



# Performance Evaluation of Allplex Respiratory Panels 1, 2, and 3 for Detection of Respiratory Viruses and Influenza A Virus Subtypes

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**ABSTRACT** The Allplex respiratory panels 1, 2, and 3 (Allplex) comprise a one-step real-time reverse transcription-PCR assay for the detection of respiratory viruses (RVs) and influenza A subtypes based on multiple detection temperature (MuDT) technology. The performance of the Allplex assay was compared with those of the AdvanSure RV real-time PCR kit (AdvanSure) and the PowerChek pandemic H1N1/H3N2/H5N1 real-time PCR kit (PowerChek) using 417 clinical respiratory specimens. In comparison with the AdvanSure assay for RV detection by each virus, the ranges of positive percent agreement, negative percent agreement, and kappa values with the Allplex assay were 82.8 to 100%, 95.5 to 100%, and 0.85 to 1.00, respectively. For influenza A virus (INF A) subtyping, the kappa values between the Allplex and PowerChek assays were 0.67 and 1.00 for the INF A H1N1-pdm09 and H3 subtypes, respectively. Uniplex PCR and sequencing for samples with discrepant results demonstrated that the majority of results were concordant with those from the Allplex assay. When testing 24 samples, the turnaround and hands-on time required to perform the Allplex assay were 4 h 15 min and 15 min, respectively. In conclusion, the Allplex assay produced results comparable to those from the AdvanSure and PowerChek assays.

**KEYWORDS** respiratory virus, multiplex real-time PCR, validation, Republic of Korea

**A**cute respiratory viral infections are common causes of substantial morbidity and mortality in pediatric and adult patients. An early detection of respiratory virus (RV) infections enables clinicians to initiate immediate therapeutic interventions that can reduce complications, antibiotic use, and unnecessary laboratory testing (1–4). Multiplex molecular assays can simultaneously detect multiple RVs, and clinical laboratories have adopted the use of various commercial assays of this type (1, 5–7).

Recently, the Allplex respiratory panels 1, 2, and 3 (Allplex; Seegene, Republic of Korea), a one-step, real-time reverse transcription-PCR (rRT-PCR) assay based on multiple detection temperature (MuDT) technology, was developed and received Conformité Européenne-*in vitro* diagnostic (CE-IVD) approval. The MuDT is a novel analytical technique that detects multiple targets in a single fluorescence channel without melting curve analysis (8). The assay is composed of three reaction tubes and targets 16 viruses and 3 influenza (INF) A virus subtypes: panel 1 includes the INF A virus (subtypes H1, H1N1-pdm09, and H3), INF B virus, and respiratory syncytial virus (RSV) types A and B; panel 2 includes adenovirus (ADV), metapneumovirus (MPV), human enterovirus (HEV), and parainfluenza virus (PIV) types 1, 2, 3, and 4; and panel 3 includes human bocavirus (BoV), coronaviruses (CoVs) OC43, 229E, and NL63, and human

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**TABLE 1** Comparisons of the Allplex respiratory panels 1, 2, and 3 and AdvanSure RV real-time RT-PCR assays for detecting respiratory viruses

Virus <sup>a</sup>	Agreement						Kappa value	
	Positive			Negative			Observed	95% CI
	%	No./total	95% CI <sup>b</sup>	%	No./total	95% CI		
ADV	82.8	24/29	63.5–93.5	99.2	383/386	97.6–99.8	0.85	0.74–0.95
BoV	100	3/3	31.0–100	99.8	411/412	98.4–99.9	0.86	0.58–1.00
CoV 229E	100	12/12	69.9–100	95.5	401/403	98.0–99.9	0.92	0.81–1.00
CoV OC43	92.3	12/13	62.1–99.6	99.7	401/402	98.4–99.9	0.92	0.81–1.00
CoV NL63	100	9/9	62.9–100	100	406/406	98.8–100	1.00	1.00
HRV	95.7	88/92	88.6–98.6	97.2	314/323	94.6–98.6	0.91	0.86–0.96
INF A	98.0	48/49	87.8–99.9	100	366/366	98.7–100	0.99	0.97–1.00
INF B	90.0	9/10	54.1–99.5	100	405/405	98.8–100	0.95	0.84–1.00
MPV	100	14/14	73.2–100	100	401/401	98.8–100	1.00	1.00
PIV 1	100	9/9	62.9–100	99.3	403/406	97.7–99.8	0.85	0.69–1.00
PIV 2	100	11/11	67.9–100	100	404/404	98.8–100	1.00	1.00
PIV 3	100	9/9	62.9–100	100	406/406	98.8–100	1.00	1.00
RSV A	100	14/14	73.2–100	99.8	400/401	98.4–99.9	0.96	0.89–1.00
RSV B	100	71/71	93.6–100	99.4	342/344	97.7–99.9	0.98	0.96–1.00

