1 **Title:** An analytical and clinical evaluation of the PathoNostics AsperGenius® Assay for the detection

2 of invasive aspergillosis and resistance to azole antifungal drugs direct from plasma samples.

3

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15

16 **ABSTRACT**

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

24 For the analytical evaluations plasma was spiked with various concentrations of *Aspergillus* genomic
25 DNA before extraction following international recommendations using two automated platforms.

26 For the clinical study, 211 samples from 10 proven/probable IA and 2 possible IA cases, and 27
27 controls were tested.

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

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

37 **INTRODUCTION**

38 Standardisation of *Aspergillus* PCR testing of blood based samples has led to the proposal to include
39 *Aspergillus* PCR in to the second revision of the EORTC/MSG consensus definitions for invasive fungal
40 disease (IFD). [1-4] This may increase demand for *Aspergillus* PCR, as it can be used, in combination
41 with other biomarker assays (Galactomannan EIA and β-D-Glucan) to improve management of
42 patients at risk of invasive aspergillosis (IA). [5] Easily attainable, quality controlled and well validated
43 assays are necessary, and commercially developed assays help in achieving these requirements.
44 Several commercial *Aspergillus* PCR assays have been developed (MycAssay Aspergillus, Renishaw
45 Fungiplex, Ademtech MycoGENIE, PathoNostics AsperGenius®) with varying degrees of clinical
46 validation. [6-10] Of particular interest, given the emergence of azole resistant strains of *A. fumigatus*,
47 are the Ademtech MycoGENIE and PathoNostics AsperGenius® assays that have the ability to detect
48 the major single nucleotide polymorphisms that infer environmentally driven resistance. Tests to
49 detect genetic mechanisms of azole resistance have been applied directly to clinical samples and
50 have the potential to overcome the limited sensitivity of conventional culture techniques. [7, 8] The
51 application of these tests to non-invasive sample types (e.g. blood) will improve clinical utility and
52 some success has been noted when testing serum. [7]
53 Recently, the European *Aspergillus* PCR initiative showed that both the analytical and clinical
54 performance of *Aspergillus* PCR was superior when testing plasma compared to serum. [3, 4] It was
55 proposed that using plasma avoided DNA trapping during clot formation, subsequently the available
56 target was greater and performance enhanced. In the previous evaluation of the PathoNostics
57 AsperGenius® assay when testing serum the sensitivity and specificity were 79% and 91%,
58 respectively, and genetic screening for resistance direct from the sample was obtained in 50% of the
59 cases.[7] It is hypothesised the testing of plasma may improve the performance of the AsperGenius®
60 assay. Nevertheless, validation when testing plasma is required to enhance the application range
61 and assay robustness.

62 This manuscript determines the analytical and clinical performance of the PathoNostics

63 AsperGenius® assay when testing plasma samples using methods in line with international

64 recommendations. [4]

65 **MATERIALS AND METHODS**

66 **Study design**

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

71

72 **Analytical Study**

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

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

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

88 **Clinical Study and Patient Population**

89 Clinical plasma samples from patients with proven, probable, possible IA, or with no evidence of
90 fungal disease (NEF) were selected. All samples had been sent as part of the care pathway
91 incorporating a well-validated "in-house" *Aspergillus* PCR. [11, 12] On completion of routine testing
92 plasma was stored at -80˚C for quality control or performance assessment purposes. The study was a
93 performance assessment of the AsperGenius® Assay and was an anonymous, retrospective
94 case/control design, not effecting patient management. Patient demographics are shown in Table 4.
95 Nucleic acid was extracted from 0.5ml of plasma using the BioMerieux EasyMag Generic 2.01
96 Protocol, following the manufacturer's instructions, with DNA eluted in 60µl. Positive (plasma
97 containing 10 genomes of *A. fumigatus* DNA) and negative (plasma only) extraction controls were
98 included in each run.

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

101

102 **PathoNostics AsperGenius® PCR amplification**

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

110 **Statistical Evaluation**

111 Analytical analysis of the AsperGenius® species PCR when testing plasma samples was performed as
112 previously described. [7] Briefly, the 100% LOD, linearity ranges and PCR amplification efficiencies
113 were calculated. Further analysis was performed correlating AsperGenius® species and resistance
114 performance so that the quantification cycle (Cq) generated by the *A. fumigatus* assay could be used
115 as a guide to the likelihood of success when performing the resistance assay.

116 When determining the clinical accuracy of the AsperGenius® species results the positivity rate in
117 samples originating from cases was compared to the false positivity rate in control samples. Clinical
118 performance was determined by the construction of 2x2 tables to calculate sensitivity, specificity,
119 positive and negative likelihood ratios and diagnostic odds ratio of the AsperGenius® species assay.
120 For all patients, only a single positive sample was required to consider the patient positive. Given the
121 case control study design, and artificially high prevalence of proven/probable IA (25.6%), predictive
122 values were not used. When required ninety-five percent confidence intervals and, *P* values (Fishers
123 exact test; *P*: 0.05) were generated to determine the significance of the difference between rates.

