Title: An analytical and clinical evaluation of the PathoNostics AsperGenius® Assay for the detection of invasive aspergillosis and resistance to azole antifungal drugs direct from plasma samples.

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Key words: Invasive aspergillosis, *Aspergillus* PCR, azole resistance determination.
**ABSTRACT**

With the proposal to include *Aspergillus* PCR in the revised EORTC/MSG definitions for fungal disease, commercially manufactured assays may be required to provide standardisation and accessibility. The PathoNostics AsperGenius® assay represents one such test that has the ability to detect a range of *Aspergillus* species and azole-resistance in *A. fumigatus*. Performance has been validated when testing BAL and serum specimens, but recent evidence suggests that testing plasma may enhance sensitivity over serum. It was decided to evaluate the analytical and clinical performance of the PathoNostics AsperGenius® assay when testing plasma.

For the analytical evaluations plasma was spiked with various concentrations of *Aspergillus* genomic DNA before extraction following international recommendations using two automated platforms. For the clinical study, 211 samples from 10 proven/probable IA and 2 possible IA cases, and 27 controls were tested.

The limit of detection when testing DNA extracted using the BioMerieux EasyMag and Qiagen EZ1 extractors was five and 10 genomes/0.5ml sample, respectively. In the clinical study, true positivity was significantly greater than false positivity (*P* < 0.0001). The sensitivity and specificity using a single positive result as significant were 80% and 77.8%, respectively. If multiple samples were required to be positive specificity was increased to 100%, albeit sensitivity reduced to 50%.

**Summary:** The AsperGenius® assay provided good clinical performance but the predicted improvement when testing plasma was not seen, possibly a result of target degradation attributed to sample storage. Prospective testing is required to determine the clinical utility of this assay, particularly the diagnosis of azole-resistant disease.
INTRODUCTION

Standardisation of *Aspergillus* PCR testing of blood based samples has led to the proposal to include

*Aspergillus* PCR in to the second revision of the EORTC/MSG consensus definitions for invasive fungal
disease (IFD).\(^1^4\) This may increase demand for *Aspergillus* PCR, as it can be used, in combination

with other biomarker assays (Galactomannan EIA and β-D-Glucan) to improve management of

patients at risk of invasive aspergillosis (IA).\(^5\) Easily attainable, quality controlled and well validated

assays are necessary, and commercially developed assays help in achieving these requirements.

Several commercial *Aspergillus* PCR assays have been developed (MycAssay Aspergillus, Renishaw

Fungiplex, Ademtech MycoGENIE, PathoNostics AsperGenius®) with varying degrees of clinical

validation.\(^6^10\) Of particular interest, given the emergence of azole resistant strains of *A. fumigatus*,

are the Ademtech MycoGENIE and PathoNostics AsperGenius® assays that have the ability to detect

the major single nucleotide polymorphisms that infer environmentally driven resistance. Tests to
detect genetic mechanisms of azole resistance have been applied directly to clinical samples and

have the potential to overcome the limited sensitivity of conventional culture techniques.\(^7^8\) The

application of these tests to non-invasive sample types (e.g. blood) will improve clinical utility and

some success has been noted when testing serum.\(^7\)

Recently, the European *Aspergillus* PCR initiative showed that both the analytical and clinical

performance of *Aspergillus* PCR was superior when testing plasma compared to serum.\(^3^4\) It was

proposed that using plasma avoided DNA trapping during clot formation, subsequently the available

target was greater and performance enhanced. In the previous evaluation of the PathoNostics

AsperGenius® assay when testing serum the sensitivity and specificity were 79% and 91%,

respectively, and genetic screening for resistance direct from the sample was obtained in 50% of the

cases.\(^7\) It is hypothesised the testing of plasma may improve the performance of the AsperGenius®

assay. Nevertheless, validation when testing plasma is required to enhance the application range

and assay robustness.
This manuscript determines the analytical and clinical performance of the PathoNostics AsperGenius® assay when testing plasma samples using methods in line with international recommendations. 4
MATERIALS AND METHODS

Study design

The study was divided into an analytical evaluation to determine the assays limit of detection (LOD), linear range and efficiency of amplification when testing plasma, and secondly, a clinical study to determine performance (sensitivity/specificity etc) when testing plasma samples from a haematology population at high risk of IA.

Analytical Study

The analytical evaluation focused on performance when detecting specimens containing genomic DNA from *A. fumigatus* or *A. terreus*. Two automated nucleic acid extraction systems were evaluated (Qiagen DSP virus kit on the EZ1 Advance XL instrument and BioMerieux Generic 2.01 Protocol on the EasyMag instrument). All nucleic acid was eluted in 60μl.

