



- (51) International Patent Classification:
C12Q 1/68 (2006.01)
- (21) International Application Number:
PCT/US2013/031774
- (22) International Filing Date:
14 March 2013 (14.03.2013)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
61/636,110 20 April 2012 (20.04.2012) US
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- (81) Designated States (*unless otherwise indicated, for every
kind of national protection available*): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP,

KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD,
ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI,
NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU,
RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ,
TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA,
ZM, ZW.

- (84) Designated States (*unless otherwise indicated, for every
kind of regional protection available*): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- *as to applicant's entitlement to apply for and be granted a
patent (Rule 4.17(ii))*
- *as to the applicant's entitlement to claim the priority of the
earlier application (Rule 4.17(iii))*

Published:

- *with international search report (Art. 21(3))*
- *with sequence listing part of description (Rule 5.2(a))*



WO 2013/158281 A1

(54) Title: COMPOSITIONS AND METHODS FOR DETECTION OF CANDIDA SPECIES

(57) Abstract: The invention features compositions and methods for the detection of *Candida* species in biological samples, such as human tissue samples. The invention features species specific nucleic acid probes that can hybridize to target nucleic acid molecules in *Candida* species and can be used in a variety of detection assays, such as fluorescence based assays or NMR based assays. The compositions and methods of the invention can be used to rapidly and accurately detect one or more *Candida* species in patients and can be used to assist in point-of-care-decisions for treating *Candida* infections.

COMPOSITIONS AND METHODS FOR DETECTION OF *CANDIDA* SPECIES

5

Cross-Reference to Related Applications

This application claims benefit of U.S. Provisional Application No. 61/636,110, filed April 20, 2012, which is hereby incorporated by reference in its entirety.

Background of the Invention

10 *Candida* species are commensal organisms of humans, usually found on the mucous membranes of the gut, oral cavity, and vaginal introitus, and in warm moist skin folds. *Candida* is the most commonly identified causative agent of oral or vaginal thrush. *Candida* can also cause life-threatening infections in hospital patients. In particular, *Candida* can cause invasive diseases in hosts with altered immunity, such as in patients with HIV infection, patients that have received organ or bone marrow
15 transplants, and in patients experiencing neutropenia after cancer immunotherapy. Patients on intensive regimens of cancer therapy, patients on prolonged broad spectrum antibiotic therapy, patients using invasive devices, and patients on prolonged hospital stays are also at high risk for such infections.

Conventional methods for identifying *Candida* species in patients include morphology and assimilation tests involving blood cultures. These tests can be time consuming, laborious, and often
20 produce false negative results, which adversely affect the targeting of anti-fungal treatment and related point-of-care decisions. In addition, these methods may require extensive, specialized equipment and highly trained operators. These methods may further be limited by their sensitivity or the number of *Candida* species that can be detected simultaneously.

Accordingly, there is a need for compositions, methods, and kits for detecting the presence of
25 *Candida* species in a biological sample in a timely, accurate, and efficient manner.

Summary of the Invention

The invention features nucleic acid probes and primers that include the sequence of any one of
30 SEQ ID NOs: 1 to 41 (e.g., nucleic acid probes having the sequence of any one of SEQ ID NOs: 1 to 10, such as any one of SEQ ID NOs: 1 to 5), and nucleic acid probes and primers having at least 90%, 95%, 97%, 99%, or more sequence identity to these probes and primers.

The nucleic acid probes and primers can be used to detect a *Candida* species (e.g., *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida glabrata*, *Candida parapsilosis*, *Candida dubliniensis*, *Candida lusitanae*, and *Candida guilliermondi*) in a biological sample. In several
35 embodiments, the nucleic acid probes of the invention include a detection moiety (e.g., a fluorescent label) that is conjugated to the probe. In other embodiments, the nucleic acid probes are conjugated to a magnetic nanoparticle. In another embodiment, the probes of the invention (e.g., SEQ ID NOs: 1 to 5)

may be molecular beacon probes that may include a fluorescent label (e.g., FAM, TAMRA, HEX, TMR, Cy3, Cy5, and other spectrally distinguishable dyes) and a quencher (e.g., DDQ-I, Dabcyl, Eclipse, Iowa Black FQ, BHQ-1, QSY-7, BHQ-2, DDQ-II, Iowa Black RQ, QSY-21, BHQ-3). In yet another embodiment, the probes of the invention (e.g., SEQ ID NOs: 1 to 5) may be a “shared-stem” molecular beacon probe. In other embodiments, the nucleic acid probes of the invention (e.g., SEQ ID NOs: 6 to 10) may be conjugated to magnetic nanoparticles.

The invention features a method for detecting at least one *Candida* species (e.g., *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida glabrata*, *Candida parapsilosis*, *Candida dubliniensis*, *Candida lusitanae*, and *Candida guilliermondi*) in a biological sample (e.g., soil, water, food, and tissue or fluid sample from an organism, such as a human). The method includes contacting the biological sample with at least one nucleic acid probe having a sequence selected from any one of SEQ ID NOs: 1 to 41 (e.g., SEQ ID NOs: 1-10, such as SEQ ID NOs: 1-5) under conditions which allow the at least one probe to hybridize to a nucleic acid molecule of the *Candida* species; and detecting hybridization between the at least one probe and the nucleic acid molecule, thereby detecting the at least one *Candida* species. In an embodiment of the method, the probe is a *Candida albicans* probe and has the sequence of SEQ ID NO: 1 or SEQ ID NO: 6; the probe is a *Candida tropicalis* probe and has the sequence of SEQ ID NO: 2 or SEQ ID NO: 7; the probe is a *Candida glabrata* probe and has the sequence of SEQ ID NO: 3 or SEQ ID NO: 8; the probe is a *Candida parapsilosis* probe and has the sequence of SEQ ID NO: 4 or SEQ ID NO: 9; the probe is a *Candida krusei* probe and has the sequence of SEQ ID NO: 5 or SEQ ID NO: 10. In several embodiments, the method features nucleic acid probes that are fluorescently labeled. These nucleic acid probes may be molecular beacon probes (e.g., probes having the sequence of any one of SEQ ID NOs: 1 to 5) that further include a quencher. According to the method of the invention, hybridization of the nucleic acid probe to target *Candida* species nucleic acid molecules produces fluorescence which may be detected in, e.g., a fluorescence based assay (e.g., a real-time or end-point PCR assay) using, e.g., an instrument capable of detecting fluorescence (e.g., a real-time or end-point thermal cycler, or a plate reader). In one embodiment, the method features one or more probes (e.g., 2, 3, 4, or 5 or more probes) that may be labeled with the same or different fluorescent labels (e.g., molecular beacon probes labeled with spectrally distinguishable fluors), and the one or more probes may each be contacted with the sample for use in the same assay, e.g., in a multiplex assay to detect up to five different *Candida* species (e.g., *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, and *Candida krusei*) in a biological sample.

The invention also features a method of using nucleic acid probes conjugated to magnetic nanoparticles (e.g., SEQ ID NOs: 6 to 10 and 13 to 31) to detect *Candida* species in, e.g., an NMR based assay (e.g., an aggregation or disaggregation assay) using an NMR instrument. The method includes contacting the biological sample with at least one nucleic acid probe having a sequence selected from any one of SEQ ID NOs: 6 to 31 (e.g., SEQ ID NOs: 6-10) under conditions which allow the at least one probe to hybridize to a nucleic acid molecule of the *Candida* species; and detecting hybridization between

the at least one probe and the nucleic acid molecule, thereby detecting the at least one *Candida* species. In an alternate embodiment of the method, probes conjugated to magnetic nanoparticles can be used in pairwise combinations in an NMR assay to detect *Candida* species, e.g., a probe having the sequence of SEQ ID NO: 6 can be used with a probe having the sequence of any one of SEQ ID NOs: 13 and 14 to
5 detect *Candida albicans*; a probe having the sequence of SEQ ID NO: 7 can be used with a probe having the sequence of any one of SEQ ID NOs: 20 to 25 to detect *Candida tropicalis*; a probe having the sequence of SEQ ID NO: 8 can be used with a probe having the sequence of any one of SEQ ID NOs: 18 and 19 to detect *Candida glabrata*; a probe having the sequence of SEQ ID NO: 9 can be used with a probe having the sequence of any one of SEQ ID NOs: 20 to 25 to detect *Candida parapsilosis*; and/or a
10 probe having the sequence of SEQ ID NO: 10 can be used with a probe having the sequence of any one of SEQ ID NOs: 15 to 17 to detect *Candida krusei*. Binding of probes to target *Candida* nucleic acid molecule can be detected by measuring the NMR relaxation rate in an NMR instrument.

The invention also features primers for DNA sequencing based detection of *Candida* species in a biological sample. In several embodiments, the primer is a *Candida krusei* primer having the sequence of
15 SEQ ID NO: 32 or SEQ ID NO: 33; the primer is a *Candida albicans* primer having the sequence of SEQ ID NO: 34 or SEQ ID NO: 35; the primer is a *Candida glabrata* primer having the sequence of SEQ ID NO: 36 or SEQ ID NO: 37; the primer is a *Candida parapsilosis* primer having the sequence of SEQ ID NO: 38 or SEQ ID NO: 39; or the primer is a *Candida tropicalis* primer having the sequence of SEQ ID NO: 40 or SEQ ID NO: 41. Sequencing may also be preceded by prior amplification using SEQ ID NO:
20 11 and SEQ ID NO: 12. Alternatively, the sequencing could be done using SEQ ID NO: 11 and SEQ ID NO: 12 and species identification conducted based on analysis of the DNA sequence (i.e., using BLAST).

The invention also features a method of using *Candida* specific primers (e.g., one or more of the DNA primers of SEQ ID NOs: 32 to 41) to detect a *Candida* species in a biological sample. The method may include use of the primers one or more of the primers for DNA sequencing. In several embodiments,
25 the primer is a *Candida krusei* primer having the sequence of SEQ ID NO: 32 or SEQ ID NO: 33; the primer is a *Candida albicans* primer having the sequence of SEQ ID NO: 34 or SEQ ID NO: 35; the primer is a *Candida glabrata* primer having the sequence of SEQ ID NO: 36 or SEQ ID NO: 37; the primer is a *Candida parapsilosis* primer having the sequence of SEQ ID NO: 38 or SEQ ID NO: 39; or the primer is a *Candida tropicalis* primer having the sequence of SEQ ID NO: 40 or SEQ ID NO: 41.

The invention also features fluorescence based methods of using the nucleic acid probes and primers described above (e.g., the nucleic acid molecules of any one or more of SEQ ID NOs: 1 to 41) to
30 detect *Candida* species in a biological sample. These methods include amplifying and sequencing target nucleic acid molecules from *Candida* species in a biological sample. In several embodiments, the methods include, e.g., a TaqMan probe based assay (e.g., real-time or end-point PCR based TaqMan
35 assay using probes having sequence of SEQ ID NOs: 1 to 5); strand-displacement probe based assays (e.g., using probes having sequence of SEQ ID NOs: 1 to 10); PCR assays using DNA binding dyes (e.g., real-time PCR based assay using SYBR[®] green dye and probes having sequence of SEQ ID NOs: 1 to

41); and *in situ* hybridization assays using fluorescently labeled species-specific probes (e.g., using probes having sequence of SEQ ID NOs 1 to 41). In several embodiments, the nucleic acid probes having the sequence of any one of SEQ ID NOs: 6-10, and 13-31 may have a detection label (e.g., a fluorescent label), such that the fluorescence detection is via a secondary step (e.g., a digoxigenin label for antibody-based detection using fluorescently labeled anti-digoxigenin antibodies, or a biotin label for detection using fluorescent streptavidin). Alternatively the detection readout may be non-fluorescent. For example, nucleic acid probes having the sequence of any one of SEQ ID NOs: 6-10 and 13-31 may have non-fluorescent detection labels, such as a radioactive isotope for autoradiographic detection, digoxigenin for antibody-based detection using a horse-radish peroxidase (HRP) conjugated anti-digoxigenin antibody, and biotin for streptavidin based detection using HRP conjugated straptividin.

In other embodiments, the fluorescence based methods for detecting the presence of at least one *Candida* species in a biological sample includes the use of molecular beacon probes (e.g., those probes having the sequences of any one of SEQ ID NOs: 1 to 41, and particularly SEQ ID NOs: 1 to 5) having a fluorescent label, which may be the same or different. Following contacting of the probe to the sample that includes nucleic acid molecules from the at least one *Candida* species and hybridization of the probe to the nucleic acid molecules, the presence of the at least one *Candida* species is detected by heating the sample through a melting temperature (T_m) of at least one of the probes to obtain a melting curve. A decrease in fluorescence of the sample at the T_m of at least one the probes indicates the presence of the at least one *Candida* species. Preferably, the molecular beacon probes used in the methods have melting temperatures that vary by at least 1°C, such that a separate melt curve can be obtained for each molecular beacon probe. In particular embodiments, e.g., in which the methods are performed using the PCR conditions set for in Example 1, a decrease in fluorescence at a T_m of ~62-67°C (e.g., ~64°C) in the presence of the probe having the sequence of SEQ ID NO: 1 indicates the presence of *Candida albicans* in the sample; a decrease in fluorescence at a T_m of ~55-61°C (e.g., ~58°C) in the presence of the probe having the sequence of SEQ ID NO: 2 indicates the presence of *Candida tropicalis* in the sample; a decrease in fluorescence at a T_m of ~65-71°C (e.g., ~67°C) in the presence of the probe having the sequence of SEQ ID NO: 3 indicates the presence of *Candida glabrata* in the sample; a decrease in fluorescence at a T_m of ~60-65°C (e.g., ~62°C) in the presence of the probe having the sequence of SEQ ID NO: 4 indicates the presence of *Candida parapsilosis* in the sample; and a decrease in fluorescence at a T_m of ~66-72°C (e.g., ~69°C) in the presence of the probe having the sequence of SEQ ID NO: 5 indicates the presence of *Candida krusei* in the sample. Many conditions may affect the T_m of a probe during a melting curve analysis, e.g., the salt(s) and salt concentration(s) present in the reaction, the presence of intercalating agents, and the pH of the reaction. Accordingly, the melting temperatures indicated above for SEQ ID NOs: 1-5 are not meant to be limiting and may differ depending upon the conditions used in the method.

In yet other embodiments, one or more of the nucleic acid probes of the invention (e.g., any one of the nucleic acid probes having the sequence of SEQ ID NOs: 1 to 10 and 13 to 41) may be used in an array or microarray based platform for detecting one or more *Candida* species in a biological sample.

Each of the methods of the invention may further include steps for processing samples (e.g., biological samples), which may include one or more of the following: mixing the sample with a lysis agent solution (e.g., a detergent or a hypotonic solution); centrifuging the sample to form a supernatant and a pellet; discarding some or all of the supernatant; and/or resuspending the pellet to form an extract containing the fungal cells. In yet other embodiments, the processing steps may further include one or more of the following: optionally washing the pellet (e.g., with TE buffer) prior to resuspending the pellet; lysing fungal cells of the extract by chemical methods (e.g., using any combination of enzymes, or detergents, or surfactants), mechanical methods (e.g., using beads such as glass beads, bead beating, use of a finned tube in combination with beads, using beads in an agitation mill, use of beads with a chelating agent, use of glass shards, use of solid particles, use of beads or solid particles with mechanical or magnetic vortex centrifugation use of ultrasound, and/or use of sonication), or methods involving temperature changes (e.g., use of heat (e.g., a temperature in the range of about 85°C to about 125°C, such as a temperature of about 95°C), use of freeze-thaw, and/or use of freeze-boil) to form a lysate; and/or placing the lysate in a detection tube. Other processing steps may optionally include amplifying nucleic acids present in a processed sample to form an amplified lysate solution; adding all or a portion of the amplified lysate solution to a detection tube that includes one or more *Candida* specific nucleic acid probes or primers and allowing contact between the target nucleic acid molecules in the lysate and the one or more *Candida* specific nucleic acid probes or primers under conditions that allow hybridization of the nucleic acid probe(s) with the target nucleic acid molecules; and detecting hybridization of the nucleic acid probe(s) or primers to the *Candida* target nucleic acid molecules whereby the detection of binding of the *Candida* species-specific probe(s) with *Candida* target nucleic acid molecules present in the biological sample indicates the presence of the *Candida* species in the biological sample.

In another embodiment, the amplified lysate solution described above may be further processed to isolate the *Candida* nucleic acid molecules, e.g., by using an ion exchange column (such as a Qiagen column), glass or silica-base reverse phase adsorption/desorption, SPRI technology, phenol-chloroform extraction and ethanol precipitation, and/or the CTAB method.

In several embodiments, each of the methods of the invention may further include an amplification step for increasing the amount of the target *Candida* nucleic acid molecules to be detected in the biological sample. The amplification may include enzymatic amplification by, e.g., a PCR reaction. Primers having the sequence of SEQ ID NOs: 11 and 12 may be used as pan-*Candida* universal forward and reverse primers, respectively, to produce a *Candida* amplicon. In other embodiments, primers having the sequences of SEQ ID NOs: 6 to 41 can be used in combination with the pan-*Candida* universal primers to produce *Candida* species amplicons. The amplification may be symmetric to

produce a double stranded amplicon or the amplification may be asymmetric to produce a single stranded amplicon.

In several embodiments, each of the methods of the invention for detecting at least one *Candida* species in a biological sample can be completed within at least about 5 hours or less (e.g., within 4.0
5 hours, 3.5 hours, 3.0 hours, 2.5 hours, 2 hours, 1.5 hours, or 1 hour or less). In still other embodiments, each of the methods of the invention can be used to detect at least one *Candida* species in a sample when present at a concentration of at least about 4 *Candida* cells/mL (e.g., 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, or 50 *Candida* cells/mL).

In another embodiment of the compositions and methods of the invention, the biological sample
10 is, e.g., a soil, water, or food sample or a tissue or fluid sample from an organism, such as a human (e.g., whole blood, sweat, tears, urine, saliva, semen, serum, plasma, cerebrospinal fluid (CSF), feces, vaginal fluid or tissue, sputum, nasopharyngeal aspirate or swab, lacrimal fluid, mucous, or epithelial swab (buccal swab), tissues, organs, bone, teeth, and tumors).

In another embodiment, the invention features a kit which includes at least one nucleic acid probe
15 or primer having a sequence selected from any of SEQ ID NOs: 1 to 41 and further includes one or more reagents for attachment of fluorescent label or magnetic nanoparticle to the probe, and/or one or more reagents for sample lysis, fungal lysis, isolation of *Candida nucleic acid*, detection of *Candida* species, and nucleic acid amplification.

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Brief Description of the Drawings

Figures 1A and 1B are schematics showing binding of molecular beacon probes to a target nucleic acid molecule. Figure 1A shows the binding of a conventional molecular beacon probe. Figure 1B shows the binding of a shared-stem molecular beacon probe of the present invention. A shared-stem molecular beacon probe is designed so that 1) a stem-loop hairpin is formed by the 5' and 3' ends of the
25 probe, 2) a fluorophore and quencher are attached to the 5' and 3' ends and sufficient quenching will occur when the hairpin structure is formed, 3) the probes have a "loop" sequence that is specific for the target *Candida* sequence to be identified in the assay, and 4) sufficient fluorophore signal may be detected upon hybridization of the molecular beacon probe to the specific target sequence of *Candida*. The molecular beacon probes are designed to have a melting temperature (T_m) that is about 5 to 10
30 degrees (e.g., ~7 degrees) higher than the annealing temperature of amplification primers that may be present with the molecular beacon probes in an assay sample, and the molecular beacon probes are thermodynamically characterized to form no self dimers or heterodimers with other probes or primers (e.g., pan-*Candida* universal PCR primers) that may be present in an assay sample. Figures 2A and 2B are graphs showing detection of *Candida krusei* in real-time PCR reactions. Figure 2A shows the
35 detection of *Candida krusei* in a sample using purified genomic DNA. Figure 2B shows the detection of *Candida krusei* in a sample prepared from a cell lysate.

Figures 3A-3F are graphs showing titration curves for detection of *Candida* species in real-time PCR reactions. Figures 3A-3C are titration curves showing detection of *Candida parapsilosis* (Figure 3A), *Candida tropicalis* (Figure 3B), and *Candida krusei* (Figure 3C) using various amounts of purified genomic DNA. Figures 3D-3F are titration curves showing detection of *Candida parapsilosis* (Figure 3D), *Candida tropicalis* (Figure 3E), and *Candida krusei* (Figure 3F) using a cell lysate.

Figures 4A-4D are graphs showing titration curves for detection of *Candida albicans* and *Candida glabrata* in real-time PCR reactions. Figures 4A and 4B are titration curves showing detection of *Candida albicans* (Figure 4A) and *Candida glabrata* (Figure 4B) using various amounts of purified genomic DNA. Figures 4C and 4D are titration curves showing detection of *Candida albicans* (Figure 4C) and *Candida glabrata* (Figure 4D) using a cell lysate.

Figures 5A-5D are graphs showing the use of multiple molecular beacon probes in a multiplex assay to detect one or more *Candida* species in a sample. In this case, the genomic DNA of *Candida parapsilosis* and *Candida tropicalis* are present in a single reaction and amplified in the presence of a single beacon (Figures 5A and 5C, SEQ ID NO: 4 and SEQ ID NO: 2, respectively labeled with FAM and HEX) or with both beacons within the same reaction and emissions are observed in parallel using two channels (Figure 5B and Figure 5D).

Figure 6 is a schematic showing alignment of *Candida* amplicon sequences that are generated by amplification using the pan-*Candida* primers. The alignment shows species-specific differences within this amplicon. Shown are sequences for *C. krusei* (pan fungal amplicon; SEQ ID NO. 42), *C. albicans* (pan fungal amplicon; SEQ ID NO: 43), *C. glabrata* (pan fungal amplicon; SEQ ID NO: 44), *C. parapsilosis* (pan candida amplicon; SEQ ID NO. 45), *C. tropicalis* (pan candida amplicon; SEQ ID NO: 46), *C. parapsilosis* (pan candida amplicon, SEQ ID NO: 47), *C. tropicalis* (pan candida amplicon, SEQ ID NO: 48), *C. lusitanae* (ITS2; SEQ ID NO: 49), *C. guilliermondi* (amplicon 2; SEQ ID NO: 50), and a majority sequence (SEQ ID NO: 51).

Figure 7 is a sequence chromatogram (SEQ ID NO:52) generated from amplification within whole blood lysate using the pan-*Candida* forward and reverse primers (SEQ ID NOs: 11 and 12), followed by a phenol/chloroform extraction, a chloroform extraction, and then subsequent Sanger Dideoxy terminator sequence analysis using Big Dye Vs.31. (Applied Biosystems, Foster City, CA).

Figures 8A-8E are graphs of titration curves showing detection of *Candida albicans* (Figure 8A), *Candida glabrata* (Figure 8B), *Candida krusei* (Figure 8C), *Candida parapsilosis* (Figure 8D), and *Candida tropicalis* (Figure 8E) in real-time PCR reactions using genomic DNA obtained from *Candida* cell lysates prepared with cells in an amount ranging from ~520 cells to ~3 cells/mL (see the data in Tables 20, 22, 24, 26, and 28, which was used to produce the graphs of Figures 8A-8E, respectively).

Figures 9A and 9B are graphs showing nucleic acid denaturation melt curves in a multiplex reaction that includes *Candida* species-specific nucleic acid molecules from, and *Candida* species-specific molecular beacon probes for, *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* (Figure 9A) and for *C. albicans* and *C. krusei* (Figure 9B). The *Candida*-specific molecular beacon probes used

in the multiplex reactions are all labeled with a HEX fluorophore. The presence of nucleic acid molecules for each *Candida* species is determined by detecting a decrease in fluorescence as the sample is heated through the melting temperature (T_m) of each *Candida* species-specific molecular beacon probe. A decrease in fluorescence is observed only when hybridized beacon probes melt off their target nucleic acid molecules and the step-loop structure of the probes reforms. No melt curve is observed when the probe-specific target nucleic acid molecules are not present in the reaction.

Detailed Description of the Invention

The invention features compositions, methods, and kits for detecting at least one *Candida* species in a variety of media and biological samples, including, for example, biofluids, tissue samples, culture samples (e.g., a blood culture), food products, water samples, and soil samples. For example, the biological sample may include samples which have been processed to remove patient tissue and/or cellular debris or samples that are substantially unprocessed, such as a whole blood sample. The biological sample may further include cell suspensions or lysates that are produced by the methods of the invention. The biological sample may further include any sample which contains at least one nucleic acid molecule of *Candida* species; or any sample in which the nucleic acid molecules of *Candida* species have been substantially isolated, enriched or purified; or any sample in which the nucleic acid molecules of *Candida* species have been amplified and enriched as an amplicon.

The methods of the invention for detecting a *Candida* species can be performed with little to no sample preparation. In addition, the methods may be performed as a singleplexed assay to detect a single *Candida* species or as a multiplexed assay that allows for detection of multiple *Candida* species in a single sample. The methods of the invention allow for rapid and accurate detection of *Candida* in a biological sample (e.g., a tissue or fluid sample from a patient), which may be used to facilitate point-of-care clinical decision making. The methods described herein can be used to provide clinically and epidemiologically meaningful targeting of anti-fungal drug therapy to patients in need thereof and to provide optimal therapeutic outcome.

The compositions of the invention for use in the detection of a *Candida* species (e.g., one or more of *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida glabrata*, *Candida parapsilosis*, *Candida dubliniensis*, *Candida lusitanae*, and *Candida guilliermondi*) in a biological sample include probes (e.g., molecular beacon probes and NMR assay probes) that can be used to detect nucleic acid molecules of the *Candida* species (e.g., in a real-time probe-based nucleic acid detection assay). The methods of the invention involve contacting a sample that includes a nucleic acid molecule of at least one *Candida* species with at least one probe that is capable of hybridizing to the nucleic acid molecule and detecting hybridization between the probe and the nucleic acid molecule. In multiplexed assays, the sample may be contacted with two or more probes (e.g., probes to any two, three, four, or five or more of the *Candida* species listed above) in order to detect hybridization between the probes and the nucleic acid molecules.

The methods of the invention may be fluorescence based detection methods using fluorescently labeled species-specific probes (e.g., molecular beacon probes), such that hybridization between the probe and the nucleic acid molecule of the *Candida* species may result in a fluorescent signal that can then be detected by an appropriate detection device (e.g., a real-time thermal cycler or other fluorescence detection devices known in the art). For example, the probes may be fluorescently labeled molecular beacon probes designed to hybridize to *Candida* species-specific target nucleic acid molecules for detection of specific *Candida* species in a biological sample. Each *Candida* species-specific probe may be labeled with a fluorescent probe that distinguishes that probe from the other *Candida* species-specific probe(s) (e.g., based on differences in the excitation or emission spectra of the different probes). Alternatively, the probes may all include the same fluorescent label, in which case distinguishing the hybridization of each *Candida* species-specific probe to its targets is determined by other techniques, such as differences between the melting temperature (T_m) of each *Candida* species-specific probe, as is discussed below.

The methods of the invention may further include amplifying nucleic acid molecules of the *Candida* species present in the biological sample prior to detection of the *Candida* nucleic acid molecules. Amplification can be used to increase the amount of the *Candida* nucleic acid molecules present in the biological sample in case the original amount of *Candida* target nucleic acid molecules in the biological sample is below the detection limit of the probe(s). Typically such amplification may be done using one or more amplification primers. Any nucleic acid amplification method known in the art (e.g., polymerase chain reaction (PCR)) may be used. Preferably, the amplification method includes the use of primers that amplify nucleic acid molecules of at least one *Candida* species present in the biological sample.