124

125 **RESULTS**

126 **Analytical Performance of the AsperGenius® species assay.**

127 When extracting DNA from plasma using the Qiagen EZ1 DSP virus kit the LOD for both the *A.*
128 *fumigatus* specific and *Aspergillus* species assays was 25 genomes/0.5ml sample using a 5µl
129 template input and 10 genomes/0.5ml sample using a 10µl template input volume (Table 1).
130 Increasing the amount of DNA template also improved reproducibility when detecting
131 5genomes/0.5ml sample but did not improve detection of 1 genome/0.5ml sample.
132 Using the BioMerieux EasyMag for DNA extraction, the LOD using a five microlitre template input for
133 both the *A. fumigatus* and *Aspergillus* spp. assays improved to five genomes/0.5ml sample,
134 compared to the equivalent volume of eluate extracted by the Qiagen EZ1 DSP virus kit (Tables 1 and
135 2). However, 4/31 replicates across all burdens generated a low level false positive *A. terreus* result
136 (Mean Ct: 42.4). Increasing the template input volume to 10µl did not improve the 100% LOD, but
137 reproducibility when detecting one genome/0.5ml sample was improved (*A. fumigatus* assay 10µl
138 template: 3/5 vs 5µl template 0/5; *Aspergillus* spp. assay 10µl template: 3/5 vs 5µl template 1/5).
139 When using the BioMerieux EasyMag to extract *A. terreus* DNA from plasma the LOD for both the *A.*
140 *terreus* specific and *Aspergillus* species targets was five genomes/0.5ml sample using 5 µl of DNA
141 template, at one genome/0.5ml sample reproducibility for both targets was 33.3%. Increasing the
142 input to 10µl per reaction lowered the LOD to one genome/0.5ml sample (Table 3).
143 For the *A. fumigatus* and *Aspergillus* spp. assays amplification was linear from 5-10000
144 genomes/0.5ml sample when testing EZ1 extracts (Figure 1a). The PCR efficiency using DNA
145 extracted from plasma by the EZ1 was 96.3% and 118.5% for the *A. fumigatus* and *Aspergillus* spp.
146 assays, respectively. When testing EasyMag extracts the linear range was also 5-10000
147 genomes/0.5ml sample for the *A. fumigatus* assay, but for the *Aspergillus* spp. assay it was 1-10000
148 genomes/0.5ml sample (Figure 1b). The PCR efficiency using DNA extracted from plasma by the
149 EasyMag was 73.8% and 119.9% for the *A. fumigatus* and *Aspergillus* spp. assays, respectively. The

150 linear range for both assays when testing *A. terreus* DNA extracted by the EasyMag was 1-10000
151 genomes/0.5ml sample (Figure 1c). The PCR efficiency testing *A. terreus* DNA extracted from plasma
152 by the EasyMag was 107.3% and 118.3% for the *A. terreus* and *Aspergillus* spp. assays, respectively.
153
154 **Analytical Performance of the AsperGenius® resistance assay.**
155 The 100% LOD for all resistance markers was 50 genomes/0.5ml sample and non-reproducible
156 detection was achieved at 25 genomes/0.5ml sample (50-75% reproducibility) 10 genomes/0.5ml
157 sample (20% reproducibility). At five genomes/0.5ml sample only the region potentially containing
158 the TR34 mutation amplified on 1/5 occasions, all other targets were consistently negative (0/5) at
159 this burden. All targets failed to amplify when testing nucleic acid extracted from samples containing
160 one genome of *A. fumigatus* DNA. This information was used to determine a minimum fungal
161 burden in a plasma sample that would permit successful amplification of the regions containing the
162 potential resistance markers. For reproducible detection of these markers the burden would need to
163 be ≥50 genomes/0.5ml sample, corresponding to a Cq value <34 cycles when detecting DNA
164 extracted by the EasyMag using the *A. fumigatus* specific assay. With non-reproducible detection of
165 resistance markers expected when testing burdens between five and <50 genomes/0.5ml sample,
166 testing *A. fumigatus* specific positive samples with Cq values between >33 and <39 cycles may result
167 in successful amplification of regions potentially harbouring mutations inferring azole resistance.
168
169 **Clinical Evaluation**
170 There were 86 samples from 12 cases of IA tested, including 10 cases of proven/probable IA (72
171 samples) and two cases of possible IA (14 samples). Unfortunately, no cases were culture positive
172 and it was not possible to derive a species level of diagnosis. The median number of samples tested
173 per case patient was seven (range 6-9). There were 125 samples from 27 patients with no evidence

174 of invasive fungal disease included as controls; the median number of extracts tested per control
175 patient was five (range 3-5).
176 The positivity rate associated for samples from proven/probable cases, was 15.3% (11/72; 95% CI:
177 8.8-23.5) and 25.0% (18/72; 95% CI: 16.4-36.1) for the *A. fumigatus* and *Aspergillus* spp. targets,
178 respectively. All 11 *A. fumigatus* positive results were concomitantly positive by the *Aspergillus* spp.
179 assay, and there were seven additional positives by the *Aspergillus* spp. assay (Figure 2). Of the
180 seven additional positive *Aspergillus* spp. assay results, four were from two patients that also had
181 other samples positive by both *A. fumigatus* and spp. assays, and three were from two patients that
182 were consistently negative by the *A. fumigatus* assay (Figure 2). The false positivity rate for samples
183 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.*
184 *fumigatus* and *Aspergillus* spp. targets, respectively. No samples (n=14) from possible patients (n=2)
185 were positive by either assay. For both the *A. fumigatus* and *Aspergillus* spp. assays the true
186 positivity for proven/probable IA cases, was significantly greater than false positivity associated with
187 the control population (*A. fumigatus* assay: Difference 15.3%, 95% CI: 8.1-25.3, *P*: <0.0001;
188 *Aspergillus* spp. assay: Difference 20.2, 95% CI: 10.1-31.6, *P*: <0.0001). There were two cases of
189 potential non-*fumigatus* disease but no positive results were generated by the *A. terreus* specific
190 assay. Given the lower PCR efficiency of the *A. fumigatus* assay it cannot be confidently determined
191 whether species positive/*A.* fumigatus negative results represent infection by species other than *A.*
192 *fumigatus*. Unfortunately, no culture data was available to provide species level identification.
193 The mean Cq value for true positive samples was 39.4 (SD: ±4.0) and 35.9 cycles (SD: ±2.5) for the *A.*
194 *fumigatus* and spp. assays, respectively. The mean Cq value for *Aspergillus* spp. false positive results
195 was 37.1 (SD: ±1.4), later than Cq values for true positives, although numbers were limited.
196 The overall combined clinical performance of the AsperGenius® assay is shown in Table 5. When
197 using a single positive PCR result to define patient positivity only 6/10 proven/probable cases were
198 positive by the *A. fumigatus* assay, compared to 8/10 by the *Aspergillus* spp. assay. Conversely,

199 specificity for the *A. fumigatus* assay was 100% (27/27) compared to 77.8% (21/27) for the

200 *Aspergillus* spp. assay, and a multiple positive PCR threshold was required to attain 100% specificity

201 for the latter.

