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Enhanced performances of the short-PCR coupled lateral flow assay in the detection of *Candida albicans* in clinical blood samples

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Abstract

Background: *Candida albicans* remains the most common fungal pathogen among the species, causing candidemia. Thus, early diagnosis is indispensable in patients with severe underlying infections.

Objectives: To develop a short-polymerase chain reaction (short-PCR) coupled with lateral flow strip (LFS) assay for the detection of *C. albicans* in clinical blood samples.

Methods: A short-PCR-LFS was enhanced to detect clinical isolates and clinical blood samples. The *ITS2* gene of *C. albicans* was amplified using the modified primers-probes to produce highly specific, dual-labeled amplicons. The sensitivity and specificity of the test system were evaluated using *C. albicans*, *Candida* spp. other than *C. albicans* and other microbial DNAs. The test system was validated by 44 clinical isolates and 51 clinical blood samples.

Results: The short-PCR-LFS revealed a high specificity for *C. albicans* with no cross-reactivity and a limit of detection (LOD) of 0.1 ng per 2 mL of blood and 2 CFU/mL using a direct colony as a template. The result was consistent with the validation by short-PCR agarose gel electrophoresis (AGE). The short-PCR-LFS assay showed all positives with all *C. albicans* relevant samples and exhibited negative for other microbial relevance samples.

Conclusions: The entire process of this system provides visual detection results less than 1 h with high sensitivity, high specificity, DNA extraction-free method, and little dependence on instruments. Thus, it can be considered as a promising method for professional use to early detect and identify clinical relevance samples of *C. albicans*.

Key words: Polymerase chain reaction, Lateral flow assay, modified primer-probes, *Candida albicans*, clinical relevance samples

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Introduction

Candida species (*Candida* spp.) are the causative agents of many life-threatening invasive infections. Acute systemic opportunistic bloodstream infections can result in multisystem organ failure. Among pathogenic *Candida* spp. in human, *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* have been reported for most infections. However, *C. albicans* remains the most common fungal pathogen among the species, causing candidemia.[1,2] Approximately 42.1 % of fungal infections and a mortality rate of 40 % have been reported, particularly under hospital condition.[3] Central venous catheters (CVC) and other indwelling medical devices are important risk factors in the development and persistence of candidemia in patients with diabetes, hemodialysis, surgery and systemic medications. The placement of CVCs or indwelling medical devices can considerably increase the risk of candidemia in hospitalized patients.[4,5] The burden of *Candida* spp. in the first positive blood culture of a bloodstream infection (BSI) episode ranges widely among patients from ≤ 1 to 10 CFU/mL. Over half of the initial blood cultures demonstrate ≤ 1 CFU/mL. Notably, subjects with *C. albicans* were more likely to have < 5 CFU/mL.[6]

Intensive candidiasis (IC) infections, *C. albicans* is the most prevalent species in the most regions of the world. Once candidemia have developed, it can spread and cause deep-seated secondary infections. Of the instances recorded in clinical settings, almost three-quarters were in the intensive care unit (ICU). Risk factors affecting mortality rates include older age, severity of the condition, use of immunosuppressive drugs, and transplantation.[7] In an immunocompromised host, IC can overcome the weak host immunity and disseminate in the bloodstream, evolving into invasive systemic candidemia.[8] Moreover, the COVID-19 pandemic increases the number of secondary invasive fungal infection and resulted in excessive ICU admissions.[9] Systemic analysis of global evidence reported that age and survival have an impact on expenditures, older and newborn patients have higher expenses because of higher morbidity.[7] Thus, early diagnosis is indispensable for providing timely therapeutic interventions in patients with severe underlying infections.

Over the past decade, standard culture methods have been used to identify disseminated systemic infections. Although this assay is the "gold standard", it requires up to 5 days for identification. Similarly, the detection of circulating antigens or antibodies is time-consuming and poorly specific or sensitive, thus delaying diagnosis.[10] Hence, this newly developed DNA-based assay will be used to provide a highly specific, sensitive, and accurate diagnosis. Polymerase chain reactions (PCRs) have become a major approach for pathogen detection. In addition to the specific DNA-based assay, several in-house and commercial PCR-based tests allow the detection of *Candida* DNA in different biospecimens. They have been suitable for the diagnosis of invasive candidemia caused by *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, and *C. krusei*.[11,12] Although PCR-based assays are highly specific, they require pre- and post-processing of the samples. These shortcomings limit the procedure. Therefore, lateral flow-integrated PCR assays are widely used for clinical diagnosis.

Candida-specific rRNA genes, the Internal Transcribed Spacer (ITS), *ITS1* or *ITS2* regions, are highly conserved formal barcodes that are frequently used for the genetic identification of *Candida*.[13] In this study, as shown in **Figure 1**, we purpose two optional ways for the detection, the DNA template could be used either being extracted from a blood sample (**Figure 1A**) or directly use a single colony picked from a selective media (**Figure 1B**). In short-PCR amplification, a pair of specific end-labeled primers was designed to provide an end-point readout of the dual-labeled amplicons. The latter was modified with fluorescein isothiocyanate (FITC) and biotin at the two ends to enhance the signals on a test strip. In principle, biotin-labeled reverse primers biotinylate the amplicons, whereas FITC-labeled forward primers dehybridize to coincidentally double-label the amplicons (**Figure 1C**). The red color signals are clearly visible to the naked eye when the probe-labeled amplicons function as bridges bound to the lateral flow strip (LFS) test line (**Figure 1D**).[14] To enhance the accuracy of detection, recent work led to the development of the short-PCR, which uses an existing PCR step to amplify higher specific and selective amplification. In brief, repeating the annealing cycles amplified the higher specific annealing and resulted in higher specific labeled target amplicons. In particular, carryover contamination and false-positive results were reduced, and the laboratory contamination generated by aerosols did not affect the reaction in this system. In this study, we thus aimed to develop a short PCR-LFS assay to enhance the specific detection of *C. albicans* as a promising method for professional use to early detect and identify *C. albicans*, allowing for the initiation of early identification after the onset of candidemia.

Methods

Bacterial strains, Yeast, Candida isolates, and clinical isolates collection

Candida albicans, non-*Candida albicans*, *Cryptococcus neoformans*, *Trichosporon* spp. and other bacteria, including 1 *Escherichia coli*, 1 *Staphylococcus aureus*, 1 *Staphylococcus epidermidis*, 1 *Bacillus cereus*, 1 *Listeria monocytogenes* and 1 *Klebsiella pneumoniae* were collected from the Research Laboratory of the Department of Clinical Microbiology, Enhancement of Safety Practice of Research Laboratory in Thailand (ESPReL), Faculty of Associated Medical Science, Khon Kaen University. The bacterial strains were cultivated on Muller Hinton agar (Himedia, Mumbai, India) and cultured with a blood agar medium (Himedia, Mumbai, India). They were then incubated at 37°C for 24 h. Forty-four clinical isolates (26 *C. albicans* and 18 *Candida* spp. other than *C. albicans* or non-*C. albicans*) were collected from the Clinical Microbiology Unit of Srinagarind Hospital, Khon Kaen University, Thailand during the period of June 2023 to June 2024. *C. albicans* and non-*C. albicans* cultures were maintained on Sabouraud Dextrose Agar (Sigma-Aldrich, U.S.A).

