Automated High Multiplex qPCR Platform for Rapid Detection of Fungal Pathogens

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Abstract

Quantitative PCR using probe-based real-time detection is restricted to few targets within a single reaction. The ICE*Plex*® System was developed to offer an automated, high multiplexing solution. 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.

Here we demonstrate that the ICE*Plex* System is capable of simultaneous detection and discrimination of fungal pathogens, *Candida albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, and *Cryptococcus neoformans*, in a single reaction. In addition, the ICE*Plex* Fungal Assay consists of an extraction control, a sensitivity control, an internal control, a pan-fungal primer set, and a calibration control. Analytical studies indicate 10-20 fungal cell sensitivity per milliliter of whole blood, good dynamic range, and high specificity.

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 16-plex ICE*Plex* Fungal Assay (12 set for yeast detection, one set for pan-fungal detection, one set for extraction control detection, one set for calibrator control, and one optional set for internal control detection), we demonstrate the performance of the ICE*Plex* System in detection of medically important fungal pathogens.

Technology Overview

Unlike any other detection system, ICE*Plex* integrates 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 ICE*Plex* System has two solid state lasers (488 nm and 639 nm excitation) and a spectrophotometer with a CCD camera, allowing two different dyes to be detected simultaneously in single well. (Figure 1)

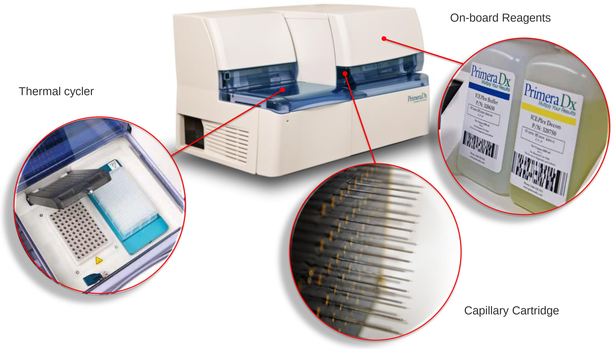


Figure 1. Components of ICE*Plex*® fully automated real-time PCR and CE system

Unlike other real-time PCR technologies, STAR (**S**calable **T**arget **A**nalysis **R**outine) technology detects and quantifies fluorescently labeled PCR products with unique sizes on the ICE*Plex* System by sequential sampling and separation on a capillary gel electrophoresis (CE) in real time. Multiple quality controls and calibrators can be embedded in the PCR reaction to ensure quality of the reported results. Built-in software automatically generates amplification curves by converting the fluorescent signals corresponding to the detected PCR amplicons once the run on ICE*Plex* is complete. A cycle threshold (C_t) for all PCR targets is reported in the Clinical Report (Figure 2).

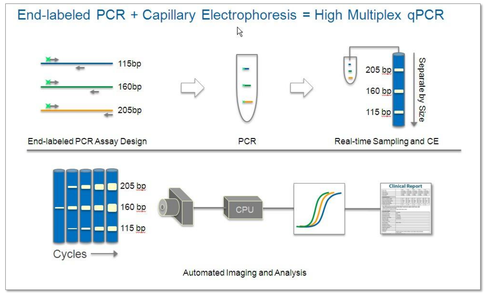


Figure 2. Principles of the multiplex real-time PCR detection on the ICE*Plex* System

Materials and Methods

Primer design: A unique region of the genome for each target in the assay was identified by bioinformatics analysis followed by homology search with 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, (1) ClustalW analyses (European Bioinformatics Institute, Cambridge, UK) of unique sequences; (2) 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 (3) primer-primer interaction analyses among different primers (Cross-Hyb, Primera Dx, Inc., Mansfield, MA).

Genomic DNA templates: Genomic DNAs of *Candida*, *Cryptococcus*, *Aspergillus*, and other microbial DNA used in the study were obtained from American Type Culture Collection (Manassas, VA) whereas human genomic DNA was obtained from Roche Applied Science (Indianapolis, IN). Sensitivity and Internal controls were custom synthesized by Integrated DNA Technologies (IDT, Inc., Coralville, IA). Calibrator control is a product from PrimeraDx, Inc (Mansfield, MA).

PCR setup and amplification conditions: The PCR reactions contained: 1X Qiagen® Multiplex PCR Master Mix with 1 U of HotStarTaq® DNA Polymerase (Qiagen, Germantown, MD), 0.2 µM of each gene-specific forward and reverse primer pairs (IDT, Inc), of which one is labeled with FAM-dye (Table 1), and 0.35 X of the Universal Calibrators (PrimeraDx). Twenty five microliter multiplex PCR reactions were subjected to thermo cycling in a standard 96-well PCR plate 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.