202 The amplification of regions harbouring potential mutations associated with azole resistance direct

203 from a sample was only successful for two patients, and neither contained the TR34/L98H or

204 TR46/T289A/Y121F mutations. Amplification was unsuccessful in a further four probable IA cases.

205

206 **DISCUSSION**

207 The performance of the PathoNostics AsperGenius® assay for the detection of *Aspergillus* DNA in
208 plasma samples was satisfactory. Both sensitivity (80%) and specificity (78%) were comparable to
209 that generated by meta-analytical reviews when testing blood, where sensitivity ranged from 84-
210 88% and specificity ranged from 75-76%. [13, 14] In the previous published evaluations of the
211 AsperGenius® assay sensitivity and specificity when testing BAL was 84% and 91%, respectively, and
212 when testing serum it was 79% and 91%, respectively. [7, 8] While sensitivity appears consistent across
213 the specimen type, specificity when testing plasma was compromised, although numbers were
214 limited in all studies. In both the serum and BAL studies optimal positivity thresholds could be
215 defined, and in the case of serum testing a threshold of 39 cycles improved specificity to 100%,
216 without compromising sensitivity. [7, 8] In the current study, it was not possible to generate a threshold
217 as false positive results had Cq values similar to true positive results from cases of aspergillosis. As
218 with serum testing, if more than one sample was positive per patient then specificity was 100%, but
219 sensitivity was duly compromised (Table 5). [7]

220 In the recent studies of the EAPCRI, it was shown that the analytical and subsequent clinical
221 performance of *Aspergillus PCR* could be improved by testing plasma over serum. [3, 4] It was
222 hypothesised that when performing the AsperGenius® assay on DNA extracted from plasma an
223 improvement in performance would have been evident. From an analytical performance this was
224 observed, comparing PCR efficiency when testing five microlitres of DNA extracted from serum and
225 plasma using the EZ1 showed the PCR efficiency for the both *A. fumigatus* and *Aspergillus* spp.
226 assays improved when testing plasma (*A. fumigatus* assay serum (72.6%) vs plasma (96.3%);
227 *Aspergillus* spp. assay serum (106%) vs plasma (118.5%).[7] Conversely, the PCR efficiency for the *A.*
228 *fumigatus* assay when testing DNA extracted using the EasyMag was superior for serum (*A.*
229 *fumigatus* assay: 97%; *Aspergillus* spp. assay: 124%) over plasma (*A. fumigatus* assay: 74%;
230 *Aspergillus* spp. assay: 120%). [7] Highlighting that PCR efficiency can be severely compromised by the

231 quality of the nucleic acid extracted and the necessity to optimise the extraction process for each
232 sample type. However, if the standard-curve of the *A. fumigatus* assay when testing DNA extracted
233 by EasyMag is examined in detail (Figure 1b) it could be argued that the detection of burdens
234 ≤10genomes/0.5ml sample is outside the linear range of the assay. Removal of these burdens from
235 the standard curve increases the coefficient of determination to 0.99 and PCR efficiency to 90%,
236 comparable to testing DNA extracted from serum by EasyMag.
237 In a previous study comparing the analytical performance of automated nucleic acid extraction
238 platforms when performing *Aspergillus* PCR, the EasyMag was associated with high quality DNA and
239 subsequent earlier Cq values, but was also associated with *Aspergillus* contamination. [15] The increase
240 in PCR efficiency when testing DNA extracted from plasma by EZ1 was not significantly associated
241 with an improved LOD for either assay, although using the EasyMag extractor and a larger DNA
242 template volume did improve recovery of lower burdens. The reproducibility of detection when
243 testing one genome/0.5ml sample extracted using the EasyMag was 60% (Table 2). There were four
244 false positive *A. terreus* results in the analytical study, whereas false positivity in the clinical study
245 was associated with the *Aspergillus* spp. target. No negative extraction control samples generated
246 false positive results. Given the different identity of the false positivity in the clinical and analytical
247 arms and the low level of overall false positivity it was felt that this was not directly associated with
248 the EasyMag extractor, as previously documented, but represented false positivity typically
249 encountered when testing clinical samples or analytical cross reactivity between *Aspergillus*
250 species.[15]
251 For all clinical samples 10µl of EasyMag extract was used for PCR amplification. This did not result in
252 improved clinical performance, with no significant improvement in sensitivity but a reduction in
253 specificity meaning the diagnostic odds ratio was less when testing plasma over serum. One
254 potential explanation for this unexpected result is that while the use of the larger volume potentially
255 increased the reproducibility of detection of the lower burdens (<10 genomes/0.5ml sample) these

256 low concentrations are more likely to be affected by sample degradation. Given the retrospective
257 nature of the study, it is hypothesised that samples containing low burdens had degraded to below
258 detectable levels minimising any benefits associated with using a larger template volume.
259 A second explanation for the lack of improvement in clinical performance is although the larger
260 input volume increases the opportunity for detecting target DNA it also increases the potential for
261 the presence of inhibitory compounds. Only two extractions exhibited total inhibition (no IC signal
262 present), a further three generated Cq values that were later than upper limit generated by the
263 manufacturer indicating a degree of partial inhibition. Of concern when interpreting the IC when
264 testing plasma or serum is the relative high concentration of IC in respect to typical *Aspergillus* PCR
265 positives in blood, and the subsequent acceptable IC Cq range proposed by the manufacturer. The
266 acceptable Cq values for the IC range between 29.5-35.0 cycles, in this study 86.2% (182/211) of
267 samples had an IC Cq value within this range, with 2.4% (5/211) of samples exhibiting partial or total
268 inhibition (Cq >35.0 cycles). A further 11.4% (24/211) of samples had an IC Cq value below the lower
269 acceptable limit (range: 26.1-29.4 cycles) and while this cannot represent inhibition it questions the
270 robustness of the IC PCR when testing DNA template input volumes greater than the 5µl
271 recommended by the manufacturer. This diversity (median IC Cq: 33.6 cycles, range: 26.1-36.4
272 cycles) makes it difficult to determine a typical (expected) reference value from which inhibition in
273 specimens can be derived. The relatively high IC concentrations, was developed for use with BAL
274 samples where fungal burdens will be greater and earlier Cq values generated. Consequently, the
275 typical IC value is significantly lower than that for *Aspergillus* PCR positives when testing serum and
276 plasma samples (typically >35 cycles). As such the effect of any inhibitory compounds on the IC PCR
277 may be less evident than that experienced on a clinical plasma sample where an inhibitory delay of
278 2-3 cycles will result in PCR negativity, but keep the IC Cq within the manufacturer's acceptable
279 range resulting in potential false negative results.

