High-throughput pathogen detection using a novel MagMAX kit and OpenArray technology

Introduction

Technologies for the detection and isolation of pathogens (such as certain bacteria, viruses, and fungi) have existed in the research and medical communities for several decades, and they continue to evolve and expand exponentially. Techniques for determining the presence of specific pathogens range from culture, viral plaque, and ELISA assays to more recently developed molecular techniques such as PCR and sequencing. Despite providing tremendous value, many of these methods are labor intensive and time consuming—some taking days or weeks to provide confirmation. In addition, sensitivity can vary, which means inconsistent and/or inconclusive results—a downside when speed and accuracy are critical.

Recently, pathogen detection methods have evolved to include nucleic acid–based tests—primarily real-time PCR [1] and next-generation sequencing—which do not have these shortcomings. There is room for further improvement, however, as these methods often rely on time-consuming sample manipulation and can require multiple kits or protocols for different sample or pathogen types.

In this two-part study using clinical research samples containing respiratory pathogens, we demonstrate the advantages of a new high-throughput and automatable workflow, which combines the speed, sensitivity, and simplicity of the Applied Biosystems™ MagMAX™ nucleic acid extraction chemistry and a Thermo Scientific™ KingFisher™ instrument with the power and flexibility of the Applied Biosystems™ TaqMan® OpenArray™ technology and QuantStudio™ 12K Flex Real-Time PCR System to deliver a single detection method for multiple sample and pathogen types.

Materials and methods

Clinical research samples

Clinical research samples positive for respiratory pathogens (>300) were acquired from multiple human sources and stored at –80°C until nucleic acid extraction could be performed. These samples covered a wide range of demographics as well as collection and storage methods (Figure 1A). Positive detection of pathogens had been determined using a variety of testing methods prior to our receipt of the samples (Figure 1B).

A. Sample types
BAL (bronchoalveolar lavage)
ETA (endotracheal aspirate)
Lithium heparin plasma
Nasopharyngeal (NP) swab
Serum
Swab
Trach
UTM; NP swab
VTM M5
VTM; bronchial
VTM; nasal/nares
VTM; nasal was
VTM; nasal throat
M4 microtest
M6 microtest

B. Test type	Initial detection test	No. of targets in panel	No. of samples
Molecular diagnostic	GenMark eSensor RVP Panel	14	206
	GenMark ePlex RP Panel	17	23
	Luminex NxTAG Respiratory Pathogen Panel	20	31
	SolGent DiaPlexC RV13 Detection PCR	13	8
	Bio-Rad Bio-Plex 2200	1	2
	Cepheid SmartCycler	1	3
	Focus Simplexa	1–3	2
	Respiratory virus panel by real-time PCR	1–3	12
Immunochromatography	Alere BinaxNOW (RSV)	1	13
	Alere BinaxNOW Influenza A&B card	2	6
	bioMérieux API/ID32	15	2
	QuickVue Influenza A+B	2	2
Staining	IFA/Manual/FA Stain	N/A	4
	Inova QUANTA-Lyser	N/A	2
Culture	Culture	N/A	12
ELISA	ELISA DSX Wampole	N/A	5

UTM: universal transport medium; VTM: viral transport medium

Figure 1. Clinical research sample information. (A) Test sample types used, covering a wide range of collection methods. **(B)** Tests used to determine the presence of pathogens.

Controls

In the first part of the study, we used intact-organism (viral and bacterial) spike-in controls for a small subset of bronchoalveolar lavage (BAL) samples as an initial test of the extraction and detection workflow.

Total nucleic acid isolation

Samples were thawed on ice, and total nucleic acid was extracted using the Applied Biosystems™ MagMAX™ Viral/Pathogen Ultra Nucleic Acid Isolation Kit, in batches of up to 96 samples. The enzyme mix was added to 200–400 µL of each sample (with or without spike-in control), and the samples were then placed onto the Thermo Scientific™ KingFisher™ Flex instrument for heated enzymatic treatment. After the incubation, a mixture containing binding solution and magnetic beads was added to each sample, and then the samples were placed back onto the instrument for the binding, washing, and elution steps. Once elution was complete, samples were stored temporarily on ice, or at –80°C for longer storage, until analysis could be performed.

