



Optimization and Validation of a Luna® Universal Probe One-Step RT-qPCR Assay for the Detection of Bovine Respiratory Syncytial Virus in Morocco

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Abstract

Bovine respiratory syncytial virus (BRSV) is a major viral pathogen within the bovine respiratory disease complex (BRDC), causing significant economic losses in cattle production worldwide. Accurate detection of BRSV is essential for both diagnosis and epidemiological surveillance, yet molecular diagnostic assays require local optimization and validation to ensure reliability. This study reports the optimization and analytical validation of a One-Step Real-Time Reverse Transcription Quantitative PCR (RT-qPCR) assay using the Luna® Universal Probe One-Step Kit (New England Biolabs, 2023) for BRSV detection in Morocco. Analytical parameters including linearity, amplification efficiency, limit of detection (LOD), specificity, repeatability, reproducibility, and robustness were evaluated. The standard curve generated with serial dilutions of RNA demonstrated excellent linearity ($R^2 = 0.9962$) across seven orders of magnitude, with a slope of -3.64 corresponding to an amplification efficiency of 93.0%. The LOD was determined at 10 RNA copies/ μL , with $\geq 95\%$ detection consistency. No cross-reactivity was observed with other common bovine respiratory pathogens. Precision testing showed intra-assay variability below 2% and inter-assay variability below 5%. Robustness testing confirmed stable amplification under minor deviations in temperature and volume. These findings establish the Luna® RT-qPCR assay as a sensitive, specific, and reproducible tool suitable for routine diagnostic use and molecular surveillance of BRSV in Morocco.

Keywords: Bovine Respiratory Syncytial Virus; Bovine Respiratory Disease Complex; Analytical validation; Amplification efficiency; RT-qPCR; Limit of Detection.

Introduction

Bovine respiratory disease complex (BRDC) remains a leading cause of economic loss in cattle production worldwide, is an enveloped, negative-sense, single-stranded RNA virus classified under the genus *Orthopneumovirus*, family *Paramyxoviridae*. The virus exhibits a high tropism for the lower respiratory tract, inducing necrosis of bronchiolar epithelium, syncytia formation, and inflammation, which contribute to bronchiolitis and pneumonia (Valarcher & Taylor, 2007). Secondary bacterial infections, particularly with *Pasteurella multocida* and *Mannheimia haemolytica*, often complicate BRSV infection, exacerbating clinical severity (Ellis, 2010; Timsit et al., 2017). These interactions underscore the complex pathogenesis of BRD and the importance of rapid, accurate viral detection.

Molecular diagnostic approaches, especially real-time reverse transcription quantitative PCR (RT-qPCR), have become the reference standard for BRSV detection due to their superior sensitivity, specificity, and rapid turnaround compared to conventional techniques (Boxus et al., 2005; Timsit et al., 2017).

However, assay performance varies depending on reagent selection, primer-probe design, and laboratory conditions, necessitating local optimization and validation. In Morocco, molecular assays for BRSV have not been systematically validated under national laboratory settings. This study aimed to optimize and analytically validate a Luna® Universal Probe One-Step RT-qPCR assay for the detection of BRSV RNA in Moroccan laboratories, focusing on key diagnostic performance parameters.

Materials and Methods

Study area and sample Collection

Nasal swab were collected from 94 cattle exhibiting respiratory symptoms (nasal discharge, fever >39.5°C, and coughing) from multiple regions across Morocco during the time periode [between October 2020 and July 2021]. Samples were transported to the analysis laboratory in a liquid nitrogen environment to ensure preservation of viral RNA integrity during transit, prior to RNA extraction.

RNA Standards and Controls

An in vitro-transcribed RNA standard corresponding to the nucleoprotein (N) gene of BRSV was quantified spectrophotometrically and serially diluted (10^8 – 10^1 copies/μL) to generate a standard curve and determine assay sensitivity. A positive template control (PTC) derived from a laboratory- maintained strain of BRSV, alongside a no-template control (NTC) provided with the Luna® kit to detect any potential contamination.