Simulated plasma samples were prepared using pooled human plasma divided into 0.5ml aliquots and spiked with various concentrations of genomic DNA from either *A. fumigatus* or *A. terreus* to achieve final burdens of 10000, 1000, 500, 100, 75, 50, 25, 10, five, one genome/0.5 ml sample. Successful detection of the higher burdens was predicted, so in order to determine accurate performance at less predictable concentrations the number of replicates was greater when testing lower burdens (Tables 1-3). To monitor for contamination during each extraction process at least one non-spiked plasma aliquot was retained to provide a negative control. To avoid airborne contamination, all required manual processes took place in a class II laminar flow cabinet.

When performing PCR amplification a five microlitre DNA template input volume was used for all burdens, with an additional 10μl input assessed for the lower burdens (<50 genomes/0.5ml sample) in an attempt to improve reproducibility of detection.
Clinical Study and Patient Population

Clinical plasma samples from patients with proven, probable, possible IA, or with no evidence of fungal disease (NEF) were selected. All samples had been sent as part of the care pathway incorporating a well-validated “in-house” Aspergillus PCR. On completion of routine testing plasma was stored at -80°C for quality control or performance assessment purposes. The study was a performance assessment of the AsperGenius® Assay and was an anonymous, retrospective case/control design, not affecting patient management. Patient demographics are shown in Table 4.

Nucleic acid was extracted from 0.5ml of plasma using the BioMerieux EasyMag Generic 2.01 Protocol, following the manufacturer’s instructions, with DNA eluted in 60µl. Positive (plasma containing 10 genomes of A. fumigatus DNA) and negative (plasma only) extraction controls were included in each run.

When performing PCR amplification a 10µl DNA template volume was used to provide optimal opportunity for detection.

PathoNostics AsperGenius® PCR amplification

For both the analytical and clinical studies the AsperGenius® species and resistance PCR testing was performed on the Qiagen Rotorgene Q High Resolution Melt Instrument. Using a final reaction volume of 25µl and following the manufacturer’s instructions, with the exception that DNA template volumes for the species assay were increased to 10µl for the clinical evaluation, and in the analytical evaluation where performance for detection of the lower burdens (<50 genomes/0.5ml sample) was compared with an input volume of five microlitres. The manufacturer recommends an input volume of five and 10µl for the species and resistance assays, respectively.
Statistical Evaluation

Analytical analysis of the AsperGenius® species PCR when testing plasma samples was performed as previously described. Briefly, the 100% LOD, linearity ranges and PCR amplification efficiencies were calculated. Further analysis was performed correlating AsperGenius® species and resistance performance so that the quantification cycle (Cq) generated by the A. fumigatus assay could be used as a guide to the likelihood of success when performing the resistance assay.

When determining the clinical accuracy of the AsperGenius® species results the positivity rate in samples originating from cases was compared to the false positivity rate in control samples. Clinical performance was determined by the construction of 2x2 tables to calculate sensitivity, specificity, positive and negative likelihood ratios and diagnostic odds ratio of the AsperGenius® species assay.

For all patients, only a single positive sample was required to consider the patient positive. Given the case control study design, and artificially high prevalence of proven/probable IA (25.6%), predictive values were not used. When required ninety-five percent confidence intervals and, P values (Fishers exact test; P: 0.05) were generated to determine the significance of the difference between rates.
RESULTS

Analytical Performance of the AsperGenius® species assay.

When extracting DNA from plasma using the Qiagen EZ1 DSP virus kit the LOD for both the A.
fumigatus specific and Aspergillus species assays was 25 genomes/0.5ml sample using a 5µl
template input and 10 genomes/0.5ml sample using a 10µl template input volume (Table 1).
Increasing the amount of DNA template also improved reproducibility when detecting
5 genomes/0.5ml sample but did not improve detection of 1 genome/0.5ml sample.

Using the BioMerieux EasyMag for DNA extraction, the LOD using a five microlitre template input for
both the A. fumigatus and Aspergillus spp. assays improved to five genomes/0.5ml sample,
compared to the equivalent volume of eluate extracted by the Qiagen EZ1 DSP virus kit (Tables 1 and
2). However, 4/31 replicates across all burdens generated a low level false positive A. terreus result
(Mean Ct: 42.4). Increasing the template input volume to 10µl did not improve the 100% LOD, but
reproducibility when detecting one genome/0.5ml sample was improved (A. fumigatus assay 10µl
template: 3/5 vs 5µl template 0/5; Aspergillus spp. assay 10µl template: 3/5 vs 5µl template 1/5).

When using the BioMerieux EasyMag to extract A. terreus DNA from plasma the LOD for both the A.
terreus specific and Aspergillus species targets was five genomes/0.5ml sample using 5 µl of DNA
template, at one genome/0.5ml sample reproducibility for both targets was 33.3%. Increasing the
input to 10µl per reaction lowered the LOD to one genome/0.5ml sample (Table 3).

