Development and Evaluation of a Molecular Assay That Simultaneously Detects Six Yeast Pathogens in a Single Tube: *C. albicans, C. glabrata, C. krusei, C. tropicalis, C. parapsilosis* and *C. neoformans.*

D. Ford, J. Carr, P. Wells, S. Girard, J. Frietas, V. Dancik, E. Garcia and V. Slepnev. Primera Dx, Mansfield, MA, USA

ABSTRACT (*updated*)

Background: The FungiQuant CCP Yeast Assay is a newly developed multiplexed PCR assay based on STAR technology (*Scalable Target Amplification Routine)*, a novel approach that integrates 'real-time' PCR and capillary electrophoresis (CE) allowing for the measurement of multiplex nucleic acids targets in a single tube with high sensitivity. Five *Candida* species (*albicans, glabrata, krusei, tropicalis and parapsilosis*) and *Cryptococcus neoformans* are specifically detected.
As an added feature, a pan-fungal primer set based on the ITS region is included to allow for the detection of unanticipated fungal species. This primer set is designed to detect virtually any fungal species.
Targets are distinguished based on amplicon length and size separation based on CE. The multiplexing power enabled by STAR technology allows inclusion of amplification and sample extraction controls **in the same tube as the sample** thereby enhancing analytical performance and result confidence.

Results of Analytical Verification:
(1) Specificity: The FungiQuant CCP Yeast assay is specific and did not show cross-reactivity or inhibition in the presence of a wide range of potentially interfering targets
(2) Precision: Over a 5-day study, precision was determined to be <30% **based on copies per reaction**
(3) Linearity: Demonstrated from 20 to >20,000 copies/reaction
(4) Sensitivity: Fewer than 10 CFU per mL whole blood

Conclusions: The FungiQuant CCP Yeast assay system employs STAR technology to enable rapid PCR measurement of five Candida species and Cryptococcus neoformans showing excellent analytical performance. This assay allows for the consolidation of yeast fungal testing of these key six pathogens into a simplified, cost-effective method.

INTRODUCTION

•Candidemia represents the fourth most common cause of nosocomial blood infections in North America (Pfaller et al, Clin Microbiol Rev 2007, 20:133)

•The associated mortality rate is from 40 – 70% often due to delay in initiation of appropriate therapy (Eggimann, P. et al (2003) Lancet Infect. Dis 3:685-702).

• While most are due to *C. albicans*, more recently there has been an increase in the incidence of *non-albicans* candidemia, especially with *C. glabrata, C. krusei, C. tropicalis* and *C. parapsilosis.*

•Identification of the causative organism responsible for bloodstream infections is critical in order to initiate appropriate therapy.

•The 'Gold Standard' for detection, blood culture, requires > 48 hours for detection and identification which can significantly delay treatment.

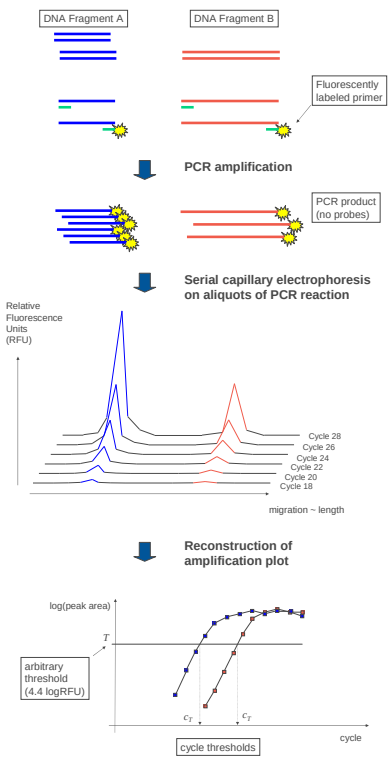
•Detection methods that expedite result generation are critical to improving patient management and therapy.

Aim: Develop a quantitative PCR-based multiplexed assay for detection of the major yeast species responsible for bloodstream infections directly from clinical samples

