Title: Diagnosis of Aspergillosis by PCR: Clinical Considerations and Technical Tips

Authors: Rosemary A. Barnes[a], P. Lewis White[b], C. Oliver Morton[c], Thomas R Rogers[d], Mario Cruciani[e], Juergen Loeffler[f] and J. Peter Donnelly[g]

Affiliations: [a] Cardiff University School of Medicine, Cardiff UK; [b] Public Health Wales Microbiology Cardiff, Cardiff, UK; [c] Western Sydney University, Sydney, Australia; [d] Clinical Microbiology, Trinity College Dublin, Ireland, [e] Center of Community Medicine and Infectious Diseases Service, ULSS 20 Verona, [f] Wuerzburg University, Wuerzburg, Germany, [g] Radboud University Medical Centre, Nijmegen, The Netherlands;

Correspondence: Dr P. Lewis White.

Address: Public Health Wales, Microbiology Cardiff, UHW, Heath Park,

                          Cardiff. UK. CF37 1EN

Telephone: +44 (0)29 2074 6581

Email: Lewis.White@wales.nhs.uk

Running title: *Aspergillus* PCR clinical and technical guidance

Key words: *Aspergillus* PCR, EAPCRI, Invasive aspergillosis

ABSTRACT

Standardization of *Aspergillus* PCR protocols has progressed and analytical validity of blood-based assays has been formally established. It remains necessary to consider how the tests can be used in practice to maximise clinical utility. To determine the optimal diagnostic strategies and influence on patient management, several factors require consideration, including the patient population, incidence of invasive aspergillosis (and other fungal disease) and the local antifungal prescribing policy. Technical issues such as specimen type, ease of sampling, frequency of testing, access to testing centres and time toreporting will also influence the use of PCR in clinical practice. Interpretation of all diagnostic tests is dependent on the clinical context and molecular assays are no exception, but with the proposal to incorporate *Aspergillus* PCR into the second revision of the consensus guidelines for defining invasive fungal disease the acceptance and understanding of molecular tests should improve.

INTRODUCTION

The European *Aspergillus* PCR initiative (EAPCRI) was formed with the aim of standardising *Aspergillus* PCR methodology in order to determine accurate analytical performance and clinical validity (www.eapcri.eu). In doing so, it has permitted the incorporation of the standardized methodology into revised guidelines for defining invasive fungal disease (IFD), with the ultimate goal of improving the diagnosis and subsequent management of patients at risk of IFD. The EAPCRI has made significant advances in standardising *Aspergillus* PCR testing of ethylenediamine tetra-acetic acid (EDTA)-whole blood, serum and plasma, determining that nucleic acid extraction procedures were the rate-limiting step governing optimal PCR performance [1-5]. A range of technical recommendations that depend on sample type have been published but there is limited information on how best to use these and interpret results in clinical practice. This review will explore the implementation of molecular diagnostic strategies and interpretation of results in different clinical contexts.

CLINICAL CONSIDERATIONS

The patient population

The performance of any test will be heavily influenced by the prevalence of disease in a population and for opportunist infections, such as invasive aspergillosis (IA), this is largely determined by the presence of several well-established risk factors (Table 1). These include neutropenia, high-dose corticosteroid treatment, graft-versus host disease and genetic predisposition. Most studies have focused on adult patients with haematological malignancies and those undergoing haematopoietic stem cell transplantation at risk of invasive disease. Other patient groups include those with other malignancies, undergoing solid organ transplantation or with critical illness requiring intensive care treatment but data from these groups are more limited, with less performance data available in paediatric populations. Preliminary data suggest performance is comparable but that the incidence of disease tends to be lower in children than in adults.

Overall, IA is an uncommon infectious disease and incidence is low, reportedly less than 5% in haematological malignancy [6]. However, there is a wide range of reported prevalence determined by the presence of risk factors and study design. Cohort studies may underestimate prevalence due to difficulties with accurate diagnosis, whereas autopsy based studies may overestimate disease as the denominator is already weighted towards disease [7, 8]. Broadly patients can be divided into low, medium and high risk (Table 1). It must be recognized that individual patients may move from one

risk category to another depending on the aggressiveness of chemotherapeutic interventions and response to treatment. The use of mould active prophylaxis, such as with posaconazole, may be expected to markedly reduce the risk in some patient groups.

The strategy

PCR can be used in two main ways. Firstly, to rule out aspergillosis and secondly, to rule in a diagnosis of IA.

Ruling out IA utilizes the high negative predictive value of the test [9]. This can be refined further according to whether the test is used as a screening test in asymptomatic patients, or as part of a fever-driven approach during febrile neutropenia that can markedly reduce empirical use of antifungal agents during refractory fever. For both approaches frequent testing is required. When testing blood, specificity of both galactomannan and β-D-glucan is higher than for PCR, while the sensitivity of PCR is higher [3]. This sensitivity confers the high negative predictive value (NPV) such that a negative test may allow the diagnosis to be excluded. Positives show good specificity but the low prevalence of disease leads to a low positive predictive value in diagnosis of IA. It is increasingly recognized that PCR positivity can reflect exposure to *Aspergillus* and may be positive long before a disease process is evident or other biomarkers are detectable [10]. Multiple positive results improve diagnostic utility and may be used initiate pre-emptive therapy and to trigger further diagnostic work-up [9]. Combining molecular and serological assays can enhance these approaches, improving specificity when both assays are positive [9].

Screening is best applied to patients in high-risk categories only (Table 1), as regularly testing of patients with lower incidences of infection is unlikely to be cost effective. Screening is only likely practical when the time to results reporting is short enough to impact on patient management (usually less than 24 hours). If turnaround times are longer, particularly if specimens are sent away to reference centres for testing, it is likely that empirical therapy may be necessary in some cases. In these instances, empiric therapy should be considered as a holding measure only until results are available enabling therapy to be stopped if tests are negative. Samples should be taken on, or before the initiation of empiric therapy.

PCR should not be used to rule out disease in patients already on mould active antifungal therapy whether as prophylaxis or empiric therapy (discussed further below). This is not only because effective chemoprophylaxis reduces the risk of disease but also because it is possible that the antifungal agents could lower the fungal burden to below the limits of detection.

Secondly, *Aspergillus* PCR can be used to rule in a diagnosis of IA in patients who have signs and symptoms of infection (Table 2). In these instances, the pre-test probability of disease is already increased. However, no single biomarker has yet shown optimal performance in ruling in the diagnosis and PCR should be combined with other antigen based biomarkers (Galactomannan or β-D-glucan). A diagnostic test can be applied during refractory febrile neutropenia or when non-specific clinical signs, (e.g. pleuritic chest pain, haemoptysis) are present and may drive pre-emptive therapy of *Aspergillus* infection before specific radiological signs of disease are manifest.

The diagnostic approach can also be applied to patients with specific radiological signs suggestive of invasive fungal disease (lung nodules, CT haloes, cavitating lesions etc.) to confirm the diagnosis and optimise targeted therapy by providing evidence of the causative organism.

