A complete workflow solution for detecting respiratory pathogens, including SARS-CoV-2, using OpenArray technology

In this report, we show that:

- Applied Biosystems™ TaqMan® Assays for respiratory pathogens meet rigorous performance criteria
- The Applied Biosystems™ TrueMark™ Respiratory Panel 2.0, OpenArray™ Plate, allows simultaneous interrogation of up to 32 respiratory pathogens, including SARS-CoV-2, and 3 controls
- The Thermo Scientific™ KingFisher™ Flex Purification System, Applied Biosystems™ TaqMan® OpenArray™ plate, and Applied Biosystems™ QuantStudio™ 12K Flex Real-Time PCR System are part of a cost-effective high-throughput workflow with minimal hands-on time

Introduction

Upper and lower respiratory tract infections are caused by a broad range of microbes, including RNA and DNA viruses, bacteria, and even fungi, and yet are often symptomatically similar. Detection of these pathogens can be challenging: immunoassays are limited to a small number of respiratory pathogens and lack sensitivity, whereas culture-based methods are labor intensive, have long turnaround times, and are prone to false-negative results due to fastidious growth in culture. While molecular testing is more sensitive, most commercially available tests are expensive, primarily focus on either viruses or bacteria, and lack the flexibility to customize target lists based on laboratory needs. In addition, concurrent prevalence of viral and bacterial pathogens is a growing concern and needs effective detection methods.

To meet the need for more comprehensive coverage of respiratory pathogens, we introduce a panel-based molecular solution that detects a wide range of respiratory viruses (including SARS-CoV-2), bacteria, and fungi in a single assay. TaqMan OpenArray plates offer a high-throughput, qPCR-based method to detect pathogenic organisms at very low concentrations. The TrueMark Respiratory Panel 2.0, OpenArray Plate, which targets 32 key respiratory pathogens, including SARS-CoV-2, is available as an inventoried panel. Alternatively, custom TaqMan OpenArray plates can be designed using flexible assay content to meet the needs of any laboratory. When combined with a KingFisher Flex Purification System and QuantStudio 12K Flex Real-Time PCR System, the TrueMark Respiratory Panel 2.0, OpenArray Plate, or a custom OpenArray plate offers a complete end-to-end solution for respiratory pathogen detection (Figure 1).

Figure 1. Workflow for detection of respiratory pathogens. The workflow shows extraction of total nucleic acid from respiratory tract samples using a KingFisher Purification System and the Applied Biosystems™ MagMAX™ Viral/Pathogen Ultra Nucleic Acid Isolation Kit, followed by preamplification and then real-time PCR analysis using the TrueMark Respiratory Panel 2.0, OpenArray Plate, on the QuantStudio 12K Flex system. Results are provided in the form of a presence or absence call for each of the respiratory pathogen targets that include bacteria and RNA and DNA viruses.

Materials and methods

Total nucleic acid isolation from respiratory tract samples

The MagMAX Viral/Pathogen Ultra Nucleic Acid Isolation Kit was used to isolate total nucleic acid (TNA) from respiratory samples. This kit was optimized for extraction of TNA from different microbe types that are found in respiratory samples (RNA and DNA viruses, bacteria, and fungi) and was shown to work well with respiratory sample types including nasopharyngeal swabs, nasopharyngeal aspirates, and bronchoalveolar lavage. TNA isolation from 96 samples using the KingFisher Flex Purification System took about 1.5 hours, with 30 minutes of hands-on time.

Detection of respiratory pathogens using TaqMan OpenArray plates

Qualified TaqMan Assay designs and target sequences for respiratory pathogens underwent thorough bioinformatics selection and analysis for high strain coverage and specificity. The assays have also undergone extensive performance testing with synthetic templates, nucleic acids extracted from whole-organism standards, and clinical research samples, to help ensure that results are accurate and reproducible with high sensitivity and specificity.

OpenArray plates provide a flexible high-throughput format for real-time PCR that utilizes a microscope slide–sized stainless steel plate with 3,072 wells for individual 33 nL reactions, where TaqMan Assays are spotted according to customer specifications. This study originally used a custom TaqMan OpenArray plate containing 44 respiratory pathogen assays plus 3 control assays (Table 1); at least 3 technical replicates were run for each assay, per plate. This custom TaqMan OpenArray plate, developed prior to the SARS-CoV-2 crisis, was used in the inclusivity, exclusivity, and concordance studies shown in this application note. We have since launched the TrueMark Respiratory Panel 2.0, OpenArray Plate, which includes targets for SARS-CoV-2, and so we have updated this study. The TrueMark Respiratory Panel 2.0, OpenArray Plate, contains assays for 32 respiratory pathogens plus 3 controls, and was used in limit of detection, linear dynamic range, and contrived sample experiments shown in this application note (Table 1). The TaqMan OpenArray plates allow 3 replicates to be run in parallel for all respiratory pathogen assays and running up to 24 samples per plate. Control assays target the Applied Biosystems™ TaqMan® Universal Extraction Control Organism *(B. atrophaeus*), TaqMan® Universal RNA Spike-In/Reverse Transcription (Xeno) Control, and human RNase P gene (*RPPH1*).