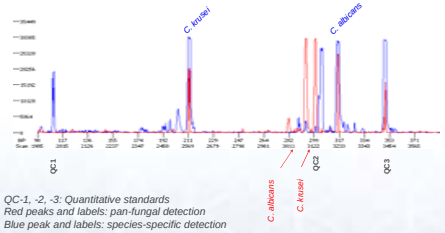
METHODS

Yeast Propagation and Isolation: A single colony for each species (*C.albicans*, ATCC 11006, *C.glabrata*, ATCC 2001, *C.krusei*, ATCC 14243, *C.neoformans*, ATCC 32045, *C. parapsilosis*, ATCC 22019, and *C.tropicalis*, ATCC 750) was selected and cultured overnight. After approximately 18 hours of growth, cells were counted using a light microscope and a hemocytometer. The yeast cells were diluted to test concentrations and spiked into healthy donor EDTA-blood.
Extraction of DNA from Blood: Yeast DNA was extracted from 3.0 mL of EDTA- blood. Red Blood cells were lysed followed by a white blood cell lysis procedure. The resulting pellet was treated with lyticase, followed by bead beating and automated nucleic acid extraction using the EasyMag Instrument. (bioMerieux, Marcy l'Etoile, France).
Multiplex PCR detection using FungiQuant CCP Yeast Assay: Extracted DNA samples were mixed with FungiQuant CCP reagents that contained primers at 250 or 500nM and quantification controls seeded at 250, 2500 and 25000 copies per reaction. PCR reactions were activated at 95C for 15 min followed by 40 cycles at 95C for 20 sec, 62C for 90sec and 72C for 60 sec. Each reaction was sampled at alternate cycles beginning at cycle 18 and continuing through cycle 40. Samples were placed in a formamide holding plate until the PCR reaction was complete. Holding plates were then denatured and analyzed on an ABI 3730XL Genetic Analyzer by capillary electrophoresis. Data collected was analyzed as described (see panel A).

A STAR Process: Multiplex PCR with CE detection

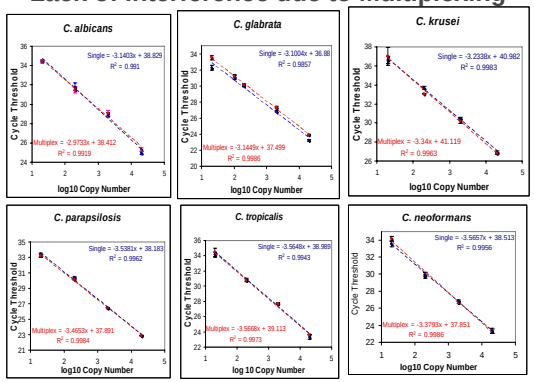


B Representative Electropherogram: *C. albicans* / *krusei* co-infection



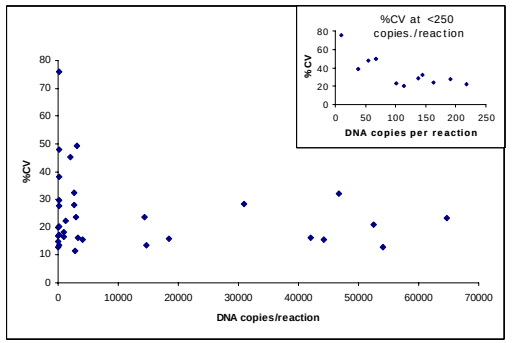
QC-1, -2, -3: Quantitative standards
Red peaks and labels: pan-fungal detection
Blue peak and labels: species-specific detection

C Primer Screening: Lack of Interference due to Multiplexing

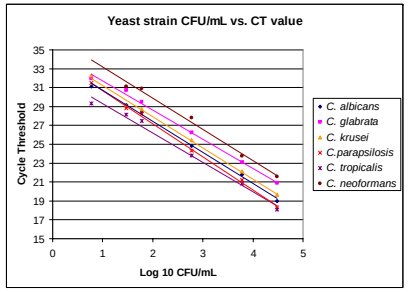


Linearity is demonstrated from 20 to 20,000 copies per reaction

D Precision: 5-day study



E Sensitivity Through Sample Prep: *Candidas*: < 10 CFU/mL blood *Cryptococcus neoformans*: <30 CFU/mL blood



F Detection of Co-Infecting Species

	CA_Specific	CA_ITS	CG_Specific	CG_ITS	CK_Specific	CK_ITS	CP_Specific	CP_ITS	CT: Specific	CT: ITS	CN_Specific	CN_ITS
CA_CG_1	85770	94070	47700	33820								
CA_CG_2	188700	244400	64050	97770								
CA_CG_3	167900	236600	51140	91860								
CA_CK_1	181600	249300			50610	8561						
CA_CK_2	228200	341100			57610	11930						
CA_CK_3	238300	381800			59600	10430						
CA_CN_1	307300	525900									6249	
CA_CN_2	360100	421300									26220	
CA_CN_3	209900	302400									5395	
CA_CP_1	225200	341000					143200	150300				
CA_CP_2	177500	271700					99570	131700				
CA_CP_3	249600	363500					143900	146200				
CA_CT_1	303500	395100							335300	560700		
CA_CT_2	324900	484400							384200	748200		
CA_CT_3	593000	767700							693800	1264000		

Results and Conclusions:

- **Simultaneous detection and quantification of 5 *Candida* species and *Cryptcoccus neoformans***
- **Excellent sensitivity at < 10 CFU/mL whole blood for *Candida spp.* and < 30 CFU/mL blood for *Cryptococcus neoformans***
- **Presence of pan-fungal primer set allows for detection of all major fungal pathogen present**

➢ ***Allows for detection, identification and quantification of targeted pathogens in one day: A clear time-to-result improvement over blood culture***

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