Additionally, diagnostic testing, may be useful for detection of breakthrough *Aspergillus* infection in patients on mould active prophylaxis or to monitor response to treatment, although data on these indications are sparse [11]. Screening, fever-driven and diagnostic strategies are illustrated in Figures 1-2.

The specimen type

The EAPCRI has validated *Aspergillus* PCR testing using whole blood serum and plasma and work on validation of bronchoalveolar lavage fluids (BALF) is underway. Less data are available for the validation of cerebrospinal fluid, urine and tissue specimens, but it is likely that the principles and critical steps identified by the EAPCRI protocols will apply.

Blood specimens are easily obtained and suited for both screening and diagnostic strategies. Whole blood processing is very labour-intensive [2] and use of serum or plasma is technically less demanding and more suited to routine laboratories [4]. Whilst marginally (but not statistically significantly) less sensitive than whole blood, serum, targeting free DNA, showed less false positivity [12]. The use of plasma as a specimen avoids the potential loss of free DNA due to trapping during clot formation and may improve clinical performance [1, 13].

Invasive specimens, such as BAL fluids and tissues, should be considered diagnostic specimens, and are preferable to blood specimens when taking a single sample for

diagnostic confirmation, particularly if therapy has already been prescribed. Here, specificity is the most important in order to confirm the diagnosis and allow targeted treatment. Meta-analyses suggest the *Aspergillus* PCR testing of BALF is clinically useful and specificity is significantly greater than galactomannan BALF testing [3, 14, 15].

TECHNICAL TIPS

Aspergillus DNA extraction from EDTA whole blood

The method for extracting nucleic acid (NA) from EDTA whole blood (WB) is shown in Figure 3. Sample volume is critical and at least 3ml of whole blood should be used [2, 5]. The requirement for this recommendation is crucial at low burdens of disease (10cfu/ml) where using 2ml and 1ml sample volumes resulted in ≥70% reductions in the reproducibility of detection compared to 3ml [5]. The only anti-coagulant suitable for samples is EDTA, as heparin has been associated with inhibition of *Aspergillus* PCR and sodium citrate vacutainers have a higher rate of *Aspergillus* contamination [16, 17]. The process targets DNA contained within intact cells. Freezing samples not only preserves free DNA but assists with erythrocyte lysis. Post erythrocyte lysis, the leucocyte pellet is lysed using SDS and proteinase K to free any phagocytosed fungal cells and release any human DNA that could interfere with PCR amplification efficiency. Human DNA will be greatly reduced by the subsequent centrifugation step. The duration of incubation is dependent on the pellet size, which is driven by the white cell count of the patient. It may be necessary to increase the proteinase K concentration or use an additional 95°C incubation step with 50mM NaOH if a pellet persists. Bead-beating is the preferred method for fungal cell lysis, and is quicker, cheaper and more efficient than enzymatic lysis (e.g. recombinant lyticase). If a bead-beater is not available each

sample can be vortexed for 30 seconds at maximum velocity. Roche MagNA Lyser ceramic green beads are recommended as they are dispensed into single use aliquots, minimising contamination risk. Post bead-beating it is essential to pulse centrifuge the tube prior to washing the bead-sample mix with a reagent compatible with the commercial NA extraction kit of choice (e.g. molecular grade water). The volume of the wash reagent should be sufficient to cover the beads, but equate to a volume that is suitable for extraction by the downstream process. Washing involves 10 agitations with a pipette ensuring the tip is at the bottom of the tube so that maximal washings can be transferred to the final extraction. A range of commercial, manual and automated extraction processes have been successfully utilised (Figure 3) but it is critical that DNA is eluted in <100µL.

Aspergillus DNA extraction from serum and plasma

The method for extracting NA from serum and plasma is shown in Figure 4. Again sample volume is critical and a minimum of 0.5ml should be used, with lesser volume associated with poorer sensitivity (*P*: 0.023) [4]. Using sample volumes <0.5ml will reduce the reproducibility of detecting 10genomes/ml (ge/ml) by 21.1% compared to a method fully compliant with EAPCRI recommendations and this difference was confirmed at burdens <10 ge/ml [4]. Theoretically, the larger the sample volume that can be practically extracted should be associated with improved detection of lower burdens. Unfortunately, in the EAPCRI study there was insufficient volume range to determine if this hypothesis was accurate [1]. The use of larger sample volumes should also be

balanced against the limitations of commercial, particularly automated, NA extraction systems and the likelihood for higher concentrations of inhibitory compounds. When performing NA extraction, a range of commercial kits, both manual and automated, have been successfully utilised (Figure 4), and by combining NA extraction with the EAPCRI recommendations a fully standardized protocol is feasible. One interesting finding of the original EAPCRI investigation into the *Aspergillus* PCR testing of serum was that two assays which were fully compliant with the EAPCRI recommendations on sample and elution volume, but provided below par positivity, were both manufactured by Promega (Wizard Genomic DNA and Maxwell) [4].

As with WB, elution volume is critical and should be <100µl. Methods using ≥100µl to elute NA showed a 37.5% reduction in the detection of 10ge/ml compared to EAPCRI compliant methods (Figure 4).

False positivity rates for the individual platforms participating in the serum study are shown in Figure 4 and appear to be spread across the range of manufacturers, indicating that contamination is likely associated with the physical process more than an individual manufacturer. However, >50% of false positivity was associated with the use of Qiagen kits, as previously noted [18].

It would appear that the EAPCRI recommendations for serum are equally applicable to plasma, but testing plasma circumvents the formation of the blood clot that has the potential to trap biomarkers. Certain compounds in plasma, such as fibrinogen, could affect PCR performance. High concentrations if present in eluted NA could interfere with $MgCl_2$ concentrations in the PCR master-mix and prevent efficient amplification [1].

Other technical considerations

Along with performance the technical complexity of any process requires consideration before implementation into routine service. Without doubt the processing of serum/plasma is less technically demanding than that of WB and allowscommercially available options to be used, eradicating the need for additional steps for cell lysis. While WB can be processed directly through commercial kits the sample volume and bead-beating requirements together with the concentration factor (≥3ml sample = <100µl eluate) render this approach impractical. The hands-on time is far longer than that for testing serum/plasma (Table 3) and the complexity means that experienced users are required for reliable extraction efficiency. The use of automated processors and protocols with less human-dependent steps reduces performance variability and standardizes turnaround times (Table 3). Fully automated procedures will carry increased equipment costs but these instruments are routine in generic molecular diagnostic laboratories and access to these settings may alleviate cost pressures.

PCR Amplification

Any *Aspergillus* PCR assay combines DNA isolation and qPCR amplification. Both steps must be compatible and optimised for sensitivity and specificity. The DNA isolation protocol is the most critical step as it ensures that target DNA is available in sufficient concentrations for amplification but contains minimal inhibitory compounds.