For the A. fumigatus and Aspergillus spp. assays amplification was linear from 5-10000
genomes/0.5ml sample when testing EZ1 extracts (Figure 1a). The PCR efficiency using DNA
extracted from plasma by the EZ1 was 96.3% and 118.5% for the A. fumigatus and Aspergillus spp.
assays, respectively. When testing EasyMag extracts the linear range was also 5-10000
genomes/0.5ml sample for the A. fumigatus assay, but for the Aspergillus spp. assay it was 1-10000
genomes/0.5ml sample (Figure 1b). The PCR efficiency using DNA extracted from plasma by the
EasyMag was 73.8% and 119.9% for the A. fumigatus and Aspergillus spp. assays, respectively. The
linear range for both assays when testing A. terreus DNA extracted by the EasyMag was 1-10000 
genomes/0.5ml sample (Figure 1c). The PCR efficiency testing A. terreus DNA extracted from plasma 
by the EasyMag was 107.3% and 118.3% for the A. terreus and Aspergillus spp. assays, respectively.

Analytical Performance of the AsperGenius® resistance assay.

The 100% LOD for all resistance markers was 50 genomes/0.5ml sample and non-reproducible 
detection was achieved at 25 genomes/0.5ml sample (50-75% reproducibility) 10 genomes/0.5ml 
sample (20% reproducibility). At five genomes/0.5ml sample only the region potentially containing 
the TR34 mutation amplified on 1/5 occasions, all other targets were consistently negative (0/5) at 
this burden. All targets failed to amplify when testing nucleic acid extracted from samples containing 
one genome of A. fumigatus DNA. This information was used to determine a minimum fungal 
burden in a plasma sample that would permit successful amplification of the regions containing the 
potential resistance markers. For reproducible detection of these markers the burden would need to 
be ≥50 genomes/0.5ml sample, corresponding to a Cq value <34 cycles when detecting DNA 
extracted by the EasyMag using the A. fumigatus specific assay. With non-reproducible detection of 
resistance markers expected when testing burdens between five and <50 genomes/0.5ml sample, 
testing A. fumigatus specific positive samples with Cq values between >33 and <39 cycles may result 
in successful amplification of regions potentially harbouring mutations inferring azole resistance.

Clinical Evaluation

There were 86 samples from 12 cases of IA tested, including 10 cases of proven/probable IA (72 
samples) and two cases of possible IA (14 samples). Unfortunately, no cases were culture positive 
and it was not possible to derive a species level of diagnosis. The median number of samples tested 
per case patient was seven (range 6-9). There were 125 samples from 27 patients with no evidence
of invasive fungal disease included as controls; the median number of extracts tested per control patient was five (range 3-5).

The positivity rate associated for samples from proven/probable cases, was 15.3% (11/72; 95% CI: 8.8-23.5) and 25.0% (18/72; 95% CI: 16.4-36.1) for the A. fumigatus and Aspergillus spp. targets, respectively. All 11 A. fumigatus positive results were concomitantly positive by the Aspergillus spp. assay, and there were seven additional positives by the Aspergillus spp. assay (Figure 2). Of the seven additional positive Aspergillus spp. assay results, four were from two patients that also had other samples positive by both A. fumigatus and spp. assays, and three were from two patients that were consistently negative by the A. fumigatus assay (Figure 2). The false positivity rate for samples from controls was 0.0% (0/125; 95% CI: 0.0-3.0) and 4.8% (6/125; 5% CI: 2.2-10.1) for the A. fumigatus and Aspergillus spp. targets, respectively. No samples (n=14) from possible patients (n=2) were positive by either assay. For both the A. fumigatus and Aspergillus spp. assays the true positivity for proven/probable IA cases, was significantly greater than false positivity associated with the control population (A. fumigatus assay: Difference 15.3%, 95% CI: 8.1-25.3, P: <0.0001; Aspergillus spp. assay: Difference 20.2, 95% CI: 10.1-31.6, P: <0.0001). There were two cases of potential non-fumigatus disease but no positive results were generated by the A. terreus specific assay. Given the lower PCR efficiency of the A. fumigatus assay it cannot be confidently determined whether species positive/A. fumigatus negative results represent infection by species other than A. fumigatus. Unfortunately, no culture data was available to provide species level identification.

The mean Cq value for true positive samples was 39.4 (SD: ±4.0) and 35.9 cycles (SD: ±2.5) for the A. fumigatus and spp. assays, respectively. The mean Cq value for Aspergillus spp. false positive results was 37.1 (SD: ±1.4), later than Cq values for true positives, although numbers were limited.