Other methods of the invention include the use of fluorescently labeled probes and/or primers of the invention for detection of at least one *Candida* species in a biological sample in fluorescence based detection assays, for example, *in situ* hybridization assays. Another detection method employs use of a double strand specific intercalating dye, such as SYBR[®] (Molecular Probes, Eugene, OR). The fluorescence based assays may be performed in conjunction with real-time PCR detection. The invention also features one or more probes and primers of the invention for use as TaqMan probes in a real-time PCR assay or an end-point PCR assay. Finally, the *Candida* specific primers of the invention (e.g., any one or more of SEQ ID NOs: 1 to 10 and 13 to 41) may be used in sequencing based assays or in array or microarray based platforms in order to detect the presence of at least one *Candida* species in a biological sample.

The methods of the invention also include NMR based assays using *Candida* species-specific probes conjugated to magnetic nanoparticles. For example, in such an assay a sample may be contacted with at least one *Candida* species-specific probe that is conjugated to a magnetic nanoparticle, such that hybridization between the probe and the nucleic acid molecule of the *Candida* species may produce an

NMR signal that may be detected by NMR spectroscopy. Other aspects of the compositions, methods, and kits of the invention are described below.

Compositions and methods for detecting *Candida* species in biological samples.

5 The invention features compositions, methods, and kits for detecting at least one *Candida* species in a biological sample. The invention features *Candida* species-specific nucleic acid probes having the sequence of any one of SEQ ID NOs: 1 to 10 and 13 to 31, and probes having at least 90%, preferably at least 95%, more preferably at least 97%, and most preferably at least 99% or more identity to these sequences, that may be employed in a number of assays to detect one or more (for e.g., 2, 3, 4, 5, 6, 7, 8,
10 or more) *Candida* species in a biological sample. For example, the probes may be used to detect one or more of *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida glabrata*, *Candida parapsilosis*, *Candida dubliniensis*, *Candida lusitanae*, and *Candida guilliermondii*, in any combination, in a biological sample.

 The methods of the invention include the use of one or more *Candida* specific probes and/or
15 primers for detecting the presence of a *Candida* target nucleic acid molecule present in a biological sample. The methods include one or more of the following steps: (a) providing a biological sample; (b) mixing the sample with a lysis agent solution; (c) centrifuging the sample to form a supernatant and a pellet; (d) discarding some or all of the supernatant; and (e) resuspending the pellet to form an extract containing the fungal cells, optionally washing the pellet (e.g., with TE buffer) prior to resuspending the
20 pellet and optionally repeating step (c); (f) lysing cells of the extract by chemical or mechanical methods to form a lysate; (g) placing the lysate of step (f) in a detection tube; (h) optionally amplifying nucleic acids therein to form an amplified lysate solution; (i) adding to the detection tube one or more *Candida* specific nucleic acid probes and contacting the target nucleic acid molecules in the lysate with one or more *Candida* specific nucleic acid probes under conditions that allow hybridization of the probe(s) (also
25 referred to as binding of the probe(s)) to the target nucleic acid molecules, and (j) detecting hybridization of the probe(s) to the *Candida* target nucleic acid molecules by methods described below. The detection of binding of the *Candida* species-specific probe(s) with *Candida* target nucleic acid molecules present in the biological sample indicates the presence of the *Candida* species in the biological sample. The methods of the invention provide rapid and accurate readouts of the presence of at least one species of
30 *Candida* in the sample. Two or more probes of the invention may be used in the methods of the invention to perform a multiplexing assay that provides for the detection of two or more *Candida* species (e.g., 2, 3, 4, or 5 or more of the *Candida* species described herein) in the biological sample.

 In certain embodiments, steps (a) through (h) are completed within at least about 5 hours or less (e.g., within 4.0 hours, 3.5 hours, 3.0 hours, 2.5 hours, 2 hours, 1.5 hours, or 1 hour or less). In particular
35 embodiments, the methods allow for (i) at least 95% correct detection at less than or equal to 5 *Candida* cells/mL in samples spiked into 50 individual healthy patient blood samples; (ii) at least 95% correct detection at less than or equal to 5 *Candida* cells/mL in samples spiked into 50 individual unhealthy

patient blood samples; (iii) greater than or equal to 80% correct detection in clinically positive patient samples (e.g., *Candida* positive by another technique, such as by cell culture) starting with 2 mL of biological sample; and/or (iv) at least 90% correct detection in biological samples containing at least 5 *Candida* cells/mL. The invention provides methods in which *Candida* species can be detected at pathogen concentration of, e.g., at least 3 *Candida* cells/mL (e.g., 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, or 50 *Candida* cells/mL) in the sample with a coefficient of variation of less than 15% (e.g., 10 cells/mL with a coefficient of variation of less than 15%, 10%, 7.5%, or 5%; or 25 cells/mL with a coefficient of variation of less than 15%, 10%, 7.5%, or 5%; or 50 cells/mL with a coefficient of variation of less than 15%, 10%, 7.5%, or 5%; or 100 cells/mL with a coefficient of variation of less than 15%, 10%, 7.5%, or 5%). The methods of the invention may be used to detect the presence of *Candida* in a biological sample having a volume in the range of, e.g., 0.05 to 10.0 mL (e.g., a biological sample having a volume in the range of 0.05 to 0.25 mL, 0.25 to 0.5 mL, 0.25 to 0.75 mL, 0.4 to 0.8 mL, 0.5 to 0.75 mL, 0.6 to 0.9 mL, 0.65 to 1.25 mL, 1.25 to 2.5 mL, 2.5 to 3.5 mL, 3.0 to 4.0 mL, or 3.0 to 10 mL).

15 Species-specific *Candida* probes of the invention

Compositions of the invention include *Candida* species-specific nucleic acid probes that can bind to target sequences within the endogenous nucleic acid sequences of specific *Candida* species. The nucleic acid probes of the invention are designed to hybridize to endogenous nucleic acid molecules of *Candida* species and to provide accurate detection of at least one *Candida* species in a biological sample tested. The nucleic acid probes can be used individually in a detection assay or two or more nucleic acid probes (e.g., 3, 4, or 5 or more probes) can be used in combination, e.g., in a multiplex fluorescence assay, or in an NMR aggregation assay, to detect one or more *Candida* species in the biological sample.

The nucleic acid probes of the invention include: 5'-GGT CAA AGT TTG AAG ATA TAC GTG GTT GAC C-3' (SEQ ID NO: 1) for detecting *Candida albicans*, 5'-CTA GCA AAA TAA GCG TTT TTG GAT GCT AG-3' (SEQ ID NO: 2) for detecting *Candida tropicalis*, 5'-CAG CAC GCA CAA AAC ACT CAC TTA TTG CTG-3' (SEQ ID NO: 3) for detecting *Candida glabrata*, 5'-GTC GAA TTT GGA AGA AGT TTT GGT TTC GAC-3' (SEQ ID NO: 4) for detecting *Candida parapsilosis*, 5'-CCT GAT TTG AGG TCG AGC TTT TTG TAT CAG G-3' (SEQ ID NO: 5) for detecting *Candida krusei*, 5'-GGT CAA AGT TTG AAG ATA TAC GTG G-3' (SEQ ID NO: 6) for detecting *Candida albicans*, 5'-CTA GCA AAA TAA GCG TTT TTG GA-3' (SEQ ID NO: 7) for detecting *Candida tropicalis*, 5'-CAG CAC GCA CAA AAC ACT CAC TTA T-3' (SEQ ID NO: 8) for detecting *Candida glabrata*, 5'-GTC GAA TTT GGA AGA AGT TTT GGT-3' (SEQ ID NO: 9), for detecting *Candida parapsilosis*, 5'-CCT GAT TTG AGG TCG AGC TTT TTG T-3' (SEQ ID NO: 10) for detecting *Candida krusei*, 5'-AATAAAATGGGCGACGCCAGAGACCGCCTT-3' (SEQ ID NO: 26) and 5'-GCATCTCCGCCTTATACCACTATCA-3' (SEQ ID NO: 27) for detecting *Candida dubliniensis*, 5'-GGTTGATATTTTCGGAGCAACGCC-3' (SEQ ID NO: 28) and 5'-GTCCTACCTGATTTGAGGGCGAAAT-3' (SEQ ID NO: 29) for detecting *Candida lusitanae*, 5'-

GCAAACGCCTAGTCCGACTAAGAGTATCACTCAATACC -3' (SEQ ID NO: 30) and 5'-
 TGTAAGGCCGGGCCAACAATACCAGAAATATCCCGC-3' (SEQ ID NO: 31) for detecting *Candida*
guilliermondi, 5'-CCGAGAGCGAGTGTTGCGAGA-3' (SEQ ID NO: 32) and 5'-
 TCTCGCAACACTCGCTCTCGG-3' (SEQ ID NO: 33) for detecting *Candida krusei*, 5'-
 5 GGTAACGTCCACCACGTATATCT-3' (SEQ ID NO: 34) and 5'-
 AGATATACGTGGTGGACGTTACC-3' (SEQ ID NO: 35) for detecting *Candida albicans*, 5'-
 GGGAGGGATAAGTGAGTGTTTTGTGCGT-3' (SEQ ID NO: 36) and 5'-
 ACGCACAAAACACTCACTTATCCCTCCC-3' (SEQ ID NO: 37) for detecting *Candida glabrata*, 5'-
 GGTACAAACTCCAAAACCTTCTTCC-3' (SEQ ID NO: 38) and 5'-
 10 GGAAGAAGTTTTGGAGTTTGTACC-3' (SEQ ID NO: 39) for detecting *Candida parapsilosis*, and 5'-
 GCTAGTGGCCACCACAATTTATTTC-3' (SEQ ID NO: 40) and 5'-
 TGAAATAAATTGTGGTGGCCACTAGC-3' (SEQ ID NO: 41) for detecting *Candida tropicalis*. The
 invention features the probes described above, as well as probes having at least 90%, 95%, 97%, 99%, or
 more sequence identity to these probes.

15 In a further embodiment, one or more of the probes described above may be used in combination
 with any one of the *Candida* species-specific probes having the sequences of SEQ ID NOs: 13 to 25,
 which are described in International Application Nos. PCT/US2011/56933 and PCT/US2011/56936, both
 of which are incorporated herein by reference. These probes include: 5'-ACC CAG CGG TTT GAG
 GGA GAA AC-3' (SEQ ID NO: 13) and 5'-AAA GTT TGA AGA TAT ACG TGG TGG ACG TTA-3'
 20 (SEQ ID NO: 14) for detecting *Candida albicans*; 5'-CGC ACG CGC AAG ATG GAA ACG-3' (SEQ
 ID NO: 15), 5'-AAG TTC AGC GGG TAT TCC TAC CT-3' (SEQ ID NO: 16) and 5'-AGC TTT TTG
 TTG TCT CGC AAC ACT CGC-3' (SEQ ID NO: 17) for detecting *Candida krusei*; 5'-CTA CCA AAC
 ACA ATG TGT TTG AGA AG-3' (SEQ ID No: 18) and 5'-CCT GAT TTG AGG TCA AAC TTA AAG
 ACG TCT G-3' (SEQ ID NO: 19) for detecting *Candida glabrata*; 5'-AGT CCT ACC TGA TTT GAG
 25 GTCNitIndAA-3' (SEQ ID NO: 20), 5'-CCG NitIndGG GTT TGA GGG AGA AAT-3' (SEQ ID NO:
 21), 5'-AAA GTT ATG AAATAA ATT GTG GTG GCC ACT AGC-3' (SEQ ID NO: 22), 5'-ACC CGG
 GGGTTT GAG GGA GAA A-3' (SEQ ID NO: 23), 5'-AGT CCT ACC TGA TTT GAG GTC GAA-3'
 (SEQ ID NO: 24), and 5'-CCG AGG GTT TGA GGG AGA AAT-3' (SEQ ID NO: 25) for detecting
 either *Candida parapsilosis* or *Candida tropicalis*. In other embodiments, reverse complement versions
 30 of one or more of the nucleic acid molecules of SEQ ID NOs 1 to 41 may also be used as probes for
 detecting *Candida* species in a biological sample or as primers for amplifying one or more *Candida* nucleic
 acid molecules in a biological sample in an amplification step.

In addition, *Candida* specific nucleic acid molecules with at least 90% sequence identity (or at
 least 90%, 95%, 97%, 99%, or more sequence identity) to the sequences of SEQ ID NOs: 1 to 41, or to
 35 the reverse complement sequences of SEQ ID NOs: 1 to 41, may be used for detecting, amplifying, or
 both amplifying and detecting *Candida* species nucleic acid molecules in a biological sample.

The *Candida* species-specific probes of the invention may also include a detection label (e.g., a fluorescent detection label) or may be conjugated to a magnetic nanoparticle for use in the methods of the invention for detecting at least one *Candida* species in a biological sample. Detection labels and magnetic nanoparticles for use in the present invention, and methods for using probes of the invention that include a detection label or that are conjugated to a magnetic nanoparticle, are described in detail below.

Composition and methods for fluorescence-based detection of *Candida* species in biological samples

The invention features *Candida* species-specific nucleic acid probes that can bind to target sequences within the endogenous nucleic acid sequences of *Candida*. These probes can be used to detect the presence of specific species of *Candida* in biological samples. The probes may be labeled with a fluorescent label (also referred to as a fluorophore) and applied to a *Candida* lysate prepared from the biological sample. Preferably, the fluorophore-labeled probe emits a fluorescent signal upon hybridization of the probe to its target sequence and a fluorescent signal is not produced by unbound probe, e.g., as described in U.S. Patent Application No. US 2010/0221710A1 (incorporated herein by reference). The fluorescent signal produced upon hybridization between probe and target nucleic acid of the *Candida* species can be detected in a fluorescence detection device (e.g., a real time thermal cycler or other detection device known in the art). Each of the *Candida* species-specific probes described herein may be differentially labeled with fluorophores with spectrally distinguishable emission spectra for use in a multiplex fluorescence detection assay. In such a multiplex assay, two or more fluorescently labeled nucleic acid probes (e.g., 3, 4, or 5 or more probes), each having a different fluorophore attached, can be used in combination to rapidly and accurately detect multiple different *Candida* species in a biological sample.

The *Candida* species-specific probes of the present invention may also be used as “molecular beacons.” Methods to prepare and use molecular beacons are fully described in, e.g., U.S. Pat. Nos. 5,118,801, 5,312,728, and 5,925,517, each of which is incorporated herein by reference. Typically, molecular beacon probes have a fluorophore attached at the 5' end of the probe and a non-fluorescent quencher attached at the 3' end of the probe. Molecular beacons further contain complementary sequences at the 5' and 3' ends allowing these two ends to hybridize. The two ends flank a middle intervening “loop” sequence. The 5' and 3' ends of the molecular beacon can hybridize to each other and form an intramolecular stem structure and the middle intervening sequence region forms a “loop.” The “loop” part of the molecular beacon can hybridize to the target nucleic acid.

Under conditions (e.g., an appropriate temperature range) when the “loop” part of the probe cannot hybridize with the target nucleic acid sequence, the 5' and 3' ends of the probe form the intramolecular stem structure. As a result, the proximity between the fluorophore and quencher causes the the quencher to absorb any fluorescence emitted by the fluorophore and no fluorescence is produced. However, under different conditions (e.g., an appropriate temperature range) that allow the “loop” part of

the probe to hybridize to the target nucleic acid sequence, the stem structure is not formed, the fluorophore is separated from the quencher and, as a result, a fluorescent signal is produced.

The molecular beacon probes of the present invention are designed to detect specific nucleic acid target sequences in *Candida* species via species-specific "loop" sequences. Use of different fluorophore labels on the 5' end of each of the species-specific probes can allow use of multiple molecular beacon probes in a multiplex assay to simultaneously and rapidly detect multiple *Candida* species in a biological sample. Alternatively, each of the *Candida* species-specific probes described herein can be prepared as molecular beacon probes and labeled with the same fluorophore or with fluorophores having overlapping emission spectra for use in a multiplex fluorescence detection assay. Since each of the *Candida* species-specific molecular beacon probes have a different melting temperature (T_m), hybridization of each of the *Candida* species-specific molecular beacon probes to their target nucleic acid molecules can be detected by assaying fluorescence through a range of temperatures that includes the T_m of each probe used.

For example, a sample containing nucleic acid molecules from a *Candida* species may be contacted with a *Candida albicans* specific molecular beacon probe having a T_m of $\sim 64^\circ\text{C}$ (e.g., a T_m of $62\text{-}67^\circ\text{C}$) and a HEX fluorophore and a *Candida glabrata* specific molecule beacon probe having a T_m of $\sim 67^\circ\text{C}$ (e.g., a T_m of $65\text{-}71^\circ\text{C}$) and a HEX fluorophore. Fluorescence in the sample may be due to binding of the *C. albicans* or the *C. glabrata* probes or both. To determine which probe(s) are bound to their target nucleic acid molecules, the temperature of the sample may be raised from, e.g., 40°C to 80°C while detecting changes in fluorescence in the sample. If *C. albicans* nucleic acid molecules are present in the sample, there will be a change in fluorescence as the sample temperature increases through 64°C . In this case, half of the *Candida albicans*-specific molecular beacon probes will dissociate from their target nucleic acid molecules, which will result in a decrease in fluorescence as the probe melts off and the stem-loop structure reforms and quenches the fluorescence of those probes. If *C. glabrata* nucleic acid molecules are present in the sample, there will be a decrease in fluorescence as the temperature is increased through 67°C . In this case, half of the *C. glabrata* probes will dissociate from their target nucleic acid molecules, which will result in a decrease in fluorescence as the probe melts off and the stem-loop structure reforms and quenches the fluorescence of those probes. There would be no melt curves in the absence of *Candida* nucleic acid molecules. Similarly, no change in fluorescence around 64°C would indicate the absence of *Candida albicans* nucleic acid molecules, while no change in fluorescence around 67°C would indicate the absence of *Candida glabrata*.

The amount of *Candida* specific target nucleic acid molecules present in a biological sample may be sufficient for detection according to the methods of the present invention (e.g., by contact with the species-specific probes of the invention) without the need for amplification of the target nucleic acid molecules. However, it may be preferable to increase the amount of target nucleic acid molecules present in the biological sample prior to detection (e.g., if the amount of target nucleic acid molecules present in the biological sample is below the detection limit of the probe(s)). In this case, an optional amplification step may be performed to amplify the target nucleic acid molecules of the *Candida* species, which will

increase the amount of the target nucleic acid molecules present in the biological sample that may be bound by the probe(s). Typically, such amplification may be performed prior to contact with the probe by using, e.g., PCR or any other amplification methods known in the art.

The amplification reaction mixture may include, e.g., (1) the target nucleic acid molecule(s) and
5 (2) forward and/or reverse amplification primers specific for the target nucleic acid molecule(s). The forward and/or reverse primers may include, for example, the sequence 5'-GGC ATG CCT GTT TGA GCG TC-3' (SEQ ID NO: 11) and the sequence 5'-GCT TAT TGA TAT GCT TAA GTT CAG CGG GT-3' (SEQ ID NO: 12), each of which is universal to multiple *Candida* species, as described in International Application Nos. PCT/US2011/56933 and PCT/US2011/56936. These primers are referred
10 to herein as "pan-*Candida* primers." Alternatively, the forward and/or reverse primers can include primers that recognize species-specific target nucleic acids and can have the sequence of any one of SEQ ID NOs: 6 to 10 and 13 to 25. The amplification produces a *Candida* amplicon in the reaction mixture. The amplification may include substantially more forward or reverse primers if the amplification is to be asymmetric, or both a forward and a reverse primer in equimolar ratios, if the amplification is to be
15 symmetric, as described in detail below.

In a further embodiment, the detection probe(s) may be added at any time during the amplification step. For example, a fluorescently labeled molecular beacon probe (or probes in a multiplex assay) may be added to a sample prior to amplification (e.g., prior to, or substantially simultaneously with, addition of the amplification primer(s)) so that during the amplification step the
20 probe binds to its target and a fluorescent signal can be detected in real time. A real-time PCR machine may be used in such an assay and allows continuous monitoring of the fluorescence every time the species-specific probe hybridizes with its target nucleic acid sequence. Alternatively, a fluorescently labeled molecular beacon probe (or probes in a multiplex assay) can be added to a sample after an amplification step, and under conditions that allow the probe to hybridize with its target *Candida* amplicon, whereby
25 hybridization of the probe to an amplicon of a *Candida* target nucleic acid molecule can be detected by recording the resulting fluorescent signal.

The detection device for detecting a fluorescent signal may include any device that can provide temperature conditions for probe hybridization and that can detect a fluorescent readout when the hybridization occurs. For example, such a device may include a thermal cycler (e.g., a real-time thermal
30 cycler). Alternatively, the device can be a fluorescent plate reader, a flow-cytometer detecting particles with conjugated capture probes, a fluorescence microscope, or a microarray-based capture/detection, each with the ability to maintain the samples at a specific temperature range and the ability to detect and record fluorescent signals.

35 ***Fluorescently labeled species-specific Candida probes***

Nucleic acid probes of the invention having the sequence of any one of SEQ ID NOs: 1 to 5 (or probes having at least 90%, 95%, 97%, or 99% or more sequence identity to the sequence of SEQ ID

NOs: 1 to 5) may be used in an assay to detect at least one *Candida* species in a biological sample.

Preferably, such probes include a detection label, such as a fluorophore. The fluorophore can be conjugated to the 5' end or the 3' end of the probe, or alternatively it can be attached internally within the probe. Fluorophores that are known in the art and can be used for conjugation to probes of the invention, as well as the different types of devices that may be used to detect such probes (e.g., different types of thermal cyclers), are described in, e.g., Table 2 of Marras (*Methods Mol. Biol.* 335:3-16, 2006 (incorporated herein by reference)). Preferred examples of a fluorophore for labeling of the probes of the invention include, for example, FAM, TAMRA, HEX, TMR, Cy3, and Cy5. For multiplexing assays utilizing multiple probes for the simultaneous detection of multiple *Candida* species, it may be preferable to attach fluorophores with spectrally distinguishable emission spectra onto each species-specific probe in order to discriminate between binding of each probe to its respective target. Alternatively, as described above, multiplex assays may also be performed using multiple different *Candida* species specific probes that are labeled with the same fluorophore. Fluorescently labeled probes may include, without limitation, TaqMan probes (also known in the art as 5' nuclease probes), strand-displacement probes, or molecular beacon probes.

The fluorescently labeled probes of the invention having sequences of SEQ ID NOS: 1 to 5 (or probes having at least 90%, 95%, 97%, or 99% or more sequence identity to the sequence of SEQ ID NOs: 1 to 5) can be molecular beacon probes that may further include a quencher attached to the probe in addition to the fluorophore, as described above. For example, the fluorophore can be attached to the 5' end of the probe and the quencher can be attached to the 3' end of the probe or vice versa. The choice of the specific quencher depends on the specific fluorophore that is attached to the probe. Examples of quenchers that can be conjugated to the molecular beacon probes of the invention include, for example, DDQ-I, Dabcyl, Eclipse, Iowa Black FQ, BHQ-1, QSY-7, BHQ-2, DDQ-II, Iowa Black RQ, QSY-21, BHQ-3. Examples of preferred fluorescent-quencher pairs used in the art are described in, e.g., Marras (*supra*; see Table 2 therein), and include, without limitations, FAM-BHQ-1, TET-BHQ-1, HEX-BHQ-1, FAM-Dabcyl, TET-Dabcyl, and HEX-Dabcyl.

Nucleic acid probes of the invention having the sequence of SEQ ID NOs: 1 to 5 (or probes having at least 90%, 95%, 97%, or 99% or more sequence identity to the sequence of SEQ ID NOs: 1 to 5) may be conventional molecular beacon probes (see Figure 1A) or "shared-stem" molecular beacon probes (see Figure 1B). Shared-stem probes have one or more of the following characteristics:

- 1) About five to ten nucleotides (for e.g., 4, 5, 6, 7, 8, or 9 nucleotides; preferably 5 or 6 nucleotides) at the 5' end that are complementary to about five to ten nucleotides (for e.g., 4, 5, 6, 7, 8, or 9 nucleotides; preferably 5 or 6 nucleotides) at the 3' end such that the 5' and 3' ends of the probe can hybridize to form a "stem" structure;

- 2) A species-specific sequence of about 17 to 30 nucleotides in length (for e.g., 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides) that resides in the middle "loop" region of

the probe, that has limited secondary structure, and that can bind to specific target sequences in the endogenous nucleic acid of each *Candida* species;

3) A shared-stem design such that the 5' end of the nucleic acid probe has the ability to hybridize to a target sequence within the endogenous nucleic acid of each *Candida* species, whereas
5 about 1 to 20 nucleotides (e.g., about 5-6 nucleotides) of the 3' stem region do not hybridize with the endogenous nucleic acid of *Candida*; and

4) A predicted stem T_m of $\sim 50^\circ\text{C}$ and a probe/target duplex T_m of between 50°C and 75°C (e.g., $\sim 60^\circ\text{C}$).

When the probe is a molecular beacon probe that includes a fluorophore and a quencher, the
10 shared-stem feature of the probe improves signal to background ratios, improves sensitivity due to increased stability of the molecular beacon probe-target duplex, potentially enhances fluorogenic signals, and reduces the secondary structure in the probe backbone.

Each of the species-specific molecular beacon probes may be labeled with a different fluorophore (e.g., FAM, TAMRA, HEX, TMR, Cy3 and Cy5), such that the molecular beacon probes may be applied
15 simultaneously to a single sample in a multiplex assay that allows simultaneous detection of multiple *Candida* species based on spectral resolution of the emission maxima for the different fluorescent labels. The molecular beacon probes are designed to minimize heterodimer formation between the beacons so the detection is amenable to multiplexing using different fluorophores, as described above.

Probes of the invention having the sequence of SEQ ID NOs: 1 to 5 (or probes having at least
20 90%, 95%, 97%, or 99% or more sequence identity to the sequence of SEQ ID NOs: 1 to 5) may be used in combination with an optional PCR based amplification step. The amplification step, when performed in the methods of the invention, may be symmetric or asymmetric PCR. Symmetric PCR can be performed using the pan-*Candida* universal primers as the forward and reverse primers, which can amplify a *Candida* amplicon to increase the amount of all *Candida* species target nucleic acid molecules
25 present in the biological sample. Symmetric PCR may also utilize the pan-*Candida* universal forward primer in combination with the complement of one or more of SEQ ID NOs: 6 to 10 and 13 to 25, or the pan-*Candida* universal reverse primer in combination with the one or more of SEQ ID NOs: 6 to 10 and 13 to 25. Asymmetric PCR can be performed using the pan-*Candida* forward or reverse primer, one or more of SEQ ID NOs: 6 to 10 and 13 to 25 as the forward primer, or one or more of the complement of
30 SEQ ID NOs: 6 to 10 and 13 to 25 as the reverse primer.

For example, *Candida* specific target nucleic acid molecules prepared from a suspension of lysed
Candida cells can be added to a tube containing an asymmetric PCR master mix that includes the pan
Candida primers and the molecular beacon probe(s). The reaction tube may then be placed in a real-time
thermal cycler machine programmed to perform multiple rounds of amplification of the target nucleic
35 acid molecules characteristic of the *Candida* species present in the mix. This allows continuous monitoring of the fluorescence every time the probe hybridizes with its target nucleic acid sequence.

The molecular beacon probes may be designed to have a T_m about 7 degrees (for e.g., 3, 4, 5, 6, 7, 8, 9, or 10 degrees) higher than the annealing temperature of the primers and are thermodynamically characterized to form no self or heterodimers with the pan-*Candida* universal primers or the primers of SEQ ID NOs: 6 to 10 and 13 to 25. The limit of detection of these molecular beacon probes is about 3
5 cells/ml (for e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, or 50 cells/mL).

