analysing wastewater from strategically selected Super-Sites, including

transportation hubs (airplane, airports, train and bus stations, port), wastewater treatment plant (WWTP), hospitals including intensive care unit (ICU), and large-event venues, using standardised procedures to generate high-quality, spatially and temporally resolved data. This protocol describes the methodological approach used to detect and quantify Ebola Bundibugyo (Bdbv-2026) in wastewater samples through a validated RT-qPCR assay, targeting the nucleoprotein gene [1]. The assay was designed by aligning 16 publicly available sequences. The wet-laboratory validation was done according to MIQE guidelines [2].

Figure 1. Methodological Framework for Wastewater-Based Detection of Ebola Bundibugyo 2026 (Bdbv-2026).



Source: EC-JRC

Metodological Framework

1 Assay Design and In Silico Validation

The RT-qPCR assay was designed using sixteen publicly available Bdbv-2026 sequences, to identify conserved genomic regions suitable for molecular detection. The nucleoprotein (NP) gene was selected as the amplification target. Candidate primers and hydrolysis probe were evaluated through in silico validation using publicly available databases including:

- Pathoplexus [1]
- National Center for Biotechnology Information (NCBI) BLAST [3]

The selected assay was subsequently used for laboratory validation.

2 Construction of Synthetic Positive Control

A synthetic positive control was developed by aligning sixteen publicly available Bdbv-2026 sequences in the genomic region corresponding to the RT-qPCR amplicon.

The resulting synthetic construct comprised 181 nucleotides and was employed for:

- Assay optimisation
- Calibration curve generation
- Determination of analytical sensitivity
- Determination of analytical quantification limits
- Evaluation of assay performance.

Synthetic genomic material for Bdbv-2026 sequence (5'-3'):

AAGGTGTCAAGCGCCTGGAGGAACTACTCCCTGCT
GCCTCAAGTGGAAAGAACATCAAGAGAACATTGG
CTGCAATGCCCGAGGAGGAAACAACAGAAGCAAA
TGCTGGACAATTTCTTTCATTTGCTAGTCTGTTTCT
CCCAAAATTGGTTGTCGGAGAAAAGGCCTGTCTGG
AGAAGGT

3.1 RT-qPCR

Real-time RT-qPCR was performed using:

- QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific)
- Luna Universal Probe One-Step RT-qPCR Kit (New England Biolabs, Ipswich, USA)
- Luna WarmStart® RT Enzyme Mix

Reaction composition (20 µl final volume):

- 10 µl Luna Universal Probe One-Step RT-qPCR reaction mix

- 1 µl Luna WarmStart® RT Enzyme Mix
- 800 nM forward primer
- 800 nM reverse primer
- 200 nM hydrolysis probe
- 5 µl nucleic acid extract
- nuclease-free water to final volume.

Positive and negative controls were included in every PCR run.

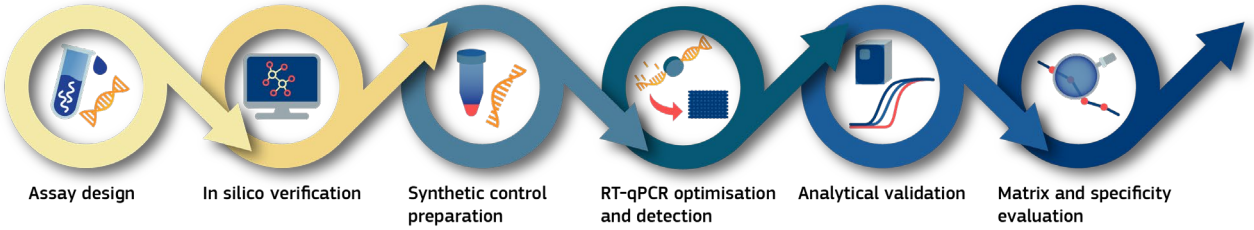
3.2 Thermal Profile

Reverse transcription: 55 °C for 10 min.

Initial denaturation: 95 °C for 10 min.

Amplification (45 cycles): 95 °C for 15 and 60 °C for 60 s.

Figure 2. Illustrative Methodological Framework.



Source: EC-JRC

Technical Specifications & Benchmarks

Box 1 and box 2 report the Bdbv RT-qPCR assay sequences, and PCR conditions. Limit of Detection (LoD) and Limit of Quantification (LoQ) are also reported.

Box 1: RT-qPCR assay target – Bdbv

Sequence (5'->3')

- F: CAAGTGGAAAGAACATCAAGAGAAC
- R: GGGAGAAACAGACTAGCAAATGA
- P: 6FAM-ATGCCCGAG/ZEN/GAGGAAACAA CAGAA-BHQ

Limit of Detection

- 6.45 copies/5 µl

Limit of Quantification

- 6.45 copies/5 µl

Box 2: RT-qPCR assay conditions

- 55 °C for 10 min, 95 °C for 10 min, 45 cycles of 95 °C for 15s and 60 °C for 1 min

Quality control and validation

The validation was performed according to MIQE recommendations and included seven analytical performance criteria.

Analytical sensitivity

The LoD was defined as the concentration where 95% of the tested concentration were positive [4].

