Journal of Microbiological Methods 250 (2026) 107697

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Validation of a standardized quantitative RT-qPCR workflow for detection and quantification of airborne respiratory viruses using gelatin membranes as sampling matrix

Irene Tellini Rusticano [*], Fabiola Berti, Carmela Calonico, Sara Boccalini [1], Angela Bechini [1]

Department of Health Sciences, University of Florence, Florence, Italy

ARTICLE INFO

Keywords:
SARS-CoV-2
Influenza A
Influenza B
RSV A/B
Analytical validation
ISO 20395
MIQE guidelines
Indoor air surveillance

ABSTRACT

Airborne microbiological monitoring is increasingly relevant for identifying and quantifying viral pathogens; however, standardized analytical workflows for respiratory viruses recovered from environmental matrices remain limited. This study reports the full analytical validation of a quantitative RT-qPCR workflow for the simultaneous detection and quantification of SARS-CoV-2, influenza A, influenza B, and RSV A/B from air samples collected on gelatin membrane filters. The protocol optimizes RNA extraction and amplification from this challenging matrix and provides a standardized procedure suitable for reproducible laboratory implementation. All validation parameters—amplification efficiency, linearity, recovery, matrix interference, limit of detection (LOD), limit of quantification (LOQ), repeatability, and reproducibility—were assessed in compliance with ISO 20395 and MIQE guidelines and met all predefined acceptance criteria. The workflow showed excellent linearity ($R^2 \geq 0.99$ for all targets), high amplification efficiency (92–103%), robust recovery (81.98–92.28%), and no significant matrix interference (±10%). LODs ranged from 5 to 9 copies/reaction, and LOQs from 8 to 10.5 copies/reaction. Precision assessments demonstrated acceptable intra- and inter-test variability (CV% ≤ 35%, SD < 0.19). Overall, this validated and standards-compliant RT-qPCR workflow provides a reliable, sensitive, and reproducible analytical tool for laboratory quantification of airborne respiratory viruses collected on gelatin membrane filters, supporting standardized virological analysis of aerosol samples.

1. Introduction

The detection and quantification of airborne pathogenic viruses, such as influenza viruses, coronaviruses, and respiratory syncytial virus, present significant methodological challenges that require robust and validated analytical approaches (Li et al., 2024; Bhardwaj et al., 2021). Airborne virus sampling and analysis are critical for environmental virology studies, yet the field suffers from a lack of harmonized protocols and standardized validation criteria (Bhardwaj et al., 2021; Puthussery et al., 2023). This absence of standardization severely limits inter-laboratory comparability and reproducibility, hindering the establishment of reliable quantitative methods for airborne viral detection (Robotto et al., 2021). A major technical obstacle lies in the sampling matrix itself. While gelatin membrane filters have demonstrated high efficiency and practicality for capturing airborne viruses (Nourmoradi et al., 2021), they represent a non-standard matrix not addressed by conventional nucleic acid extraction kits. The gelatin matrix poses unique challenges for viral genome recovery, requiring specific pre-treatment protocols and extraction optimization to ensure efficient lysis, nucleic acid release, and compatibility with downstream molecular assays. Without adequate validation of these critical pre-analytical steps, the reliability and reproducibility of quantitative results remain compromised. Furthermore, the lack of validated quantitative RT-qPCR workflows specifically designed for airborne viral matrices represents a significant gap in environmental virology methods (Yoshinaga et al., 2025). Most existing RT-qPCR protocols have been developed and validated for clinical specimens (e.g., nasopharyngeal swabs, sputum) but their direct application to air samples collected on gelatin filters has not been systematically validated. This methodological gap includes insufficient characterization of analytical performance parameters such as limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, and matrix effects specific to gelatin-based air

* Corresponding author at: Department of Health Sciences, University of Florence, Viale Morgagni 48, 50134 Florence, Italy.
E-mail address: irene.tellinirusticano@unifi.it (I.T. Rusticano).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.mimet.2026.107697
Received 23 December 2025; Received in revised form 2 August 2026; Accepted 5 September 2026
Available online 6 September 2026

sampling (Bustin et al., 2009). The absence of standardized procedures has resulted in fragmented and poorly comparable datasets across studies, limiting the advancement of airborne virology as a reliable analytical discipline (Bhardwaj et al., 2021; Robotto et al., 2021). There is an urgent need for method development and validation following internationally recognized guidelines, such as ISO standards for molecular diagnostic methods and the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines, to ensure transparency, reproducibility, and analytical robustness (Bustin et al., 2009). To our knowledge, this study provides one of the first comprehensive analytical validations of a multiplex quantitative RT-qPCR workflow for the simultaneous detection and quantification of airborne respiratory viruses collected on gelatin membrane filters, performed in accordance with ISO 20395 and MIQE guidelines. The present study addresses these methodological gaps by developing and validating a comprehensive analytical procedure for the simultaneous detection and quantification of multiple respiratory viruses in air samples. Specifically, we focused on SARS-CoV-2, influenza A and B viruses, and respiratory syncytial virus (RSV) A/B. Our approach integrated gelatin membrane filter sampling with optimized viral RNA extraction protocols and multiplexed quantitative RT-qPCR, following ISO standards and MIQE guidelines. The validation process included systematic evaluation of pre-analytical variables (gelatin membrane treatment and nucleic acid extraction efficiency), analytical performance parameters (sensitivity, specificity, linearity, precision, and accuracy), and matrix-specific interferences. This work contributes to the standardization of airborne virus detection methods, providing a validated workflow that enhances reproducibility and inter-laboratory comparability in environmental virology studies.

