

## Original article

# Optimization and performance evaluation of PCR primers for human respiratory viruses detection

Kittima Phutthawong<sup>1</sup>, Ariya Khamwut<sup>2</sup>, Sasiprapa Anoma<sup>3</sup>, Siwaporn Boonyasuppayakorn<sup>3</sup>, Sunchai Payungporn<sup>2,\*</sup>

<sup>1</sup>Medical Biochemistry Program, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

<sup>2</sup>Center of Excellence in Systems Microbiology, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

<sup>3</sup>Center of Excellence in Applied Medical Virology, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

## Abstract

**Background:** Respiratory viral infections are a main cause of acute respiratory disease and contribute to morbidity worldwide. The wide diversity of viruses presents challenges for comprehensive detection.

**Objectives:** To develop, optimize, and evaluate polymerase chain reaction (PCR) primers targeting genomic regions of human respiratory viruses, including Adenovirus, Bocavirus, Parvovirus B19, Influenza virus, Respiratory syncytial virus, Metapneumovirus, Rhinovirus, Enterovirus A71, Coxsackievirus A16, Parainfluenza virus, Measles virus, Mumps virus, and Coronaviruses.

**Methods:** Genome reference sequences (2019–2025) were obtained from NCBI and BV-BRC databases. Nucleotide sequences were aligned using MEGA X to identify conserved regions for each virus and designed as primers with degenerate bases to support sequence variation based on standard parameters, which consist of melting temperature (55°C–68°C), GC content (35.0%–65.0%), amplicon length (380–700 bp), and predicted secondary structure using the OligoAnalyzer™ and Oligos. Primer specificity was assessed using sequence alignment and similarity searches using the NCBI BLAST to minimize off-target sequences. Then, gradient PCR was performed to optimize the annealing temperature, and the limit of detection was evaluated using a 10-fold serial dilution of the standard plasmid containing the specific gene of each virus.

**Results:** This study demonstrated that 16 primer pairs with suitable thermodynamic properties exhibited minimal secondary structures. Preliminary results showed that all primers successfully produced PCR products of the expected amplicon size, without cross-amplification with human cDNA samples. The limit of detection ranged from 10<sup>2</sup> to 10<sup>5</sup> copies/μL.

**Conclusion:** These findings provide a preliminary result of suitable candidate primers for human respiratory viral detection.

**Keywords:** assay development, PCR, primer design, respiratory virus

**\*Correspondence to:** Sunchai Payungporn, Center of Excellence in Applied Medical Virology, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.  
E-mail: sp.medbiochemcu@gmail.com

**Received:** 1 May 2026

**Revised:** 25 June 2026

**Accepted:** 2 July 2026

Acute respiratory infections (ARIs) are highly prevalent worldwide, causing significant morbidity, mortality, and economic impact on healthcare systems. The study reported more than 17.2 billion episodes globally, with an incidence rate of 162,485 cases per 100,000 population in 2021. <sup>(1)</sup> Furthermore, in Thailand, the incidence of non-COVID-19 acute respiratory infections has risen following the relaxation of COVID-19 control measures. In 2023, the virus was detected in 78.5% of patients with all-age acute respiratory infections, particularly children, who were at the highest risk of infection. Infection of the respiratory tract often spreads to become more severe lower respiratory tract infections, which is the cause of most of the mortality. <sup>(2-4)</sup> Recent epidemiological studies reported that respiratory infection-related hospitalizations with unknown etiology after the COVID-19 pandemic are associated with distinct seasonal and demographic patterns, leading to difficulty in diagnosis. <sup>(4)</sup> Patients with upper respiratory infections can often recover by themselves. However, viral-bacterial and viral-viral co-infections, such as RSV with influenza A, rhinovirus with adenovirus, and *Mycoplasma pneumoniae*, can constitute major factors in serious illnesses. <sup>(5,6)</sup>

Current upper respiratory infection diagnoses rely mostly on symptom-based clinical assessment and patient medical history. However, this approach may lead to an imprecise or unknown etiology. Laboratory diagnostic methods such as viral culture and serological assays are time-consuming, <sup>(7)</sup> while multiplex real-time PCR (qPCR) assays are typically limited to detecting a restricted number of pathogens. <sup>(8)</sup> Therefore, molecular approaches capable of detecting multiple pathogens simultaneously are important for improving diagnosis, surveillance, and molecular epidemiological investigations.

Previous studies have developed multiplex PCR panels and metagenomic sequencing approaches for detecting multiple respiratory viruses. <sup>(9,10)</sup> Most existing primer sets are designed to generate relatively short amplicons for qPCR, which provides high amplification efficiency and sensitivity. Amplicons for qPCR may offer limited taxonomic resolution for pathogen identification compared to sequencing-based detection. However, PCR primers suitable for sequencing-based detection across multiple respiratory virus groups remain relatively limited. Therefore, continued development of updated primer designs is still needed.

