HDPCR™ RV6 RUO Assay

Introduction

In December of 2019, an outbreak of pneumonia due to a previously unidentified virus was reported to the World Health Organization. The etiology of this outbreak was identified as a novel coronavirus (SARS-CoV-2), which has since been implicated as the cause of a global pandemic referred to as COVID-19. As the pandemic progresses, knowledge relating to the dynamics of how this novel coronavirus will interact with other common respiratory illness is evolving. The United States Centers for Disease Control and Prevention (US CDC) states that seasonal influenza epidemics known as "Flu Season" occur annually during late fall and winter in the United States, and traditionally coincides with increases in other respiratory illnesses including RSV, human coronaviruses, and others[1].

The winter of 2020/2021 yielded extraordinarily low influenza activity. According to the US CDC, only 0.2% of all respiratory samples tested by US clinical laboratories were positive for influenza virus; this is in stark contrast with the three seasons prior, which reported positivity rates between 26.2 and 30.3%[2]. There are many speculations regarding the cause(s) of the nearly non-existent flu season in 2020/2021, including the effect of SARS-CoV-2 precautions such as social distancing, masking, at home, work, and schooling, along with travel restrictions. However, it is also possible that these precautions coincided with a cyclically low flu season, yielding an unprecedented season[3]. There are speculations as to what the 2021/2022 influenza season has in store with continued changes related to the COVID-19 pandemic including vaccination coverage, decreased immunity to other respiratory viruses due to lack of exposure in the prior year, and natural fluctuations in infectivity of these viruses.

A lack of predictability in historically seasonal influenza like illnesses colliding with the persistence of SARS-CoV-2 must be considered in the context of potential co-infections. There are multiple reports of SARS-CoV-2 co-infections. A study of 213 COVID-19 patients from the beginning of the pandemic in Wuhan, China evidenced that 45.5% of this cohort were co-infected with Influenza A and SARS-CoV-2[4]. Similarly, a recent meta review that suggests 10% of SARS-CoV-2 infections had a secondary viral infection[5]. The incidence of co-infection with influenza or other respiratory viruses likely depends on the prevalence of these viruses in circulation[6]; however, co-infections with Influenza A are specifically interesting when considering recent reports that infection with Influenza A can augment the ability of SARS-CoV-2 to enter host cells and increase infectivity[7].

Respiratory syncytial virus (RSV) is another seasonal virus that manifests with influenza like symptoms and is the most common cause of bronchiolitis and pneumonia in children younger than 1 year of age in the United States[8]. It was noted both in Australia and the United States that there were spikes in RSV infections in the spring and summer of 2021 which is inconsistent with the historic peaks observed in fall and winter[9]. This RSV activity had such large, localized spikes, that the CDC issued a Health Alert Network Advisory released 10 June 2021 warning of interseasonal RSV activity and recommending that patients with respiratory symptoms be tested for RSV if SARS-CoV-2 results were negative[10].

The persistence of circulating SARS-CoV-2, in conjunction with common seasonal illnesses that share symptoms, demonstrate the need to detect and differentiate SARS-CoV-2 from common causes of respiratory illnesses such as influenza and RSV. The CDC gained emergency use authorization (EUA) for a combined Influenza/SARS-CoV-2

multiplex assay in July 2020, stating that combination of Flu and COVID-19 testing could increase efficiencies, help with reagent shortages, and inform public health officials. Additionally, templates outlining studies necessary for submission for emergency use authorization available from the FDA include criteria for multianalyte testing. The precedent being set by federal public health authorities in conjunction with overlapping seasonality, potentials for intervention[11], and necessary epidemiological mapping all underscore the need for tests that differentiate between Influenza, COVID-19, and RSV.