PCR-Target

Testing conducted by EAPCRI has indicated that qPCR assays for IA should target multi-copy genes. Single copy genes are not recommended as they are not as sensitive as ribosomal targets [2]. The most common targets are the 18S, 28S and internal transcribed spacer (ITS) regions, with best results obtained by targeting the 28S gene in terms of analytical specificity [19]. qPCR assays should utilise a probe, this increases cost and complicates design but these are outweighed by the benefits to assay specificity. Hydrolysis probes (Taqman), scorpion and hybridisation FRET probes have all been tested and all perform well [2]. Hybridisation FRET probes, used by Light-Cycler, can generate melt curves that can be useful for distinguishing between positive samples if they have been generated by different species [20].

It is important to decide on the target range of a PCR assay. Some centres may focus on a single species per assay, several related species per assay, or a pan-fungal approach. Pan-fungal amplification followed by sequencing of the PCR product gives certainty of identification but is potentially costlier than the other strategies and can delay results. There are also issues with targeting multiple species, which has been reviewed by the EAPCRI [19]. *Aspergillus* is a polyphyletic genus. Some *Aspergillus* species are more closely related to *Penicillium* than to other *Aspergillus* species. Therefore, one cannot produce a truly pan-*Aspergillus* assay without amplification of non-target species. However, in multicentre testing the EAPCRI found that assays targeting the *Aspergillus* genus were preferable and most reliable for detecting *A. fumigatus* at low DNA concentrations, and that the benefits of a broader detection range were more significant than any potential cross reactivity [19].

Template and assay volume

It is necessary to determine an optimum template and final assay volume. Increasing template volumes increase the probability of amplification of low abundance fungal DNA. However, large template volumes increase the transfer of PCR inhibitors. It is important to maximise the amount of template added while ensuring sufficient assay volume to minimise the influence of PCR inhibitors. Larger template volumes may require a greater volume of master-mix and there is a financial cost associated with increased assay volumes so a balance is required. Generally, the use of larger template DNA volumes have improved performance, provided these assays maintain the template DNA volume at ≤ 30% of the total assay volume [2, 19].

Testing analytical sensitivity and specificity

In any laboratory, it is essential to determine the analytical sensitivity and specificity of the assay to ensure appropriate performance. Testing analytical sensitivity involves spiking samples (blood, serum, or plasma) with fungal DNA or conidia; this examines the effectiveness of the overall assay from DNA isolation through to PCR amplification. This ensures that the assay functions effectively in the relevant laboratory. A DNA calibrator for assessing *Aspergillus* PCR assay performance was described by Lyon et al and is optimal for assays that target serum/plasma or for testing qPCR elements [21]. A calibrator allows for inter-lab comparisons and quality control. For assays that target intact fungal cells, such as whole blood DNA isolations, panels can be obtained independently through QCMD or EAPCRI [3].

Analytical specificity testing determines detection range of target species and identifies potential cross-reactions between the PCR assay and non-target species. Amplification of common environmental fungi will make it difficult to interpret PCR results without sequencing of the PCR product, which increases cost and time to result.

Contamination and controls

The successful implementation of a PCR-based strategy for diagnosis of IA is dependent on the effective control and identification of sources of contamination. Studies have shown that blood vacutainers may contain fungal DNA contamination [17], this creates the problem of false positives across certain specimen types. This may be solved through application of a Cq cut-off but since positivity is regularly at the functional limits of qPCR background contamination may mask actual fungal infection.

Reagents in kits for both automated and manual DNA isolation had previously been found to be contaminated with fungal DNA. False positivity and contamination remain a concern when performing *Aspergillus* PCR and have been linked to many sources including commercial extraction platforms [1, 17, 18, 22, 23]. This may be because of manufacturing practices that neglect the need to exclude fungal contamination. It is important to identify suppliers whose reagents are negative for fungal contamination. This also extends to oligonucleotide suppliers, where monitoring for differences in analytical performance due to batch variation by performing acceptance testing of quantified target is recommended.

To ensure the validity of *Aspergillus* PCR assays it is essential to include the controls. Ideally the negative DNA extraction control will be a vacutainer filled with blood from a healthy donor. This will help ensure that the DNA isolation procedure and vacutainer are both free from contamination. This may be impractical over the long-term and is difficult to monitor due to variations in batches of vacutainer. Consequently, a negative control in the form a tube containing just the extraction reagents can be used to monitor for extraction-borne contamination. A positive DNA extraction control, consisting of donor EDTA whole blood spiked with conidia (*ca* 20 conidia ml^{-1}) or donor serum/plasma spiked with 20 genome equivalents of *Aspergillus* DNA ensures that the DNA isolation protocol is functioning at the required level.

DNA isolation should be spatially separated from where the PCR assays are prepared with unilateral flow from DNA extraction, through PCR preparation to PCR amplification. PCR reagents and master mixes should be prepared in a lateral-flow hood that is separate from where template DNA is added. Micro-aerosols from template DNA or PCR amplified DNA can contaminate the laboratory environment and lead to false positive results.

An internal control (IC) PCR is required and can take the form of a spiked master-mix to monitor for PCR inhibition. For whole blood extractions, it is feasible to introduce spores from bacteria (e.g. *Bacillus* sp.) at the beginning of the extraction process to monitor the efficiency of the entire extraction process as well as inhibition. Alternatively, quantified DNA can be incorporated post bead-beating to monitor the efficiency of the final clean-up in addition to inhibition. Any IC PCR should be at a concentration similar to that of

the suspected target but provide reproducible detection (e.g. Cq: 35 cycles). Using human DNA as an indicator for inhibition is not recommended due to the varying concentrations exhibited across patient samples. Given that the only likely DNA source in serum/plasma is free DNA (DNAemia) then extraction protocols permit the incorporation of an IC target into the sample prior to extraction allowing the efficiency of extraction in each sample to be monitored and, in principle, removing the necessity for a positive extraction control. The IC should take the form of quantified DNA and should follow the target and concentration recommendations outlined for WB. The qPCR assay should also include no template controls (NTC) and positive amplification controls to ensure that the PCR reagents are clean and functioning to specification.

Commercial Assays

Instead of establishing an in-house assay, there are now a number of commercially available PCR assays for the diagnosis of IA and IFD. These allow for inter-laboratory standardisation, reduced preparation time in diagnostic centres and independent quality control of the reagents. Products include MycAssay Aspergillus (Microgen Bioproducts Ltd), AsperGenius (Pathonostics), MycoReal Aspergillus (Ingenetix GmbH), Affigene Aspergillus tracer (Cepheid), Bruker Fungiplex Aspergillus (Previously Renishaw Fungiplex), Aspergillus spp Q-PCR Alert (Nanogen), and Septifast (Roche). To date, while clinical validity (sensitivity/specificity) of these assays is generally favourable, evaluation of clinical utility is limited and there have been few head to head evaluations [24-31]. The development of commercial PCR assays with the ability to detect markers associated with azole resistance offer a marked advantage for the management of disease where culture positivity is limited [24-26, 32].