All samples in this clinical research study were tested using our optimized protocol for respiratory pathogen profiling, which utilizes a preamplification step to maximize sensitivity, with the added benefit of sample conservation. Synthetic templates or purified genomic nucleic acid samples were first reverse-transcribed and preamplified as follows: 5 µL of each sample was combined with 2.5 µL of Applied Biosystems™ TaqPath™ 1-Step RT-qPCR Master Mix, CG, and 2.5 µL of Applied Biosystems™ TaqMan® PreAmp Pool, Respiratory Tract Microbiota (for the custom OpenArray plates) or TrueMark Respiratory Panel 2.0 PreAmp Primers (for the TrueMark Respiratory Panel 2.0), then reverse-transcribed and amplified for 14 cycles. Preamplified samples were diluted 1:10 with nuclease-free water, and then 2.5 µL of each diluted sample was combined with 2.5 µL of Applied Biosystems™ TaqMan® OpenArray™ Real-Time PCR Master Mix in a well of an OpenArray™ 384-Well Sample Plate. Each reaction was transferred using the Applied Biosystems™ QuantStudio™ 12K Flex AccuFill™ System to subarrays on the TaqMan OpenArray plate. Plates were then run on the QuantStudio 12K Flex Real-Time PCR System, and data were analyzed by the instrument software.

For details on sample extraction, target and control assays, and running experiments, refer to the application guide “Respiratory Tract Microbiota Profiling Experiments v2: TaqMan Assays for respiratory pathogens profiling experiments using OpenArray plates” (**Pub. No. MAN0019506**).

Table 1. Categorization of respiratory pathogens. Assays highlighted in blue are included on the TrueMark Respiratory Panel 2.0, OpenArray Plate.

Organism type	Nucleic acid type	Assay ID	Assay name	Organism name
Virus	DNA	Vi99990001_po	AdV_1of2	Adenovirus 1/2*
		Vi99990002_po	AdV_2of2	Adenovirus 2/2*
		Vi99990003_po	HBoV	Human bocavirus
		Vi06439647_s1	HHV3	Human herpesvirus 3 (HHV3—varicella zoster virus)
		Vi06439675_s1	HHV4	Human herpesvirus 4 (HHV4—Epstein-Barr virus)
		Vi06439643_s1	HHV5	Human herpesvirus 5 (HHV5—cytomegalovirus)
		Vi06439627_s1	HHV6	Human herpesvirus 6 (HHV6)
	RNA	Vi06439671_s1	CoV_229E	Human coronavirus 229E
		Vi06439674_s1	CoV_HKU1	Human coronavirus HKU1
		Vi06439673_s1	CoV_NL63	Human coronavirus NL63
		Vi06439646_s1	CoV_OC43	Human coronavirus OC43
		Vi06439631_s1	EV_pan	Human enterovirus (pan assay)
		Vi06439669_s1	EV_D68	Human enterovirus D68
		Vi99990004_po	hMPV	Human metapneumovirus (hMPV)
		Vi06439642_s1	hPIV1	Human parainfluenza virus 1 (hPIV1)
		Vi06439672_s1	hPIV2	Human parainfluenza virus 2 (hPIV2)
		Vi06439670_s1	hPIV3	Human parainfluenza virus 3 (hPIV3)
		Vi99990005_po	hPIV4	Human parainfluenza virus 4 (hPIV4)
		Vi99990006_po	HPeV	Human parechovirus
		Vi99990014_po	RSVA	Human respiratory syncytial virus A (RSVA)
		Vi99990015_po	RSVB	Human respiratory syncytial virus B (RSVB)
		Vi99990007_po	RV_1of2	Human rhinovirus 1/2*
		Vi99990008_po	RV_2of2	Human rhinovirus 2/2*
		Vi99990011_po	Flu_A_pan	Influenza A
		Vi99990009_po	Flu_A_H1	Influenza A/H1-2009
		Vi99990010_po	Flu_A_H3	Influenza A/H3
		Vi99990012_po	Flu_B_pan	Influenza B
		Vi99990013_po	Measles	Measles virus
		Vi06439644_s1	MERS_CoV	Middle East respiratory syndrome coronavirus (MERS)
		Vi06439657_s1	Mumps	Mumps virus
		Vi06439634_s1	SARS_CoV	Severe acute respiratory syndrome coronavirus (SARS)
		Vi07918636_s1	SARS-CoV2S	Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); S protein gene**
		Vi07918637_s1	SARS-CoV2N	Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); N protein gene**
Bacterium	DNA	Ba06439624_s1	Bordetella	*Bordetella bronchiseptica, parapertussis,* or *pertussis*
		Ba06439621_s1	B.holmesii	*Bordetella holmesii*
		Ba06439623_s1	B.pertussis	*Bordetella pertussis*
		Ba06439616_s1	C.pneumoniae	*Chlamydophila pneumoniae*
		Ba06439618_s1	C.burnetii	*Coxiella burnetii*
		Ba06439625_s1	H.influenzae	*Haemophilus influenzae*
		Ba04932083_s1	K.pneumoniae	*Klebsiella pneumoniae*
		Ba06439617_s1	L.pneumophila	*Legionella pneumophila*
		Ba06439622_s1	M.catarrhalis	*Moraxella catarrhalis*
		Ba06439620_s1	M.pneumoniae	*Mycoplasma pneumoniae*
		Ba04646259_s1	S.aureus	*Staphylococcus aureus*
		Ba06439619_s1	S.pneumoniae	*Streptococcus pneumoniae*
Fungus	DNA	Fn06439626_s1	P.jirovecii	*Pneumocystis jirovecii*
Control	RNA	Ac00010014_a1	Xeno	Xeno RNA control
	DNA	Hs04930436_g1	RPPH1	Ribonuclease P RNA component H1
		Ba06596576_s1	B.atrophaeus	*Bacillus atrophaeus* or *subtilis,* subspecies *globigii*