Real-time PCR analysis

Before analysis with real-time PCR, the extracted nucleic acid was first preamplified using Applied Biosystems™ TaqPath™ 1-Step RT-qPCR Master Mix, CG, to maximize sensitivity: 5 µL of sample was combined with the master mix and Applied Biosystems™ TaqMan® respiratory tract microbiota (RTM) primer pool for 14 cycles of preamplification. Once preamplification was complete, the samples were diluted 1:10 with nuclease-free water and stored on ice. Then 2.5 µL of the diluted preamplified sample was combined with 2.5 µL of the Applied Biosystems™ TaqMan® OpenArray™ Real-Time PCR Master Mix and placed in the Applied Biosystems™ OpenArray™ AccuFill™ instrument to prepare the Applied Biosystems™ TaqMan® OpenArray™ Respiratory Tract Microbiota (RTM) Plate (Version A). Once prepared, the plates were run on the Applied Biosystems™ QuantStudio™ 12K Flex instrument and the data analyzed.

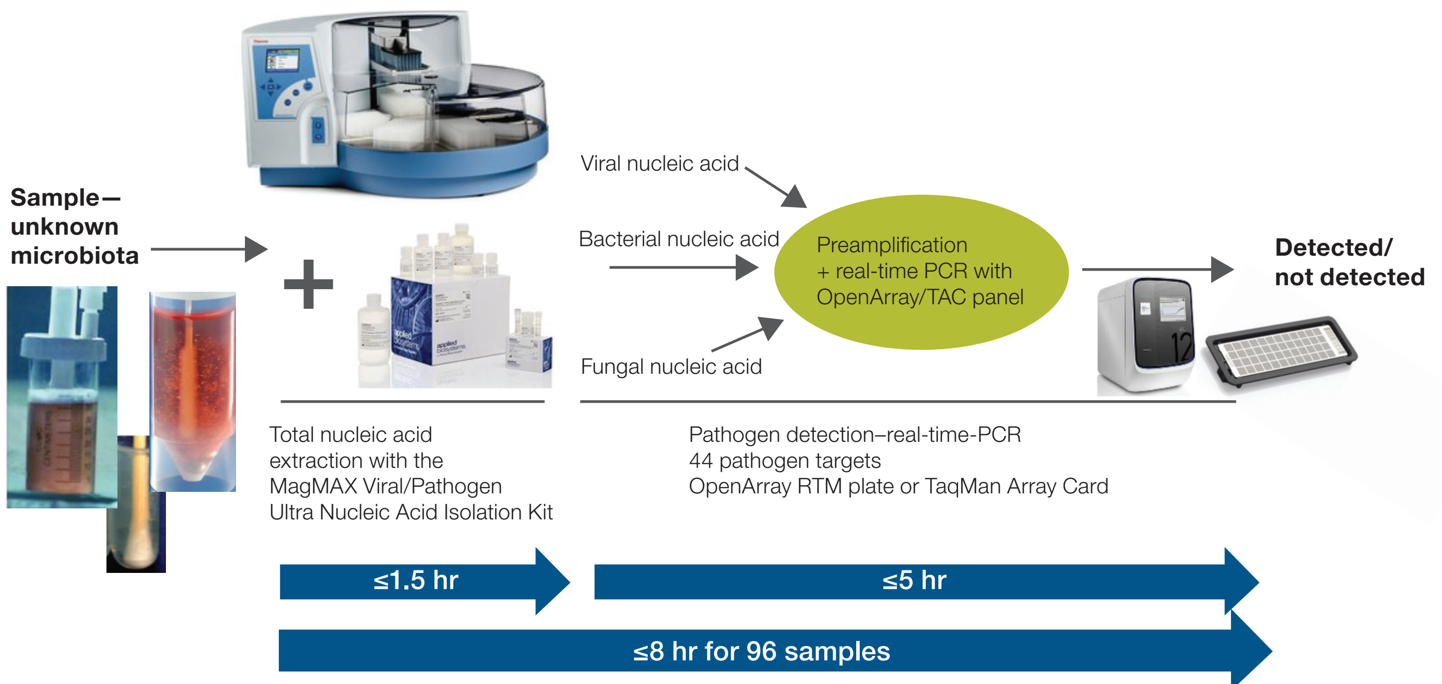


Figure 2. Workflow for detection of respiratory tract pathogens. The workflow shows extraction of total nucleic acid from samples, using the MagMAX Viral/Pathogen Ultra Nucleic Acid Isolation Kit, followed by preamplification and real-time PCR analysis using OpenArray or Applied Biosystems™ TaqMan® Array Card (TAC) panels. Results provide a detected/not detected call for each of 44 pathogen targets that include bacteria, RNA and DNA viruses, and fungi.