Analysis of qPCR

PCR amplification should be performed in duplicate as a minimum, and even a single positive replicate should be considered potentially significant. Periodic sequencing of positive results is necessary to ensure that analytical specificity has not been compromised and monitoring for genetic drift in isolates of *Aspergillus* is recommended to ensure PCR assays have not been undermined by mutations within the target region.

The use of next generation (whole genome) sequencing will also be useful for monitoring evolution of the organism.

In terms of amplification, a Cq threshold of 43 cycles will generate sensitivity and specificity of 85.6% and 94.7%, respectively and any positive ≤34 cycles is associated with 100% specificity [4]. However, it is important to remember that Cq values will likely vary between assays. Indeed, different real-time amplification platforms will generate Cq values using different algorithms and even the same assay on different platforms can provide differing Cq values.

PERFORMANCE COMPARISONS

The benefits of compliance with the EACPRI recommendations have been independently verified by meta-analysis of *Aspergillus* PCR methods testing blood specimens [33]. In this study, there was a trend towards improved sensitivity but a significant improvement in specificity when using EAPCRI compliant methods. One drawback of the study was it did not differentiate between assays testing different blood fractions and so it was not possible to identify an optimal specimen for screening purposes [33].

An overview of pooled performance from three recent manuscripts comparing compliant methodology for testing serum, WB and plasma is shown in table 4. Plasma PCR testing appears optimal (DOR: 30 – more than double any other sample). Even in populations where the incidence (pre-test probability) of IA is high at 15%, PCR

negativity reduces this probability to 2% for all specimen types (Table 4). Predictably, when applying the effects of non-compliance, as determined in the analytical studies, to the clinical performance, all assays show a reduction in sensitivity and their ability to exclude disease when negative. This is most noticeable when testing WB. For a typical population with incidence of 5-10% WB PCR negativity using non-compliant methodology only reduced probability of disease by 1-2%, making it difficult to argue against the use of empirical therapy. On the basis of specificity, no single assay, whether compliant or not, was sufficient to confirm a diagnosis of IA (Table 4). In the original manuscripts from which the performance data was collated, it was concluded that combining molecular testing with serological biomarkers (GM-EIA or β-D-glucan) was necessary to rule in disease. This has been supported by a systematic review and meta-analysis evaluating combined *Aspergillus* PCR and GM-EIA testing [3, 34]. One interesting finding when comparing biomarker testing in an animal model of IA was that while increasing GM-EIA index and β-D-glucan concentration corresponded with disease progression, PCR positivity of serum or WB reflected exposure and the potential to provide earliest evidence of infection [35]. Nevertheless, the study also confirmed the requirement for combination biomarker testing.

Interpretive criteria

Decisions may be based on single or multiple results. As with all available biomarkers, the relatively high number of false positive results combined with the low prevalence, limit the ability to predict disease such that multiple positive results or preferably the combination of different biomarker results are required to rule in disease [36] whereas a

single negative result may be adequate to exclude disease, at that time point. For PCR, a systematic review reported a mean sensitivity and specificity of 80.5% (95% CI; 73.0 to 86.3) and 78.5% (67.8 to 86.4) for a single positive test result, and 58.0% (36.5 to 76.8) and 96.2% (89.6 to 98.6) for two consecutive positive test results [37]. Combination testing appears to show the best clinical utility resulting in improved and earlier diagnosis, reduced use of antifungal agents (predominantly a reduction in empiric use) and in one study a reduction in fungal related death [10, 38, 39]. Since different markers may be detectible during the evolution of infection from exposure through to colonisation and then invasive disease, simultaneous presence of different biomarkers in the same specimens is not a pre-requisite for diagnostic criteria [35]. Similarly, levels of biomarkers may be at the limits of detection particularly during the early stages of infection and specimens may be intermittently positive. Multiple positives taken during an individual period of risk may be more useful than consecutive positives.

Availability of testing and time to results

Not all centres will have facilities to perform testing at their own institution and may be required to send specimens away to reference centres. For all strategies (screening, fever-driven and diagnostic) results need to be available within 24-48 hours if they are to directly influence patient management and enable pre-emptive and targeted therapy. Without this turn-around time, empirical antifungal therapy will remain an integral part of care for patients at risk with refractory fever. However, risk stratification should still be employed and the decision to stop empirical drugs should be taken if results are subsequently found to be negative [40]. The influence of transit conditions requires

consideration, when sending samples to other centres for testing. For GM-EIA and β-D-

Glucan the biomarker targets are relatively stable, whereas for PCR samples,

degradation needs to be avoided by sending samples chilled, or preferably frozen.

Influence of antifungal exposure

It has been reported that the use of effective mould active antifungal agents whether for prophylaxis or empirical therapy will reduce the sensitivity of biomarker assays. Most data exist for galactomannan detection [41] but there is also a suggestion from studies that PCR will be similarly affected [42, 43].

The range of different agents used and the interpretation of effective prophylaxis hamper studies. Mould-active azoles include itraconazole, voriconazole, posaconazole and now isavuconazole, although only posaconazole has a strong evidence base for the prevention of aspergillosis [44] and therapeutic monitoring is required for most triazoles [45]. Prophylaxis with echinocandins and also intermittent dosing regimens of liposomal amphotericin B are also used, albeit with a limited evidence base.

It is unlikely that prophylactic antifungal drugs affect true analytical sensitivity and specificity of the assay, but rather they reduce the incidence of disease (and hence pre-test probability) to levels that impact on utility and prevent a screening strategy from being cost-effective or viable. Theoretically, assays may still be useful to detect breakthrough infections and also for monitoring response to therapy but practical data are limited. The effect of antifungal drugs on biomarker levels in different specimen types (BALF versus blood) has been less studied, but it seems likely that any reduction in levels would be greatest in circulatory samples.

CONCLUDING REMARKS

The advances made in Europe and Australia have gone a long way in providing a standard for PCR diagnosis of aspergillosis and allowed clinical evaluation in

haematological populations. This has resulted in widespread agreement that PCR is a useful tool in diagnosis and should contribute to consensus definitions [3]. Lack of an FDA recommended assay has limited clinical usage in many countries. Recent guidelines are couched with caution although they still make a strong recommendation for use of *Aspergillus* PCR 'in conjunction with other diagnostic tests and the clinical context' [46]. Understanding the clinical context remains the key factor and increasing understanding and awareness of this should bring about more widespread acceptance of aspergillus PCR.

REFERENCES

1.   Loeffler J, Mengoli C, Springer J, et al. Analytical Comparison of In Vitro-Spiked Human Serum and Plasma for PCR-Based Detection of Aspergillus fumigatus DNA: a Study by the European *Aspergillus* PCR Initiative. *J. Clin Microbiol*. 2015; **53**: 2838-2845.

2.   White PL, Perry MD, Loeffler J, et al. Critical Stages of Extracting DNA from *Aspergillus fumigatus* in Whole-Blood Specimens. *J. Clin Microbiol*. 2010; **48**: 3753-3755.

3.   White PL, Wingard JR, Bretagne S, et al. *Aspergillus* Polymerase Chain Reaction: Systematic Review of Evidence for Clinical Use in Comparison With Antigen Testing. *Clin Infect Dis*. 2015; **61**: 1293-1303.