The amplification reaction may also be performed using pairwise combinations of one of the universal primers and a primer selected from any one of SEQ ID NOs: 13 to 25. The concentration of one of the primers may be in excess compared to the other for asymmetric PCR. Such pairwise combinations may be designed based on the desired *Candida* species amplicon and the genomic position
10 of the probe and the primer sequences. Such pairwise combinations may include, without limitation, the universal forward primer in combination with: a) a reverse primer having the reverse complement of primer of SEQ ID NO: 13 for amplifying a *Candida albicans* amplicon, b) a reverse primer having the reverse complement sequence of SEQ ID NOs: 20 to 25 for amplifying a *Candida tropicalis* or *Candida parapsilosis* amplicon, c) a reverse primer having the reverse complement sequence of SEQ ID NOs: 18
15 or 19 for amplifying a *Candida glabrata* amplicon, and d) a reverse primer having the reverse complement sequence of SEQ ID NOs: 15 to 17 for amplifying a *Candida krusei* amplicon.

Alternatively, the universal reverse primer may be used in combination with: a) a forward primer having the sequence of SEQ ID NO: 13 for amplifying a *Candida albicans* amplicon, b) a forward primer having the sequence of SEQ ID NOs: 20 to 25 for amplifying a *Candida tropicalis* or *Candida
20 parapsilosis* amplicon, c) a forward primer having the sequence of SEQ ID NOs: 18 or 19 for amplifying a *Candida glabrata* amplicon, and d) a forward primer having the sequence of SEQ ID NOs: 15 to 17 for amplifying a *Candida krusei* amplicon.

Alternate methods for detecting Candida species using probes and primers of the invention

In a further embodiment of the invention, all probes and primers described above can be used by
25 methods known in the art for detecting, amplifying and sequencing target nucleic acid molecules from *Candida* species in samples. For example, probes having the sequence of any one of SEQ ID NOs: 6 to 10, and 13-41 can be used to detect one or more *Candida* species in a biological sample in one or more of the following methods: a) a TaqMan probe based assay (e.g., real-time PCR based TaqMan assay), b)
30 strand-displacement probe based assays, c) PCR assays using DNA binding dyes (e.g., real-time PCR based assay using SYBR[®] green dye), d) *in situ* hybridization assays using fluorescently labeled species-specific probes, e) sequencing based assays (e.g., dideoxy-sequencing using probes having SEQ ID NOs: 32 to 41), and f) array or microarray based assays. Probes having the sequence of any one of SEQ ID
NOs: 6-10, and 13-41 may have detection labels such that the fluorescence detection is via a secondary
35 step. In such cases, the detection labels on the probe may include, without limitation, digoxigenin label for antibody-based detection using fluorescent labeled anti-digoxigenin antibodies, or biotin label for detecting using fluorescent streptavidin. Probes having the sequence of any one of SEQ ID NOs: 6-10,

and 13-41 may also have non-fluorescent detection labels including, but not limited to, radioactive isotopes for autoradiographic detection, digoxigenin for antibody-based detection using a horse-radish peroxidase (HRP) or alkaline phosphatase conjugated anti-digoxigenin antibody, and biotin for streptavidin based detection using HRP or alkaline phosphatase conjugated streptavidin. Non-fluorescent methods for detecting probe binding, such as autoradiographic detection, HRP- or alkaline phosphatase-based detection, colorimetric or chemiluminescent detection, and detection using array or microarray platforms are well known in the art and can be adapted by those of skill in the art to the methods described herein for detecting *Candida* species in a biological sample.

10 Compositions and methods for detecting *Candida* species in an NMR based assay

An alternate method for detecting *Candida* species in a biological sample may include the use of an NMR based assay using species-specific probes that are designed to be compatible with such assays. Use of NMR based assays to detect *Candida* species are described in, e.g., International Application No. PCT/US2011/56933 and International Application No. PCT/US2011/56936.

15 The present invention features probes having the sequence of any one of SEQ ID NOs: 6 to 10 and 26 to 31 that are conjugated to magnetic nanoparticles for use in an NMR based assay for detecting at least one *Candida* species in a biological sample. These nucleic acid probes of the invention include, e.g., 5'-GGT CAA AGT TTG AAG ATA TAC GTG G-3' (SEQ ID NO: 6) for detecting *Candida albicans*, 5'-CTA GCA AAA TAA GCG TTT TTG GA-3' (SEQ ID NO: 7) for detecting *Candida tropicalis*, 5'-CAG CAC GCA CAA AAC ACT CAC TTA T-3' (SEQ ID NO: 8) for detecting *Candida glabrata*, 5'-GTC GAA TTT GGA AGA AGT TTT GGT-3' (SEQ ID NO: 9), for detecting *Candida parapsilosis*, 5'-CCT GAT TTG AGG TCG AGC TTT TTG T-3' (SEQ ID NO: 10) for detecting *Candida krusei*, 5'- AATAAAATGGGCGACGCCAGAGACCGCCTT-3' (SEQ ID NO: 26) and 5'- GCATCTCCGCCTTATACCACTATCA-3' (SEQ ID NO: 27) for detecting *Candida dubliniensis*, 5'- GGTTGATATTTCCGAGCAACGCC-3' (SEQ ID NO: 28) and 5'- GTCCTACCTGATTTGAGGGCGAAAT-3' (SEQ ID NO: 29) for detecting *Candida lusitanae*, and 5'- GCAAACGCCTAGTCCGACTAAGAGTATCACTCAATACC -3' (SEQ ID NO: 30) and 5'- TGTAAGGCCCGGCCAACAATACCAGAAATATCCCGC-3' (SEQ ID NO: 31) for detecting *Candida guilliermondi*. Probes having sequences with at least 90%, e.g., at least 95%, 97%, and 99% or more sequence identity to the sequences of SEQ ID NOs: 6 to 10 and 26 to 31 and that are bound to nanoparticles are also included in the present invention.

As further described in International Application No. PCT/US2011/56933 and International Application No. PCT/US2011/56936, the NMR assay may be an "aggregation assay" in which magnetic nanoparticles can include one or more populations having a first probe and a second probe conjugated to their surface, the first probe operative to bind to a first segment of the target nucleic acid and the second probe operative to bind to a second segment of the target nucleic acid. Upon probe hybridization to the target nucleic acid, the magnetic particles form aggregates.

The terms “aggregation” and “aggregate” are used interchangeably in the context of the magnetic particles described herein and mean that two or more magnetic particles are brought into close proximity to one another as a result of target nucleic acid molecule binding by the nanoparticle-conjugated probes. Hybridization between probe and target nucleic acid may be detected in a nuclear magnetic resonance (NMR) instrument by measuring the “NMR relaxation rate,” as described in International Application No. PCT/US2011/56933 and International Application No. PCT/US2011/56936. NMR relaxation rate refers to a measurement of any of the following in a sample: T_1 , T_2 , T_1/T_2 hybrid, $T_{1\rho}$, $T_{2\rho}$, and T_2^* . The methods of the invention are designed to produce an NMR relaxation rate characteristic of whether a target nucleic acid from *Candida* is present in a biological sample. In some instances the NMR relaxation rate is characteristic of the quantity of target nucleic acid present in the sample. In addition to the “aggregation” assay described above, the probes can be used in “disaggregation” assays, according to methods described in International Application No. PCT/US2011/56933 and International Application No. PCT/US2011/56936.

The *Candida* lysate containing the target nucleic acid may be contacted with magnetic nanoparticles conjugated to species specific probes having the sequence of any one or more of SEQ ID NOs: 6 to 10 and 26 to 31, or their complements, and used in combination with magnetic nanoparticles conjugated with species-specific probes having the sequence of SEQ ID NOs: 13 to 25. For example, the probe of SEQ ID NO: 6 may be used in combination with a probe having the sequence of any one of SEQ ID NO: 13 or 14 for detecting *Candida albicans*; the probe of SEQ ID NO: 7 may be used in combination with a probe having the sequence of any one of SEQ ID NOs: 20 to 25 for detecting *Candida tropicalis*; the probe of SEQ ID NO: 8 may be used in combination with a probe having the sequence of any one of SEQ ID NO: 18 or 19 for detecting *Candida glabrata*; the probe of SEQ ID NO: 9 may be used in combination with a probe having the sequence of any one of SEQ ID NOs: 20 to 25 for detecting *Candida parapsilosis*; and the probe of SEQ ID NO: 10 may be used in combination with a probe having the sequence of any one of SEQ ID NOs: 15 to 17 for detecting *Candida krusei*.

The NMR based detection methods may also include an optional amplification step, which may be performed to amplify the target nucleic acid prior to or substantially during contacting of the biological sample with the probe-nanoparticle conjugate. The amplification step of this method may include one or more of the following steps:

- (a) performing one or more cycles of amplification with one or more of the *Candida* specific PCR primers described above in a detection tube,
- (b) addition of the probe-nanoparticle conjugate,
- (c) exposing the amplification reaction mixture, or an aliquot thereof, to conditions permitting the aggregation or disaggregation of the superparamagnetic particles,
- (d) detecting hybridization between probes and target nucleic acid by NMR and measuring the NMR signal from the detection tube,
- (e) repeating steps (a)-(d) until a desired amount of amplification is obtained; and

- (f) on the basis of the result of step (d), quantifying the amplicons present at the corresponding cycle of amplification.

The amplification can be performed using combinations of the pan-*Candida* universal primers. Optionally, probes having sequences of any one or more of SEQ ID NOs: 6 to 10 and 13 to 31 may also be used as forward and/or reverse primers (in a symmetric or an asymmetric amplification reaction) or in combination with one of the pan-*Candida* primers (in a symmetric or an asymmetric amplification reaction) to produce a *Candida* amplicon. The amplification may be performed using pairwise combinations of one of the universal primers and a primer selected from any one of SEQ ID NOs: 6 to 10 and 13 to 31 (or its complement). Such pairwise combinations may be designed based on the desired *Candida* species amplicon and the genomic positions of the probe and the primer sequences to be used. Such pairwise combinations may include, without limitation, use of the universal forward primer in combination with: a) a reverse primer having the complement of any one of SEQ ID NOs: 6 or 13 for amplifying a *Candida albicans* amplicon; b) a reverse primer having the complement sequence any one of SEQ ID NOs: 7, 9, or 20 to 25 for amplifying a *Candida tropicalis* or *Candida parapsilosis* amplicon; c) a reverse primer having the complement sequence of any one of SEQ ID NOs: 8, 18, or 19 for amplifying a *Candida glabrata* amplicon; d) a reverse primer having the complement sequence of any one of SEQ ID NOs: 10 or 15 to 17 for amplifying a *Candida krusei* amplicon; e) a reverse primer having the complement sequence of any one of SEQ ID NO: 26 or 27 for amplifying a *Candida dubliniensis* amplicon; f) a reverse primer having the complement sequence of any one of SEQ ID NO: 28 or 29 for amplifying a *Candida lusitanae* amplicon; g) a reverse primer having the complement sequence of any one of SEQ ID NO: 30 or 31 for amplifying a *Candida guilliermondi* amplicon.

Alternatively, the universal reverse primer may be used in combination with: a) a forward primer having the sequence of any one of SEQ ID NO: 6 or 13 for amplifying a *Candida albicans* amplicon; b) a forward primer having the sequence of any one of SEQ ID NOs: 7, 9, or 20 to 25 for amplifying a *Candida tropicalis* or *Candida parapsilosis* amplicon; c) a forward primer having the sequence of any one of SEQ ID NOs: 8, 18, or 19 for amplifying a *Candida glabrata* amplicon; d) a forward primer having the sequence of any one of SEQ ID NOs: 10 or 15 to 17 for amplifying a *Candida krusei* amplicon; e) a forward primer having the sequence of any one of SEQ ID NOs: 26 or 27 for amplifying a *Candida dubliniensis* amplicon; f) a forward primer having the sequence of SEQ ID NO: 28 or 29 for amplifying a *Candida lusitanae* amplicon; g) a forward primer having the sequence of any one of SEQ ID NO: 30 or 31 for amplifying a *Candida guilliermondi* amplicon.

The probe-nanoparticle conjugate can be added to the amplification mix prior to, or during, the PCR reaction, such that the probe-nanoparticle conjugates are present during the PCR reaction and under appropriate conditions can bind with the target nucleic acid. Alternatively, the probe-nanoparticle conjugate can be added to the *Candida* lysate after the amplification step to detect the amplified *Candida* target nucleic acid.

The term “magnetic particle” refers to particles including materials of high positive magnetic susceptibility such as paramagnetic compounds, superparamagnetic compounds, and magnetite, gamma ferric oxide, or metallic iron. The magnetic nanoparticles may have a mean diameter of from 150 nm to 1200 nm (for e.g., 150, 250, 350, 450, 550, 650, 750, 850, 950, 1050, 1150, and 1200 nm) and are typically a composite material including multiple metal oxide crystals and an organic matrix. The magnetic particles of the invention may have a surface decorated with functional groups that can be conjugated to, e.g., one or more nucleic acid probes of having the sequence of any one of SEQ ID NOs: 6 to 10 and 13-31, their complements, or probes having at least 90%, 95%, 97%, 99%, or more sequence identity to the sequences of SEQ ID NOs: 6 to 10 and 13-31 and their complements. The base particle for use in the methods of the invention can be any of the commercially available particles further described in, e.g., Table 2 of the International Application No. PCT/US2011/56933. The magnetic nanoparticle maybe modified using protein blockers, as described in the International Application No. PCT/US2011/56933, in order to reduce non-specific binding.

The nucleic acid probes of the invention can be linked to the metal oxide of the nanoparticle through covalent attachment to a functionalized polymer or to non-polymeric surface-functionalized metal oxides. In the latter method, the magnetic particles can be synthesized according to the method of, e.g., Albrecht et al., (*Biochimie*, 80:379, 1998; incorporated herein by reference), which includes coupling dimercapto-succinic acid to the iron oxide to provide a carboxyl functional group.

Where the probes of the invention are attached to magnetic particles via a functionalized polymer associated with the metal oxide, preferably the polymer is hydrophilic. In certain embodiments, the probe-conjugated nanoparticles may be made using probes that have terminal amino, sulfhydryl, or phosphate groups and superparamagnetic iron oxide magnetic particles bearing amino or carboxy groups on a hydrophilic polymer. Several methods for synthesizing carboxy and amino derivatized-magnetic particles are known in the art.

Probes of the invention may also be attached to a magnetic nanoparticle via ligand-protein binding interaction, such as biotin-streptavidin, in which the ligand is covalently attached to the oligonucleotide and the protein to the particle, or vice versa. This approach can allow for more rapid reagent preparation for NMR assays.

30 **Amplification of nucleic acid molecules of *Candida***

As is discussed above, the methods of the invention can optionally include an amplification step in which specific regions of the *Candida* genome are amplified to form a *Candida* species amplicon.

The terms “amplification” or “amplify” or derivatives thereof as used herein to mean one or more methods known in the art for copying a target or template nucleic acid, thereby increasing the number of copies of the target or template nucleic acid. Amplification may be exponential or linear. Amplification may also be asymmetric (only the sense or antisense strand is copied) or symmetric (both the sense and

antisense strands are copied). Nucleic acid molecules amplified in this manner form an “amplified region” or “amplicon.”

The amplification reaction increases the number of copies of *Candida* target nucleic acid molecules present in a *Candida* lysate solution. Preferably, amplification of the *Candida* target nucleic acid molecules produces a lysate solution that includes from 40% (w/w) to 95% (w/w) target nucleic acid (e.g., from 40 to 60%, from 60 to 80%, or from 85 to 95% (w/w) target nucleic acid) and from 5% (w/w) to 60% (w/w) nontarget nucleic acid (e.g., from 5 to 20%, from 20 to 40%, or from 40 to 60% (w/w) nontarget nucleic acid). The species-specific nucleic acid probes described above can be added to the lysate solution (prior to or after amplification) for hybridization to the target nucleic acid molecules and for detection of specific *Candida* species present in the biological sample.

In the context of the present invention, amplification produces *Candida* species amplicons. For example, as shown in Figure 6, amplification with the pan-*Candida* primers produces an amplicon which has regions which differ in sequence between *Candida* species and regions which share a common sequence between all *Candida* species. The regions that are different can be used to differentiate between *Candida* species.

Primers for amplification can be readily designed by those skilled in the art to target a specific template nucleic acid sequence. In certain preferred embodiments, the resulting amplicons are short and allow for rapid cycling and generation of copies. The size of the amplicon can vary as needed to provide the ability to discriminate target nucleic acids from non-target nucleic acids. For example, amplicons can be less than about 1,000 nucleotides in length. Desirably the amplicons are from 100 to 500 nucleotides in length (e.g., 100 to 200, 150 to 250, 300 to 400, 350 to 450, or 400 to 500 nucleotides in length).

While the exemplary methods described hereinafter relate to amplification using polymerase chain reaction (“PCR”), numerous other methods are known in the art for amplification of nucleic acids (e.g., isothermal methods, rolling circle methods). Alternate methods for amplification are described in further detail in, e.g., the International Application No. PCT/US2011/56933 and International Application No. PCT/US2011/56936.

Amplification may be performed by methods including a polymerase chain reaction (PCR) and the amplification reaction mixture may include, e.g., (1) the target nucleic acid molecule(s), (2) forward and/or reverse amplification primers specific for the target nucleic acid molecule(s), and (3) a detection probe(s) that can bind to target sequences within the amplified target nucleic acid (the amplicon). In certain embodiments, the invention features the use of polymerase enzymes compatible with complex biological samples such as whole blood, e.g., NEB Hemoklentaq, DNAP Omniklentaq, Kapa Biosystems whole blood enzyme, Thermo-Fisher Finnzymes Phusion enzyme.

In certain embodiments, the primers can include the pan-*Candida* primer sequences where the forward and reverse primer have the sequence 5'-GGC ATG CCT GTT TGA GCG TC-3' (SEQ ID NO: 11) and the sequence 5'-GCT TAT TGA TAT GCT TAA GTT CAG CGG GT-3' (SEQ ID NO: 12), respectively. In other embodiments, specific *Candida* species amplicons may be amplified by the use of

appropriate forward and reverse primers that bind to and produce species-specific *Candida* amplicons, as described above.

In certain embodiments the amplification may be asymmetric, in which the concentration of one of the primers in the reaction is higher than the concentration of the other primer. For example, the concentration of the forward amplification primer may be about 4 times (e.g., 2, 3, 4, 5, 10, 20, 50, times) the concentration of the reverse primer, or vice-versa. This reaction condition results in preferential amplification of single stranded amplicons. In other embodiments, the amplification may be symmetric. In this case, the PCR reaction may be performed using equal concentrations of the forward and reverse primers, which results in double stranded amplicons. Methods for performing asymmetric and symmetric PCR are known in the art (see, for example, Poddar, *Molecular and Cellular Probes* 14: 25-32, 2000, incorporated herein by reference). In one particular example, the pan-*Candida* universal forward and/or reverse primers can be used in pairwise combinations with any one of the primers having the sequence of SEQ ID NOs: 6-10 and 13-31, or their complements, to produce species-specific *Candida* amplicons.

15 **Biological samples that can be tested for the presence of a *Candida* species**

The compositions, methods, and kits featured in the invention may be used to detect at least one (and preferably two or more) *Candida* species that may be present in a biological sample. By biological sample is meant a variety of media including, but not limited to, biofluids, tissue samples, culture samples (e.g., a blood culture), food products, water samples, and soil samples that contain one or more species of *Candida* cells. For example, the biological sample can be a sample which has been processed to remove cellular and tissue debris and patient nucleic acids, or the biological sample may be a sample that has been processed to partially or fully isolate *Candida* cells from the sample. The biological sample may include, e.g., cell suspensions or cell lysates. The biological sample may also be one that has been substantially unprocessed, such as a whole blood sample. The biological sample may also be any sample in which the nucleic acid molecules of *Candida* have been substantially isolated, enriched by amplification or other methods, or purified. The biological sample can be any sample that contains at least one genome equivalent of *Candida*.

Typically, the sample is human in origin, but alternatively it may be non-human in origin (such as from an animal). The biological sample is preferably a fluid sample, and can typically comprise a body fluid, but may also be a solid tissue sample. The sample may be, e.g., whole blood, sweat, tears, urine, saliva, semen, serum, plasma, cerebrospinal fluid (CSF), feces, vaginal fluid, sputum, nasopharyngeal aspirate or swab, and lacrimal fluid. The sample can also be mucous or epithelial swab (buccal swab), tissues, organs, bone, teeth, and tumors.

The compositions, methods, and kits of the invention can also be used to monitor and diagnose infectious disease in a multiplexed, automated, no sample preparation system. Examples of pathogens that may be detected using the devices, kits, and methods of the invention include, e.g., *Candida*, e.g., *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis*, *C. lusitanae*, and *C.*

guilliermondi. The methods of the invention can be used to identify and monitor the pathogenesis of disease in a subject, to select therapeutic interventions, and to monitor the effectiveness of the selected treatment.

The methods of the invention can also be used to monitor sepsis or septic shock. Sepsis and septic shock are serious medical conditions that are characterized by a whole-body inflammatory state (systemic inflammatory response syndrome or SIRS) and the presence of a known or suspected infection. Sepsis is defined as SIRS in the presence of an infection, septic shock is defined as sepsis with refractory arterial hypotension or hypoperfusion abnormalities in spite of adequate fluid resuscitation, and severe sepsis is defined as sepsis with organ dysfunction, hypoperfusion, or hypotension. To determine whether a patient has sepsis, it is necessary to identify the presence of a pathogen and to identify bacterial or fungal origin. To most effectively treat a patient, the earliest initiation of appropriate therapy is important to a satisfactory outcome. Antimicrobial and other treatments for sepsis rely on the classification of pathogens at multiple levels, including the identification of an agent as 1) bacterial, viral, fungal, parasitic or otherwise; 2) gram positive, gram negative, yeast, or mold, 3) species, and 4) susceptibility.

Each of these levels of specificity improves the time to initiation of appropriate therapy, and each step further down the track will lead to a narrowing of therapeutic agents to the most specific set. Without absolute susceptibility data, empiric approaches to care rely on the information available about the pathogen (at whichever level) and the pattern of pathogen frequency and susceptibility trends in the hospital or another site of care. Thus, certain categories of pathogens are frequently presumed to be causative until there are more data to refine the pairing of pathogen and therapy. The methods of the present invention can be used to facilitate early diagnosis of fungal (e.g., *Candida*) infection, and thus can provide improved therapeutic outcomes in patients diagnosed using such methods.

Biological sample lysis methods

In any of the methods of the invention for detection of *Candida* species in a biological sample, the disruption of patient cells present in the biological sample can be carried out using a lysis agent (e.g., a lysis buffer, a hypotonic buffer, or a nonionic detergent). Lysis buffers which can be used in the methods of the invention include, without limitation, isotonic solutions of ammonium chloride (optionally including carbonate buffer and/or EDTA), and hypotonic solutions. Alternatively, the lysis agent can be aqueous solution of one or more detergents (e.g., nonionic, ionic, polymeric, and zwitterionic, such as, (e.g., nonyl phenoxy polyethoxy ethanol (NP-40), 4-octylphenol polyethoxylate (Triton-X100), Brij-58), or related surfactants, and mixtures thereof). The lysis agent disrupts at least some of the sample cells, allowing a large fraction of certain components to be separated. For example, if the biological sample is whole blood, the lysis agent disrupts at least some of the red blood cells present in the sample, allowing a large fraction of certain components of whole blood (e.g., certain whole blood proteins) to be separated (e.g., as supernatant following centrifugation) from the *Candida* fungal cells present in the whole blood sample. The *Candida* fungal cells can then be optionally washed to further remove whole blood lysed

debris from the sample. The lysed sample may then be centrifuged to produce a supernatant and a pellet. The resulting pellet containing the *Candida* cells may be reconstituted to form a suspension of *Candida* cells, which may be further treated as discussed below.

5 Fungal lysis methods

In any of the methods of the invention for detection of *Candida* species in a biological sample, the isolated *Candida* cells in suspension may be lysed using either chemical or mechanical methods known in the art in order to release the target nucleic acid from the *Candida* cells. For example, chemical lysis methods include, without limitation, treatment of fungal cells with one or more enzymes, such as
10 Zymolyase (see, e.g., Park et al., *J. Clin Microbiol.* 38: 2829-2839, 2000, incorporated herein by reference), or detergents, or surfactants. Alternatively, *Candida* cells may be lysed using mechanical methods known in the art. For example, mechanical lysis methods include, without limitation, use of solid particles, sand, or glass shards, use of glass beads as described in, e.g., Hirose et al., *Biotechnology Techniques* 13: 571-575, 1999 (incorporated herein by reference), use of beads with sonication or
15 mechanical vortex centrifugation or magnetic vortex centrifugation as described in, e.g., U.S. Patent No. 7,723,095 (incorporated herein by reference), bead beating described in International Application No. PCT/US2011/56933 and International Application No. PCT/US2011/56936, use of a finned tube, e.g., use of a finned tube with beads added to the tube for lysing the sample, as described in U.S. Patent Application No. 61/601,842 (incorporated herein by reference), use of ultrasound as described in, e.g.,
20 U.S. Patent No. 6,686,195 (incorporated herein by reference), heating the sample or applying high pressure as described in, e.g., Chisti and Moo-Young, *Biotechnology/The Science and Business*, Chapter 13, Harwood Academic Publishers, 1999 (incorporated herein by reference), use of solid particles in the presence of chelating agents as described in, e.g., U.S. Patent No 7,494,771 B2 (incorporated herein by reference), use of high speed agitation bead mills as described in, e.g., Kula and Schutte, *Biotechnology Progress* 3(1): 31, 1987 (incorporated herein by reference), use of cell wall breaking devices as described
25 in, e.g., U.S. Patent No. 4,295,613 (incorporated herein by reference), and freeze-boil and freeze-thaw methods (see, e.g., Griffiths et al., *J. Med. Microbiol.* 55: 1187-1191, 2006, incorporated herein by reference).

When beads are used to lyse the *Candida* cells, the beads may be 0.5 mm glass beads, 0.7 mm
30 silica beads, 0.1 mm silica beads, 0.7 mm silica beads, yttrium stabilized zirconium oxidized beads, or a mixture of differently sized beads made of inert material (see, e.g., Curran and Evans, *J. Bacteriol.* 43(2): 125, 1942; and Lamanna and Mallette, *J. Bacteriol.* 67(4): 503, 1954, incorporated herein by reference). Typically, the beads are added to the tube containing the *Candida* cells in suspension and the tube is placed in a vortexer (e.g., a Biospec bead beater) and vortexed at maximum speed for 5-15 minutes (e.g.,
35 5 minutes) so that the *Candida* cells are lysed and the endogenous target nucleic acids are released.

If desired, the lysate can be further processed to purify the *Candida* nucleic acid molecules by methods known in the art which include, without limitations, use of an ion exchange columns, e.g., an

anion exchange column, such as a Qiagen column, phenol-chloroform extraction and ethanol precipitation methods as described in Maniatis *et al.* "Molecular Cloning- A laboratory manual" Cold Spring Harbor Press, 1982 (incorporated herein by reference), use of fungal DNA extraction kits as described in, e.g., Fredricks *et al.*, *J. Clin. Microbiol.* 43 (10): 5122-5128, 2005 (incorporated herein by reference), use of the "CTAB method" as described in Zhang *et al.*, *FEMS Microbiology Letters* 145(2): 261-265, 1996 (incorporated herein by reference), and spray technology.