2. Materials and methods

Given the use of gelatin membranes as the air sampling medium, these filters were included in the method validation process to exclude any potential interference arising from their use. Therefore, the membranes were treated as an integral part of the analytical procedure, simulating real sampling conditions by spiking them with known concentrations of target viruses.

2.1. Methodological optimization of analytical protocol

The general analytical protocol for the collection and identification of pathogens in air samples has been optimized to overcome critical problems associated with the extraction and amplification of viral genetic material from gelatin membranes. These problems could occur since the membranes themselves can release residues that interfere with PCR techniques and, at the same time, retain viral particles, leading to an underestimation of their quantification. Protocol optimization has therefore the objective to improve analytical sensitivity and specificity, offering a reliable approach for this type of analysis. The developed protocol comprises three main phases:

i) Membrane Treatment: Gelatin membranes were spiked with known quantities of microbiological targets to simulate real samples. This step allowed testing the efficacy of subsequent extraction and quantification procedures while controlling experimental variables.

ii) Extraction: The nucleic acid extraction protocol was optimized to ensure efficient recovery of genetic material and minimize potential inhibitions.

iii) Quantification**:** A qualitative multiplex RT-qPCR kit, the TrueMark™ SARS-CoV-2, Flu A, Flu B, RSV A/B Select Panel (Cat. No. A59526, Thermo Fisher Scientific, Waltham, MA, USA), was modified and adapted for quantitative analysis by establishing specific standard curves to obtain reproducible and comparable quantitative data.

2.2. Reference materials

AcroMetrix™ multi-analyte SARS-CoV-2, Flu A/B, RSV A/B Control (Thermo Fisher Scientific), a multi-analyte control at 10,000 copies/mL, was used as a positive RT-qPCR control.

Field strains of SARS-CoV-2, Flu A and B, and RSV A/B, extracted from quantified biological samples, were used for setting up standard curves. These strains were provided by the Meyer Children's Hospital IRCCS (SARS-CoV-2, RSV A/B) and the Microbiology and Virology Laboratory of the Department of Experimental and Clinical Medicine (Flu A and B), and stored as stock solutions at −80 °C.

2.3. Preparation of spiked controls

Gelatin membranes were prepared and spiked under sterile conditions to simulate sample matrices for method validation. Target analytes at defined concentrations were first added according to the specific validation parameter under investigation (e.g., recovery, matrix effects, sensitivity, precision). Each gelatin membrane was aseptically transferred into a sterile 15-mL conical tube. Three millilitres of DNase/RNase-free water pre-warmed to 37 °C were then added to dissolve the membrane. Samples were vortexed for 10 s and incubated at 37 °C for 10 min. The vortexing and incubation steps were repeated once, after which the samples were vortexed again and immediately processed for RNA extraction.

2.4. RNA extraction protocol

Viral RNA was extracted using the PureLink™ RNA Mini Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Since this kit was not originally validated for gelatin membrane samples, the manufacturer's protocol was optimized in collaboration with Thermo Fisher Scientific technical support. Preliminary method-development experiments were performed using the manufacturer's original RNA extraction protocol to evaluate its suitability for gelatin membrane samples. These experiments revealed consistent overestimation of viral copy numbers following RT-qPCR analysis of positive controls added to gelatin membrane extracts, while matrix blanks remained negative for all viral targets. Based on these findings, the extraction protocol was optimized by modifying the lysis and washing steps prior to formal analytical validation. The results of these preliminary optimization experiments are reported in Supplementary Table S4. Briefly, the volume of PureLink™ Lysis Buffer was increased from 210 μL (recommended for sample volumes $\leq$500 μL) to 250 μL per sample to improve gelatin membrane solubilization and facilitate viral RNA release. In addition, one extra wash step using Wash Buffer II (WII, ethanol-containing) was included to reduce potential PCR inhibitors associated with the gelatin matrix and improve RNA purity. All other extraction steps, including incubation conditions, were performed according to the manufacturer's instructions. RNA purity was assessed spectrophotometrically using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA). RNA integrity was evaluated by agarose gel electrophoresis using the *E*-Gel™ Power Snap Electrophoresis System (Thermo Fisher Scientific, USA) prior to RT-qPCR analysis. The effectiveness of these modifications was evaluated by comparing RNA recovery rates with those obtained using the unmodified protocol on standardized samples.

2.5. Amplification protocol

RT-qPCR analysis was performed using the TrueMark™ SARS-CoV-2, FLU A, FLU B, RSV A/B Select Panel (Cat. No. A59526, Thermo Fisher Scientific) on a QuantStudio 5 thermocycler (Thermo Fisher Scientific). Each reaction mix had a total volume of 25 μL, consisting of 12 μL of Master Mix and 13 μL of extracted RNA. For each RT-qPCR run, a positive amplification control and a no-template control (NTC) were included to verify assay performance and monitor potential

contamination. In addition, a matrix blank consisting of a dissolved gelatin membrane without the addition of viral targets was processed through the entire RNA extraction workflow and subsequently included in each RT-qPCR run as a negative extraction control to verify the absence of contamination and assess any potential matrix-related interference.

Amplification was carried out according to the manufacturer's recommended thermal profile. Data analysis was performed using QuantStudio Design & Analysis software (v1.5.2), interpreting results based on Cycle threshold (Ct) values relative to standard curves.

2.6. Validation parameters

All validation experiments were performed using gelatin membranes, either in their original form or as extracts (matrix blank), spiked with known concentrations of the target analytes (SARS-CoV-2, Flu A and B, RSV A/B) to simulate real analysis conditions. This approach was chosen to determine the following validation parameters:

Recovery: Assessed using a certified multi-analyte control to determine extraction efficiency from the sampling matrix.

Matrix Interference: Evaluated to verify whether gelatin membranes affected the quantification of target analytes and/or inhibited PCR amplification.