* For adenoviruses and rhinoviruses, two assays are required for full strain coverage. For additional details on each assay, go to **thermofisher.com/taqman**.

** For SARS-CoV-2 detection, assays for the spike (S) protein and nucleocapsid (N) protein genes each have high coverage of known strains.

Results

Sensitivity and linear dynamic range of TaqMan Assays

The sensitivity, efficiency, and linear dynamic range (LDR) were evaluated using serial dilutions of the Applied Biosystems™ TrueMark™ Respiratory Panel 2.0 Amplification Control, which is a linearized plasmid DNA control containing all target and control sequences. Preamplification and real-time PCR were performed on the amplification control without sample preparation, using the same optimized protocol used for respiratory tract samples and organism control samples. The amplification control dilution series, with input concentrations ranging from 10^5 to 0.1 copies/µL, was tested on custom TaqMan OpenArray plates covering 44 respiratory pathogen assays plus control assays (data not shown) and on the TrueMark Respiratory Panel 2.0, OpenArray Plate, which contains the SARS-CoV-2 assays (Figure 2).

We achieved high sensitivity (limit of detection, LOD) down to 1–10 copies/µL of input per reaction for all respiratory pathogen assays (Figure 2), with minimal variation at lower concentrations. All assays demonstrated a LDR of 5 orders of magnitude (10^5 to 1 copies/µL) where R^2 was greater than 0.99 and PCR efficiency was very close to 100%. Representative LDR data plots are shown for four viral and two bacterial targets in Figure 3.

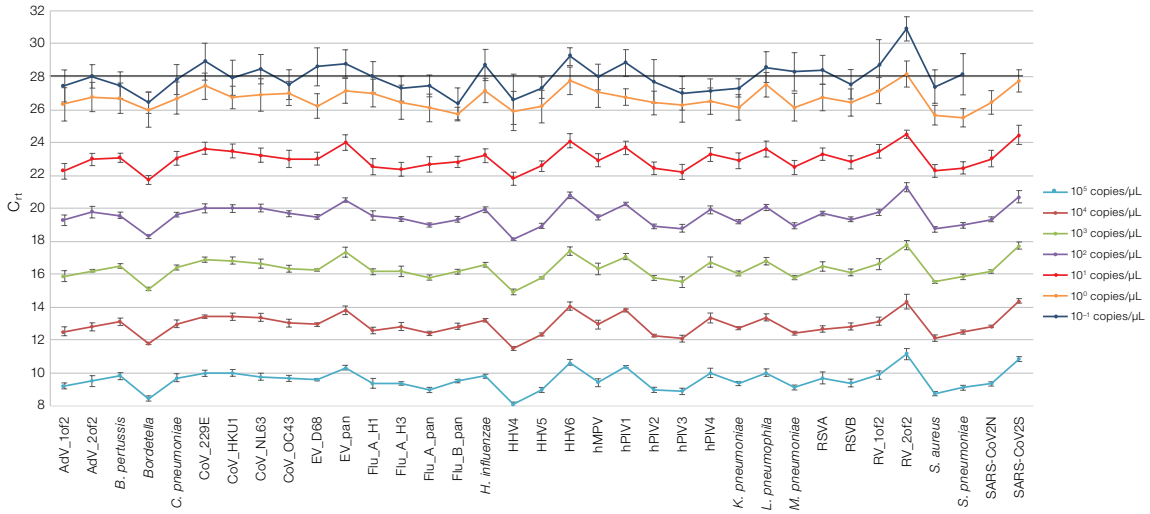

Figure 2. Limit of detection using the TrueMark Respiratory Panel 2.0 Amplification Control. Serial dilutions of 10^5 copies/µL down to 0.1 copies/µL of the amplification control were tested using the optimized protocol for preamplification plus real-time PCR with the TrueMark Respiratory Panel 2.0, OpenArray Plate (format 112), containing 35 respiratory pathogen assays. Three technical replicates were run for each concentration. All assays were able to detect down to 1–10 copies/µL of target input using a C_{rt} threshold of 28. Note: The RV_2of2 assay shows higher C_{rt} values and standard deviations than the RV_1of2 assay, as it is mismatched by 1 nucleotide with the amplification control rhinovirus sequence.