4.   White PL, Mengoli C, Bretagne S, et al. Evaluation of *Aspergillus* PCR Protocols for Testing Serum Specimens. *J. Clin Microbiol*. 2011; **49**: 3842-3848.

5.   White PL, Bretagne S, Klingspor L, et al. Aspergillus PCR: One Step Closer to Standardization. *J. Clin Microbiol*. 2010; **48**: 1231-1240.

6.   Pagano L, Caira M, Picardi M, et al. Invasive Aspergillosis in Patients with Acute Leukemia: Update on Morbidity and Mortality—SEIFEM-C Report. *Clin Infect Dis*. 2007; **44**: 1524-1525.

7.   Chamilos G, Luna M, Lewis RE, et al. Invasive fungal infections in patients with hematologic malignancies in a tertiary care cancer center: an autopsy study over a 15-year period (1989-2003). *Haematologica* 2006; **91**: 986-989.

8.   Lewis RE, Cahyame-Zuniga L, Leventakos K, et al. Epidemiology and sites of involvement of invasive fungal infections in patients with haematological malignancies: a 20-year autopsy study. *Mycoses*. 2013; **56**: 638-645.

9.   Barnes RA, Stocking K, Bowden S, et al. Prevention and diagnosis of invasive fungal disease in high-risk patients within an integrative care pathway. *J Infect*. 2013; **67**: 206-214.

10.   Meije Y, Aguado JM, Cuenca-Estrella M. Silent and prolonged *Aspergillus* DNAemia in oncohematological patients receiving antifungal prophylaxis: a new phenomenon with clinical implications. *BMT*. 2011; **46**: 1016-1017.

11.   Duarte RF, Sanchez-Ortega I, Cuesta I, et al. Serum Galactomannan-Based Early Detection of Invasive Aspergillosis in Hematology Patients Receiving Effective Antimold Prophylaxis. *Clin Infect Dis*. 2014; **59**: 1696-1702.

12.   Springer J, Morton CO, Perry M, et al. Multicenter Comparison of Serum and Whole-Blood Specimens for Detection of *Aspergillus* DNA in High-Risk Hematological Patients. *J Clin Microbiol.* 2013; **51**: 1445-1450.

13.   McCulloch E, Ramage G, Jones B, et al. Don't throw your blood clots away: use of blood clot may improve sensitivity of PCR diagnosis in invasive aspergillosis. *J Clin Pathol*. 2009; **62**: 539-541.

14.   Sun W, Wang K, Gao W, et al. Evaluation of PCR on bronchoalveolar lavage fluid for diagnosis of invasive aspergillosis: a bivariate meta-analysis and systematic review. *PLoS One*. 2011; **6**: e28467.

15.   Tuon F. A systematic literature review on the diagnosis of invasive aspergillosis using polymerase chain reaction (PCR) from bronchoalveolar lavage clinical samples. *Rev Iberoam Micol.* 2007; **24**: 89-94.

16.   Garcia ME, Blanco JL, Caballero J, Gargallo-Viola D. Anticoagulants interfere with PCR used to diagnose invasive aspergillosis. *J Clin Microbiol*. 2002; **40**: 1567-1568.

17.   Harrison E, Stalhberger T, Whelan R, et al. *Aspergillus* DNA contamination in blood collection tubes. *Diag Microbiol Infect Dis.* 2010; **67**: 392-394.

18.   Jaeger EEM, Carroll NM, Choudhury S, et al. Rapid detection and identification of *Candida*, *Aspergillus*, and *Fusarium* species in ocular samples using nested PCR. *J Clin Microbiol*. 2000; **38**: 2902-2908.

19.   Morton CO, White PL, Barnes R, et al. Determining the analytical specificity of PCR-based assays for the diagnosis of IA : what is *Aspergillus*? *Med Mycol*. 2017; **55**: 402-413

20.   Springer J, Loeffler J, Heinz W, et al. Pathogen-Specific DNA Enrichment Does Not Increase Sensitivity of PCR for Diagnosis of Invasive Aspergillosis in Neutropenic Patients. *J Clin Microbiol*. 2011; **49**: 1267-1273.

21.   Lyon GM, Abdul-Ali D, Loeffler J *et al*. Development and evaluation of a calibrator material for nucleic acid-based assays for diagnosing aspergillosis. *J Clin Microbiol*. 2013; **51**: 2403-2405.

22.   Perry MD, White PL, Barnes RA. Comparison of four automated nucleic acid extraction platforms for the recovery of DNA from *Aspergillus fumigatus*. *J Med Microbiol*. 2014; **63**: 1160-1166.

23.   Rimek D, Garg AP, Haas WH, Kappe R. Identification of contaminating fungal DNA sequences in zymolyase. *J Clin Microbiol*. 1999; **37**: 830-831.

24   White PL, Posso RB, Barnes RA. Analytical and Clinical Evaluation of the PathoNostics AsperGenius Assay for Detection of Invasive Aspergillosis and Resistance to Azole Antifungal Drugs during Testing of Serum Samples. *J Clin Microbiol.* 2015; **53**: 2115-2121.

25.   Chong GM, van der Beek MT, von dem Borne PA, et al. PCR-based detection of Aspergillus fumigatus Cyp51A mutations on bronchoalveolar lavage: a multicentre validation of the AsperGenius assay® in 201 patients with haematological disease suspected for invasive aspergillosis. *J Antimicrob Chemother.* 2016; **71**: 3528-3535.

26.   Chong GL, van de Sande WW, Dingemans GJ, et al. Validation of a New *Aspergillus* Real-Time PCR Assay for Direct Detection of *Aspergillus* and Azole

Resistance of Aspergillus fumigatus on Bronchoalveolar Lavage Fluid. *J Clin Microbiol*. 2015; **53**: 868-874.

27.   Danylo A, Courtemanche C, Pelletier R, Boudreault AA. Performance of MycAssay Aspergillus DNA real-time PCR assay compared with the galactomannan detection assay for the diagnosis of invasive aspergillosis from serum samples. *Med Mycol*. 2014; **52**: 577-583.

28.   Guinea J, Padilla C, Escribano P, et al. Evaluation of MycAssay™ Aspergillus for diagnosis of invasive pulmonary aspergillosis in patients without hematological cancer. *PLoS One*. 2013; **8**: e61545.

29.   White PL, Perry MD, Moody A, Follett SA, Morgan G, Barnes RA. Evaluation of analytical and preliminary clinical performance of Myconostica MycAssay Aspergillus when testing serum specimens for diagnosis of invasive Aspergillosis. *J Clin Microbiol*. 2011; **49**: 2169-2174.

30.   Torelli R, Sanguinetti M, Moody A, et al. Diagnosis of invasive aspergillosis by a commercial real-time PCR assay for Aspergillus DNA in bronchoalveolar lavage fluid samples from high-risk patients compared to a galactomannan enzyme immunoassay. *J Clin Microbiol*. 2011; **49**: 4273-4278.

