



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



Contents lists available at ScienceDirect

# Diagnostic Microbiology and Infectious Disease

journal homepage: [www.elsevier.com/locate/diagmicrobio](http://www.elsevier.com/locate/diagmicrobio)

## Verification of the Abbott Alinity m Resp-4-Plex assay for detection of SARS-CoV-2, influenza A/B, and respiratory syncytial virus

Annie Cheng<sup>a</sup>, Stefan Riedel<sup>a,b</sup>, Ramy Arnaout<sup>a,b,c</sup>, James E. Kirby<sup>a,b,\*</sup>

<sup>a</sup> Department of Pathology, Beth Israel Deaconess Medical Center, Boston, MA, USA

<sup>b</sup> Harvard Medical School, Boston, MA, USA

<sup>c</sup> Division of Clinical Informatics, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA, USA

### ARTICLE INFO

#### Article history:

Received 8 April 2021

Revised in revised form 20 September 2021

Accepted 16 October 2021

Available online 22 October 2021

#### Keywords:

SARS-CoV-2

Influenza

Respiratory syncytial virus

Multiplex

Nucleic acid amplification technology

Molecular diagnostics

### ABSTRACT

COVID-19 symptomatology may overlap with other circulating respiratory viruses that may also cause severe disease and for which there are specific and potentially life-saving treatments. The Abbott Alinity m Resp-4-Plex assay is a multiplex PCR assay that simultaneously detects and differentiates infection with SARS-CoV-2, influenza A, influenza B, and respiratory syncytial virus (RSV). We characterized its accuracy, precision, and analytical sensitivity. All were found to be robust for measures examined. In the context of sample-to-answer, near random access automation on the Alinity m platform, we believe that the Resp-4-Plex assay offers significant utility in addressing the current needs of the SARS-CoV-2 pandemic and future needs during anticipated endemic circulation of SARS-CoV-2 with other respiratory viruses.

© 2021 The Author(s). Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

### 1. Introduction

SARS-CoV-2, influenza A/B, and RSV may cause respiratory infection with significant morbidity and mortality. Respiratory disease signs and symptoms for these viruses overlap, and, therefore, it is not possible to reliably differentiate between them on clinical grounds alone, especially early during the course of disease. SARS-CoV-2 and influenza, especially, have significant implications in terms of transmission inside and outside of hospital settings and therefore require reliable methods for diagnosis. RSV, although primarily thought of as a serious pathogen in young children, can also cause bronchiolitis and pneumonia in adults. Depending on the stage of illness, there are therapeutics with varying efficacy for SARS-CoV-2, influenza, and RSV. Therefore, sensitive detection and differentiation of these viruses are valuable clinical determinations.

With social distancing and masking during the COVID pandemic, the circulation of influenza and other respiratory viruses almost ceased in many locations (Olsen et al., 2006). Presumably, however, with less than 100% vaccine efficacy for influenza, waning immunity to circulating respiratory viruses over a large population cohort, and reopening of society, circulation of influenza and RSV may rebound and even exceed normal levels for a period of time (Baker et al., 2021). At the same time, SARS-CoV-2 will likely become endemic,

potentially adopting a seasonal cycle with enhanced transmission during the winter, as observed in the United States during the winters of 2020 and 2021 (Byun et al., 2021). It will therefore be critical to be able to test both patients and symptomatic staff for high consequence respiratory pathogens to avert potential nosocomial transmission and to identify the most advantageous therapeutic options for patients with serious illness.