Kits for detection of *Candida* species

Kits of the invention may include one or more probes and primers having the sequence of any of SEQ ID NOs: 1 to 41 (e.g., SEQ ID NOs: 1 to 5) for detecting *Candida* species in biological samples. The kits may further include instructions for using one or more of the probes and primers in methods for detecting a *Candida* species (e.g., *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida glabrata*, *Candida parapsilosis*, *Candida dubliniensis*, *Candida lusitaniae*, and *Candida guilliermondii*) in a biological sample.

In addition, the kits may include any combination of reagents for sample lysis, fungal cell lysis, amplification of *Candida* nucleic acid molecules, and reagents for detection of *Candida* species appropriate for fluorescence detection and/or NMR based detection.

The kits may also include one or more reagents for fluorescently labeling a probe or probes (e.g., probes having SEQ ID NOs: 1 to 5) or the kits may include a probe or probes that are already fluorescently labeled (e.g., the probes of the kits may be molecular beacon probes having the sequence of any one of SEQ ID NOs: 1 to 5). The kits may also include reagents for conjugating nanoparticles to a probe or probes of the invention (e.g., a probe having the sequence of any one of SEQ ID NOs: 6 to 10 and 13 to 31) or the kits may include a probe or probes that are already conjugated to nanoparticles for use in NMR based detection assays.

Uses for *Candida* specific molecular beacon probes

The *Candida* specific molecular beacon probes described herein can be used in several ways, including the following:

- a) If intact fungal cells can be enriched and/or partially or fully purified from a biological matrix (e.g., a biological sample, such as whole blood, serum, sputum, urine, etc.), the *Candida* specific molecular beacon probes can be used in a method to detect and identify a fungal species in the biological matrix;
- b) If fungal DNA can be isolated from any biological sample, the *Candida* specific molecular beacon probes can be used to detect the *Candida* DNA, as well as identify the particular fungal species;
- c) In a rapid and convenient method for fungal identification in a post blood-culture sample. This could be through direct detection, using the *Candida* specific molecular beacon probes, of

Candida within a small aliquot (~5-50 uL) of the blood culture media and/or removal of 5 mL or less of the media, centrifugation and removal of the supernatant, and resuspension of the cells within TE. This potentially could be done at earlier time points during blood culture incubation to maximize time to result;

- 5 d) In a robust and quantitative assay for quantification of fungal cells used to generate *Candida* spiked whole blood samples from frozen cell bullets;
- e) In a high throughput and sensitive tool to quantify and ensure lot to lot reproducibility and stability of *Candida* external controls; and
- 10 f) In a high throughput and sensitive orthogonal assay to monitor the enzymatic activity of a polymerase for use in an amplification reaction using *Candida* specific nucleic acid molecules and/or to monitor the activity and reliability of a PCR master mix (lot to lot reproducibility, shelf life stability, material compatibility, and ship stability).

15 The following examples are meant to illustrate the invention. They are not meant to limit the invention in any way.

Examples

Example 1: Detection of Candida krusei using a species-specific molecular beacon probe in a real-time PCR assay.

20 A general protocol for detecting *Candida* species using fluorescently labeled molecular beacon probes (e.g., SEQ ID NOs: 1 to 5) is described below. For preparation of *Candida* cell lysate, 1 mL of PBS, TE, or YPD broth containing spiked *Candida* cells at concentrations ranging from 10^4 to 1 *Candida* cells were added to a tube containing 300 mg of yttrium stabilized zirconium oxide beads. The cells were harvested via centrifugation at 6000G for 5 minutes and the supernatant was removed. The cells were

25 then washed with 150 to 1500 μ L of TE buffer and harvested again via centrifugation at 6000G. 100 μ L of TE buffer was added to the tubes and the tube was placed in a vortexer (Biospec bead beater) at maximum speed for 5 minutes. The resulting *Candida* lysate was removed and transferred to a clean 1.7 mL polypropylene tube, and was either used directly in a real-time PCR reaction or was further processed using a Qiagen kit to isolate the *Candida* genomic DNA containing the target nucleic acid molecules.

30 An asymmetric master mix was prepared using pan-*Candida* forward and reverse primers, as shown in Table 1.

Table 1.

Component	per 100 uL reaction
Nuclease free water*	17.33
5X reaction Buffer	20
10 mM mix	2
100 uM Forward Primer	0.3
100 uM Reverse Primer	0.075
100 uM Molecular beacon	0.3
Hemoklentaq	10
sum	50.00

5 The molecular beacon probe (at a concentration of 300 nM; the molecular beacon probe may be used at a concentration of ~100-600 nM) was added directly to the asymmetric master mix. Fifty microliters of cell lysate or diluted genomic DNA was added directly to the master mix in a 96-well plate and the plate was loaded onto a real-time PCR thermal cycler (i.e. Roche LightCycler). The real-time cycling parameters used for asymmetric amplification are described in Table 2.

10

Table 2.

<p>Pre-incubation: 95C, 5 minutes, ramp rate: 4.4 degrees C/sec</p> <p>Amplification-45 cycles Denaturation: 95C, 20 sec, ramp rate: 4.4 degrees C/sec Annealing: 60C, 40 sec, ramp rate: 2.2 degrees C/sec Elongation: 68C, 30 sec, ramp rate: 4.4. degrees C/sec</p> <p>Melt Curve 95C, 1 minute, ramp rate: 4.4 degrees C/sec 40C, 1 minute, ramp rate: 2.2 degrees C/sec 80C, continuous, ramp rate: 0.29 degrees C/sec</p>	15
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20 In this example, a molecular beacon probe specific for *Candida krusei* (having the sequence of SEQ ID NO: 5) was used. Real-time PCR data are provided below for reactions containing 0 to 50,000 genomic equivalents of isolated *Candida krusei* genomic DNA added as template to each reaction. Four replicates for each reaction condition were performed and the mean Cp values (cross-over point), standard deviation of Cp (Std. Cp) and CV (coefficient of variation, n = 3 or n = 4) were calculated for
 25 each experiment described below. The average amplification efficiency and the PCR efficiency for the reactions were also calculated in each case to monitor that the results were not affected by amplification artifacts.

As shown in Table 3, and Figure 2A, the *Candida krusei* molecular beacon probe was used successfully to detect the target nucleic acid molecule in the genomic DNA isolated from *Candida krusei*. The “ND” indicates “not detected” and no Cp was reported.

5 Table 3.

<u>C. krusei genomic equivalents/rx</u>	<u>Cp1</u>	<u>Cp2</u>	<u>Cp3</u>	<u>Cp4</u>	<u>Average</u>	<u>Std Cp</u>	<u>CV</u>
50,000	21.36	21.29	21.34	21.5	21.37	0.09	0.4%
5,000	24.91	24.86	24.94	24.87	24.90	0.04	0.1%
500	28.23	28.27	28.11	28.33	28.24	0.09	0.3%
50	31.81	31.74	31.78	31.59	31.73	0.10	0.3%
5	35.09	34.98	34.95	35.19	35.01	0.11	0.3%
no template	ND	ND	ND	ND	ND		
amplification efficiency=	1.96						
PCR efficiency=	96%						

The *Candida krusei* specific molecular beacon probe was also used to detect *C. krusei* in a *Candida* lysate prepared as described above. Real-time PCR data are provided below for PCR reactions containing *Candida* lysate prepared using different concentrations of *Candida* cells. The average amplification efficiency and the PCR efficiency for the reactions were also calculated to determine whether use of a lysate as the source of the genetic material affected the amplification and PCR efficiency.

As seen in Table 4 below, the amplification and PCR efficiencies when lysate was used are similar to the results obtained when isolated genomic DNA template was used (see Table 3). Thus, genetic material from a *Candida* cell lysate can be directly used in a PCR reaction to amplify *Candida* target nucleic acid molecules. Also, as shown in Figure 2B, *Candida krusei* was successfully detected in a sample prepared as a *Candida* lysate using the *C. krusei* specific molecular beacon probe.

Table 4.

<u>cells/rx</u>	<i>C. krusei</i>							<u>Average</u>	<u>Std Cp</u>	<u>CV</u>
	1rst run				2nd run					
	<u>Cp1</u>	<u>Cp2</u>	<u>Cp3</u>	<u>Cp4</u>	<u>Cp1</u>	<u>Cp2</u>	<u>Cp3</u>			
400	27.98	27.96	27.88	27.9	28.01	28.03	27.96	27.96	0.05	0.2%
40	31.26	31.25	31.46	31.24	31.53	31.51	31.51	31.39	0.14	0.4%
4	34.34	34.13	33.96	34.5	34.34	34.86	34.59	34.39	0.30	0.9%
amplification efficiency=	2.05									
PCR efficiency=	105%									

Additional data showing the successful use of a HEX-labeled *C. krusei* probe with genomic *Candida krusei* DNA are shown in Table 5 and Figure 3C, and additional data showing the successful use of a HEX-labeled *C. krusei* probe with *Candida krusei* are shown in Table 6 and Figure 3F. The volume of diluted genomic DNA or lysate added in these PCR reactions was 100 ul instead of 50 ul as in the reactions described above.

10 Table 5.

<u>copies/rx</u>	<i>C. krusei</i> gDNA titration_300nM HEX						
	<u>Cp1</u>	<u>Cp2</u>	<u>Cp3</u>	<u>Cp4</u>	<u>Average</u>	<u>Std Cp</u>	<u>CV</u>
10,000	24.77	24.84	24.8	24.8	24.79	0.04	0.1%
1,000	28.34	28.29	28.3	28.4	28.32	0.04	0.1%
100	31.79	31.8	31.8	31.9	31.81	0.03	0.1%
10	35.07	35.01	35.2	35.1	35.08	0.07	0.2%
no template					ND	ND	ND
amplification efficiency	1.95						
PCR efficiency	95%						

Table 6.

<u>genomic equivalent/rx</u>	<u>Log</u>	<i>C. krusei</i> cell lysate_300nM HEX						
		<u>Cp1</u>	<u>Cp2</u>	<u>Cp3</u>	<u>Cp4</u>	<u>Average</u>	<u>Std Cp</u>	<u>CV</u>
1,000	3.0	27.81	27.59	27.9	27.8	27.76	0.12	0.4%
100	2.0	31.21	31.1	31.2	31.1	31.15	0.05	0.2%
10	1.0	34.72	34.88	34.9	34.9	34.86	0.10	0.3%
no cell lysate				17.9	12.3	15.05	3.96	26.3%
amplification efficiency		1.91						
PCR efficiency		91%						

Example 2: Detection of *C. parapsilosis* using a species-specific molecular beacon probe in a real-time PCR assay.

A HEX labeled *C. parapsilosis* probe (having the sequence of SEQ ID NO: 4) was used at 300 nM concentration in a real-time PCR assay that was performed according to the parameters described in Table 1 above. 100 µl of diluted genomic DNA was added to the PCR reaction mix and 45 cycles of amplification were performed with an annealing temperature of 55°C. A titration using different amounts of genomic DNA template was performed. Real-time PCR data are provided in Table 7 below for reactions containing 0 to 10,000 copies of genomic DNA per reaction. Four replicates for each reaction condition were performed and the average Ct values were calculated. The average amplification efficiency and the PCR efficiency for the reactions were also calculated. Table 7 and Figure 3A show that the *C. parapsilosis* molecular beacon probe was used successfully to detect the target nucleic acid molecule using genomic DNA isolated from *Candida parapsilosis*.

Table 7.

<i>C. parapsilosis</i> gDNA titration 300nM HEX								
<u>copies/rx</u>	<u>Log</u>	<u>Cp1</u>	<u>Cp2</u>	<u>Cp3</u>	<u>Cp4</u>	<u>Average</u>	<u>Std Cp</u>	<u>CV</u>
10,000	4.0	25.01	24.96	24.99	25.09	25.01	0.06	0.2%
1,000	3.0	28.45	28.5	28.44	28.48	28.47	0.03	0.1%
100	2.0	31.84	31.81	31.85	31.83	31.83	0.02	0.1%
10	1.0	35.27	35.06	34.99	34.74	35.02	0.22	0.6%
no template						ND	ND	ND
amplification efficiency		1.99						
PCR efficiency		99%						

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Next, a similar assay was performed using a *Candida parapsilosis* lysate rather than genomic DNA. A titration was performed with 100 ul of a *Candida* lysate prepared with different concentrations of *C. parapsilosis* cells. Real-time PCR data are provided below (Table 8) for PCR reactions containing *Candida* lysate having 0 to 1000 (i.e., 0, 10, 100, or 1,000) genomic equivalents of *Candida parapsilosis* genomic DNA per reaction. As shown in Table 8 and Figure 3D, *Candida parapsilosis* was successfully detected using the *C. parapsilosis* specific molecular beacon probe at all genomic equivalents prepared using a *Candida* lysate.

20

Table 8.

		<i>C.parapsilosis</i> cell lysate 300nM HEX						
<u>genomic equivalent/rx</u>	<u>Log</u>	<u>Cp1</u>	<u>Cp2</u>	<u>Cp3</u>	<u>Cp4</u>	<u>Average</u>	<u>Std Cp</u>	<u>CV</u>
1,000	3.0	28.61	28.65	28.73	28.66	28.66	0.05	0.2%
100	2.0	31.88	31.89	31.97	31.93	31.92	0.04	0.1%
10	1.0	34.91	34.96	34.9	34.97	34.94	0.04	0.1%
no cell lysate								
amplification efficiency		2.08						
PCR efficiency		108%						

Example 3: Detection of *C. tropicalis* using a species-specific molecular beacon probe in a real-time PCR assay.

A HEX labeled *C. tropicalis* probe (having the sequence of SEQ ID NO: 2) was used at 300 nM concentration in a real-time PCR assay that was performed according to the parameters described in Table 1 above. 100 µl of diluted genomic DNA was added to the PCR reaction mix and 45 cycles of amplification were performed with an annealing temperature of 55 °C. A titration using different amounts of genomic DNA template was performed. Real-time PCR data are provided in Table 9 for reactions containing 0 to 10,000 (i.e., 0, 100, 1,000, and 10,000) copies of genomic DNA per reaction. Four replicates for each reaction condition were performed and the average Ct values were calculated. The average amplification efficiency and the PCR efficiency for the reactions were also calculated. Table 9 and Figure 3B show that the *C. tropicalis* molecular beacon probe was used successfully to detect the target nucleic acid molecule using genomic DNA isolated from *Candida tropicalis*.

Table 9.

		<i>C. tropicalis</i> gDNA titration 300nM HEX						
<u>copies/rx</u>		<u>Cp1</u>	<u>Cp2</u>	<u>Cp3</u>	<u>Cp4</u>	<u>Average</u>	<u>Std Cp</u>	<u>CV</u>
10,000		25.41	25.03	24.99	25.26	25.17	0.20	0.8%
1,000		28.79	28.72	28.82	28.69	28.76	0.06	0.2%
100		32.21	32.23	32.25	32.32	32.25	0.05	0.1%
10		35.11	35	34.96	35.38	35.11	0.19	0.5%
no template						ND	ND	ND
amplification efficiency		2.00						
PCR efficiency		100%						

Next, a similar assay was performed using a *Candida tropicalis* lysate rather than genomic DNA. A titration was performed with 100 ul of a *Candida* lysate prepared with different concentrations of *C. tropicalis* cells. Real-time PCR data are provided below (Table 10) for PCR reactions containing

Candida lysate having 0 to 1000 (i.e., 0, 10, 100, or 1,000) genomic equivalents of *Candida tropicalis* genomic DNA per reaction. As shown in Table 10 and Figure 3E, *Candida tropicalis* was successfully detected using the *C. tropicalis* specific molecular beacon probe at all genomic equivalents prepared using a *Candida* lysate.

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Table 10.

		<i>C. tropicalis</i> cell lysate 300nM HEX						
<u>genomic equivalent/rx</u>	<u>Log</u>	<u>Cp1</u>	<u>Cp2</u>	<u>Cp3</u>	<u>Cp4</u>	<u>Average</u>	<u>Std Cp</u>	<u>CV</u>
1,000	3.0	28.57	28.67	28.59	28.61	28.61	0.04	0.2%
100	2.0	31.99	31.92	32.06	32.05	32.01	0.06	0.2%
10	1.0	35.07	34.57	34.55	34.46	34.66	0.28	0.8%
no cell lysate						ND	ND	ND
amplification efficiency		2.14						
PCR efficiency		114%						

Example 4: Detection of C. albicans using a species-specific molecular beacon probe in a real-time PCR assay.

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A HEX labeled *C. albicans* probe (having the sequence of SEQ ID NO: 1) was used at 300 nM concentration in a real-time PCR assay that was performed according to the parameters described in Table 1 above. 100 µl of diluted genomic DNA was added to the PCR reaction mix and 45 cycles of amplification were performed with an annealing temperature of 60 °C. A titration using different amounts of genomic DNA template was performed. Real-time PCR data are provided in Table 11 for reactions containing 0 to 10,000 copies (i.e., 0, 10, 100, 1,000, or 10,000) of genomic DNA per reaction. Four replicates for each reaction condition were performed and the average Ct values were calculated. The average amplification efficiency and the PCR efficiency for the reactions were also calculated. Table 11 and Figure 4A show that the *C. albicans* molecular beacon probe was used successfully to detect the target nucleic acid molecule using genomic DNA isolated from *Candida albicans*.

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Table 11.

		<i>C. albicans</i> genomic DNA						
copies/rx	Log	Cp1	Cp2	Cp3	Cp4	Average	Std Cp	CV
10,000	4.0	24.57	24.71	24.7	25	24.75	0.18	0.7%
1,000	3.0	28.01	28.15	28.19	28.45	28.20	0.18	0.7%
100	2.0	31.59	31.63	31.65	31.86	31.68	0.12	0.4%
10	1.0	34.86	34.14	35.02	35.75	34.94	0.66	1.9%
no template						ND	ND	ND
amplification efficiency		1.97						
PCR efficiency		97%						

Next, a similar assay was performed using a *Candida albicans* lysate rather than genomic DNA. A titration was performed with 100 ul of a *Candida* lysate prepared with different concentrations of *C. albicans* cells. Real-time PCR data are provided below (Table 12) for PCR reactions containing *Candida* lysate which provide 0 to 400 (i.e., 0, 4, 40, or 400) genomic equivalents of *Candida albicans* genomic DNA per reaction. As shown in Table 12 and Figure 4C, *Candida albicans* was successfully detected using the *C. albicans* specific molecular beacon probe at all genomic equivalents prepared using a *Candida* lysate.

10

Table 12:

		<i>C. albicans</i> cell lysate						
cells/rx	Log	Cp1	Cp2	Cp3	Cp4	Average	Std Cp	CV
400	2.6	28.59	28.68	28.66	28.68	28.65	0.04	0.1%
40	1.6	32.15	32.1	32.02	32.02	32.07	0.06	0.2%
4	0.6	34.84	34.86	34.66	34.86	34.81	0.10	0.3%
no cell lysate				16.27		16.27	ND	ND
amplification efficiency		2.11						
PCR efficiency		111%						

Example 5: Detection of *C. glabrata* using a species-specific molecular beacon probe in a real-time PCR assay.

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A HEX labeled *C. glabrata* probe (having the sequence of SEQ ID NO: 3) was used at 300 nM concentration in a real-time PCR assay that was performed according to the parameters described in Table 1 above. 100 µl of diluted genomic DNA was added to the PCR reaction mix and 45 cycles of amplification were performed with an annealing temperature of 60 °C. A titration using different amounts of genomic DNA template was performed. Real-time PCR data are provided in Table 13 for

20

reactions containing 0 to 10,000 (i.e., 0, 10, 100, 1,000, or 10,000) copies of genomic DNA per reaction. Four replicates for each reaction condition were performed and the average Ct values were calculated. The average amplification efficiency and the PCR efficiency for the reactions were also calculated. Table 13 and Figure 4B show that the *C. glabrata* molecular beacon probe was used successfully to detect the target nucleic acid molecule using genomic DNA isolated from *Candida glabrata*.

Table 13.

copies/rx	Log	<i>C.glabrata</i> genomic DNA						
		Cp1	Cp2	Cp3	Cp4	Average	Std Cp	CV
10,000	4.0	26.55	26.38	26.38	26.46	26.44	0.08	0.3%
1,000	3.0	30.02	30.05	30.02	30.18	30.07	0.08	0.3%
100	2.0	33.02	32.97	32.78	32.84	32.90	0.11	0.3%
10	1.0	36.47	34.22	36.73	36.72	36.04	1.22	3.4%
no template						ND	ND	ND
amplification efficiency		2.07						
PCR efficiency		107%						

Next, a similar assay was performed using a *Candida glabrata* lysate rather than genomic DNA.

10 A titration was performed with 100 ul of a *Candida* lysate prepared with different concentrations of *C. glabrata* cells. Real-time PCR data are provided below (Table 14) for PCR reactions containing *Candida* lysate which provide 0 to 400 (i.e., 0, 4, 40, or 400) genomic equivalents of *Candida glabrata* genomic DNA per reaction. As shown in Table 14 and Figure 4D, *Candida glabrata* was successfully detected using the *C. glabrata* specific molecular beacon probe at all genomic equivalents prepared using a
15 *Candida* lysate.

Table 14.

cells/rx	Log	<i>C. glabrata</i> cell lysate						
		Cp1	Cp2	Cp3	Cp4	Average	Std Cp	CV
400	2.6	29	29.15	29.24	28.95	29.09	0.13	0.5%
40	1.6	32.07	32.11	31.92	32.02	32.03	0.08	0.3%
4	0.6	35.09	34.8	34.7	34.84	34.86	0.17	0.5%
no cell lysate						ND	ND	ND
amplification efficiency		2.22						
PCR efficiency		122%						

The above data in Tables 4 to 14 and in Figures 3 and 4 demonstrate that each of the species specific probes can be used to successfully detect specific *Candida* species using either purified genomic DNA as an input in a real-time PCR assay or, alternatively, using a fungal cell lysate as an input in the assay.

Example 6: Multiplexed real-time PCR assay for detecting multiple Candida species in a sample.

For a multiplexed molecular beacon assays, 300 nM of each species-specific beacon probe (0.3uL per 100 uL reaction) were added to the PCR master mix. The *C. parapsilosis* and *C. tropicalis* molecular beacons (SEQ ID NO: 4 and SEQ ID NO: 2, respectively) are labeled with a FAM and a HEX fluor, respectively. The beacons are multiplexed in the reaction and detected in their respective channels (488-533 nm for FAM) and (488-610 for HEX). To ensure that the amplification efficiencies were similar between reactions containing a single beacon and reactions containing multiplexed beacons, *C. tropicalis* and *C. parapsilosis* genomic DNA was titrated into reactions at concentrations spanning 1E4 down to 10 copies per 20 uL reaction volume. These reactions included either a single beacon for detection (shown in Table 15 for *C. parapsilosis* and Table 17 for *C. tropicalis*) or the two beacons in the same reaction but detected with different channels (Table 16 and Table 18). In both cases the amplification efficiencies were similar for both the single detection reactions and multiplexed detection reactions with no measurable impact on detection sensitivity. Thus, both *C. tropicalis* and *C. parapsilosis* can be detected within a single PCR reaction and potentially all five beacons could be multiplexed in a single reaction enabling detection of the five most clinically relevant *Candida* species within the same well.

Table 15.

Channel	483-533	<i>C. parapsilosis</i> detection single beacon					
<u>copies/rx</u>	Log	Cp1	Cp2	Cp3	Average	Std Cp	CV
10000	4	21.58	21.53	21.5	21.53667	0.040415	0.2%
1000	3	25.01	24.96	24.97	24.98	0.026458	0.1%
100	2	28.59	28.64	28.58	28.60333	0.032146	0.1%
10	1	31.89	31.81	32	31.9	0.095394	0.3%
no template					ND		
amplification efficiency=		1.9					
PCR efficiency=		93.2%					

5 Table 16.

Channel	483-533	<i>C. parapsilosis</i> detection multiplexed reaction					
<u>copies/rx</u>	Log	<u>Cp1</u>	<u>Cp2</u>	<u>Cp3</u>	<u>Average</u>	<u>Std Cp</u>	<u>CV</u>
10000	4	22.61	22.51	22.48	22.53333	0.068069	0.3%
1000	3	26.01	25.96		25.985	0.035355	0.1%
100	2	29.55	29.29	29.28	29.37333	0.15308	0.5%
10	1	32.62	32.67	32.98	32.75667	0.195021	0.6%
no template					ND		
amplification efficiency=		1.96					
PCR efficiency=		96.0%					

Table 17.

Channel	488-610	<i>C. tropicalis</i> detection single beacon					
<u>copies/rx</u>	Log	Cp1	Cp2	Cp3	Average	Std Cp	CV
10000	4	21.44	21.47	21.59	21.5	0.079373	0.4%
1000	3	24.63	24.77	24.67	24.69	0.072111	0.3%
100	2	28.23	28.01	28.08	28.10667	0.112398	0.4%
10	1	31.92	31.65	31.86	31.81	0.141774	0.4%
no template					ND		
amplification efficiency=		1.95					
PCR efficiency=		95.3%					

Table 18.

5

Channel	488-610	<i>C. tropicalis</i> detection multiplexed reaction					
<u>copies/rx</u>	Log	Cp1	Cp2	Cp3	Average	Std Cp	CV
10000	4	21.55	21.57	21.55	21.55667	0.011547	0.1%
1000	3	24.96	24.89	24.99	24.94667	0.051316	0.2%
100	2	28.72	28.5	28.51	28.57667	0.124231	0.4%
10	1	32.12	31.84	31.89	31.95	0.149332	0.5%
no template					ND		
amplification efficiency=		1.94					
PCR efficiency=		94.0%					

Example 7: DNA sequencing-based detection of Candida species.

A probe having the sequence of SEQ ID NO: 32 (specific to *C. krusei*) was contacted with nucleic acid molecules that were amplified from within whole blood lysate using the pan-*Candida* forward and reverse primers (SEQ ID NOs: 11 and 12), followed by conducting a phenol/chloroform extraction, a chloroform extraction, and then conducting Sanger Dideoxy Sequencing using Big Dye Terminators on an AB 3730 capillary sequencing instrument. The resulting chromatogram, which is shown in Figure 7, demonstrates that this method can also be used to of detect *Candida* species using the probes and primers of this invention.

The sequencing-based detection method described above can also be performed using a probe having the sequence of SEQ ID NO: 33 to detect *C. krusei*; a probe having the sequence of SEQ ID NO: 34 or 35 to detect *C. albicans*; a probe having the sequence of SEQ ID NO: 36 or 37 to detect *C. glabrata*; a probe having the sequence of SEQ ID NO: 38 or 39 to detect *C. parapsilosis*; or a probe having the sequence of SEQ ID NO: 40 or 41 to detect *C. tropicalis*.

Example 8: Detection of C. albicans using a species-specific molecular beacon probe in a real-time PCR assay.

A FAM labeled *C. albicans* probe (having the sequence of SEQ ID NO: 1) was used at 300 nM concentration in a real-time PCR assay that was performed according to the parameters described in Table 1 above. 100 µl of diluted genomic DNA was added to the PCR reaction mix and 45 cycles of amplification were performed with an annealing temperature of 60 °C. A titration using different amounts of genomic DNA template was performed. Real-time PCR data are provided in Table 19 for reactions containing 0 to 50,000 copies (i.e., 0 (no template), 5, 50, 500, 5,000, or 50,000) of genomic DNA per reaction. Four replicates for each reaction condition were performed and the average Cp values were calculated. The average amplification efficiency (2.03) and the PCR efficiency (103%) for the reactions were also calculated. Table 19 shows that the *C. albicans* molecular beacon probe was used successfully to detect the target nucleic acid molecule using genomic DNA isolated from *C. albicans*.