The PCR development typically starts from *in silico* primer design, followed by optimization, evaluation, and clinical validation. *In silico* analysis uses bioinformatics tools to assess optimized primer quality and predict potential problems such as secondary structures, primer dimer formation, and non-specific amplification. In addition, there is a need to design primer sets and update them to accommodate rapid viral genome mutation. Thus, the purpose of this study was to design, optimize, and evaluate PCR primers for the amplification of genomic regions from various human respiratory viruses, which potentially improves the accuracy of viral identification and is useful for future molecular epidemiology studies.

## Materials and methods

### *Viral genome sequence collection and primer design*

Whole-genome sequences of each virus were retrieved, particularly between 2019 and 2025, from reference databases including the National Center for Biotechnology Information (NCBI) and the Bacterial and Viral Bioinformatics Resource Center (BV-BRC). The lists of human respiratory viruses and candidate genes were summarized in **Table 1**. Complete FASTA sequences of each family were aligned to identify conserved regions within viral genes across different viral genomes for viral species classification. Multiple sequence alignments were conducted using MEGA X with the Clustal W algorithm. Consensus sequences generated for each viral family were used to design virus-specific primers using Primer-BLAST. Primer design parameters will include a length of 18–25 nucleotides, GC content of 35%–60%, and an amplicon size of 380–700 bp. All primers had a melting temperature ( $T_m$ ) of 55°C–66°C and demonstrated high specificity to the target virus, without predicted similarity with *Homo sapiens* (taxid: 9606) using Human RefSeqGene sequences database and other pathogen genomes based on BLAST searches against The NCBI database. Additionally, those primers were generated combined with IUPAC codes, with less than 5 positions mismatch, and avoid a 5-base at the 3' ends for degenerate primers to enable broad applicability across different strains within each viral family. Primer secondary structures, including hairpins ( $\Delta G > -3$  kcal/mol), self-dimers ( $\Delta G > -5$  kcal/mol), were evaluated using the OligoAnalyzer, and primer-dimers were assessed using the Oligos software (**Table 2**).

**Table 1.** Respiratory viruses in the PCR panel.

| Family                  | Virus                                                                                   | Abbreviation             | Target gene* |
|-------------------------|-----------------------------------------------------------------------------------------|--------------------------|--------------|
| <i>Adenoviridae</i>     | - Human adenovirus (1, B1, B3, B114, 7, 21a, 55, E4, D37, 41)- Human mastadenovirus A-F | HAdV<br>HAdV-A to HAdV-F | Hexon        |
| <i>Parvoviridae</i>     | - Human bocavirus 1, 2                                                                  | HBoV-1, HBoV-2           | NS1          |
|                         | - Human parvovirus B19                                                                  | B19V                     | NS1          |
| <i>Orthomyxoviridae</i> | - Influenza A (H1N1, H3N2, H5N1, H7N9, H9N2)                                            | IAV                      | M            |
|                         | - Influenza B (Victoria, Yamagata)                                                      | IBV                      | PB2          |
|                         | - Influenza C                                                                           | ICV                      | PB2          |
| <i>Pneumoviridae</i>    | - Respiratory syncytial virus A, B                                                      | RSV-A, RSV-B             | N            |
|                         | - Human Metapneumovirus                                                                 | hMPV                     | F to M2      |
| <i>Picornaviridae</i>   | - Human Rhinovirus A-C                                                                  | HRV-A to HRV-C           |              |
|                         | - Enterovirus A71                                                                       | EV-A71                   | 5' UTR       |
|                         | - Coxsackievirus A6, A10, A16                                                           | CV-A6, CV-A10, CV-A16    |              |
| <i>Paramyxoviridae</i>  | - Human parainfluenza viruses 1                                                         | hPIV-1                   | RdRp         |
|                         | - Human parainfluenza viruses 2                                                         | hPIV-2                   | HN           |
|                         | - Human parainfluenza viruses 3                                                         | hPIV-3                   | RdRp         |
|                         | - Human parainfluenza viruses 4                                                         | hPIV-4                   | M            |
|                         | - Measles                                                                               | -                        | P/V/C        |
|                         | - Mumps                                                                                 | -                        | F            |
| <i>Coronaviridae</i>    | - Severe acute respiratory syndrome coronavirus 2                                       | SARS-CoV-2               |              |
|                         | - Middle East respiratory syndrome coronavirus                                          | MERS-CoV                 | RdRp         |
|                         | - Human Coronavirus OC43, HKU1                                                          | HCoV-OC43, HKU1          |              |
|                         | - Human Coronavirus 229E, NL63                                                          | HCoV-229E, NL63          | RdRp         |

