Using Next Generation qPCR Technology for Detection of Seven Different Sexually Transmitted Infectious Agents in One Reaction

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Abstract

Simultaneous detection and discrimination of seven different sexually transmitted infectious agents is very difficult to do using ordinary probe-based real-time PCR based systems. Taking advantage of the high multiplexing capabilities of the ICE*Plex*® System, we have very quickly designed a prototype sexually transmitted disease (STD) detection panel. The ICE*Plex* System combines PCR thermal cycling with dynamic, sequential amplicon separation and multi-color quantitative detection by capillary electrophoresis (CE) in a single integrated system. Unlike probe-based qPCR, the ICE*Plex* System directly measures amplicon accumulation through incorporation of labeled primers.

Using this high multiplexing platform, we show excellent detection of four different classes of microorganisms: fungi, bacteria, parasite, and virus. The PrimeraDx Multiplex Sexually Transmitted Infection (STI) panel consists of primers that can detect *Candida albicans*, *Candida glabrata*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis* as well as Herpes Simplex Virus type 1 (HSV-1) and type 2 (HSV-2). Moreover, because this next generation qPCR system is capable of high multiplex, we were able to include an internal control, a pan-fungal primer set, a pan-bacterial primer set, and a calibration control. We show high concordance with clinical isolates and excellent sensitivity.

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 PrimeraDx 11-plex STI panel, we demonstrate the performance of the ICE*Plex* System in detection of sexually transmitted infectious agents in one single assay in one well.

Technology Overview

The ICE*Plex* System is the first real-time PCR system that combines a PCR thermal cycler that accommodates a standard 96-well PCR plate, a capillary electrophoresis (CE) system with a replaceable CE cartridge, and fluidic pumps with on-board reagents. The system is also capable of simultaneous detection of two dyes in a single PCR reaction because it has two solid state lasers (488 nm and 639 nm excitation) and a spectrophotometer with CCD camera. (Figure 1)

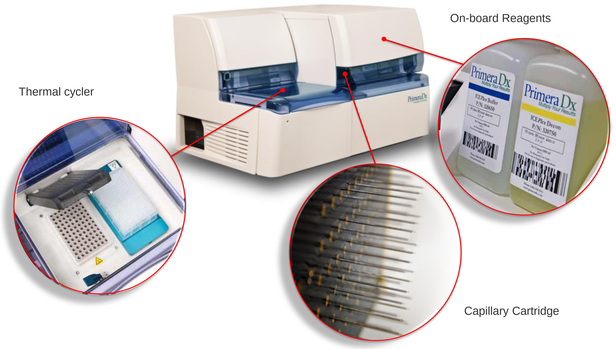


Figure 1. Components of ICE*Plex*® fully automated real-time PCR and CE system

STAR technology enables generation of fluorescently labeled PCR products with unique sizes on the ICE*Plex* System, and their detection by means of capillary gel electrophoresis (CE) in real time. Taking advantage of the multiplex capability of the ICE*Plex* System, embedded size calibrators and controls can be detected simultaneously in a single reaction. Amplification curves are generated for each and every PCR target. The built-in software automatically calculates cycle thresholds (C_ts) for all detected PCR targets and reports them in the Clinical reports (Figure 2).

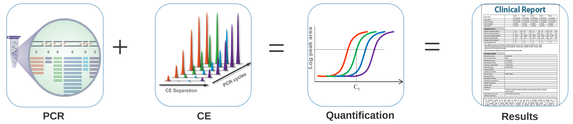


Figure 2. Principles of the multiplex real-time PCR detection on the ICE*Plex*® System

Introduction

Detecting more than four micobes in a single tube by real-time PCR is challenging because of lack of availability of sensitive and reliable detection methods. Sexually transmitted diseases are caused by multiple pathogens belonging to bacteria, virus, fungi, and parasites. For example, Genital herpes is caused by herpes simplex viruses 1 and 2, *Gonorrhoea* is caused by *Neisseria gonorrhoeae*, *Chlamydia* is caused by *Chlamydia trachomatis, Trichomoniasis* is caused by *Trichomonas vaginalis, Candidiasis* is caused by *Candida albicans and C glabrata,* etc. Here, we demonstrate that the new PrimeraDx Multiplex STI panel is able to detect different classes of pathogens in a single PCR reaction on the ICE*Plex* System.

Materials and Methods

Primer design: A unique region of the genome for each microbial 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 sequences, primer design on the consensus sequences (Steve Rozen and Helen J. Skaletsky (2000) Primer3 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: Purified genomic DNAs of *Candida albicans*, *Candida glabrata*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis* as well as HSV-1 and -2 were obtained from American Type Culture Collection (Manassas, VA). Copy numbers were deduced based on the genome size and concentration of the template DNAs.

PCR setup and amplification conditions: Multiplex PCR reactions were assembled and 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 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 thermal cycling on the ICE*Plex* System.