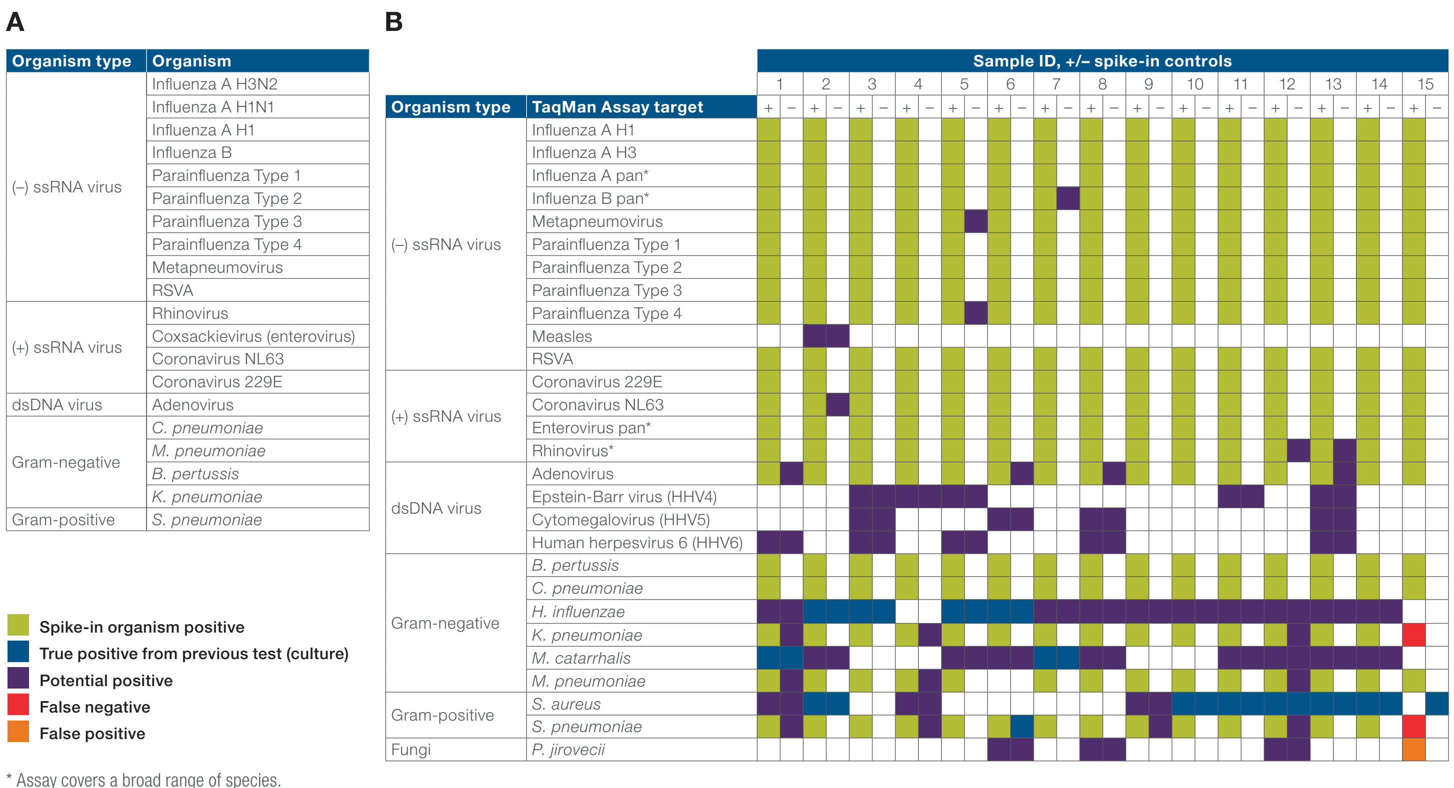
Results

Identification of positives and false negatives

Total nucleic acid was extracted from 15 BAL samples with and without intact-organism spike-in controls and then analyzed by real-time PCR using the OpenArray RTM plate (Figure 3). As expected, spike-in samples showed positive detection for corresponding targets in the controls (green), with only two false negatives (red) and one false positive (orange)—a ~99% accuracy rate. True positives, as previously determined by culture, were also successfully detected in both the +/– spike-in samples (blue), as well as a range of potential positive targets not previously confirmed (purple).

Isolation and analysis with and without spike-in controls

Based on the success of part one, total nucleic acid was isolated from the remaining samples (consisting of a range of types) in batches of 96 samples, using the same extraction and OpenArray plate analysis workflow but without any spike-in control organisms. Results were then compared to previous detection results provided by the vendor. Expected positive samples as well as target information from the molecular test panels and the OpenArray RTM plate can be seen in Figure 4A. When comparing just the molecular test results, the MagMAX/OpenArray RTM workflow showed a ~91% concordance rate with previously confirmed targets as well as positive detection of 68 targets not already detected, despite their being present in the test panels (Figure 4B). Similarly, when comparing results from all previous tests, the MagMAX/OpenArray RTM workflow again showed ~91% concordance rate with previously confirmed targets. We also saw detection of a large number of additional targets that weren't detected