A multiplex testing option for high-consequence testing would address these specific diagnostic needs. The Abbott Alinity m Resp-4-Plex assay in March 2021 received emergency use authorization designation for detection of SARS-CoV-2, influenza A, influenza B and RSV. Approved sample types are either a nasopharyngeal swab collected by a healthcare provider or a nasal swab specimen self-collected in a healthcare setting submitted in viral transport medium or saline. The multiplex, reverse-transcription real-time PCR assay targets the RdRp and N genes of SARS-CoV2; the matrix gene of influenza A; the nonstructural 1 gene of influenza B; and the matrix gene of RSV. An internal control is spiked into each sample in the form of armored RNA encoding a segment of the hydroxypyruvate reductase gene from the pumpkin plant, *Cucurbita pepo*; it controls for appropriate extraction and amplification in each reaction. Each amplicon is detected by a real-time probe with a distinct fluorophore with the exception that probes for both SARS-CoV-2 targets are detected with the same fluorophore. The primers and probes for the SARS-CoV-2 targets are the same as those used in the singleplex Abbott RealTime SARS-CoV-2 and Alinity m SARS-CoV-2 assays, whose performance

\* Corresponding author.

E-mail address: [jekirby@bidmc.harvard.edu](mailto:jekirby@bidmc.harvard.edu) (J.E. Kirby).

characteristics have been examined in prior literature (Arnaout et al., 2021; Hirschhorn et al., 2021; Smith et al., 2020). Cycle threshold numbers (Ct) determined on the Alinity m instrument in the Resp-4-Plex assay are determined based on a fluorescence cutoff. They are used along with the inflection point of the amplification curve at the maximum amplification efficiency (the max ratio) (Shain and Clemens, 2008) for qualitative assessment of target positivity and negativity (personal communication, Joshua Kostera, Abbott Molecular).

Here, we describe characterization of accuracy, precision, and limit of detection of the Alinity m Resp-4-Plex assay determined as part of normal quality assurance activities prior to adoption for clinical use in our clinical laboratory.

## 2. Materials and methods

### 2.1. Patients and samples

Samples tested were nasopharyngeal flocked swab samples transported in universal transport medium (for all samples other than those initially tested for SARS-CoV-2) or saline (for SARS-CoV-2 comparisons). Specimens were collected from an almost exclusively adult population with an average age of 54 years (S.D. +/- 21 years). There was no preselection of patients based on demographics or sex; however, on later analysis it was found that there was a skew to higher female samples represented (64% female, 36% male). All but one specimen selected based on comparator positivity for influenza A and B were obtained from the 2019-2020 winter influenza season. SARS-CoV-2 positive specimens were from January 2021 or earlier. Negative specimens were from 2020 and 2021. Specimens positive for respiratory viruses other than those detected in the Resp-4-Plex assay were obtained during the years 2015-2021. Specimens were stored for less than 72 hours at 4°C prior to initial testing and then frozen at -80°C. Samples went through a total of 2 freeze-thaw cycles during the process of aliquoting for this study. Samples were maintained frozen at -80°C until the day of testing by Resp-4-Plex and supplementary methods.

### 2.2. Accuracy

Results from prior determinations either by Cepheid Xpert® Xpress Flu; direct fluorescent antigen testing for influenza A/B, RSV, adenovirus, and parainfluenza 1, 2 and 3 and/or detection in shell vial culture on R-Mix-Too monolayers (Quidel Corporation, San Diego, CA) were performed using the D3 Ultra DFA Respiratory Screening and Identification Kit, (Quidel) and/or reference laboratory testing at Eurofin ViraCor using the laboratory-developed TEM-PCR assay (Han et al., 2006) were considered predicate comparator assays as enumerated. Samples were divided for Resp-4-Plex testing on the 2 Alinity m instruments at our institution. As there was no difference in performance on these identical systems, results are presented in aggregate. Discrepant resolution included repeat testing of samples as available using Resp-4-Plex on the second Alinity m platform and/or using the Cepheid Xpert® Xpress SARS-CoV-2/Flu/RSV assay. Data for individual sample is listed in Table S1.