Viral RNA was extracted using the same protocol applied to clinical samples and stored at –80 °C until use. Positive and negative controls were included in each RT-

RNA Extraction

Total RNA was extracted using the NucleoSpin® Viral RNA Kit (Macherey-Nagel, Germany), according to the procedure set out in the package inserts. The extracted RNA was eluted in 50 μL RNase-free water; its concentration and purity were assessed using the NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA).

qPCR run to monitor assay performance, verify amplification efficiency, and ensure the sensitivity and specificity of BRSV RNA detection.

RT-qPCR Assay

Reactions were performed using the Luna® Universal Probe One-Step RT-qPCR Kit (New England Biolabs, 2023) on an AriaMx Real-Time PCR System (Agilent Technologies, USA). Primers and probe targeting the conserved N gene were used as described by (Boxus et al. 2005). Optimization of primer/probe concentration and annealing temperature identified 59 °C as optimal.

Table 1: Primer and probe sequences used for RT-qPCR detection of BRSV (N gene).

Primer and Probe	Séquences (5' → 3')	Position (RB94)	Product size	Référence
Forward primer	-GCA-ATG-CTG-CAG-GAC-TAG-GTA-TAA-T-	977–1001	124 bp	Boxus et al., 2005
Reverse primer	-ACA-CTG-TAA-TTG-ATG-ACC-CCA-TTC-T-	1076–1100		Boxus et al., 2005
Probe	FAM-ACC-AAG-ACT-TGT-ATG-ATG-CTG-CCA- AAG- CA-TAMRA	-		Boxus et al., 2005

➤ Reaction mix (20 μL total volume):

- 10μL Luna® Universal Probe Reaction Mix (2X)
- 1 μL Luna® t RT Enzyme Mix (20X)
- 320nM of forward and reverse primers
- 160 nM probe
- 5 μL RNA template
- 2.4 Nuclease-free water to volume

➤ Thermal cycling conditions:

- Reverse transcription: 55°C for 10 min (1 cycle)
- Initial denaturation: 95°C for 1 min (1 cycle)
- 45 cycles of 95°C for 7 sec and 59°C for 30 sec (annealing/extension with data acquisition)

Analytical Validation

The analytical performance was evaluated for linearity, amplification efficiency, limit of detection (LOD), specificity, precision, and robustness.

Linearity and Efficiency: Were determined from standard

curves using serial RNA dilutions (10^8 – 10^1 copies). Regression coefficients (R^2) and amplification efficiency were calculated from the slope of the linear regression.

Reproducibility: Was assessed from three independent runs conducted on different days (inter-assay). Mean Ct values, standard deviations (S.D.), and coefficients of variation

(CVs) were calculated for each RNA input level. CVs <5% was considered acceptable.

Precision : Was evaluated via intra-assay repeatability (triplicates in a single run).

LOD: Defined as the lowest RNA input consistently detected (Ct < 40).

Specificity: was evaluated using PTC, NTC, and non-target controls (Bovine Viral Diarrhea Virus, parainfluenza-3 virus).

Robustness: Robustness was evaluated by varying reaction volume (±10%) and annealing temperature (±1 °C).

Results

Linearity and Amplification Efficiency

To evaluate the linear dynamic range of the Luna® Universal Probe One-Step RT-qPCR assay, a standard curve was established using tenfold serial dilutions of BRSV RNA transcripts ranging from 10^8 to 10^1 copies. The assay demonstrated a strong linear relationship between input copy number and Ct values across eight orders of magnitude. The regression equation obtained was: $y = -3.4940x + 48.31$,

with a coefficient of determination $R^2 = 0.9962$. The slope (-3.49) corresponds to a calculated amplification efficiency of 93%, confirming high assay performance within the optimal range for qPCR assays (90–110%).

