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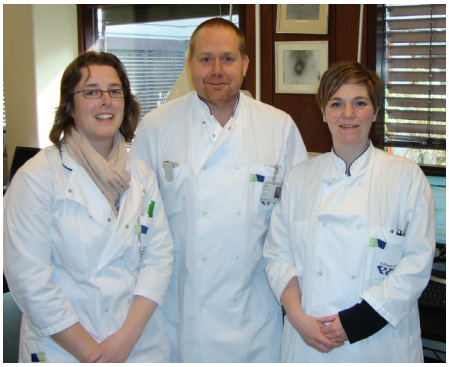
Parainfluenza Virus Research Using a Multiplex Real-Time RT-PCR Method and the ViiA™ 7 Real-Time PCR System



LABORATORY FOR MEDICAL MICROBIOLOGY AND IMMUNOLOGY, ST. ELISABETH HOSPITAL, TILBURG, THE NETHERLANDS

Left to right: Hèlen van Raak, John Rossen, PhD, and Astrid van Steen

The Laboratory for Medical Microbiology and Immunology performs both microbiological and serological analyses for the mid of Brabant (The Netherlands) and for specific analyses outside of that region. The lab performs tests for the detection of microorganisms (including bacteria, fungi, yeast, viruses, and parasites) that cause infectious disease in humans. Approaches used to analyze these organisms include: direct identification and isolation of the organisms (culture, antigen tests); identification of antibodies against the organisms (serology); and molecular techniques to identify the organisms by targeting their DNA/RNA.

APPLICATION

Virus Research and Differentiation

TECHNOLOGIES

ViiA™ 7 Real-Time PCR System
7500 Fast Real-Time PCR System
TaqMan® Universal PCR Master Mix

Abstract

Real-time PCR-based molecular virology testing requires assays that are sensitive, specific, and that can distinguish between virus types. Here, researchers from the Laboratory for Medical Microbiology and Immunology, St. Elisabeth Hospital, Tilburg, The Netherlands, used a multiplex reverse transcription PCR (m-RT-PCR) assay in a research-only study to detect and differentiate human parainfluenza viruses (HPIVs 1–4) in 30 samples derived from human nasopharyngeal washes. The assay was performed using either the new Applied Biosystems® ViiA™ 7 Real-Time PCR System or the Applied Biosystems® 7500 Fast Real-Time PCR System. The data indicate that the ViiA™ 7 Real-Time PCR System produces results equivalent to those of the 7500 Fast Real-Time PCR System and offers several new features that allow increased productivity.

Introduction

Human parainfluenza viruses (HPIVs) are nonsegmented RNA viruses that are members of the Paramyxoviridae family [1]. HPIV type 1 (HPIV-1) and HPIV-3 belong to the Respirovirus (former Paramyxovirus) genus, while HPIV-2 and HPIV-4 (consisting of subtypes A and B [2]) belong to the Rubulavirus genus. These pathogens cause upper and lower respiratory tract illnesses including pneumonia, croup, and bronchiolitis in infants and children.

The availability of HPIV-specific detection assays is important because other respiratory pathogens cause similar illnesses. In recent years, molecular methods have resulted in sensitive, specific, and rapid detection of respiratory viruses [3]. In this research-only study, a multiplex real-time RT-PCR assay to detect and differentiate parainfluenza viruses (HPIVs 1–4) was tested on 30 human samples using the Applied Biosystems® 7500 Fast Real-Time PCR System. The assay was also performed using the new Applied Biosystems® ViiA™ 7 Real-Time PCR System to evaluate data reproducibility on the two systems.

Materials and Methods

Figure 1 shows the workflow used to analyze 30 samples for HPIVs.

Primer Design

Primer and probe sequences were selected using Primer Express® software (Applied Biosystems) from an alignment of nucleotide sequences of HPIV-1, HPIV-2, HPIV-3, and HPIV-4. Highly conserved regions were selected for each virus (Table 1). Parainfluenza virus 1, 2, and 3 primers and probes were designed on the hemagglutinin-neuraminidase gene [4]. Parainfluenza virus 4 primer and probe sequences were designed on the nucleocapsid gene [5].

Nucleic Acid Extraction

Nasopharyngeal washes were collected, and 200 µL of each sample was used to isolate DNA and RNA using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics), as described by the manufacturer. Purified nucleic acid was eluted in a final volume of 100 µL.