Table 19:

C. albicans detection in singleplex reactions

copies/rx	Log	Cp1	Cp2	Cp3	Cp4	Average	Std Cp	CV
50000	5	21.71	21.86	21.9	21.9	21.84	0.09	0.4%
5000	4	25.24	25.06	25.23	25.27	25.20	0.09	0.4%
500	3	28.66	28.64	28.72	28.68	28.68	0.03	0.1%
50	2	31.84	31.87	31.88	31.84	31.86	0.02	0.1%
5	1	34.39	35	35	35	34.80	0.31	0.9%
no template		ND	ND	ND	ND			

slope -3.2566
 0.31
 amplification efficiency= 2.03
 PCR efficiency= 103%

Next, a similar assay was performed using a *C. albicans* lysate rather than genomic DNA. To determine the ability of the molecular beacons to detect *Candida* within crude cell lysates, we prepared lysate from *Candida* cells spiked in 1x phosphate buffered saline (PBS) buffer. *Candida* cells were quantified using a Coulter counter and were spiked into PBS at concentrations ranging from ~520 cells/mL to ~3 cells/mL. The *in-vitro* spiked solutions were added to a 1 mL polypropylene tube containing 300 mg of yttrium stabilized zirconium oxide beads. The cells were harvested via centrifugation at 6000xG for 5 minutes and the supernatant was removed. 100 uL of 1xTE was added to the tubes and the tubes were subjected to bead beating, as described herein, by placement in a vortexer (Biospec bead beater) at maximum speed for 5 minutes. Fifty microliters of lysate was then transferred into an asymmetric PCR master mix containing the molecular beacon probe, as described in Example 1.

Real-time PCR data are provided below (Table 20) for PCR reactions containing *C. albicans* genomic DNA prepared from *C. albicans* lysate using the indicated number of cells/mL. The reactions were set up using lysates prepared over multiple days not the same sample lysate detected over multiple days, thus the variation in the entire process is shown. As shown in Table 20 and Figure 8A, *C. albicans* was successfully detected using the *C. albicans* specific molecular beacon probe, and semi-quantitative detection of *C. albicans* was possible using as few as ~3 cells/mL.

Table 20:

		<i>C. albicans</i> cells/mL									
		520	400	280	52	40	28	5.2	4	2.8	
operator	date	2.716003	2.6021	2.44716	1.716	1.6021	1.4472	0.699	0.6021	0.44735803	0
NP	5/1/2012		28.47			32.83					ND
			28.45			32.95					ND
			28.45			32.89					ND
			28.47			32.86					ND
NP	3/20/2012		28.59			32.15			34.84		ND
			28.68			32.1			34.86		ND
			28.66			32.02			34.66		ND
			28.68			32.02			34.86		ND
NP	3/30/2012	28.36	28.75	29.15	31.64	31.81	32.42		34.03	34.66	ND
		28.35	28.79	29.18	31.67	31.73	32.44	34.03	33.76	34.77	ND
		28.45	28.77	29.24	31.64	31.78	32.5	34.12	34.09		ND
		28.29	28.7		31.9	31.79		33.97	34.18		ND
NP	3/28/2012	28.48	28.77	29.15	31.83	31.8	32.69	34.88	34.54	34.99	ND
		28.5	28.8	29.16	31.62	31.68	32.66	34.57	34.21	34.94	ND
		28.32	28.78	29.16	31.56	31.7	32.44	34.77	34.53	34.9	ND
		28.28	28.78	29.15	31.71	31.73	32.78	34.32	34.55	34.81	ND
NP	4/19/2012	28.14	28.65	29.25	31.8	31.75	32.59	34.08	34.18	34.29	ND
		28.12	28.57	29.28	31.75	31.84	32.63	33.98	34.03	34.42	ND
		28.03	27.24	29.25	31.58	31.64	32.58	33.9	34.23	34.55	ND
		28.11	28.64	29.21	31.61	31.72	32.49		34.23	34.31	ND
mean		28.28583	28.585	29.1982	31.693	32.04	32.565	34.262	34.361	34.664	
sd		0.155649	0.3384	0.04916	0.1068	0.4547	0.1175	0.3561	0.3356	0.2600855	
cv		0.55%	1.18%	0.17%	0.34%	1.42%	0.36%	1.04%	0.98%	0.75%	

The above data demonstrate that the *C. albicans* molecular beacon probe can be used to successfully detect *C. albican* using either purified genomic DNA as an input in a real-time PCR assay or, alternatively, using a fungal cell lysate as an input in the assay.

Example 9: Detection of C. glabrata using a species-specific molecular beacon probe in a real-time PCR assay.

A HEX labeled *C. glabrata* probe (having the sequence of SEQ ID NO: 3) was used at 300 nM concentration in a real-time PCR assay that was performed according to the parameters described in Table 1 above. 100 μ l of diluted genomic DNA was added to the PCR reaction mix and 45 cycles of amplification were performed with an annealing temperature of 60 $^{\circ}$ C. A titration using different amounts of genomic DNA template was performed. Real-time PCR data are provided in Table 21 for reactions containing 0 to 50,000 copies (i.e., 0 (no template), 5, 50, 500, 5,000, or 50,000) of genomic DNA per reaction. Four replicates for each reaction condition were performed and the average Cp values were calculated. The average amplification efficiency (2.23) and the PCR efficiency (123%) for the reactions were also calculated. Table 21 shows that the *C. glabrata* molecular beacon probe was used successfully to detect the target nucleic acid molecule using genomic DNA isolated from *C. glabrata*.

Table 21:

C. glabrata detection in singleplex reactions

copies/rx	Log	Cp1	Cp2	Cp3	Cp4	Average	Std Cp	CV
50000	5	23.72	23.78	23.68	23.71	23.72	0.04	0.2%
5000	4	27.1	27.18	27.22	27.24	27.19	0.06	0.2%
500	3	30.56	30.67	30.64	30.51	30.60	0.07	0.2%
50	2	33.17	33.46	33.44	33.55	33.41	0.16	0.5%
5	1	35	35	35	35	35.00	0.00	0.0%
no template		ND	ND	ND	ND			

slope -2.87

0.35

amplification efficiency= 2.23

PCR efficiency= 123%

Next, a similar assay was performed using a *C. glabrata* lysate rather than genomic DNA according to the assay described in Example 8.

Real-time PCR data are provided below (Table 22) for PCR reactions containing *C. glabrata* genomic DNA prepared from *C. glabrata* lysate using the indicated number of cells/mL. The reactions were set up using lysates prepared over multiple days not the same sample lysate detected over multiple days, thus the variation in the entire process is shown. As shown in Table 22 and Figure 8B, *C. glabrata* was successfully detected using the *C. glabrata* specific molecular beacon probe, and semi-quantitative detection of *C. glabrata* was possible using as few as ~3 cells/mL.

Table 22:

<i>C. glabrata</i> cells/ml		520	400	280	52	40	28	5.2	4	2.8	
operator	date	1.716003	2.60206	3.447158	1.716003	1.60206	3.447158	0.69827	0.60206	3.447158	0
NP	3/25/2012	28.43	28.75	29.05	31.77	31.91	32.39	34.27	34.53	34.71	NG
		28.55	28.76	28.94	31.85	31.95	32.39	34.07	34.54	34.97	ND
		28.3	28.59	29.09		31.98	32.31	34.08	34.67	34.86	ND
		28.51		28.68	31.85		32.58			34.81	ND
		28.92	29.06	29.44	31.57	31	32.23	32.76	33.45	34.24	ND
		28.82	28.95	29.35	31.69	31.68	32.46	33.92	33.65	34.94	NG
		28.83	28.91	29.29	31.54	31.95	32.17	34.27		34.38	NG
			29.26			32.83					ND
NP	5/10/2012				32.95					ND	
			29.75			32.89				ND	
			29.74			32.86				ND	
CW	5/10/2012		29.65			32.98				NG	
			29.75			32.88				NG	
			29.79			33.03				ND	
NP	3/20/2012		29.74			33.06				ND	
			28.69			32.17			35.19	ND	
			28.81			32.18			35.42	ND	
			28.84			32.55			35.24	NG	
	mean	28.62571	28.83667	29.26286	31.71333	31.745	32.36143	33.895	34.168	34.70143	
	sd	0.231722	0.168721	0.255976	0.137502	0.380986	0.138856	0.57183	0.571244	0.283515	
	cv	0.81%	0.59%	0.87%	0.43%	1.20%	0.43%	1.69%	1.57%	0.82%	

The above data demonstrate that the *C. glabrata* molecular beacon probe can be used to successfully detect *C. glabrata* using either purified genomic DNA as an input in a real-time PCR assay or, alternatively, using a fungal cell lysate as an input in the assay.

Example 10: Detection of *C. krusei* using a species-specific molecular beacon probe in a real-time PCR assay.

A HEX labeled *C. krusei* probe (having the sequence of SEQ ID NO: 6) was used at 300 nM concentration in a real-time PCR assay that was performed according to the parameters described in Table 1 above. 100 µl of diluted genomic DNA was added to the PCR reaction mix and 45 cycles of amplification were performed with an annealing temperature of 60 °C. A titration using different amounts of genomic DNA template was performed. Real-time PCR data are provided in Table 23 for reactions containing 0 to 50,000 copies (i.e., 0 (no template), 5, 50, 500, 5,000, or 50,000) of genomic DNA per reaction. Four replicates for each reaction condition were performed and the average Cp values were calculated. The average amplification efficiency (2.05) and the PCR efficiency (105%) for the reactions were also calculated. Table 23 shows that the *C. krusei* molecular beacon probe was used successfully to detect the target nucleic acid molecule using genomic DNA isolated from *C. krusei*.

Table 23:

C. krusei detection in singleplex reactions

copies/rx	Log	Cp1	Cp2	Cp3	Cp4	Average	Std Cp	CV
50000	5	22.17	22.3	22.15		22.21	0.08	0.4%
5000	4	25.77	25.86	25.75	25.85	25.81	0.06	0.2%
500	3	29.15	29.03	29.21	29.2	29.15	0.08	0.3%
50	2	32.17	32.25	32.2	32.34	32.24	0.07	0.2%
5	1	35	35	35	35	35.00	0.00	0.0%
no template		ND	ND	ND	ND			

slope -3.2019

0.31

amplification efficiency= 2.05

PCR efficiency= 105%

Next, a similar assay was performed using a *C. krusei* lysate rather than genomic DNA according to the assay described in Example 8.

Real-time PCR data are provided below (Table 24) for PCR reactions containing *C. krusei* genomic DNA prepared from *C. krusei* lysate using the indicated number of cells/mL. The reactions were set up using lysates prepared over multiple days not the same sample lysate detected over multiple days, thus the variation in the entire process is shown. As shown in Table 24 and Figure 8C, *C. krusei* was successfully detected using the *C. krusei* specific molecular beacon probe, and semi-quantitative detection of *C. krusei* was possible using as few as ~3 cells/mL.

Table 24:

<i>C. krusei</i> cells/ml.		520	400	280	52	40	28	5.2	4	2.8		
operator	date	3.716003	2.60206	3.447158	1.716003	1.60206	1.447158	0.68897	0.60206	0.447156	C	
NP	3/21/2012		27.81			31.21			34.72		ND	
			27.59			31.1			34.88		ND	
			27.88			31.17			34.91		ND	
NP	3/23/2012		27.77			31.12			34.94		ND	
			28.71			31.9			34.81		ND	
			28.83			31.92			34.97		ND	
NP	3/23/2012		28.75			31.85			34.88		ND	
			28.69			31.32			32.8		ND	
			27.83	28.01	28.43	31.19	31.53	31.67	33.47	34.34	34.28	ND
NP	3/30/2012		27.77	28.03	28.36	30.88	31.51	31.69	33.66	34.26	34.16	ND
			27.84	27.96	28.36	30.84	31.51	31.74	33.2	34.59	34.43	ND
			27.24	27.98		30.82	31.26			33.95	34.34	ND
			27.38	27.96		30.76	31.25			33.92	34.13	ND
			27.27	27.88		30.85	31.45			33.65	33.96	ND
			27.32	27.9		30.79	31.24			33.78	34.5	ND
NP	5/10/2012		28.2			31.75					ND	
			28.22			31.72					ND	
			28.2			31.74					ND	
CW	5/10/2012		28.35			31.75					ND	
			28.48			31.59					ND	
			28.45			31.73					ND	
NP	3/20/2012		28.37			31.7					ND	
			28.56			31.6					ND	
			27.74			30.92				34.84		ND
NP	3/20/2012		27.82			31.97			34.72		ND	
			27.91			31.22				34.32		ND
mean		27.49571	28.14316	28.38333	30.87571	31.42233	31.7	33.44333	34.402667	34.25714		
std		0.307184	0.364662	0.040415	0.14409	0.279148	0.026056	0.231157	0.6403399	0.187146		
cv		1.12%	1.30%	0.14%	0.47%	0.89%	0.11%	0.69%	1.86%	0.55%		

The above data demonstrate that the *C. krusei* molecular beacon probe can be used to successfully detect *C. krusei* using either purified genomic DNA as an input in a real-time PCR assay or, alternatively, using a fungal cell lysate as an input in the assay.

Example 11: Detection of C. parapsilosis using a species-specific molecular beacon probe in a real-time PCR assay.

A HEX labeled *C. parapsilosis* probe (having the sequence of SEQ ID NO: 5) was used at 300 nM concentration in a real-time PCR assay that was performed according to the parameters described in Table 1 above. 100 µl of diluted genomic DNA was added to the PCR reaction mix and 45 cycles of amplification were performed with an annealing temperature of 60 °C. A titration using different amounts of genomic DNA template was performed. Real-time PCR data are provided in Table 25 for reactions containing 0 to 50,000 copies (i.e., 0 (no template), 5, 50, 500, 5,000, or 50,000) of genomic DNA per reaction. Four replicates for each reaction condition were performed and the average Cp values were calculated. The average amplification efficiency (2.10) and the PCR efficiency (110%) for the reactions were also calculated. Table 25 shows that the *C. parapsilosis* molecular beacon probe was used

successfully to detect the target nucleic acid molecule using genomic DNA isolated from *Candida parapsilosis*.

Table 25:

C. parapsilosis detection in singleplex rx

copies/rx	Log	Cp1	Cp2	Cp3	Cp4	Average	Std Cp	CV
50000	5	22.55	22.43	22.47	22.45	22.48	0.05	0.2%
5000	4	25.91	25.85	25.9	25.72	25.85	0.09	0.3%
500	3	29.2	29.2	29.25	29.25	29.23	0.03	0.1%
50	2	32.13	32.16	32.15	32.31	32.19	0.08	0.3%
5	1	35	35	34.53	35	34.84	0.24	0.7%
no template		ND	ND	ND	ND			

slope -3.1079
0.32
amplification efficiency= 2.10
PCR efficiency= 110%

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Next, a similar assay was performed using a *C. parapsilosis* lysate rather than genomic DNA according to the assay described in Example 8.

Real-time PCR data are provided below (Table 26) for PCR reactions containing *C. parapsilosis* genomic DNA prepared from *C. parapsilosis* lysate using the indicated number of cells/mL. The reactions were set up using lysates prepared over multiple days not the same sample lysate detected over multiple days, thus the variation in the entire process is shown. As shown in Table 26 and Figure 8D, *C. parapsilosis* was successfully detected using the *C. parapsilosis* specific molecular beacon probe, and semi-quantitative detection of *C. parapsilosis* was possible using as few as ~3 cells/mL.

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Table 26:

<i>C. parapsilosis</i> cells/		520	400	280	52	40	28	5.2	4	2.8	0
operator	date	2.718003	2.60206	2.447159	1.716003	1.60306	1.447158	0.72897	0.60206	0.447158	0
NP	3/23/2012		28.71			31.99			35.06		ND
			28.71			31.93			34.89		ND
			28.63			31.89			35.25		ND
NP	4/2/2012	28.3	28.59	29.02	31.3	31.36	31.67	33.37	33.27	33.49	ND
		28.24	28.55	29.03	31.33	31.16	31.74	33.1	33.22	33.47	ND
		28.28	28.6	29.05	31.32	31.23	31.65	33.17	33.19	33.17	ND
		28.21	28.52	29.05	31.2	31.14	31.65	32.92	33.06	33.3	ND
		28.05	28.28	28.8	29.49	30.75	29.88	29.11	32.18	32.01	ND
		28.05	28.3	28.77	30.81	30.61	30.72	32.22	32.16	32.43	ND
		28.06	28.16	28.88	30.86	30.53	31.25	31.64	32.03	31.47	ND
NP	4/30/2012		28.9			31.19					ND
			28.94			31.35					ND
			28.9			31.21					ND
			28.93			31.49					ND
NP	5/11/2012		28.66			31.07					ND
			28.68			31.12					ND
			28.7			31.11					ND
			28.67			31.11					ND
CW	5/11/2012		28.94			31.17					ND
			29.02			31.2					ND
			28.96			31.23					ND
			29.01			31.24					ND
mean	28.2575	28.68333	29.04	31.2875	31.93667	31.6775	33.14	33.99143	33.3575		
sd	0.040311	0.046188	0.018257	0.059657	0.050332	0.04272	0.186011	1.013138	0.1513		
cv	0.14%	0.16%	0.06%	0.19%	0.16%	0.13%	0.56%	2.98%	0.45%		

The above data demonstrate that the *C. parapsilosis* molecular beacon probe can be used to successfully detect *C. parapsilosis* using either purified genomic DNA as an input in a real-time PCR assay or, alternatively, using a fungal cell lysate as an input in the assay.

Example 12: Detection of C. tropicalis using a species-specific molecular beacon probe in a real-time PCR assay.

A HEX labeled *C. tropicalis* probe (having the sequence of SEQ ID NO: 2) was used at 300 nM concentration in a real-time PCR assay that was performed according to the parameters described in Table 1 above. 100 µl of diluted genomic DNA was added to the PCR reaction mix and 45 cycles of amplification were performed with an annealing temperature of 60 °C. A titration using different amounts of genomic DNA template was performed. Real-time PCR data are provided in Table 27 for reactions containing 0 to 50,000 copies (i.e., 0 (no template), 5, 50, 500, 5,000, or 50,000) of genomic DNA per reaction. Four replicates for each reaction condition were performed and the average Cp values were calculated. The average amplification efficiency (2.11) and the PCR efficiency (111%) for the reactions were also calculated. Table 27 shows that the *C. tropicalis* molecular beacon probe was used successfully to detect the target nucleic acid molecule using genomic DNA isolated from *C. tropicalis*.

Table 27:

C. tropicalis detection in singleplex rx								
copies/rx	Log	Cp1	Cp2	Cp3	Cp4	Average	Std Cp	CV
50000	5	22.81	22.81	22.23	22.72	22.64	0.28	1.2%
5000	4	26.23	26.08	26.23	26.13	26.17	0.08	0.3%
500	3	29.35	29.37	29.31	29.31	29.34	0.03	0.1%
50	2	32.59	32.26	32.14	32.34	32.33	0.19	0.6%
5	1	35	35	35	35	35.00	0.00	0.0%
no template		ND	ND	ND	ND			

slope -3.09
0.32
amplification efficiency= 2.11
PCR efficiency= 111%

Next, a similar assay was performed using a *C. tropicalis* lysate rather than genomic DNA according to the assay described in Example 8.

Real-time PCR data are provided below (Table 28) for PCR reactions containing *C. tropicalis* genomic DNA prepared from *C. tropicalis* lysate using the indicated number of cells/mL. The reactions were set up using lysates prepared over multiple days not the same sample lysate detected over multiple days, thus the variation in the entire process is shown. As shown in Table 28 and Figure 8E, *C. tropicalis* was successfully detected using the *C. tropicalis* specific molecular beacon probe, and semi-quantitative detection of *C. tropicalis* was possible using as few as ~3 cells/mL.

Table 28:

<i>C. tropicalis</i> cells/mL		520	400	280	52	40	28	5.2	4	2.8	
operator	date	2.716003	2.60206	2.447158	1.716003	1.60206	1.447158	0.69897	0.60206	0.447158	0
NP	4/30/2012		28.94			31.1					ND
			28.85			30.98					ND
			28.96			31.04					ND
			28.92			30.91					ND
NP	3/23/2012		28.71			31.9			34.81		ND
			28.83			31.92			34.97		ND
			28.75			31.85			34.88		ND
NP	4/3/2012		28.68			31.32			32.8		ND
		28.36	28.72	29.15	31.64	31.28	32.42		32.81	34.66	ND
		28.35	28.65	29.18	31.67	31.42	32.44	34.03	32.68	34.77	ND
		28.45	28.68	29.24	31.64	31.55	32.5	34.12	32.8		ND
NP	4/4/2012	27.87	28.56	28.86	30.49	30.53	31	32.22	32.04	32.73	ND
		27.85	28.56	29.02	30.5	30.77	31.06	31.83	31.96	32.77	ND
		27.82	28.54	28.99	30.43	30.67	31.24	31.99		32.84	ND
NP	5/11/2012		28.61			30.93					ND
			28.66			30.84					ND
			28.66			30.96					ND
			28.65			30.97					ND
CW	5/11/2012		28.87			30.86					ND
			28.93			30.96					ND
			28.92			30.98					ND
NP	3/21/2012		28.98			31.02					ND
			28.57			31.99			35.07		ND
			28.67			31.82			34.57		ND
			28.58			32.06			34.55		ND
		28.61			32.05			34.46		ND	
mean		28.11667	28.73158	29.07667	31.06167	31.152222	31.77667	32.838	33.30556	33.554	
sd		0.298239	0.132173	0.135745	0.645025	0.4114044	0.745913	1.138143	1.228455	1.061267	
cv		1.06%	0.46%	0.47%	2.08%	1.32%	2.35%	3.47%	3.69%	3.16%	

The above data demonstrate that the *C. tropicalis* molecular beacon probe can be used to successfully detect *C. tropicalis* using either purified genomic DNA as an input in a real-time PCR assay or, alternatively, using a fungal cell lysate as an input in the assay.

Example 13: Detection of Candida albicans in a multiplex reaction with Candida albicans, Candida krusei, and Candida glabrata.

The molecular beacon probes can also be used to successfully detect *Candida* target nucleic acid molecules in a multiplexed reaction that includes nucleic acid molecules from two or more *Candida* species.

A multiplex reaction was performed using nucleic acid molecules (genomic DNA) from *C. albicans*, *C. krusei*, and *C. glabrata*. 300 nM of each *Candida* species-specific beacon probe (corresponding to SEQ ID NOs: 1, 6, and 3, respectively; 0.3uL per 100 uL reaction) were used in a real-time PCR assay that was performed according to the parameters described in Table 1 above. The *C. albicans*, *C. krusei*, and *C. glabrata* molecular beacons are each labeled with spectrally distinguishable fluorophors that are detected in their respective channels. 100 µl of diluted genomic DNA was added to the PCR reaction mix and 45 cycles of amplification were performed with an annealing temperature of 60

°C. A titration using different amounts of genomic DNA template was performed. Real-time PCR data are provided in Table 29 for reactions containing 0 to 50,000 copies (i.e., 0 (no template), 5, 50, 500, 5,000, or 50,000) of genomic DNA per reaction. Four replicates for each reaction condition were performed and the average Cp values were calculated. The average amplification efficiency (2.00) and the PCR efficiency (100%) for the reactions were also calculated. Table 29 shows that the *C. albicans* molecular beacon probe was used successfully to detect the *C. albicans* target nucleic acid molecules using genomic DNA isolated from *C. albicans*, even in the presence *C. krusei* and *C. glabrata* nucleic acid molecules, as well as molecular beacons for all of these *Candida* species. Notably, the amplification efficiency and PCR efficiency in the multiplex reaction was similar to that observed in the singleplex reaction (see Example 8 and Table 19). Thus, the limit of detection using the molecular beacon probes is not changing even when they are used in combination. Similarly, the presence of multiple probes in the sample is not affecting the efficiency of the nucleic acid amplification reaction.

Table 29:

C. albicans detection in multiplex rx (C. albicans, C. krusei, and C. glabrata multiplex)							
copies/rx	Cp1	Cp2	Cp3	Cp4	Average	Std Cp	CV
50000	21.69	21.76	21.05	21.68	21.55	0.33	1.5%
5000	25.1	25.14	25.08	25.02	25.09	0.05	0.2%
500	28.7	28.55	28.51	28.52	28.57	0.09	0.3%
50	31.68	31.77	31.92		31.79	0.12	0.4%
5	34.45	35	34.93	35	34.79	0.27	0.8%
no template	ND	ND	ND	ND			
slope	-3.32						
	0.30						
amplification efficiency=	2.00						
PCR efficiency=	100%						

Example 14: Detection of *C. glabrata* in a multiplex reaction with *C. albicans*, *C. krusei*, and *C. glabrata*.

A molecular beacon probe for *C. glabrata* (SEQ ID NO: 3) can be used to successfully detect *C. glabrata* nucleic acid molecules in a multiplex reaction that also includes nucleic acid molecules from *C. albicans* and *C. krusei*. The multiplex reaction was performed as described in Example 13. Real-time PCR data are provided in Table 30 for reactions containing 0 to 50,000 copies (i.e., 0 (no template), 5, 50, 500, 5,000, or 50,000) of genomic DNA per reaction. Four replicates for each reaction condition were performed and the average Cp values were calculated. The average amplification efficiency (2.02) and the PCR efficiency (102%) for the reactions were also calculated. Table 30 shows that the *C. glabrata* molecular beacon probe was used successfully to detect *C. glabrata* target nucleic acid molecules using genomic DNA isolated from *C. glabrata*, even in the presence *C. albicans* and *C. krusei* nucleic acid molecules, as well as molecular beacons for all of these *Candida* species. Notably, the amplification

efficiency and PCR efficiency in the multiplex reaction was similar to that observed in the singleplex reaction (see Example 9 and Table 21).

Table 30:

C. glabrata detection in multiplex rx (C. albicans, C. krusei, and C. glabrata multiplex)							
copies/rx	Cp1	Cp2	Cp3	Cp4	Average	Std Cp	CV
50000	23.65	23.79	23.25	23.75	23.61	0.25	1.0%
5000	27.08	27.12	26.92	27.03	27.04	0.09	0.3%
500	30.55	30.49		30.54	30.53	0.03	0.1%
50	33.24	33.3	33.49	33.38	33.35	0.11	0.3%
5		35	35		35.00	0.00	0.0%
no template	ND	ND	ND	ND			
slope	-3.27						
	0.31						
amplification efficiency=	2.02						
PCR efficiency=	102%						

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Example 15: Detection of *C. krusei* in a multiplex reaction with *C. albicans*, *C. krusei*, and *C. glabrata*.

A molecular beacon probe for *Candida krusei* (SEQ ID NO: 6) can be used to successfully detect *C. krusei* nucleic acid molecules in a multiplex reaction that also includes nucleic acid molecules from *C. albicans* and *C. glabrata*. The multiplex reaction was performed as described in Example 13. Real-time PCR data are provided in Table 26 for reactions containing 0 to 50,000 copies (i.e., 0 (no template), 5, 50, 500, 5,000, or 50,000) of genomic DNA per reaction. Four replicates for each reaction condition were performed and the average Cp values were calculated. The average amplification efficiency (2.12) and the PCR efficiency (112%) for the reactions were also calculated. Table 31 shows that the *C. krusei* molecular beacon probe was used successfully to detect *C. krusei* target nucleic acid molecules using genomic DNA isolated from *C. krusei*, even in the presence *C. albicans* and *C. glabrata* nucleic acid molecules, as well as molecular beacons for all of these *Candida* species. Notably, the amplification efficiency and PCR efficiency in the multiplex reaction was similar to that observed in the singleplex reaction (see Example 10 and Table 23).

