

Development and Validation of a Microsphere-Based Luminex Assay for Rapid Identification of Clinically Relevant *Aspergilli*[∇]

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A Luminex-based assay for the rapid identification of *Aspergillus* species was designed, optimized, and validated with 131 clinical isolates of *Aspergillus fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, *A. ustus*, and *A. versicolor*. The six species-specific probes were directed toward the internal transcribed spacer 1 (ITS-1) region and tested in a multiplex format with results generated within 6 h. Species identifications generated by the *Aspergillus* Luminex assay were 100% concordant with results from comparative sequence analyses of the ITS-1 region and showed excellent specificity. The *Aspergillus* Luminex assay is a rapid, relatively simple method that may prove to be a useful diagnostic tool for rapid *Aspergillus* identification in clinical laboratory settings.

Correct *Aspergillus* species identification may impact therapeutic decision making since previous studies have clearly demonstrated species-specific differences in antifungal susceptibilities (1, 8, 15, 17, 18, 21, 24). Such identification strategies, if available in a multiplexed format, can reduce time and labor in clinical microbiology laboratories. Luminex xMAP (Luminex Corp., Austin, TX) is a microsphere-based multiplexing system where microspheres are internally dyed with various proportions of red and infrared fluorescent dyes, producing different spectral addresses detected by two lasers. User-designed species-specific probes can then be bound to these microspheres and tested in a 96-well format using the biotinylated PCR amplicons with hybridization reactions quantified by the fluorescence of the reporter molecule streptavidin-R-phycoerythrin (SAPE; Molecular Probes, Carlsbad, CA) (13).

The Luminex xMAP technology has been previously employed for genotyping a wide range of microorganisms, including fungi. Bovers et al. developed a Luminex assay based on the intergenic spacer 1 region for the identification of clinically relevant *Cryptococcus spp.* (6). In studies by Diaz et al., the Luminex platform was used to differentiate between clinically relevant *Cryptococcus*, *Malassezia*, and *Trichosporon* species, and in addition, the investigators employed a mini-cluster probe for identification of new species in these genera based on the intergenic spacer 1, D1/D2, and internal transcribed spacer 1 (ITS-1) regions (10–12). Similarly, Das et al. employed the Luminex assay for the identification and differentiation of six clinically relevant *Candida* species based on the ITS-2 region (9). In another study, the nucleotide variation in the RNA polymerase II second largest subunit B2 was exploited to design a Luminex assay for genotyping human pathogenic fusaria (20).

In the present study, we designed and validated a rapid identification method using the Luminex xMAP technology to

identify six clinically important *Aspergillus* species: *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, *A. ustus*, and *A. versicolor*. The *Aspergillus* Luminex assay displayed good specificity and, as designed, can be used for multiplexed and high-throughput detection of clinically relevant aspergilli.

MATERIALS AND METHODS

Aspergillus isolates. Two different panels of aspergilli were used in this study. The first panel consisted of 44 *Aspergillus* isolates that represented previously validated and type isolates from the culture collections of the Centers for Disease Control and Prevention (CDC), Atlanta, GA, and the National Center for Agricultural Utilization Research, U.S. Department of Agriculture, Peoria, IL, respectively (Table 1). This panel of isolates (denoted as reference isolates for the purposes of this study) was used for the initial Luminex assay development. Once the assay conditions were established using the reference aspergilli, the *Aspergillus* Luminex assay was tested on an additional set of 131 clinical *Aspergillus* isolates that included 89 *A. fumigatus*, 17 *A. flavus*, 12 *A. niger*, 4 *A. terreus*, 3 *A. ustus*, and 6 *A. versicolor* isolates. All the clinical aspergilli were obtained from the Mycotic Diseases Branch Culture Collection, CDC, Atlanta, GA.

Genomic DNA, PCR, and sequencing of the ITS-1 region. All isolates were stored frozen and subcultured on Sabouraud's dextrose agar plates before DNA extraction was performed. Genomic DNA extraction was performed as described previously (16), and the PCRs were performed with the ITS primers ITS 600 F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS 600 R (5'-TCCTCCGCTTATTGATATGC-3'). The cycling conditions were as follows: 95°C for 3 min, 35 cycles of 95°C for 45 s, an annealing step at 57°C for 30 s, an extension step at 72°C for 3 min, and a final extension step at 72°C for 10 min. The presence and size of amplicons were verified on a gel, fragments were purified using the ExoSAP-IT PCR purification kit (USB Corporation, Cleveland, OH), and the forward and reverse fragments were sequenced with the PCR primer sets as described elsewhere (5).