Limit of Detection (LOD) and Limit of Quantification (LOQ): Determined to assess method sensitivity and precision at low concentrations.

Precision: Analyzed through repeated tests under different experimental conditions to determine method reproducibility.

Efficiency: Determined using standard curves from scalar dilutions of titrated viral strains to verify the method's ability to correctly quantify analytes.

Linearity: Verified to demonstrate proportionality between measured signal and target virus concentration across a broad range.

The operating methods and acceptability criteria for each validation parameter are described in detail in Table 1.

2.6.1. Determination of extraction phase recovery percentage

Extraction recovery was evaluated for each target pathogen to determine the efficiency of viral RNA extraction from gelatin membranes. A multi-analyte control with known target pathogen concentrations was spiked onto gelatin membranes and extracted. The experiment was conducted at three concentration levels (low, medium, high), with triplicates for each level. Viral copy numbers were determined via RT-qPCR by comparing obtained Ct values with previously generated standard curves, in compliance with ISO/IEC 17025:2017 principles for cost and reagent optimization (International Organization for Standardization (ISO), 2017). The percentage recovery was calculated by comparing the measured mean copy number to the expected copy number for each level, and a mean value of recovery was determined.

2.6.2. Evaluation of matrix interference on RT-qPCR

Matrix interference from the gelatin membrane on the RT-qPCR reaction was evaluated according to ISO 20395:2019 guidelines (International Organization for Standardization (ISO), 2019). A titrated multi-analyte control was added to extracted gelatin membrane blanks (matrix blank) and then analyzed by RT-qPCR. Triplicate analyses were performed under three experimental conditions: 1) matrix blank spiked with multi-analyte control (extracted gelatin membranes without viral target, spiked before PCR), 2) multi-analyte control only (without matrix), 3) matrix blank only (extracted gelatin membranes without control addition). Copy numbers were determined by comparing Ct values with validated standard curves, aligning with ISO 17025 optimization criteria (International Organization for Standardization (ISO), 2017). While ISO 20395:2019 states that absence of interference is verified when the sum of copies from conditions 2 and 3 equals that of condition 1, it does not specify an acceptability range (International Organization for Standardization (ISO), 2019). Therefore, a predefined acceptance threshold of ±10% relative to the theoretical sum of expected viral copies was prospectively adopted as a conservative, method-specific operational criterion to identify potentially relevant matrix effects, in accordance with the principles of the MIQE Guidelines and ISO/IEC 17025:2017. (Bustin et al., 2009; International Organization for Standardization (ISO), 2017).

2.6.3. Determination of limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) was defined as the lowest target concentration at which ≥95% of replicates yield a positive result. The limit of quantification (LOQ) was defined as the lowest concentration measurable with acceptable accuracy and precision. Precision was expressed as the coefficient of variation (CV%) calculated on absolute copy numbers, rather than on Ct values, to facilitate comparison with other measurement techniques.

LOD and LOQ were determined in a single experiment for each viral target, following scientific literature guidelines, particularly the MIQE Guidelines (Bustin et al., 2009). The number of replicates was chosen to balance statistical robustness with reagent optimization, as suggested by Newman and Maritz (Newman and Maritz, 2017). Unlike other experiments, field strains with known titers were used for LOD/LOQ determination to minimize concentration variations. For each target, five scalar dilutions were prepared and analyzed in five independent replicates. The quantification was performed by comparing standard curves,

Table 1
Summary of parameters and acceptability criteria for method validation.

Parameter	Number of Replicates	Acceptability Criteria	Normative References/Guidelines
Extraction Recovery	3 concentrations (low, medium, high), each in triplicate	% Recovery: comparison between expected and measured copies (no specific threshold, global mean calculated)	(International Organization for Standardization (ISO), 2017).
Matrix Interference	Triplicates for each of the 3 experimental conditions	Absence of interference: sum (cond. 2 + cond. 3) = cond. 1 ± 10%	(Bustin et al., 2009; International Organization for Standardization (ISO), 2019).
Limit of Detection (LOD)	5 scalar dilutions × 5 replicates	≥ 95% of positive replicates	(Bustin et al., 2009; Newman and Maritz, 2017; Forootan et al., 2017).
Limit of Quantification (LOQ)	5 scalar dilutions × 5 replicates	CV% (absolute copies) < 35%	(Bustin et al., 2009; Newman and Maritz, 2017; Forootan et al., 2017).
Precision – Repeatability (intra-assay)	Triplicates in the same session	CV% < 35% (absolute copies) and SD < 0.19 (log copies)	(Bustin et al., 2009; Newman and Maritz, 2017).
Precision – Reproducibility (inter-assay)	Triplicates on 3 different days	CV% < 35% (absolute copies) and SD < 0.19 (log copies)	(Bustin et al., 2009; Newman and Maritz, 2017).
Amplification Efficiency (E)	Scalar dilutions in triplicate	Slope: −3.1 to −3.6 (90–110%). $R^2 \geq 0.99$	(International Organization for Standardization (ISO), 2019).
Linearity	Scalar dilutions in triplicate	Slope: 0.95–1.05 $R^2 \geq 0.99$. Intercept ≈ 0	(International Organization for Standardization (ISO), 2019).

Note: Coefficient of Variation (CV), Standard Deviation (SD), coefficient of determination of the linear regression (R^2).

selected from the three replicates with the lowest variability (Forootan et al., 2017).

2.6.4. Determination of precision (repeatability and reproducibility)

Precision was assessed as both intra-assay (within-run repeatability) and inter-assay (between-run reproducibility), in accordance with the recommendations of Newman and Maritz (Newman and Maritz, 2017) and the MIQE guidelines (Bustin et al., 2009). Three control samples at different positivity levels (high, medium, low) were prepared by spiking multi-analyte control into extracted gelatin membrane blanks.