The overall combined clinical performance of the AsperGenius® assay is shown in Table 5. When using a single positive PCR result to define patient positivity only 6/10 proven/probable cases were positive by the A. fumigatus assay, compared to 8/10 by the Aspergillus spp. assay. Conversely,
specificity for the *A. fumigatus* assay was 100% (27/27) compared to 77.8% (21/27) for the
*Aspergillus* spp. assay, and a multiple positive PCR threshold was required to attain 100% specificity
for the latter.

The amplification of regions harbouring potential mutations associated with azole resistance direct
from a sample was only successful for two patients, and neither contained the TR34/L98H or
TR46/T289A/Y121F mutations. Amplification was unsuccessful in a further four probable IA cases.
DISCUSSION

The performance of the PathoNistics AsperGenius® assay for the detection of Aspergillus DNA in plasma samples was satisfactory. Both sensitivity (80%) and specificity (78%) were comparable to that generated by meta-analytical reviews when testing blood, where sensitivity ranged from 84-88% and specificity ranged from 75-76%. In the previous published evaluations of the AsperGenius® assay sensitivity and specificity when testing BAL was 84% and 91%, respectively, and when testing serum it was 79% and 91%, respectively. While sensitivity appears consistent across the specimen type, specificity when testing plasma was compromised, although numbers were limited in all studies. In both the serum and BAL studies optimal positivity thresholds could be defined, and in the case of serum testing a threshold of 39 cycles improved specificity to 100%, without compromising sensitivity. In the current study, it was not possible to generate a threshold as false positive results had Cq values similar to true positive results from cases of aspergillosis. As with serum testing, if more than one sample was positive per patient then specificity was 100%, but sensitivity was duly compromised (Table 5).

In the recent studies of the EAPCRI, it was shown that the analytical and subsequent clinical performance of Aspergillus PCR could be improved by testing plasma over serum. It was hypothesised that when performing the AsperGenius® assay on DNA extracted from plasma an improvement in performance would have been evident. From an analytical performance this was observed, comparing PCR efficiency when testing five microlitres of DNA extracted from serum and plasma using the EZ1 showed the PCR efficiency for the both A. fumigatus and Aspergillus spp. assays improved when testing plasma (A. fumigatus assay serum (72.6%) vs plasma (96.3%); Aspergillus spp. assay serum (106%) vs plasma (118.5%). Conversely, the PCR efficiency for the A. fumigatus assay when testing DNA extracted using the EasyMag was superior for serum (A. fumigatus assay: 97%; Aspergillus spp. assay: 124%) over plasma (A. fumigatus assay: 74%; Aspergillus spp. assay: 120%). High PCR efficiency can be severely compromised by the
quality of the nucleic acid extracted and the necessity to optimise the extraction process for each sample type. However, if the standard-curve of the \textit{A. fumigatus} assay when testing DNA extracted by EasyMag is examined in detail (Figure 1b) it could be argued that the detection of burdens ≤10 genomes/0.5ml sample is outside the linear range of the assay. Removal of these burdens from the standard curve increases the coefficient of determination to 0.99 and PCR efficiency to 90%, comparable to testing DNA extracted from serum by EasyMag.

In a previous study comparing the analytical performance of automated nucleic acid extraction platforms when performing \textit{Aspergillus} PCR, the EasyMag was associated with high quality DNA and subsequent earlier Cq values, but was also associated with \textit{Aspergillus} contamination. The increase in PCR efficiency when testing DNA extracted from plasma by EZ1 was not significantly associated with an improved LOD for either assay, although using the EasyMag extractor and a larger DNA template volume did improve recovery of lower burdens. The reproducibility of detection when testing one genome/0.5ml sample extracted using the EasyMag was 60% (Table 2). There were four false positive \textit{A. terreus} results in the analytical study, whereas false positivity in the clinical study was associated with the \textit{Aspergillus} spp. target. No negative extraction control samples generated false positive results. Given the different identity of the false positivity in the clinical and analytical arms and the low level of overall false positivity it was felt that this was not directly associated with the EasyMag extractor, as previously documented, but represented false positivity typically encountered when testing clinical samples or analytical cross reactivity between \textit{Aspergillus} species.\textsuperscript{15}