previously—most likely a combination of targets not included in the previous panels, false negatives, false positives, and background flora in the sample. Subsets of these additional targets were confirmed positive by orthogonal testing methods, indicating that a majority are likely true positive samples.



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Organism type	Organism
(–) ssRNA virus	Influenza A H3N2
	Influenza A H1N1
	Influenza A H1
	Influenza B
	Parainfluenza Type 1
	Parainfluenza Type 2
	Parainfluenza Type 3
	Parainfluenza Type 4
	Metapneumovirus
	RSVA
(+) ssRNA virus	Rhinovirus
	Coxsackievirus (enterovirus)
	Coronavirus NL63
	Coronavirus 229E
dsDNA virus	Adenovirus
Gram-negative	*C. pneumoniae*
	M. pneumoniae
	B. pertussis
	K. pneumoniae
Gram-positive	*S. pneumoniae*

* Assay covers a broad range of species.

Figure 3. Target detection in BAL samples with or without spike-in control organisms. (A) Intact organisms spiked into the lavage samples prior to nucleic acid extraction and analysis. **(B)** Detection results after real-time PCR with preamplification.

Conclusions

With our new workflow, which uses the MagMAX Viral/Pathogen Ultra Nucleic Acid Isolation Kit with a KingFisher instrument, and TaqMan OpenArray technology on the QuantStudio 12K Flex Real-Time PCR System, we successfully extracted nucleic acid from a range of clinical research respiratory samples and then matched detection results with those of existing tests (both molecular and other) on the market. In addition, the compatibility of the nucleic acid extraction with different pathogen types and the expanded target coverage of the OpenArray RTM plate and assays allow for increased detection capability with minimal increase in time-to-results.