Repeatability (Intra-Assay): each sample was analyzed in triplicate within the same analytical session.

Reproducibility (Inter-Assay): each sample was analyzed in triplicate across three independent days.

2.6.5. Determination of amplification efficiency (E) and linearity

Amplification efficiency (E) was evaluated by generating standard curves for each virus using serial dilutions of titrated field strains spanning a broad concentration range, with each dilution analyzed in triplicate. Mean Ct values were calculated, and viral copy numbers were converted to a logarithmic scale (log_{10} copies) to establish the linear relationship between the initial nucleic acid quantity and the Ct value.

Amplification efficiency (E) was calculated from the slope (m) of the linear regression between Ct and log_{10} copies, in accordance with ISO 20395: 2019 (International Organization for Standardization (ISO), 2019). For each target, the standard error (SE) and confidence interval (CI) of the regression were also determined, as required by ISO 20395:2019. To this end, the following formulas were applied to calculate the standard error (SE) of the efficiency, and the 95% confidence interval (CI) (International Organization for Standardization (ISO), 2019).

$$SE_E = SE_b * \frac{((1+\bar{E})*\ln(10))}{(\bar{b}^2)} \quad CI = \bar{E} \pm t_{0.95,n-2} * SE_E$$

where $\bar{E}$, mean amplification efficiency; $\bar{b}$, mean slope of the regression; SE_b, standard error of the slope; $t_{0.95,n-2}$, critical value of the Student's t distribution at 95% confidence with n − 2 degrees of freedom; n, number of replicates.

Linearity was assessed by evaluating the relationship between expected (log_{10}expected copies) and measured (log_{10}measured copies) nucleic acid quantities from standard curves. Linear regression analysis was performed using log_{10} expected copies as the independent variable and log_{10} measured copies as the dependent variable. The coefficient of determination (R^2) was used to evaluate the degree of linear correlation.

2.6.6. Absolute quantification

The target-specific standard curves generated for amplification efficiency assessment were also used for absolute quantification. Ct values were converted into absolute copy numbers using the inverse equation of the corresponding linear regression:

$$\text{Copies} = 10^{\wedge}((\text{Ct} - \text{b})/\text{m})$$

where m is the slope and b is the intercept of the target-specific calibration curve. The regression parameters (slope, intercept, amplification efficiency and R^2) for each viral target are reported in Fig. 1. The regression equations were implemented in dedicated Microsoft Excel worksheets to automatically convert Ct values into the corresponding absolute copy numbers, ensuring a standardized calculation procedure.