For all clinical samples 10μl of EasyMag extract was used for PCR amplification. This did not result in improved clinical performance, with no significant improvement in sensitivity but a reduction in specificity meaning the diagnostic odds ratio was less when testing plasma over serum. One potential explanation for this unexpected result is that while the use of the larger volume potentially increased the reproducibility of detection of the lower burdens (<10 genomes/0.5ml sample) these
low concentrations are more likely to be affected by sample degradation. Given the retrospective
nature of the study, it is hypothesised that samples containing low burdens had degraded to below
detectable levels minimising any benefits associated with using a larger template volume.
A second explanation for the lack of improvement in clinical performance is although the larger
input volume increases the opportunity for detecting target DNA it also increases the potential for
the presence of inhibitory compounds. Only two extractions exhibited total inhibition (no IC signal
present), a further three generated Cq values that were later than upper limit generated by the
manufacturer indicating a degree of partial inhibition. Of concern when interpreting the IC when
testing plasma or serum is the relative high concentration of IC in respect to typical *Aspergillus* PCR
positives in blood, and the subsequent acceptable IC Cq range proposed by the manufacturer. The
acceptable Cq values for the IC range between 29.5-35.0 cycles, in this study 86.2% (182/211) of
samples had an IC Cq value within this range, with 2.4% (5/211) of samples exhibiting partial or total
inhibition (Cq >35.0 cycles). A further 11.4% (24/211) of samples had an IC Cq value below the lower
acceptable limit (range: 26.1-29.4 cycles) and while this cannot represent inhibition it questions the
robustness of the IC PCR when testing DNA template input volumes greater than the 5μl
recommended by the manufacturer. This diversity (median IC Cq: 33.6 cycles, range: 26.1-36.4
cycles) makes it difficult to determine a typical (expected) reference value from which inhibition in
specimens can be derived. The relatively high IC concentrations, was developed for use with BAL
samples where fungal burdens will be greater and earlier Cq values generated. Consequently, the
typical IC value is significantly lower than that for *Aspergillus* PCR positives when testing serum and
plasma samples (typically >35 cycles). As such the effect of any inhibitory compounds on the IC PCR
may be less evident than that experienced on a clinical plasma sample where an inhibitory delay of
2-3 cycles will result in PCR negativity, but keep the IC Cq within the manufacturer’s acceptable
range resulting in potential false negative results.
In addition to inhibitory compounds the presence of interfering substances should also be considered. In a previous EAPCRI study the presence of fibrinogen in plasma was proposed to have the potential to influence magnesium concentration, which is critical to optimal PCR performance. It is possible that fibrinogen is present in nucleic acid eluates and this could interfere with PCR amplification. Using the larger input volume this could have affected the performance of the AsperGenius® assay and could explain the wide ranging IC Cq values, explaining why the mean Cq values for 2/5 simulated samples extracted using the Qiagen EZ1 and amplification performed using 10µl of template had very late IC Cq values (Table 1).

Although the use of 10µl of EasyMag eluate improved the detection of low burdens PCR efficiency using 10µl template was not calculated as the range of burdens tested using a 10µl input was limited to 1log. A further limitation of the study it was not possible to perform a direct comparison with the previous serum study and the samples included were different. With hindsight it may have been wise to perform the plasma testing using five microlitres of template, as the improvement in efficiency over serum was confirmed and this volume was used for the previous serum study. Currently, the AsperGenius® assay is only validated for in vitro diagnostic testing of BAL samples and this has implications when interpreting positive results from blood samples. The positivity threshold for the species assay when testing BAL is <36 cycles, when testing blood this is likely too early with a median Cq of 35.9 cycles for clinical PCR positives and 11/18 (61.1%) having Cq values ≥36.0 cycles. It is important to remember that when testing blood specimens by Aspergillus PCR the strategy is to exclude disease using a negative result generated by frequent screening with a highly sensitive assay, subsequently a Cq threshold is not essential, albeit at the expense of false positive results.

The regions potentially associated with azole resistance were only successfully amplified from two cases of IA. Given the costs associated with both the AsperGenius® species multiplex (Approx. $1000/50 reactions) and the AsperGenius® resistance multiplex (Approx. $1600/50 reactions of both species and resistance multiplex) it may be difficult to justify the costs associated with direct from
plasma resistance testing. However, if direct resistance testing was only applied to samples strongly
positive by the species assay then wastage associated through failed amplification could be limited.

Costs for screening with the species assay could be offset by reductions in the unnecessary use of
antifungal therapy, as seen in other studies where *Aspergillus* PCR, in combination with
galactomannan ELISA, was shown to reduce empirical therapy. ¹⁶⁻¹⁸

To conclude, the PathoNostics AsperGenius® assay can be used to perform PCR testing on plasma
and will provide performance that is comparable to testing serum. Unexpectedly, the predicted
improvements in clinical performance associated with plasma testing were not seen, possibly a
result of the retrospective study design or the impact of larger concentration of
inhibitory/interfering compounds. Considering the latter, the current IC for the PathoNostics
AsperGenius® assay showed too much variability to confidently predict inhibition, although this
could be a result of using a larger template volume. The study also highlights the necessity to
individually evaluate PCR assays when testing different specimen types. Assays will have varying
master-mix compositions and reaction kinetics, which may not be optimal across samples and
subsequent eluate make-up. The clinical utility of commercially available *Aspergillus* PCR assays,
such the AsperGenius® assay, require prospective evaluation with particular reference to the impact
of potential early diagnosis of azole-resistant disease on patient management.