### 2.3. Analytical sensitivity

For limit-of-detection (LoD) studies, the AccuPlex SARS-CoV-2, Flu A/B and RSV Verification Panel (catalog #0505-0183, LGC SeraCare, Milford, MA) with individual members at defined equal concentrations, quantified by digital droplet PCR, were initially diluted to 1E4, 5E3, 1E3, 5E2, 2E2, 1E2, 5E1, 2.5E1, 1E1 and 0.5E1 target-amplicon genome copies per mL and tested by Resp-4-Plex in quadruplicate. Each panel member consists of either part or the entire genome of the target virus cloned into a replication-incompetent Sindbis virus. The Sindbis virus is an enveloped, single-stranded, RNA genome virus

that serves as a surrogate and control for all processes in the assay including extraction. The linearity of amplification of each assay was determined by least-squares linear regression based on Ct values obtained from each screening concentration, and PCR efficiency was determined using slope of the regression line using the ThermoFisher calculator: <https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/qpcr-efficiency-calculator.html>. Three dilutions bracketing the potential limit of detection cutoff were tested again with twenty replicates each to establish the LoD for each analyte. The 95% detection threshold and confidence intervals were extrapolated by logistic regression in Prism 9 for MacOS (GraphPad, San Diego, CA). LoD analysis was performed on a single Alinity m instrument.

## 3. Results

### 3.1. Accuracy

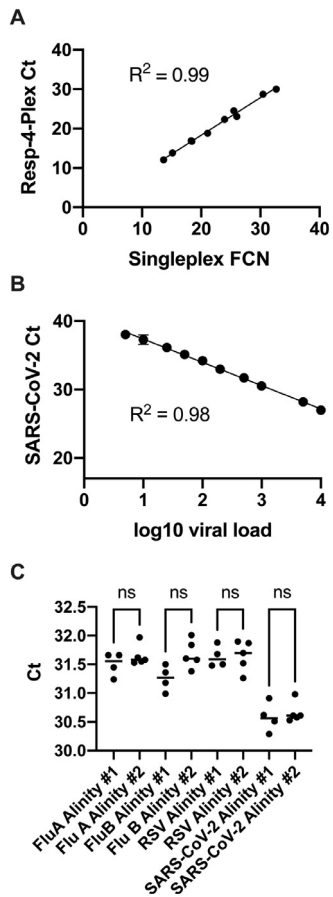
To determine accuracy, the Resp-4-Plex assay was run using clinical samples that were previously determined to be positive for the constituent viruses. Ten samples previously determined to be SARS-CoV-2 positive using the Alinity m SARS-CoV-2 singleplex assay were tested using Resp-4-Plex. All were positive for SARS-CoV-2. Although the Ct values for SARS-CoV-2 on Resp-4-Plex were a mean -1.81 +/- 0.58 lower than fractional cycle numbers on the singleplex assay, the assays were highly correlated with one another ( $R^2 = 0.99$ ) (Fig. 1A).

Twelve samples previously determined to be influenza A positive by Cepheid Xpress Flu were tested using Resp-4-Plex. All tested positive for influenza A. A single sample tested with Resp-4-Plex also yielded a positive RSV result with a high Ct value of 32.46 (see limit of detection analysis below). The test was repeated using Resp-4-Plex on the second Alinity m instrument and gave qualitatively and quantitatively almost identical results. The sample was then tested using the Cepheid Xpress SARS-CoV-2/Flu/RSV assay and was influenza A positive, RSV negative. The latter result could therefore either represent an Xpress RSV false negative based on low viral load or a Resp-4-Plex false positive.

Ten samples previously determined to be influenza B positive using the Cepheid Xpress Flu assay were tested using Resp-4-Plex. All tested positive for influenza B. A single sample tested with Resp-4-Plex also yield a positive RSV result. The test was repeated using Resp-4-Plex on the second Alinity m instrument and gave similar results. The sample was then tested using the Cepheid Xpress SARS-CoV-2/Flu/RSV assay and was influenza B positive, RSV positive. This sample was resolved as an influenza B/RSV true positive.

Ten samples previously determined to be RSV positive using a combination of direct fluorescence antigen testing and/or shell vial culture and a single sample determined to be RSV positive by TEM-PCR were tested using Resp-4-Plex. All were positive for RSV.