<sup>a</sup>ADV, adenovirus; BoV, bocavirus; CoV, coronavirus; HRV, human rhinovirus; INF, influenza virus; MPV, metapneumovirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus.

<sup>b</sup>CI, confidence interval.

rhinovirus (HRV). The Allplex assay combined with the automated workstation Microlab Nimbus IVD (Hamilton, Reno, NV, USA) comprises a fully automated system from specimen handling to result, including nucleic acid extraction, real-time PCR setup, and data analysis with the Seegene viewer (Seegene).

The aims of this study were to evaluate the analytical performance of the Allplex assay and to compare the performance with those of the AdvanSure RV real-time PCR kit (AdvanSure; LG Life Sciences, Republic of Korea) and the PowerChek pandemic H1N1/H3N2/H5N1 real-time PCR kit (PowerChek; Kogene Biotech, Republic of Korea) for RV detection and INF A virus subtyping, respectively.

## RESULTS

**Distribution of respiratory viruses.** Two of the 417 specimens included in this study generated invalid results in the Allplex assay and were omitted from analysis due to insufficient amounts of residual specimens for repeat testing. The results of the remaining 415 specimens were analyzed in this study. After discrepancy resolution, at least one RV was detected in 302 specimens (247 with a single virus, 48 with two viruses, 5 with three viruses, and 2 with four viruses) by either of the two assay types (the Allplex assay or the AdvanSure assay).

**Comparison of the Allplex and AdvanSure assays.** A total of 296 (71.3%) and 300 (72.3%) samples were Allplex- and AdvanSure-positive, respectively. As shown in Table 1, the ranges for positive and negative agreement between the Allplex and AdvanSure assays were 82.8 to 100% and 95.5 to 100%, respectively. The kappa values for the two methods ranged from 0.85 to 1.00.

Discordant results were observed in 32 specimens, and two samples had two discrepant viruses; thus, 34 discrepancies were detected. Among the 32 specimens, 13 (40.6%) were polyviral specimens. A total of 22 results were Allplex positive and AdvanSure negative. Among the 22 results, 90.9% (20/22) were confirmed positive by uniplex PCR and sequencing (Table 2). Of the 12 Allplex-negative and AdvanSure-positive results, 50.0% (6/12) were confirmed positive by uniplex PCR and sequencing. Overall, the uniplex PCR and sequencing for the samples with discordant results demonstrated that a majority (76.5% [26/34]) of the results were concordant with those from the Allplex assay.

The AdvanSure assay gave false-positive HRV results for two samples, of which one sample showed cross-reactivity between HRV and HEV. The Allplex assay correctly detected HEV in the sample.

**TABLE 2** Resolution of discordant results between the Allplex respiratory panels 1, 2, and 3 and the AdvanSure RV real-time RT-PCR assays

Virus <sup>a</sup>	Discordant results		No. of positive results in monoplex PCR and sequencing
	Allplex respiratory panels 1, 2, and 3/AdvanSure RV real-time RT-PCR	No. of samples	
ADV	+/-	3	3
ADV	-/+	5	2
BoV	+/-	1	1
CoV 229E	+/-	2	2
CoV OC43	+/-	1	0
CoV OC43	-/+	1	1
INF A	-/+	1	0
INF B	-/+	1	1
PIV 1	+/-	3	3
HRV	+/-	9	9
HRV	-/+	4	2
RSV A	+/-	1	1
RSV B	+/-	2	1

<sup>a</sup>ADV, adenovirus; BoV, bocavirus; CoV, coronavirus; HRV, human rhinovirus; INF, influenza virus; PIV, parainfluenza virus; and RSV, respiratory syncytial virus.