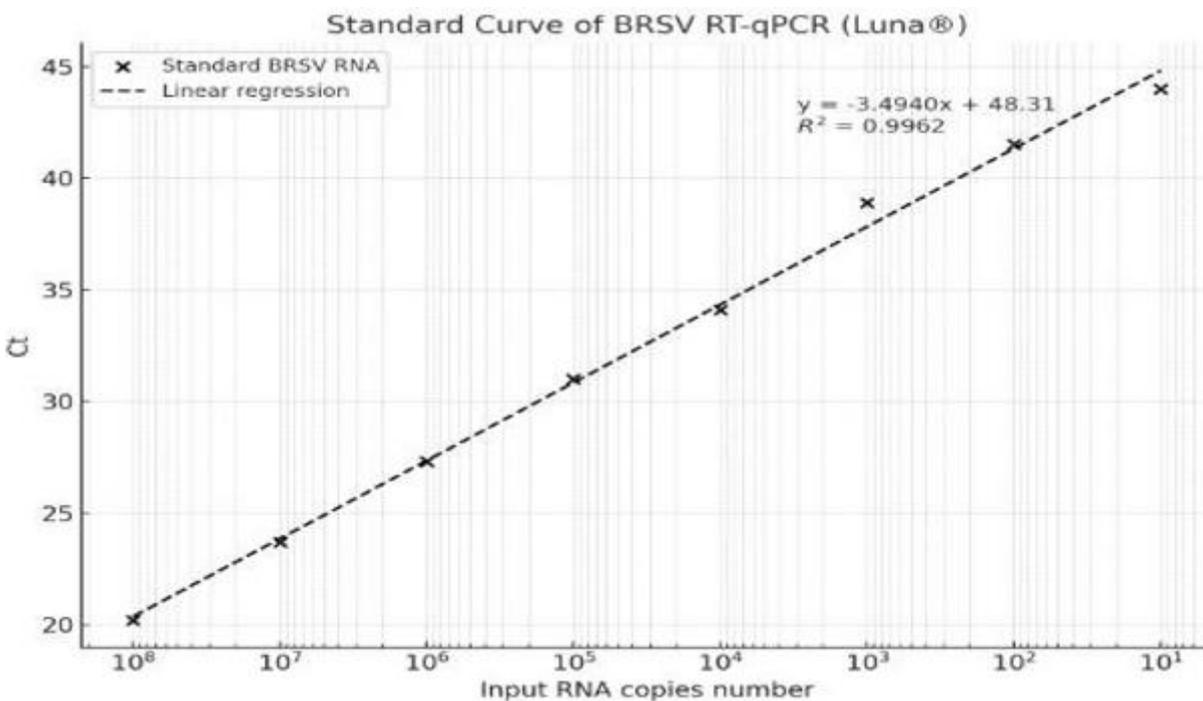


Figure 1. Standard curve of the Luna® RT-qPCR assay for BRSV detection using serial dilutions (10^8 – 10^1 RNA copies).

These results confirm that the assay is both sensitive and reliable, with consistent performance across a wide dynamic range suitable for quantitative applications.

Reproducibility

The reproducibility of the Luna® Universal Probe One-Step RT-qPCR assay was evaluated using tenfold serial dilutions of in vitro-transcribed BRSV RNA (10^8 – 10^3 copies). Standard curves were generated across three independent experimental runs performed on different days. The mean threshold cycle (Ct) values, standard deviations (S.D.), and coefficients of variation (CV) are summarized in table 2.

Table 2. Reproducibility of the Luna® RT-qPCR assay for BRSV detection.