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Table 31:

C. krusei detection in multiplex rx (C. albicans, C. krusei, and C. glabrata multiplex)

copies/rx	Cp1	Cp2	Cp3	Cp4	Average	Std Cp	CV
50000	22.18	22.34	22.12	22.24	22.22	0.09	0.4%
5000		25.74	25.66	25.68	25.69	0.04	0.2%
500	29.13	29.26	29.38	28.93	29.18	0.19	0.7%
50	32.14	32.09	32.18	32.22	32.16	0.06	0.2%
5	34.36	33.68	35	35	34.35	0.63	1.8%
no template	ND	ND	ND	ND			

slope -3.07
 0.33
 2.12
 112%

5 *Example 16:* Detection of *C. parapsilosis* in a multiplex reaction with *C. parapsilosis* and *C. tropicalis*.

A molecular beacon probe for *Candida parapsilosis* (SEQ ID NO: 4) can be used to successfully detect *C. parapsilosis* nucleic acid molecules in a multiplex reaction that also includes nucleic acid molecules from *C. tropicalis*. The multiplex reaction was performed using nucleic acid molecules (genomic DNA) from *C. parapsilosis* and *C. tropicalis*. 300 nM of each *Candida* species-specific beacon probe (corresponding to SEQ ID NOs: 4 and 2, respectively; 0.3uL per 100 uL reaction) were used in a real-time PCR assay that was performed according to the parameters described in Table 1 above. 100 µl of diluted genomic DNA was added to the PCR reaction mix and 45 cycles of amplification were performed with an annealing temperature of 60 °C. A titration using different amounts of genomic DNA template was performed. Real-time PCR data are provided in Table 32 for reactions containing 0 to 50,000 copies (i.e., 0 (no template), 5, 50, 500, 5,000, or 50,000) of genomic DNA per reaction. Four replicates for each reaction condition were performed and the average Cp values were calculated. The average amplification efficiency (2.07) and the PCR efficiency (107%) for the reactions were also calculated. Table 32 shows that the *C. parapsilosis* molecular beacon probe was used successfully to detect *C. parapsilosis* target nucleic acid molecules using genomic DNA isolated from *C. parapsilosis*, even in the presence *C. tropicalis* nucleic acid molecules, as well as molecular beacons for both of these *Candida* species. Notably, the amplification efficiency and PCR efficiency in the multiplex reaction was similar to that observed in the singleplex reaction (see Example 11 and Table 25).

Table 32:

C. parapsilosis detection in multiplex rx (C. tropicalis, C. parapsilosis)

copies/rx	Cp1	Cp2	Cp3	Cp4	Average	Std Cp	CV
50000	22.03	22.33	22.47	22.47	22.33	0.21	0.9%
5000	25.83	25.86	25.81	25.81	25.83	0.02	0.1%
500	29.17	29.16	29.18	29.13	29.16	0.02	0.1%
50	32.03	32.11	32.29	32.1	32.13	0.11	0.3%
5	35	35	35	35	35.00	0.00	0.0%
no template	ND	ND	ND	ND			

slope -3.17
 0.32
 2.07
 107%

Example 17: Detection of C. tropicalis in a multiplex reaction with C. parapsilosis and C.

5 *tropicalis.*

A molecular beacon probe for *C. tropicalis* (SEQ ID NO: 2) can be used to successfully detect *C. tropicalis* nucleic acid molecules in a multiplex reaction that also includes nucleic acid molecules from *C. parapsilosis*. The multiplex reaction was performed as described in Example 16. Real-time PCR data are provided in Table 33 for reactions containing 0 to 50,000 copies (i.e., 0 (no template), 5, 50, 500, 5,000, 10 or 50,000) of genomic DNA per reaction. Four replicates for each reaction condition were performed and the average Cp values were calculated. The average amplification efficiency (2.11) and the PCR efficiency (111%) for the reactions were also calculated. Table 33 shows that the *C. tropicalis* molecular beacon probe was used successfully to detect *Candida tropicalis* target nucleic acid molecules using genomic DNA isolated from *C. tropicalis*, even in the presence *C. parapsilosis* nucleic acid molecules, as 15 well as molecular beacons for both of these *Candida* species. Notably, the amplification efficiency and PCR efficiency in the multiplex reaction was similar to that observed in the singleplex reaction (see Example 12 and Table 27).

Table 33:

C. tropicalis detection in multiplex rx (C. tropicalis, C. parapsilosis)							
copies/rx	Cp1	Cp2	Cp3	Cp4	Average	Std Cp	CV
50000	22.75	22.75	22.94	22.43	22.72	0.21	0.9%
5000	26.1	26.05	26.33	26.09	26.14	0.13	0.5%
500	29.19	29.45	29.51	29.29	29.36	0.15	0.5%
50	32.26	32.24	32.69	32.15	32.34	0.24	0.7%
5	35	35	35	35	35.00	0.00	0.0%
no template	ND	ND	ND	ND			
slope	-3.08						
	0.33						
	2.11						
	111%						

Example 18: Detection of *Candida* species using a heat denatured fungal lysate.

- 5 To determine if the molecular beacon probes could be used for the detection of *Candida* cells directly using a 95°C heat denaturation step to lyse the *Candida* cells instead of mechanical lysis, such as by bead beating, we spiked 50 uL of TE containing Coulter counted *Candida* cells directly into a 96-well plate. We then added the molecular beacon probe-containing master mix on top of the cells and conducted an RT-PCR using the parameters described in Example 1 above.
- 10 Real-time PCR data are provided below (Tables 34-38) for PCR reactions, each of which contains *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* genomic DNA prepared from heat denatured lysates containing the indicated number of cells. As shown in Tables 34-38, the *Candida* specific molecular beacon probes (*C. albicans*: SEQ ID NO: 1; *C. glabrata*: SEQ ID NO: 3; *C. krusei*: SEQ ID NO: 6; *C. parapsilosis*: SEQ ID NO: 4; and *C. tropicalis*: SEQ ID NO: 2) successfully
- 15 detected the indicated *Candida* nucleic acid molecules following lysis by heat denaturation. Detection was possible even up to 10 cells.

Table 34: Detection of *C. albicans* in fungal lysate

C. albicans						
cells	Cp1	Cp2	Cp3	Average	Std Cp	CV
1000	37.18	36.78	37.4	37.12	0.31	0.8%
100	ND	40	40	40.00	0.00	0.0%
50	40	39.09	ND	39.55	0.64	1.6%
10	40	ND	ND	40.00		
no template	ND	ND	ND			

Table 35: Detection of *C. glabrata* in fungal lysate

C. glabrata						
cells	Cp1	Cp2	Cp3	Average	Std Cp	CV
1000	34	33.99	34.31	34.10	0.18	0.5%
100	37.61	36.63	36.26	36.83	0.70	1.9%
50	40	36.85	39.43	38.76	1.68	4.3%
10	ND	40	40	40.00	0.00	0.0%
no template	ND	ND	ND			

Table 36: Detection of *C. krusei* in fungal lysate

C. krusei						
cells	Cp1	Cp2	Cp3	Average	Std Cp	CV
1000	29.53	28.98	29.00	29.17	0.31	1.1%
100	32.51	32.54	32.26	32.44	0.15	0.5%
50	33.28	33.48	33.54	33.43	0.14	0.4%
10	35.74	35.76	36.11	35.87	0.21	0.6%
no template	ND	ND	ND			

5 Table 37: Detection of *C. parapsilosis* in fungal lysate

C. parapsilosis						
cells	Cp1	Cp2	Cp3	Average	Std Cp	CV
1000	36.16	35.63	35.35	35.71	0.41	1.2%
100	37.78	38.59	39.19	38.52	0.71	1.8%
50	39.54	40	38.28	39.27	0.89	2.3%
10	ND	40	ND			
no template	ND	ND	ND			

Table 38: Detection of *C. tropicalis* in fungal lysate

C. tropicalis						
cells	Cp1	Cp2	Cp3	Average	Std Cp	CV
1000	32.93	33.37	33.2	33.17	0.22	0.7%
100	36.03	36.23	36.82	36.36	0.41	1.1%
50	37.14	37.74	37.58	37.49	0.31	0.8%
10	40	40	40	40.00	0.00	0.0%
no template	ND	40	ND			

As positive controls, the same *Candida* nucleic acid molecules prepared via the PCR described above were detected using *Candida* specific probes (*C. albicans*: SEQ ID NOs: 13 and 14; *C. glabrata*: SEQ ID NOs: 17 and 18; *C. krusei*: SEQ ID NOs: 15 and 16; *C. parapsilosis*: SEQ ID NOs: 21 and 22; and *C. tropicalis*: SEQ ID NOs: 19 and 20) conjugated to magnetic nanoparticles for use in an NMR based assay, as is described above. In short, the hybridization induced agglomeration assays is performed by aliquoting 15 µL of the amplification reaction into 0.2 mL thin walled PCR tubes and incubating within a sodium phosphate hybridization buffer (4×SSPE) with the indicated pairs of oligonucleotide probe derivatized nanoparticles at a final iron concentration of 0.2 mM iron per reaction. Hybridization reactions were incubated for 3 minutes at 95°C followed by 30 minutes incubation at 60°C within a shaking incubator set at an agitation speed of 1000 rpm (Vortemp, LabNet International). Hybridized samples are then placed in a 37°C heating block to equilibrate the temperature to that of the MR reader for 3 minutes. Each sample is then subjected to a 5 second vortexing step (3000 rpm) and inserted into the MR reader for T2 measurement. The baseline T2 signal is ~30-40 msec, thus a signal <45 indicates no target DNA is present.

As shown in Tables 39-43, there is 97% concordance between the detected *Candida* cells via the molecular beacon probes and the T2 detection reactions run on the RT-PCR generated amplicons. We observe one instance in which *C. glabrata* nucleic acid molecules from the sample prepared using 10 cells was not detected via RT-PCR but was detected with the NMR-based T2 assay (compare Cp1 in Table 35 to Rep1 in Table 40), and one sample in which *C. tropicalis* nucleic acid molecules from the sample prepared using 10 cells was detected via RT-PCR but not by the NMR-based T2 assay (compare Cp1 in Table 38 to Rep1 in Table 43). These discordant results are highlighted in the tables below.

Table 39:

C. albicans RT-PCR product, T2 detection

Rep1	Rep2	Rep3	Average	Std Cp	CV
253.8	239.06	253.78	248.88	8.50	3.4%
36.09	223.18	250.38	236.78	19.23	8.1%
251.33	233.13	36.72	242.23		0.0%
220.19	36.62	36.17	220.19		0.0%
36.91	36.58	38.53	37.34	1.04	2.8%

Table 40:

C. glabrata RT-PCR product, T2 detection

Rep1	Rep2	Rep3	Average	Std Cp	CV
102.97	98.49	96.11	99.19	3.48	3.5%
117.8	121.86	112.59	117.42	4.65	4.0%
125.25	108.89	180.44	138.19	37.49	27.1%
273.68	139.8	189.81	201.10	67.65	33.6%
35.16	35.19	36.64	35.66	0.85	2.4%

Table 41:

C. krusei RT-PCR product, T2 detection

Rep1	Rep2	Rep3	Average	Std Cp	CV
105.92	116.9	104.72	109.18	6.71	6.1%
106.36	118.04	107.04	110.48	6.56	5.9%
110.11	119.5	109.77	113.13	5.52	4.9%
122.32	128.94	119.36	123.54	4.91	4.0%
35.63	35.26	37.55	36.15	1.23	3.4%

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Table 42:

C. parapsilosis RT-PCR product, T2 detection

Rep1	Rep2	Rep3	Average	Std Cp	CV
303.44	307.97	279.39	296.93	15.36	5.2%
343.76	327.83	361.66	344.42	16.92	4.9%
390.33	464.65	315.78	390.25	74.44	19.1%
32.27	448.74	32.66			
31.98	32.02	32.94	32.31	0.54	1.7%

10

Table 43:

C. tropicalis RT-PCR product, T2 detection

Rep1	Rep2	Rep3	Average	Std Cp	CV
279.39	471.34	441.24	397.3233	103.2362	26.0%
361.66	438.41	435.62	411.90	43.53	10.6%
315.78	452.44	436.73	401.65	74.78	18.6%
32.66	475.71	530.58	346.32	273.02	78.8%
36.04	35.07	35.36	35.49	0.50	1.4%

Example 19: Detection of *Candida* species using melt-curve analysis.

Because the T_m of the *Candida* species-specific molecular beacon probes differ by at least 1 degree, it is possible to multiplex the beacon probes with the same fluorophore and deconvolute the signals using melt curve analysis alone. Molecular beacon probes for *C. albicans* (SEQ ID NO: 1), *C. glabrata* (SEQ ID NO: 3), *C. krusei* (SEQ ID NO: 6), *C. parapsilosis* (SEQ ID NO: 4), and *C. tropicalis* (SEQ ID NO: 2) were all labeled with a HEX fluorophore and added at 300 nM concentration to a single sample that included target nucleic acid molecules for each of the *Candida* species.

As shown in Figures 9A and 9B, the presence of target nucleic acid molecules for the indicated *Candida* species targets was determined within the sample simply by looking for the presence of the species-specific melt curve and using the T_m as a means of species identification. Use of the melt-curve analysis abrogates the need to use multiple different fluorophores in a single multiplexed reaction and simplifies the instrument design. It should be noted that there is no melt curve when the target nucleic acid molecule is not present in the reaction. The T_m is the temperature at which a decrease in fluorescence is observed and this only occurs when the hybridized beacon probe is melted off the target and the beacon probe stem-loop structure reforms.

Example 20: Molecular beacon analysis using a post-blood culture.

To perform a blood culture analysis, up to 10 ml of a blood sample from a patient suspected of having a blood systemic infection are incubated in a blood culture bottle. The blood culture media typically are composed to promote the growth of either aerobic or anaerobic pathogens. Depending on the specific requirements for growth of the suspected pathogen, culture media is composed of a number of nutrients that feed pathogens and resins that absorb antibiotics/antifungals that might be present in the blood specimen and inhibit growth. When a pathogen reaches a concentration of 10^6 to 10^8 CFU/mL, it produces enough carbon dioxide through its normal metabolic pathways to activate the blood culture detection system, thus signaling a positive blood culture for the original sample. At this time, a sample from this culture bottle is aliquoted onto a species-specific detection methodology, which may be a culture plate or another system that identifies the pathogenic species and assesses its susceptibility to

antimicrobials (e.g., Vitek 2, Biomerieux, Durham, NC). Species identification is typically performed over a day, although it can be significantly longer.

In order to expedite the species identification of *Candida* that may be present in a blood culture, a singleplex or multiplex reaction for detecting one or more *Candida* species, according to the methods described in, e.g., Examples 1-19 above, can be used. The protocol involves adding a sample from the positive blood culture bottle to the singleplex or multiplex reaction, centrifuging the sample to pellet the pathogen cells, which may be optionally washed one or more times to remove interferents from the blood culture media, lysing the cells and using the nucleic acid molecules from the cells in a molecular beacon protocol described in one or more of Examples 1-19 above. This process would rapidly and with high fidelity determine the presence and species identification of one or more *Candida* species in the blood sample; a positive result indicates *Candida* infection in the patient.

Species identification could rapidly assist in appropriate administration of antifungals as several *Candida* species are more resistant to fluconazole (e.g., *Candida glabrata* and *Candida krusei*).

Example 21: Use of the molecular beacon assay to detect *Candida* in a whole blood sample.

The *Candida* specific molecular beacon probes described herein can be used to detect the presence of *Candida* in a whole blood sample from a patient suspected of having a *Candida* infection. Generally, the protocol involves i) obtaining a 1-10 mL sample of whole blood from the patient; ii) lysing the red blood cells (e.g., using an ammonium chloride iso-osmotic solution, a detergent lysis solution, or a hypotonic lysis solution); iii) centrifuging the lysed sample, e.g., at 3,000g to 12,000g for 5 minutes to pellet the *Candida* cells; iv) removing and discarding the supernatant; v) washing the pellet with an equal volume of TE (equivalent to the blood specimen volume) or less; vi) centrifuging the resuspended *Candida* cells at 3,000g to 12,000g for 5 minutes to pellet the *Candida* cells; vii) removing and discarding the supernatant; viii) optionally repeating steps v)-vii); ix) adding 50-150 uL of 1xTE; x) adding 300 mg of 500 to 800 micron beads (e.g., silica or zirconium oxide (yttrium stabilized) beads); xi) vortexing at maximum power (3000 rpm) for 5-10 minutes to lyse the *Candida* cells; xii) aliquoting 10 to 50 uL of the cell lysate into a molecular beacon probe-containing PCR mastermix on, e.g., a 96 well plate (see, e.g., Example 1); and xiii) running an RT-PCR reaction and detecting the presence of *Candida* according to one or more of the methods described in Examples 1-19.

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Example 22: Use of the molecular beacon assay to detect *Candida* in a urine sample.

The *Candida* specific molecular beacon probes described herein can be used to detect the presence of *Candida* in a urine sample from a patient suspected of having a *Candida* infection.

Generally, the protocol involves i) obtaining a 1-10 mL sample of urine from the patient; ii) centrifuging the sample at 3,000g to 12,000g to pellet the *Candida* cells; iii) removing and discarding the supernatant; iv) washing the pellet with an equal volume of TE or less (equivalent to the urine specimen volume); v) centrifuging at 3,000g to 12,000g for 5 minutes to pellet the *Candida* cells; vi) removing and discarding the supernatant; vii) optionally repeating steps iv)-vi); viii) adding 50-150 uL of 1xTE; ix) adding 300 mg of 500 to 800 micron beads (e.g., silica or zirconium oxide (yttrium stabilized) beads); x) vortexing at maximum power (3000 rpm) for 5-10 minutes to lyse the *Candida* cells; xi) aliquoting 10 to 50 uL of cell lysate into molecular beacon probe-containing PCR mastermix on, e.g., a 96 well plate (see, e.g., Example 1); and xii) running an RT-PCR reaction and detecting the presence of *Candida* according to one or more of the methods described in Examples 1-19.

15 Other Embodiments

While certain novel features of this invention shown and described below are pointed out in the annexed claims, the invention is not intended to be limited to the details specified, since a person of ordinary skill in the relevant art will understand that various omissions, modifications, substitutions and changes in the forms and details of the invention illustrated and in its operation may be made without departing in any way from the spirit of the present invention. No feature of the invention is critical or essential unless it is expressly stated as being “critical” or “essential.”

Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed in the scope of the present invention.

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

Claims

1. A nucleic acid probe comprising the sequence of SEQ ID NO: 1.
2. A nucleic acid probe comprising the sequence of SEQ ID NO: 2.
3. A nucleic acid probe comprising the sequence of SEQ ID NO: 3.
4. A nucleic acid probe comprising the sequence of SEQ ID NO: 4.
5. A nucleic acid probe comprising the sequence of SEQ ID NO: 5.
6. The nucleic acid probe of any one of claims 1 to 5, wherein said probe comprises a detection moiety that is conjugated to the probe.
7. The nucleic acid probe of claim 6, wherein said detection moiety comprises a fluorescent label.
8. The nucleic acid probe of any one of claims 1 to 5, wherein said probe is a molecular beacon comprising a fluorescent label and a quencher.
9. A method for detecting at least one *Candida* species in a biological sample, said method comprising:
 - a) contacting said sample with at least one nucleic acid probe having a sequence selected from any one of SEQ ID NOs: 1 to 5 under conditions which allow the at least one probe to hybridize to a nucleic acid molecule of said *Candida* species; and
 - b) detecting hybridization between said at least one probe and said nucleic acid molecule, thereby detecting said at least one *Candida* species.
10. The method of claim 9, wherein the probe is a *Candida albicans* probe and has the sequence of SEQ ID NO: 1.
11. The method of claim 9, wherein the probe is a *Candida tropicalis* probe and has the sequence of SEQ ID NO: 2.
12. The method of claim 9, wherein the probe is a *Candida glabrata* probe and has the sequence of SEQ ID NO: 3.

13. The method of claim 9, wherein the probe is a *Candida parapsilosis* probe and has the sequence of SEQ ID NO: 4.
14. The method of claim 9, wherein the probe is a *Candida krusei* probe and has the sequence of SEQ ID NO: 5.
15. The method of claim 9, wherein said probe has a sequence selected from any one of SEQ ID NOs: 1 to 5.
16. The method of claim 15, wherein said probe comprises a detection moiety that is conjugated to the probe.
17. The method of claim 16, wherein said detection moiety comprises a fluorescent label.
18. The method of claim 17, wherein said probe is a molecular beacon that further comprises a quencher.
19. The method of claim 9, wherein said method comprises contacting said sample with two or more probes having sequences selected from any of SEQ ID NOs: 1 to 5, whereby two or more *Candida* species selected from the group consisting of *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, and *Candida krusei* are detected.
20. The method of claim 9, wherein said method comprises contacting said sample with up to five probes having sequences selected from any of SEQ ID NOs: 1 to 5, whereby up to five *Candida* species selected from the group consisting of *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, and *Candida krusei* are detected.
21. The method of claim 19 or 20, wherein each said probe is differentially labeled.
22. The method of claim 19 or 20, wherein each said probe comprises the same label.
23. The method of any one of claims 9 to 22, wherein the biological sample is selected from the group consisting of whole blood, sweat, tears, urine, saliva, semen, serum, plasma, cerebrospinal fluid (CSF), feces, vaginal fluid or tissue, sputum, nasopharyngeal aspirate or swab, lacrimal fluid, mucous, or epithelial swab (buccal swab), tissues, organs, bones, teeth, and tumors.
24. The method of any one of claims 9 to 23, wherein the biological sample is a human sample.

25. The method of any one of claims 9 to 24, wherein at least one cell in said biological sample is lysed using a hypotonic solution or a detergent, wherein optionally, said lysis step is followed by centrifuging the lysed sample and removing the resulting supernatant.
26. The method of any one of claims 9 to 25, wherein at least one cell of said *Candida* species is lysed by chemical lysis.
27. The method of claim 26, wherein said chemical lysis comprises treatment with one or more enzymes.
28. The method of any one of claims 9 to 25, wherein at least one cell of said *Candida* species is lysed by mechanical lysis.
29. The method of claim 28, wherein said mechanical lysis comprises using any one or more of a finned tube, small particles, ultrasound, freeze-boil, freeze-thaw, and a bead.
30. The method of claim 29, wherein said bead comprises a glass bead.
31. The method of any one of claims 9 to 24, wherein at least one cell of said *Candida* species is lysed by heat denaturation.
32. The method of claim 31, wherein said heat denaturation comprises heating said at least one cell to about 95°C.
33. The method of any one of claims 9 to 32 further comprising amplifying said nucleic acid molecule of said *Candida* species prior to said detecting step.
34. The method of claim 33, wherein said amplifying comprises enzymatic amplification.
35. The method of claim 33 or 34, wherein said amplifying comprises asymmetric amplification.
36. The method of any one of claims 9 to 35, wherein said probe comprises a 5' end and a 3' end, wherein said 5' end is capable of hybridizing to said nucleic acid molecule, and wherein about 1 to 20 nucleotides of said 3' end of said probe do not hybridize to said nucleic acid molecule.
37. The method of any one of claims 9 to 36 further comprising placing a container comprising a composition resulting from said contacting step into a device capable of detecting said hybridization between said probe and said nucleic acid molecule.

38. The method of any one of claims 9 to 37, wherein each said probe comprises a fluorescent label and said hybridization is detected by heating said sample through a melting temperature (T_m) of at least one said probe to obtain a melting curve, wherein a decrease in fluorescence of said sample at the T_m of at least one said probe indicates the presence of said at least one *Candida* species.
39. The method of claim 38, wherein a decrease in fluorescence at a T_m of the probe having the sequence of SEQ ID NO: 1 indicates the presence of *Candida albicans* in the sample.
40. The method of claim 38, wherein a decrease in fluorescence at a T_m of the probe having the sequence of SEQ ID NO: 2 indicates the presence of *Candida tropicalis* in the sample.
41. The method of claim 38, wherein a decrease in fluorescence at a T_m of the probe having the sequence of SEQ ID NO: 3 indicates the presence of *Candida glabrata* in the sample.
42. The method of claim 38, wherein a decrease in fluorescence at a T_m of the probe having the sequence of SEQ ID NO: 4 indicates the presence of *Candida parapsilosis* in the sample.
43. The method of claim 38, wherein a decrease in fluorescence at a T_m of the probe having the sequence of SEQ ID NO: 5 indicates the presence of *Candida krusei* in the sample.
44. The method of any one of claims 9 to 43, wherein said hybridization is detected by fluorescence of said probe using a thermal cycler.
45. The method of any one of claims 9 to 44, wherein detection of said at least one *Candida* species in said sample can be completed within 3 hours.
46. The method of any one of claims 9 to 45, wherein the method is capable of detecting said at least one *Candida* species in said sample when present at a concentration of at least 3 cells/ml.
47. A kit comprising at least one nucleic acid probe having a sequence selected from any of SEQ ID NOs: 1 to 5.
48. The kit of claim 47 further comprising one or more reagent for sample lysis, fungal lysis, detection of *Candida* species, and nucleic acid amplification.