**Comparison of the Allplex assay with the PowerChek assay for INF A virus subtyping.** A total of 48 specimens with INF A virus were included in the analysis. As shown in Table 3, the kappa values for the two methods were 0.67 and 1.00 for INF A H1N1-pdm09 and the H3 subtype, respectively. Discordant results were observed in four specimens, of which three were subtyped H1N1-pdm09 by only the Allplex assay. The remaining specimen was subtyped H1N1-pdm09 by only the PowerChek assay. Uniplex PCR and sequencing confirmed that all discordant specimens were INF A H1N1-pdm09.

**Analytical performance and workload analysis for the Allplex assay.** The detection limits for the Allplex assay for RVs were 50 copies/reaction, except for PIV type 1 (10 copies/reaction), PIV type 4, and MPV (10<sup>3</sup> copies/reaction). In the analytical specificity test, all 21 different microorganisms gave negative results, and nonspecific positive reactions were not observed (Table 4). For the 24 samples, the turnaround time (TAT) and hands-on time (HOT) required for performing the Allplex assay were 4 h 15 min and 15 min, respectively.

**DISCUSSION**

In the last decade, advances in molecular diagnostic testing have led to changes in clinical microbiology laboratories (9–11). There is a wide array of molecular technologies emerging for the detection of RVs from clinical specimens. Some of these technologies have potential for high-throughput batch testing and others will allow for rapid near-patient testing (6, 7, 12, 13). Random-access testing, such as with the BioFire Film Array respiratory panel (bioMérieux, Marcy-l’Etoile, France), may be suitable for laboratories handling small numbers of specimens due to a limited capacity, but also has a rapid TAT and a simple work process (7, 13–15). On the other hand, high-throughput batch testing may be useful for laboratories performing large numbers of assays. The Allplex assay is best suited for batch testing, which is capable of handling

**TABLE 3** Comparison of the Allplex respiratory panels 1, 2, and 3 and the PowerChek pandemic H1N1/H3N2/H5N1 real-time PCR assays for influenza A virus subtyping

Influenza A virus subtype	Agreement						Kappa value	
	Positive			Negative			Observed	95% CI
	%	No./total	95% CI <sup>a</sup>	%	No./total	95% CI		
H1N1-pdm09	97.5	39/40	85.3–99.9	62.5	5/8	25.9–89.8	0.67	0.37–0.97
H3	100	4/4	39.6–100	100	44/44	90.0–100	1.00	1.00

<sup>a</sup>CI, confidence interval.

**TABLE 4** Analytic specificity for the Allplex respiratory panels 1, 2, and 3

Microbial species or type	Strain	Result
<i>Bordetella pertussis</i>	ATCC BAA-589D	Negative
<i>Chlamydophila pneumoniae</i>	Clinical isolate	Negative
<i>Enterobacter aerogenes</i>	ATCC 15038D	Negative
<i>Enterobacter cloacae</i>	ATCC 13047	Negative
<i>Fusobacterium nucleatum</i>	ATCC 25586D-5	Negative
<i>Haemophilus influenzae</i>	ATCC 51907D	Negative
<i>Klebsiella oxytoca</i>	ATCC 700324D	Negative
<i>Klebsiella pneumoniae</i>	ATCC 700721D-5	Negative
<i>Legionella pneumophila</i>	ATCC 41782	Negative
<i>Mycoplasma pneumoniae</i>	ATCC 29342	Negative
<i>Proteus mirabilis</i>	ATCC 12453D	Negative
<i>Pseudomonas aeruginosa</i>	ATCC 47085D	Negative
<i>Pseudomonas fluorescens</i>	ATCC 49642	Negative
<i>Staphylococcus aureus</i>	ATCC 700699D-5	Negative
<i>Serratia marcescens</i>	ATCC 27137D-5	Negative
<i>Streptococcus agalactiae</i>	ATCC BAA-611D	Negative
<i>Streptococcus mitis</i>	ATCC 3556	Negative
<i>Streptococcus pneumoniae</i>	ATCC BAA-255D	Negative
<i>Candida albicans</i>	ATCC 14053	Negative
Human herpesvirus 1	ATCC VR-260	Negative
Human herpesvirus 2	ATCC VR-734	Negative

