Development of a High Multiplex Respiratory Infectious Disease Detection Panel Using Next Generation qPCR Technology

Kiran Madanahally Divakar and Lilly Kong. PrimeraDx, Mansfield, MA 02048

Abstract

Quantitative PCR using FRET-based real-time detection is restricted to very few targets within a single reaction. The ICE*Plex*® System offers an automated, high multiplexing solution. The ICE*Plex* System integrates PCR thermal cycling with dynamic, sequential amplicon separation by capillary electrophoresis (CE), and multi-color quantitative detection. Unlike probe-based qPCR, the ICE*Plex* System directly measures amplicon accumulation through incorporation of labeled primers.

Here we demonstrate that the ICE*Plex* system is capable of simultaneous detection and discrimination of important respiratory infectious agents belonging to Influenza and Respiratory Syncytial Virus (RSV). Primera Dx Multiplex Respiratory panel consists of primers that can detect and differentiate seasonal influenza strains, swine influenza, novel influenza strains, as well as RSV A and RSV B. The Multiplex Respiratory panel also contains primers that can detect drug resistant strains of Influenza. In addition, the assay consists of an internal control, extraction control, and a calibration control.

The ICE*Plex* System is an automated bench top instrument that has overcome many of the limitations of traditional real-time PCR based multiplexing. Using this high multiplex respiratory panel, we demonstrate the performance of the ICE*Plex* System in detection and discrimination of seasonal influenza strains, swine influenza, novel influenza strains, and drug resistant mutant strains.

Technology

The ICE*Plex* System is a fully automated real time PCR platform that combines an amplication module (thermocycler) and a detection module (a capillary electrophoresis cartridge, two solid state lasers with excitation maximum at 488 nm and 639 nm and a spectrophotometer with CCD camera). All ICE*Plex* System reagents are kept on board of the platform enabling an easy consumable maintenance (Figure 1).

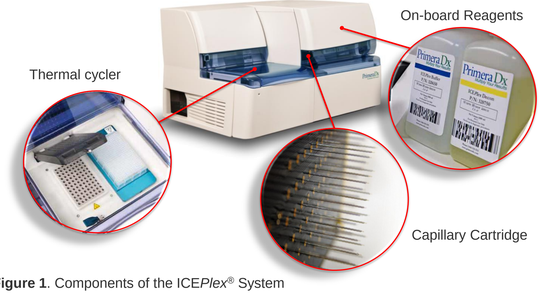

Figure 1. Components of the ICE*Plex*® System

The ICE*Plex* System generates fluorescently labeled PCR products (amplicons) which are separated based on their different sizes by capillary gel electrophoresis (CE). Accumulation of the fluorescent amplicons is monitored in real time by in house developed software that converts the fluorescent signal into amplication curves and calculates cycle thresholds (C_ts) for all PCR targets. The combination of PCR and CE enables simultaneous detection and quantification of multiplex targets in single reaction with accuracy similar to real-time PCR methods (Figure 2).

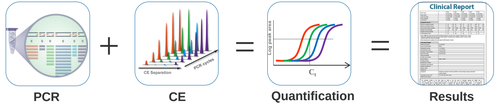

Figure 2. Multiplex real-time PCR detection on the ICE*Plex*® System

Introduction

Influenza virus causes highly contagious respiratory infection that can easily spread and cause considerable morbidity and mortality. It is major public health concern. Main causal agents of Influenza infection are Influenza virus A and B, and most of the Influenza pandemics are associated with Influenza A. Influenza A can infect human, animal, and birds, and can recombine to create novel Influenza strains that can be resistant to Oseltamivir (Tamiflu) and Zanamivir (Relenza). Another important viral agent that causes acute respiratory tract infection especially in young children and infants is respiratory syncytial virus (RSV), A and B. Here, we developed a multiplex respiratory panel that simultaneously and rapidly detects and discriminates Influenza A and B, Influenza A subtypes of public concerns and seasonal and pandemic importance, drug resistant (Oseltamivir) mutations, and RSV A and B.