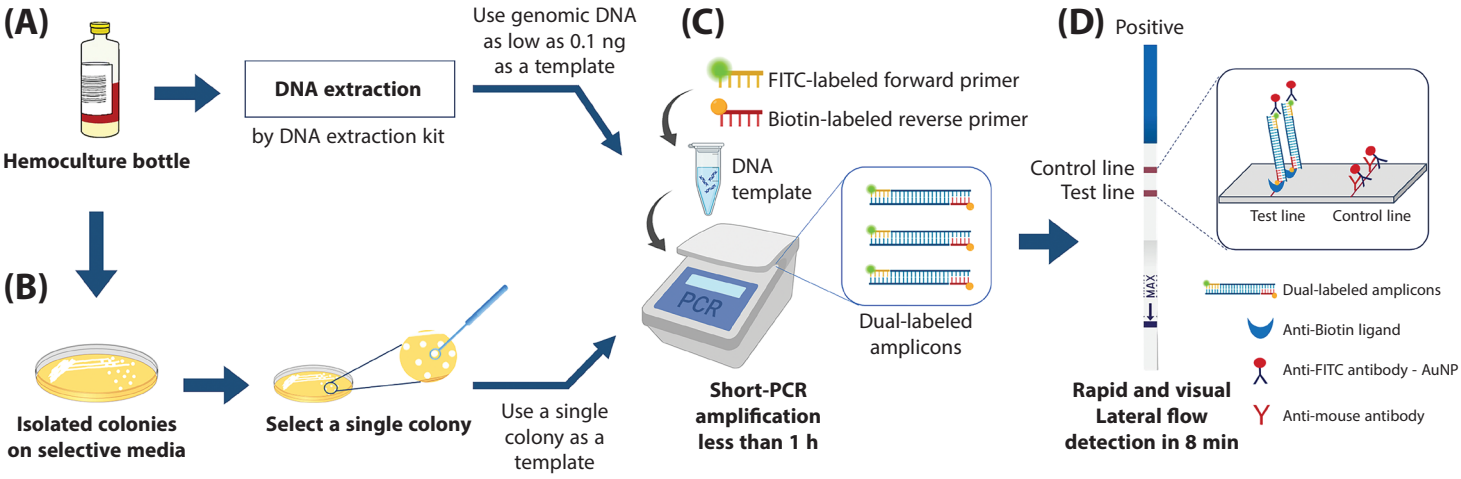

Figure 1. Schematic of the short-PCR-LFS testing workflow for the detection of *C. albicans*. The use of DNA extraction method and using extracted DNA as a template (A). The use of a direct single colony as a DNA template (B). The DNA template was amplified by short-PCR (C). The dual-labeled amplicons were detected by the naked eyes on the test strips (D). Some part of the schematic diagram was created by Biorender (http://www.biorender.com).

Clinical blood samples

Fifty-one leftover hemoculture bottles were collected from patients with candidemia that were obtained from Clinical Microbiology Unit of Srinagarind Hospital, Khon Kaen University, Thailand and Khon Kaen Hospital, Khon Kaen, Thailand during the period of May 2024 to September 2024. All procedures were performed according to the protocol approved by the Khon Kaen University Ethics Committee (project number HE611605). All methods were carried out in accordance with the Declaration of Helsinki and the approved protocol and guidelines. The need for informed consent was waived due to secondary use of leftover samples for research purposes by the Khon Kaen University Ethics Committee for Human Research (KKUEC). The leftover hemoculture bottles were stored at 4°C until they were aliquoted, but not for longer than 7 days. The clinical information about sample collection, ages, gender of patients and the culture laboratory results are listed in **Table 1**. The hospital laboratory employed hemoculture, Gram stain microscopic examination, and a microbial identification system based on MALDI-TOF mass spectrometry to identify the microbial agents.

Table 1. The clinical data of all 51 septicemia patients.

Code	Gender	Age (Y)	Specimens	Sample date	Culture confirmed date	Microbial agents
C1	Female	71	blood	12/5/2024	16/5/2024	*C. glabrata*
C2	Female	39	blood	6/5/2024	7/5/2024	*C. glabrata*
C3	Female	39	blood	7/5/2024	10/5/2024	*C. glabrata*
C4	Male	69	blood	3/5/2024	5/5/2024	*C. albicans*
C5	Male	67	blood	3/5/2024	5/5/2024	*C. albicans*
C6	Male	45	blood	19/5/2024	21/5/2024	*C. albicans*
C7	Male	86	blood	26/5/2024	28/5/2024	*C. albicans*
C8	Male	66	blood	13/5/2024	15/5/2024	*C. albicans*
C9	Female	63	blood	17/5/2024	18/5/2024	*C. tropicalis*
C10	Female	63	blood	17/5/2024	18/5/2024	*C. tropicalis*
C11	Male	44	blood	22/5/2024	24/5/2024	*C. albicans*
C12	Female	63	blood	25/5/2024	27/5/2024	*C. albicans*
C13	Male	100	blood	15/5/2024	17/5/2024	*C. afermentans*
C14	Male	44	blood	22/5/2024	25/5/2024	*C. albicans*
C15	Female	75	blood	23/5/2024	27/5/2024	*C. albicans*
C16	Male	66	blood	13/5/2024	15/5/2024	*C. albicans*

Table 1. (Continued)

Code	Gender	Age (Y)	Specimens	Sample date	Culture confirmed date	Microbial agents
C17	Female	75	blood	27/5/2024	29/5/2024	*C. albicans*
C18	Female	63	blood	17/5/2024	18/5/2024	*C. tropicalis*
C19	Male	44	blood	22/5/2024	25/5/2024	*C. albicans*
C20	Male	44	blood	22/5/2024	25/5/2024	*C. albicans*
C21	Male	52	blood	9/6/2024	9/6/2024	*C. albicans*
C22	Male	63	blood	17/6/2024	19/6/2024	*C. albicans*
C23	Male	72	blood	15/6/2024	15/6/2024	*C. parapsilosis*
C24	Male	62	blood	22/6/2024	24/6/2024	*C. albicans*
C25	Male	42	blood	25/6/2024	25/6/2024	*C. albicans*
C26	Male	75	blood	19/6/2024	19/6/2024	*C. neoformans*
C27	Male	43	blood	1/7/2024	3/7/2024	*C. albicans*
C28	Female	58	blood	13/7/2024	14/7/2024	*C. tropicalis*
C29	Female	58	blood	13/7/2024	14/7/2024	*C. tropicalis*
C30	Female	58	blood	13/7/2024	14/7/2024	*C. tropicalis*
C31	Male	22	blood	15/7/2024	18/7/2024	*C. glabrata*
C32	Male	64	blood	15/7/2024	18/7/2024	*C. albicans*
C33	Male	52	blood	18/7/2024	22/7/2024	*C. glabrata*
C34	Male	32	blood	22/7/2024	24/7/2024	*Candida* spp. other than *C. albicans*
C35	Female	2D	blood	10/8/2024	12/8/2024	*C. albicans*
C36	Male	17	blood	17/8/2024	19/8/2024	*C. albicans*
C37	Female	68	blood	23/8/2024	25/8/2024	*C. orthopsilosis*
C38	Female	83	blood	27/8/2024	29/8/2024	*C. albicans*
C39	Female	0	blood	28/8/2024	29/8/2024	*C. tropicalis*
C40	Male	72	blood	2/9/2024	4/9/2024	*Listeria monocytogenes*
C41	Male	41	blood	8/9/2024	9/9/2024	*C. tropicalis*
C42	Male	55	blood	8/9/2024	9/9/2024	*C. tropicalis*
C43	Male	55	blood	8/9/2024	9/9/2024	*C. tropicalis*
C44	Female	1D	blood	8/9/2024	9/9/2024	*C. tropicalis*
C45	Male	64	blood	9/9/2024	10/9/2024	*C. albicans*
C46	Male	55	blood	3/9/2024	4/9/2024	*C. tropicalis*
C47	Female	82	blood	12/9/2024	13/9/2024	*C. dubliniensis*
C48	Female	3D	blood	13/9/2024	13/9/2024	*C. tropicalis*
C49	Female	1D	blood	10/9/2024	11/9/2024	*C. tropicalis*
C50	Male	55	blood	3/9/2024	4/9/2024	*C. tropicalis*
C51	Male	55	blood	3/9/2024	4/9/2024	*C. tropicalis*