Eighteen samples previously determined to be negative for viruses by direct fluorescent antigen testing and/or shell vial culture, 5 of which were also negative by TEM-PCR, were tested using Resp-4-Plex. Sixteen samples were negative for the viruses detected by Resp-4-Plex. One sample tested with Resp-4-Plex yield as positive result for influenza B with a high Ct value of 34.2. The test was repeated using Resp-4-Plex on the second Alinity m instrument and gave similar results. The sample was then tested using the Cepheid Xpress SARS-CoV-2/Flu/RSV assay and was influenza B positive. A second sample (also negative by TEM-PCR) yielded a positive result for influenza A with Resp-4-Plex with a high Ct value of 36.53 and a positive result for RSV with a lower Ct value of 21.82. The test was repeated using Resp-4-Plex on the second Alinity m instrument and gave similar results. The sample was then tested using the Cepheid Xpress SARS-CoV-2/Flu/RSV assay and was influenza A negative (however, with a Ct value of 38.6, endpoint fluorescence of 142) and



**Fig. 1.** Performance of the Resp-4-Plex Assay. (A) Cycle threshold (Ct) results from the SARS-CoV-2 test in the Alinity m Resp-4-Plex multiplex assay and fractional cycle numbers (FCN) (Shain and Clemens, 2008) from the Alinity m SARS-CoV-2 singleplex assay were highly correlated when testing patient samples spanning the analytical measurement range of these tests. (B) Amplification of the SARS-CoV-2 targets in the Resp-4-Plex assay was log-linear when examined in the range at and above the limit of detection. Data points shown are the mean and standard deviation of Ct values for 4 replicate measurements. (C) Data points for positive control Ct values for each individual assay in Resp-4-Plex, tested on 4 to 5 separate days on 2 separate Alinity instruments, were highly correlated and not statistically (n.s.) different between instruments.

RSV positive (Ct value of 23.4). The sample was resolved as likely true positive on Resp-4-Plex for influenza A and RSV.

Six samples positive for adenovirus or parainfluenza virus 1, 2, or 3 by direct fluorescent antigen testing, 2 samples positive for adenovirus or parainfluenza virus by shell vial culture, 1 sample positive for enterovirus by TEM-PCR and 3 samples positive for low pathogenicity coronaviruses by TEM-PCR were tested using Resp-4-Plex. All samples were negative for the viruses detected by Resp-4-Plex.

### 3.2. Analytical sensitivity

For SARS-CoV-2, the LoD screen demonstrated excellent linearity with  $R^2 = 0.98$  and a 97% PCR efficiency (Fig. 1B). The manufacturer's claimed LoD was confirmed through 20 replicates at 50, 25, and 10 copies/mL using the SeraCare reference material, yielding 100%, 100%, and 80% detection, respectively. The LoD was therefore  $\leq 25$  copies per mL with an Ct value of  $35.72 \pm 0.54$  at 25 copies/mL. The logistic regression was nonconvergent and therefore an extrapolated LoD could not be established.

For influenza A, the LoD screen demonstrated excellent linearity with  $R^2 = 0.97$  and 93% PCR efficiency. The manufacturer's claimed LoD was confirmed through twenty replicates at 50, 25, and 10 copies/mL using the SeraCare reference material, yielding 100%,

80% and 30% detection, respectively. The LoD was therefore  $\leq 50$  copies per mL with a Ct value of  $37.18 \pm 0.67$  at 50 copies/mL. By logistic regression, the LoD was 47 copies/mL (95% confidence interval  $\sim 25$ -125 copies/mL).