Figure 1

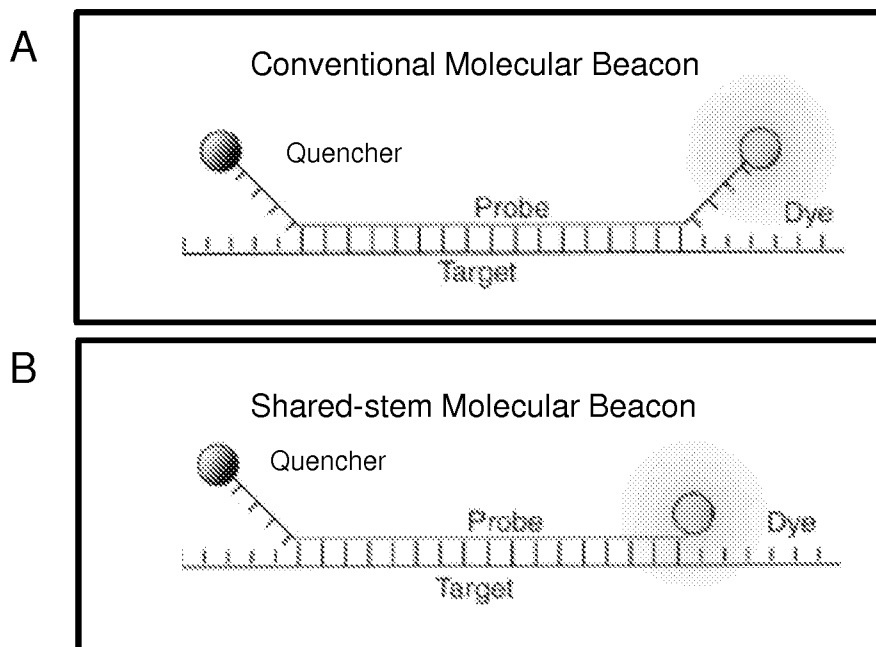


Figure 2

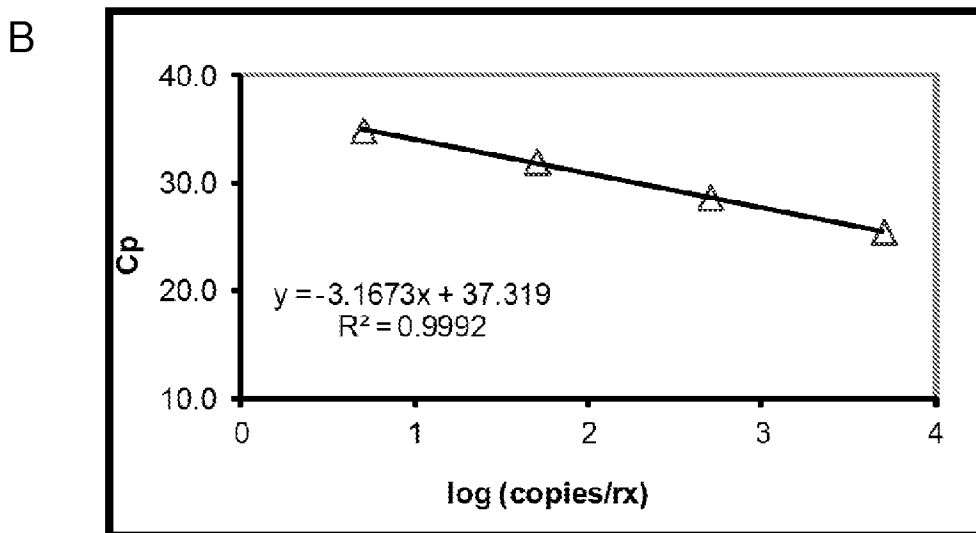
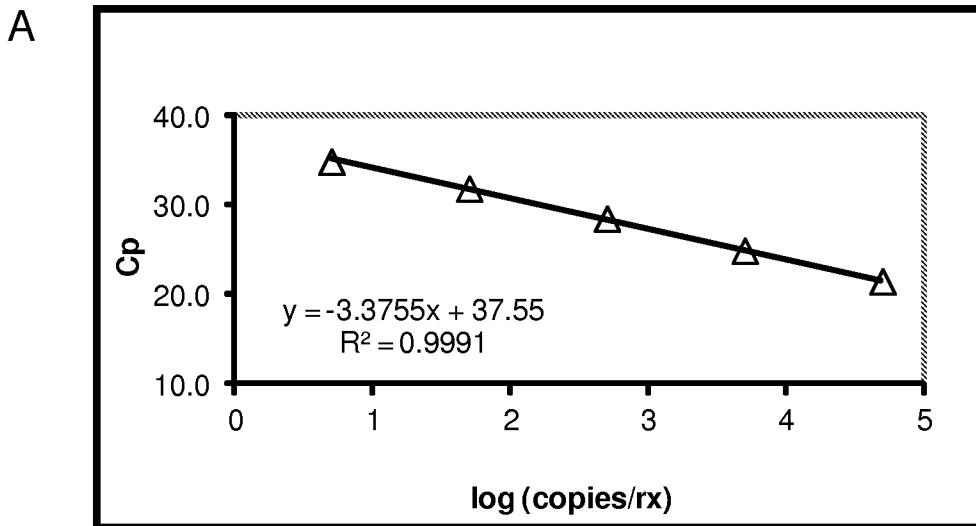


Figure 3

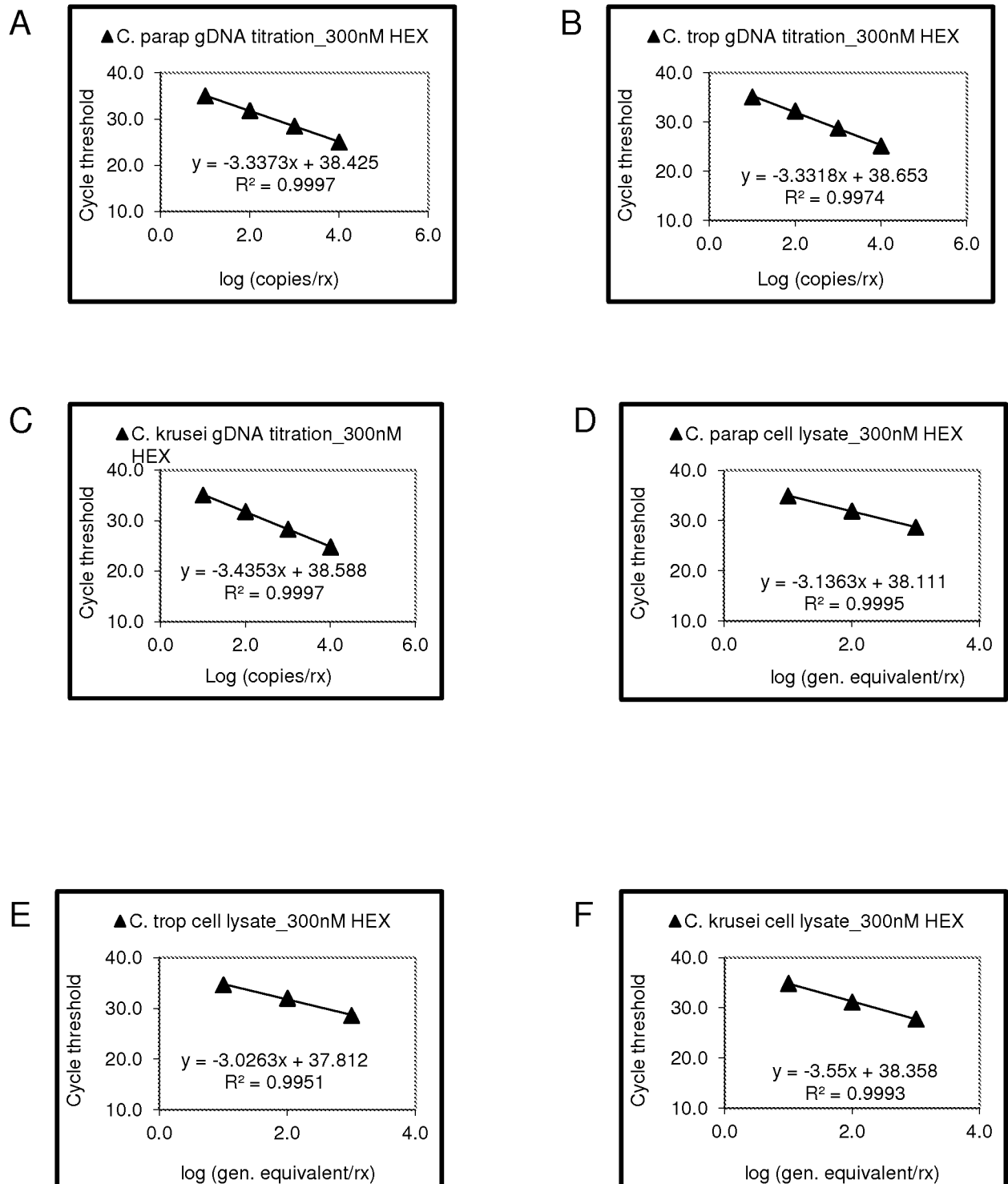
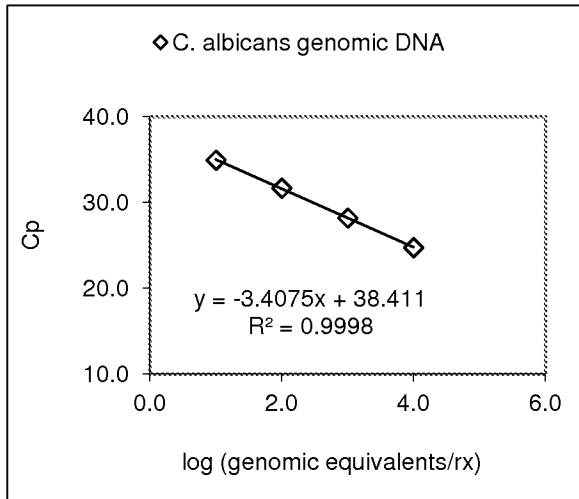
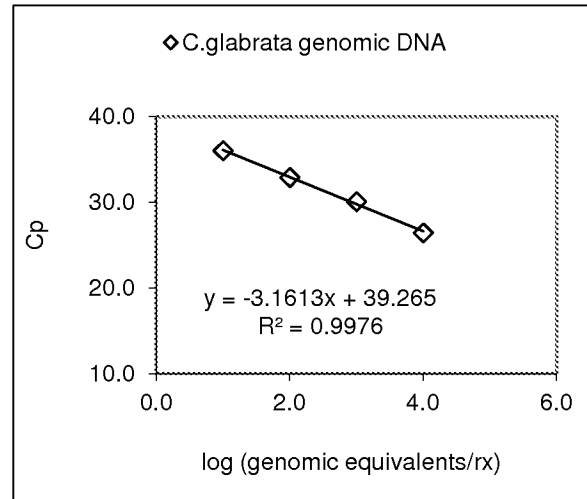


Figure 4

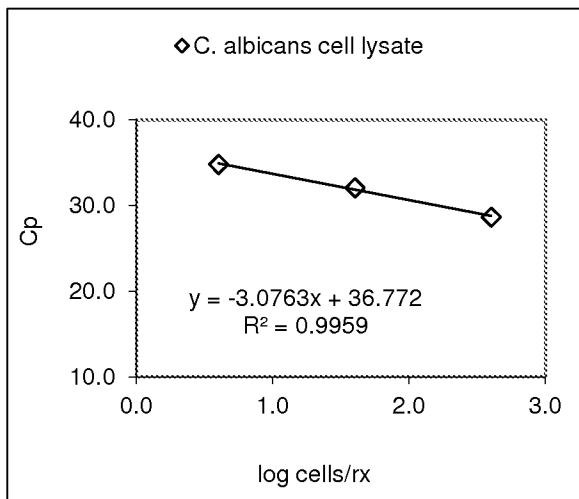
A



B



C



D

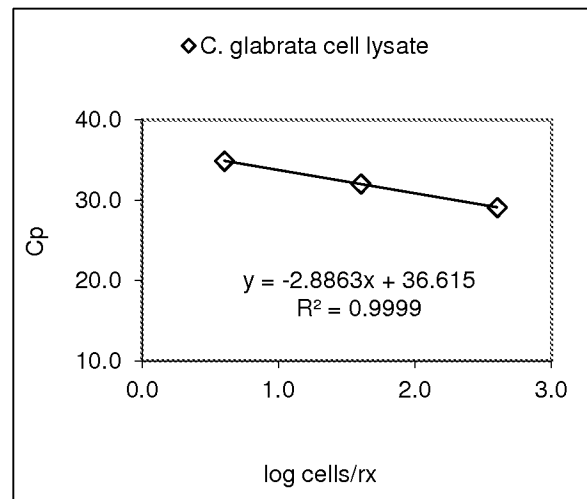
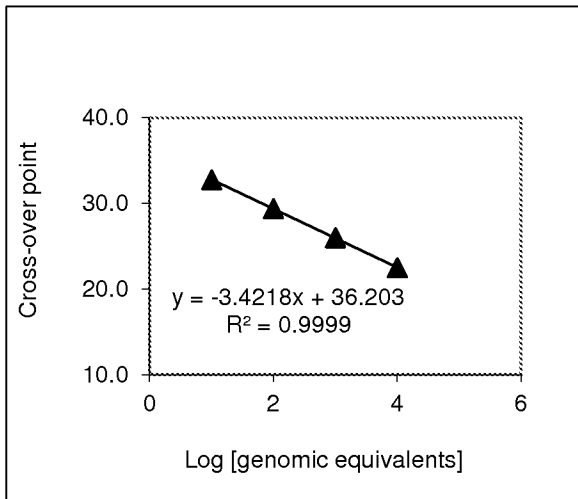
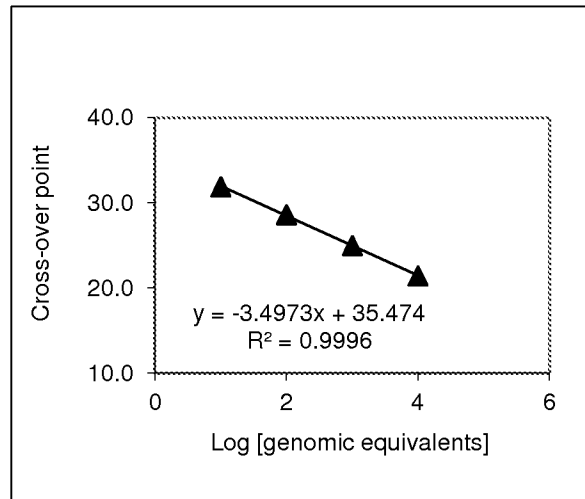


Figure 5

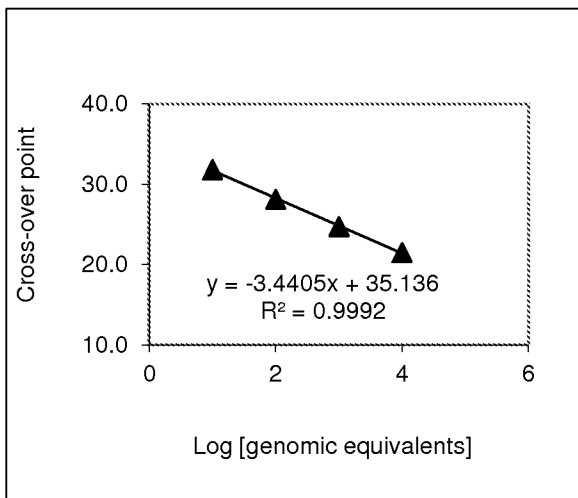
A



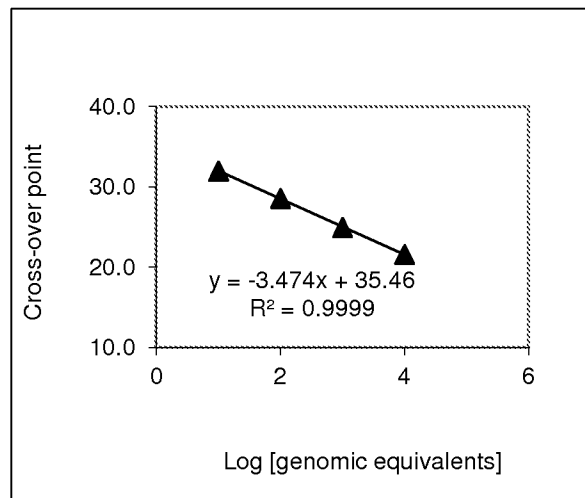
B



C



D



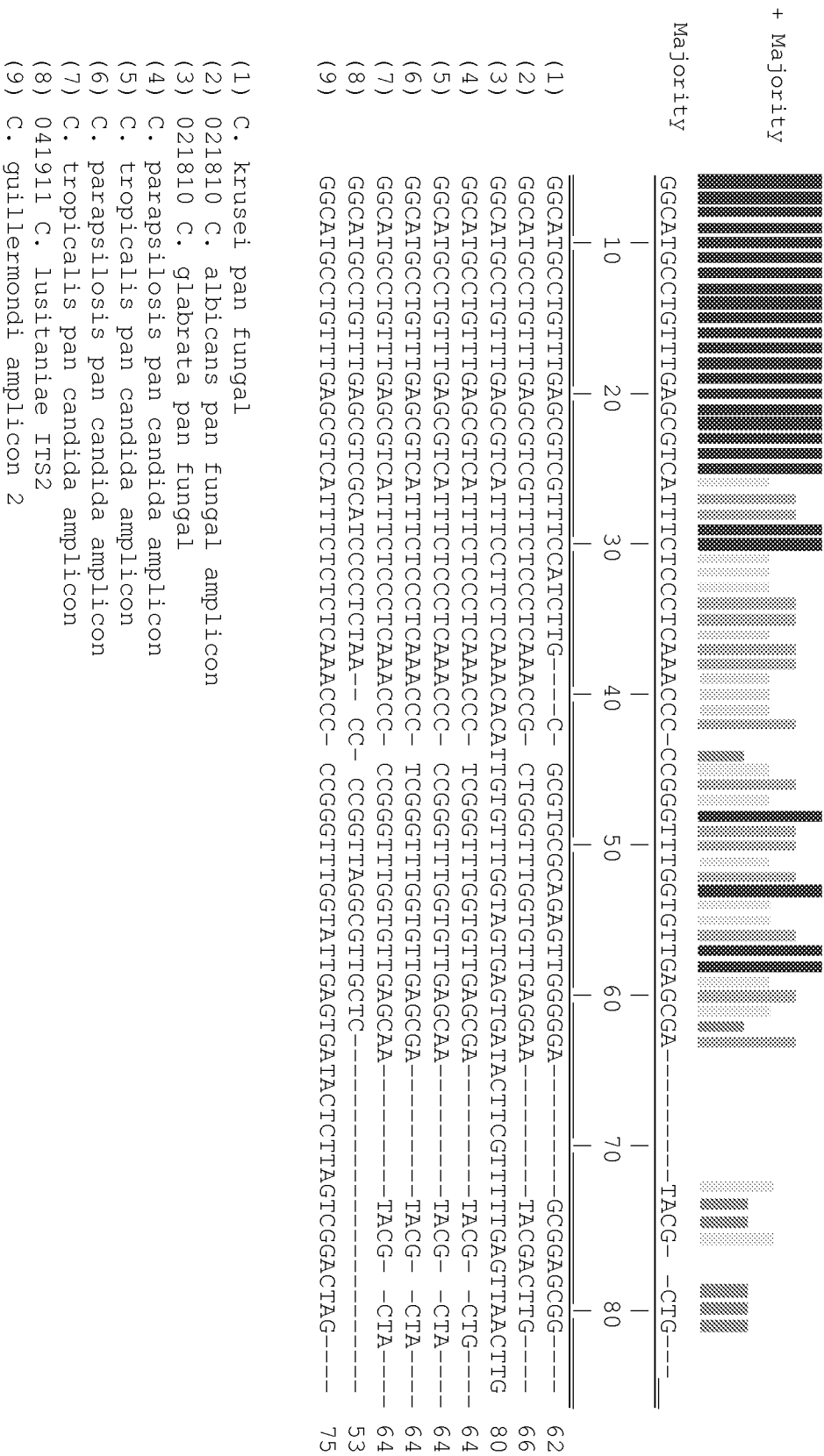


FIGURE 6

FIGURE 6 (con't)

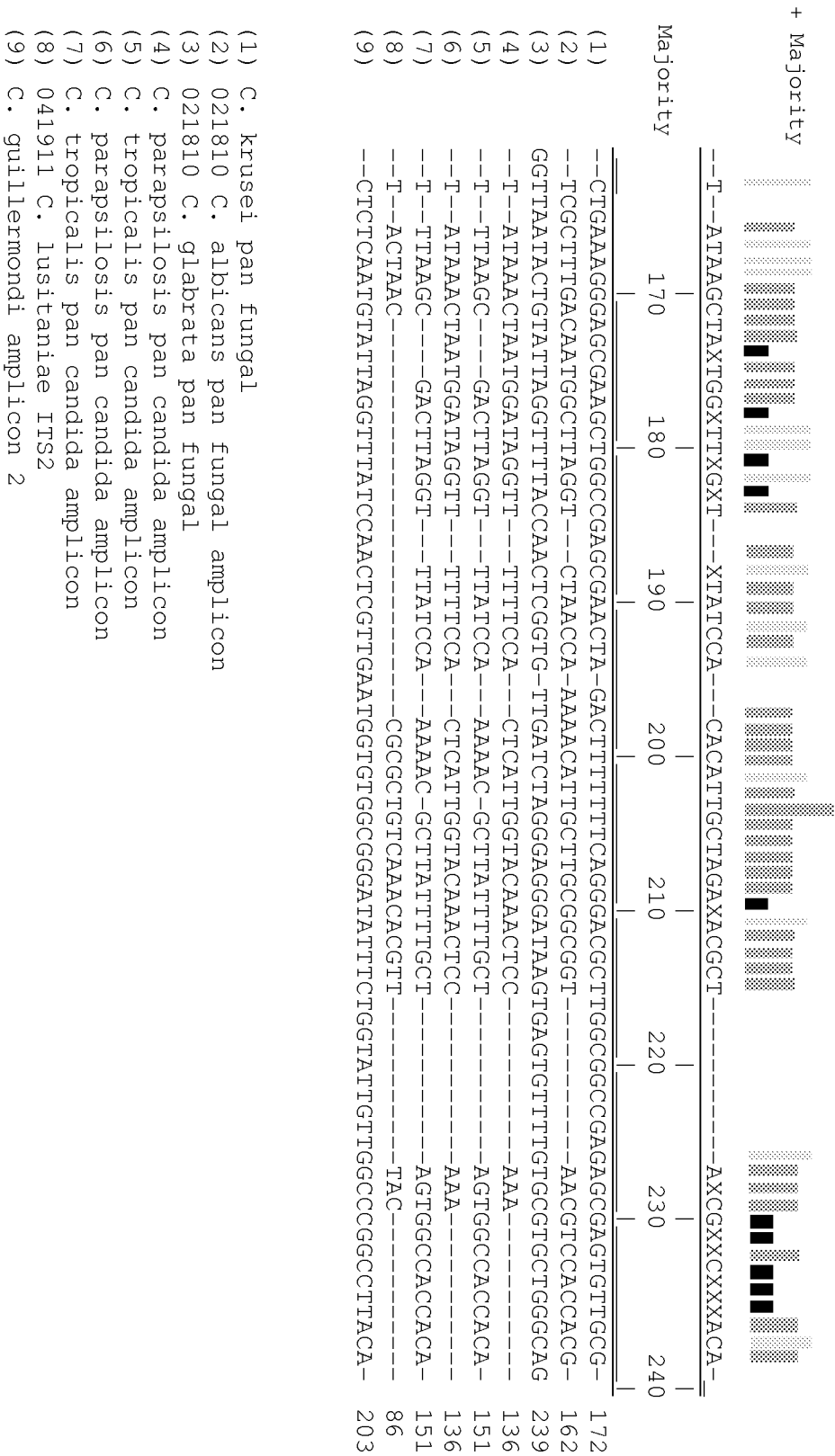
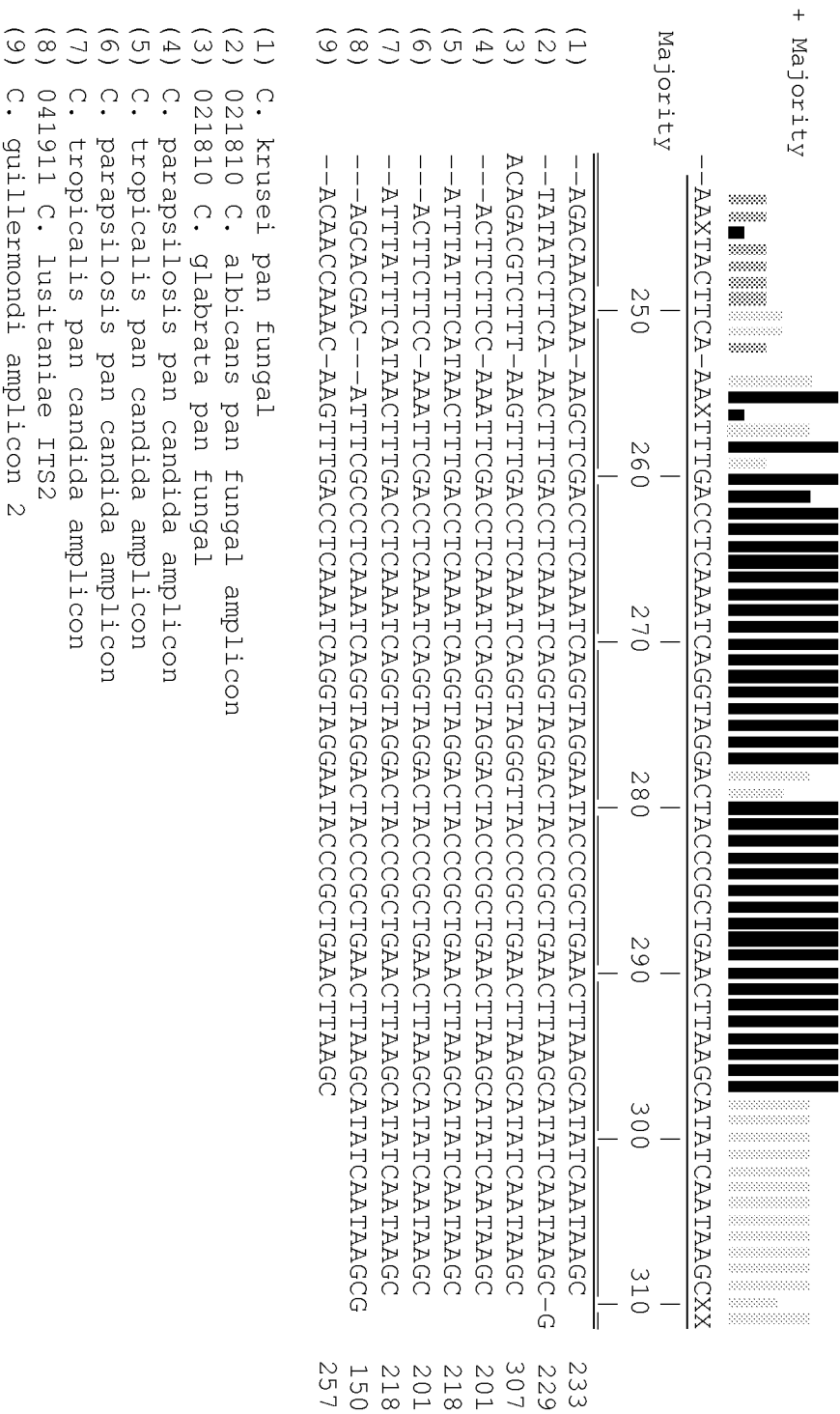


FIGURE 6 (con't)