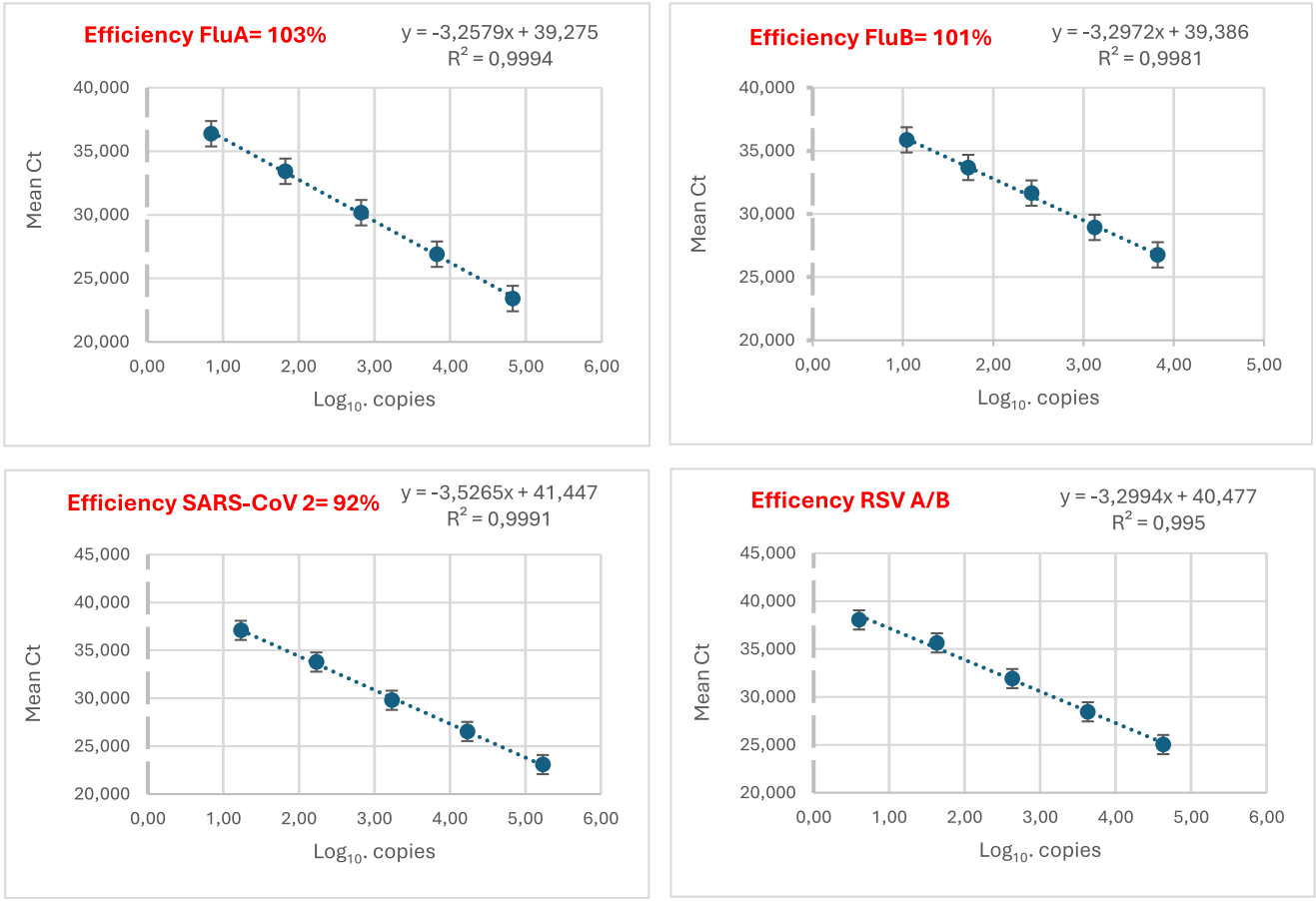

Fig. 1. Standard curves and amplification efficiencies for Flu A, Flu B, SARS-CoV-2 and RSV A/B.

3. Results

3.1. Recovery

Recovery was calculated as the mean percentage of quantified value versus expected input across three concentration levels. Overall, the analytical recovery demonstrated robust and consistent efficiency for all pathogens and concentration levels tested. For SARS-CoV-2, the overall recovery was 92.18%, with values ranging from 87.17% to 101.89%. For Flu A, the mean recovery was 82.25%; although a slightly lower value was observed at the highest concentration (73.67%), acceptable results were obtained at medium (87.72%) and low (85.35%) levels. Flu B showed the most stable performance, with an average recovery of 92.28%. Finally, RSV A/B showed an overall recovery of 81.98%, with values ranging from 72.51% to 86.95%. Details of the individual tests are available in Supplementary Table S1: Individual recovery results by virus and concentration level.

3.2. Matrix interference

For all analyzed targets, the number of copies detected in matrix blank spiked with the multi-analyte control was within ±10% of the theoretical sum of copies measured in the multi-analyte control alone and the matrix blank alone. This indicates that gelatin membrane matrix did not significantly interfere with PCR analysis for any of the pathogens, confirming the suitability and robustness of the extraction and amplification method for air sample analysis. Detailed results for each viral target and control condition are reported in Supplementary Table S2: Detailed results of matrix interference tests for each viral target and control condition.

3.3. Limit of detection (LOD) and limit of quantification (LOQ)

The sensitivity of the method was confirmed by LOD and LOQ values that fall within a narrow range across all viral targets, with all results meeting the predefined acceptability criteria (Table 1). Specifically, SARS-CoV-2 showed the lowest LOD value (5 copies/ reaction) and a LOQ of 10.5 copies/reaction, corresponding to a CV% of 25% at the LOQ level. Flu A exhibited a LOD of 8.5 copies/reaction and a LOQ of 8.5 copies/reaction, with a CV% of 24%, while Flu B showed comparable values (LOD 8 copies/reaction; LOQ 8 copies/reaction with a CV% of 29%. RSV A/B presented slightly higher thresholds (LOD and LOQ of 9 copies/reaction) and a CV% of 34%, still within the acceptance criterion. Detailed results of individual tests for each viral target are provided in Supplementary Table S3: Detailed results of LOD and LOQ determination for each viral target.

3.4. Precision (repeatability and reproducibility)

Intra-assay precision (Table 2) demonstrated high reproducibility across all viral targets and concentration levels. SD of $\log_{10}$ copies were consistently low, ranging from 0.025 to 0.077, well below the predefined acceptability criterion of 0.19. CV% calculated on Ct values were also within limits (< 35%), with values between 6% and 17% across the different viruses and concentrations. These results confirm excellent repeatability of the assay under identical experimental conditions.

Inter-assay precision (Table 3), assessed across multiple independent runs, likewise met the established acceptance criteria. SD values remained below 0.19 for all pathogens, ranging from 0.069 to 0.117 depending on the viral target and concentration level. As expected, CV% values were slightly higher than intra-assay results but consistently below the 35% threshold.

Specifically, CV% ranged from 16% to 26% for SARS-CoV-2 and influenza viruses, and up to 25% for RSV A/B at the lowest concentration level.

3.5. Amplification efficiency

The analysis of amplification efficiency for all targets (SARS-CoV-2, Flu A and B, and RSV A/B) demonstrated full compliance with the predefined criteria (Table 4). The corresponding standard curves are presented in Fig. 1, showing the strong linear correlation and consistent amplification efficiency across all viral targets.

All slopes obtained from the calibration curves were within the acceptable range (−3.6 to −3.1), confirming the expected relationship between copy number and Ct values. R^2 were consistently ≥0.99, indicating excellent linearity of the standard curves and a robust correlation between Ct values and analyte concentrations. Amplification efficiencies ranged from 92% to 103%, fully within the acceptance range of 90%–110%, thereby confirming optimal target amplification.

3.6. Linearity

Linearity parameters for all targets met the predefined acceptance criteria, confirming the method's proportional response across the tested concentration range (Table 5).

Particularly, the slopes of the regression lines were consistently equal to 1.000, demonstrating perfect proportionality between expected and measured quantities. R^2 were all ≥0.99, confirming the strong linear correlation throughout the range. Intercept values varied only between 0.000 and 0.004, remaining very close to zero and thus indicating the absence of significant systematic error in quantification.

Compliance with the MIQE guidelines is documented in the completed MIQE checklist provided as Supplementary Table S4.

4. Discussion

Airborne surveillance of respiratory viruses has advanced substantially, yet the field still lacks fully validated, end-to-end quantitative workflows that comply with recognized international standards and enable robust cross-study comparison. Several methodological reviews have highlighted that variability in sampling media, extraction procedures, and RT-qPCR reporting formats remains a major obstacle to

Table 2
Summary of intra-assay precision results for SARS-CoV-2 FLU A, FLU B and RSV A/B.