280 In addition to inhibitory compounds the presence of interfering substances should also be
281 considered. In a previous EAPCRI study the presence of fibrinogen in plasma was proposed to have
282 the potential to influence magnesium concentration, which is critical to optimal PCR performance. [4]
283 It is possible that fibrinogen is present in nucleic acid eluates and this could interfere with PCR
284 amplification. Using the larger input volume this could have affected the performance of the
285 AsperGenius® assay and could explain the wide ranging IC Cq values, explaining why the mean Cq
286 values for 2/5 simulated samples extracted using the Qiagen EZ1 and amplification performed using
287 10µl of template had very late IC Cq values (Table 1).
288 Although the use of 10µl of EasyMag eluate improved the detection of low burdens PCR efficiency
289 using 10µl template was not calculated as the range of burdens tested using a 10µl input was limited
290 to 1log. A further limitation of the study it was not possible to perform a direct comparison with the
291 previous serum study and the samples included were different. With hindsight it may have been
292 wise to perform the plasma testing using five microlitres of template, as the improvement in
293 efficiency over serum was confirmed and this volume was used for the previous serum study. [7]
294 Currently, the AsperGenius® assay is only validated for in vitro diagnostic testing of BAL samples and
295 this has implications when interpreting positive results from blood samples. The positivity threshold
296 for the species assay when testing BAL is <36 cycles, when testing blood this is likely too early with a
297 median Cq of 35.9 cycles for clinical PCR positives and 11/18 (61.1%) having Cq values ≥36.0 cycles. It
298 is important to remember that when testing blood specimens by *Aspergillus* PCR the strategy is to
299 exclude disease using a negative result generated by frequent screening with a highly sensitive
300 assay, subsequently a Cq threshold is not essential, albeit at the expense of false positive results.
301 The regions potentially associated with azole resistance were only successfully amplified from two
302 cases of IA. Given the costs associated with both the AsperGenius® species multiplex (Approx.
303 $1000/50 reactions) and the AsperGenius® resistance multiplex (Approx. $1600/50 reactions of both
304 species and resistance multiplex) it may be difficult to justify the costs associated with direct from

305 plasma resistance testing. However, if direct resistance testing was only applied to samples strongly
306 positive by the species assay then wastage associated through failed amplification could be limited.
307 Costs for screening with the species assay could be offset by reductions in the unnecessary use of
308 antifungal therapy, as seen in other studies where *Aspergillus* PCR, in combination with
309 galactomannan ELISA, was shown to reduce empirical therapy. [16-18]
310 To conclude, the PathoNostics AsperGenius® assay can be used to perform PCR testing on plasma
311 and will provide performance that is comparable to testing serum. Unexpectedly, the predicted
312 improvements in clinical performance associated with plasma testing were not seen, possibly a
313 result of the retrospective study design or the impact of larger concentration of
314 inhibitory/interfering compounds. Considering the latter, the current IC for the PathoNostics
315 AsperGenius® assay showed too much variability to confidently predict inhibition, although this
316 could be a result of using a larger template volume. The study also highlights the necessity to
317 individually evaluate PCR assays when testing different specimen types. Assays will have varying
318 master-mix compositions and reaction kinetics, which may not be optimal across samples and
319 subsequent eluate make-up. The clinical utility of commercially available *Aspergillus* PCR assays,
320 such the AsperGenius® assay, require prospective evaluation with particular reference to the impact
321 of potential early diagnosis of azole-resistant disease on patient management.
322
323 **Conflicts of Interest**
324 **PLW** is a founding member of the EAPCRI, received project funding from Myconostica, Luminex,
325 Renishaw diagnostics and Bruker, was sponsored by Myconostica, MSD, Launch, Bruker and Gilead
326 Sciences to attend international meetings, provided consultancy for Renishaw Diagnostics Limited
327 and is a member of the advisory board and speaker bureau for Gilead Sciences.
328 **RAB** is a founding member of the EAPCRI, received an educational grant and scientific fellowship
329 award from Gilead Sciences and Pfizer, is a member of the advisory board and speaker bureau for

330 Gilead Sciences, MSD, Astellas, and Pfizer, and was sponsored by Gilead Sciences and Pfizer to

331 attend international meetings.

332 **RBP** has no conflicts of interest

333 **REFERENCES**

334 1) **White PL, Bretagne S, Klingspor L, Melchers WJG, McCulloch E, Schulz B, Finnstrom N,**
335 **Mengoli C, Barnes RA, Donnelly JP, Loeffler J on behalf of the European *Aspergillus* PCR**
336 **Initiative.** 2010. *Aspergillus* PCR: one step closer to standardization. J Clin Microbiol.
337 **48**:1231-1240.

338 2) **White PL, Mengoli C, Bretagne S, Cuenca-Estrella M, Finnstrom N, Klingspor L, Melchers**
339 **WJ, McCulloch E, Barnes RA, Donnelly JP, Loeffler J; European Aspergillus PCR Initiative**
340 **(EAPCRI).** 2011. Evaluation of Aspergillus PCR protocols for testing serum specimens. J Clin
341 Microbiol. **49**:3842-3848.

342 3) **White PL, Barnes RA, Springer J, Klingspor L, Cuenca-Estrella M, Morton CO, Lagrou K,**
343 **Bretagne S, Melchers WJG, Mengoli C, Donnelly JP, Heinz WJ, Loeffler J**. 2015. The clinical
344 performance of Aspergillus PCR when testing serum and plasma - a study by the European
345 Aspergillus PCR Initiative. J Clin Microbiol. **53**:2832-2837.