In the current environment, research tests that are easy to adopt, cost effective, and have flexible throughput are necessary to gaining more information on the interplay between COVID and other seasonal respiratory viruses that manifest with similar symptoms. Additionally, outbreak mapping has increased relevance on the heels of a global pandemic[12].ChromaCode's HDPCR™ RV6 RUO Assay is a real time PCR test that provides qualitative detection and differentiation of SARS-CoV-2, Influenza A, Influenza B, and RSV A/B from common upper respiratory research samples. The RV6 RUO assay utilizes ChromaCode's proprietary High Definition PCR (HDPCR) to allow for multiplexing targets within a single color-channel with existing qPCR instrumentation. HDPCR's unique marriage of chemistry and data science provide a flexible and low-cost approach to epidemiological surveillance mapping. Herein, we present preliminary data based on testing of prospective and retrospective respiratory samples to establish the performance of the RV6 RUO Assay.

Methods

Materials and Methods Overview

The HDPCR RV6 RUO Assay was evaluated with samples that were extracted using the Thermo Scientific™ KingFisher™ Flex Extraction System and the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (Thermo Scientific). All extractions utilized 180 µL sample with 20 µL of Sample Processing Control (SPC) spiked in for a total of 200 µL sample with an elution volume of 50 µL. Thermal cycling was run on several Applied Biosystems™ instruments, including the 7500 Fast, 7500 Fast Dx, QuantStudio™ 5 96-well, 0.2 mL, QuantStudio 5 384-well, QuantStudio 7 96-well, and QuantStudio 12K Flex 384-well. Analytical studies were performed at ChromaCode in Carlsbad, CA. Natural sample evaluations were performed at The Medical College of Wisconsin, Milwaukee, WI.

Samples

Analytical studies for the RV6 RUO Assay were performed using negative matrix, were nasopharyngeal samples collected in M4RT or STM (Medshenker) that had been originally tested for all targets on the RV6 RUO panel. For natural sample characterization, a combination of frozen retrospective (n=168) and fresh prospective samples (n=400) was tested on a standard of care assay with either the Roche SARS-CoV-2/Flu, or standalone SARS-CoV-2 EUA Assay on the Cobas® 6800, VERIGENE® RP Flex, or Cepheid Xpert® Xpress SARS-CoV-2/Flu/RSV.

Results

Analytical Sensitivity

The analytical sensitivity (Limit of Detection or LoD) of the HDPCR RV6 RUO Assay was determined using a two-stage approach. In the first stage, a range finding study, was performed by testing 5 replicates of serial dilutions of each virus spiked into pooled negative nasopharyngeal swab (NPS) matrix. After establishment of a preliminary LoD in the first stage, the second stage (LoD confirmation) confirmed the LoD by extraction of 20 replicates. The LoD was defined as the lowest concentration where a minimum of 19/20 replicates were detected. The results of Stage 2 confirmation are presented in Table 1, indicating the established LoD for the RV6 RUO Assay when performed on the Applied Biosystems 7500 Fast Dx platform.

To ensure equivalent performance on other qPCR instruments, five (5) concentrations (3x, 2x, 1.5x, 1x, and 0.5x LoD) for each target were diluted to the desired concentration in negative NPS matrix and evaluated in a two-stage approach. In the first stage, range finding was completed with five (5) replicates. In the second stage, the limit of detection was

confirmed with twenty (20) replicates apart from the QuantStudio 5 (384-well) which was confirmed with sixty (60) replicates. The limit of detection was defined as the lowest concentration where ≥95% of samples evaluated were correctly identified.

The results from this study determined that all instruments evaluated and seen in Table 2 perform equivalently to the 7500 Fast Dx (≤ 3X away from the established LoD).