31.   White PL, Hibbitts SJ, Perry MD, et al. Evaluation of a commercially developed semi-automated PCR-SERS assay for the diagnosis of invasive fungal disease. *J Clin Microbiol*. 2014; **52**: 3536-3543.

32.   Montesinos I, Argudín MA, Hites M, et al. Culture based methods and molecular tools for azole-resistant *Aspergillus fumigatus* detection in a Belgian University hospital. *J Clin Microbiol*. 2017 Epub Ahead of print.

33.   Arvanitis M, Ziakas PD, Zacharioudakis IM, et al. PCR in diagnosis of invasive aspergillosis: a meta-analysis of diagnostic performance. *J Clin Microbiol*. 2014; **52**: 3731-3742

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

35.   White PL, Wiederhold NP, Loeffler J, et al. Comparison of Nonculture Blood-Based Tests for Diagnosing Invasive Aspergillosis in an Animal Model. *J Clin Microbiol.* 2016; **54**: 960-966.

36.   Pini P, Bettua C, Orsi CF, et al. Evaluation of serum (1 -> 3)-beta-D-glucan clinical performance: kinetic assessment, comparison with galactomannan and evaluation of confounding factors. *Infection.* 2016; **44**: 223-233.

37.   Cruciani M, Mengoli C, Loeffler J, et al. Polymerase chain reaction blood tests for the diagnosis of invasive aspergillosis in immunocompromised people. C*ochrane data syst rev.* 2015; **10**: CD009551-CD.

38.   Aguado JM, Vazquez L, Fernandez-Ruiz M, et al. Serum Galactomannan Versus a Combination ofGalactomannan and Polymerase Chain Reaction-Based Aspergillus DNA Detection for Early Therapy of Invasive Aspergillosis in High-Risk Hematological Patients: A Randomized Controlled Trial. *Clin Infect Dis.* 2015; **60**: 405-414.

39.   Rogers TR, Morton CO, Springer J, et al. Combined real-time PCR and galactomannan surveillance improves diagnosis of invasive aspergillosis in high risk patients with haematological malignancies. *Brit J Haematol.* 2013; **161**: 517-524.

40.   Agrawal S, Hope W, Sinko J, Kibbler C. Optimizing management of invasive mould diseases. *JAC.* 2011; **66**: i45-i53.

41.   Marr KA, Laverdiere M, Gugel A, Leisenring W. Antifungal therapy decreases sensitivity of the *Aspergillus* galactomannan enzyme immunoassay. *Clin Infect Dis*. 2005; **40**: 1762-1769.

42.   McCulloch E, Ramage G, Rajendran R, et al. Antifungal treatment affects the laboratory diagnosis of invasive aspergillosis. *J Clin Pathol*. 2012; **65**: 83-86.

43.   Springer J, Lackner M, Nachbaur D, et al. Prospective multicentre PCR-based *Aspergillus* DNA screening in high-risk patients with and without primary antifungal mould prophylaxis. *Clin Microbiol Infect.* 2016; **22**: 80-86.

44.   Cornely OA, Maertens J, Winston DJ, et al. Posaconazole vs. fluconazole or itraconazole prophylaxis in patients with neutropenia. *New Eng J Med.* 2007; **356**: 348-359.

45.   Ashbee HR, Barnes RA, Johnson EM, et al. Therapeutic drug monitoring (TDM) of antifungal agents: guidelines from the British Society for Medical Mycology. *JAC*. 2014; **69**: 1162-1176.

46.   Patterson TF, Thompson GR, Denning DW, et al. Practice Guidelines for the Diagnosis and Management of Aspergillosis: 2016 Update by the Infectious Diseases Society of America. *Clin Infect Dis*. 2016; **63**: e1-e60.

47.   Pagano L, Caira M, Candoni A, et al. The epidemiology of fungal infections in patients with hematologic malignancies: the SEIFEM-2004 study. *Haematologica*. 2006; **91**:1068-1075.

48.   Weinberger M, Elattar I, Marshall D, et al. Patterns of infection in patients with aplastic anemia and the emergence of *Aspergillus* as a major cause of death. *Medicine*. 1992; **71**: 24-43.

49.   Singh N, Paterson DL. *Aspergillus* infections in transplant recipients. *Clin Microbiol Rev*. 2005; **18**: 44-69.

50.   Koulenti D, Garnacho-Montero J, Blot S. Approach to invasive pulmonary aspergillosis in critically ill patients. *Curr Opin Infect Dis*. 2014; **27**: 174-183.

51.   Prentice AG, Barnes RA, Rogers TRF, et al. Therapy of deep fungal infection in haematological malignancy. *JAC*. 1997; **40**: 779-788.

52.   Springer J, White PL, Hamilton S, et al. Comparison of Performance Characteristics of *Aspergillus* PCR in Testing a Range of Blood-Based Samples in Accordance with International Methodological Recommendations. *J Clin Microbiol*. 2016; **54**: 705-711.

53.   White PL, Barnes RA, Springer J, et al. Clinical Performance of *Aspergillus* PCR for Testing Serum and Plasma: a Study by the European Aspergillus PCR Initiative. J Clin Microbiol. 2015; **53**: 2832-2837.

Table 1. Underlying clinical conditions and the associated risk of invasive aspergillosis

Condition (Age limits)	Approximate incidence of disease %	Risk Category	Reference
Haematological malignancy (>16yo)			
Acute myeloid leukaemia and myelodysplastic syndrome	8-12	High	47
Acute lymphatic leukaemia	4-6	Moderate	47
Chronic Myeloid leukaemia	2.5	Low	47
Chronic lymphatic leukaemia	0.5	Low	47
Lymphoma	1	Low	47
Hodgkin's Disease	0.3	Low	47
Multiple myeloma	0.3	Low	47
Aplastic Anaemia (1-75yo)	15	High	48
Stem cell transplantation (NS)			
autologous	2–6	Low	49
allogeneic	5–26	High	49

Solid organ transplantation (NS)			
Kidney/pancreas	0-4	Low	49
liver	1-7	Low/moderate	49
Heart/lung	1-15	Moderate/High	49
Small bowel	0-10	Limited data	59
Critical Illness (NS)	0.3-6	Low	50

Key:

YO: years old

NS: not specified

Table 2. Clinical signs, symptoms and conditions associated with invasive aspergillosis [adapted from Prentice et al [51]]

Clinical signs, symptoms and conditions associated with Invasive aspergillosis
Any non-resolving fever despite antibiotics during prolonged, severe neutropenia or immunosuppression
Symptoms and signs of new, resistant or progressive lower respiratory tract infection, e.g. pleuritic pain, pleural rub
Prolonged, severe lymphopenia in chronic graft versus host disease (GVHD) and immunosuppression
Symptoms and signs of progressive upper respiratory tract infection
Periorbital swelling
Maxillary swelling and tenderness
Palatal necrosis or perforation*
Focal neurological or meningeal irritation symptoms and signs with fever
Unexplained mental changes with fever
Papular or nodular skin lesions
Intra-ocular signs of systemic fungal infection