*D: Day

Preparation of Candida and Bacterial DNA

A single colony from the overnight inoculum was suspended in sterile distilled water. For the short-PCR assay, DNA was extracted for primer validation screening, short-PCR cycle optimization, short-PCR-LFS test specificity, and a cross-reaction study. The DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the Quick Start Protocol cat. No. 51304/06. The concentration of extracted DNA was measured at 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, U.S.A). Thereafter, the extracted genomic DNA was stored at -20°C for use as the DNA template in short-PCR amplifications.

Preparation of clinical blood samples for short-PCR-LFS test

To remove the erythrocytes from blood, a 1 volume of patient's blood samples were incubated with 1 volume of lysis solution (1N NaOH, 0.2M citrate and 0.4M *N*-acetylcysteine) on a shaker for 20 min. The lysate was centrifuged at 1,800 × g for 15 min. This removal step was performed twice. The erythrocyte-free pellet was washed in 2 mL of 20 mM Tris-HCl (pH 8.3), centrifuged at 3,000 × g for 10 min and resuspended in 180 µL of Qiagen ATL lysis buffer (QIAGEN, Hilden, Germany) and 20 µL of proteinase K. The DNA extraction was slightly modified from the QIAamp DNA Blood Mini Kit's instruction. The mixture was vortexed and incubated at 65°C for 1 h. The 200 µL of Qiagen buffer AL was added in the mixture and heated at 70°C for another 10 min. The DNA extraction was then applied with QIAamp's protocol. After DNA extraction from blood, the columns were washed twice with 50 mM EDTA before washing with buffer AW. For DNA elution step, the 30 µL of preheated (at 70°C) Qiagen buffer AE was added to a column, incubated at 70°C for 5 min, and centrifuged at 16,000 × g for 1 min. This step was eluted twice and the purified DNA was kept at -20°C until use.[15]

Primers-probes design and Short-Polymerase Chain Reaction (short-PCR) amplification

The primers were manually designed using Primer3Plus and Primer-Blast program to be specific for a conserved region of the *ITS2* gene in accordance with the TwistAmp™ design manual (TwistDX, Cambridge, UK). The ITS2.1 primer was used as a reference primer.[16] The ITS2.2 Forward primer (5′-TTTTATCAACTTGTCACACCAGATTAT-TAC-3′) was modified with oligo synthesis 5′ FITC and the reverse primer (5′-CACGAATATCTGCAATTCATATTACGTATC-3′) with modified oligo 5′ Biotin.

For the *Candida* isolate colony, a starting of 5 CFU/reaction (2×10^2 CFU/mL) of the culture was used as a template. For genomic DNA extracted from blood, samples subjected to short-PCR were used with various concentration. The short-PCR amplification was performed using the PCR machine (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, U.S.A). A 25 µL of PCR reaction mixture contained 12.5 µL of 2X PCRBIO Taq Mix Red reagents (PCR Biosystems, U.K.), 1 µL of 10 µM forward (0.5 µL) mixed with reverse (0.5 µL) primers, 2 µL of DNA template and adjusted to reach a final volume of 25 µL with nuclease-free water containing 5% Dimethyl sulfoxide (DMSO). The optimal amplification condition performed by initial denaturation at 95°C for 2 min followed by 30 cycles of the denaturation at 96°C for 15 sec, annealing at 57°C for 1 min and terminated with final extension at 72°C for 5 min. The PCR amplicons were then separated by 2% agarose gel electrophoresis (Mupid-EXU Gel Electrophoresis System, Tokyo, Japan) loading in a 1X TBE buffer at 100 V for 35 min.

Sensitivity, Detection Limit, and specificity of the short-PCR-LFS assay

For the use of clinical isolates, the *Candida* colony was ten-fold serially diluted from 2×10^2 to 2×10^{-2} CFU/mL. Furthermore, the genomic DNAs extracted from five positive blood samples were pooled and ten-fold diluted from 10 ng, 1 ng, 0.1 ng and 0.01 ng and then were used in short-PCR to determine the detection limits. To evaluate the interspecies specificity, the genomic DNAs from *Cryptococcus neoformans*, *Trichosporon* spp., *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Listeria monocytogenes*, and *Klebsiella pneumoniae* were tested. Non-template control (NTC) was used as a negative control.

Applicability and validation of the short-PCR-LFS system

A 10 µL of PCR amplicons were mixed in a microtube with 60 µL of running buffer (5 mM Phosphate buffer (pH 7.4), 1 % Triton X, 0.1 % Bovine Serum Albumin (BSA), and 5 % v/v DMSO). The mixture was dropped onto the sample pad of a lateral flow strip (KBiosciences, Thailand). A positive result was defined when the test and control lines were visually detected or as negative when only a colored band on the control line was visible. After 8 min of incubation, the test line intensity was measured and imaged by a rapid lateral flow strip reader (RapidScan ST5-D, Pacific Image Electrodes, Taiwan). An affordable personal smartphone (Vivo Y50, Vivo, Guangdong, China) was optional utilized to capture the strip. The main camera with auto-focus mode and natural daylight or bright white light were recommended to improve color accuracy. However, to minimize variations in the light background intensity that could impact the resolution of the images, the smartphone used throughout the experiment must be the same. The sensitivity (TP/(TP + FN) × 100), specificity (TN/(TN + FP) × 100), positive predictive value (PPV), negative predictive value (NPV) and 95% confidence interval (CI) were calculated using the free software, MedCalc Software Ltd. Diagnostic test evaluation calculator. https://www.medcalc.org/calc/diagnostic_test.php (Version 23.1.3; accessed January 15, 2025). TP, FP, FN and TN are true positive, false positive, false negative and true negative, respectively.

Results

Specificity and sensitivity of short-PCR and short-PCR-LFS system

To evaluate the specificity of the short-PCR-LFS assay for *C. albicans* detection, the extracted DNA from *C. albicans* and other pathogens were performed. The short-PCR amplicons at 30 short-PCR cycles were amplified and detected as a clear target band in both short-PCR (**Figure 2A**) and short-PCR-LFS assays (**Figure 2B**). No cross-reactions with other pathogens were founded, indicating the specificity of the short-PCR-LFS assay.

The sensitivity of *C. albicans* detection in clinical blood samples was evaluated. The limit of detection (LOD) of both short-PCR-AGE (**Figure 3A**) and short-PCR-LFS (**Figure 3B**) was 0.1 ng per 2 mL of clinical human blood. For quantitative analysis, calibration curves of the test line intensities with different concentrations of *C. albicans* DNA were generated. The calibration curves were fitted linearly with a correlation coefficient (R^2) of 0.9254 as shown in **Figure 3C**. The results supported good linearity between the test-line intensity and *C. albicans* DNA concentration at y = 1561.9x – 970.57. Furthermore, a single colony of *C. albicans* with no prior DNA extraction was also determined. The LOD of both short-PCR and short-PCR-LFS was 2 CFU/mL and fitted linearly with a correlation coefficient (R^2) of 0.983.