Table 1. HDPCR RV6 RUO Analytical Sensitivity

Reportable	Strain or Isolate	Results
Influenza A	Influenza A H3N2 (A/Perth/16/2009)	0.01 $TCID_{50}$/mL
	Influenza A H1N1 (A/New Caledonia/20/1999)	0.11 $TCID_{50}$/mL
	Influenza A H1N1 2009 (A/NY/01/09)	0.33 $TCID_{50}$/mL
Influenza B	Influenza B (B/Brisbane/60/2008)	0.01 $TCID_{50}$/mL
	Influenza B Yamagata_Florida/04/06	0.03 $TCID_{50}$/mL
RSV	Respiratory Syncytial Virus A (2006 Isolate)	1.320$TCID_{50}$/mL
	Respiratory Syncytial Virus B CH93(18)-18	0.03 $TCID_{50}$/mL
SARS-CoV-2	2019-nCoV/USA-WA1/2020	10 $TCID_{50}$/mL

Table 2. HDPCR RV6 RUO Instrument Compatibility

PCR Platform	Software Version	Well Format	Plate Name	Part Number	Manufacturer
QuantStudio 7	1.3	96 well (0.1 mL Fast)	MicroAmp™ EnduraPlate™ Optical 96-Well Clear Reaction Plates with Barcode	4483485/ 4483494	Applied Biosystems
QuantStudio 5	1.5.1	384 well	MicroAmp Optical 384-Well Reaction Plate with Barcode	4309849/ 4326270	Applied Biosystems
QuantStudio 12K Flex	1.3	384 well	MicroAmp Optical 384-Well Reaction Plate	4309849/ 4326270	Applied Biosystems
QuantStudio 5	1.5.1	96 well (0.2 mL)	MicroAmp Optical 96-Well Reaction Plate with Barcode	4306737/ 4326659	Applied Biosystems
7500 Fast	2.3	96 well (0.1 mL)	MicroAmp EnduraPlate Optical 96-Well Fast Clear Reaction Plates with Barcode	4483485/ 4483494	Applied Biosystems

In Silico Inclusivity

The primer and probe sequences for the two independent regions of SARS-CoV-2 detected by the HDPCR RV6 Assay were evaluated in silico. US sequences for SARS-CoV-2 (beginning with hCoV-19/USA) made up about 20% (82,862/412,614) of all sequences in the EpiCov section in GISAID as of February 1st, 2021. Greater than 98.0% of sequences screened have 100% homology to oligonucleotides for respective targets based on a bioinformatics assessment.

SARS-CoV-2 variants of concerns (UK/Alpha variant (B.1.1.7), South Africa/Beta variant (B.1.351), Brazil/Gamma variant (P.1), India/Delta variant (B.617.2), and recently the Omicron variant (B.1.529)) were analyzed independently in the in silico analysis. All above variants have 100% homology to SARS-CoV-2 RdRp and E gene oligonucleotides, with the exception of one (1) mismatch to the South Africa variant in the middle of the reverse primer for the E gene. This mismatch is not predicted to impact sensitivity since both the RdRp and E gene targets are sufficient for detection.

In silico analysis using sequences from NCBI and GISAID run on February 1, 2021, indicated that greater than 99.0% of Flu A, Flu B, RSV A, and RSV B sequences with a host human are predicted to be detected. Influenza A sequences from swine and avian sources are predicted to be detected in greater than 95% of the sequences evaluated.

Analytical Specificity

Analytical specificity was performed on the HDPCR RV6 Assay with viral, bacterial, fungal targets and pooled nasal wash. All targets were tested with three (3) replicates. Viral exclusivity strains were tested at 1 $X10^5$ TCID50/mL or the highest concentration achievable, based on the stock tube concentration. Bacterial and fungal exclusivity strains were tested at 10^6 CFU/mL. The same targets were tested for specificity as were tested in microbial interference (Table 5) and the analytical specificity was 100%.

Interfering Substances

The HDPCR RV6 Assay was tested in the presence of potentially interfering substances that may be present in a respiratory sample. The study evaluated four (4) RV6 targets: Influenza A, Influenza B, RSVA and SARS-CoV-2 at 3X LoD. The samples were diluted to 3X LoD in pooled negative NPS matrix containing relevant concentrations of the inhibitory substances (listed in Table 3).

All expected target present and target absent replicates in the study were called correctly for each interfering substance.