Here we have demonstrated the usefulness of this workflow for detection of respiratory pathogens, but it can also be applied to other areas of infectious disease research that would benefit from a fast and comprehensive detection method.

Reference

1. Caliendo AM (2011) Multiplex PCR and emerging technologies for the detection of respiratory pathogens. *Clin Infect Dis* 52 Suppl 4:S326-30.

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Organism type	Target	Expected positive	Molecular test targets	OpenArray targets
(–) ssRNA virus	Influenza A	◊	•	Δ
	Influenza A H1	◊	•	Δ
	Influenza A H3	◊	•	Δ
	Influenza B	◊	•	Δ
	Parainfluenza Type 1	◊	•	Δ
	Parainfluenza Type 2	◊	•	Δ
	Parainfluenza Type 3	◊	•	Δ
	Parainfluenza Type 4	◊	•	Δ
	Metapneumovirus	◊	•	Δ
	RSV	◊	•	Δ
	RSVA	◊	•	Δ
	RSVB	◊	•	Δ
	Measles			Δ
	Mumps			Δ
(+) ssRNA virus	Human parechovirus (HPeV)			Δ
	Enterovirus	◊	•	Δ
	Rhinovirus	◊	•	Δ
	Coronavirus OC43		•	Δ
	Coronavirus 229E		•	Δ
	Coronavirus NL63		•	Δ
	Coronavirus HKU1		•	Δ
dsDNA virus	Adenovirus	◊	•	Δ
	Varicella zoster virus (HHV3)			Δ
	Epstein-Barr virus (HHV4)			Δ
	Cytomegalovirus (HHV5)			Δ
	Human herpesvirus 6 (HHV6)			Δ
ssDNA virus	Human bocavirus		•	Δ
Gram-negative	*B. pertussis*	◊		Δ
	B. holmesii			Δ
	C. burnetii			Δ
	Bordetella			Δ
	H. influenzae	◊		Δ
	C. pneumoniae		•	Δ
	M. pneumoniae		•	Δ
	K. pneumoniae			Δ
	M. catarrhalis			Δ
Gram-positive	*S. aureus*			Δ
	S. pneumoniae			Δ
Fungi*	*P. jirovecii*			Δ

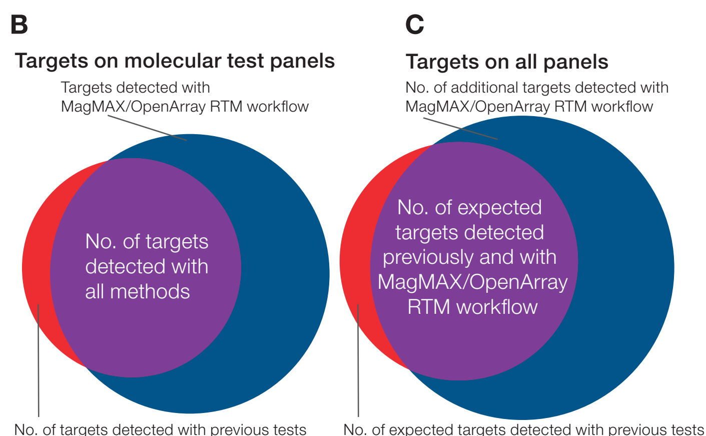

Figure 4. Target detection in clinical research samples. (A) List of targets by name and organism type. Diamonds indicate the expected positive detections based on previous test results; circles indicate the targets on the molecular test panels (vs. detection by immunochromatography or culture/staining); triangles indicate the targets on the OpenArray RTM plates. **(B)** Venn diagram showing comparison of results from previous molecular panels and results from the MagMAX/OpenArray RTM workflow. **(C)** Venn diagram showing comparison of results from all previous test methods and the OpenArray RTM workflow.

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