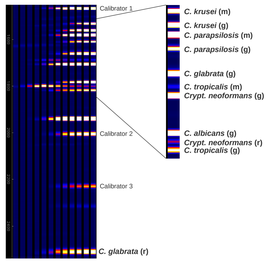
Nucleic acid purification: Nucleic acids of yeast cells after lysis were purified/extracted using NucliSENS® easyMag® according to the manufacturer's instructions.

Primer Name	Primer Sequence	Primer Name	Primer Sequence
>ALBICANS_M_F	ACTGTAGCTGAGCGTAAGACATTAGGTTAT	>CA_RPS_F	ACGTTGATGTTGCCATTCCA
>ALBICANS_M_R	ATAGTAACTCCGGGTGCGAAAGGTATAA	>CA_RPS_R	ATATGCTTCTTCGACGGCATTTTTG
>GLABRATA_R_F	ATATCGTTGGGGGAACGCTCT	>CG_CIT_F	ATCTGCCAAGTCGACCCTAACG
>GLABRATA_R_R	AGCTTGCGCTCGTGTCCC	>CG_CIT_R	ATATCCGACCAAGTGTGTGGTGTG
>KRUISII_M_F	AGCTTTACCCCTCTGAATTTTCGT	>CK_ABC_F	ATGGATAGCGAGGACGGCGTG
>KRUISII_M_R	CCACCGGCACGCTTTGT	>CK_ABC_R	GCCTGGCTTGACCCATCCGT
>PARAPSILOSIS_M_F	TCTGAAGGTTGTACGAAATGGGGAAAAA	>CP_SCR3_F	AGACCATTGCCAACAAAAGCTCC
>PARAPSILOSIS_M_R	CAGAAGACCCTAGTATCGCTGAACCAATTT	>CP_SCR3_R	ATCTGCACCAGCTTGAGCGT
>TROPICALIS_M_F	ATATCAACCATCTCCAGCCCCATC	>CP_CIT_F	AGCCAGCCAAAGCTGAAGAAG
>TROPICALIS_M_R	ATATTTCGTCACCGTTGACCTCCA	>CP_CIT_R	GGGCTTTTGGCAATTCCTTT
>NEOFORMANS_R_F	ATCAACGGATCTCTTGGCTTCCACAT	>CN_CIT_F	ATGAGGACGCCTCTGACGAGAAA
>NEOFORMANS_R_R	ACCCAAATCCAAGTCCAACAGGTAATAAAA	>CN_CIT_R	ATAGCAGTGTATCGGGGGTCCT
Pan-fungal_ITS34_F	CGTGAATCATCGAATCTTTGAACGCAC	Extraction control_pombe_F	ATGGGTATCCGATTCCGTGGTT
Pan-fungal_ITS34_R	GGTTTCTTTTCCTCCGCTTATTGATATGC	Extraction control_pombe_R	GGGCAACGGTCAATGAGTTC
Internal control_F	GGAAGGGTCTGCATCACAAGATCCT		
Internal control_R	CATGAATGGTGTGAGCTTGGTGAACA		

Table 1. Primer sequences of the gene-specific primers for different targets in the ICE*Plex* Fungal Assay

Results

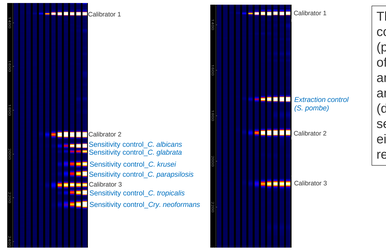
Simultaneous detection of fungal targets in one reaction



The ICE*Plex* Fungal Assay can simultaneously detect and differentiate six major fungal pathogens. Each fungal species is detected by two amplicons: one is from single-copy region and the other from multi-copy region on chromosomal or mitochondrial genome (Fig.3). Based on calibration control copy number, assay can perform approximate quantification.

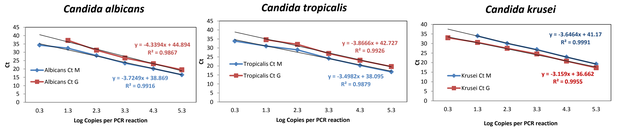
Figure 3. Simultaneous detection of single- and multi-copy targets in the ICE*Plex* Fungal Assay.

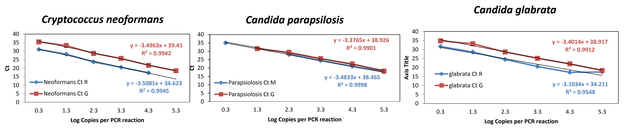
Embedded calibration, extraction and sensitivity controls



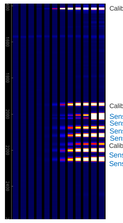
The ICE*Plex* Fungal Assay contains a sensitivity control (positive control for the presence of primers in the PCR reaction), an extraction control (*S. pombe*), and an optional Internal Control (data not shown here). The sensitivity control can be used either alone or in each PCR reaction.