### PCR optimization for the detection of human respiratory viruses

Viral DNA fragments derived from each viral target gene were synthesized and inserted into a pUC57 vector (Beijing Tsingke Biotech, China). These templates were verified by Sanger sequencing and then used as a template ( $10^6$  copies/ $\mu$ L) for PCR amplification of each viral target gene. PCR assays were optimized using gradient PCR to adjust a suitable annealing temperature ranging from 55°C–64°C. The optimized condition consists of final reagent concentrations as follows: 1X standard *Taq* reaction buffer, 200  $\mu$ M dNTPs, 0.2  $\mu$ M of each primer, 0.025 U/ $\mu$ L of *Taq* DNA polymerase (NEB™, USA),  $10^6$  copies/ $\mu$ L of plasmid templates, and DEPC-adjusted volume to 12.5  $\mu$ L. PCR amplification was carried out in the Eppendorf Mastercycler Nexus GSX1 thermal cycler. PCR conditions included 1 cycle of initial denaturation at 95°C for 2 minutes, followed by 40 cycles of amplification (denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 68°C for 1 minute), followed by 1 cycle of final extension at 68°C for 5 minutes. PCR products were subsequently visualized using 2% gel electrophoresis with BIO-Print gel documentation (Vilber, France).

### Non-specific amplification against human genes

For non-specific amplification, the assay was evaluated against RNA extracted from saliva and nasal swabs of healthy individuals ( $n = 6$ ) at King Chulalongkorn Memorial Hospital. This protocol has been approved by the Institutional Review Board (IRB) (IRB No. 0059/69), Faculty of Medicine, Chulalongkorn University. RNA was extracted using Quick-RNA Viral Kit (Zymo Research, USA) according to the manufacturer's instructions. Extracted RNA was reverse transcribed into cDNA using random hexamers and Revert Aid Reverse Transcriptase (Thermo Scientific™, USA) following the manufacturer's protocol. The human cDNAs were used as a template for non-specific testing of the assay. The human GAPDH gene was amplified using Primer GAPDH\_F85; GTGAAGGTCGGAG TCAACGG and GAPDH\_R603; GTTGTTCATGG ATGACCTTGGC from a previous study.<sup>(11, 12)</sup> PCR amplification was performed under the optimized PCR conditions of each primer pair, whereas the GAPDH primer was amplified at an annealing temperature ( $T_a$ ) of 60°C. GAPDH amplification served as an internal control for cDNA quality assessment.

Table 2 . List of designed primer sets for respiratory virus detection.

