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Development and Evaluation of a Calibrator Material for Nucleic Acid-Based Assays for Diagnosing Aspergillosis

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Twelve laboratories evaluated candidate material for an *Aspergillus* DNA calibrator. The DNA material was quantified using limiting-dilution analysis; the mean concentration was determined to be 1.73×10^{10} units/ml. The calibrator can be used to standardize aspergillosis diagnostic assays which detect and/or quantify nucleic acid.

Despite advances in the science of disease diagnosis, including that of infectious diseases, the diagnosis of invasive aspergillosis (IA) remains challenging. Molecular methods have not been widely used diagnostically due to the lack of standardization and validation of these tests (1). Without biologic standards, assay comparison and calibration become very difficult.

The *A*spergillus *Te*chnology *C*onsortium (AsTeC) is an NIH-contracted consortium which was established to develop and maintain a repository of prospectively collected clinical samples from patients at high risk for developing IA. In addition, AsTeC was also established to evaluate prospective diagnostic assays for IA. In order to appropriately evaluate and compare novel diagnostic assays, it was necessary to establish a reference standard. Herein, we describe a collaborative effort with the Invasive Aspergillosis Animal Models (IAAM) group to develop a nucleic-acid material which can be used to standardize diagnostic assays which target *Aspergillus* DNA.

Aspergillus DNA was prepared following modification of a previously published method (2). Conidia were collected from potato dextrose (PD) plates and then suspended in two 300-ml aliquots of half-strength PD broth. Following overnight shaking (225 rpm at 30°C), the cultures were centrifuged and pellets were washed with water. Each pellet was resuspended in 20 ml of spheroplasting buffer (1 M sorbitol, 0.1 M EDTA, pH 8.0, and 10 μl of beta-mercaptoethanol diluted to 10 ml with H_2O) and incubated with zymolyase (0.1g) for 1 h at 30°C. Following centrifugation, the pellet was resuspended in lysis buffer (10 mM EDTA, 10 mM Tris, and 0.5% SDS) and incubated with 500 μl proteinase K at 50°C for 1 h. Following this incubation, a 1× volume of phenol was added, and incubation was continued at 50°C for 30 min. The aqueous layer was repeatedly ex-

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TABLE 1 Real-time PCR assays and results for the 12 laboratories

Testing center	Amplification method	Gene target	Vol of DNA added to reaction mixture (μl)	Limiting dilution	Calibrator concn (U/ml)
1	Real-time PCR, TaqMan	18S rRNA	5	1.58×10^{-8}	1.27×10^{10}
2	Real-time PCR, TaqMan	18S rRNA	10	3.8×10^{-9}	2.63×10^{10}
3	Real-time PCR, TaqMan	18S rRNA	5	9.06×10^{-9}	2.21×10^{10}
4	Real-time PCR, TaqMan	18S rRNA	10	2.75×10^{-8}	3.64×10^{9}
5	Real-time PCR, TaqMan	18S rRNA	10	5.83×10^{-9}	1.72×10^{10}
6	Real-time PCR, TaqMan	28S rRNA	25	3.00×10^{-9}	1.33×10^{10}
7	Real-time PCR, FRET[a]	28S rRNA	10	5.96×10^{-9}	1.68×10^{10}
8	Real-time PCR, FRET	18S rRNA	10	1.38×10^{-8}	7.25×10^{9}
9	Real-time PCR, molecular beacon probes	ITS1 region	2	2.71×10^{-8}	1.85×10^{10}
10	Real-time PCR, TaqMan	28S rRNA	5	1.00×10^{-8}	2.00×10^{10}
11	Real-time PCR, TaqMan	ITS region	10	3.08×10^{-9}	3.25×10^{10}
12	Real-time PCR, TaqMan	28s rRNA	7.5	7.84×10^{-9}	1.70×10^{10}

[a] FRET, fluorescent resonance energy transfer.

TABLE 2 Qualitative results from the 12 participating testing centers

Dilution	No. of replicates positive/total no. tested) for testing center:											
	1	2	3	4	5	6	7	8	9	10	11	12
1×10^{-9}	1/10	2/10	0/10	0/10	2/10	2/10	1/10	0/10	0/10	1/10	3/10	1/10
3×10^{-9}	3/10	4/10	2/10	0/10	4/10	7/10	3/10	2/10	1/8	2/10	6/10	3/10
1×10^{-8}	4/10	10/10	8/10	1/10	8/10	9/10	9/10	7/10	1/8	6/10	10/10	9/10
3×10^{-8}	8/10	10/10	9/10	7/10	10/10	10/10	10/10	8/10	7/8	9/10	9/10	10/10
1×10^{-7}	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	9/10
1×10^{-6}	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10

tracted with hot phenol until the aqueous layer was clear. Chloroform was added to the supernatant; after separation, DNA was precipitated overnight at −20°C using a 2.5× volume of 100% ethanol and a 0.1× volume of sodium acetate, pelleted, washed twice with 70% ethanol, and air dried. The resuspended pellet was treated with RNase for 45 min and then reextracted with phenol-chloroform and precipitated using 100% ethanol and sodium acetate followed by 70%-ethanol washes. The quality of the purified DNA was assessed by spectrophotometry (expected $A_{260}/A_{280} = 1.7$ to 1.9).