The LoQ was defined as the lowest concentration presenting a coefficient of variation below 35% [5].

Accuracy

Accuracy was evaluated by spiking wastewater concentrates with positive control material.

- Percentage recovery: 73–126%
- Average recovery: 100%
- Standard deviation: 16%
- Relative error: 0.6–27%
- Average relative error: 14%
- Standard deviation: 8%

Repeatability (Intra-assay variance)

Testing was conducted at the LoQ concentration in concentrated raw wastewater samples.

- Standard deviation (Cq): 0.130–0.771

- Average: 0.453
- Standard deviation: 0.195
- Coefficient of variation (%CV): 0.8–6%
- Average: 2%
- Standard deviation: 2%

Reproducibility (Inter-assay variance)

Assessment was conducted using three different qPCR platforms and different analytical runs.

- Coefficient of variation (CV): 19–26%
- Average: 23%
- Standard deviation: 3%

Given that the testing was done at the lowest concentration (LoQ) and spiked in concentrated raw wastewater samples, a CV value of 30% was accepted for reproducibility.

Linearity and Amplification Efficiency

Calibration curves contained between five and seven concentration points.

- PCR efficiency: 90–95%
- Average: 92%
- Standard deviation: 2%
- Linearity (R^2): 0.993–1.000
- Average: 0.998
- Standard deviation: 3×10^{-3}

Robustness and Matrix Effects

Robustness was assessed by varying annealing temperatures between 54 °C and 64 °C

No significant variation in Cq values was observed. Matrix effects were evaluated by analysing concentrated wastewater samples originating from:

a) Hospitals
b) Wastewater treatment plants, including wastewater receiving high loads of industrial effluents with a known concentration of positive control.

Inhibition testing through decimal dilution demonstrated $\Delta Cq < 2$, indicating negligible PCR inhibition.

Specificity and Limitation

Specificity was evaluated using banked samples expected to be negative for Bdbv-2026. It was not conducted against other members of the Ebola family due to the lack of available controls

Results demonstrated:

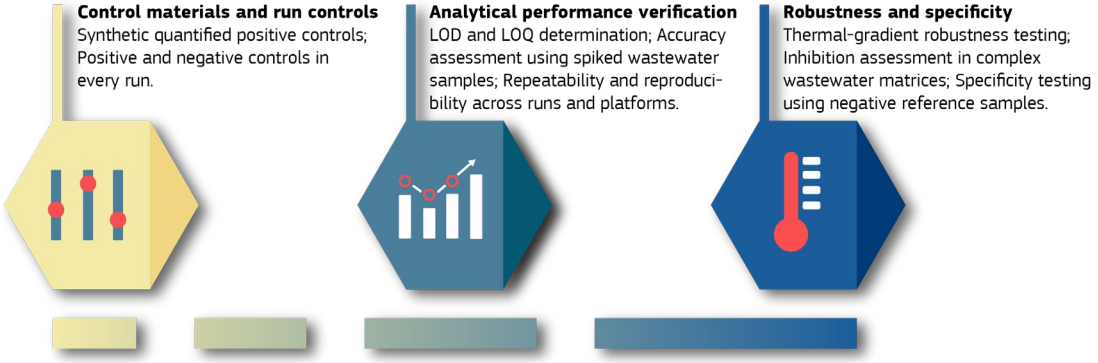
- Absence of false-positive amplification
- Absence of non-specific amplification
- 100% agreement with expected results.

Flagship Actions

To ensure reliable implementation of the assay, several quality assurance measures were incorporated:

- Use of synthetic quantified positive controls
- Inclusion of positive and negative controls in every run
- Verification of assay sensitivity through LoD and LoQ determination
- Assessment of accuracy through spiked wastewater samples
- Evaluation of repeatability and reproducibility across analytical runs and platforms
- Robustness testing through thermal-gradient experiments
- Inhibition assessment in complex wastewater matrices
- Specificity evaluation using negative reference samples.

Figure 3. Flagship Actions for Quality Assurance



Source: EC-JRC

References

[1] Pathoplexus, 2026. https://pathoplexus.org/ebola-bdbv/search, last accessed: 29 May 2026.

[2] Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., et. al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009 Apr;55(4):611-22. doi: https://10.1373/clinchem.2008.112797.

[3] National Center for Biotechnology Information, 2026. Basic Local Alignment Search Tool. https://blast.ncbi.nlm.nih.gov/Blast.cgi, last accessed: 29 May 2026.

[4] Bustin, S. A., Ruijter, J. M., van den Hoff, M. J. B., Kubista, M., Pfaffl, M. W., Shipley, G. L., Tran, N., Rödiger, S., Untergasser, A., Mueller, R., Nolan, T., Milavec, M., Burns, M. J., Huggett, J. F., Vandesompele, J., Wittwer, C.T. MIQE 2.0: Revision of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments Guidelines. Clin Chem. 2025 Jun;71(6):634-51. https://10.1093/clinchem/hvaf043.

[5] Klymus, K. E., Merkes, C. M., Allison, M. J. Reporting the limits of detection and quantification for environmental DNA assays. Environmental DNA. 2020;2(3):271–282. https://10.1002/edn3.29.

Acknowledgments

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