| No. | Viral target               | Primer name     | Sequence (5' → 3')*      | Position (Start-End) | Accession no. | GC (%) | Mean T <sub>m</sub> (°C) | Hairpin ΔG (kcal/mol) | Product size (bp) |
|-----|----------------------------|-----------------|--------------------------|----------------------|---------------|--------|--------------------------|-----------------------|-------------------|
| 1   | IAV                        | IAV_M_F         | ATGAGYCTTCWACCGAGGT      | 1-20                 | PV261493      | 47.5   | 61.4                     | 0.05                  | 649               |
|     |                            | IAV_M_R         | TCATHGCMTHACCATYTGCT     | 628-649              |               | 48.5   | 65.8                     | 0.98                  |                   |
| 2   | IBV                        | IBV_PB2_F       | ACCAAGGGYGAYATGGCAACA    | 193-214              | PV349139      | 50.0   | 66.9                     | -1.38                 | 507               |
|     |                            | IBV_PB2_R       | GAACTCAGCTGATGTTGCTCC    | 679-699              |               | 52.4   | 63.0                     | -0.85                 |                   |
| 3   | ICV                        | ICV_PB2_F       | ATATCCARGGTGTCAGAGCAGT   | 1070-1091            | OQ733373      | 47.7   | 64.0                     | 0.92                  | 671               |
|     |                            | ICV_PB2_R       | ATTGTCCTGTTCTCCTAGCCT    | 1720-1740            |               | 52.4   | 65.0                     | 0.99                  |                   |
| 4   | HCoV-229E, NL63            | AlphaCoV_RdRp_F | CTTATATGATGCCTRTTATGGGT  | 697- 719             | MT797664      | 37.0   | 59.2                     | -0.45                 | 496               |
|     |                            | AlphaCoV_RdRp_R | GTACTCAAWGCWGCACAGAA     | 1172- 1192           |               | 45.2   | 61.8                     | 0.77                  |                   |
| 5   | SARS-CoV-2, MERS-CoV, HCoV | BetaCoV_RdRp_F  | GGTAATGCTGCTATYASHGATTAT | 14651- 14674         | OK114442      | 36.8   | 60.6                     | 0.77                  | 540               |
|     |                            | BetaCoV_RdRp_R  | ATCACAYTTAGGRTAATCCCAACC | 15161- 15184         |               | 41.7   | 62.4                     | 0.32                  |                   |
| 6   | HBoV                       | HBoV2_NS1_F     | GAACATCTKCTGAGGTAGGAG    | 1095- 1116           | PP625120      | 52.3   | 62.4                     | -1.57                 | 595               |
|     |                            | HBoV2_NS1_R     | TACACTTWCWGGTTTCHACCCA   | 1668-1689            |               | 47.0   | 64.3                     | -0.29                 |                   |
| 7   | B19V                       | HBoB19_NS_F     | CTTGGTGGTCTGGGATGAAGGT   | 1457- 1478           | MZ695225      | 54.5   | 65.8                     | 0.94                  | 520               |
|     |                            | HBoB19_NS_R     | GCGCGGGTTTCAGTGTTC       | 1957-1976            |               | 65.0   | 67.8                     | 0.27                  |                   |
| 8   | HAdV                       | HAdVs_Hexon_F   | ATHCCHTCKCGVAACTGGGC     | 20900-20919          | MW748653      | 59.2   | 66.1                     | 1.82                  | 638               |
|     |                            | HAdVs_Hexon_R   | AAGTTRCVGGARAABGGDATGCG  | 21515-21537          |               | 50.7   | 66.4                     | 2.61                  |                   |
| 9   | hMPV                       | hMPV_F_M_F      | CRTGYAAAGTCAGCACAGGAAG   | 4143- 4164           | MZ851793      | 50.0   | 63.5                     | 0.05                  | 675               |
|     |                            | hMPV_F_M_R      | ATCWGGCCAACTCCAGTAATTG   | 4796- 4817           |               | 45.5   | 62.6                     | -0.05                 |                   |
| 10  | hPIV-1, hPIV-3             | hPIV 1,3_RdRp_F | TGGGCWTCAGACCCCHTATTCAT  | 11553- 11574         | MT210644      | 47.0   | 64.2                     | -1.47                 | 698               |
|     |                            | hPIV 1,3_RdRp_R | TGWGCTCWCWATCTTTCATCAGT  | 12228- 12250         |               | 43.5   | 63.6                     | -0.35                 |                   |
| 11  | hPIV-4                     | hPIV 4_M_F      | ACAACTTRGCGGAAAGCTCACG   | 3622- 3642           | LC823197.1    | 52.4   | 64.4                     | 0.37                  | 528               |
|     |                            | hPIV 4_M_R      | TYTGGCTGCTGGGACAGAATG    | 4128- 4149           |               | 54.8   | 65.7                     | -3.26                 |                   |
| 12  | hPIV-2                     | hPIV 2_HN_F     | TCATACAAATGGGACGCCTAAAT  | 418- 439             | OP672250      | 40.9   | 62.0                     | -1.04                 | 592               |
|     |                            | hPIV 2_HN_R     | TTATGTTGGGATGKTTGGGAT    | 988-1009             |               | 38.6   | 61.5                     | 1.24                  |                   |
| 13  | RSV                        | RSV_N_F         | ARATGGCTCTTAGCAAAAGTCA   | 1137- 1157           | LC741420.1    | 40.5   | 61.0                     | -1.94                 | 537               |
|     |                            | RSV_N_R         | GACCTGATCTATCTCCTGCTG    | 1653- 1673           |               | 52.4   | 61.0                     | -0.3                  |                   |
| 14  | Mumps                      | Mumps_HN_F      | GCATTGTACCCGATTAGTAT     | 5075- 5094           | PQ157298.1    | 40.0   | 57.8                     | 0.86                  | 656               |
|     |                            | Mumps_HN_R      | TTCTCAGCGTAAGTGATGTTGC   | 5709- 5730           |               | 45.5   | 63.0                     | -0.63                 |                   |
| 15  | HRV, EV-A71, CVs           | Picomar_5UTR_F  | CAAGYACTYCTGTWCCCCG      | 111-130              | MN228693.1    | 57.5   | 63.1                     | 0.78                  | 396               |
|     |                            | Picomar_5UTR_R  | GAAACACGGACACCCAAAGTAG   | 485-506              |               | 50.0   | 63.1                     | -0.72                 |                   |
| 16  | Measles                    | measles_AVC_F   | TGGAGAGACGCGATGACGACG    | 2055- 2074           | OR290097      | 60.0   | 65.2                     | -1.86                 | 620               |
|     |                            | measles_AVC_R   | CAGATCCGGGTGCTCCACTCTG   | 2653- 2674           |               | 59.1   | 65.9                     | -0.8                  |                   |

\*IUPAC nucleotide codes; Y = C/T, R = A/G, W = A/T, S = G/C, M = A/C, K = G/T, V = A/G/C, H = A/C/T, D = A/G/T, B = C/G/T

