APPLICATION NOTE

Detection and Subtyping of Viral Pathogens Using Custom qPCR Assays on the Biomark X9 System

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 PCR and 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 common upper respiratory pathogens in up to 48 samples from a single run using 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 2 hr 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. Assays can be added to multiple chip inlets to generate replicate datapoints per sample, which increases confidence in results. Additional assays can be added easily to detect more pathogens or to further subtype each.

The current list of targeted pathogens (viral targets) tested includes influenza A, influenza B, respiratory syncytial virus (RSV), SARS-CoV-2, human coronaviruses HKU1, NL63, OC43 and 229E, human metapneumovirus, enterovirus and human parechovirus. Influenza A subtypes H1N1 and H3N2 and RSV subtypes A and B were also included for additional subtyping.

Materials and methods

Well-characterized, commercially available samples for the viral targets were purchased from ZeptoMetrix (see Appendix A). Pathogen-specific primers and probes were purchased from IDT. Equal volumes of control material and donor saliva were mixed together and diluted in PBS, and RNAsecure (Thermo Fisher Scientific, 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 min to extract the viral RNA. A targeted 1-step reverse transcription (RT)/preamplification was performed in triplicate on a standard thermocycler using Standard BioTools Advanta™ RT-Preamp Master Mix (102-0419), and the preamplified DNA was diluted 1:5 in DNA Suspension Buffer (Teknova, T0227). The diluted DNA was mixed with Advanta PCR MM (102-0420) and 20X GE Sample Loading Reagent (85000735) and added to the sample inlets of a Standard BioTools 48.48 Dynamic Array™ IFC-X Real-Time PCR. Individual probe-based assays and RNase P (as an internal control) were mixed with 2X Assay Loading Reagent (85000736) and added in duplicate to the assay inlets of the 48.48 IFC-X (integrated fluidic circuit for the Biomark X9 System). 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.

For a more detailed description of the methods used in this study, view our technical note: **Viral Pathogen Detection with Custom Assays Using the 48.48 IFC-X and the Biomark X9 System**.

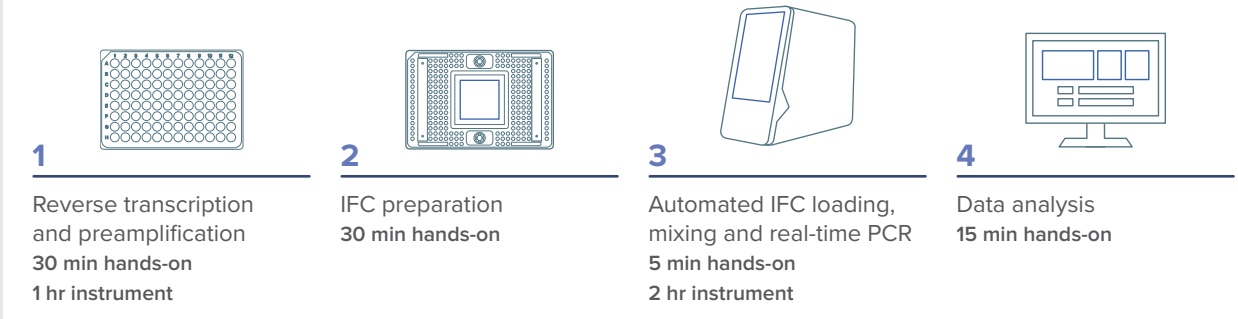

Figure 1. Workflow and instrumentation

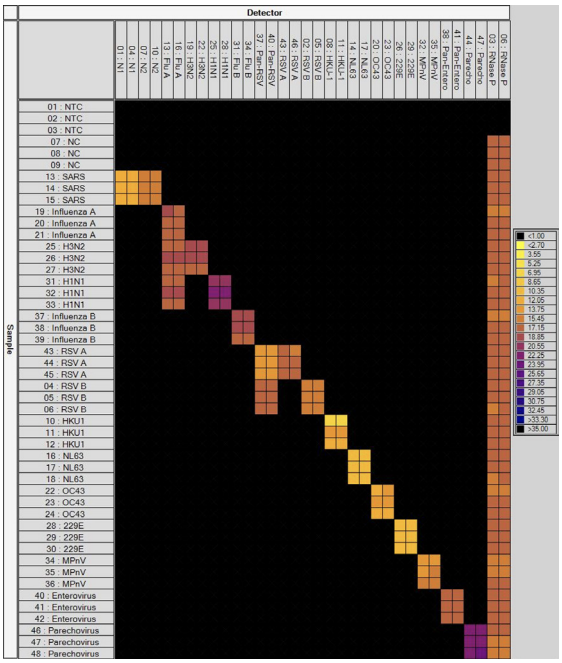

Figure 2. IFC amplification and heat map. See Appendix A for which control tubes correspond to the controls represented in this heat map.

Results

Successful amplification and identification of viral targets was achieved using the 48.48 IFC-X 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 strain), no cross-reactivity is observed with the H1N1 assay. One of the features of Standard BioTools Real-Time PCR Analysis Software is the ability to automatically execute custom interpretive scripts to generate a report that identifies which pathogen is detected in a sample. For more information on this, please contact techsupport@standardbio.com.