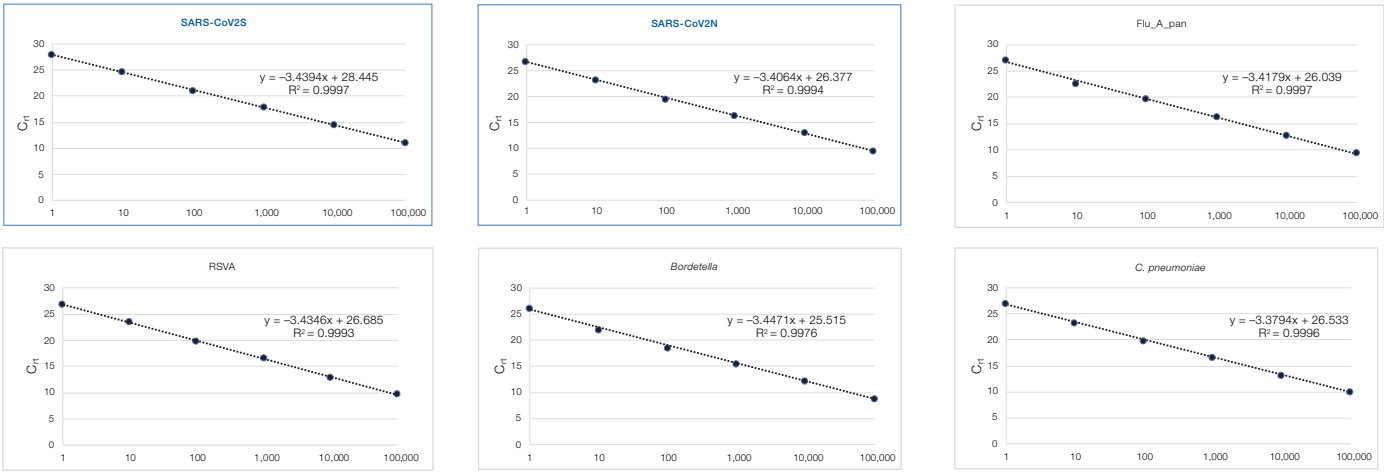

Figure 3. LDR results for representative TaqMan Assays targeting respiratory pathogens. Data from dilutions (10^5 copies/µL down to 1 copy/µL) of the TrueMark Respiratory Panel 2.0 Amplification Control shown in Figure 2 were used to calculate the LDR for the respiratory pathogen assays. Plots for 6 representative assays are shown. All assays demonstrated an LDR of 5 orders of magnitude where R^2 was greater than 0.99 and PCR efficiency was approximately 100%.

The workflow LOD was also determined for a subset of the viral and bacterial respiratory target organisms, using low concentrations of 22 commercially available, enumerated whole organisms that were spiked into viral transport medium. TNA was extracted using the MagMAX Viral/Pathogen Ultra Nucleic Acid Isolation Kit, and sample aliquots underwent reverse transcription and preamplification followed by real-time PCR on the custom TaqMan OpenArray plate containing assays for 44 respiratory tract pathogens; the SARS-CoV-2 assays were not included in this study, since it took place prior to the SARS-CoV-2 crisis. Two-fold dilution series covering the estimated LOD per organism were tested for a total of 12 replicates, and LOD values were calculated by probit regression analysis. LODs were confirmed by testing an additional 12 extractions at 0.25X, 1X, and 4X LOD concentrations. The LOD results shown in Table 2 are similar to those provided in the literature for respiratory pathogens detected by other qPCR platforms.

Table 2. LODs of TaqMan Assays for respiratory pathogens on TaqMan OpenArray plates.

