

## Development of a Nested Qualitative Real-Time PCR Assay To Detect *Aspergillus* Species DNA in Clinical Specimens

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**We adapted a nested *Aspergillus* PCR to a nested qualitative real-time format on a LightCycler system. An evaluation using 134 clinical specimens showed that the real-time PCR assay significantly reduced the time for results to be made available without compromising sensitivity and was less labor-intensive. Its use will aid in the rapid diagnosis of invasive aspergillosis.**

In recent years, real-time PCR assays have been developed for the rapid diagnosis of invasive aspergillosis (IA) (1–9, 11). Compared with conventional gel-based PCR methods, real-time methods allow rapid DNA amplification and product detection and quantification of fungal load. The potential for contamination with previously amplified products is reduced, since the PCR tubes do not have to be reopened for post-PCR analysis (3, 6). However, real-time PCR assays are less sensitive than nested PCR assays (1, 11), and the majority detect only the most common pathogenic species, *Aspergillus fumigatus*.

In this study, we developed a nested *Aspergillus*-specific PCR assay into a nested qualitative real-time PCR assay for use with a LightCycler (LC) system (Roche Diagnostics, Australia). The first-round PCR is performed on a block-based PCR machine, while the second-round amplification and the product detection are carried out simultaneously using an *Aspergillus* genus-specific TaqMan probe and an LC instrument. We compared the performance of the assay with that of our routine nested PCR assay by using the same clinical specimens from a random population of immunosuppressed hematology patients at high risk for IA.

**Probe design.** The *Aspergillus*-specific probe was designed using Primer Express software, version 1.0 (Applied Biosystems, Foster City, CA), and Oligo Explorer software, version 1.1.0 (T. Kuulasma, University of Kuopio, Kuopio, Finland [http://www.uku.fi/~kuulasma/OligoSoftware]). The probe was designed within a conserved region of the 18S rRNA genes (GenBank database) of *A. fumigatus* (AB008401), *A. flavus* (AF18SRR), *A. nidulans* (AB008403), *A. niger* (AN18SRR), *A. ochraceus* (AB008405), *A. terreus* (AB008409), *A. ustus* (AB002072), and *A. versicolor* (AB008411). This region (5'-TTG TTA AAC CCT GTC GTG CTG GGG ATA GAG-3') was not homologous to the 18S rRNA genes of other sequenced pathogenic fungi or mammalian DNA. The fluorogenic probe was labeled at the 5' end with FAM (6-carboxy-fluorescein) and at the 3' end with TAMRA (6-carboxy-tetra-

methyl-rhodamine) and synthesized by Applied Biosystems, Foster City, CA.

**DNA extraction.** A GenElute Mammalian DNA kit (Sigma-Aldrich Co., St. Louis, MO) was used for all DNA extractions. Tissue specimens were extracted according to the manufacturer's instructions. Blood samples (500  $\mu$ l) were lysed with 3 volumes of erythrocyte lysis buffer (0.155 M NH<sub>4</sub>Cl, 0.01 M NH<sub>4</sub>HCO<sub>3</sub>, and 0.1 mM EDTA [pH 7.4]) (10) for 10 min at –20°C and centrifuged at 6,600 rpm for 10 min. Cerebrospinal fluid, ascitic fluid, and bronchoalveolar lavage (BAL) specimens were centrifuged at 13,000 rpm for 10 min. Supernatants were discarded and the pellets resuspended in 200  $\mu$ l of sorbitol buffer (1 M sorbitol, 100 mM EDTA, and 0.1% 2-mercaptoethanol) (13) and 200 U of lyticase (Sigma-Aldrich Co.). After incubation at 37°C for 60 min, spheroplasts were precipitated by centrifugation at 7,600 rpm for 5 min, resuspended in 180  $\mu$ l of lysis solution T (GenElute Mammalian DNA kit) and 20  $\mu$ l of proteinase K (Sigma-Aldrich Co.), and incubated at 55°C for 60 min. DNA was extracted according to the manufacturer's instructions with a final elution volume of 120  $\mu$ l.