For influenza B, the LoD screen demonstrated excellent linearity with an  $R^2 = 0.97$  and a 101% PCR efficiency. The manufacturer's claimed LoD was confirmed through twenty replicates at 50, 25, and 10 copies/mL using the SeraCare reference material, yielding 100%, 90%, and 70% detection, respectively. The LoD was therefore  $\leq 50$  copies per mL with a Ct value of  $35.94 \pm 0.73$  at 50 copies/mL. By logistic regression, the LoD was 36 copies/mL (95% confidence interval  $\sim 18$ -178 copies/mL).

For RSV, the LoD screen demonstrated excellent linearity with an  $R^2 = 0.97$  and 97% PCR efficiency. The manufacturer's claimed LoD was confirmed through twenty replicates at 50, 25, and 10 copies/mL using the SeraCare reference material, yielding 100%, 90% and 75% detection, respectively. The LoD was  $\leq 50$  copies per mL with an Ct value of  $35.75 \pm 0.70$  at 50 copies/mL. By logistic regression the LoD was 39.8 copies/mL (95% confidence interval  $\sim 18$ -1,000 copies/mL).

### 3.3. Precision

Intrarun precision was determined by using pooled samples, positive for each of the 4 viruses, and a negative pool. Each pool was tested in quadruplicate on each of the 2 Alinity m instruments with each individual test for each specific virus performed on each of the Assay Processing Units (APU) #1 through #4 on each instrument, so that every APU was tested during precision testing. Inter-run precision was verified by testing the pools for each virus on each Alinity m instrument again on an additional 2 separate days. Qualitative intrarun and inter-run precision results were 100% correlated.

Although Resp-4-Plex is a qualitative assay, we also assessed quantitative precision by comparison of Ct values in replicates. Coefficients of variation (CV) varied from 0.6% to 2.5% across all viruses tested on both instruments in intrarun precision comparisons and from 0.4% to 2.9% in inter-run precision comparisons. The precision of the Ct values for positive controls run during the validation on Alinity m #1 ( $n = 4$ ) and Alinity m #2 ( $n = 5$ ) instruments, run once per day of testing, was also examined. CVs on individual instruments for each assay were all less than 1%. There was no statistical difference between the positive control Ct values run on Alinity #1 and Alinity #2 for the 4 viruses (Fig. 1C), with significance considered  $P \leq 0.05$ , with comparisons performed using the Kruskal-Wallis test. Therefore, reproducibility of both instruments appeared essentially identical for the Resp-4-Plex assay.

## 4. Discussion

The Resp-4-Plex assay is a welcome addition to targeted respiratory panel options that will be necessary in a post-COVID world. It appeared highly accurate, sensitive, and precise. The 3 discrepancies with original comparator methods, specifically detection of co-infections, could be attributed to enhanced detection by Resp-4-Plex. This was because: (1) the second virus was detected on repeat testing by Resp-4-Plex on a second Alinity m platform, (2) the second virus was detected by the alternative Cepheid respiratory panel, and/or (3) the second virus had a very high Ct value, near its limit of detection, and therefore plausibly may have been below the limit of detection of the comparator assays. Coinfections with influenza and RSV are relatively rare, but their detection in our study may reflect the overlapping seasonality during sample collection periods and/or high acuity of patients (Hardick et al., 2006). Overall, our experience was consistent with qualitative detection data described in the EUA product insert, although our data set was significantly smaller in size.

Notably, the LoD for each individual virus was robust with high amplification efficiencies even in the context of a multiplex assay and testing of quality control material in which all 4 targets were present in equivalent amounts. The multiplex and singleplex SARS-CoV-2 assays were also extremely well correlated ( $R^2 = 0.99$ ). Furthermore, the Ct values were also log-linearly correlated with the quantitative standard at and above the limit of detection of the assays with excellent PCR efficiencies. The log-linear performance of the Resp-4-Plex assays suggest that future conversion to a quantitative readout, i.e., a viral load, should easily be supported (Arnaout et al., 2021) and under certain circumstances may provide additional utility for patient management.