The identities of all 175 *Aspergillus* isolates were confirmed by comparative sequence analysis of the ITS-1 and ITS-2 regions.

***Aspergillus* species-specific Luminex probe design.** Sequences generated from the ITS-1 region of the 44 reference *Aspergillus* isolates were aligned using the software ClustalW, and candidate regions specific to each species were identified for the Luminex probe design. Each species-specific Luminex probe was designed to have at least a 2-nucleotide difference compared to other probes and was 21 to 25 mer in length. The six species-specific probes were AF (*A. fumigatus*), AL (*A. flavus*), AN (*A. niger*), AT (*A. terreus*), AU (*A. ustus*), and AV (*A. versicolor*) (Table 2). The stability, melting temperature, and other factors for each probe and ITS-1 complement were evaluated using the software Oligo (Molecular Biology Insights and BioMath, Cascade, CO).

Probe coupling to microspheres. The species-specific Luminex probes AF, AL, AN, AT, AU, and AV (designed as detailed above) were covalently coupled to carboxylated microspheres 130, 131, 132, 133, 134, and 135, respectively, as previously described with some modifications (9). In brief, 2.5×10^6 of each

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TABLE 1. *Aspergillus* isolates used in the Luminex assay as the reference panel

Organism	Isolate no.	Source
<i>Aspergillus fumigatus</i>	B5230	CDC
	B6028	CDC
	B6029	CDC
	B6030	CDC
	NRRL5109	USDA
	NRRL5517	USDA
<i>Aspergillus flavus</i>	B5906	CDC
	B5912	CDC
	B5913	CDC
	B5915	CDC
	B5916	CDC
	B1000	CDC
	NRRL506	USDA
	NRRL1957	USDA
	B6064	CDC
	NRRL326	USDA
<i>Aspergillus niger</i>	NRRL330	USDA
	NRRL348	USDA
	NRRL363	USDA
	NRRL566	USDA
	B6446	CDC
	B6448	CDC
	B6525	CDC
	B6413	CDC
<i>Aspergillus terreus</i>	B6450	CDC
	B5964	CDC
	NRRL15722	USDA
	B5628	CDC
	B5650	CDC
	B6134	CDC
	NRRL1974	USDA
	NRRL4688	USDA
	NRRL4876	USDA
	NRRL5077	USDA
<i>Aspergillus ustus</i>	B6120	CDC
	B6570	CDC
	B6574	CDC
	B4642	CDC
	NRRL4791	USDA
	NRRL20734	USDA
<i>Aspergillus versicolor</i>	B6120	CDC
	B6570	CDC
	B6574	CDC
	B4642	CDC
	NRRL4791	USDA
	NRRL20734	USDA

microsphere set was transferred to a low-binding microcentrifuge tube (Eppendorf, Westbury, NY). The microspheres were centrifuged (Eppendorf) for 3 min at $\geq 8,000 \times g$. After the supernatant was removed, avoiding the pellet, 25 μ l of 2-(*N*-morpholino) ethanesulfonic acid (MES) (Sigma, St. Louis, MO), 30 mg/ml of EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride] (Pierce, Milwaukee WI), and 500 picomoles of each species-specific probe were coupled to the designated bead region. The solution(s) was shaken in the dark at room temperature for 30 min and 30 mg/ml EDC was added again, followed by a second 30-min incubation period. The microspheres were washed with 500 μ l of 0.02% Tween (Sigma, St. Louis, MO) and centrifuged for 3 min at $\geq 8,000 \times g$, and the supernatant was removed and 500 μ l 0.1% lauryl sulfate added (Sigma, St. Louis, MO). Finally, this solution was centrifuged for 3 min at $\geq 8,000 \times g$, the supernatant was removed, and 50 μ l of Tris-EDTA buffer (TE) was added (Sigma, St. Louis, MO). The microspheres coupled with probes were stored at 4°C in the dark until ready for use.

Assay to confirm the binding of *Aspergillus* probes to microspheres. The species-specific Luminex probes that were bound to designated microspheres were tested to confirm that the probes were bound to the respective microspheres. First, a biotinylated reverse probe that complemented the species-specific Luminex probes was designed. A working solution containing 3 μ l of each species-specific microsphere set was diluted to 1 ml with 1.5 \times TMAC (5 M tetramethyl ammonium chloride–20% Sarkosyl–1 M Tris-HCl [pH 8.0]–0.5 M EDTA [pH 8.0]–dH₂O) (Sigma, St. Louis, MO). In a 96-well conical plate (Corning, Corning, NY), 10 μ l of the probe complement of each Luminex species-specific probe was added to each well, followed by 33 μ l of bead solution and 7 μ l of TE buffer to bring the final solution volume to 50 μ l per well; the appropriate negative controls, consisting of wells containing TE buffer and microsphere solution, were included. The plates were sealed with microseal film (Bio-Rad, Hercules, CA) and heated to 94°C for 5 min for initial denaturation, followed by hybridization at 52°C for 30 min. After 30 min, the plate was centrifuged at $\geq 8,000 \times g$ for 2 min, the supernatant was carefully removed to avoid the pelleted product, and 75 μ l of SAPE (4 mg/ml) in 1 \times TMAC was