**Conflicts of Interest**

**PLW** is a founding member of the EAPCRI, received project funding from Myconostica, Luminex,
Renishaw diagnostics and Bruker, was sponsored by Myconostica, MSD, Launch, Bruker and Gilead
Sciences to attend international meetings, provided consultancy for Renishaw Diagnostics Limited
and is a member of the advisory board and speaker bureau for Gilead Sciences.

**RAB** is a founding member of the EAPCRI, received an educational grant and scientific fellowship
award from Gilead Sciences and Pfizer, is a member of the advisory board and speaker bureau for
Gilead Sciences, MSD, Astellas, and Pfizer, and was sponsored by Gilead Sciences and Pfizer to attend international meetings.

RBP has no conflicts of interest.
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Table 1. Analytical performance of the PathoNostics AsperGenius® species assay when testing *A. fumigatus* genomic DNA extracted from plasma samples using the Qiagen EZ1 Advance XL instrument.

<table>
<thead>
<tr>
<th>Fungal load (genomes/0.5ml sample)</th>
<th><em>A. fumigatus</em></th>
<th><em>Aspergillus</em> spp.</th>
<th><em>A. terreus</em></th>
<th>Internal Control</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Positives/total</td>
<td>Mean C&lt;sub&gt;q&lt;/sub&gt; (SD)</td>
<td>Positives/total</td>
<td>Mean C&lt;sub&gt;q&lt;/sub&gt; (SD)</td>
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<td>DNA template Volume: μL</td>
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<td>0/5</td>
<td>-</td>
</tr>
</tbody>
</table>

* One sample was deemed inhibitory to PCR amplification, as such only 12 replicates were included in the analysis of the *A. fumigatus*, *A. terreus* and *Aspergillus* species assays, whereas 12/13 replicates are shown for the corresponding internal control PCR.
Table 2. Analytical performance of the PathoNostics AsperGenius® species assay when testing *A. fumigatus* genomic DNA extracted from plasma samples using the BioMerieux EasyMag instrument.