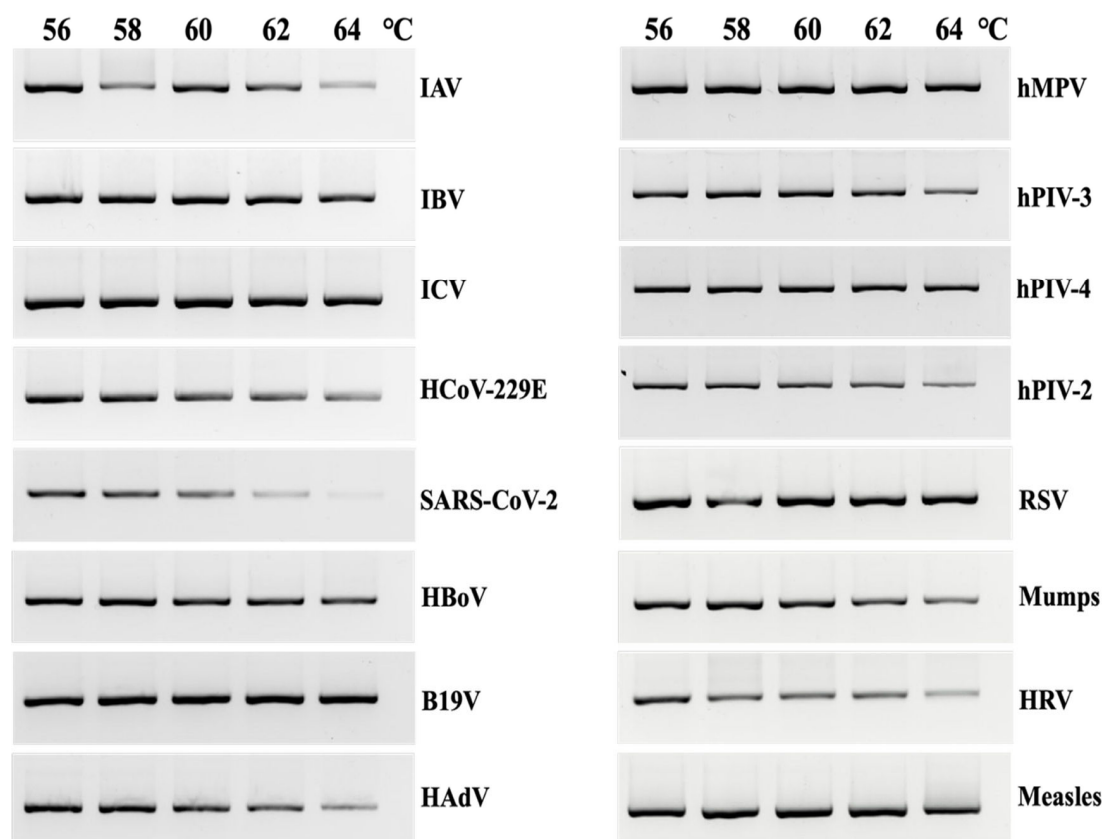


**Figure 1.** Gradient PCR optimization of the sixteen primers using annealing temperatures ranging from 56°C to 64°C.

### Limit of detection

The limit of detection was evaluated using a 10-fold serial dilution ( $10^6$  to 10 copies/ $\mu$ l) of the synthesized template containing the specific gene of each virus as a template, under optimized PCR conditions. PCR products were then visualized using 2% agarose gel electrophoresis. The lowest concentration of template that yields a positive band represents the limit of detection of the assay.

## Results

### Primer design and *in silico* evaluation

Primers were designed based on predefined criteria, including melting temperature (59.2°C–67.8°C), GC content (36.8%–65.0%), amplicon length (396–698 bp), and predicted secondary structures. Candidate primers were evaluated *in silico* for potential hairpin formation ( $\Delta G = -3.26$ – $-2.61$  kcal/mol) and thermodynamic stability. A total of 16 primer pairs were designed to provide broad coverage of the major human respiratory viruses (**Table 2**). In addition, all primer sequence similarity searches against nucleotide databases did not match the human genome (*Homo sapiens*).

### PCR optimization for the detection of human respiratory viruses

The gradient PCR optimization results for the 16 primer pairs are shown in **Figure 1**. Agarose gel electrophoresis demonstrated that an annealing temperature of 56°C produced clear amplification bands with low primer-dimer formation compared to other tested temperatures. In addition, this temperature provided the most consistent amplification across all primer panels. Therefore, 56°C was selected as the optimal annealing temperature for PCR amplification of all primer pairs. Each primer pair was subsequently evaluated using PCR amplification to confirm successful amplification of products. The PCR results showed clear bands corresponding to the predicted amplicon sizes (**Figure 2A**).