a maximum of 96 rRT-PCRs per batch. The assay has definitive strengths in terms of workload (HOT and ease of handling) compared with other recently available batch-testing techniques (7, 16, 17). The application of the Microlab Nimbus IVD, an automated liquid handler, reduces the HOT to 15 min to test 24 samples. Furthermore, the Allplex assay minimizes the possibility of contamination, since it is a one-step rRT-PCR process that does not require the manipulation of postamplification material (18).

In this study, the Allplex assay demonstrated results equivalent to those of the AdvanSure and PowerChek assays. The majority of the discrepant results were concordant with the results from the Allplex assay after discrepancy resolution. All discordant results that were confirmed positive by uniplex PCR and sequencing were associated with high cycle threshold ( $C_T$ ) values, suggesting low viral genome loads (data not shown).

One sample showed cross-reactivity between HRV and HEV in the AdvanSure assay, while the Allplex assay correctly detected HEV in the sample. Results from previous studies demonstrated that the similarities between the HRV and HEV sequences enabled cross-amplification in molecular assays (19, 20). Thus, a positive result for any HRV or HEV would indicate the detection of HRV/HEV in many commercially available RV panels (7, 17, 19). However, differentiating between HRV and HEV infections is important in outbreak areas and for epidemiology (19, 21). One strength of the Allplex assay is that it enables specific identification of HRVs and HEVs. Although we did not evaluate the diagnostic accuracy for HEV detection in this study, there were six specimens that were HEV positive. All of these specimens were confirmed by uniplex PCR and sequencing. Except for the result from one sample with a low viral load, all results were confirmed positive (data not shown).

The limitations of this study include the low numbers of specific virus-positive specimens and INF A virus subtypes. This prevented a robust evaluation of diagnostic performance. Further studies are needed to investigate the diagnostic accuracy for detecting HEV and PIV type 4. Another limitation is that we could not assess the diagnostic accuracy, including sensitivity and specificity, because a consistent reference standard was not applied.

In conclusion, the Allplex assay produced results comparable to those of the AdvanSure and PowerChek assays. The Allplex assay showed a higher concordance with the results of uniplex PCR and sequencing compared with those of the comparative methods. Thus, the Allplex assay is a potentially useful tool for detecting RVs in clinical laboratories where high-throughput batch testing is required.

## MATERIALS AND METHODS

**Clinical specimens.** The institutional review board of the Samsung Medical Center approved this study. For this study, 417 clinical respiratory specimens were obtained via a total of 260 nasopharyngeal swabs, 148 nasopharyngeal aspirates, and 9 fluid samples from bronchoalveolar lavage that were collected from patients with symptoms of respiratory tract infections between December 2015 and July 2016. Patients' ages ranged from 7 days to 91 years. A total of 260 specimens (62.4%) were obtained from pediatric patients. These samples were analyzed with the AdvanSure assay. All specimens were stored at  $-70^{\circ}\text{C}$  until testing with the Allplex assay with or without the PowerChek assay.

**Allplex respiratory panels 1, 2, and 3.** Nucleic acids were extracted from 450- $\mu\text{l}$  stored specimens with 10  $\mu\text{l}$  bacteriophage MS2 added as an exogenous internal control using the StarMag 96 virus kit (Seegene). The final elution volume was 100  $\mu\text{l}$  for each sample. The extracted nucleic acid (8  $\mu\text{l}$ ) was added to a tube containing 17  $\mu\text{l}$  of one-step RT-PCR premix. The Microlab Nimbus IVD system automatically performed the nucleic acid processing and PCR setup. One-step rRT-PCR was conducted using the CFX96 system (Bio-Rad, Hercules, CA, USA). The mixture was incubated at  $50^{\circ}\text{C}$  for 20 min for the reverse transcription step, which was followed by denaturation at  $95^{\circ}\text{C}$  for 15 min and 45 cycles of PCR (10 s at  $95^{\circ}\text{C}$ , 1 min at  $60^{\circ}\text{C}$ , 10 s at  $72^{\circ}\text{C}$ ). Fluorescence was detected at two temperatures ( $60^{\circ}\text{C}$  and  $72^{\circ}\text{C}$ ), and a positive test result was defined as a well-defined exponential fluorescence curve that crossed the  $C_{\text{T}}$  at a value of  $<42$  for individual targets.