Twelve clinical and research laboratories in the United States and Europe were enlisted for determining the quantity of DNA. Each laboratory received a blinded panel of specimens consisting of 10 replicates of calibrator dilutions of 1×10^{-6}, 1×10^{-7}, 1×10^{-8}, 3×10^{-8}, 1×10^{-9}, and 3×10^{-9} and 5 negative aliquots (Tris-EDTA [TE] buffer).

The concentration of the undiluted specimen was determined by limiting-dilution analysis. This method has been described previously for establishing international standard material for hepatitis C and B viruses (3, 4). The effects of repeated freeze-thaw cycles and prolonged −80°C storage on the integrity of the DNA were assessed at dilutions of 10^{-5} and 10^{-6}. Ten aliquots were tested at each time point and each freeze-thaw cycle. Each aliquot was tested in a real-time PCR assay as previously described by the IAAM group (6).

The characteristics of the 12 real-time PCR assays used by the participating laboratories are shown in Table 1. Of the 60 negative samples (5 samples × 12 laboratories), there was one false-positive result. Table 1 shows the results of limiting-dilution analysis and calibrator concentration for each laboratory; the results are reported as units/ml and are not corrected for gene copy number. Neither the platform, the specific target, nor the volume of material added to the assay appeared to be correlated with apparent sensitivity of the assay. Table 2 shows the qualitative reporting of all participating centers. The concentration of the calibrator determined by the 12 laboratories ranged from 3.64×10^9 to 3.25×10^{10} U/ml, with a mean concentration of 1.73×10^{10} U/ml (standard deviation [SD], 0.78×10^{10} units/ml). This mean value was the assigned concentration for the undiluted calibrator material.

The results of the stability study performed at the central laboratory are shown in Tables 3 and 4. There was little change in crossing-threshold (C_T) values over the 2-year storage period. The mean and SD C_T values for the 10^{-5} dilution were 30.4 (0.2) for time zero and 30.5 (0.2) after 2 years of storage. These two values are within the variability of PCR assays. Similar results were seen for the more dilute 10^{-6} sample. Based on C_T values, the DNA was stable for up to 2 years when storage was done at −80°C. Table 4 shows the results of testing after repeated freeze-thaw cycles. These data show that the calibrator material is also stable over at least 10 freeze-thaw cycles.

A candidate material was created by purifying DNA from *Aspergillus fumigatus* strain AF293, and an arbitrary "unit" value was assigned to the material based on limiting-dilution studies performed in 12 laboratories in the United States and Europe. Despite apparent differences in qualitative sensitivities of assays, the analysis produced remarkably similar results, with a standard deviation of 0.78×10^{-10} $\log_{10}$ across the 12 participating laboratories. This work builds upon that of the EAPCRI (2, 5), who demonstrated that standardization of extraction techniques led to improved sensitivity performance of PCR for detecting *Aspergillus* conidia. The development of this DNA standard will allow improved interlaboratory comparison of techniques and fungal load values.

This study is limited in that all assays used target in some way the ribosomal DNA (rDNA) of *Aspergillus*. Clearly, this is an attractive target for developing sensitive PCR assays, since there are typically 30 to 90 copies of the rDNA present in most isolates of *Aspergillus* (6). However, while we can assign an arbitrary "unit" value to our material, it is difficult to extrapolate this to a genome equivalent given the variable number of

TABLE 3 Stability of AF293 DNA calibrator when stored at −80°C[a]

Dilution and statistic	C_T value for storage time					
	Time zero	1 mo	3 mo	6 mo	1 yr	2 yr
10^{-5}						
Mean	30.4	30.5	30.6	31.0	30.8	30.5
SD	0.2	0.3	0.3	0.3	0.5	0.2
10^{-6}						
Mean	34.3	34.5	34.8	34.4	34.3	34.2
SD	0.8	0.6	0.8	0.6	0.6	0.5

[a] All values are the crossing-threshold (C_T) values obtained by real-time PCR. For references purposes, a value of 3.3 C_T is equivalent to a 1-$\log_{10}$ change in the quantity of DNA. All experiments were performed by the central laboratory.

TABLE 4 Stability of AF293 DNA calibrator when taken through multiple freeze-thaw cycles[a]

Dilution and statistic	C_T value for freeze-thaw cycle:					
	0	1	2	3	5	10
10^{-5}						
Mean	30.4	30.5	30.6	30.6	31.1	30.8
SD	0.2	0.3	0.1	0.2	0.3	0.2
10^{-6}						
Mean	34.3	34.3	34.7	34.0	34.7	34.5
SD	0.8	0.8	0.7	0.4	0.4	0.6

[a] See footnote *a* of Table 3.

copies of the DNA per genome. Since the candidate material is DNA, it could not be used to assess methods of extraction from clinical specimens, which could add considerable variability to quantitative results.

Individuals and institutions interested in obtaining calibrator material should contact the corresponding author.

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