### Non-specific amplification against human genes

For the non-specific amplification evaluation, pooled human cDNAs from healthy specimens were tested against the 16 candidate primer pairs. The results showed that no nonspecific PCR product bands

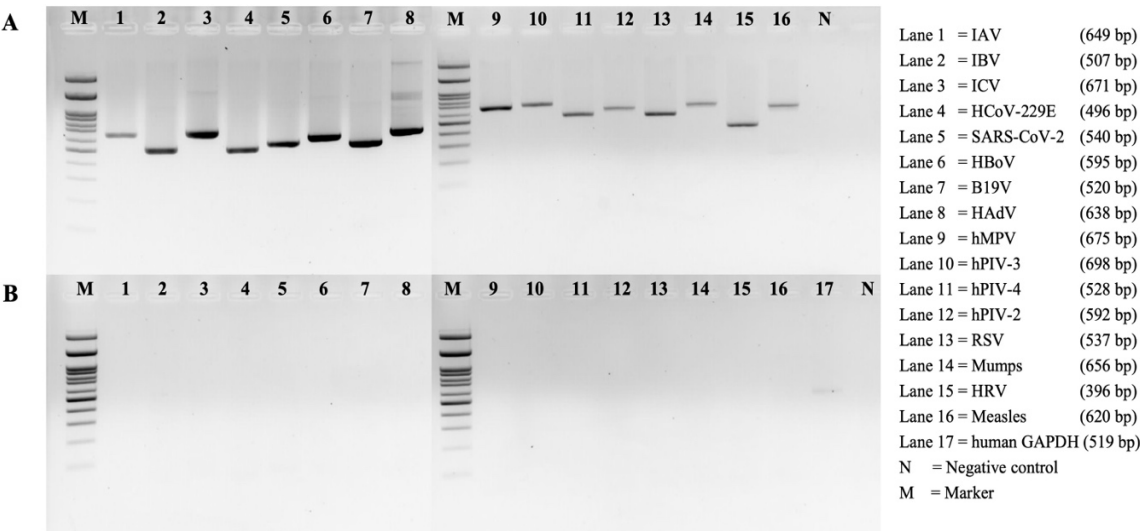


**Figure 2.** PCR specificity and non-specific amplification analysis. (A) Specific amplification of each primer pair using synthesized plasmid templates as positive controls. (B) Evaluation of non-specific amplification using healthy human cDNA, with GAPDH as an internal control.

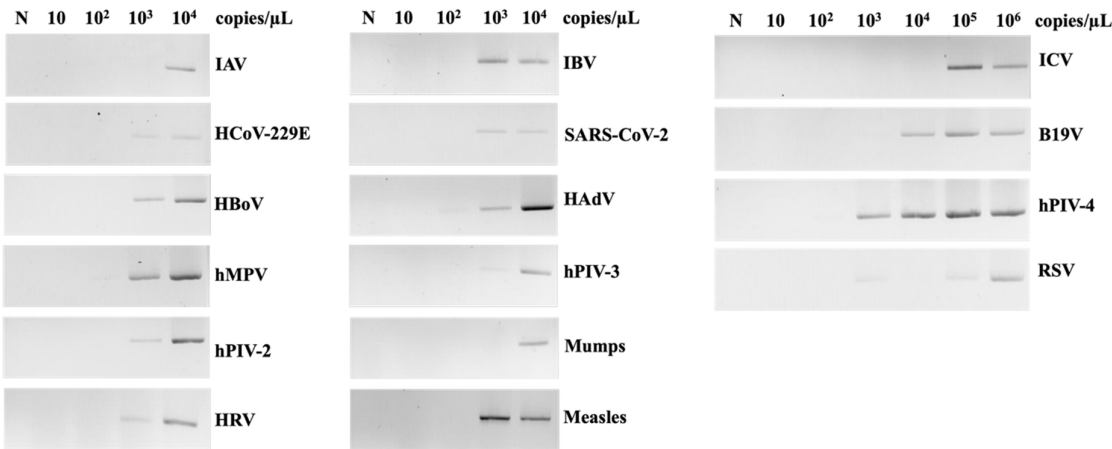


**Figure 3.** Limit of detection (LoD) analysis of each primer pair using synthesized templates at different template concentrations.

were observed for each primer pair. GAPDH gene amplification was included as an internal control to confirm cDNA quality. The GAPDH primer successfully generated the expected amplicon (Figure 2B), confirming the absence of non-specific amplification in tested human cDNA at the selected 56°C annealing temperature.