Reverse Transcription

Following sample preparation, cDNA was synthesized using MultiScribe™ Reverse Transcriptase (Applied Biosystems) and random hexamers according to the manufacturer's instructions.

Multiplex Real-Time PCR

HPIV detection was performed in parallel using a multiplex real-time PCR assay specific for HPIVs 1–4. Assays were performed in triplicate. To help ensure reliable results, each plate included a negative isolation control and a positive amplification control. Real-time PCR was performed on the 7500 Fast Real-Time PCR System using the standard instrument protocol. The experiments were repeated on the ViiA™ 7 Real-Time PCR System.

Results and Discussion

Researchers at the Laboratory for Medical Microbiology and Immunology currently use an Applied Biosystems® 7500 Fast Real-Time PCR System. This platform is widely used in virology and microbiology laboratories in the Netherlands and enables the use of several dyes, including Cy®5, for multiplex real-time PCR analysis. This multiplexing capability allows researchers to expand the number of targets amplified in a single reaction and increase their throughput. The Applied Biosystems® ViiA™ 7 Real-Time PCR System incorporates the new OptiFlex™ System, which offers a higher level of multiplexing capability with six decoupled excitation and emission filters for detection of up to 21 colors (Table 2).

Figure 2 shows the amplification of HPIVs 1–4 using the multiplex real-time RT-PCR assay and the ViiA™ 7 Real-Time PCR System. The multiplex strategy was efficient, enabling high throughput and fast results. In addition, this approach limits the complexity of the assay, which can ultimately reduce costs and complexity of the validation process.

Results obtained on the 7500 Fast Real-Time PCR System using a 96-well Fast block were reproducible on the ViiA™ 7 Real-Time PCR System using a 384-well block (Table 3). The difference in the threshold cycle (C_t) value observed is due to the differences in plate format and sample volume. The new ViiA™ 7 Real-Time PCR System has a user-friendly software interface that made plate setup and results analysis easy to learn. In addition, the software data analysis assigns quality control flags, which gave more confidence in the results interpretation.

Features	ViiA™ 7 Real-Time PCR System
Block Configurations	96-well, Fast 96-well, 384-well, TaqMan® Array Micro Fluidic Cards
Run Time	30 minutes expected (Fast 96-well) 35 minutes (384-well)
Resolution	Down to 1.5-fold change for singleplex reaction
Excitation Source	OptiFlex™ System with halogen lamp
Detection Channels	Decoupled—6 emission, 6 excitation
21 CFR Part 11	Optional software module
Remote Monitoring	Available to monitor up to 4 instruments in real time and the status of up to 15 instruments
Data Export Format	User configurable: *.xls, *.txt, and 7900 formats

Nucleic Acid Extraction	Reverse Transcription	Multiplex Real-Time PCR Amplification
• Total nucleic acid was purified from nasopharyngeal washes.	• Each 200 µL reaction contained: – 80 µL eluted RNA – 20 µL 10X RT buffer – 5.5 mM $MgCl_2$ – 500 µM each dNTP – 2.5 µM random hexamers – 0.4 U/µL RNase Inhibitor – MultiScribe™ Reverse Transcriptase • Following incubation for 10 min at 25°C, RT was carried out for 30 min at 48°C, followed by RT inactivation for 5 min at 95°C.	• Each 25 µL reaction contained: – 10 µL cDNA – 12.5 µL 2X TaqMan® Universal PCR Master Mix – 300–900 nM forward and reverse primers – 75–200 nM each probe • Real-time PCR was performed on either the 7500 Fast Real-Time PCR System or the ViiA™ 7 Real-Time PCR System.

Figure 1. Multiplex Real-Time RT-PCR Assay Workflow.

