Broad-Spectrum Detection and Subtyping of Respiratory Pathogens Using an Expanded 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 25 common upper respiratory pathogens in up to 48 or 96 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 integrated fluidic circuit (IFC) 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 IFC's 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 IFC'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 easily added 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, enterovirus D68, human parainfluenza viruses 1–4, adenovirus, rhinovirus, measles, *Bordetella pertussis* and *Streptococcus pneumoniae*. Influenza A subtypes H1N1, H3N2 and H5N1 and RSV subtypes A and B were also included for additional subtyping. Control materials sourced from ZeptoMetrix, genomic RNA provided by ATCC, synthetic controls from Twist and gBlocks synthetic controls from IDT were evaluated using the Standard BioTools™ 48.48 IFC-X, comprising 2,304 individual nanoliter reaction chambers, and the 96.96 Dynamic Array™ IFC, comprising 9,216 individual nanoliter reaction chambers. These different IFC formats were utilized to test for precise detection and concurrent subtyping, where applicable.

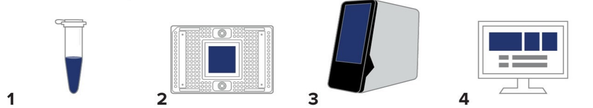
Materials and methods

Well-characterized, commercially available samples for viral and bacterial targets (PN NATRST-BIO, NATRVP-NNS and NATEVPOS-6MC, 0810164CFHI) were purchased from ZeptoMetrix. *Streptococcus pneumoniae* (33400D-5), enterovirus D68 strain US/MO/14-18947 (VR-1823D) and synthetic human coronavirus strain HKU1 RNA (VR-3262SD) were purchased from ATCC. gBlocks synthetic controls from IDT were designed for measles wild type (wt) and vaccine strain (vac) and transcribed by *in vitro* transcription. 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 pathogen nucleic acid. A targeted one-step reverse transcription/preamplification, cDNA dilution, sample and assay premixes, and IFC loading were set up following the Viral Pathogen Detection with Custom Assays Using the 48.48 Dynamic Array IFC-X and the Biomark X9 System Technical Note. Reagent prep volumes for the sample premix setup were doubled for the 96.96 Dynamic Array IFC and loaded according to the Biomark X9 System User Guide. Data was analyzed using Standard BioTools Real-Time PCR Analysis Software.

* Synthetic controls were spiked into inactivated saliva that followed the same heat inactivation process as detailed above.

Figure 1. Workflow and instrumentation

A. Biomark X9 System workflow



B. Instrumentation

Results

All pathogen targets were successfully and accurately amplified and identified using the 48.48 IFC-X and 96.96 Dynamic Array IFC on the Biomark X9 System. Figure 2 below displays the 96.96 Dynamic Array IFC infrastructure and how it works. The Standard BioTools Real-Time PCR Analysis Software processes the data and provides outputs for result interpretation. Amplification curves demonstrate assay performance and support simultaneous subtyping (for example, influenza A, Figure 3) and multi-target identification (for example, *Bordetella* spp., Figure 4). Its heat map feature displays a color-coding system for Ct values for each reaction chamber, highlighting the specificity of detected pathogens and the RNase P internal control (Figure 5). The software also enables data export for custom report generation using the data collected from the heat map.

Figure 2. IFC infrastructure

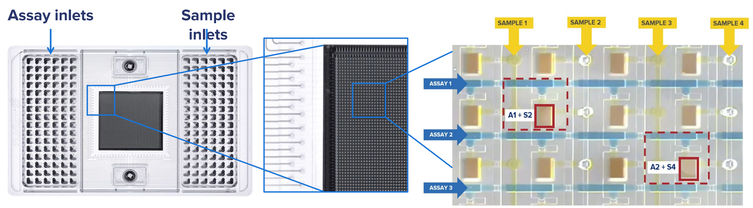


Figure 2. The infrastructure of the 96.96 Dynamic Array IFC. Individual assays and samples are loaded into their respective inlets and the Biomark X9 System automates the reaction assembly at a nanoliter scale. Each sample is processed with every assay in dedicated reaction chambers, allowing for single fluorescent channel probes, simplifying assay panel design.

