Timely Detection and Subtyping of Upper Respiratory Pathogens Using a Microfluidics-Based Workflow

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Introduction

Timely detection and accurate identification of infectious pathogens including subtypes is key in developing tools used by public health programs to conduct outbreak surveillance and management. This can help shorten outbreak duration and support identification of the causative agent(s) so that appropriate corrective measures can be designed and implemented. Molecular methods such as polymerase chain reaction (PCR) and next-generation sequencing (NGS) routinely contribute to the effort by allowing multiple pathogens to be genomically profiled at the same time, and, in some cases, without a dependency on isolate culture. This effectively reduces the time and testing needed to identify pathogens present in samples collected from an outbreak.

In this study, we demonstrate proof of concept and describe a microfluidics-based protocol designed to detect and identify subtypes of 16 common upper respiratory pathogens in up to 48 samples from a single run. The protocol employs an automated workflow starting with nucleic acid mixed with or derived from saliva (extraction-free). The use of nanoliter-scale microfluidic reactions conserves precious reagents while reducing plastic waste and enabling sustainable lab operations.

Following preparation of sample and assay mixes, which are dispensed into a microfluidic chip that is subsequently loaded onto the Biomark™ X9 System for High-Throughput Genomics for processing, results are available in two hours without manual intervention. Each of the chip's 48 assay inlets connects with an independent reaction chamber, which enables all assays to share a common fluorophore and thermal profile while preventing assay-to-assay interference associated with multiplex reactions. Based on the chip's open architecture, assays can be added to multiple inlets to generate replicate datapoints per sample, which can increase confidence in results. Additional assays can be added easily to detect more pathogens or to further subtype pathogens of particular interest.

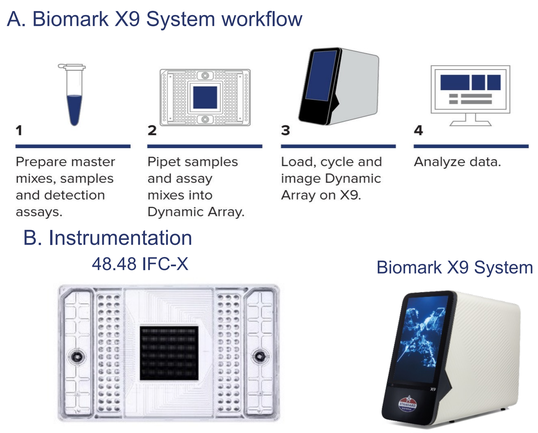
The current list of targeted pathogens tested includes influenza A, influenza B, respiratory syncytial virus (RSV), SARS-CoV-2, human coronaviruses HKU1, NL63, OC43 and 229E, human metapneumovirus, enterovirus, human parainfluenza viruses 1–4, adenovirus and rhinovirus. Influenza A subtypes H1N1, H3N2 and H5N1 and RSV subtypes A and B were also included for additional subtyping. Commercially available control material from ZeptoMetrix was tested and accurate detection of expected targets was demonstrated, including further subtyping of influenza A H1N1, H3N2 and RSV A and B. Extracted nucleic acid from avian influenza-positive pasteurized milk was obtained from the University of Wisconsin. The microfluidics-based workflow described offers a rapid, scalable and environmentally sustainable solution for pathogen detection and subtyping, with significant potential to enhance public health response during outbreaks.

Materials and methods

Well-characterized, commercially available samples for the viral targets (PN NATRVP2.1-BIO, NATFVP-NNS and NATEVP-C) were purchased from ZeptoMetrix. Equal volumes of control material and donor saliva were mixed and RNAsecure RNase Inactivation Reagent (Thermo Fisher Scientific, PN AM7005) was added to the saliva-control mixtures to a final concentration of 1x. The mixtures were subjected to heat denaturation at 90 °C for 10 minutes to extract the viral RNA*. A targeted one-step reverse transcription/preamplification was performed on a standard thermocycler using Standard BioTools™ Advanta™ RT PA MM (RT-preamplification master mix), and the cDNA was diluted 1:5 in DNA Suspension Buffer (Teknova, PN T0227). The diluted cDNA was mixed with Advanta PCR MM and sample loading reagent and loaded into the sample inlets of a Standard BioTools 48.48 Dynamic Array™ IFC-X Real-Time PCR. Individual probe-based assays for the viral targets and RNase P as an internal control were mixed with assay loading reagent and added to the assay inlets of the 48.48 IFC-X. The IFC was placed in a Biomark X9 System for sample-assay mixing, cycling and data capture. Data was analyzed using Standard BioTools Real-Time PCR Analysis Software.