Pathogen	SD High Pos.	SD Pos.	SD Low Pos.	CV% High	CV% Pos.	CV% Low
SARS-CoV-2	0.032	0.050	0.033	7%	11%	8%
Flu A	0.077	0.039	0.059	17%	9%	14%
Flu B	0.025	0.035	0.055	6%	8%	13%
RSV A/B	0.033	0.056	0.034	8%	13%	8%

Note: Standard deviations (SD) and coefficients of variation (CV%) were calculated from intra-assay replicates at three concentration levels; CV (%) was calculated from absolute copy numbers; SD refers to log10 copies number.

Table 3
Summary of inter-assay precision results for SARS-CoV-2, FLU A, FLU B and RSV A/B.

Pathogen	SD High Pos.	SD Pos.	SD Low	CV% High	CV% Pos.	CV% Low
SARS-CoV-2	0.069	0.108	0.083	16%	23%	18%
Flu A	0.089	0.117	0.086	20%	26%	19%
Flu B	0.087	0.085	0.084	20%	19%	18%
RSV A/B	0.090	0.074	0.114	16%	17%	25%

Note: Inter-assay standard deviations (SD) and coefficients of variation (CV%) were derived from multiple independent runs across different days; CV (%) was calculated from absolute copy numbers; SD refers to log10 copies number.

Table 4
Summary of Amplification Efficiency Parameters: slope, R^2, efficiency and confidence intervals for SARS-CoV-2, influenza A and B viruses, and RSV A/B.

Parameter	SARS-CoV-2	FluA	Flu B	RSV A/B	Acceptability Criteria
Slope (m)	−3.526	−3.258	−3.297	−3.299	−3.6 to −3.1
R^2	0.999	0.999	0.998	0.995	≥0.99
Efficiency	92%	103%	101%	101%	90%–110%
SE Efficiency	1.07	1.08	1.80	2.91	/
95% CI	89–95	100–106	95–107	92–110	/

Note: Slope and R^2 values were calculated from the standard curve. Efficiency and confidence intervals derived from replicate analysis of serial dilutions.

Table 5
Linearity assessment of the assay for detection of SARS-CoV-2, FLU A, FLU B, and RSV A/B.

Pathogen	Slope (m)	R^2	Intercept	Acceptability Criteria
SARS-CoV-2	1.000	0.999	0.003	Slope: 0.95–1.05; R^2: ≥0.99. Intercept: 0
Influenza A virus	1.000	0.999	0.002	Slope: 0.95–1.05; R^2: ≥0.99. Intercept: 0
Influenza B virus	1.000	0.998	0.004	Slope: 0.95–1.05; R^2: ≥0.99. Intercept: 0
RSV A/B	1.000	0.995	0.000	Slope: 0.95–1.05; R^2: ≥0.99. Intercept: 0

Note: Values are derived from standard curve regression using serial dilutions of quantified RNA standards.

comparability and interpretation, with most investigations focusing on detection feasibility rather than formal analytical validation—particularly for filter-based methods, where matrix effects and recovery are seldom assessed against predefined acceptance criteria (Bhardwaj et al., 2021; Robotto et al., 2021; Borges et al., 2021; Dinoi et al., 2022; Silva et al., 2022).

This persistent lack of harmonization hinders meaningful comparison between studies and limits the development of shared frameworks for environmental and public health surveillance. Similar concerns have recently been raised regarding the absence of common validation schemes for viral bioaerosol detection and quantification (Justen et al., 2025). Recent methodological developments, such as the COPMAN-Air workflow proposed by Yoshinaga et al. (), have further improved the sensitivity of airborne viral RNA detection, highlighting the growing interest in quantitative air surveillance. However, comprehensive analytical validation according to internationally recognized validation frameworks remains limited (Yoshinaga et al., 2025).

In response to this gap, the present study provides, to our knowledge, one of the first comprehensive analytical validations of a multiplex quantitative RT-qPCR workflow for airborne respiratory viruses collected on gelatin membrane filters, performed in accordance with ISO 20395 and MIQE guidelines. Unlike previous studies primarily focused on method development or analytical performance evaluation, our workflow was systematically validated using predefined acceptance criteria for recovery, matrix interference, amplification efficiency, linearity, precision, LOD and LOQ. (Bustin et al., 2009; International Organization for Standardization (ISO), 2019).

All analytical parameters, including recovery, linearity, precision, and limits of detection and quantification (LOD/LOQ), met the predefined acceptance criteria, confirming the robustness, sensitivity, and reproducibility of the method (Bustin et al., 2009; Newman and Maritz, 2017; Forootan et al., 2017). Amplification efficiencies ranged from 92% to 103%, with slopes between −3.6 and − 3.1 and $R^2 \geq 0.99$, demonstrating a strong correlation between target concentration and Ct values (International Organization for Standardization (ISO), 2019). Linearity analyses yielded slopes of 1.000, intercepts between 0.000 and 0.004, and $R^2 \geq 0.99$, confirming the ability to quantify viral targets across a broad concentration range, a key requirement for environmental monitoring where viral loads may vary widely (International Organization for Standardization (ISO), 2019).