* Also a indicator of mucormycosis

Table 3. Technical considerations when extracting nucleic acid from blood based samples

Parameter	Sample type					
	Serum		**Plasma**		**WB**	
Process	Manual	Fully automated	Manual	Fully automated	Manual	Semi-automated
Processing time (h)	1-2	1	1-2	1	4-5	4
Hands-on time (h)	1-2	0.3	1-2	0.3	3-4	2-3
Complexity [a]	Low	Very low	Low	Very low	High	Medium
Equipment Requirements [b]	Basic	Advanced/Specific	Basic	Advanced/Specific	Basic	Advanced/Specific
Generic Applicability [c]	Good	Excellent	Good	Excellent	Poor	Fair
Relative	Lowe	Higher	Lowe	Higher	Highe	Highest

Cost [d]	st	st	r

Key:

[a] Very low: basic training to run specific instrument; Low: experience of molecular based methods; Medium: specific training in respect to WB processing and basic training to run specific instrument; High: specific training in respect to WB processing in combination with experience of molecular based methods.

[b] Basic: General laboratory equipment (safety cabinets, pipettes, microfuges, heating blocks etc); Advanced/specific: Automated nucleic acid extraction platform required.

[c] General applicability to refers to the suitability of the method in a generic molecular diagnostics laboratory

[d] Costs include the requirement to purchase automated nucleic acid equipment but exclude labour costs.

Table 4. The theoretical effect of non-compliance with the EAPCRI recommendations when PCR testing whole blood, serum and plasma. The clinical performance for non-compliant methods has been adjusted using the differences between compliant and non-compliant protocols noted in the original analytical studies of WB and serum.[4, 5] For plasma testing it has been assumed that non-compliance would have the same effect on performance as it did on serum testing. The sensitivity and specificity represent pooled data derived from three recent EAPCRI compliant studies [12, 52, 53].

Performance Parameter (Population = 1000)	Sample type/Compliance of NA extraction protoc													
	WB – Compliant			WB– Non-compliant			Plasma - Compliant			Plasma - Non-compliant			Serum Compl	
Incidence of IA (%)	5	10	15	5	10	15	5	10	15	5	10	15	5	10
Sensitivity (%; 95% CI)	79 (67-88)			48			93 (79-98)			80			76 (66-	
Specificity (%; 95% CI)	79 (66-87)			64			69 (58-78)			70			77 (68-	
PPV (%)	17	30	40	7	13	19	14	25	35	12	23	32	15	27
NPV (%)	99	97	95	96	92	89	99	99	98	98	97	95	98	97
LR +tive	3.76			1.33			3.00			2.67			3.30	
LR -tive	0.27			0.81			0.10			0.29			0.3	

	DOR	13.93	1.64	30	9.21	10.6

KEY:

NA:	Nucleic Acid
WB:	Whole blood
IA:	Invasive aspergillosis
PPV:	Positive predictive value
NPV:	Negative predictive value
LR:	Likelihood ratio
DOR:	Diagnostic odds ratio

Figure 1. Screening strategy for high risk patients not receiving mould active prophylaxis and fever-driven strategy incorporating Aspergillus PCR and antigen testing
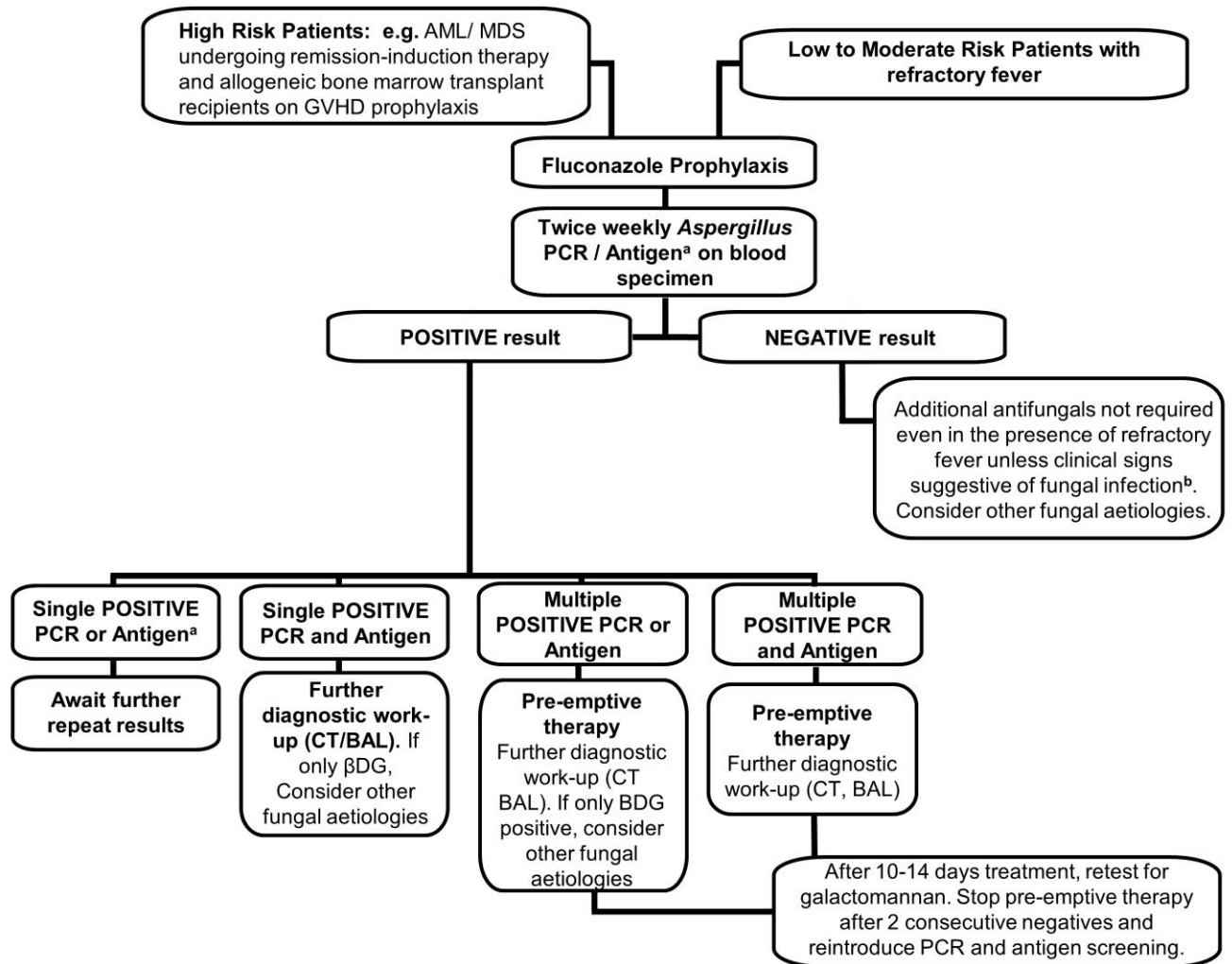
[a] Current antigen tests available: Galactomannan, Beta D glucan, (lateral flow device)   [b] See table 2