Several limitations of the study should be noted. The number of samples examined was relatively small, limiting the power of the study. Several comparator assays were used based on our current clinical practice. Furthermore, samples were frozen between initial testing by the comparator and later testing using the Resp-4-Plex method. It is possible that storage and freeze-thaw may have resulted in specimen degradation, although we did not observe lower detection using the Resp-4-Plex method relative to comparators.

Taken together, we verified the performance characteristics of a new multiplex respiratory panel assay on the Alinity m molecular system. This platform notably provides high throughput, sample-to-answer, random access and semi-batch functionality with a 115-minute sample-to-answer turnaround time for prioritized specimens; and an ability to load and perform multiple different tests at the same time. We believe this assay and platform will be especially useful in fulfilling future needs in situations where SARS-CoV-2, influenza A, influenza B and RSV circulate at significant levels and where these viruses need to be detected and differentiated for optimal patient management.

#### Author contribution

Annie Cheng conceptualized and performed experiments. Stefan Riedel reviewed and edited manuscript and aided in formal analysis. Ramy Arnaout reviewed and edited the manuscript and aided in formal analysis.

#### Acknowledgments

We thank the Abbott Molecular Group for providing test reagents and LGC SeraCare control material for this work under a COVID-19 Diagnostics Evaluation Agreement. RA was a recipient of grant support from Abbott Molecular under a clinical study agreement.

#### Declaration of competing interest

The authors report no conflicts of interest relevant to this article.

#### Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.diagmicrobio.2021.115575.

#### References

- Arnaout R, Lee RA, Lee GR, Callahan C, Cheng A, Yen CF, et al. The limit of detection matters: the case for benchmarking severe acute respiratory syndrome coronavirus 2 testing. *Clin Infect Dis* 2021;73:e3042–6. doi: 10.1093/cid/ciaa1382.
- Baker RE, Park SW, Yang W, Vecchi GA, Metcalf CJE, Grenfell BT. The impact of COVID-19 nonpharmaceutical interventions on the future dynamics of endemic infections. *Proc Natl Acad Sci U S A* 2020;117:30547–53. doi: 10.1073/pnas.2013182117.
- Byun WS, Heo SW, Jo G, et al. Is coronavirus disease (COVID-19) seasonal? A critical analysis of empirical and epidemiological studies at global and local scales. *Environ Res* 2021;196:110972. doi: 10.1016/j.envres.2021.110972.
- Han J, Swan DC, Smith SJ, et al. Simultaneous amplification and identification of 25 human papillomavirus types with Templex technology. *J Clin Microbiol* 2006;44:4157–62. doi: 10.1128/jcm.01762-06.
- Hardick J, Shaw-Saliba K, McBryde B, et al. Identification of pathogens from the upper respiratory tract of adult emergency department patients at high risk for influenza complications in a pre-Sars-CoV-2 environment. *Diagn Microbiol Infect Dis* 2021;100: 115352. doi: 10.1016/j.diagmicrobio.2021.115352.
- Hirschhorn JW, Kegl A, Dickerson T, et al. Verification and validation of SARS-CoV-2 assay performance on the Abbott m2000 and alinity m systems. *J Clin Microbiol* 2021;59:e03119–20. doi: 10.1128/jcm.03119-20.
- Olsen SJ, Azziz-Baumgartner E, Budd AP, et al. Decreased influenza activity during the COVID-19 pandemic - United States, Australia, Chile, and South Africa, 2020. *MMWR Morb Mortal Wkly Rep* 2020;69:1305–9. doi: 10.15585/mmwr.mm6937a6.
- Shain EB, Clemens JM. A new method for robust quantitative and qualitative analysis of real-time PCR. *Nucleic Acids Res* 2008;36:e91. doi: 10.1093/nar/gkn408.
- Smith KP, Cheng A, Chopelas A, et al. Large-scale, in-house production of viral transport media to support SARS-CoV-2 PCR testing in a multihospital health care network during the COVID-19 pandemic. *J Clin Microbiol* 2020;58:e00913–20. doi: 10.1128/jcm.00913-20.