Materials and methods

Primer design: Unique region of the genome for each target in the assay was identified by bioinformatics analysis followed by Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (Bethesda, MD). Geneious™ Pro software (Auckland, New Zealand) was used to carry-out ClustalW analysis (European Bioinformatics Institute, Cambridge, UK) of unique sequence, primer design on the consensus sequences (Steve Rozen and Helen J. Skaletsky (2000) Primer 3 on the WWW for general users and for biologist programmers), and primer-primer interaction among different primers (Cross-Hyb, Primera Dx, Inc., Mansfield, MA).

Genomic DNA templates: Synthetic oligonucleotides (IDT, Inc., Coralville, IA) that carry out the unique sequences for the different targets were used as templates to develop the panel. Influenza A, B, RSV A and B, A/H1, A/H3, N1-human, and N2 were tested using the RNA (data not shown).

PCR setup and amplification conditions: Multiplex PCR amplifications were performed in the 96 wells of a standard PCR plates. The PCR reactions contained: 1X Qiagen® Multiplex PCR Master Mix and 1 U of HotStarTaq® DNA Polymerase from Qiagen (Germantown, MD), 0.2 µM of gene specific forward and reverse primer pairs (IDT, Inc., Coralville, IA), of which one primer is labeled with FAM-dye (table 1) and 0.35X of the Universal Calibrators (PrimeraDx, Inc., Mansfield, MA). Twenty five microliter multiplex PCR reactions were subjected to thermocycling on the ICE*Plex* System.

PCR amplification conditions were as follow:

- 98° C for 10 minutes
- 16 cycles at 60° C for 20 sec., 72° C for 30 sec. and 96° C for 20 sec.
- 20 cycles at 64° C for 110 sec., 72° C for 150 sec., 96° C for 10 sec.

Organisms	Primers	Sequences
Influenza A	Forward	/56-FAM/TCATCCCGTCAGGCCCCCTC
	Reverse	GGGCACGGTGAGCGTGAACA
Influenza B	Forward	/56-FAM/AGTGGGGAACACACCTTCGGCG
	Reverse	GTCCTCCTGGTGCCTTTTCCGC
Influenza A/H1	Forward	/56-FAM/ATATACCCCAAGCCAAGTTCATGGCCC
	Reverse	TGCCCCACAGCACGAGGACT
Influenza A/H3	Forward	/56-FAM/CACATACGGGGCCTGTCCCA
	Reverse	ATACCATCCACCATTCCCTCCCAACC
Influenza A/H5	Forward	/56-FAM/ACCATCGGGGAGTGCCCCAAA
	Reverse	ATA TAA ATACCATTCCCTGCCATCCTCCC
Influenza A/H7	Forward	/56-FAM/TGTGGGGAAGTGCCCTCGGT
	Reverse	ATCGATGAGGCCCTCCCATCC
Influenza A/H9	Forward	/56-FAM/ACAAGGGGCCTTGGCCCTATCA
	Reverse	GGGGCATAAATCACCCACCCGC
Influenza A/N1-human	Forward	/56-FAM/ATGGGGCCGCCTCGTACAAA
	Reverse	TCACTGTGCCAGTGTCTGGGT
Influenza A/N2	Forward	/56-FAM/AGGCTTTCCGCTGGTGGGGA
	Reverse	GGGTCCGATAAGGGGTCCTACCAC
SNP I117V	Forward	TTCCAAGGGGGATGTGCCTGTTGTA
	Reverse	/56-FAM/ TGTGAGGGCTTCTGTCTTTGACAGTC
SNP I314V	Forward	CGTGCTATATATAAATATGGCAGATTATAGAGAT
	Reverse	/56-FAM/GCCCCTCTCTCGTTATTAGGATCCC
RSVA	Forward	/56-FAM/ACCTCACCCAGAATCCCCAGC
	Reverse	GGCGTTGTTTTGTGGTGGG
RSVB	Forward	/56-FAM/ACCCACAGCATCCGAGCCCT
	Reverse	ATAACTCCATGGTTATTTGCCCCAG