**Limit of detection**

The analytical sensitivity of the designed primer sets ranged from 10<sup>2</sup> to 10<sup>5</sup> copies/μL, depending on the target virus. The lowest detection limit (10<sup>2</sup> copies/μL) was observed for the Human adenovirus (HAdV) and Human metapneumovirus (hMPV) primer sets. Most primer sets, including those targeting Influenza

B virus (IBV), Alphacoronaviruses (HCoV-229E), Betacoronaviruses (SAR-CoV-2), Human bocavirus (HBoV), Human parainfluenza viruses 3 (hPIV-3), Human parainfluenza virus 2 (hPIV-2), Human parainfluenza virus 4 (hPIV-4), Respiratory syncytial virus (RSV), Human rhinovirus (HRV), and Measles virus, demonstrated detection limits of 10<sup>3</sup> copies/μL. Detection limits of 10<sup>4</sup> copies/μL were observed for Influenza A virus (IAV), Human parvovirus B19 (B19V), and Mumps virus primer sets, while the Influenza C virus (ICV) primer set showed the lowest analytical sensitivity, with a detection limit of 10<sup>5</sup> copies/μL. Detailed sensitivity results for each primer pair are shown in Figure 3.

## Discussion

This study demonstrated that conserved genomic regions across multiple respiratory viruses can be identified and used to design primer sets based on standard primer design criteria. A total of 16 primer pairs were generated to cover seven viral families commonly associated with human respiratory infections. However, designing universal primers to cover high genetic variability requires some compromise to target variable regions, so this study's criteria differed from standard parameters. (e.g., GC content < 40%,  $\Delta G > -2$  kcal/mol). Furthermore, the incorporation of degenerated bases to avoid mismatch between primer and viral sequence variation leads to unstable melting temperatures ( $T_m$ ).<sup>(13)</sup> Additionally, annealing temperature ( $T_a$ ) is normally 5°C less than  $T_m$ . Therefore, the primer with higher  $T_m$ , such as an adenovirus primer pair, exhibits a mean calculated  $T_m$  above 66°C, but the optimal annealing temperature is 56°C because of the presence of multiple degenerated positions. These findings suggested that primer sets were capable of targeting a wide range of respiratory viruses with no preliminary cross-reactivity in the tested human cDNA. Therefore, primers were suitable for agarose gel electrophoresis-based detection and might be applied for downstream sequencing applications.

For most viral groups, including Influenza A virus, Influenza B virus, Influenza C virus, Human bocavirus, Human parvovirus B19, Human metapneumovirus, Human parainfluenza virus 4, Human parainfluenza virus 2, Respiratory syncytial virus, Measles virus, and Mumps virus, PCR products can be differentiated by agarose gel electrophoresis. However, some viruses, such as Human coronavirus 229E, Human coronavirus NL63, Severe acute respiratory syndrome coronavirus 2, Middle East respiratory syndrome coronavirus, Human coronavirus OC43, Human coronavirus HKU1, Human adenovirus, Human parainfluenza virus 1, Human parainfluenza virus 3, Human rhinovirus, Enterovirus A71, and Coxsackievirus, could not be clearly distinguished due to similar product sizes. Additional sequencing analysis is required for accurate viral identification. From a previous study, next-generation sequencing analysis was performed to detect four human seasonal coronaviruses (Human coronavirus OC43, Human coronavirus HKU1, Human coronavirus 229E, and Human coronavirus NL63).<sup>(14)</sup> The sequencing data can be used for viral identification, phylogenetic analysis, mutation tracking,

and molecular epidemiological surveillance. Moreover, Influenza A and Influenza B virus primers were designed from relatively conserved genomic regions, including the matrix (M) and polymerase basic protein 2 (PB2) genes, to support broad viral detection. They may not provide sufficient resolution for strain-level identification. Additional analysis of subtype-associated genes, such as the hemagglutinin (HA) and neuraminidase (NA) genes, might be required for more accurate influenza strain characterization.<sup>(15)</sup>

Overall, these primers could support the broad screening of multiple respiratory viruses from a minimal sample volume. Future coupling of this initial targeted amplification with rapid sequencing technologies could significantly reduce turnaround times compared to traditional viral culture or serological assays.<sup>(16)</sup> This integrated approach would provide comprehensive species identification, ultimately supporting timely clinical decision-making and patient management.

However, this study has some limitations. First, primer design was performed *in silico*, and experimental evaluation was conducted using synthesized plasmid templates. Thus, this study represents a preliminary analytical evaluation. The predicted performance might not fully reflect clinical sample conditions. Further evaluation using clinical specimens is required to confirm the performance and diagnostic applicability. Second, the analytical specificity was tested against a limited number of human cDNA samples, which only provides an initial *in vitro* baseline. Lastly, the limit of detection for Influenza C virus was relatively high ( $10^5$  copies/ $\mu$ L), reflecting a sensitivity trade-off in broad-range viral screening. Since Influenza C has low epidemiological significance,<sup>(17)</sup> future development plans might remove Influenza C from the panel to optimize resources. Despite these limitations, this study provides an important framework for further clinical evaluation and validation of PCR-based respiratory virus detection using clinical specimens.

## Conclusion

This study designed candidate PCR primers targeting major human respiratory viruses. The designed primer sets provide a preliminary result for further optimization and evaluation of PCR-based assays for respiratory virus detection in laboratory and clinical applications.