*The H5N1 positive sample was not subjected to heat denaturation nor mixed with saliva.

Figure 1. Workflow and instrumentation



Results

Successful amplification and identification of all viral targets were achieved using the 48.48 IFC-X on the Biomark X9 System. The unique design of the 48.48 IFC-X features 2,304 individual nanoliter reaction chambers delivering multiplex data output in simple singleplex design and setup. The heat map depicts data for each reaction chamber and highlights amplified viral and RNase P internal control targets using cycle threshold (Ct) values, which are color-coded (Figure 2). The heat map data shows specificity of the assays as indicated by no cross-reactivity with the other pathogens analyzed in the panel. In instances in which an additional subtyping assay was used, the subtype was positively identified and did not cross-react with other subtypes within the same species. For example, influenza A has a pan-universal gene target that identifies a sample as positive for influenza A, but with the unique design of the IFC, each influenza A positive sample can be simultaneously subtyped when subtyping assays are added for their detection. The heat map data and amplification curves (Figure 3) further show that for influenza A H1, no cross-reactivity is observed with the H1N1 pandemic strain and assay. One of the features of Standard BioTools Real-Time PCR Analysis Software is the ability to write custom interpretive scripts to generate a report that identifies which pathogen is detected in a sample. An example of an output file is depicted in Figure 4. The use of replicate assays loaded in the IFC can be leveraged in the interpretive software to give greater precision of the call in the interpretive report.

Figure 2. IFC amplification heat map

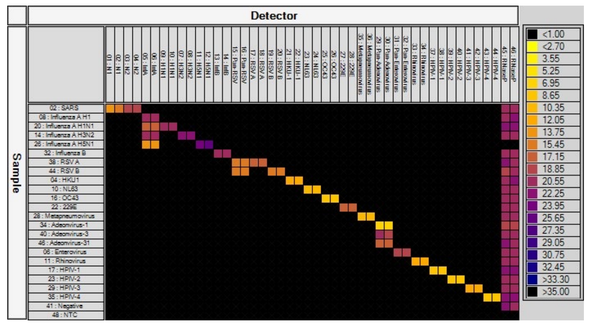


Figure 2. Heat map of targeted amplification showing each sample (rows) tested by each targeted assay in duplicate (columns). Each square represents an individual reaction chamber and has been automatically color-coded according to the legend on the right.