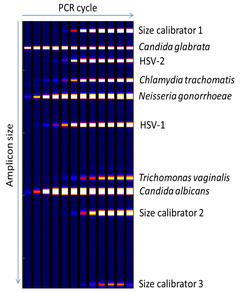
PCR amplification conditions were as follow:

- 98° C for 10 minutes
- 18 cycles at 56° C for 20 sec., 72° C for 30 sec. and 96° C for 20 sec.
- 24 cycles at 66° C for 90 sec., 72° C for 140 sec., 96° C for 10 sec.

Organism	Forward Primer	Reverse Primer
Neisseria gonorrhoeae	*(FAM)*-GACTTTGTCGAACGCAGTCA	GGCGGCATTAATTTGAGTGT
Chlamydia trachomatis	*(FAM)*-CTTTGCGCACAGACGATCTA	CTCTCCCATTTCTCCCCACAA
Candida albicans	*(FAM)*-ACTGTAGCTGAGCGTAAGACATTAGGTTAT	ATAGTAACTCCGGGTGCGAAAGGTATAA
Candida glabrata	GGAGCAGGTATTGGTATTGGTATCGTATTC	*(FAM)*-CCTGTAGCTTCTGATAAAGCCATTCCTAAA
Trichomonas vaginalis	*(FAM)*-AGCTCAACGACTGCCTCAAT	CTTCGAAATCAGCGCATGTA
Herpes Simplex Virus 1	*(FAM)*-ACTTTGACGAGGCCAAGCTA	CACCGTCTTTGTTGGGAACT
Herpes Simplex Virus 2	*(FAM)*-CCGCGGCCAACCATCACCAT	GACGCGACGGCCACCTTCTC

Table 1. Primer sequences of the gene specific primers for different targets in PrimeraDx Multiplex STI panel

Results



Our results demonstrated that the PrimeraDx Multiplex STI panel simultaneous detects all seven targets in one reaction on the ICE*Plex* System . **Figure 3** shows all seven targets separated based on amplicon size along with three size calibrators. Data on pan-fungal, pan-bacteria and internal control are not shown. The reaction consisted of multiplexed primers and multiplexed targets in a single tube.

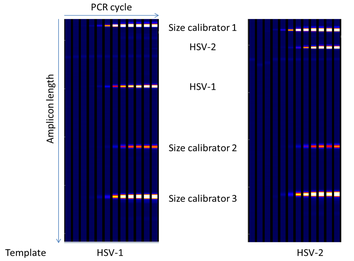
Figure 3. Simultaneous detection of all seven STI targets in a single reaction. Multiplex capability of the ICE*Plex*® system enables detection of 3 size calibrators in every reaction.

The sensitivity of the PrimeraDx Multiplex STI panel was determined to be a single copy per reaction for some of the targets in the panel (table 2)

Copies/reaction	*Chlamydia trachomatis*	*Neisseria gonorrhoeae*	*Candida albicans*	*Candida glabrata*	HSV-1	HSV-2
1000	100%	100%	100%	100%	100%	100%
100	100%	100%	100%	100%	100%	100%
10	66%	100%	100%	100%	41%	25%
1	33%	100%	100%	100%	0%	0%

Table 2. Detection rate of the Multiplex STI panel. The detection rate was not tested with *Trichomonas vaginalis*

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



The PrimeraDx Multiplex STI panel showed high specificity in detection of the HSV-1 and HSV-2. **Figure 4** shows specificity of HSV-1 and HSV-2 primers to their specific targets. The experimental reaction consisted of multiplexed primers and either HSV-1 or HSV-2 target in a single tube. Results indicated no cross-reactivity between HSV-1 and HSV-2 primers.

Figure 4. High specificity in detection of HSV-1 and -2 in a single PCR reaction.

Specificity Organism	Test Result
Mycoplasma hominis	No cross reactivity
Gardnerella vaginalis	No cross reactivity
Haemophilus ducreyi	No cross reactivity
Mobiluncus mulieris	No cross reactivity
Staphylococcus aureus	No cross reactivity
Pseudomonas aerugenosa	No cross reactivity
Aspergillus brasiliensis	No cross reactivity
Escherichia coli	No cross reactivity
Bacillus subtilis	No cross reactivity

Table 3. High specificity of Primera Dx Multiplex STI panel

No cross-reactivity was observed when the multiplexed primers were tested against bacterial and fungal genomic DNA at 1e5 copies per reaction (Table 3).

Conclusions

1. **The Multiplex STI panel can detect multiple microbial targets belonging to different classes: virus, fungi, bacteria, and parasite.**
2. **We show strong differentiation between HSV-1 from HSV-2.**
3. **The demonstration assay has good sensitivity.**
   - ***N gonorrhoeae, C albicans, C glabrata* can be detected (100%) at single copy level in PCR reaction.**
   - **HSV-1, HSV-2, and *C trachomatis* are detected (100%) at 100 copies per PCR reaction.**
4. **The STI panel is specific and did not detect other microbes tested. The *C. trachomatis* primers detected serovar H, E, D; other serovars not tested (data not shown).**
5. **The results showed high concordance with similar detection methods on clinical samples (data not shown).**

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