## Acknowledgement

This project is funded by The National Research Council of Thailand (NRCT) and Chulalongkorn University (N42A680278), Ratchadapisek Somphot Fund, Faculty of Medicine, Chulalongkorn University, and the H.M. the King Bhumibhol Adulyadej's 72<sup>nd</sup> Birthday Anniversary Scholarship. We also thank for supporting from the Center of Excellence in Systems Microbiology members. We thank internship students from Burapha University and Catholic University of Lyon (UCLy) for their participation in primer design.

## Conflict of interest statement

All authors declare that this research was conducted in the absence of any commercial, financial, or personal relationships that could have appeared to influence the work reported in this paper.

## Data sharing statement

All data generated or analyzed during the present study are included in this published article. Further details are available for noncommercial purposes from the corresponding author on reasonable request.

## References

1. GBD 2021 Upper Respiratory Infections Otitis Media Collaborators. Global, regional, and national burden of upper respiratory infections and otitis media, 1990–2021: a systematic analysis from the Global Burden of Disease Study 2021. *Lancet Infect Dis* 2025; 25:36–51.
2. World Health Organization. Pneumonia in children [Internet]. 2022 [cited 2025 Nov 11]. Available from: <https://www.who.int/news-room/fact-sheets/detail/pneumonia>
3. Inna P, Suntronwong N, Sinsulpsiri S, Srimanewiroon S, Poovorawan Y. Viral etiology associated with acute respiratory tract infection patients in Bangkok, Thailand. *Cureus* 2024; 16:e66897.
4. Scaglione G, Canuti M, Offer M, Breschi V, Piralla A, Baldanti F, et al. Burden and outcomes of severe lower respiratory tract infections with unknown etiology: a retrospective observational study on epidemiological trends over an 8-year period (2016–2024). *Infect Dis Ther* 2025; 14:1075–87.
5. Babawale PI, Guerrero-Plata A. Respiratory viral coinfections: insights into epidemiology, immune response, pathology, and clinical outcomes. *Pathogens* 2024; 13:316.
6. Zhang Y, Zhao J, Zou X, Fan Y, Xiong Z, Li B, et al. Severity of influenza virus and respiratory syncytial virus coinfections in hospitalized adult patients. *J Clin Virol* 2020; 133:104685.
7. Zhang S, Zhang W, Tang YW. Molecular diagnosis of viral respiratory infections. *Curr Infect Dis Rep* 2011; 13:149–58.
8. Seder I, Téllez RC, Zhang J, Zheng T, McCleary S, El Mouatassim S, et al. Fast and sensitive multiplexed diagnostic system enabled by real-time solid-phase PCR assay. *NPJ Biosens* 2026; 3:17.
9. Chen J, Xu F. Application of nanopore sequencing in the diagnosis and treatment of pulmonary infections. *Mol Diagn Ther* 2023; 27:685–701.
10. Guo Y, Li Z, Li L, Li S, Sun L, Yang X, et al. A dual-process of targeted and unbiased nanopore sequencing enables accurate and rapid diagnosis of lower respiratory infections. *EBioMedicine* 2023; 98:104858.
11. Chanchaem P, Poovorawan Y, Payungporn S. Complete genome characterization and phylogenetic analysis of WU polyomavirus in Thai pediatric patients with respiratory tract infections in 2013. *J Med Assoc Thai* 2015; 98 Suppl 1:S29–35.
12. Thongpan I, Suntronwong N, Vichaiwattana P, Wanlapakorn N, Vongpunsawad S, Poovorawan Y. Respiratory syncytial virus, human metapneumovirus, and influenza virus infection in Bangkok, 2016–2017. *PeerJ* 2019; 7:e6748.
13. Rose TM, Schultz ER, Henikoff JG, Pietrokovski S, McCallum CM, Henikoff S. Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. *Nucleic Acids Res* 1998; 26:1628–35.
14. Musaeva T, Fadeev A, Pisareva M, Eder V, Ksenafontov A, Korzhanova M, et al. Development of primer panels for whole-genome amplification and sequencing of human seasonal coronaviruses: hCoV-OC43, hCoV-HKU1, hCoV-229E, and hCoV-NL63. *Viruses* 2024; 17:13.
15. Liu J, Zhang X, Li S, Ding X. Genome-wide identification of characteristic nucleotide fragments for surveillance and subtype typing of influenza A viruses. *DIT* 2025; 3:100056.
16. Girma A. Biology of human respiratory syncytial virus: current perspectives in immune response and mechanisms against the virus. *Virus Res* 2024; 350: 199483.
17. Shimizu K, Kawakami C, Matsuzaki Y, Fujisaki S, Nagata S, Morita H, et al. Monitoring Influenza C and D viruses in patients with respiratory diseases in Japan, January 2018 to March 2023. *Influenza Other Respir Viruses* 2024; 18:e13345.