TABLE 2. *Aspergillus* species-specific Luminex probes directed to the ITS-1 region of the rRNA

Probe	Target	Probe sequence (5'–3')
AF	<i>Aspergillus fumigatus</i>	GAAAGTATGCAGTCTGAGTTGAT
AL	<i>Aspergillus flavus</i>	CACCCGTGTTACTGTACCTTAG
AN	<i>Aspergillus niger</i>	AACACGAACACTGTCTGAAAGCGT
AT	<i>Aspergillus terreus</i>	AACATGAACCCCTGTTCTGAAAGCT
AU	<i>Aspergillus ustus</i>	CTGAGCTTGATACAAGCAAAC
AV	<i>Aspergillus versicolor</i>	AGTGATGCAGTCTGAGTCTGAATAT

added. For the final hybridization of SAPE, the plate was heated at 52°C for 10 min, read on the Luminex200 using MasterPlexCT (Miraibio, San Francisco, CA), and analyzed using MasterPlex GT analysis software (Miraibio, San Francisco, CA) as detailed below in the section on data analysis.

PCR primer design for *Aspergillus* isolates. After the Luminex species-specific probes were designed and bound to the microspheres, three sets of PCR primers that would yield amplicon lengths amenable to the Luminex assay were designed to amplify 100-bp, 250-bp, and 600-bp portions of the ITS regions using the following respective sequences: ITS 100 F (5'-GGAAGTAAAAGTCGTAAC AAG 3') and ITS 100 R (5'-GAGATCCA/GTTGTTGAAAGTTT-3'); ITS 250 F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS 250 R (5'-GCTGCGT TCTTCATCGATGC-3'); and ITS 600 F (5'-GGAAGTAAAAGTCGTAACA AGG-3') and ITS 600 R (5'-TCCTCCGCTTATTGATATGC-3'). Each reverse primer was labeled with a biotin molecule, and PCR amplicons for the reference aspergilli were generated using the primer pairs. The PCR conditions were as described in the section on genomic DNA. After PCR, 10 microliters of the PCR product was added to a 96-well conical plate (Corning), and the Luminex assay was performed as described earlier (in the section on coupling confirmation) to determine the appropriate amplicon length that yields efficient hybridization with the species-specific Luminex probes.

The reproducibility of the Luminex assay was assessed as follows. PCR products obtained from the reference aspergilli ($n = 44$) were tested on the Luminex platform, and aliquots of the PCR amplicons were frozen on day 1. On days 2 and 3, the frozen aliquots were thawed and tested in the Luminex assay in independent runs. Additionally, on day 4, genomic DNA was extracted again from the reference *Aspergillus* isolates and subjected to PCR and Luminex analyses. The results from all four experiments were analyzed to evaluate the hybridization efficiency of the biotin-labeled PCR products after storage and to compare the results of two independent Luminex assays performed on the same batch of isolates.

Validation of the *Aspergillus* Luminex assay. Once the assay parameters (including amplicon size, PCR conditions, and coupling confirmation) were established, the *Aspergillus* Luminex assay was employed to genotype a panel of 131 *Aspergillus* clinical isolates. The species identifications of the 131 aspergilli were also derived employing comparative sequence analyses of the ITS regions. For the Luminex assay, the genomic DNA of all aspergilli was subjected to PCR using primer pairs ITS 250 F and ITS 250 R; 10 μ l of the PCR product, 33 μ l of bead solution containing the six *Aspergillus* species-specific probes, and 7 μ l of TE buffer were added per well, and the assay conditions were exactly as described for coupling confirmation. The negative control consisted of the microsphere solution (33 μ l) and TE buffer (17 μ l) with no target DNA.

Data analysis. The data were acquired using the MasterPlexCT system and analyzed using the MasterPlex GT software. Individual sets of microspheres were analyzed by a dual laser system, and the median fluorescence intensity (MFI) value was calculated. The MFI represents the median signal intensity measured per microsphere set. The signal-to-background ratio represents the MFI signals of positive controls versus the background fluorescence of samples containing all components except the amplicon target. A positive signal was defined as an MFI signal that is at least twice the background level after subtraction of the background.