Table 3. HDPCR RV6 RUO Interfering Substances Evaluation

Substance Name	Brand Name/Active Ingredient	Concentration Evaluated
Mucin	Mucin/Purified mucin protein	60 µg/mL
Neo-synephrine (vasopressin/decongestant drops)	4-Way Nasal Spray/phenylephrine	15% (vol/vol)
Decongestant nasal spray	Zicam Extreme Congestion/Oxymetazoline	15% (vol/vol)
Zicam Allergy Relief	Zicam/Galphimia glauca, Histaminum hydrochloricum, Luffa operculata, Sulphur	5% (vol/vol)
Saline nasal spray	Sunmark/Sodium Chloride	15% (vol/vol)
Nasal corticosteroid	Rhinocort/Budesonide	5% (vol/vol)
Throat lozenges	Halls/Menthol	0.68 g/mL or 1/18 lozenge, crushed
Zanamivir (anti-viral)	Zanamivir	3.3 to 5 mg/mL
Tobramycin (systemic antibacterial)	Tobramycin	4.0 µg/mL
Mupirocin (antibiotic ointment)	Mupirocin	6.6 to 10 mg/mL
Oseltamivir phosphate (Tamiflu)	Oseltamivir phosphate	7.5 to 25 mg/mL
Human blood, EDTA anticoagulated	NA	2% (vol/vol)
Human genomic DNA	NA	50 ng/reaction

Competitive Microbial Interference

The HDPCR RV6 RUO Assay was tested to evaluate the competitive microbial interference (Co-presence) of the RV6 targets using eighteen (18) different target combinations. In each condition a target was at low concentration (3X LoD) and high concentration (≥10^4 $TCID_{50}$/mL). Three (3) replicates at each condition were evaluated; if less than three (3) replicates were positive a 3-fold higher concentration of the strain at LoD was evaluated. The study evaluated co-infection on both the 7500 Fast Dx and QuantStudio 5 (384-well).

All targets in a simulated co-infection were correctly identified at the LoD value reported in (Table 4). RSV A performance is impacted near LoD when in a co-infection with high concentration Influenza A and SARS-CoV-2 samples.

Table 4. HDPCR RV6 RUO Competitive Interference

Target at LoD	Interferent	7500 Fast Dx	QuantStudio 5 (384)
Influenza B	Influenza A	3X	3X
RSV A	Influenza A	81X	81X
RSV B	Influenza A	3X	3X
SARS-CoV-2	Influenza A	3X	3X
Influenza A	Influenza B	3X	3X
RSV A	Influenza B	3X	9X
RSV B	Influenza B	3X	3X
SARS-CoV-2	Influenza B	3X	3X
Influenza A	RSV A	3X	3X
Influenza B	RSV A	3X	3X
SARS-CoV-2	RSV A	3X	3X
Influenza A	RSV B	3X	3X
Influenza B	RSV B	3X	3X
SARS-CoV-2	RSV B	3X	3X
Influenza A	SARS-CoV-2	3X	9X
Influenza B	SARS-CoV-2	3X	9X
RSV A	SARS-CoV-2	27X	27X
RSV B	SARS-CoV-2	3X	3X

Microbial Interference Evaluation

The HDPCR RV6 RUO assay was tested in the presence of microorganisms that are frequently found in NPS samples or with pooled nasal wash to ensure proper identification of the RV6 targets. A total of twenty-two (22) viruses and twenty (20) eleven spiked pools were evaluated. Each microorganism was shown previously to not cause false positive detection in the Exclusivity: Analytical Specificity Study. Each pool was spiked with an RV6 Assay targets to a final concentration of 3x LoD. All RV6 assay targets were detected at 3x LoD in the presence of the forty-two (42) microbial interference organisms and the pooled nasal wash (Table 5)

Table 5. *Microbial Interference Evaluation: Detection of Flu A, Flu B, RSV, and SARS-CoV-2*