Recovery experiments demonstrated efficient viral RNA extraction and quantification without significant loss. Mean recovery values (82–92%) were consistent with previous studies reporting that recovery efficiency for airborne virus detection is strongly influenced by the sampling device, extraction protocol, and analytical workflow employed (Ladhani et al., 2017). Matrix interference was negligible, confirming the suitability of gelatin membranes as an analytical matrix (Bustin et al., 2009; International Organization for Standardization (ISO), 2019). These results highlight the workflow's capability not only for detection but also for reliable quantification, which is essential for exposure assessment and surveillance of viral circulation in real-world environments. Precision results met MIQE-aligned acceptance thresholds under both repeatability and reproducibility conditions (CV% < 35%; SD < 0.19 log copies) (Bustin et al., 2009; Newman and Maritz, 2017). The high intra- and inter-assay reproducibility, characterized by low variability, underscores the method's consistency and potential for routine implementation.

LOD and LOQ determinations further confirmed the method's ability to detect and quantify airborne viruses at low concentrations. Although all four viral targets met the predefined analytical acceptance criteria, RSV exhibited comparatively greater variability at low target concentrations than the other targets. Increased variability is expected for all RT-qPCR assays as measurements approach the analytical detection limit owing to stochastic effects associated with low-copy-number sampling. However, target-specific analytical characteristics may have contributed to the observed RSV performance. In particular, the commercial assay simultaneously detects both RSV-A and RSV-B, which are genetically distinct viruses. Consequently, sequence variability may have a greater influence on primer/probe binding and amplification efficiency than for assays targeting a single viral genome. Since a commercial multiplex RT-qPCR panel was used, the primer/probe sequences and their individual analytical performance could not be evaluated.

Comparable sensitivity ranges have been reported in recent integrated air sampling–qPCR studies (Yoshinaga et al., 2025), though these lacked formal validation or acceptance benchmarks. Although some recovery values were marginally lower, the overall dataset demonstrates a technically sound, quantitatively stable workflow that transforms air samples into reliable viral copy-number estimates suitable for routine environmental monitoring. When positioned against prior literature, our findings supply the missing analytical validation layer repeatedly called for by methodological reviews, effectively converting detection-oriented assays into standardized measurement systems (Robotto et al., 2021; Borges et al., 2021; Dinoi et al., 2022; Silva et al., 2022).

While previous field and device comparison studies often focused on relative sampler performance, few have included complete intra-laboratory validation with explicit acceptance criteria. For example, a recent HVAC-duct study in student dormitories compared air-sampling methods but emphasized detection feasibility rather than MIQE/ISO-based quantitative validation; our work complements such studies by introducing standards-based calibration, precision, and sensitivity benchmarks (Sousan et al., 2024). We acknowledge that the validation was conducted within a single laboratory using a single RNA extraction

kit and a single real-time PCR platform (QuantStudio 5). Therefore, inter-laboratory and cross-platform studies are required to confirm the reproducibility and broader applicability of the proposed workflow.

These findings support the potential of the validated workflow as a transferable and reproducible tool for real-time indoor virus surveillance in settings such as hospitals, schools, and workplaces, enabling early outbreak detection, evaluation of filtration and air purification systems, and evidence-based public health decision-making.

By adhering to international standards (ISO and MIQE), the method ensures comparability, reproducibility, and traceability across laboratories, fostering broader implementation and harmonized practices.

Ultimately, this work bridges analytical microbiology with environmental health practice, promoting multidisciplinary collaboration among researchers, regulators, and policymakers to strengthen biohazard management, infection prevention, and public health protection.

4.1. Future perspectives

This study serves as a proof of concept for a robust and quantitative multiplex RT-qPCR method validated in strict accordance with international standards. It represents an important step toward the harmonization of microbiological procedures for environmental monitoring - a field that still lacks universally accepted analytical protocols.

Further studies using authentic environmental samples collected under different real-world conditions are needed to evaluate the effects of environmental inhibitors and confirm the broader applicability of the workflow.

Gelatin membranes, used as sampling supports, showed notable advantages: high viral recovery, easy handling, and full solubility that enables direct nucleic acid extraction. Coupled with multiplex quantitative RT-qPCR, this approach supports simultaneous detection and quantification of multiple viral targets. Generating quantitative rather than qualitative data is crucial for exposure assessment, risk estimation, and trend analysis in indoor environments.

A major future step will be the development of a regulatory framework for airborne virus detection based on standardized validation criteria. Harmonized analytical protocols could form the foundation for evidence-based public health decision-making, especially in sensitive indoor settings such as hospitals, schools, and healthcare wards, where quantitative monitoring can inform infection prevention and improve indoor air quality management.

5. Conclusion

This study provides a comprehensive validation of a quantitative multiplex RT-qPCR method for the detection and quantification of respiratory viruses in air samples. The method fulfills internationally recognized ISO 20395 and MIQE acceptance criteria, confirming its reliability and reproducibility. Optimization of the membrane treatment, RNA extraction, and amplification steps resulted in a consistent and accurate workflow capable of generating quantitative data essential for epidemiological surveillance and biological risk assessment.

Unlike most previous approaches focused solely on qualitative detection, this work delivers a comprehensively validated ISO/MIQE-compliant quantitative workflow for SARS-CoV-2, influenza A/B, and RSV A/B from gelatin air samples, aligned with international standards. By offering reproducible and quantitative results, the method represents both a technical advancement and a practical contribution toward the establishment of harmonized protocols for airborne virus monitoring.

Future inter-laboratory validation will be crucial to support regulatory recognition and facilitate standardized application in indoor environments such as healthcare facilities, schools, and public venues. Adoption of the proposed protocol in environmental and occupational health surveillance could enhance the capacity to assess infection risks, evaluate filtration system efficiency, and inform preventive strategies.

By addressing key gaps in environmental virus monitoring, this study lays the foundation for harmonized airborne pathogen surveillance, supporting public health preparedness, cross-sectoral risk management, and research on the interactions between air quality and infectious disease transmission.