Organism (strain)	Assay name	LOD (units/mL)*	95% confidence interval
Adenovirus (1)	AdV_1of2	7.81×10^{0}	(2.09×10^{0}, 2.92×10^{1})
Coronavirus 229E	CoV_229E	1.40×10^{-1}	(4.11×10^{-2}, 4.77×10^{-1})
Coronavirus NL63	CoV_NL63	2.94×10^{-3}	(1.02×10^{-3}, 8.44×10^{-3})
Coronavirus OC43	CoV_OC43	4.15×10^{-1}	(8.96×10^{-2}, 1.92×10^{0})
Enterovirus type 68 (2007 isolate)	EV_D68	4.01×10^{-2}	(1.26×10^{-2}, 1.28×10^{-1})
Enterovirus type 71 (2003 isolate)	EV_pan	6.95×10^{-2}	(1.70×10^{-2}, 2.85×10^{-1})
Human metapneumovirus (IA-2002)	hMPV	7.83×10^{-2}	(2.25×10^{-2}, 2.73×10^{-1})
Influenza A H1N1 (A/Brisbane/59/07)	Flu_A_pan	6.48×10^{-3}	(2.49×10^{-3}, 1.69×10^{-2})
Influenza A H1N1pdm (NY/03/09)	Flu_A_H1	4.87×10^{-3}	(1.27×10^{-3}, 1.87×10^{-2})
Influenza A H1N1pdm (NY/03/09)	Flu_A_pan	1.63×10^{-2}	(1.14×10^{-3}, 2.32×10^{-1})
Influenza A H3 (A/Wisconsin/67/05)	Flu_A_H3	1.91×10^{-2}	(4.45×10^{-3}, 8.21×10^{-2})
Influenza A H3 (A/Wisconsin/67/05)	Flu_A_pan	1.31×10^{-2}	(4.64×10^{-3}, 3.70×10^{-2})
Influenza B (B/Florida/04/06)	Flu_B_pan	6.11×10^{-2}	(1.96×10^{-2}, 1.91×10^{-1})
Parainfluenza 1	hPIV1	2.58×10^{-3}	(1.40×10^{-3}, 4.79×10^{-3})
Parainfluenza 2	hPIV2	2.70×10^{0}	(8.99×10^{-1}, 8.14×10^{0})
Parainfluenza 3	hPIV3	2.83×10^{0}	(9.13×10^{-1}, 8.75×10^{0})
Parainfluenza 4	hPIV4	1.85×10^{0}	(7.60×10^{-1}, 4.51×10^{0})
Respiratory syncytial virus A (2006 isolate)	RSVA	8.48×10^{-2}	(2.15×10^{-2}, 3.34×10^{-1})
Respiratory syncytial virus B (CH93(18)-18)	RSVB	2.62×10^{-1}	(6.86×10^{-2}, 1.00×10^{0})
Rhinovirus/enterovirus (1A)	RV_1of2	1.49×10^{-1}	(4.08×10^{-2}, 5.43×10^{-1})
Bordetella parapertussis (E595)	*Bordetella*	2.16×10^{4}	(3.74×10^{3}, 1.25×10^{5})
Bordetella pertussis (E431)	*B. pertussis*	9.87×10^{3}	(2.31×10^{3}, 4.21×10^{4})
Bordetella pertussis (E431)	*Bordetella*	9.63×10^{3}	(2.37×10^{3}, 3.91×10^{4})
Legionella pneumophila (Philadelphia)	*L. pneumophila*	2.12×10^{2}	(6.71×10^{1}, 6.70×10^{2})
Mycoplasma pneumoniae (M129)	*M. pneumoniae*	8.71×10^{0}	(1.38×10^{0}, 5.49×10^{1})

* LOD units are $TCID_{50}$/mL for viruses and CFU/mL for bacteria.

High specificity of TaqMan Assays for respiratory pathogens

TaqMan Assays for respiratory pathogens have undergone rigorous bioinformatic analysis to help ensure maximum strain coverage while minimizing the potential for off-target cross-reactivity. Each assay, with the exception of the SARS-CoV-2 assays (which were developed during the SARS-CoV-2 crisis and after this cross-reactivity study was done), has been tested with target and nontarget genomic RNA or DNA isolated from target organisms (nucleic acid acquired from ATCC) in our inclusivity panel (Table 3). The inclusivity panel covers 29 of 42 (69%) respiratory pathogen targets; missing from this analysis were unculturable and biosafety level 3 and 4 organisms.

The respiratory pathogen assays provided highly specific results when screened simultaneously against the available subset of respiratory pathogen genomes on TaqMan OpenArray plates (Table 4). Testing against nontarget organisms in an exclusivity panel also demonstrated no cross-reactivity of the respiratory pathogen assays with closely related species and other respiratory microbes (Table 5 and data not shown).

Table 3. Respiratory pathogen inclusivity controls.

Organism type	Nucleic acid type	Organism	ATCC ID*
Virus	DNA	Adenovirus C	VR-846D
		Adenovirus E	VR-1572D
		HHV3	VR-1367DQ
		HHV5	VR-538DQ
		Human coronavirus 229E	VR-740D
		Human coronavirus OC43	VR-1558D
	RNA	Enterovirus D68	VR-1823D
		Enterovirus 71	VR-1432DQ
		Rhinovirus B	VR-1663DQ
		Influenza A virus (H1N1)	VR-1736D
		Influenza B virus (BY)	VR-1813D
		Measles virus	VR-24D
		Mumps virus	VR-106D
		Parainfluenza virus 1 (PIV1)	VR-94D
		Parainfluenza virus 2 (PIV2)	VR-92D
		Parainfluenza virus 3 (PIV3)	VR-93D
		Parainfluenza virus 4b (PIV4b)	VR-1377D
		Respiratory syncytial virus A (RSVA)	VR-1540D
		Respiratory syncytial virus B (RSVB)	VR-1803D
Bacterium	DNA	*Bordetella bronchiseptica*	BAA-588D-5
		Bordetella holmesii	51541_D2
		Bordetella parapertussis	BAA-587D-5
		Bordetella pertussis	9797D-5
		Chlamydophila pneumoniae	VR-1360D
		Haemophilus influenzae	51907DQ
		Klebsiella pneumoniae	700721DQ
		Legionella pneumophila	33152DQ
		Moraxella catarrhalis	25240D-5
		Mycoplasma pneumoniae	15531D
		Staphylococcus aureus	BAA-1718DQ
		Streptococcus pneumoniae	700669DQ

* Genomic nucleic acid controls were sourced from ATCC.

