A Two-Hour Microfluidics-Based Detection Workflow for Concurrent Detection and Identification of Common Upper Respiratory Pathogens

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INTRODUCTION

Timely detection and accurate identification of infectious pathogens are 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) allow 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 four upper respiratory pathogens in up to 48 samples from a single run using an automated workflow starting with nucleic acid 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 an 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.

The current list of targeted pathogens includes influenza A, influenza B, respiratory syncytial virus and SARS-CoV-2 and can be further expanded and customized using the open architecture of the IFC. Each of the IFC'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.

MATERIALS AND METHODS

Well-characterized, commercially available samples for the viral targets [NATtrol™ SARS-CoV-2 External Run Control (PN NATSARS(COV2)-ERC) and Flu Verification Panel (PN NATFVP-NNS)] were purchased from ZeptoMetrix®. Equal volumes of control material and donor saliva were mixed together and RNA*secure*™ 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. 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 followed by a custom interpretative script.

Figure 1: workflow and instrumentation

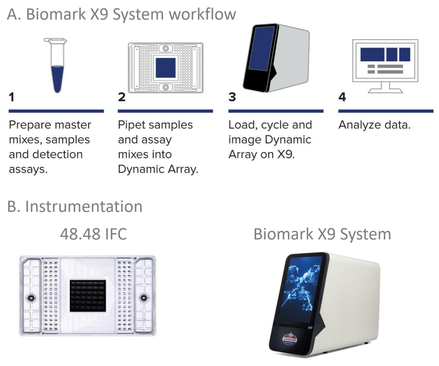

Figure 2: IFC amplification heat map

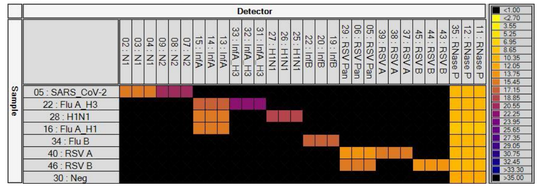

Figure 2: Heat map of targeted amplification showing each sample (rows) tested by each targeted assay in triplicate (columns). Each square represents an individual reaction chamber and has been automatically color-coded according to the legend on the right.

Figure 3: amplification curves

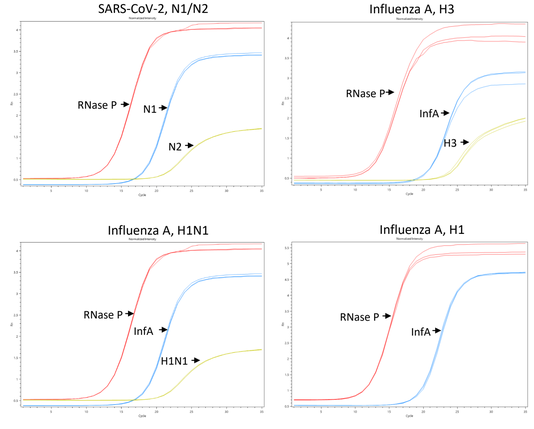

Figure 3: Amplification curves for each control tested. Each assay was run in triplicate and the curves exhibit the high degree of reproducibility for each assay designed and tested.

Figure 4: custom interpretive software

Sample Name	N1	N2	InfA	InfB	RSV Pan	RNase P	COVID-19	InfA	InfB	RSV
SARS_CoV-2	+	+	-	-	-	+	**Detected**	Not Detected	Not Detected	Not Detected
Flu A_H3	-	-	+	-	-	+	Not Detected	**Detected**	Not Detected	Not Detected
H1N1	-	-	+	-	-	+	Not Detected	**Detected**	Not Detected	Not Detected
Flu A_H1	-	-	+	-	-	+	Not Detected	**Detected**	Not Detected	Not Detected
Flu B	-	-	-	+	-	+	Not Detected	Not Detected	**Detected**	Not Detected
RSV A	-	-	-	-	+	+	Not Detected	Not Detected	Not Detected	**Detected**
RSV B	-	-	-	-	+	+	Not Detected	Not Detected	Not Detected	**Detected**
Neg	-	-	-	-	-	+	Not Detected	Not Detected	Not Detected	Not Detected

Figure 4: Interpretive software for samples and assays tested. Individual assays are marked with "+" when amplified and "-" when not amplified. The software returns "Detected" when all criteria for positive identification are met for the specific disease/pathogen. This version of the software was written to identify targets but not subtyping. The software is flexible: Users can add additional targets and subtyping features.

RESULTS

Successful amplification and identification of viral targets were achieved using the 48.48 IFC on the Biomark X9 System. Heat map data depicts the reaction chambers that amplified viral targets using cycle threshold (Ct) values, which are color-coded (Figure 2). Additionally, 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 subtype assay was used, the subtype was positively identified and did not cross-react with other subtypes within the same species. For example, a swine flu (H1N1) assay was designed to specifically react with H1N1 and not other H1 subtypes. The heat map data and amplification curves (Figure 3) show that for influenza A H1, no cross-reactivity is observed with the H1N1 assay. One of the features of the 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 (Figure 4). The use of replicate assays loaded in the IFC is leveraged in the interpretive software to give greater confidence in the call of Detected vs. Not Detected output in the interpretive report.

CONCLUSION

In this study, we show the ability to quickly screen for the most common respiratory pathogens 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 can be done with just a few microliters of sample and master mix, cutting down on cost. Since the IFC digitizes the samples and assays into individual reaction chambers, assay design is simplified, and cross-reactivity is less of a concern than in multiplex detection systems. The IFCs are open, allowing for flexibility in the assays an end user wishes to test as well as the ability to quickly add or subtract assays on a per needed basis. The ability to perform replicate assays within the same IFC reduces variability and gives greater confidence in the data collected before reporting. Further studies for this panel will include using extracted viral genome, the addition of more targets and subtyping assays and a more detailed interpretive software that will further report the subtype for pathogens that require it.

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