**First-round PCR.** Oligonucleotide primer pairs (AFU 7S, AFU 7AS; AFU 5S, AFU 5AS) (10) were used in the first- and second-round PCR amplification reactions, respectively. Reactions were performed in a final volume of 25  $\mu$ l. The first-round PCR mix contained 1 $\times$  PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% gelatin) (Applied Biosystems), 1 mM MgAc, 200  $\mu$ M of the deoxynucleoside triphosphates (dNTPs) (Roche Diagnostics), 1.2  $\mu$ M concentrations of primers AFU 7S (5'-CGGCCCTTAAATAGC CCG) and AFU 7AS (5'-GACCGGGTTTGACCAACTTT), 1.25 U Taq DNA polymerase (Applied Biosystems), and 10  $\mu$ l of DNA. DNA amplification was performed on an Eppendorf Mastercycler gradient thermocycler (Eppendorf AG, Germany) by incubation at 94°C for 2 min, followed by 30 amplification cycles of 94°C for 20 s, 65°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min.

**Second-round PCRs. Conventional assay.** The reaction mix contained 1 $\times$  TTH buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.01% Tween 20, 0.01% gelatin, 0.01% NP-40), 3 mM MgAc, 200  $\mu$ M of dNTPs, 0.4  $\mu$ M of primers AFU 5S (5'-AGGGCCAGCGGTACATCACCTTG) and AFU

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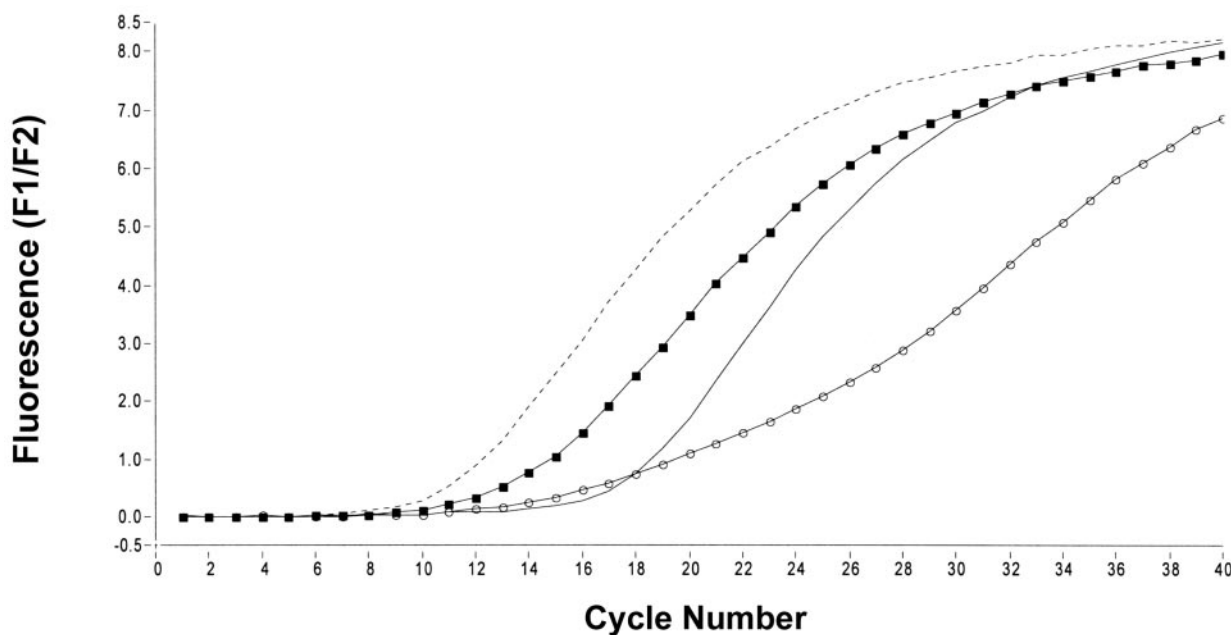


FIG. 1. Amplification profiles of DNA extracted from *A. flavus* (■), *A. fumigatus* (—), *A. nidulans* (---), and *A. terreus* (○). F1, LC channel 1 (530 nm); F2, LC channel 2 (640 nm).