Table 4. Specificity testing of respiratory pathogen assays with the ATCC inclusivity panel.*

	Adenovirus C	Adenovirus E	*Bordetella holmesii*	*Bordetella pertussis*	*Bordetella parapertussis*	*Bordetella bronchiseptica*	*Chlamydophila pneumoniae*	Coronavirus 229E	Coronavirus OC43	Enterovirus D68	Enterovirus 71	Influenza A (H1N1)	Influenza B	*Haemophilus influenzae*	HHV3	HHV5	PIV1	PIV2	PIV3	PIV4b	*Klebsiella pneumoniae*	*Legionella pneumophila*	*Moraxella catarrhalis*	*Mycoplasma pneumoniae*	Measles	Mumps	RSVA	RSVB	Rhinovirus B	*Staphylococcus aureus*	*Streptococcus pneumoniae*
AdV_1of2	17.98																														
AdV_2of2		19.84																													
B. holmesii			17.44																												
B. pertussis				17.96																											
Bordetella				17.71	17.87	17.79																									
C. pneumoniae							15.09																								
CoV_229E								22.87																							
CoV_OC43									16.35																						
EV_D68										16.80																					
EV_pan										23.14	21.85																				
Flu_A_H1												16.70																			
Flu_A_pan												14.76																			
Flu_B_pan													15.87																		
H. influenzae														17.69																	
HHV3															14.48																
HHV5																15.68															
hPIV1																	16.76														
hPIV2																		15.96													
hPIV3																			15.72												
hPIV4																				16.56											
K. pneumoniae																					14.94										
L. pneumophila																						15.76									
M. catarrhalis																							15.75								
M. pneumoniae																								15.16							
Measles																									16.72						
Mumps																										16.76					
RSVA																											14.66				
RSVB																											27.45	16.63			
RV_1of2										18.04	19.70																		17.40		
RV_2of2																													18.29		
S. aureus																														13.68	
S. pneumoniae																															13.63

* Genomic RNA or DNA at 10^3 copies/µL from 31 ATCC cultivatable respiratory pathogens were simultaneously screened against all respiratory pathogen assays (with the exception of SARS-CoV-2 assays) on TaqMan OpenArray plates. The microbial genomic samples are listed in columns, and the target assays are listed in rows. The assays specifically amplified their intended targets, and no significant off-target amplifications were detected. The shaded boxes contain the average C_{rt} values (N = 3, C_q calculated by the relative threshold method) for each assay–sample combination that passed recommended filtration criteria for respiratory pathogen assays run with the protocol for preamplification plus qPCR (where $C_{rt} \leq 28$, AmpScore ≥1.2, and C_q confidence ≥0.7). Note that the *Bordetella pertussis* sample is detected by both the *B. pertussis* and *Bordetella* assays, and the influenza A (H1N1) sample is detected by both the Flu_ A_H1 and Flu_A_pan assays. The enterovirus D68 sample is detected by both the EV_D68 and EV_pan assays, though at a much lower efficiency (C_{rt} difference of several cycles) with the EV_pan assay, which does not detect all enterovirus D68 samples. The RV_1of2 assay detects the rhinovirus B sample as well as both enterovirus D68 and 71 samples. This is expected, as the RV_1of2 assay detects both rhinovirus and enterovirus strains whereas the EV_D68 and EV_pan assays are specific for enterovirus strains. The RSVA sample is specifically detected by the RSVA assay and is also detected at a much lower efficiency (C_{rt} difference of ~13) by the RSVB assay, due to the high sequence relatedness of RSVA and RSVB.

Table 5. Respiratory pathogen exclusivity controls.

Organism type	Nucleic acid type	Organism	ATCC ID*	Near neighbor or environment
Virus	DNA	Vaccinia virus	VR-1508D	Human respiratory pathogen
	RNA	Rubella virus	VR-315D	Human respiratory pathogen
		Rotavirus	VR-2018DQ	Human gastroenteric pathogen
Bacterium	DNA	*Psychrobacter cryohalolentis*	BAA-1226D-5	*Moraxella catarrhalis*
		Pasteurella multocida	700806	*Haemophilus influenzae*
		Raoultella planticola	33531	*Klebsiella pneumoniae*
		Achromobacter xylosoxidans	27061	*Bordetella bronchiseptica, pertussis, parapertussis, or holmesii*
		Blastomyces dermatitidis	26199D-2	Human respiratory pathogen
		Corynebacterium diphtheriae	ATCC 700971D-5	Human respiratory pathogen
		Burkholderia cepacia	ATCC 25416D-5	Human respiratory pathogen
		Neisseria meningitidis	ATCC 700532D-5	Human respiratory pathogen
		Cryptococcus neoformans	MYA-565D-5	Human respiratory pathogen
		Staphylococcus saprophyticus	ATCC 15305D-5	Human respiratory pathogen
		Streptococcus mitis	ATCC 49456D-5	Human respiratory pathogen
Fungus		*Aspergillus fumigatus*	ATCC 1022D	Human respiratory pathogen

* Genomic nucleic acid controls were sourced from ATCC.