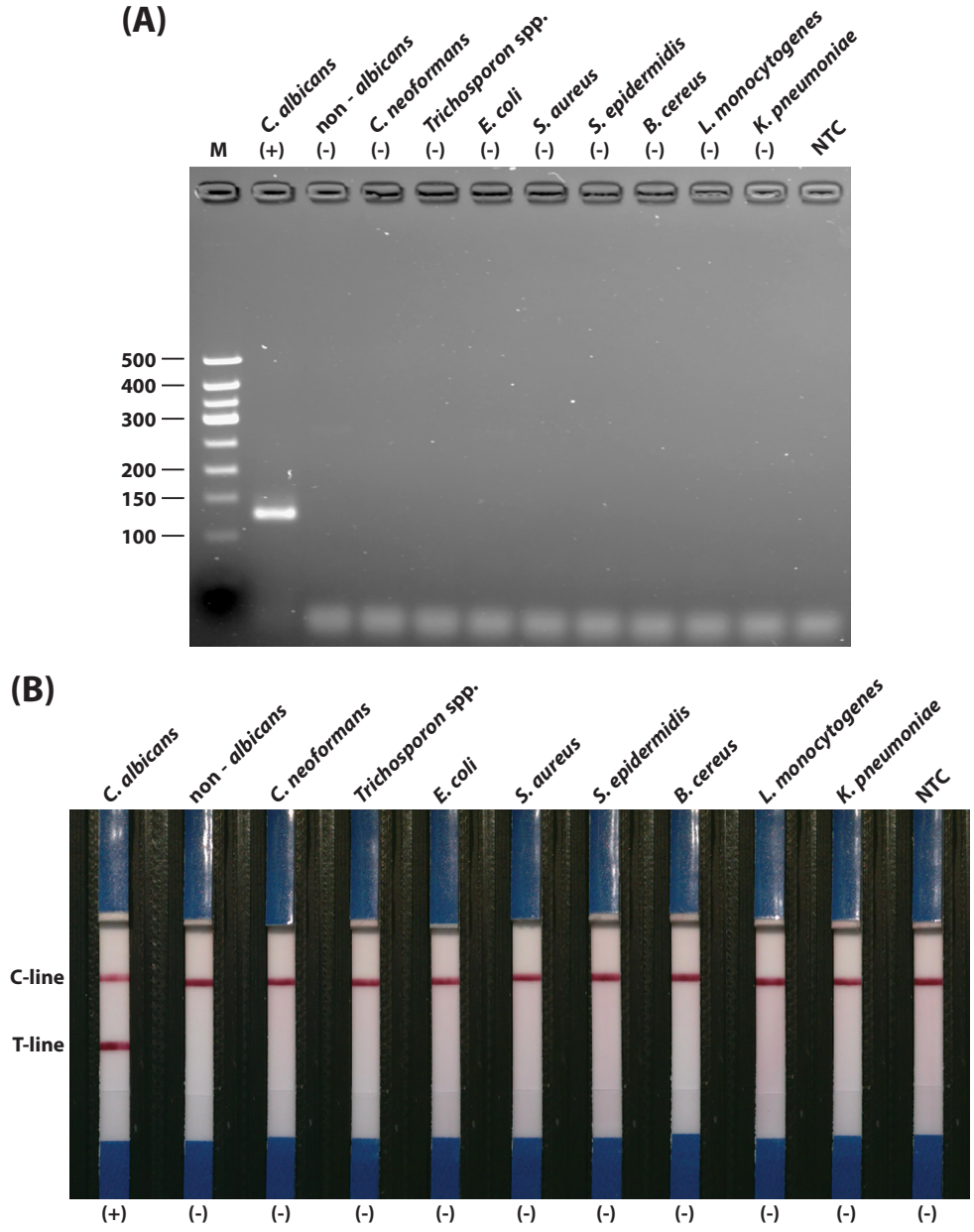

Figure 2. Specificity of the short-PCR-LFS assay and cross-reaction study. Short-PCR-AGE assay (A). Short-PCR-LFS test (B). Non-*C. albicans* represents *Candida* spp. other than *C. albicans*. NTC was non-template negative control (nuclease-free water).

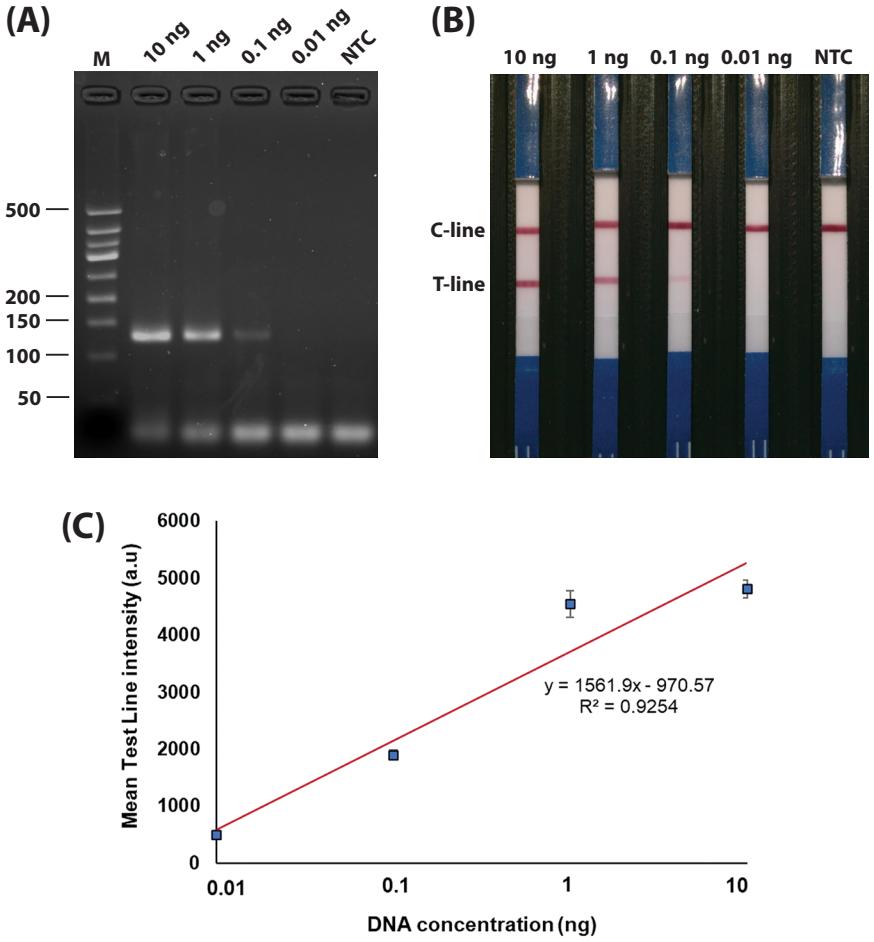

Figure 3. Sensitivity determination and detection limit of the short-PCR-LFS assay performed with clinical blood samples. Short-PCR-AGE (A). Short-PCR-LFS (B). Calibration curve of the test-line intensity of the LFS with a rapid lateral flow strip reader (RapidScan ST5-D) (C). Test-line color intensities were collected from at least five replicates. NTC was non-template negative control (nuclease-free water).