5AS (5'-GGGRGTCGTTGCCAACCYCYCCTGA), 0.5 U DYNzyme DNA polymerase (Finnzymes, Finland), and 2  $\mu$ l of the first-round PCR product. The amplification conditions were 96°C for 2 min, followed by 30 cycles of 96°C for 20 s, 65°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination. A negative control of molecular biology-grade water and a control to exclude the presence of inhibitory substances were included for each specimen. The PCR assay and/or DNA extraction was repeated if any controls failed. To minimize contamination, extractions and first- and second-round PCRs were performed in three separate rooms. Gels were run in a fourth room, with a unidirectional work flow between all rooms.

**Real-time assay.** The second-round real-time PCRs were performed in glass capillaries (Roche Diagnostics) in a 20- $\mu$ l final volume and contained 1 $\times$  LC FastStart reaction mix (containing FastStart *Taq* DNA polymerase, reaction buffer, dNTPs, and 1 mM MgCl<sub>2</sub>) (Roche Diagnostics), 3.5 mM MgCl<sub>2</sub>, 0.5  $\mu$ M of each primer, 0.1  $\mu$ M *Aspergillus*-specific probe, and 2  $\mu$ l of the first-round PCR product. Cycling parameters on the LC machine were 95°C for 10 min, followed by 35 cycles of 95°C for 0 s, 65°C for 5 s, and 72°C for 15 s, and a final cooling at 40°C for 30 s. Fluorescence data calculated by Light Cycler were collected at the end of each 65°C annealing step with a single fluorescence acquisition for each capillary. A sample was designated positive for *Aspergillus* species DNA when there was an exponential increase in fluorescence during the first 30 cycles of PCR amplification (crossing point,  $\leq 30$ ). Samples with crossing points of  $>30$  cycles were repeated to verify the results. The sensitivity of the nested real-time assay was determined by extracting DNA from blood seeded with

serial dilutions of either *A. fumigatus*, *A. flavus*, *A. nidulans*, or *A. terreus* conidia ( $10^4$  to  $10^0$  CFU/ml).

The *Aspergillus*-specific probe hybridized to DNA extracted from all four *Aspergillus* spp. (Fig. 1). The sensitivities of both the real-time and conventional PCR assays were equivalent to 10 CFU of conidia/ml of blood for all *Aspergillus* spp. (shown for the nested real-time assay in Fig. 2).

In total, 134 specimens from 39 patients, including whole-blood ( $n = 124$ ), cerebrospinal fluid ( $n = 2$ ), BAL ( $n = 2$ ), ascitic fluid ( $n = 1$ ), and fresh tissue ( $n = 5$ ) specimens, were tested. Nine (6.7%) specimens (8 blood and 1 BAL) were positive by both the conventional and real-time nested PCR assays, 125 (93.3%) were negative in both, and no results were discordant. The real-time PCR protocol alone was applied to six of the nine positive specimens. Only two of the six were positive under this protocol, indicating that the single step real-time PCR assay was less sensitive than the nested method.

The conventional nested PCR assay required up to 5 h 30 min for the first- and second-round PCR amplifications, agarose gel electrophoresis, ethidium bromide staining, and result analysis. The nested real-time PCR assay was less labor-intensive. Furthermore, the first- and second-round PCR amplifications were completed and results were available in less than 3 h.