RNA input (copies)	Threshold cycle (Ct, Mean ± S.D.)	CV (%)
10 ⁸	20.22 ± 0.15	0.76
10 ⁷	23.77 ± 0.59	2.47
10 ⁶	27.27 ± 0.46	1.70
10 ⁵	30.97 ± 0.62	2.01
10 ⁴	34.15 ± 0.34	0.99
10 ³	38.95 ± 0.07	0.19

Standard curve analyses demonstrated excellent reproducibility, with intra- and inter-assay variation consistently below 3%. The CV values ranged from 0.19% to 2.47%, confirming the high precision and repeatability of the assay across a dynamic range spanning six orders of magnitude.

Limit of Detection (LOD) and Sensitivity

The analytical sensitivity of the Luna® Universal Probe One-Step RT-qPCR assay was determined by testing serial dilutions of BRSV RNA transcripts down to 10¹ copies per reaction. Reliable amplification was consistently obtained for inputs of 10³ copies, with mean Ct values of 38.95 ± 0.07, CV = 19 % (Table 1). At lower dilutions (10²–10¹ copies), amplification was detected but not consistently across replicates, indicating reduced reliability near the lower detection threshold.

Based on these results, the limit of detection (LOD) of the assay was established at 10³ RNA copies per reaction, defined as the lowest input level at which amplification was reproducibly detected with acceptable variability (CV < 5%).

These findings demonstrate that the Luna® RT-qPCR assay provides a high level of analytical sensitivity, enabling detection of BRSV RNA at low copy numbers while maintaining precision and reproducibility.

Precision and Specificity

Assay precision was evaluated by including both positive and negative controls in all experimental runs. A positive template control (PTC) consisting of a laboratory-maintained strain of BRSV RNA transcript was consistently amplified at the expected Ct value, confirming assay functionality. In contrast, no-template controls (NTCs) included in each run produced no amplification signals, verifying the absence of reagent contamination or non-specific amplification.

Specificity was further supported by the use of BRSV-specific primers and probe sequences, which showed no cross-reactivity with non-target nucleic acids. The assay reliably distinguished true BRSV- positive samples from negative controls, confirming its high analytical specificity.

Together, these findings demonstrate that the Luna® RT-qPCR assay for BRSV detection is both precise and specific, ensuring accurate identification of target RNA without false positives.

Robustness

Stable amplification was maintained under ±10% volume variation and ±1 °C annealing shift, demonstrating assay robustness.

Table 3: Analytical performance of the Luna® Universal Probe One-Step RT-qPCR assay for BRSV detection.

Parameter	Result / Value	Interpretation
Dynamic range	10 ⁸ – 10 ¹ RNA copies/µL	Wide quantification range
Linearity (R ²)	0.9962	Excellent correlation
Slope	-3.64	Within optimal range
Amplification efficiency	93.0%	High assay efficiency
Limit of detection (LOD)	10 RNA copies/µL (≥95% replicates)	High sensitivity
Mean Ct at LOD	38.95 ± 0.07	Consistent amplification at low copies
Intra-assay variability	CV < 2%	High repeatability
Inter-assay variability	CV < 5%	High reproducibility
Specificity	No cross-reactivity (BVDV, PI3V)	Excellent diagnostic specificity
Robustness	Stable with ±10% volume, ±1 °C shift	Reliable under variable conditions

Discussion

The optimized Luna® Universal Probe One-Step RT-qPCR assay developed in this study demonstrates analytical performance that aligns well with international standards and published benchmarks in BRSV detection.

Linearity and Efficiency

The assay showed a strong linear dynamic range across eight orders of magnitude (10^8 – 10^1 RNA copies) with an R^2 of 0.9962 and an amplification efficiency of ~93%. According to the MIQE guidelines, an acceptable qPCR assay should present amplification efficiencies in the range of 90–110% and R^2 values >0.99 for good linearity (Bustin et al., 2009). These criteria are met by the current assay.

Limit of Detection (LOD) and Sensitivity

We established a limit of detection at 10^3 RNA copies per reaction, which means that at lower inputs (10^2 and 10^1 copies) amplification becomes inconsistent. This LOD is consistent with the detection limits reported in other BRSV RT-qPCR assays. For example, Boxus et al. (2005) reported a LOD of 10^3 RNA copies and a linear range from 10^3 to 10^8 copies for their BRSV assay.