Accurate identification of respiratory pathogens in clinical research samples

The sensitivity, specificity, and accuracy of the respiratory pathogen assays on TaqMan OpenArray plates (excluding the SARS-CoV-2 assays, which were developed following completion of this large study with clinical research samples) were further examined by testing with ~400 purchased clinical research samples that were previously characterized for respiratory pathogens by various methods, including immunoassay, culture, and nucleic acid tests. The overall detection rate of the indicated respiratory pathogens in these samples by our assays was very high. Shown in Table 6 are the results of concordance analysis conducted for the ~400 samples in this set that had been characterized by commercial nucleic acid tests. Samples covering 17 key respiratory viruses were included in this study. A high positive percent agreement of over 97% with other detection platforms was observed.

Table 6. Clinical research sample testing: agreement with other nucleic acid test methods.

Pathogen	True positive*	False negative**	Positive percent agreement (%)
Adenovirus	31	1	96.88
Coronavirus 229E	2	0	100.00
Coronavirus HKU1	1	0	100.00
Coronavirus OC43	1	0	100.00
Influenza A	6	0	100.00
Influenza A/H1	7	0	100.00
Influenza A/H3	54	0	100.00
Influenza B	28	0	100.00
hMPV	25	1	96.15
hPIV	2	0	100.00
hPIV1	20	1	95.24
hPIV2	10	1	95.24
hPIV3	45	1	97.83
RSV	6	0	100.00
RSVA	40	1	97.56
RSVB	58	1	98.31
Rhinovirus, enterovirus	75	5	93.75
Total	411	12	97.16

* Call from the vendor. Note: Four hPIV3 samples were mischaracterized by a commercial vendor as hPIV1 samples, as confirmed by qPCR, Sanger sequencing, and Ion Torrent™ next-generation sequencing (NGS). An additional 5 samples sold as influenza B samples by a commercial vendor were characterized as negative for influenza B and positive for either RSVA (2 samples), RSVB, influenza A H1, or no target, as confirmed by qPCR, Sanger sequencing, Ion Torrent NGS, and an orthogonal commercial molecular test platform. The orthogonally confirmed characterizations are tallied in the true-positive column for these samples.

** Call not detected by the respiratory pathogen assays.

Our collection of TaqMan assays for respiratory pathogen detection includes other viral, bacterial, and fungal assays that are not included in the reference tests in our concordance study. In many of the clinical research samples, the respiratory pathogen assays detected additional targets that were either present or not present in the reference tests. To determine the veracity of these calls, Sanger sequencing was performed on over 200 additional targets as well as concordant target controls. However, 50 sequencing attempts did not generate sequencing results, either because the target was present in low amounts (e.g., high C_{rt} values) or because the sequencing primers did not detect the target. All 172 targets that generated sequencing results confirmed the identities of the targets that were detected by the respiratory pathogen assays (data not shown).

Following the launch of the TrueMark Respiratory Panel 2.0, OpenArray Plate, which includes assays for SARS-CoV-2, concordance testing was done using contrived samples. Inactivated noninfectious SARS-CoV-2 viral lysate (ZeptoMetrix) or inactivated intact respiratory organisms (NATtrol™ RP Multimarker 2 panel from ZeptoMetrix) were spiked into negative sample matrices (20 nasopharyngeal swabs and 20 nasal washes) to prepare contrived samples, which were processed through the entire workflow and run on the TrueMark Respiratory Panel 2.0, TaqMan OpenArray Plate. All organisms were detected, and assay cross-reactivity between SARS-CoV-2 and other coronaviruses or respiratory organisms was not observed (Table 7).

Table 7. TrueMark Respiratory Panel 2.0, OpenArray Plate, tested with contrived samples.

Pathogen	True positive (TP)	False negative (FN)	False positive (FP)	True negative (TN)*	Positive percent agreement (PPA) (%)	Negative percent agreement (NPA) (%)	Overall percent agreement (OPA) (%)
Influenza A	40	0	0	120	100	100	100
Influenza B	40	0	1	119	100	99.17	99.38
hPIV2	40	0	1	119	100	99.17	99.38
hPIV3	40	0	0	120	100	100	100
RSVA	40	0	0	120	100	100	100
Bordetella	40	0	0	120	100	100	100
B. pertussis	40	0	0	120	100	100	100
CoV_OC43	40	0	0	120	100	100	100
CoV_NL63	40	0	0	120	100	100	100
CoV_229E	40	0	0	120	100	100	100
SARS-CoV-2	40	0	0	120	100	100	100

* Three samples per matrix were used for TN results, which were used to calculate the NPA and OPA results.

Of note, 180 of the clinical research respiratory samples tested with respiratory pathogen assays on the TaqMan OpenArray plates were also tested on Applied Biosystems™ TaqMan® Array Cards. Results were highly concordant between the tests, demonstrating functional equivalence of the respiratory pathogen assays between formats. For more information, see the application note **"A complete workflow solution for detecting respiratory pathogens, including SARS-CoV-2, using TaqMan Array Cards"**.