Evaluation of the short-PCR-LFS system for the detection of C. albicans from clinical isolates

To confirm the practical applicability of this short-PCR-LFS system, forty-four clinical isolates (26 *C. albicans* and 18 *Candida* spp. other than *C. albicans*) were subjected to the short-PCR-LFS and conventional AGE assays. Twenty-six clinical isolates of *C. albicans* (positive, n = 26) showed a detection rate of 100 % (26/26) as shown in **Figure 4A and 4B**. The use of a direct colony as the DNA template, in an amount corresponding to the detection limit, was intended for use as a visual yardstick (red line), which is a comparative measure to indicate which signal could be positive. If the positive control is intended to be used to confirm the proper operation of the LFS, more DNA templates could be applied to produce a more intense signal.[17] Test-line quantification confirmed that sample no. CA1 to CA26 were positive for *C. albicans* amount load not less than the LOD. All the tested isolates showed positive signals with various intensities above that of the positive control (PC-LOD) (**Figure 4C**). Eighteen samples (non-CA1 to non-CA18) that were *Candida* spp. other than *C. albicans* (negative; n = 18) showed very weak signals below the PC-LOD (**Figure 4F**). The short-PCR-LFS had the same accuracy as the conventional approach, according to all detection results, which were consistent with those of the AGE assay (**Figure 4D and 4E**).

Detection of C. albicans in clinical blood samples using short-PCR-LFS system

The fifty-one patient's blood samples were used to detect by both assays. For short-PCR-AGE, all 22 cases were positive for *C. albicans* and 28 cases for *Candida* spp. other than albicans (non-albicans) were negative (**Figure 5A**). For short-PCR-LFS, all 23 cases were positive for *C. albicans*, while 28 cases of non-albicans exhibited negative results (**Figure 5B**). All tested *C. albicans* cases showed positive signals with various intensities above that of the positive control (PC-LOD), while *Candida* spp. other than *C. albicans* showed very weak signals below the PC-LOD (**Figure 5C**). MedCalc Software calculation of all 51 clinical blood cases testing showed 95.65% sensitivity (95%CI, 78.05 to 99.89) and 100% PPV (95%CI, 84.56 to 100) for the detection of *C. albicans*. Non-*C. albicans* cases were observed as negative results, with 100% specificity (95%CI, 87.66 to 100) and 96.55% NPV (95%CI, 80.46 to 99.48).

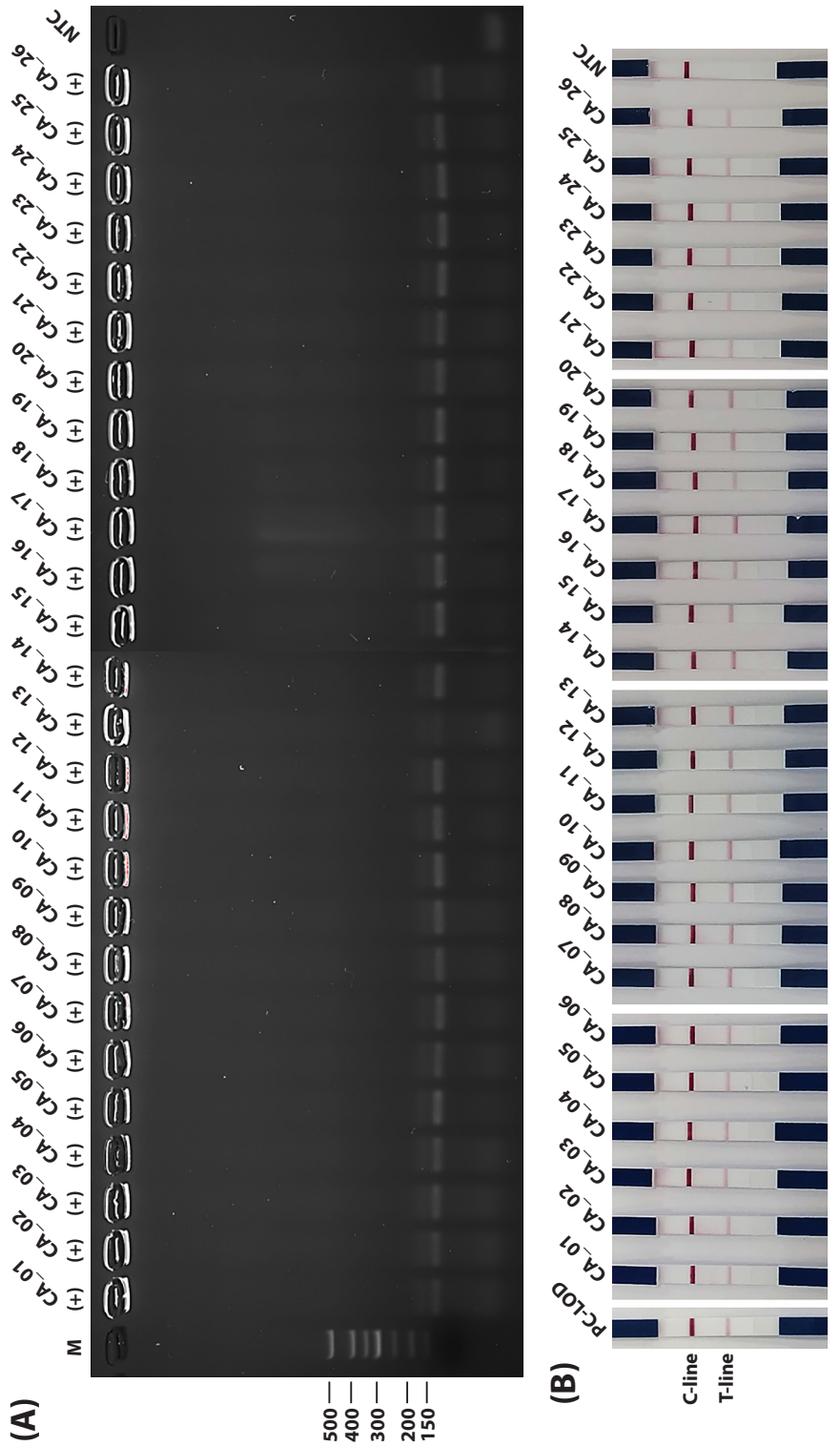

Figure 4. Validation of the LFS specificity and detection of *C. albicans* from 44 clinical isolates. Short-PCR products from a direct colony of *C. albicans* clinical isolates: tested on the AGE (A). on the LFS (B). Test-line intensities (a.u.) (C). Non-*C. albicans*: tested on the AGE (D). on the LFS (E). Test-line intensities (a.u.) (F). NTC indicates the non-template negative control (nuclease-free water). PC-LOD indicates a positive control at the limit of detection (LOD) value (2 CFU/mL). The red line (yardstick) represents the quantity of DNA template that corresponds to the LFS's detection limit, which produced a positive signal.