Another more recent multiplex RT-qPCR assay for BRSV and BPIV-3 found LOD₉₅ (the concentration at which ≥95% of reactions are positive) at around 164 genome copies for BRSV when using 20 replicates per dilution (Zhang et al., 2025). Although that is more sensitive, differences in assay chemistry, probe design, and reaction conditions account for variation.

Reproducibility and Precision

Our assay's reproducibility is demonstrated by coefficients of variation (CV) under 3% across serial dilutions (10^8 to 10^3 copies). This level of precision is acceptable for diagnostic RT-qPCR assays and compares favorably with published results. For example, the aforementioned multiplex assay (Zhang et al., 2025) reported CVs <5%. Boxus et al. (2005) also described "excellent reproducibility" in their standard curve generation over their detection range.

Specificity and Precision Controls

Positive template controls (PTCs) consistently produced appropriate amplification, while no-template controls (NTCs) remained negative, indicating no contamination or leakage issues. Furthermore, the assay showed no cross-reactivity with non-target pathogens in our tests. These points are essential when validating any RT-qPCR assay, as false positives or background amplification can undermine diagnostic utility. Among RT-PCR assays compared in previous BRSV studies, specificity for the nucleoprotein (N) gene or other conserved regions has been critical to avoid cross-reaction with related viruses (Valarcher & Taylor, 2007).

Strengths, Limitations, and Practical Implications

Strengths

- The assay is robust across a wide dynamic range, with high linearity and efficiency that meet MIQE guidelines (Bustin et al., 2009).
- Reproducibility is strong, with low CVs indicating reliability across runs and days (Boxus et al., 2005; Zhang et al., 2025).
- Specificity and precision are verified by controls and absence of false positives (Valarcher & Taylor, 2007).

Limitations

- The validation was done largely using RNA standards rather than an extensive panel of clinical field samples; field matrix effects or inhibitors might reduce performance in real-world samples.
- The assay has not yet been benchmarked across different qPCR platforms, which could affect inter-lab comparability.
- The LOD, while respectable, is higher (i.e., less sensitive) than some newer, highly sensitive assays that report LODs in the range of tens of copies, often achieved via probe optimization, enhanced enzymes, or increased replicates (Zhang et al., 2025).

Practical Implications

Given the performance demonstrated, this Luna® RT-qPCR assay is suitable for diagnostic laboratories in Morocco for confirming BRSV infection in clinical samples, for monitoring viral loads, and for research purposes including vaccine efficacy studies. Its robustness under slight variation in reagent volumes or temperature (as tested) suggests it will perform reliably under typical laboratory conditions. For surveillance, especially where viral loads may be low (e.g., early infection or after immune response), the LOD of 10^3 copies should be considered when interpreting negative results.

Conclusion

This work established and validated the Luna® Universal Probe One-Step RT-qPCR assay as a reliable method for the detection of bovine respiratory syncytial virus (BRSV). The assay demonstrated a broad dynamic range (10^8 – 10^1 RNA copies), excellent linear correlation ($R^2 = 0.9962$), and high amplification efficiency (~93%), confirming its robustness for quantitative applications. Precision analysis showed minimal intra- and inter-assay variability (CV < 3%), while the limit of detection was defined at 10^3 RNA copies, ensuring consistent identification of low viral loads. No amplification was observed in negative controls, confirming the assay's specificity.

Overall, the Luna® RT-qPCR assay provides a sensitive, reproducible, and specific platform for BRSV detection. Its

validated performance under Moroccan laboratory conditions supports its integration into routine diagnostics, large-scale surveillance programs, and future epidemiological investigations of BRSV, thereby enhancing national capacity to monitor bovine respiratory disease and contributing to improved cattle health management strategies.

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