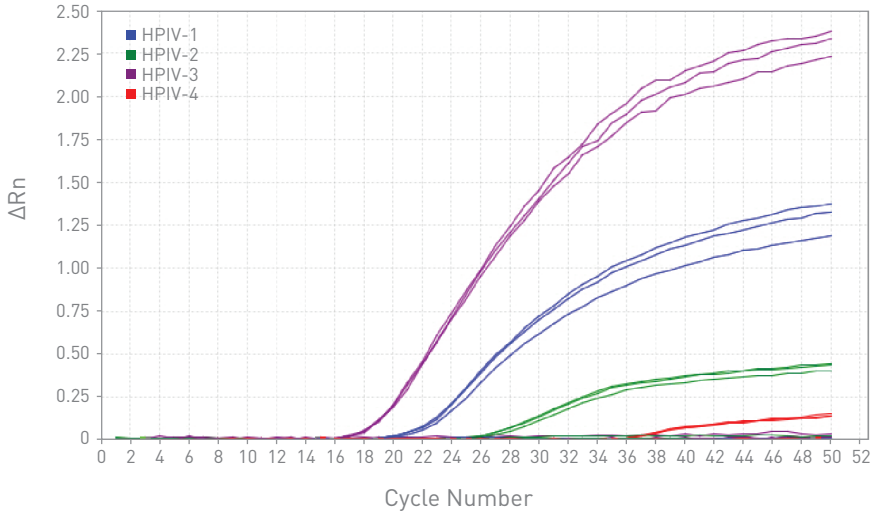


Figure 2. Linear Amplification Plot Showing Detection of HPIVs 1–4 Using the Multiplex RT-PCR Assay on the ViiA™ 7 Real-Time PCR System. The differences in primer and probe concentrations used for each target explain the diverse amplification exponential trends.

Table 1. Primer and Probe Sequences for Target Genes. DFO = Dragon Fly Orange; YY = Yakima Yellow. BHQ1 and BHQ2 are two FRET-based quenchers (Black Hole Quencher).

Parainfluenza Virus 1	
PIV1-F2	AAA AAC TTA GGG TTA AAG ACA ATC CA
PIV1-R2	GCC AGA TGT RTG TCY TTC CTG CTG GT
PIV1-2-DFO-BHQ2	DFO- CAAACGATGGCTGAAAAAGGGA -BHQ2
Parainfluenza Virus 2	
PIV2-F2	CCA TTT ACC TAA GTG ATG GAA
PIV2-R2a	CGT GGC ATA ATC TTC TTT TT
PIV2-R2b	TGT GGC ATA ATC TTC TTT CT
PIV2-2-YY-BHQ1	YY- AATCGCAAAAGCTGTTCAGTCAC -BHQ1
Parainfluenza Virus 3	
PIV3-F2	CAG GAA GCA TTG TRT CAT CTG T
PIV3-R2	ATA GTG TGT AAT GCA GCT YGT
PIV3-2-FAM-BHQ1	FAM- ACCCAGTCATAACTTACTCAACAGCAAC -BHQ1
Parainfluenza Virus 4	
PIV4-F1	CAA AYG ATC CAC AGC AAA GAT TC
PIV4-R1	ATG TGG CCT GTA AGG AAA GCA
PIV4-1-Cy5-BHQ2	Cy5- GTATCATCATCTGCCAAATCGGCAATTAAACA -BHQ2

Table 2. Filter Combinations and Dye Compatibility on the ViiA™ 7 Real-Time PCR System.

Channel	Dye Examples	Excitation Filter	Emission Filter
1	**FAM™, SYBR®, SYTO® 9 (MeltDoctor™),** fluorescein, SYPRO® Orange	470 ± 15 nm	520 ± 15 nm
2	**VIC®,** JOE™, TET™, HEX™	520 ± 10 nm	558 ± 12 nm
3	**TAMRA™, NED™,** BODIPY® TMR-X	550 ± 10 nm	586 ± 10 nm
4	**ROX™,** Texas Red®	580 ± 10 nm	623 ± 14 nm
5	LIZ™	640 ± 10 nm	682 ± 14 nm
6	Alexa Fluor®, Joda-4	662 ± 10 nm	711 ± 12 nm

Dyes indicated in **boldface** are Applied Biosystems calibration dyes.

Table 3. Comparison of Results Using the ViiA™ 7 Real-Time PCR System and the 7500 Fast Real-Time PCR System.

Target Gene	7500 Fast Real-Time PCR System—Positive Samples	ViiA™ 7 Real-Time PCR System—Positive Samples
HPIV-1	7	7
HPIV-2	1	1
HPIV-3	22	22
HPIV-4	3	3

Conclusion

The multiplex real-time RT-PCR assay presented here provides a robust, sensitive, and easily automatable workflow to detect HPIVs 1–4. Results obtained using the 7500 Fast Real-Time PCR System were reproducible on the ViiA™ 7 Real-Time PCR System. In addition, the ViiA™ 7 System's user-friendly interface and data analysis features offered advantages to the workflow and additional confidence in the results. This multiplex assay may be a valuable approach for HPIV detection in clinical research settings.

Acknowledgments

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