CRediT authorship contribution statement

Irene Tellini Rusticano: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Conceptualization. **Fabiola Berti:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology. **Carmela Calonico:** Writing – review & editing, Methodology, Investigation. **Sara Boccalini:** Writing – review & editing, Supervision, Project administration. **Angela Bechini:** Writing – review & editing, Supervision, Project administration.

Funding

This research did not receive specific funding. It was conducted as part of the institutional activities of the Department of Health sciences, University of Florence.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors gratefully acknowledge Dr. Alessandro Calamai, General Director of Erail and President of Erail Engineering Corp., for the support provided in the realization of the method validation project. The authors also thank the Meyer Children's Hospital IRCCS for providing reference strains of SARS-CoV-2 and RSV A/B, and the Microbiology and Virology Laboratory, Department of Experimental and Clinical Medicine, for providing reference strains of Influenza A and B viruses. The authors further acknowledge the Fondazione per la Ricerca of the University of Florence (UNIFI), with special thanks to Prof. Marco Pierini and Dr. Alessandro Monti for their valuable support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2026.107697.

Data availability

All raw analytical data and calculation sheets used for result processing are available in the Supplementary Material accompanying this article.

References

Bhardwaj, J., Hong, S., Jang, J., Han, C.H., Lee, J., Jang, J., 2021. Recent advancements in the measurement of pathogenic airborne viruses. J. Hazard. Mater. 420, 126574. https://doi.org/10.1016/j.jhazmat.2021.126574.

Borges, J.T., Nakada, L.Y.K., Maniero, M.G., Guimarães, J.R., 2021. SARS-CoV-2: a systematic review of indoor air sampling for virus detection. Environ. Sci. Pollut. Res. Int. 28 (30), 40460–40473. https://doi.org/10.1007/s11356-021-13001-w.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55 (4), 611–622. https://doi.org/10.1373/clinchem.2008.112797.

Dinoi, A., Feltracco, M., Chirizzi, D., Trabucco, S., Conte, M., Gregoris, E., Barbaro, E., La Bella, G., Ciccarese, G., Belosi, F., La Salandra, G., Gambaro, A., Contini, D., 2022. A review on measurements of SARS-CoV-2 genetic material in air in outdoor and

indoor environments: implication for airborne transmission. Sci. Total Environ. 809, 151137. https://doi.org/10.1016/j.scitotenv.2021.151137.
Forootan, A., Sjöback, R., Björkman, J., Sjögreen, B., Linz, L., Kubista, M., 2017. Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). Biomol. Detect. Quantific. 12, 1–6. https://doi.org/10.1016/j.bdq.2017.04.001.
International Organization for Standardization (ISO), 2017. ISO/IEC 17025:2017 – General Requirements for the Competence of Testing and Calibration Laboratories. ISO, Geneva.
International Organization for Standardization (ISO), 2019. ISO 20395:2019 – Biotechnology – Requirements for Evaluating the Performance of Quantification Methods for Nucleic Acid Target Sequences – qPCR and dPCR. ISO, Geneva.
Justen, L.J., Grimm, S.L., Esvelt, K.M., Bradshaw, W.J., 2025. Indoor air sampling for detection of viral nucleic acids. J. Aerosol Sci. 187, 106549. https://doi.org/10.1016/j.jaerosci.2025.106549.
Ladhani, L., Pardon, G., Meeuws, H., van Wesenbeeck, L., Schmidt, K., Stuyver, L., van der Wijngaart, W., 2017. Sampling and detection of airborne influenza virus towards point-of-care applications. PLoS One 12 (3), e0174314. https://doi.org/10.1371/journal.pone.0174314.
Li, B., Zhang, J., Wang, Y., et al., 2024. Multi-scenario surveillance of respiratory viruses in aerosols. Environ. Int. 165, 107367. https://doi.org/10.1016/j.envint.2024.107367.
Newman, H., Maritz, J., 2017. Basic overview of method validation in the clinical virology laboratory. Rev. Med. Virol. 30, e1940. https://doi.org/10.1002/rmv.1940.
Nourmoradi, H., Mohammadi-Moghadam, F., Hemati, S., Kakaei, H., 2021. Virus-sampling technologies in different environments. In: Environmental and Health Management of Novel Coronavirus Disease (COVID-19), pp. 41–63. https://doi.org/10.1016/B978-0-323-85780-2.00010-X.
Puthussery, J.V., Wattrell, C.G., Brown, M.J., et al., 2023. Real-time environmental surveillance of SARS-CoV-2 aerosols. Nat. Commun. 14, 39419. https://doi.org/10.1038/s41467-023-39419-z.
Robotto, A., Quaglino, P., Lembo, D., Morello, M., Brizio, E., Bardi, L., Civra, A., 2021. SARS-CoV-2 and indoor/outdoor air samples: a methodological approach to have consistent and results. Environ. Res. 195, 110847. https://doi.org/10.1016/j.envres.2021.110847.
Silva, P.G., Branco, P.T.B.S., Soares, R.R.G., Mesquita, J.R., Sousa, S.I.V., 2022. SARS-CoV-2 air sampling: a systematic review on the methodologies for detection and infectivity. Indoor Air 32 (8), e13083. https://doi.org/10.1111/ina.13083.
Sousan, S., Boatman, M., Johansen, L., Fan, M., Roper, R.L., 2024. Comparing and validating air sampling methods for SARS-CoV-2 detection in HVAC ducts of student dorms. Environ. Pollut. 343, 123164. https://doi.org/10.1016/j.envpol.2023.123164.
Yoshinaga, T., Ando, Y., Sato, Y., Kishida, T., Kitajima, M., 2025. Development of COPMAN-Air method for high-sensitivity detection of SARS-CoV-2 in air. Sci. Rep. 15, 14340. https://doi.org/10.1038/s41598-025-99365-2.