346 4) **Loeffler J, Mengoli C, Springer J, Bretagne S, Cuenca Estrella M, Klingspor L, Lagrou K,**
347 **Melchers WJG, Morton CO, Barnes RA, Donnelly JP, White PL.** 2015. Analytical comparison
348 of in vitro spiked human serum and plasma for the PCR-based detection of Aspergillus
349 fumigatus DNA – a study by the European Aspergillus PCR Initiative. J Clin Microbiol.
350 **53**:2838-2845.

351 5) **Arvanitis M, Anagnostou T, Mylonakis E.** 2015. Galactomannan and Polymerase Chain
352 Reaction-Based Screening for Invasive Aspergillosis Among High-Risk Hematology Patients: A
353 Diagnostic Meta-analysis. Clin Infect Dis. **61**:1263-1272.

354 6) **White PL, Hibbitts SJ, Perry MD, Green J, Stirling E, Woodford L, McNay G, Stevenson R and**
355 **Barnes RA**. 2014. Evaluation of a commercially developed semi-automated PCR-SERS assay
356 for the diagnosis of invasive fungal disease. J Clin Microbiol. **52**:3536-3543.

357 7) **White PL, Posso RB, Barnes RA.** 2015. Analytical and Clinical Evaluation of the PathoNostics
358 AsperGenius Assay for Detection of Invasive Aspergillosis and Resistance to Azole Antifungal
359 Drugs during Testing of Serum Samples. J Clin Microbiol. **53**:2115-2121.
360 8) **Chong GL, van de Sande WW, Dingemans GJ, Gaajetaan GR, Vonk AG, Hayette MP, van**
361 **Tegelen DW, Simons GF, Rijnders BJ**. 2015. Validation of a New Aspergillus Real-Time PCR
362 Assay for Direct Detection of Aspergillus and Azole Resistance of Aspergillus fumigatus on
363 Bronchoalveolar Lavage Fluid. J Clin Microbiol. **53:**868-874.
364 9) **White PL, Perry MD, Moody A, Follett SA, Morgan G, Barnes RA**. 2011. Evaluation of
365 analytical and preliminary clinical performance of Myconostica MycAssay Aspergillus when
366 testing serum specimens for diagnosis of invasive Aspergillosis. J Clin Microbiol. **49**:2169-
367 2174.
368 10) **Guinea J, Padilla C, Escribano P, Muñoz P, Padilla B, Gijón P, Bouza E**. 2013. Evaluation of
369 MycAssay™ Aspergillus for diagnosis of invasive pulmonary aspergillosis in patients without
370 hematological cancer. PLoS One. **8**:e61545.
371 11) **Barnes RA, Stocking K, Bowden S, Poynton MH, White PL**. 2013. Prevention and diagnosis
372 of invasive fungal disease in high-risk patients within an integrative care pathway. J Infect.
373 **67**:206–214
374 12) **White PL, Linton CJ, Perry MD, Johnson EM, Barnes RA**. 2006. The evolution and evaluation
375 of a whole blood polymerase chain reaction assay for the detection of invasive aspergillosis
376 in hematology patients in a routine clinical setting. Clin Infect Dis. **42**:479-486.
377 13) **Arvanitis M, Ziakas PD, Zacharioudakis IM, Zervou FN, Caliendo AM, Mylonakis E**. 2014.
378 PCR in diagnosis of invasive aspergillosis: a meta-analysis of diagnostic performance. J Clin
379 Microbiol. **52**:3731-3742
380 14) **Mengoli C, Cruciani M, Barnes RA, Loeffler J and Donnelly JP**. 2009. Use of PCR for diagnosis
381 of invasive aspergillosis: systematic review of and meta-analysis. Lancet Infect Dis. **9**:89-96.

382 15) **Perry MD, White PL, Barnes RA**. 2014. Comparison of four automated nucleic acid
383 extraction platforms for the recovery of DNA from Aspergillus fumigatus. J Med Microbiol.
384 **63:**1160-1166.
385 16) **Morrissey CO, Chen SC, Sorrell TC**, **Milliken S, Bardy PG, Bradstock KF, Szer J, Halliday CL,**
386 **Gilroy NM, Moore J, Schwarer AP, Guy S, Bajel A, Tramontana AR, Spelman T, Slavin MA;**
387 **Australasian Leukaemia Lymphoma Group and the Australia and New Zealand Mycology**
388 **Interest Group.** 2013. Galactomannan and PCR versus culture and histology for directing use
389 of antifungal treatment for invasive aspergillosis in high-risk haematology patients: a
390 randomised controlled trial. Lancet Infect Dis. **13**(6):519-28.
391 17) **Aguado JM, Vázquez L, Fernández-Ruiz M, Villaescusa T**, **Ruiz-Camps I, Barba P, Silva JT,**
392 **Batlle M, Solano C, Gallardo D, Heras I, Polo M, Varela R, Vallejo C, Olave T, López-Jiménez**
393 **J, Rovira M, Parody R, Cuenca-Estrella M; PCRAGA Study Group.; Spanish Stem Cell**
394 **Transplantation Group.; Study Group of Medical Mycology of the Spanish Society of**
395 **Clinical Microbiology and Infectious Diseases.; Spanish Network for Research in Infectious**
396 **Diseases.** 2014. Serum galactomannan versus a combination of galactomannan and PCR-
397 based *Aspergillus* DNA detection for early therapy of invasive aspergillosis in high-risk
398 hematological patients: a randomized controlled trial. Clin Infect Dis. **60**(3):405-414.
399 18) **Barnes RA, Stocking K, Bowden S, Poynton MH, White PL**. 2013. Prevention and diagnosis
400 of invasive fungal disease in high-risk patients within an integrative care pathway. J Infect.
401 **67**(3):206-14
402 19) **De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, Pappas PG,**
403 **Maertens J, Lortholary O, Kauffman CA, Denning DW, Patterson TF, Maschmeyer G, Bille J,**
404 **Dismukes WE, Herbrecht R, Hope WW, Kibbler CC, Kullberg BJ, Marr KA, Muñoz P, Odds**
405 **FC, Perfect JR, Restrepo A, Ruhnke M, Segal BH, Sobel JD, Sorrell TC, Viscoli C, Wingard JR,**
406 **Zaoutis T, Bennett JE, European Organization for Research and Treatment of**

407 **Cancer/Invasive Fungal Infections Cooperative Group, National Institute of Allergy and**
408 **Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group.** 2008. Revised
409 definitions of invasive fungal disease from the European Organization for Research and
410 Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National
411 Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus
412 Group. Clin Infect Dis **46**:1813–1821.