Figure 3. Influenza A amplification curves

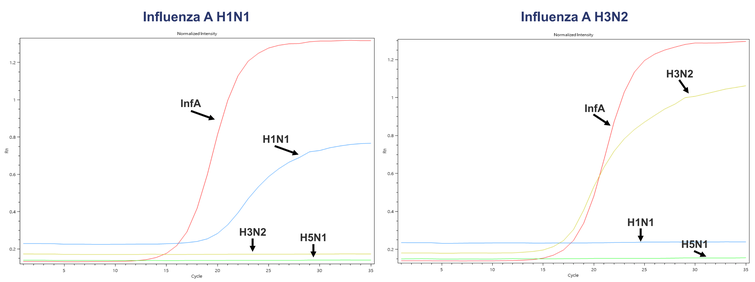


Figure 3. Amplification curves for influenza A H1N1 and H3N2 feature the specificity of each influenza A assay on the IFC.

Figure 4. *Bordetella* spp. identification

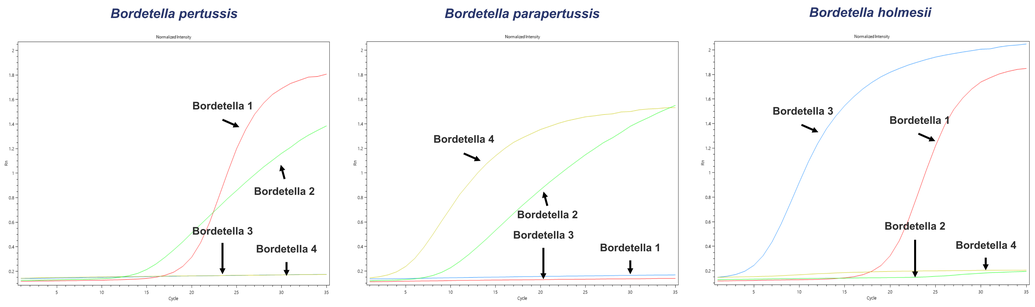


Figure 4. Amplification curves for the *Bordetella* spp. targets. The amplification plots illustrate how each *Bordetella* species can be accurately and efficiently identified and differentiated from one another, facilitating the prompt detection of potential outbreaks. Specifically, Bordetella 1 + Bordetella 2 correspond to *B. pertussis*; Bordetella 2 + Bordetella 4 correspond to *B. parapertussis*; and Bordetella 1 + Bordetella 3 indicate *B. holmesii*.

Figure 5. IFC amplification heat maps

Figure 5a. 48.48 IFC

Figure 5b. 96.96 IFC

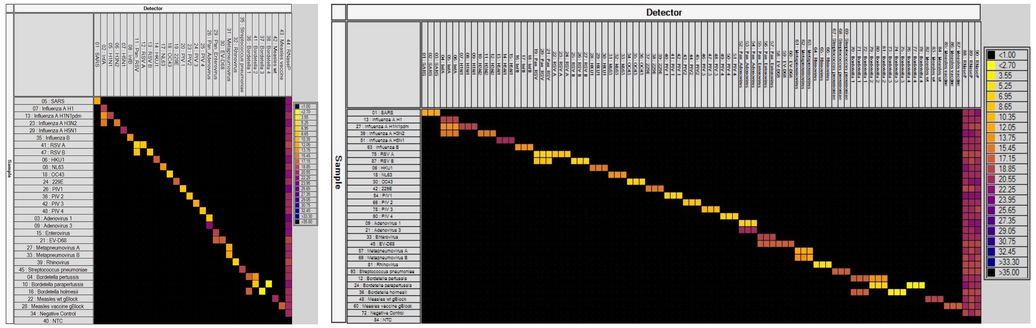


Figure 5. Heat maps of targeted amplification, with each row representing an individual sample tested in duplicate by each targeted assay shown in columns. Each square corresponds to a single reaction chamber and is automatically color-coded based on the legend provided on the right. The identical RT-PA samples were applied across both IFC formats. Comparable laboratory procedures are employed for both IFC formats, enabling rapid transition between formats if sample volume or target pathogens rise.

CONCLUSIONS

Key points of Standard BioTools microfluidic technology

- Enables rapid, simultaneous screening of multiple pathogens and eliminates reflex testing required for subtyping
- The integrated fluidic circuit (IFC) reduces sample and reagent volumes for overall cost savings
- The open platform allows for adding, removing or replicating assays with ease and little optimization to respond to new variants
- Individual reaction chambers allow for a single fluorescent channel with single assay detection, eliminating cross-reactivity concerns between similar pathogens
- Multiple IFC formats facilitate rapid adjustment to throughput requirements in response to an outbreak

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