RESULTS

***Aspergillus* Luminex assay development.** *Aspergillus* species-specific Luminex probes directed to the ITS-1 region were designed and are listed in Table 2. The six species-specific probes AF, AL, AN, AT, AU, and AV were attached to the designated microspheres, and probe coupling was confirmed by coupling confirmation assays (data not shown).

TABLE 3. Specificity of probes used to detect clinically important *Aspergillus* species in the multiplex format

Target DNA ^a	MFI with standard error for indicated probe					
	AF	AL	AN	AT	AU	AV
<i>A. fumigatus</i> (7)	568 ± 22	5	5	5	5	5
<i>A. flavus</i> (8)	0	550 ± 30	0	0	0	0
<i>A. niger</i> (7)	17	17	845 ± 55	17	17	17
<i>A. terreus</i> (8)	9	9	9	384 ± 25	9	9
<i>A. ustus</i> (7)	30	30	30	30	680 ± 23	30
<i>A. versicolor</i> (7)	7	7	7	7	7	1,427 ± 54

^a The number of isolates included in the validation panel is indicated within parentheses.

Using three different sets of primers targeted to amplify 100 bp, 250 bp, and 600 bp of the ITS regions, the effect of amplicon size on MFI signals was assessed for the reference *Aspergillus* isolates. Hybridization signals of less than twice the background level and higher cross-reactivity to some species-specific probes were observed with the 100-bp amplicon. While the 600-bp amplicon product generated an MFI of less than twice the background level, variable hybridization to the target DNA was observed, thus impacting reproducibility. An amplicon length of 250 bp generated optimal and reproducible data with no cross-reactivity with other probes (data not shown). Thus, the primer set that yielded the 250-bp amplicon length (primers ITS 250 F and ITS 250 R) was selected and employed in the *Aspergillus* Luminex assay.

Each of the species-specific *Aspergillus* probes was designed to target the respective *Aspergillus* target DNA, thus yielding high MFIs with the respective target DNA but MFIs less than twice the background level with nontarget DNA. As can be seen from Table 3, the Luminex probes AF, AL, AN, AT, AU, and AV hybridized with their respective targets, *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, *A. ustus*, and *A. versicolor*, yielding MFIs that ranged from a mean MFI of 384 (*A. terreus*) to 1,427 (*A. versicolor*). In spite of this species-specific variability in MFI values, each of the probes produced MFIs that were more than twice the background level, thus generating an MFI that was, on average, 167% higher than the background.

When the assay was tested for reproducibility, it was found that MFIs of the PCR products decreased after being subjected to a freeze-thaw cycle compared to the MFIs of the fresh PCR products. For instance, for all *A. fumigatus* isolates, the mean MFIs on days 1, 2, and 3 were 895, 735, and 690, respectively, after freezing and thawing, whereas the fresh PCR products for all *A. fumigatus* isolates (tested on day 4) generated a mean MFI of 815. Thus, although the mean MFIs decreased over the course of 3 days and varied between the two PCR assays, the MFIs were always at least twice the background level. This was a consistent trend observed for all *Aspergillus* reference isolates that included representative type isolates for each species (data not shown).

Luminex assay validation. Once the Luminex assay parameters were determined using the reference *Aspergillus* panel as described above, the Luminex assay (a patent has been applied for for this probe set) was tested with a set of 131 sequence-confirmed *Aspergillus* isolates. All the target PCR amplicons hybridized to their species-specific Luminex probes, and the species identifications generated by the Luminex assay corre-

lated 100% with the identities generated by a comparative sequence identification of the ITS regions.

DISCUSSION

Over the last several years, molecular methods, including the rolling-cycle amplification, repetitive sequence-based PCR, PCR-restriction enzyme, reverse line blot assay, and DNA microarray methods, have been evaluated for *Aspergillus* species identification (7, 14, 19, 22, 25). Although these methods have been demonstrated to be useful for species identification, most of these methods (except the reverse line blot assay) are not amenable to multiplexing. In addition, some of the methods, such as DNA microarrays, are expensive to perform and require sophisticated analyses to interpret the results. DNA sequence-based methods are considered the gold standard for fungal species identification and have been employed increasingly for the identification of *Aspergillus* species. However, comparative sequence-based methods can be labor-intensive and time-consuming and cannot be multiplexed.