<table>
<thead>
<tr>
<th>Fungal load (genomes/0.5ml sample)</th>
<th><em>A. fumigatus</em></th>
<th><em>Aspergillus</em> spp.</th>
<th><em>A. terreus</em></th>
<th>Internal Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positives/total</td>
<td>Mean C&lt;sub&gt;q&lt;/sub&gt; (SD)</td>
<td>Positives/total</td>
<td>Mean C&lt;sub&gt;q&lt;/sub&gt; (SD)</td>
</tr>
<tr>
<td>10000</td>
<td>1/1</td>
<td>24.72</td>
<td>1/1</td>
<td>23.86</td>
</tr>
<tr>
<td>1000</td>
<td>1/1</td>
<td>27.93</td>
<td>1/1</td>
<td>26.55</td>
</tr>
<tr>
<td>500</td>
<td>1/1</td>
<td>28.85</td>
<td>1/1</td>
<td>27.23</td>
</tr>
<tr>
<td>100</td>
<td>1/1</td>
<td>31.26</td>
<td>1/1</td>
<td>29.43</td>
</tr>
<tr>
<td>50</td>
<td>3/3</td>
<td>33.24 (0.98)</td>
<td>3/3</td>
<td>30.75 (0.81)</td>
</tr>
<tr>
<td>25</td>
<td>3/3</td>
<td>34.31 (1.79)</td>
<td>3/3</td>
<td>31.20 (0.61)</td>
</tr>
<tr>
<td>10</td>
<td>5/5</td>
<td>37.5 (1.63)</td>
<td>5/5</td>
<td>32.5 (0.41)</td>
</tr>
<tr>
<td>5</td>
<td>5/5</td>
<td>38.0 (2.66)</td>
<td>5/5</td>
<td>33.28 (0.73)</td>
</tr>
<tr>
<td>1</td>
<td>0/5</td>
<td>-</td>
<td>1/5</td>
<td>35.35</td>
</tr>
<tr>
<td>0</td>
<td>0/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
</tr>
<tr>
<td>DNA template Volume: 30 μL</td>
<td>10</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>---------------------------</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>1/1</td>
<td>5/5</td>
<td>3/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>35.82</td>
<td>38.65 (1.90)</td>
<td>40.67 (2.14)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>34.46</td>
<td>35.80 (1.15)</td>
<td>36.94 (1.45)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>33.15</td>
<td>30.56 (1.00)</td>
<td>31.59 (1.77)</td>
<td>32.60 (1.25)</td>
</tr>
<tr>
<td>Fungal load (genomes/0.5ml sample)</td>
<td>A. fumigatus</td>
<td>Aspergillus spp.</td>
<td>A. terreus</td>
<td>Internal Control</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>--------------</td>
<td>----------------</td>
<td>-----------</td>
<td>-----------------</td>
</tr>
<tr>
<td>0/1 Positives/total Mean C_q (SD)</td>
<td>1/1 24.30</td>
<td>1/1 26.10</td>
<td>1/1 30.21</td>
<td></td>
</tr>
<tr>
<td>0/1 Positives/total Mean C_q (SD)</td>
<td>1/1 27.40</td>
<td>1/1 29.14</td>
<td>1/1 32.49</td>
<td></td>
</tr>
<tr>
<td>0/1 Positives/total Mean C_q (SD)</td>
<td>1/1 27.98</td>
<td>1/1 30.23</td>
<td>1/1 33.37</td>
<td></td>
</tr>
<tr>
<td>0/1 Positives/total Mean C_q (SD)</td>
<td>2/2 30.14 (0.04)</td>
<td>2/2 32.48 (0.05)</td>
<td>2/2 33.82 (0.21)</td>
<td></td>
</tr>
<tr>
<td>0/1 Positives/total Mean C_q (SD)</td>
<td>2/2 30.34 (0.38)</td>
<td>2/2 32.69 (0.39)</td>
<td>2/2 30.86 (1.51)</td>
<td></td>
</tr>
<tr>
<td>0/1 Positives/total Mean C_q (SD)</td>
<td>2/2 31.30 (0.18)</td>
<td>2/2 33.67 (0.06)</td>
<td>2/2 33.43 (0.36)</td>
<td></td>
</tr>
<tr>
<td>0/1 Positives/total Mean C_q (SD)</td>
<td>3/3 31.87 (0.34)</td>
<td>3/3 34.27 (0.35)</td>
<td>3/3 32.71 (1.38)</td>
<td></td>
</tr>
<tr>
<td>0/1 Positives/total Mean C_q (SD)</td>
<td>3/3 33.39 (0.16)</td>
<td>3/3 36.16 (0.49)</td>
<td>3/3 32.74 (0.53)</td>
<td></td>
</tr>
<tr>
<td>0/1 Positives/total Mean C_q (SD)</td>
<td>3/3 34.33 (0.38)</td>
<td>3/3 36.89 (0.29)</td>
<td>3/3 34.22 (0.45)</td>
<td></td>
</tr>
<tr>
<td>0/1 Positives/total Mean C_q (SD)</td>
<td>1/3 35.61</td>
<td>1/3 38.18</td>
<td>3/3 33.55 (0.69)</td>
<td></td>
</tr>
<tr>
<td>0/1 Positives/total Mean C_q (SD)</td>
<td>0/3 -</td>
<td>0/3 -</td>
<td>3/3 33.59 (1.59)</td>
<td></td>
</tr>
<tr>
<td>DNA template volume: 10 μl</td>
<td>10</td>
<td>0/3</td>
<td>-</td>
<td>3/3</td>
</tr>
<tr>
<td>--------------------------</td>
<td>----</td>
<td>-----</td>
<td>---</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0/3</td>
<td>-</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0/3</td>
<td>-</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0/3</td>
<td>-</td>
<td>0/3</td>
</tr>
</tbody>
</table>
### Table 4. Patient demographics and diagnosis of IA according to the revised EORTC-MSG definitions