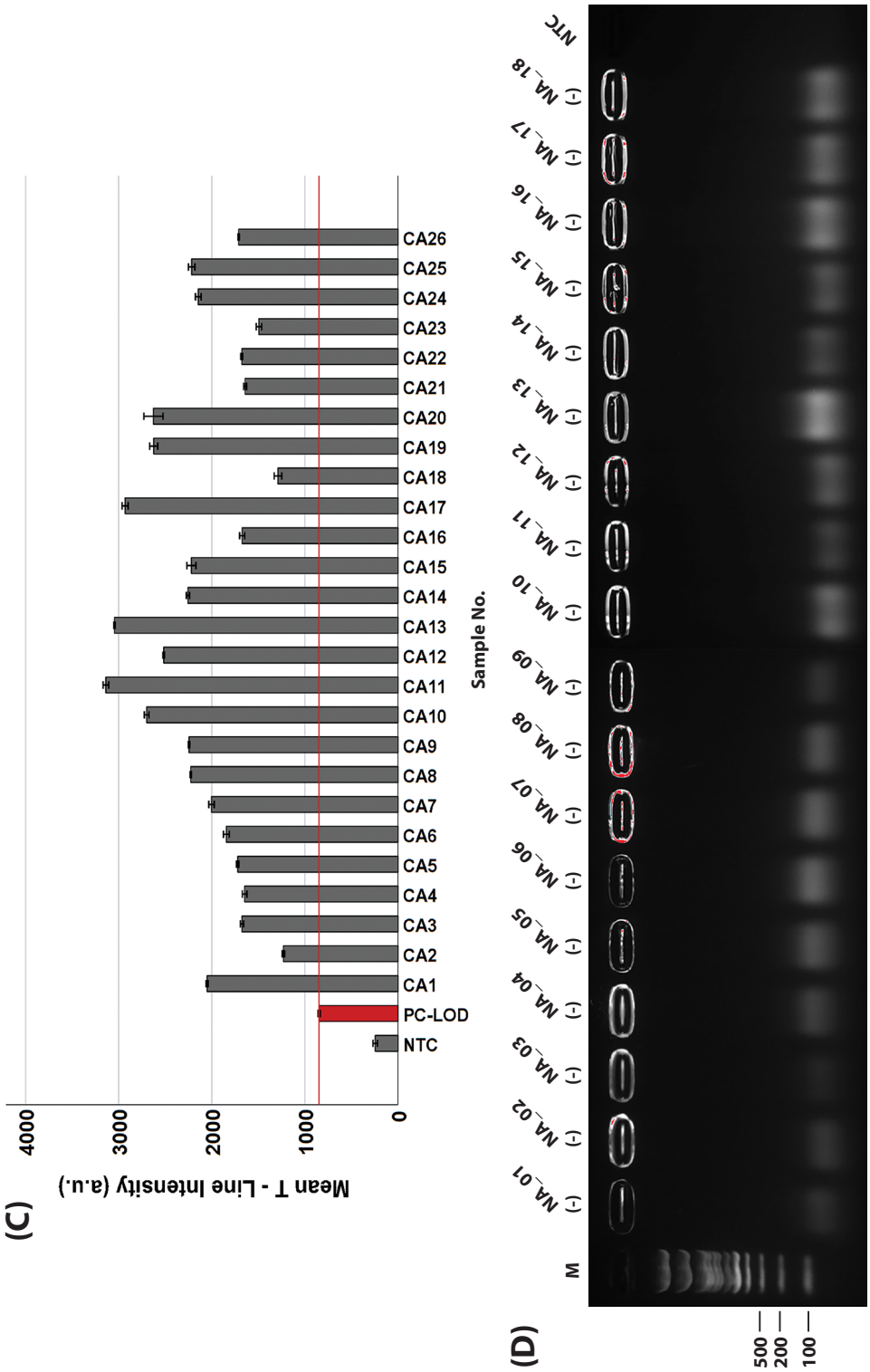

Figure 4. (Continued)

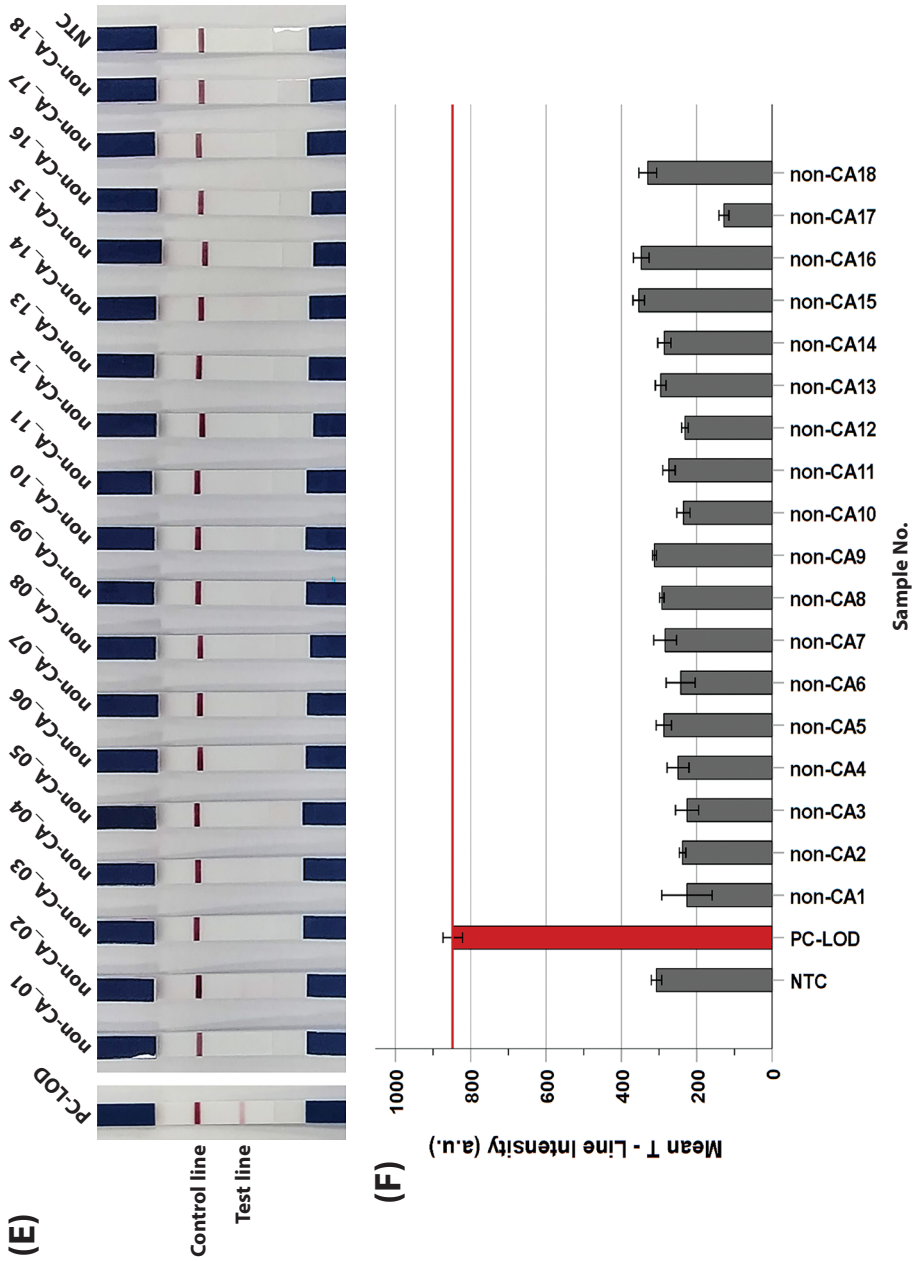

Figure 4. (Continued)