The microsphere-based Luminex xMAP technology builds on the principles of flow cytometry and enzyme immunoassay, resulting in a sensitive, specific genotyping method that is rapid and has the additional flexibility of a multiplex format. To this end, an *Aspergillus* Luminex assay was designed and validated for the rapid identification of six medically important aspergilli, *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, *A. ustus*, and *A. versicolor*, from culture. The results demonstrated that the assay was specific to the target DNA, was easy to perform, and had a rapid turnaround time of about 6 h (not including DNA extraction).

Previous studies have suggested that the probe GC content and length and the length of the PCR amplicon can influence hybridization profiles, thereby impacting the successful outcome of the Luminex assay (13). A probe GC content of 30 to 50% was optimal for this study. In addition, all species-specific probes were designed to be 21 to 25 mer in length, and this yielded superior hybridization. One parameter that needed optimization for the current assay was the PCR amplicon length. Longer amplicons may inhibit hybridization due to steric hindrance, but in some studies, larger amplicon targets have been shown to be efficient for specific hybridization (13). Diaz and Fell assessed the effect of amplicon length on hybridization efficiency by utilizing three sets of primers generating amplicons 490 to 600 bp, 650 to 875 bp, and 950 to 1,200 bp (11). For the most part, these investigators found a lower

hybridization signal with the shortest amplicon target and a higher hybridization signal with amplicon targets longer than 600 bp (11). In this study, an evaluation of three different amplicon sizes demonstrated that a 250-bp amplicon length provided optimal hybridization for all isolates within a given *Aspergillus* species. Additionally, our study also demonstrated that the *Aspergillus* Luminex assay yielded lower but reproducible results with PCR amplicons that had been freeze-thawed over time as well as between two independent assays.

After the conditions of the *Aspergillus* Luminex assay were optimized with the reference panel of isolates (that included type isolates), the assay was tested on an additional set of 131 *Aspergillus* clinical isolates. There was 100% correlation between the results of the *Aspergillus* Luminex assay and the identification derived by the comparative sequence analysis method, thus yielding an assay specificity of 100%. Currently, the *Aspergillus* Luminex assay includes only six probes and, as designed, can identify the predominant *Aspergillus* species that cause invasive aspergillosis (IA). Numerous studies have demonstrated that IA is caused predominantly by these six *Aspergillus* species, with one large multicenter study showing that 56% of IA was due to *A. fumigatus*, 18.7% was caused by *A. flavus*, 8% was caused by *A. niger*, 16% was caused by *A. terreus*, and 1.3% was caused by *A. versicolor*. Although multiple different *Aspergillus* species can exist in the environment and, in theory, can cause IA, the Luminex assay was designed to identify the relevant species that may be recovered from clinical specimens and would serve as a first line of identification. For instance, if the six-probe *Aspergillus* Luminex assay is used for the identification of an unknown *Aspergillus* isolate in a clinical microbiology laboratory and there is no hybridization with the target DNA (because it is a species not included in the six-probe panel), the target DNA can then be sequenced as a second-step strategy for identification.

The *Aspergillus* species-specific probes were directed to the ITS-1 locus, as this region has been demonstrated to be useful for species complex-level identification within this genus (4). However, the ITS locus is not suitable for the identification of individual species within the species complex; for instance, the ITS locus cannot discriminate between species within the *A. fumigatus* complex that includes the newly described species *A. lentulus* and other species such as *A. udagawae*, *A. thermomutatus*, and *A. fumigatus* (3). Thus, with the current Luminex panel, DNA from these isolates will hybridize to the *A. fumigatus* probe and will therefore be identified as *A. fumigatus* complex. Recent studies have demonstrated that comparative sequence analyses of protein-coding regions such as that for β tubulin provide enough discrimination to differentiate taxa within the *Aspergillus* species complexes (2, 23). For such levels of identification, an additional set of probes directed to the β tubulin or any other suitable locus or loci can be designed and added to the Luminex panel. Up to 100 different *Aspergillus* probes can be used on the Luminex platform; thus, in theory, 100 different genotypes can be distinguished using this assay.

As designed, the *Aspergillus* Luminex assay can be used for the identification of isolates grown as pure culture. Other studies have employed Luminex assays for the direct detection of pathogens from clinical specimens (10), and it remains to be seen if the *Aspergillus* Luminex assays can be used as a diagnostic tool as well. In this center, the cost of sequencing meth-

ods is as low as \$7 per sample, and though, at this time, the cost of the Luminex assay is greater, time and labor can serve as a definite trade-off. With continued and increased use of the Luminex technology, the cost of the assay may decrease, thus truly providing clinical microbiology laboratories with a technology that is high throughput as well as economical. In summary, a rapid, specific, and multiplex method, the *Aspergillus* Luminex assay, is described for the identification of various clinically important aspergilli.

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