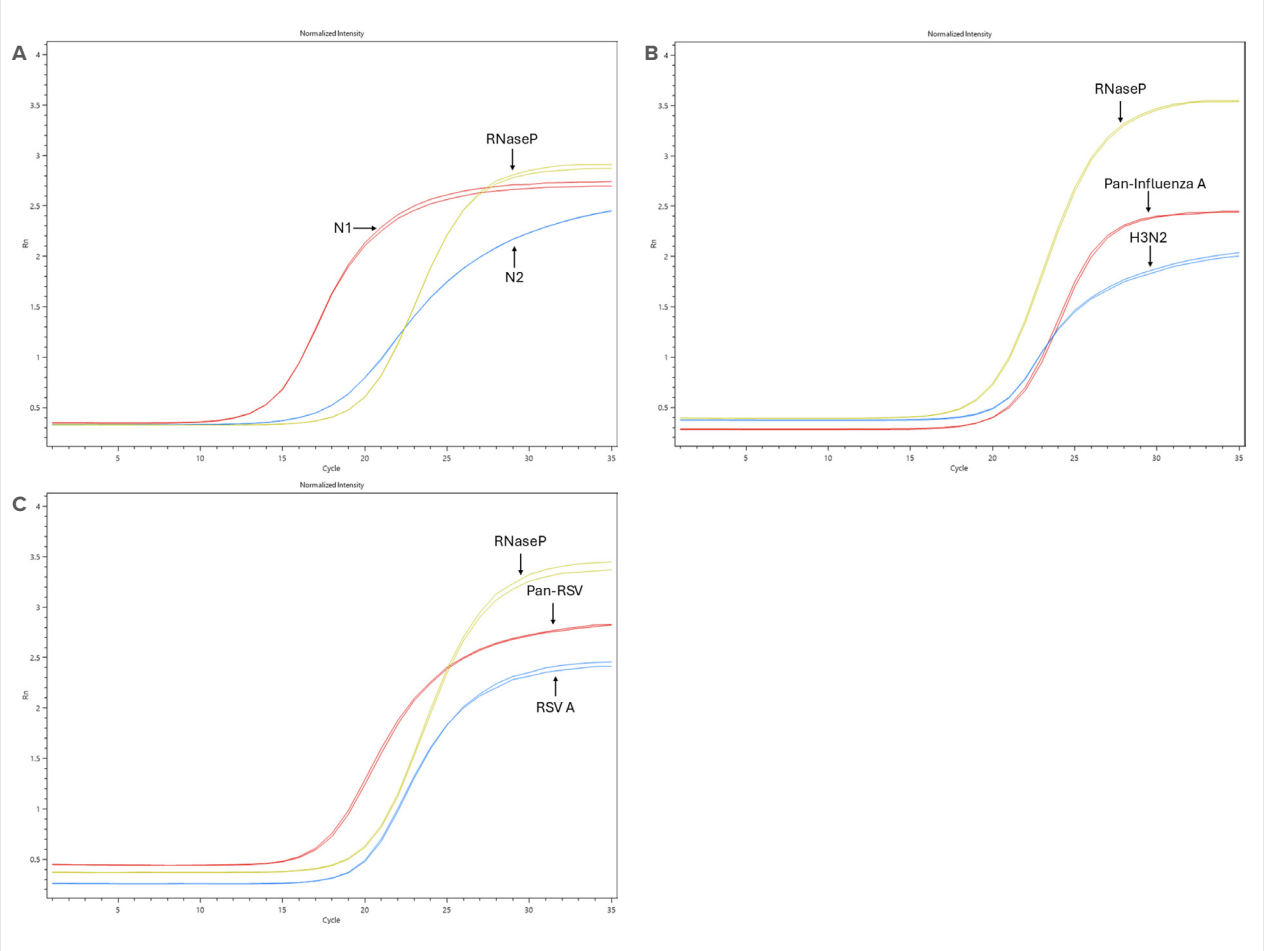

Figure 3. Amplification plots. (A) SARS-CoV-2 (B) Influenza A H3N2 (C) RSV A

Conclusion

In this study, we show the ability to quickly screen for the most common respiratory pathogens, including 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 1 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 utilize an open architecture, 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.

Appendix A

Controls used from ZeptoMetrix

NATtrol SARS-Related Coronavirus 2 (SARS-CoV-2) External Run Control (NATSARS(COV2)-ERC)

- Includes SARS-CoV-2 (USA-WA1/2020)

NATtrol Flu Verification Panel (NATFVP-NNS)

- Influenza AH1 (A/New Caledonia/20/99)
- Influenza AH3 (A/Brisbane/10/07)
- Influenza A H1N1 (A/NY/02/09)
- Influenza B (B/Florida/02/06, CH93(18)-18)
- Respiratory Syncytial Virus A (N/A)
- Respiratory Syncytial Virus B (CH93(18)18)

NATtrol Respiratory Verification Panel 2 (NATRVP2-QIA)

- Adenovirus (N/A)
- Coronavirus HKU-1 (Recombinant)
- Coronavirus NL63 (N/A)
- Coronavirus OC43 (N/A)
- Coronavirus 229E (N/A)
- Metapneumovirus 8 (Peru6-2003)

NATtrol EV Panel (NATEVP-C)

- Coxsackievirus Type A16 (N/A)*
- Parechovirus Type 1 (Harris)

* For the data generated in the heat map, Coxsackievirus Type A16 was used. However, the EV Panel has 4 additional enterovirus positive controls, and all have been positively detected with this panel.

Learn more at
standardbio.com/area-of-interest/pathogen-detection

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LAB-00015 Rev 02 072025
Viral Pathogen Detection with Custom Assays Application Note