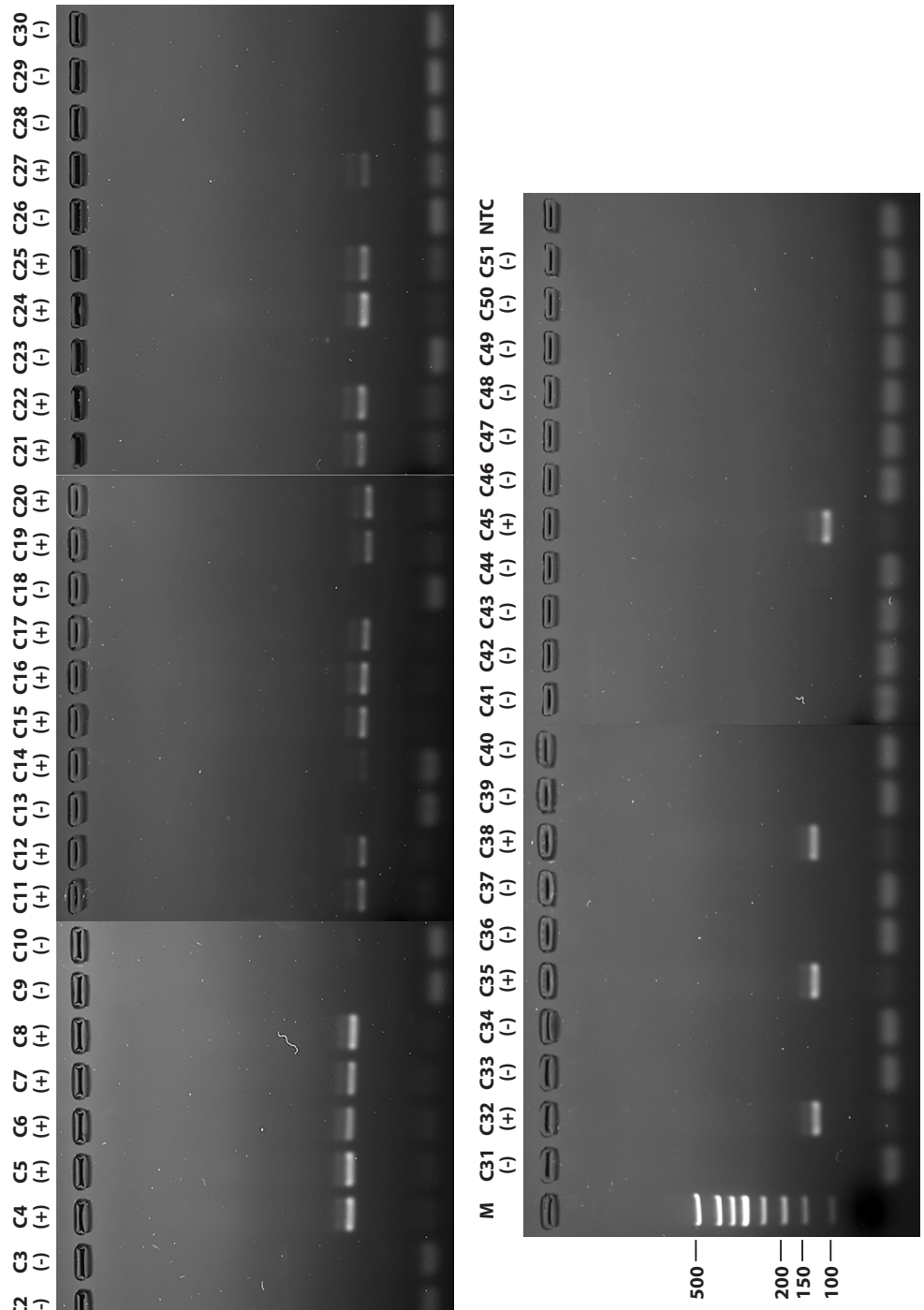

Figure 5. Validation of the short-PCR-LFS specificity and detection of *C. albicans* from all 51 clinical blood samples. Tested on the AGE (A). on the LFS (B). Test-line intensities (a.u.) (C). NTC and P indicates the non-template negative control (nuclease-free water) and positive control, respectively. PC-LOD indicates a positive control at the limit of detection (LOD) value (0.1 ng per 2 mL of clinical blood sample). The red line (yardstick) represents the quantity of DNA template that corresponds to the LFS's detection limit, which produced a positive signal.

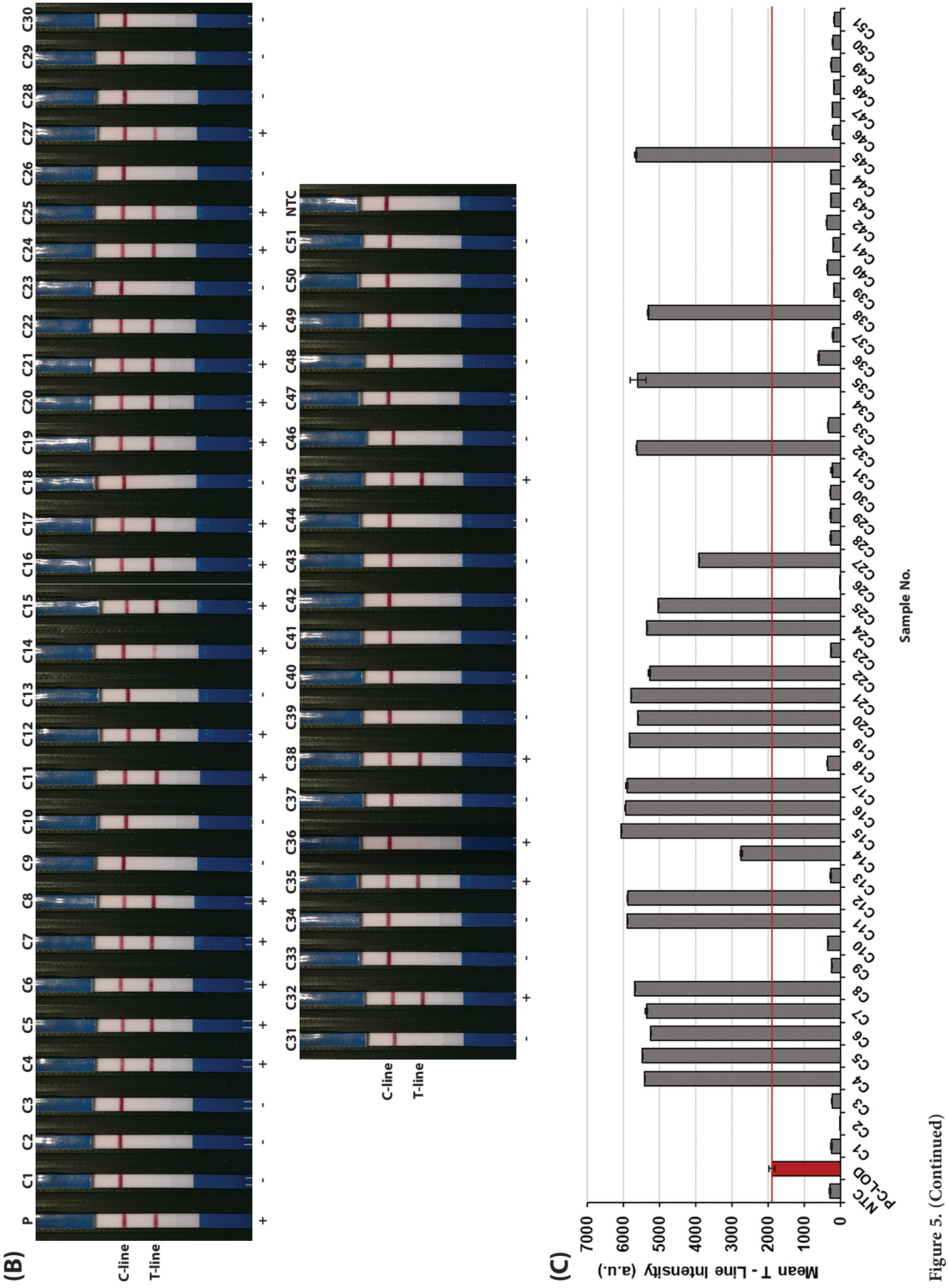

Figure 5. (Continued)