Sensitivity and dynamic range of the ICE*Plex* Fungal Assay





Specificity of the ICE*Plex* Fungal Assay



No cross-reactivity was observed with the following genomic DNAs when tested at 100,000 genomic copies per PCR reaction

Candida lusitaniae 42720D-5
Candida kefyr
Aspergillus brasiliensis 16404D-2
Neisseria meningitidis BAA-335D-5
Aspergillus nidulans 38163D-2
Aspergillus flavus 20430D-2
Aspergillus fumigatus 118
Aspergillus brasiliensis SN26 9642D-2
Cryptococcus albidus IFF-8274C
Mycoplasma pneumoniae 15531D
Human Adenovirus 4 strain RI-67
Streptococcus pyogenes MGAS 10394
Borrelia burgdorferi strain B31
Human Adenovirus 8 strain: Trim
Campylobacter jejuni subsp: adenoid 71
Enterococcus faecalis V583
Toxoplasma gondii RH
Human Adenovirus 12 strain: Huie
Human Adenovirus 11 strain: Slobitiski
HSV-2 Strain MS
Human T-cell Leukemia virus II in pBR322,
Listeria monocytogenes strain EGDE
Fusarium solani FIV/74
Clostridium perfringens 13124D-5
Neisseria meningitidis BAA-335D-5
Streptococcus pneumoniae TIGR4
Mycoplasma hominis strain pg21
Staphylococcus aureus subsp: *aureus*
Mobilincus mulieris strain BV 64-5
Escherichia coli 8739D-5
Chlamydia trachomatis H strain: UW-43/CX
Bacillus subtilis 168
Neisseria gonorrhoeae 53422D
Ureaplasma parvum strain 27
Haemophilus ducreyi 35000 HP
Gardenerella vaginalis 49145D-5
Enterococcus faecalis 9027D-5
Pseudomonas aeruginosa 9027D-5
Bacillus subtilis Spizizenii, HSV1 MCINTYRE
Trichomonas vaginalis C-1:NH
Chlamydia trachomatis serovar D
Mycoplasma hominis strain PG21

Sample preparation method for whole blood

Lysis

- 3 ml whole blood + 9 ml ***water*** and ***invert tube 5-10 times***
  - ***4,000 rpm*** for ***2 min*** (tabletop)
- Resuspend pellet in 9 ml ***water*** and ***invert tube 5-10 times***
  - ***4,000 rpm*** for ***2 min*** (tabletop)
- Resuspend pellet in 1 ml ***Protease Solution*** (30 µl Proteinase K in RBC Lysis Buffer), ***15 min*** at ***60 °C***
  - ***14,000 rpm*** for ***2 min*** (microcentrifuge)
- Resuspend pellet in 0.2 ml Lyticase Solution (20U Lyticase in 50mM Tris-Cl, pH 7.6, 1 mM EDTA, 0.2% β-mercaptoethanol), 30 min at 37 °C
  - ***No centrifugation***
- Add ***0.3ml Bead-Beating Buffer*** (50mM Tris-HCl, pH 7.6, 25 mM EDTA, 0.2% β-mercaptoethanol) for bead-beating
  - ***4 m/sec for 60 sec*** on FastPrep-24
  - Harvest the lysate and ***rinse the beads with 0.6 5ml*** buffer for a final 1ml sample volume

Extraction

- DNA isolation on easyMAG (Specific B/Whole Blood Protocol), 50 µl elution

Conclusions

- The ICE*Plex* Fungal Assay consists of yeast-specific primers and primers for all controls, including a calibration control, a sensitivity control, an internal control, and an extraction control.
- No cross-reactivity was detected with gram-negative bacteria, gram-positive bacteria, other fungal genomic DNA mixes or human genomic DNA.
- No major multiplex primer interactions were observed. The PCR efficiencies of each fungal target primer set were comparable between single and multiplex formats (data not shown).
- Primers and PCR chemistry could detect single copies of DNA in PCR reaction. For most targets, assay showed five-dynamic range with PCR efficiencies ranging from 70-110%.
- Sample preparation method indicated effective removal of red and white blood cells. Further, fungal cell lysis method indicated a lysis efficiency of 90-100% and DNA extraction efficiency of 40-60% (data not shown).
- Study demonstrated the capability of the ICE*Plex* system to simultaneously detect major yeast pathogens in a single-tube multiplex format.

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