413 **Table 1.** Analytical performance of the PathoNostics AsperGenius® species assay when testing *A. fumigatus* genomic DNA extracted from plasma samples

414 using the Qiagen EZ1 Advance XL instrument.

	Fungal load (genomes/ 0.5ml sample)	PathoNostics AsperGenius® target							
		A. fumigatus		*Aspergillus* spp.		*A.terreus*		Internal Control	
		Positives/total	Mean C_q (SD)	Positives/total	Mean C_q (SD)	Positives/total	Mean C_q (SD)	Positives/total	Mean C_q (SD)
DNA template Volume: 5µL	10000	7/7	27.21 (0.63)	7/7	26.18 (0.56)	0/7	-	7/7	31.33 (2.02)
	1000	7/7	30.16 (0.54)	7/7	29.07 (0.37)	0/7	-	7/7	32.57 (2.62)
	500	7/7	31.37 (0.61)	7/7	30.30 (0.35)	0/7	-	7/7	32.79 (2.26)
	100	7/7	34.6 (1.55)	7/7	32.60 (0.40)	0/7	-	7/7	33.53 (2.44)
	75	9/9	34.26 (0.75)	9/9	33.14 (1.23)	0/9	-	9/9	32.65 (2.18)
	50	9/9	35.47 (1.13)	9/9	34.15 (0.70)	0/9	-	9/9	33.19 (2.72)
	25	12/12*	37.29 (1.40)	12/12*	35.41 (1.24)	0/12*	-	12/13*	33.30 (1.87)
	10	11/15	37.62 (1.63)	12/15	35.33 (0.80)	0/15	-	15/15	30.67 (3.82)

	5	4/15	38.94 (1.23)	5/15	36.53 (0.78)	0/15	-	15/15	32.95 (2.41)
	1	3/15	39.82 (1.29)	4/15	37.14(0.78)	0/15	-	15/15	32.39 (2.56)
	0	0/15	-	0/15	-	0/15	-	15/15	30.49 (2.81)
DNA template Volume: 10 µL	25	3/3	37.57 (1.22)	3/3	34.60	3/3	-	3/3	39.14 (1.05)
	10	10/10	38.69 (2.07)	10/10	35.86	10/10	-	10/10	32.45 (1.79)
	5	5/10	42.62 (4.23)	5/10	37.66	5/10	-	10/10	31.73 (2.72)
	1	2/10	40.40 (1.13)	2/10	36.95	2/10	-	10/10	32.09 (2.15)
	0	0/5	-	0/5	-	0/5	-	5/5	38.99 (3.08)

415 ***** 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

416 *Aspergillus* species assays, whereas 12/13 replicates are shown for the corresponding internal control PCR.

417

418 **Table 2.** Analytical performance of the PathoNostics AsperGenius® species assay when testing *A. fumigatus* genomic DNA extracted from plasma samples

419 using the BioMerieux EasyMag instrument.

420

	Fungal load (genomes/ 0.5ml sample)	PathoNostics AsperGenius® target							
		A. fumigatus		*Aspergillus* spp.		*A.terreus*		Internal Control	
		Positives/total	Mean C_q (SD)	Positives/total	Mean C_q (SD)	Positives/total	Mean C_q (SD)	Positives/total	Mean C_q (SD)
DNA template volume: 5µL	10000	1/1	24.72	1/1	23.86	1/1	41.95	1/1	29.68
	1000	1/1	27.93	1/1	26.55	0/1	-	1/1	32.25
	500	1/1	28.85	1/1	27.23	0/1	-	1/1	31.67
	100	1/1	31.26	1/1	29.43	0/1	-	1/1	32.43
	50	3/3	33.24 (0.98)	3/3	30.75 (0.81)	1/3	39.39	3/3	33.33 (0.67)
	25	3/3	34.31 (1.79)	3/3	31.20 (0.61)	1/3	42.10	3/3	33.57 (0.80)
	10	5/5	37.5 (1.63)	5/5	32.5 (0.41)	0/5	-	5/5	31.86 (0.61)
	5	5/5	38.0 (2.66)	5/5	33.28 (0.73)	0/5	-	5/5	33.27 (0.82)
	1	0/5	-	1/5	35.35	1/5	43.90	5/5	33.48 (1.00)
	0	0/5	-	0/5	-	0/5	-	5/5	31.83(1.56)

421

DNA template Volume: 10 µL	10	1/1	35.82	1/1	34.46	0/5	-	1/1	33.15
	5	5/5	38.65 (1.90)	5/5	35.80 (1.15)	0/5	-	5/5	30.56 (1.00)
	1	3/5	40.67 (2.14)	3/5	36.94 (1.45)	0/5	-	5/5	31.59 (1.77)
	0	0/5	-	0/5	-	0/5	-	5/5	32.60 (1.25)

424 **Table 3.** Analytical performance of the PathoNostics AsperGenius® species assay when testing *A. terreus* genomic DNA extracted from plasma samples using

425 the BioMerieux EasyMag instrument.

426

	Fungal load (genomes/ 0.5ml sample)	PathoNostics AsperGenius® target							
		A. fumigatus		*Aspergillus* spp.		*A.terreus*		Internal Control	
		Positives/total	Mean C_q (SD)	Positives/total	Mean C_q (SD)	Positives/total	Mean C_q (SD)	Positives/total	Mean C_q (SD)
DNA template volume: 5µL	10000	0/1	-	1/1	24.30	1/1	26.10	1/1	30.21
	1000	0/1	-	1/1	27.40	1/1	29.14	1/1	32.49
	500	0/1	-	1/1	27.98	1/1	30.23	1/1	33.37
	100	0/2	-	2/2	30.14 (0.04)	2/2	32.48 (0.05)	2/2	33.82 (0.21)
	75	0/2	-	2/2	30.34 (0.38)	2/2	32.69 (0.39)	2/2	30.86 (1.51)
	50	0/2	-	2/2	31.30 (0.18)	2/2	33.67 (0.06)	2/2	33.43 (0.36)
	25	0/3	-	3/3	31.87 (0.34)	3/3	34.27 (0.35)	3/3	32.71 (1.38)
	10	0/3	-	3/3	33.39 (0.16)	3/3	36.16 (0.49)	3/3	32.74 (0.53)
	5	0/3	-	3/3	34.33 (0.38)	3/3	36.89 (0.29)	3/3	34.22 (0.45)
	1	0/3	-	1/3	35.61	1/3	38.18	3/3	33.55 (0.69)
	0	0/3	-	0/3	-	0/3	-	3/3	33.59 (1.59)