Figure 2 Diagnostic strategy incorporating Aspergillus PCR and antigen testing

Patients with symptoms suggestive of fungal infection (Table 2) or CT evidence of possible disease including patients on mould active prophylaxis with suspected breakthrough infection

Aspergillus PCR and antigen from blood and specimens from suspected sites of infection (respiratory [BALF], CSF sinus apspirate etc)

POSITIVE result

NEGATIVE result

Commence antifungal treatment

Consider other fungal aetiologies
Extend diagnostic work up

After 12-14 days treatment:

- Monitor galactomannan to assess response
- If CT normal or non-specific:
- stop treatment when negative
- If CT confirms IFD stop after 2 consecutive negatives one week apart
- Consider need for secondary prophylaxis

Figure 3 The EAPCRI method for extracting cell associated Aspergillus DNA from EDTA whole blood

Protocol	**Notes**	**Rationale**

≥ 3 mL EDTA whole blood =

100% detection of 10 CFU/mL

→ **Using Smaller volumes reduces sensitivity**

1mL EDTA blood = 100% reduction in detection
2mL EDTA blood = 70% reduction in detection

→ **Freezing blood assists in erythrocyte lysis and limits DNA degradation.**

Anticoagulants: heparin is inhibitory to PCR amplification and citrates is associated with high levels of *Aspergillus* contamination

X2 Hypotonic erythrocyte lysis Buffer: Incubate at RT for 10 min and centrifuge at 3000*g* for 15 min, decant supernatant

→ **Removal of erythrocyte lysis =** 10% reduction in detection

→ **Removes haemoglobin that is inhibitory to PCR. Commercial Option:** Promega Cell Lysis Buffer

Leukocyte lysis: lysis buffer with proteinase K, incubate at 56-65°C for up to 1 hr. Centrifuge at 7500*g* for 10 min and decant supernatant

→ **Removal of leukocyte lysis =**
- 60% reduction in detection
- PCR Inhibition

→ **Reduces human DNA concentration and releases any phagocytosed fungal target**

Fungal Cell Lysis: Bead beating (approx. 10 beads) for 30 s using a vortex mixer or a bead beater

→ **Removal of Bead Beating =** 100 % reduction in detection of fungal DNA

→ **Bead beating is quicker, cheaper and more efficient at lysing fungal cells than enzymatic lysis**

Wash Beads and complete DNA extraction using commercially available assay

Elution ≤ 100 µL = 100% detection of 10 CFU/mL

→ **Elution in 200 µL =** 60% reduction in detection
Elution in 100 µL = 50% reduction in detection

→ **Commercial kits compatible with EAPCRI recommendations:**
Manual: Qiagen Qiamp Blood, Zymo Research ZR Fungal/Bacterial DNA, Roche High Pure Template PCR.
Automated: MagNA Pure Total NA, Qiagen EZ1 Tissue, BioMerieux EasyMag, Cepheid GeneXpert
Semi-automated: Roche Septifast

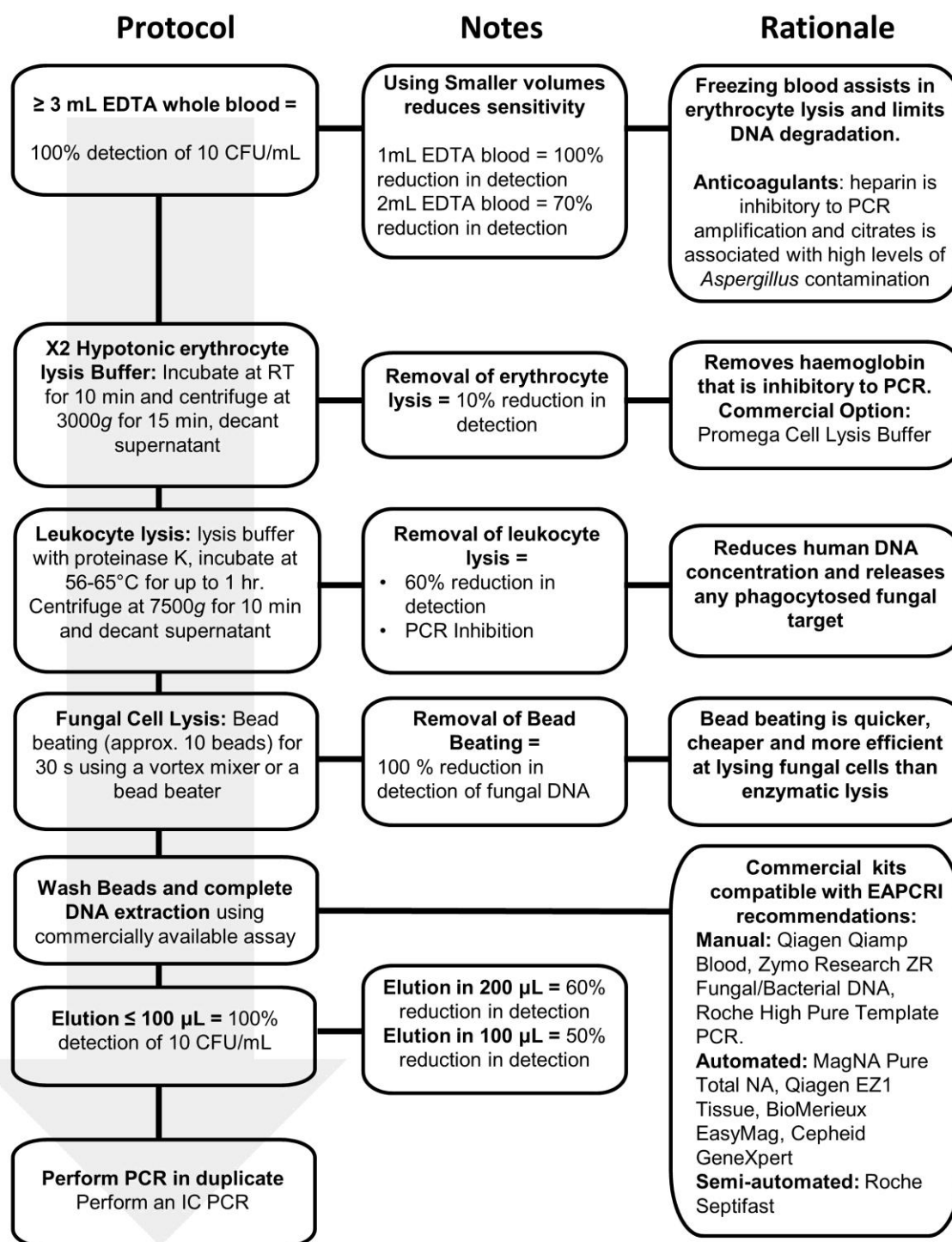**Perform PCR in duplicate**
Perform an IC PCR

Figure 4 The EAPCRI method for extracting cell associated Aspergillus DNA from serum/plasma