Discussion

Isolation of *C. albicans* is a laborious fungal culture method used in conventional diagnostic procedures. In addition, physicochemical and morphological identifications consume a large number of chemical reagents, require cumbersome experimental steps, and increase exposure to toxic reagents.[18] DNA-based approaches have been used instead of conventional assays to differentiate the species among *Candida*.[19] Molecular diagnostics, culture-independent identification, can be applied to shorten time to result. Since, pathogen levels in blood of BSI patients can be as low as 1-10 CFU/mL.[20] Thus, the greater sensitivity of PCR-based test should be associated with an *in vitro* detection limit of ≤ 10 CFU/mL.[6] This study successfully developed a rapid short-PCR-LFS assay with high sensitivity and specificity compared to the gold standard culture method for *C. albicans* detection. A single direct colony, DNA extraction-free method, was optional way to use as a DNA template in our short-PCR-LFS assay to obtain the test results with an LOD of 2 CFU/mL. On the other hand, the second way for detecting and identifying *C. albicans* could be the use of an extracted DNA from the leftover clinical blood samples as a template as low as 0.1 ng/2 mL in our system.

The length of the amplicon containing the *ITS2* region can serve as a reference for fungal identification in clinical specimens.[21] Thus, a specific target in the length of the *ITS2* region was identified in this study to make a specific diagnosis of pathogenic *C. albicans* isolated from blood in leftover hemoculture bottle. Compared to short-PCR-AGE, the developed short-PCR-LFS was less dependent on equipment and more rapid. The designed *ITS2* primers-probes allowed the differentiation of *C. albicans* from the other pathogens without cross-reactivity, which is consistent with numerous previous studies.[21–23]

The TwistAmp® kit has been developed to provide users with a more flexible set of reagents for isothermal amplification, known as the recombinase polymerase amplification (RPA) assay. RPA is a recently developed thermostatic amplification method that uses recombinase to open double-stranded DNA and facilitate the binding of primers to the DNA target.[24] Similar to PCRs, RPA amplified the target DNA using two primer pairs. The design of the RPA primer pairs is similar to that of PCR primers, and primers developed for PCR often work well in RPA reactions. In this study, the target gene was effectively amplified by short-PCR and RPA (data not shown) assays using the same specific *ITS2* primers-probes in line with the TwistAmp manuals. Previous studies have reported that using the same primers for PCR and RPA assays resulted in high specificity and sensitivity,[25,26] similar to our work. However, few commercial RPA kits are conducive to large-scale detection and are prone to nonspecific amplification. Thus, we suggest using the short-PCR-LFS assay for the detection of *C. albicans* because of the availability of commercial kits, affordability of PCR instruments and consumables, and wide range of applications. Moreover, the PCR reagents are relatively inexpensive and ideal for quantitative purposes.[27]

When the short-PCR products were identified with the LFS, the short-PCR-LFS assay was shown to be more rapid, precise, and efficient. Our accelerated method could detect *C. albicans* 7 times faster than traditional PCR-AGE method in a processing time less than 1 h, as compared to the latter. Furthermore, we used this assay with *Candida* spp. other than *C. albicans* (non-*C. albicans*) and it did not result in false positives. Increased specific annealing, and hence increased specific amplification, are the benefits of this short-PCR over traditional PCR-AGE. Additionally, by regulating the amplification time, carryover contamination and false-positive results may be decreased.[28] Similar to a previous study, the amplification time was limited to 40 min to reduce false-positive results.[23] Furthermore, aerosol-generated laboratory contamination did not interfere with the process in this system.

The results of LFS detection of double-labeled amplicons in this study were in agreement with previous research that revealed the high specificity of the PCR-Lateral Flow Immunoassay (PCR-LFIA).[29,30] The biotin-labeled reverse primers biotinylated the amplicons, whereas the FITC-labeled forward primers dehybridized to coincidental double-label amplicons. Thereafter, for detection with the LFS, FITC- and biotin-labeled amplicons were moved along the strip and bound to the AuNP-labeled anti-FITC antibody in the presence of running buffer containing 5% DMSO. DMSO is an additive which helps reduce non-specific background signals and improve clarity of the test lines. The complexes were immobilized with streptavidin via biotin complexation in the test-line zone. This binding produces a visible red signal which promotes the rapid visualization on the LFS[31,32] (**Figure 1**). To make the reading of the LFS more precise, the results of color quantification read by a rapid strip reader were further enhanced and recommended for subsequent applications.

For the clinical applicability of the short-PCR-LFS test, all 44 clinical isolates and 51 clinical blood samples were analyzed using both short-PCR-LFS and conventional assay. Considerably advantages of this developed assay, PCR provides high analytical sensitivity, while LFA provides rapid diagnostic speed, simple and economic procedures.[33] So far, in agreement with previous studies, the PCR-based LFA tests have been utilized for the detection and identification in various living species due to the characteristics of high sensitivity and strong specificity, including species-specific PCR, DNA hybridization and sequencing.[29,34,35] One more consideration that needs to be addressed is a specificity of the designed primers. The assay might become less specific if nonspecific sample preamplification takes place. To minimize nonspecific sample preamplification, optimizing the primer design and assay conditions are crucial, which is a promising way to maintain specificity of the assay.[33] As a result, sequence-specific PCR primers were employed at a highly specific position of the *ITS2* gene, which amplified only the targeted gene of *C. albicans* that had this position, while the primer failed to amplify in those of non-*C. albicans*.

This further confirms the specificity of this signature designed primers for *C. albicans*. This assay was much faster than the labor-intensive gold standard assay. It is more convenient to identify positive or negative results using the red-line signal on a test strip. In particular, a linear relationship was observed between color intensity and *C. albicans* concentration displaying a good linearity. This indicated that the detection sensitivity of this LFS system had no adverse effect by the presence of unpurified DNA or other cellular components when using a direct colony of *C. albicans* as a template for the reaction.

Conclusion

The short PCR-LFS assay developed in this study provides rapid visualization for *C. albicans* molecular diagnostic detection. This system yields visual detection results less than 1 h with high sensitivity, specificity, and accuracy, and little dependence on instruments. This method can be applied for professional use to early detect and identify clinically relevant *C. albicans*.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author Contributions

- P.T. (Patcharaporn Tippayawat), O.S., B.S., and S.N. conceptualized and designed the experiments.
- O.S. and B.S. wrote the manuscript.
- L.W., C.S. and R.P.K. collected and provided the clinical samples.
- O.S., B.S., C.P., N.S., P.T. (Patsara Thongmee) performed the experiments.
- P.T. (Patcharaporn Tippayawat), O.S., B.S., and C.P. analyzed the data.
- P.T. (Patcharaporn Tippayawat) and J.D. supervised and provided funding.
- P.T. (Patcharaporn Tippayawat), M.W., A.L., R.T., A.C., J.D., and A.S. supervised, reviewed, edited, and proofread the manuscript.
- All the authors reviewed and approved the final version of the manuscript.
- All the authors have read and agreed to the submitted version of the manuscript.

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