DNA template volume: 10µL	10	0/3	-	3/3	36.45 (0.56)	3/3	35.09 (0.44)	3/3	34.46 (0.27)
	5	0/3	-	3/3	33.47 (0.09)	3/3	36.11 (0.18)	3/3	33.96 (0.19)
	1	0/3	-	3/3	32.52 (0.35)	3/3	39.23 (0.69)	3/3	34.76 (0.84)
	0	0/3	-	0/3	-	0/3		2/3	34.06 (2.01)

429 **Table 4.** Patient demographics and diagnosis of IA according to the revised EORTC-MSG definitions [19]

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

430

431 **Key:** **AA:** Aplastic Anaemia

432 **AML:** Acute Myeloid Leukaemia

433 **ALL:** Acute lymphoblastic Leukaemia

434 **MDS:** Myelodysplastic syndrome

435 **Lymphoma:** Hodgkins, Non-Hodgkins and Burkitts Lymphoma

436 **IPA:** Invasive pulmonary aspergillosis

437 **N/A:** Not applicable

438 **Table 5.** Clinical Performance of AsperGenius® Species assay when testing serum from haematology
439 with proven/probable IA (n=10), possible IA (n=2) and with no evidence of fungal disease (NEF,
440 n=27). Performance represents a combination of results for the *A. fumigatus* specific and the broad
441 range *Aspergillus* species assays, as in a clinical scenario a positive result in either assay would carry
442 significance.

Parameter	Population			
	Proven/Probable IA vs NEF		Proven/Probable/Possible IA vs NEF	
	Single Positive Threshold	Multiple (≥2) positive threshold	Single Positive Threshold	Multiple (≥2) positive threshold
Sensitivity (n/N, %, 95% CI)	8/10, 80.0% (49.0-94.3)	5/10, 50.0% (23.7-76.3)	8/12, 66.7% (39.1-86.2)	5/12, 41.7% (19.3-68.1)
Specificity (n/N, %, 95% CI)	21/27, 77.8% (59.2-89.4)	27/27 100%, (87.5-100)	21/27, 77.8% (59.2-89.4)	27/27 100%, (87.5-100)
LR +tive	3.6	>500*	3.0	>417*
LR -tive	0.26	0.5	0.43	0.44
DOR	14.0	>1000*	7.0	>947.7*

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

444 **Key:**

445 **IA: Invasive Aspergillosis**

446 **NEF: No evidence of fungal disease**

447 **LR: Likelihood ratio**

448 **DOR: Diagnostic odds ratio**

449

450

451 **Figure 1.** Standard curves for the PathoNostics AsperGenius® *A. fumigatus* and *Aspergillus* species

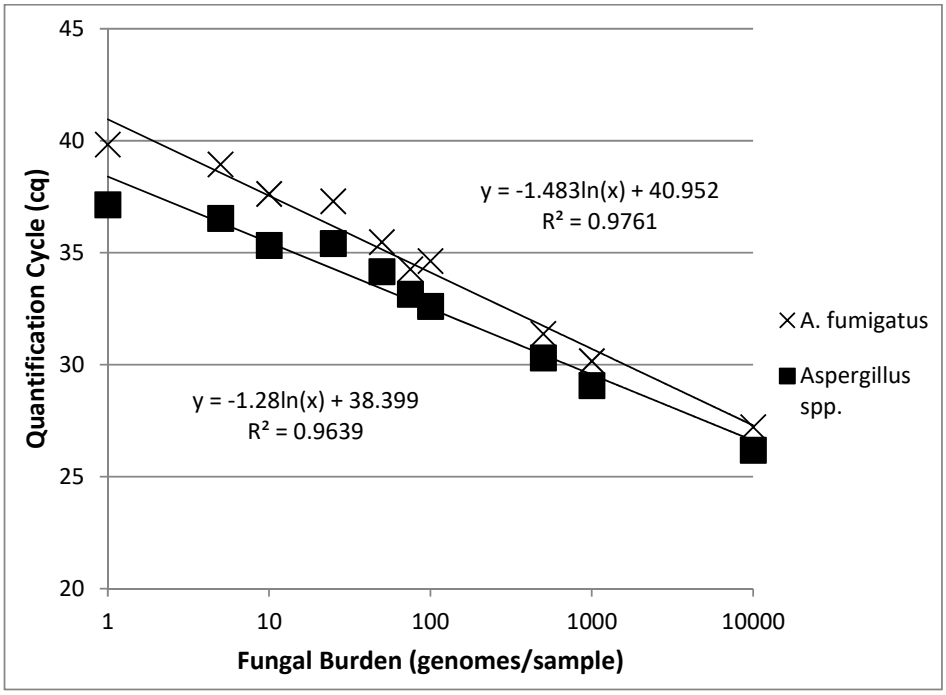
452 assays testing *A. fumigatus* genomic DNA extracted from plasma samples by a) Qiagen EZ1 and b)

453 BioMerieux EasyMag automated extractors, and c) the *A. terreus* and *Aspergillus* species assays

454 testing *A. terreus* genomic DNA extracted from plasma samples by BioMerieux EasyMag automated