Organism	Final Testing concentration	Inf A 3x LoD	Inf B 3x LoD	RSV 3x LoD	SARS-CoV-2 3 x LoD
hMPV A1	1.17 X 10^4 $TCID_{50}$/mL	Y*	Y	Y	Y
Adenovirus C1/Adenoid 71	4.17 X 10^4 $TCID_{50}$/mL	Y	Y	Y	Y
Adenovirus 7a	4.17 X 10^4 $TCID_{50}$/mL	Y	Y	Y	Y
Coronavirus 229E	5.62 X 10^3 $TCID_{50}$/mL	Y	Y	Y	Y
Coronavirus OC43	4.17 X 10^4 $TCID_{50}$/mL	Y	Y	Y	Y
Coronavirus NL63	1.41 X 10^4 $TCID_{50}$/mL	Y	Y	Y	Y
Coronavirus HKU1	1.00 X10^6 genome copies/rxn	Y	Y	Y	Y
Coxsackievirus B4	1.00 X 10^5 $TCID_{50}$/mL	Y	Y	Y	Y
Cytomegalovirus	1.86 X 10^3 $TCID_{50}$/mL	Y	Y	Y	Y
Enterovirus 68 (2007 isolate)	1.00 X 10^5 $TCID_{50}$/mL	Y	Y	Y	Y
Enterovirus 71	1.00 X 10^5 $TCID_{50}$/mL	Y	Y	Y	Y
Epstein Barr Virus	1.00 X 10^5 $TCID_{50}$/mL	Y	Y	Y	Y
Human Rhinovirus Type 1A	1.00 X 10^6 genome copies/rxn	Y	Y	Y	Y
MERS-CoV (inactivated)	1.17 X 10^4 $TCID_{50}$/mL	Y	Y	Y	Y
SARS-1 (inactivated)	7.33 x 10^2 genome equivalents /rxn	Y	Y	Y	Y
Measles virus	4.17 X 10^4 $TCID_{50}$/mL	Y	Y	Y	Y
Mumps virus	2.45 x 10^4 $TCID_{50}$/mL	Y	Y	Y	Y
Parainfluenza Type 1	5.01 X 10^4 $TCID_{50}$/mL	Y	Y	Y	Y
Parainfluenza Type 2	1.00 X 10^5 $TCID_{50}$/mL	Y	Y	Y	Y
Parainfluenza Type 3	1.70 X 10^4 $TCID_{50}$/mL	Y	Y	Y	Y
Parainfluenza Type 4	4.17 X 10^4 $TCID_{50}$/mL	Y	Y	Y	Y
Parechovirus	1.00 X 10^5 $TCID_{50}$/mL	Y	Y	Y	Y
Bordetella pertussis	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Chlamydia pneumoniae	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Parainfluenza Type 4	4.17 X 10^4 $TCID_{50}$/mL	Y	Y	Y	Y
Parechovirus	1.00 X 10^5 $TCID_{50}$/mL	Y	Y	Y	Y
Bordetella pertussis	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Chlamydia pneumoniae	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Haemophilus influenzae	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Legionella pneumophila	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Mycobacterium tuberculosis (avirulent strain)	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Mycoplasma pneumoniae	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Pseudomonas aeruginosa	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Neisseria meningitidis	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Neisseria elongata	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Escherichia coli	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Moraxella catarrhalis	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Corynebacterium diphtheriae	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Lactobacillus plantarum	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Streptococcus pneumoniae	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Streptococcus pyogenes	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Streptococcus salivarius	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Pneumocystis jirovecii (PJP), S. cerevisiae	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Staphylococcus aureus (MSSA)	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Staphylococcus epidermidis	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
Candida albicans	1.00 X 10^6 CFU/mL	Y	Y	Y	Y
pooled nasal wash	NA	Y	Y	Y	Y
Negative NPS matrix	NA	Y	Y	Y	Y