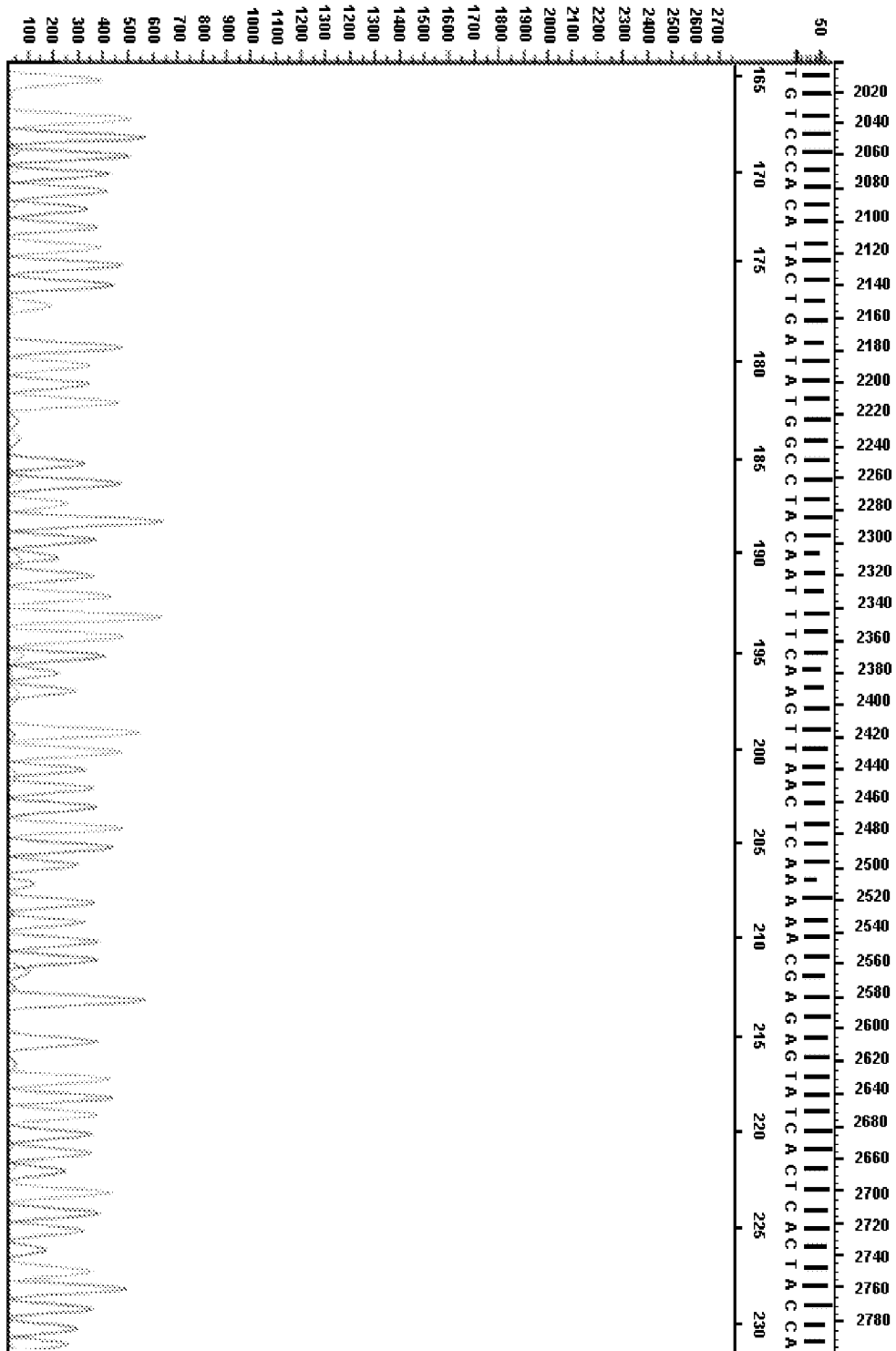


FIGURE 7

Figure 8A

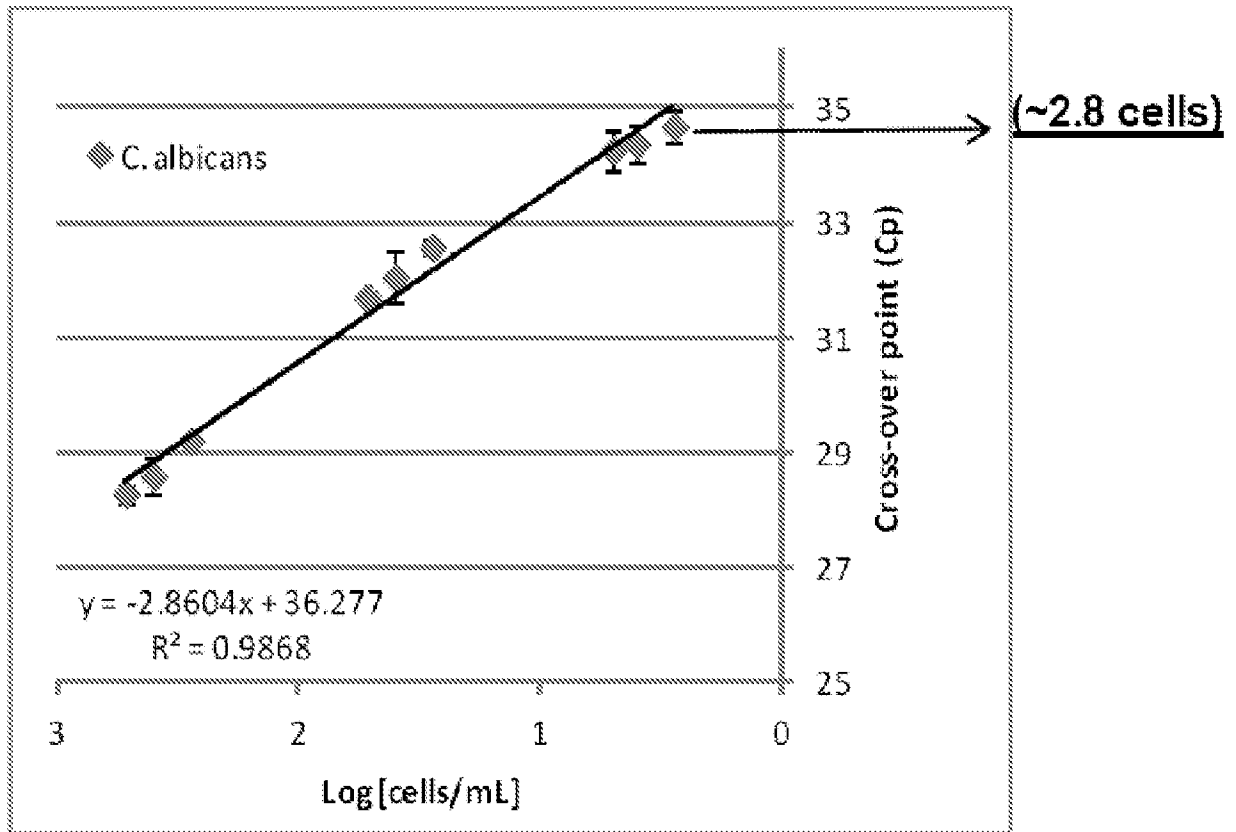


Figure 8B

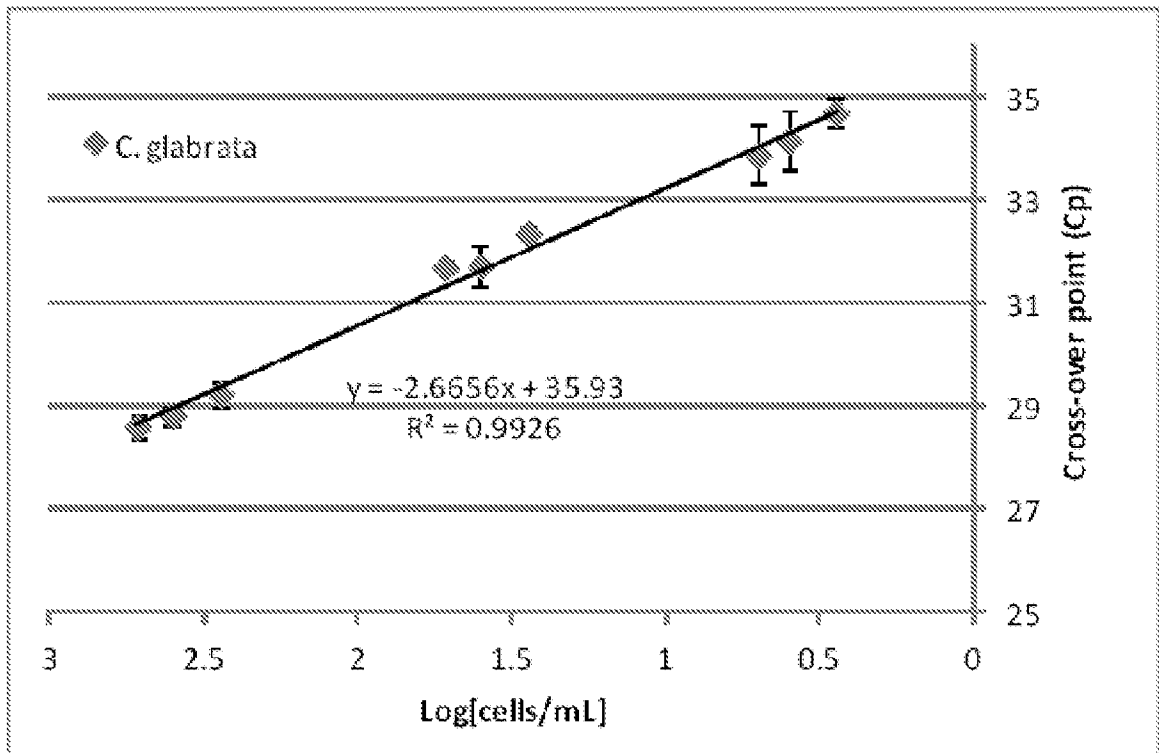


Figure 8C

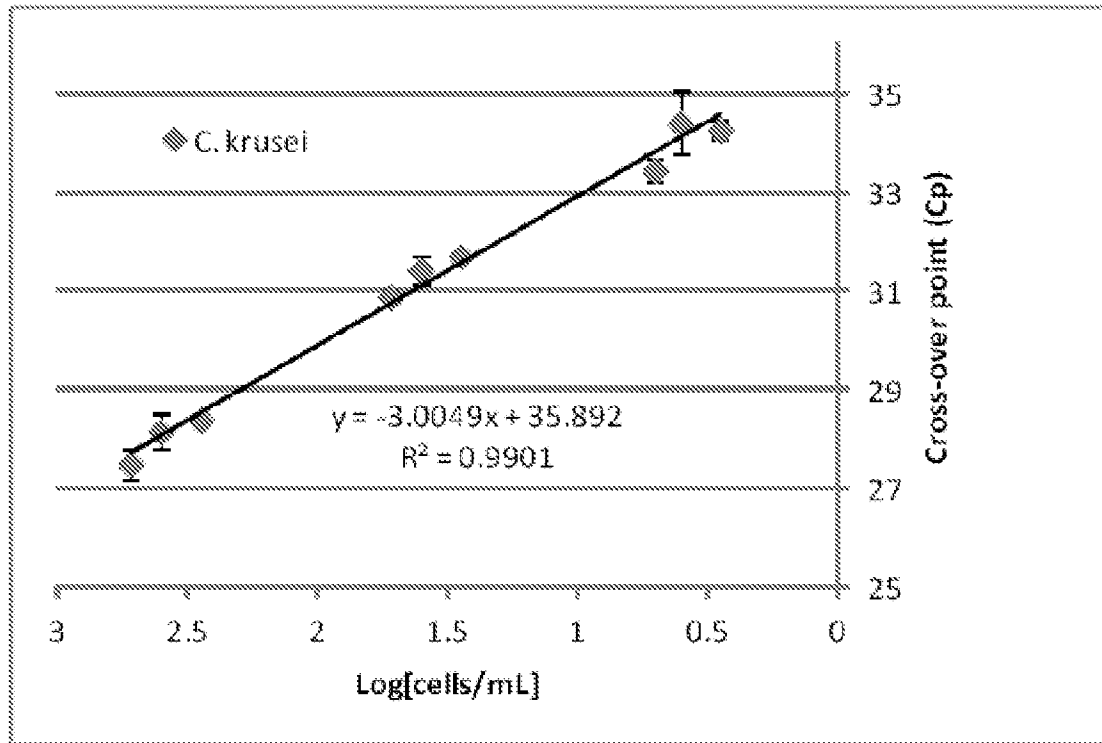


Figure 8D

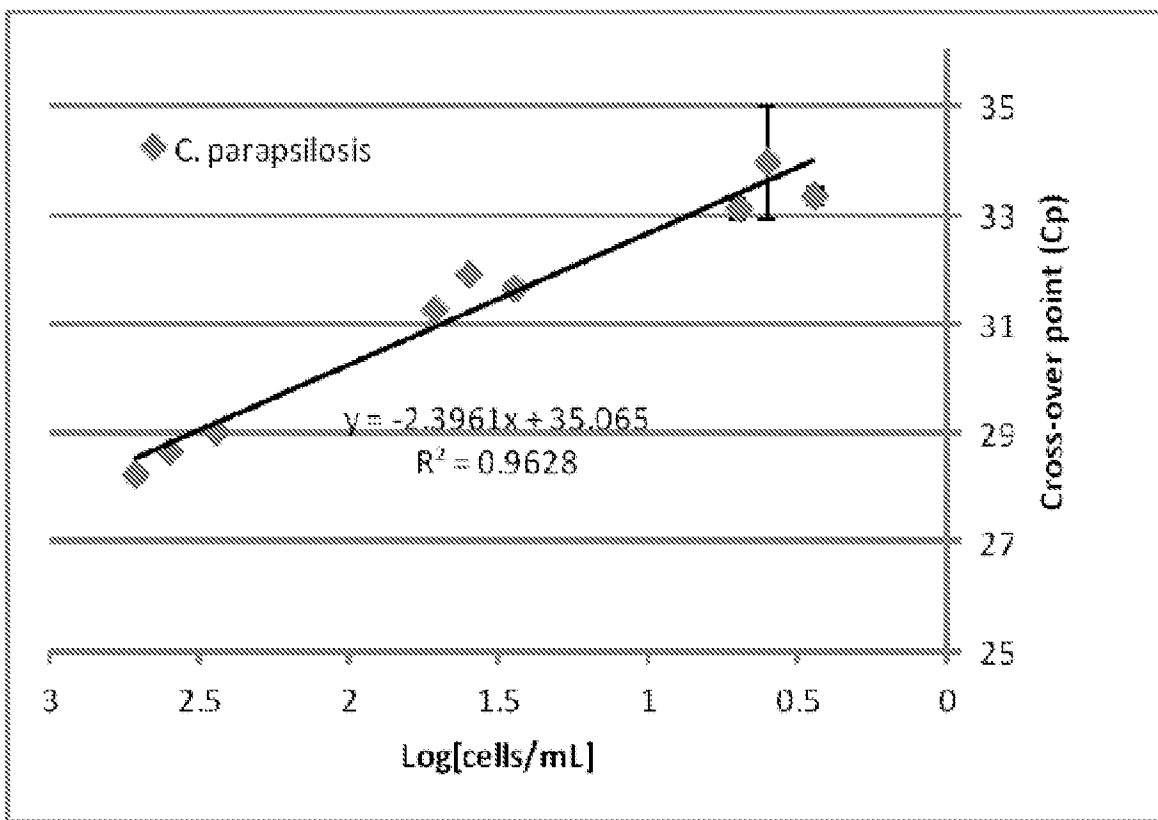


Figure 8E

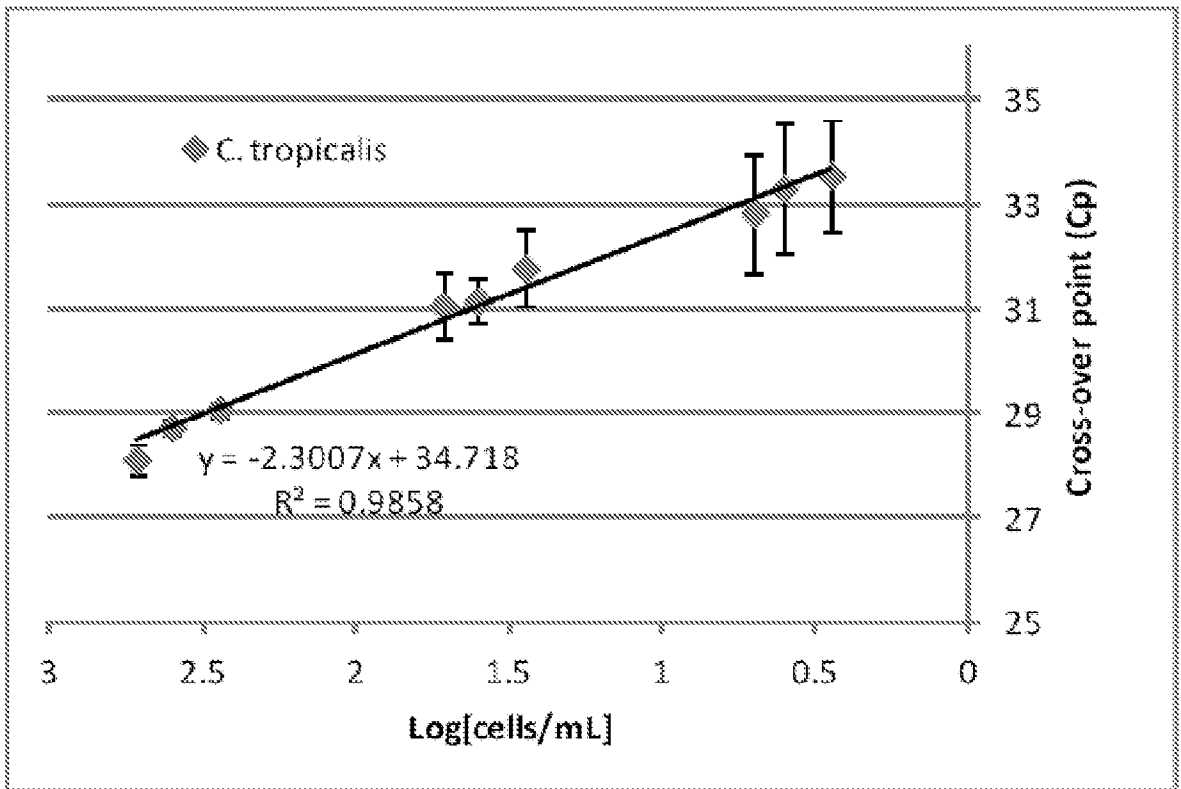
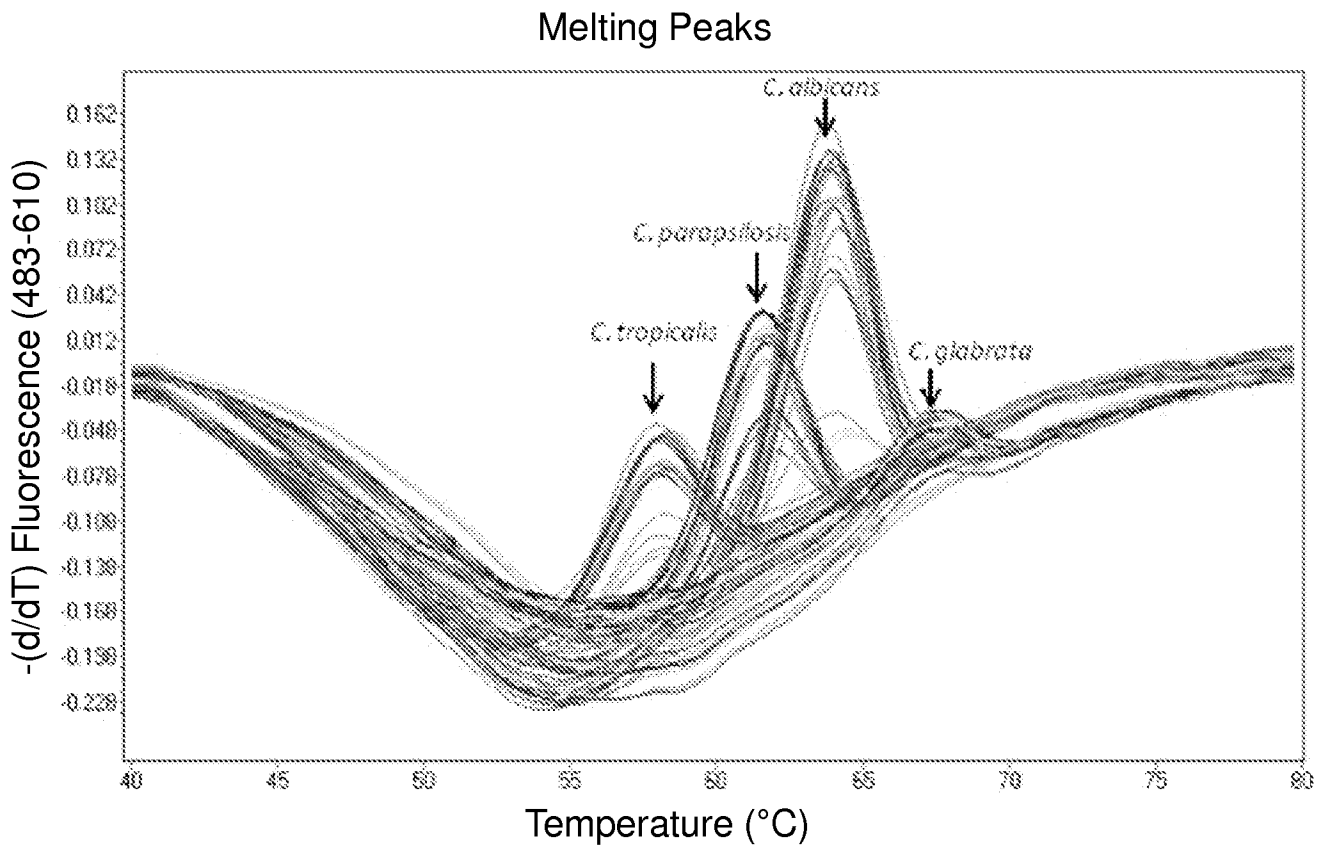
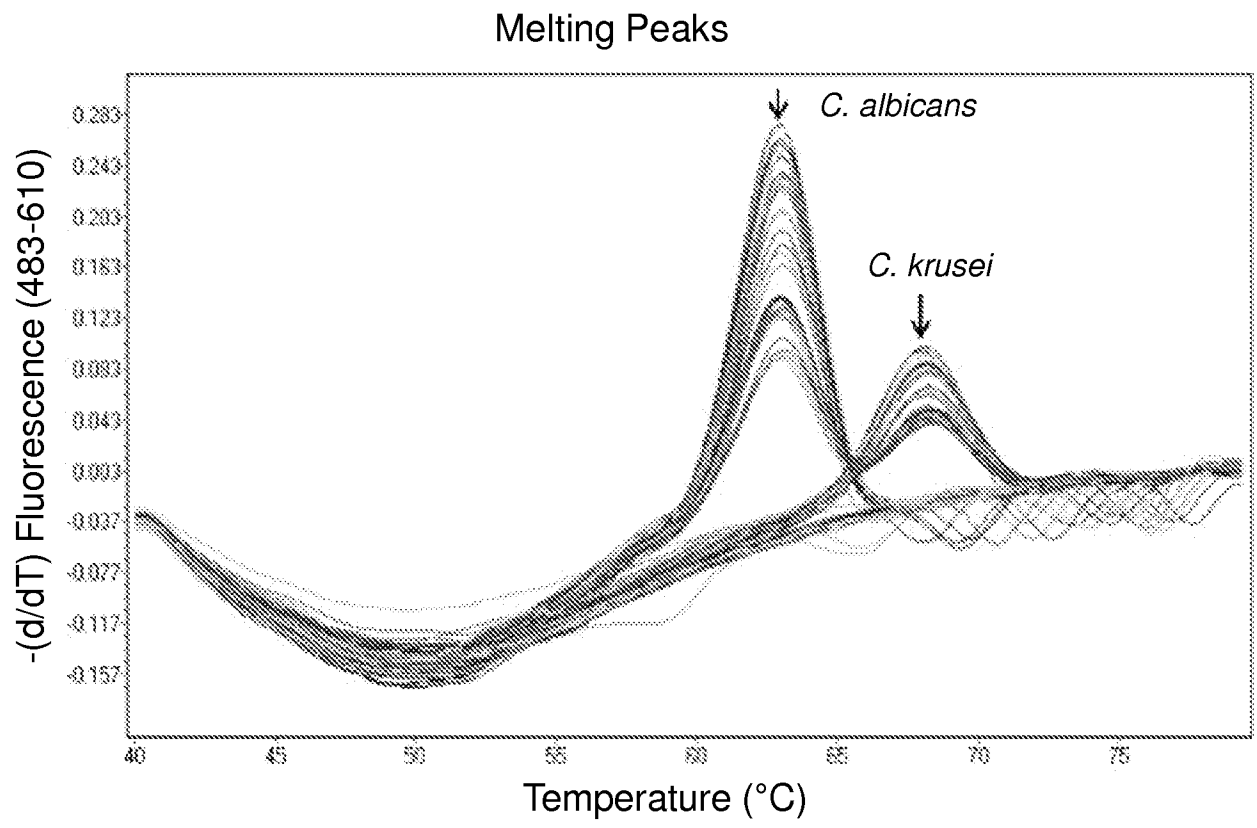


Figure 9



A

Figure 9



B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 13/31774

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - C12Q 1/68 (2013.01) USPC - 435/6.15 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC(8): C12Q 1/68 (2013.01) USPC: 435/6.15 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched USPC: 453/6.11, 6.15; 536/24.32, 24.3 (text search) Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Electronic data bases: Patbase; Google Scholar; GenCore sequence search (NT) Search terms: Candida, Candida albicans, detection, oligonucleotide probe, fluorescence, label, quencher qPCR		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6,242,178 B1 (LOTT et al.) 5 June 2001 (05.06.2001). Especially col 4 ln 25-32, col 6 ln 45-64.	1, 6-10 and 15-18
A	US 2006/0234219 A1 (OHNO et al.) 19 October 2006 (19.10.2006). Especially SEQ ID NO: 18.	1, 6-10 and 15-18
A	US 2010/0129821 A1 (FREDRICKS et al.) 27 May 2010 (27.05.2010). Especially SEQ ID NO: 71.	1, 6-10 and 15-18
<input type="checkbox"/> Further documents are listed in the continuation of Box C.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 16 July 2013 (16.07.2013)		Date of mailing of the international search report 25 JUL 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201		Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 13/31774

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.: 23-48
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1, 6-10, 15-22, drawn to a nucleic acid probe of SEQ ID NO:1 and a method for detecting *Candida* species in a biological sample using said nucleic acid probe. Please note that claims 19-22 will be searched only if additional sequences are elected for search.

Group II: Claims 2, 6-9, 11, 15-22, drawn to a nucleic acid probe of SEQ ID NO:2 and a method for detecting *Candida* species in a biological sample using said nucleic acid probe. Please note that claims 19-22 will be searched only if additional sequences are elected for search.

-----Please see continuation in supplemental box-----

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Claims 1, 6-10, 15-22, limited to SEQ ID NO:1 (i.e. claims 1, 6-10, 15-18)

Remark on Protest

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 13/31774

Continuation of:

Box No. III Observations where unity of invention is lacking

Group III: Claims 3, 6-9, 12, 15-22, drawn to a nucleic acid probe of SEQ ID NO:3 and a method for detecting *Candida* species in a biological sample using said nucleic acid probe. Please note that claims 19-22 will be searched only if additional sequences are elected for search.

Group IV: Claims 4, 6-9, 13, 15-22, drawn to a nucleic acid probe of SEQ ID NO:4 and a method for detecting *Candida* species in a biological sample using said nucleic acid probe. Please note that claims 19-22 will be searched only if additional sequences are elected for search.

Group V: Claims 5, 6-9, 14, 15-22, drawn to a nucleic acid probe of SEQ ID NO: 5 and a method for detecting *Candida* species in a biological sample using said nucleic acid probe. Please note that claims 19-22 will be searched only if additional sequences are elected for search.

The inventions listed as Groups I-V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

A special technical feature of each of the inventions listed as Groups I-V is the specific *Candida* nucleic acid probe recited therein, not required by any other group. Significant structural similarities cannot readily be ascertained among the *Candida* nucleic acid probes.

Common Technical Features

The feature shared by Groups I-V is a nucleic acid probe for detecting *Candida* species in a biological sample, and a method comprising contacting a sample with the nucleic acid probe and detecting hybridization to detect at least one *Candida* species.

However, this shared technical feature does not represent a contribution over prior art, because the shared technical feature is anticipated/made obvious by US 6,242,178 B1 to Lott et al. (hereinafter 'Lott').

Lott discloses a nucleic acid probe for detecting *Candida* species in a biological sample (abstract, col 4, ln 25-32). Lott further discloses a method for detecting at least one *Candida* species in a biological sample (col 6, ln 45-47), said method comprising: a) contacting said sample with at least one nucleic acid probe under conditions which allow the at least one probe to hybridize to a nucleic acid molecule of said *Candida* species (col 6, ln 47-60); and b) detecting hybridization between said at least one probe and said nucleic acid molecule, thereby detecting said at least one *Candida* species (col 6, ln 60-64).

As the common technical feature was known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups. Further, without significant structural similarities, the *Candida* nucleic acid probes do not have a shared technical feature. In the absence of a shared special technical feature, the inventions lack unity with one another.

Therefore, Groups I-V lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 13/31774

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

a. (means)

on paper

in electronic form

b. (time)

in the international application as filed

together with the international application in electronic form

subsequently to this Authority for the purposes of search

2. In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

GenCore ver 6.4.1 SEQ ID NO: 1