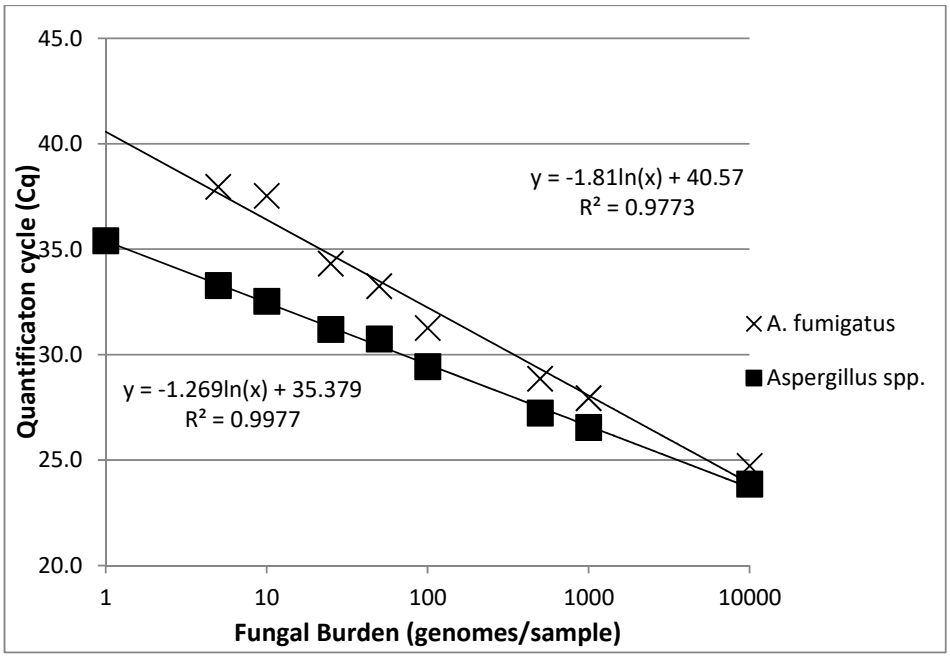
455 extractor.

A.



456

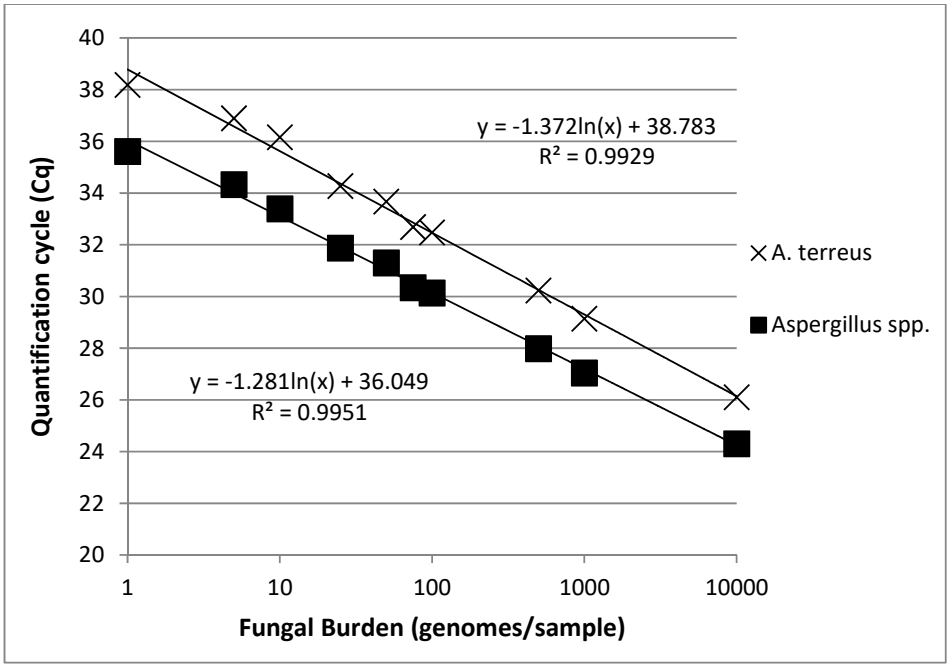
B.



457

458

C.


40
38
36
34
32
30
28
26
24
22
20
Quantification cycle (Cq)
1
10
100
1000
10000
Fungal Burden (genomes/sample)
y = -1.372ln(x) + 38.783
R² = 0.9929
y = -1.281ln(x) + 36.049
R² = 0.9951
A. terreus
Aspergillus spp.

459

460 **Figure 2.** PathoNostics AsperGenius® PCR positivity according to sampling for the cases of proven/probable invasive aspergillosis. Grey cells represent

461 positive results with the number representing the corresponding Cq value.

Patient (EORTC/MSG diagnosis)[19]	Sample 1		2		3		4		5		6		7		8	
	Afumi	Asp	Afumi	Asp	Afumi	Asp	Afumi	Asp	Afumi	Asp	Afumi	Asp	Afumi	Asp	Afumi	Asp
1 (Probable IA)	-	-	-	-	-	-	-	-	38.0	37.1	37.0	36.1	37.6	36.5	NT	NT
2 (Probable IA)	-	-	-	37.1	-	-	-	-	-	33.1	40.4	32.6	-	36.0	NT	NT
3 (Probable IA)	-	33.2	-	34.7	-	-	-	-	-	-	-	-	-	-	-	-
4 (Probable IA)	-	-	-	-	-	42.2	-	-	-	-	-	-	-	-	NT	NT
5 (Prob Asp Sin)	37.7	36.2	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT
6 (Probable IA)	-	-	-	37.0	-	-	-	-	-	-	-	-	35.8	34.7	NT	NT
7 (Probable IA*)	-	-	44.8	38.7	-	-	-	-	-	-	-	-	-	-	-	-
8 (Probable IA)	34.3	31.8	44.7	36.8	37.4	34.7	46.3	37.6	-	-	-	-	NT	NT	NT	NT
9 (Probable IA)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT
10 (Prov Asp Sin)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT

462

463

464 **Key**:

465 **Afumi**: Pathonostics AsperGenius® *A. fumigatus* assay

466 **Asp**: PathoNostics AsperGenius® Species assay

467 **IA:** Invasive aspergillosis

468 **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

469 was the last sample to be tested. It was excluded to avoid presentation difficulties.

470 **Prob Asp Sin**: Probable *Aspergillus* sinusitis

471 **Prov Asp Sin**: Proven Aspergillus sinusitis

472 **NT**: No sample tested

473 -: Assay was negative

474

475

476