*Y indicates detection at 3x LoD concentration of analyte

Natural Sample Evaluation

A natural sample evaluation was performed at Medical College of Wisconsin with residual, de-identified nasopharyngeal swab samples in commercially available UTM/VTM utilizing an ABI 7500 Fast Dx as the qPCR instrument. A combination of frozen retrospective (n=168) and fresh prospective samples (n=400) were tested on a standard of care assay with either the Roche SARS-CoV-2/Flu, or standalone SARS-CoV-2 EUA Assay on the Cobas® 6800, VERIGENE® RP Flex, or Cepheid Xpert® Xpress SARS-CoV-2/Flu/RSV. Target performance is displayed in Table 6, where all targets showed greater than 94% sensitivity and 99% specificity.

Table 6. HDPCR RV6 RUO Natural Sample Evaluation

Sample	True Positive	True Negative	False Positive	False Negative	Sensitivity (95% CI)	Specificity (95% CI)
Influenza A	51	400	0	0	100% (91.3-100)	100% (98.8-100)
Influenza B	29	422	0	0	100% (85.4-100)	100% (98.3-100)
RSV A/B	49	90	0	1	98.0% (88.0-99.9)	100 (94.9-100)
SARS-CoV-2	48	458	2	3	94.1 % (82.8-98.5)	99.6% (98.3-99.9)

Discussion

ChromaCode's HDPCR multiplexing technology empowers the large install base of Applied Biosystems Real-Time PCR (qPCR) instrumentation to perform multiplex testing at a very low cost with no instrument or software modifications. HDPCR couples traditional TaqMan® chemistry with proprietary data-science based algorithms, reducing unwanted variability inherent in PCR reactions as well as thermal and optical variability from qPCR hardware. ChromaCode can encode multiple analytes into a single-color channel and differentiate targets by varying end-point signal intensity for a given target. All HDPCR tests have an identical workflow to traditional qPCR assays and utilize ChromaCode's and remotely accessible ChromaCode Cloud software.

Without clarity of the composition of respiratory viruses in circulation during this and subsequent years' respiratory seasons, and as SARS-CoV-2 transitions from a pandemic to potentially an endemic virus, insights into infection rates and potential for co-infections will be key in epidemiologic research. A reliable tool that differentiates Influenza A, Influenza B, RSV, and SARS-CoV-2 can enable laboratories to perform this critical research. The data in this manuscript demonstrated the ability for the RV6 RUO Assay to specifically identify RNA from top respiratory viruses from upper respiratory samples. The assay is inclusive of all variants of interest and concern for SARS-CoV-2 and includes 2 different gene targets from SARS-CoV-2 to ensure coverage as the virus continues to mutate. RV6 RUO proved to be robust in the face of common interfering substances and performed with high sensitivity and specificity in a cohort of natural samples. Additionally, the analytical accuracy of the assay was maintained in simulated co-infections of differing viral loads, a key to further research in this arena.

Additionally, the ability for the RV6 RUO assay to be run on a variety of common qPCR instruments allows for laboratories to expand the utility of their existing testing equipment with no additional capital expenditures. The consistent performance between a 96-well instrument and a 384-well instrument allows for peace of mind when facing potential increased testing needs based on infection rates. The data being analyzed on the ChromaCode Cloud™

allows for results interpretation and streamlined reporting that can be accessed from anywhere.

Conclusions

The HDPCR respiratory short panel inclusive of COVID-19 demonstrates ability to serve as powerful research tool for this innovative technology. Without full clarity on the future of SARS-CoV-2 infection patterns and potential co-infections, it is important to have a cost-effective tool to enable laboratories to study these phenomena. The flexibility of the research assay including the ability to be used on 96-well and 384-well qPCR instrumentation and different extraction platforms coupled with the flexibility of the ChromaCode Cloud, which can enable selective target reporting and can be accessed from anywhere, provide a research solution to help laboratories and researchers to start reacting to all phases of the COVID pandemic.

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