In addition to testing with clinical research samples, TaqMan OpenArray plates were tested with whole-organism proficiency test controls from Quality Control for Molecular Diagnostics (QCMD). Three panels of QCMD samples, which consisted of both negative and positive controls covering 17 common respiratory pathogens, were used to evaluate the accuracy of the TaqMan Assays (Table 8). All control organisms were detected for 100% concordance.

Table 8. TaqMan OpenArray plates tested with QCMD proficiency test control samples.

QCMD control identity	Sample count	TaqMan OpenArray plate result
AdV type 1	1	Detected
Coronavirus NL63	2	Both detected
Coronavirus OC43	1	Detected
Enterovirus 68	1	Detected
hMPV	3	All detected
Influenza type A (H1N1)	1	Detected*
Influenza type A	3	All detected
Influenza type B	2	Both detected
Parainfluenza type 1	1	Detected
RSV type A	2	Both detected
RSV type B	2	Both detected
Rhinovirus	2	Both detected
Bordetella pertussis	1	Detected
Haemophilus influenzae	2	Both detected
Legionella pneumophila	2	Both detected
Mycoplasma pneumoniae	1	Detected
Streptococcus pneumoniae	2	Both detected
Negative	3	Confirmed
Overall	32	All detected

* The influenza type A (H1N1) sample was detected only by the Flu_A_pan assay and not the Flu_A_H1 assay. Flu assays were developed for strains from 2013 onward to capture circulating strains; the Flu_A_H1 assay detects the 2009 pandemic H1N1 strain but may not detect other older strains. Sanger sequencing analysis of the QCMD influenza type A (H1N1) sample showed sequence mismatches with the Flu_A_H1 assay probe binding site, explaining the lack of detection. The sequence matched that of an influenza A H1 strain from 2008 that was not considered in assay design.

Conclusions

- The TrueMark Respiratory Panel 2.0, OpenArray Plate, provides an accurate, reliable workflow for identification of a broad range of common and opportunistic respiratory pathogens, including SARS-CoV-2.
- The TaqMan Assays for respiratory pathogens demonstrated accurate performance in numerous tests for sensitivity and specificity with different sample types.
- The MagMAX Viral/Pathogen Nucleic Acid Isolation Ultra Kit, optimized for microbial sample preparation, provides an automated solution for extracting total nucleic acid that can be analyzed using the TrueMark Respiratory Panel 2.0, OpenArray Plate.
- The TrueMark Respiratory Panel 2.0, OpenArray Plate, offers a low-cost solution for simultaneous detection of viral and bacterial respiratory pathogens.

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Ordering information

Product	Quantity	Cat. No.
TrueMark Respiratory Panel 2.0		
TrueMark Respiratory Panel 2.0, OpenArray Plate (112-assay format, 24 samples)*	1 plate	A49044
TrueMark Respiratory Panel 2.0 PreAmp Primers	1 x 1 mL	A49049
TrueMark Respiratory Panel 2.0 Amplification Control	5 x 50 µL	A48101
Custom OpenArray plate and controls		
TaqMan OpenArray Real-Time PCR Plate with Inventoried Assays (56-assay format, 48 samples)	1 (10 pack)	4471125
TaqMan OpenArray Real-Time PCR Plate with Inventoried Assays (112-assay format, 24 samples)	1 (10 pack)	4471126
TaqMan OpenArray Real-Time PCR Plate with Inventoried Assays (168-assay format, 16 samples)	1 (10 pack)	4471127
TaqMan Universal RNA Spike-In/Reverse Transcription (Xeno) Control	5 x 200 µL	A39179
TaqMan Universal Extraction Control Organism (*B. atrophaeus*)	3 vials/kit	A39180
Master mixes		
TaqPath 1-Step RT-qPCR Master Mix, CG	5 x 1 mL	A15299
TaqMan OpenArray Real-Time PCR Master Mix	1 x 5 mL	4462164
Instrumentation and sample preparation		
KingFisher Flex Purification System with 96 Deep-Well Head	1 system	5400630
MagMAX Viral/Pathogen Ultra Nucleic Acid Isolation Kit	100 preps	A42356
QuantStudio 12K Flex Real-Time PCR System with OpenArray Block	1 system	4472380
QuantStudio 12K Flex AccuFill System	1 system	4471021
Veriti 96-Well Thermal Cycler	1 system	4375786

* This inventoried plate contains 35 assays for 32 key respiratory pathogens and for the TaqMan Universal Extraction Control Organism (*B. atrophaeus*), TaqMan Universal RNA Spike-In/Reverse Transcription (Xeno) Control, and human RNase P gene (*RPPH1*). Plates contain 3 replicates of each respiratory pathogen assay and the Xeno assay, and 2 replicates of the *B. atrophaeus* and RNase P assays. Up to 23 samples and 1 control sample can be run per plate. Further details are available in the application guide (**Pub. No. MAN0019506**).

Find out more at **thermofisher.com/respiratorypanel**

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