**AdvanSure RV real-time PCR.** The AdvanSure assay is a Ministry of Food and Drug Safety (MFDS) of Korea-approved one-step rRT-PCR assay for detecting RV pathogens. The assay detects 14 different RVs, namely CoV 229E, CoV NL63, CoV OC43, PIV types 1 to 3, INF types A and B, RSV types A and B, HRV, MPV, ADV, and BoV. Procedures were performed according to the manufacturer's instructions as previously described (16). Briefly, nucleic acids from 200  $\mu\text{l}$  of clinical samples were extracted into an elution volume of 100  $\mu\text{l}$  by the TanBead Smart LabAssist-32 extraction system (Taiwan Advanced Nanotech, Inc., Taiwan). The rRT-PCR was conducted in a final volume of 20  $\mu\text{l}$  using the Slan real-time PCR detection system (LG Life Science).

**PowerChek pandemic H1N1/H3N2/H5N1 real-time PCR kit.** The PowerChek assay is MFDS approved and is a CE-IVD-certified one-step rRT-PCR assay for IFN A virus subtyping. The assay detects INF A subtypes H1N1-pdm09, H3N2, and H5N1. Nucleic acids were extracted from 140- $\mu\text{l}$  stored specimens and eluted in 50  $\mu\text{l}$  using the QIAamp DSP viral RNA minikit (catalog no. 61904; Qiagen, Hilden, Germany) according to the manufacturer's instructions. rRT-PCR was performed in a total volume of 20  $\mu\text{l}$  (15  $\mu\text{l}$  PCR mixture and 5  $\mu\text{l}$  template RNA) using the 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). Procedures were performed according to the manufacturer's instructions.

**Comparisons of the Allplex assay with the AdvanSure and PowerChek assays.** Specimens with discordant results between the Allplex assay and the comparative methods (the AdvanSure or PowerChek assays) were confirmed by uniplex PCR and sequencing in a blinded manner. Primers identical to those of the Allplex and comparative methods were used for uniplex PCR and sequencing. Since the AdvanSure assay does not detect HEV and PIV type 4, we excluded those results from the comparisons with the two assays.

**Analytical sensitivity and specificity of the Allplex assay.** Serially diluted plasmids containing the target gene were used to determine the analytical sensitivity. The pUC19 vector was used for plasmid DNA preparation. Serial dilutions of the prepared plasmid DNA were made from  $10^5$  to 1 copies per reaction to determine the analytical sensitivity of the assay. MPV, BoV, and CoV NL63 samples were isolated from patients, and their sequences were confirmed by direct sequencing. All other standard strains were obtained from American Type Culture Collection (ATCC). Totals of 21 or 24 replicates of each dilution step were performed. The lower detection limit was defined as the lowest concentration that was detected in  $\geq 95\%$  of the replicates.

Cross-reactivity of the Allplex assay was assessed using 21 different pathogens (Table 4). The DNA was extracted from supplied samples and assayed with the Allplex assay using the same procedures that were used for sample processing.

**Workflow analysis.** Allplex TAT and HOT were evaluated for 24 samples. TAT was defined as the time interval between when the laboratory received the sample and when the final results were produced. HOT was defined as the time the technician spent on sample preparation.

**Statistical analysis.** Statistical analysis was performed using the VassarStats website (<http://vassarstats.net/>). Interrater agreement statistics (kappa calculation) were used to compare the detections of RVs between the Allplex assay and the comparative methods.

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