Table 1. Gene specific primers for the different PCR targets in the multiplex respiratory panel

Results

Simultaneous detection of Influenza A and B, and Influenza A strain variants in single PCR reaction

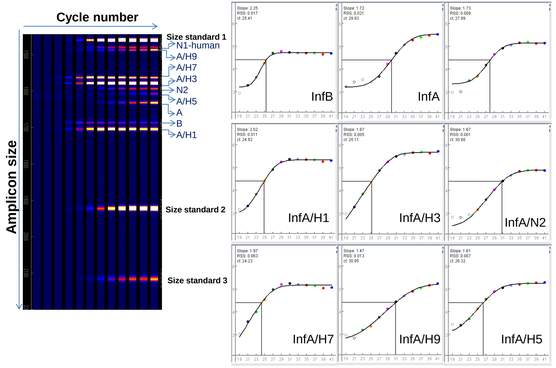

Figure 3. The multiplex respiratory panel can detect and discriminate Influenza A, B, and Influenza A strain variants.

The Multiplex Respiratory panel can simultaneously detect and differentiate RSV A and RSV B

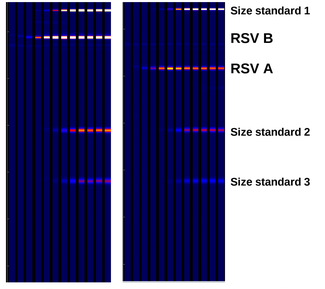

Figure 4. The Multiplex Respiratory panel can detect and discriminate respiratory syncytial virus A and B strains.

PDx Multiplex Respiratory panel can detect and discriminate Tamiflu drug resistant SNP

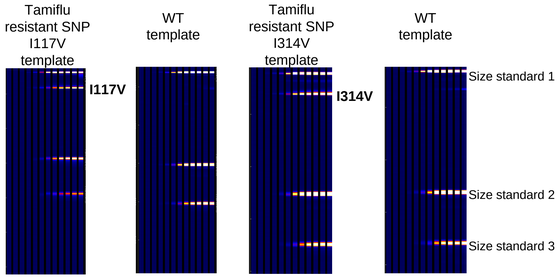

Figure 5. PDx Multiplex Respiratory panel can detect and discriminate Tamiflu drug resistant SNP, I117V and I314V, and thus helps in personalized medicine.

The PrimeraDx Multiplex Respiratory panel can detect TamiFlu resistant N2 subunit of avian and swine

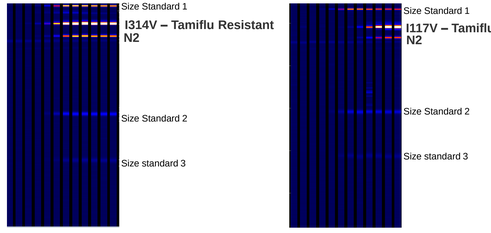

Figure 6. The Multiplex Respiratory panel simultaneously detects and discriminate SNP variant and the subtype

Conclusions

1. **Pilot study demonstrates ICE*Plex* ability to detect multiple respiratory infectious agents, Influenza A and B, RSV A and B, Influenza A subtyping, and identify drug resistant SNP variants.**
2. **Primers have been tested against gram-negative bacteria, gram-positive bacteria, and fungal genomic DNA mixes for any cross-reactivity. The primers showed specificity with their templates.**
3. **The multiplex respiratory panel run time is less than three hours.**

ICE*Plex* system and PDx Multiplex Respiratory panel have not been approved by the FDA for IVD. This information is for demonstration purpose only.

PrimeraDx
The Multiplex PCR Company