The aim of this study was to transfer a nested *Aspergillus*-specific PCR assay onto the LC platform to realize the potential benefits of a real-time PCR platform while retaining the sensitivity of the nested method. To date, the majority of published real-time PCR assays are specific for *A. fumigatus*, yet in Australia, 10 to 15% of IA cases are due to other *Aspergillus* species (Australian Mycoses Interest Group, unpublished data). These species will not be detected by *A. fumigatus*-specific assays, as was demonstrated recently in a case of IA due to *A. terreus* (8). Real-time assays are less sensitive than

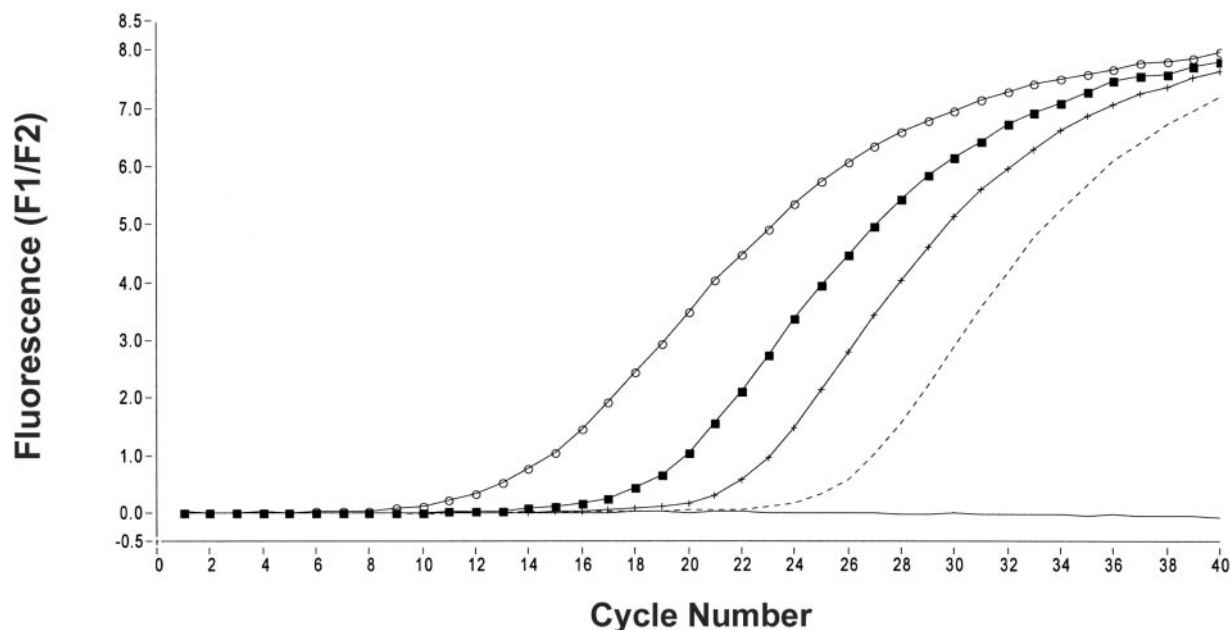


FIG. 2. Amplification profiles of DNA extracted from blood samples spiked with serial dilutions of *Aspergillus* conidia at concentrations of  $10^4$  (○),  $10^3$  (■),  $10^2$  (+),  $10^1$  (---), and  $10^0$  (—) CFU per ml. F1, LC channel 1 (530 nm); F2, LC channel 2 (640 nm).

nested PCR assays, including in our hands (see above and references 1, 4, 11, and 12). However, their rapidity and reduced potential for contamination make them an attractive option for a diagnostic laboratory.

To transfer the conventional nested PCR assay across to the LC platform, the second-round PCR conditions were modified, and a fluorescent-labeled *Aspergillus*-specific probe was incorporated into the amplification mix. This significantly reduced labor costs and the duration of the assay without compromising sensitivity or specificity. The use of sealed capillary tubes for the second-round reaction and the absence of post-PCR product manipulation also reduced the risk of contamination due to amplicon carryover.

We conclude that the adaptation of a nested PCR assay developed for the rapid diagnosis of IA from blood, BAL, and tissue specimens to a real-time PCR platform is a significant advance. This platform should be applicable to the diagnosis of other invasive infections.

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