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Proven/Probable IA (n=10)</th>
<th>Possible IA (=2)</th>
<th>NEF (n=27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/Female</td>
<td>6/4</td>
<td>1/1</td>
<td>15/12</td>
</tr>
<tr>
<td>Median age (range)</td>
<td>60.5 (25-74)</td>
<td>- (18-51)</td>
<td>56 (21-76)</td>
</tr>
<tr>
<td>Underlying condition (N)</td>
<td>AML (7)</td>
<td>AML (1)</td>
<td>AML (17)</td>
</tr>
<tr>
<td></td>
<td>ALL (2)</td>
<td>ALL (1)</td>
<td>Lymphoma (6)</td>
</tr>
<tr>
<td></td>
<td>MDS (1)</td>
<td>AA (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALL (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MDS (1)</td>
<td></td>
</tr>
<tr>
<td>Allogeneic Stem cell</td>
<td>6</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>transplantation (N)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fungal Prophylaxis (N)</td>
<td>Fluconazole (9)</td>
<td>Fluconazole (2)</td>
<td>Fluconazole (15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Voriconazole (1)</td>
</tr>
<tr>
<td>Fungal Disease</td>
<td>Proven <em>Aspergillus</em> Sinusitis (1)</td>
<td>Possible IPA (2)</td>
<td>N/A</td>
</tr>
<tr>
<td>Manifestation (N)</td>
<td>Probable IPA (6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probable IPA/Sinusitis (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probable Sinusitis (1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key:
- **AA**: Aplastic Anaemia
- **AML**: Acute Myeloid Leukaemia
- **ALL**: Acute lymphoblastic Leukaemia
- **MDS**: Myelodysplastic syndrome
- **Lymphoma**: Hodgkins, Non-Hodgkins and Burkitts Lymphoma
- **IPA**: Invasive pulmonary aspergillosis
- **N/A**: Not applicable
Table 5. Clinical Performance of AsperGenius® Species assay when testing serum from haematology with proven/probable IA (n=10), possible IA (n=2) and with no evidence of fungal disease (NEF, n=27). Performance represents a combination of results for the A. fumigatus specific and the broad range Aspergillus species assays, as in a clinical scenario a positive result in either assay would carry significance.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proven/Probable IA vs NEF</td>
</tr>
<tr>
<td></td>
<td>Single Positive</td>
</tr>
<tr>
<td></td>
<td>Threshold</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>8/10, (n/N, %, 95% CI)</td>
</tr>
<tr>
<td>Specificity</td>
<td>21/27, (n/N, %, 95% CI)</td>
</tr>
<tr>
<td>LR +tive</td>
<td>3.6</td>
</tr>
<tr>
<td>LR -tive</td>
<td>0.26</td>
</tr>
<tr>
<td>DOR</td>
<td>14.0</td>
</tr>
</tbody>
</table>

*To overcome infinity the parameter determined using a specificity value of 99.9%

Key:
IA: Invasive Aspergillosis
NEF: No evidence of fungal disease
LR: Likelihood ratio
DOR: Diagnostic odds ratio
Figure 1. Standard curves for the PathoNostics AsperGenius® A. fumigatus and Aspergillus species assays testing A. fumigatus genomic DNA extracted from plasma samples by a) Qiagen EZ1 and b) BioMerieux EasyMag automated extractors, and c) the A. terreus and Aspergillus species assays testing A. terreus genomic DNA extracted from plasma samples by BioMerieux EasyMag automated extractor.
C.

Quantiﬁcation Results for C. elegans and Aspergillus spp.

For C. elegans, the quantiﬁcation cycle (Cq) is given by the equation:

\[ y = -1.372\ln(x) + 38.783 \]

with a correlation coefﬁcient of \( R^2 = 0.9929 \).

For Aspergillus spp., the quantiﬁcation cycle (Cq) is given by the equation:

\[ y = -1.281\ln(x) + 36.049 \]

with a correlation coefﬁcient of \( R^2 = 0.9951 \).
Figure 2. PathoNostics AsperGenius® PCR positivity according to sampling for the cases of proven/probable invasive aspergillosis. Grey cells represent positive results with the number representing the corresponding Cq value.

<table>
<thead>
<tr>
<th>Patient (EORTC/MSG diagnosis)</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Afumi</td>
</tr>
<tr>
<td>1 (Probable IA)</td>
<td>-</td>
</tr>
<tr>
<td>2 (Probable IA)</td>
<td>-</td>
</tr>
<tr>
<td>3 (Probable IA)</td>
<td>-</td>
</tr>
<tr>
<td>4 (Probable IA)</td>
<td>-</td>
</tr>
<tr>
<td>5 (Prob Asp Sin)</td>
<td>37.7</td>
</tr>
<tr>
<td>6 (Probable IA)</td>
<td>-</td>
</tr>
<tr>
<td>7 (Probable IA*)</td>
<td>-</td>
</tr>
<tr>
<td>8 (Probable IA)</td>
<td>34.3</td>
</tr>
<tr>
<td>9 (Probable IA)</td>
<td>-</td>
</tr>
<tr>
<td>10 (Prob Asp Sin)</td>
<td>-</td>
</tr>
</tbody>
</table>

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Key:

Afumi: Pathonostics AsperGenius® *A. fumigatus* assay

Asp: PathoNostics AsperGenius® Species assay

IA: Invasive aspergillosis

Probable IA*: Patient had a total of nine samples tested, the one additional sample tested was negative by both the *A. fumigatus* and species assays and was the last sample to be tested. It was excluded to avoid presentation difficulties.

Prob Asp Sin: Probable *Aspergillus* sinusitis

Prov Asp Sin: Proven *Aspergillus* sinusitis

NT: No sample tested

-: Assay was negative