Figure 3. Influenza A amplification curves

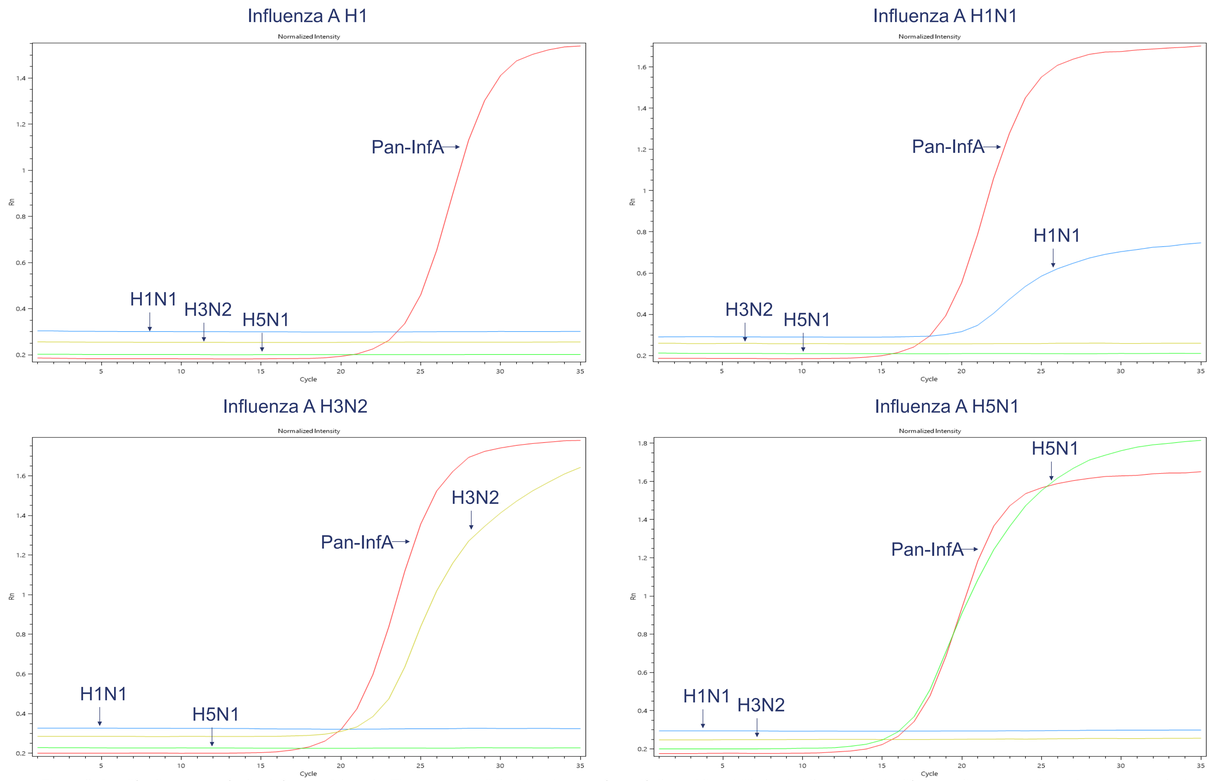


Figure 3. Amplification curves for the influenza A targets. The curves exhibit a high degree of specificity and the ability to simultaneously identify the pathogen and its subtype when subtyping assays are included.

Figure 4. Custom interpretive software

Sample Name	Detected	Sample Name	N1	N2	InfA	H1N1	H3N2	H5N1	InfB	RSV Pan	RSV A	RSV B	HKU1	NL63	OC43	229E	PIV1	PIV2	PIV3	PIV4	Adenovirus	Enterovirus	Metapneumovirus	Rhinovirus	RNase P
Sample 1	SARS	SARS	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Sample 2	FluA	FluA	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Sample 3	FluA (H1N1)	H1N1	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Sample 4	FluA (H3N2)	H3N2	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Sample 5	FluA (H5N1)	H5N1	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Sample 6	FluB	FluB	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Sample 7	RSV (A)	RSV A	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Sample 8	RSV (B)	RSV B	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
Sample 9	HKU1	HKU1	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
Sample 10	NL63	NL63	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+
Sample 11	OC43	OC43	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
Sample 12	229E	229E	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+
Sample 13	Parainfluenza Virus-1	PIV1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+
Sample 14	Parainfluenza Virus-2	PIV2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+
Sample 15	Parainfluenza Virus-3	PIV3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
Sample 16	Parainfluenza Virus-4	PIV4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+
Sample 17	Adenovirus	Ad1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
Sample 18	Adenovirus	Ad3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
Sample 19	Adenovirus	Ad31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
Sample 20	Enterovirus	Entero	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+
Sample 21	Metapneumovirus	Meta	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
Sample 22	Rhinovirus	Rhino	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Negative		Negative	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
NTC		NTC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 4. Interpretive software for samples and assays tested. Individual assays are marked with "+" when amplified and "-" when not amplified. When all criteria for positive identification are met, the software returns which pathogen is detected and which subtype, when applicable [for example, FluA (H5N1)]. The software is flexible: Users can add/remove targets and subtypes.

Conclusion

In this study, we have shown the ability to quickly screen for 16 common respiratory pathogens, plus influenza A and RSV subtypes, in a single run. The power of Standard BioTools microfluidic technology is in its ability to quickly test any sample for a multitude of targets in singleplex and in one fluorescent channel. This is achieved with just a few microliters of sample and master mix, cutting down on cost. Additionally, since the IFC digitizes the samples and assays into individual reaction chambers, assay design is simplified and the risk of cross-reactivity associated with multiplex detection systems is mitigated. IFCs utilize an open architecture that makes it easy to change, add, subtract and replicate assays on an as-needed basis. This feature can prove to be vital when outbreaks occur, enabling quick pathogen detection and identification for epidemiological tracking. The ability to simultaneously subtype a pathogen cuts down on time and resources during an outbreak. The ability to perform replicate assays within the same IFC reduces variability and gives greater confidence in the data collected before reporting. Further studies are planned to explore the use of an extracted viral genome, as well as the addition of assays targeting bacterial pathogens and subtypes of interest.

Acknowledgements

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