PCR – PLATFORMS, STRENGTHS AND WEAKNESSES

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Abstract
As highlighted in the 1st Advances Against Aspergillosis Conference the development and use of molecular methods to aid in diagnosis of invasive aspergillosis is extensive, but consensus over an optimal method has not been achieved (1). With high sensitivities and specificities the benefits of *Aspergillus* PCR assays are clear and the use of real-time PCR platforms for quantifying fungal burden, reducing the sample turn around time and minimising the opportunity for contamination have further enhanced their clinical relevance.
Apart from contamination (2) the other weaknesses of *Aspergillus* PCR methods are often less evident, albeit possibly widespread:

1) Choice of specimen (BAL, CSF, Serum or Whole Blood).
The wrong choice may limit target availability and/or introduce false positive and false negative results due to colonisation or contamination and PCR inhibition, respectively.
2) Extraction Procedure (Manual or Automated).
The performance of any PCR assay is dependent on the quality of the DNA extracted and the use of a robust and reproducible extraction procedure is paramount as the variation in the quality and quantity of DNA released by different methods can be great.
3) PCR assay design.
In designing a PCR assay time must be taken to minimise oligonucleotide cross hybridisation. Non-specific oligonucleotides may generate false positive results. However, the use of non-specific primers in a PCR assay utilising a specific probe may lead to the generation of false negative results.

4) PCR Platform
The choice of PCR platform is critical. The use of real-time platforms over block-based thermocyclers is widely preferred, although the choice of real-time platform can also be significant (3).
5) PCR results
Result interpretation in a clinical context is also important. It is generally accepted that two consecutive, reproducible positive PCR results are required in determining a true positive result. In a clinical setting this may be difficult with patients receiving antifungal therapy possibly reducing, already low, fungal burden below reproducible PCR thresholds.

In conclusion PCR is a useful tool to aid in the diagnosis of invasive aspergillosis, although it is essential that an optimal method be agreed to allow inclusion in future consensus diagnosis criteria. It should be used in conjunction with other methods (e.g. GM ELISA and HRCT) to enhance the opportunity for detection of this devastating infection.

References

1) Buchheidt D and Hummel M. Aspergillus polymerase chain reaction (PCR) diagnosis. *Med Mycol*. 2005; **43**(Suppl 1): 139-145.
2) Loeffler J, Hebart H, Bialek R, Hagmeyer L, Schmidt D, Sertey FP, Hartmann M, Eucker J and Einsele H. Contaminations occurring in fungal PCR assays. *J Clin Microbiol*. 1999; **37**(4): 1200-1202.
3) White PL, Barton R, Guiver M, Linton CJ, Wilson S, Smith M *et al.* on behalf of the UK Fungal PCR consensus group. A consensus on fungal PCR diagnosis? – A UK-

Ireland evaluation of PCR methods for detection of systemic fungal infections. Submitted for Publication 2006.

National Public Health Service for Wales
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PCR – Platforms, Strengths and Weaknesses

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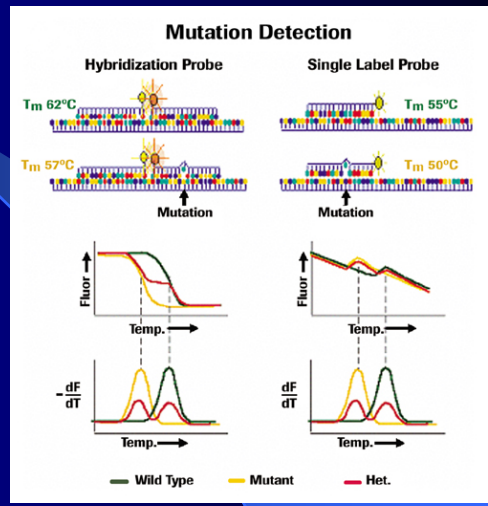
The clinical use of *Aspergillus* PCR

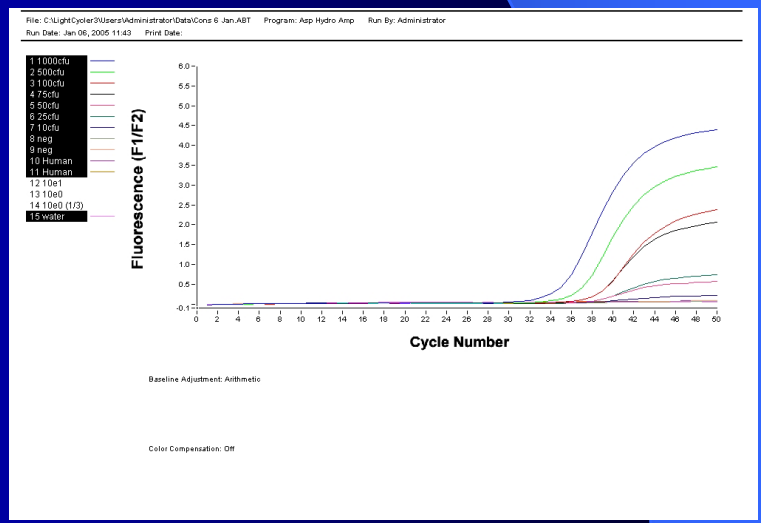
- Widespread
  - Up to 2004: 45 papers[1]
    - Sensitivities: 40 – 100%
    - Specificities: 64 – 100%
  - 2004 to date: 19 papers
    - Sensitivities: 12 – 100%
    - Specificities: 75 – 100%
- Lack of standardisation
- A consensus PCR method is essential
  - Multi-centre collaboration
  - Evaluate DNA extraction and PCR amplification

[1]Buchheidt and Hummel, 2005 Med Mycol 43 S139-145

Benefits

- Benefits of real-time PCR[1]:
  - Minimal contamination risk
  - No post amplification handling
    - UDG treatment
  - Fast turn-around time
  - High Sensitivity and Specificity
    - Using fluorescently labelled probes
  - Quantification
    - Monitor fungal burden
    - Therapeutic response





[1]Bretagne and Costa, 2006 Clin Chim Acta 363 221-228

Limiting Factors

Contamination[1]

Frequency[2]

Inhibitors[3]

Sample type

Target/Volume[4]

Patient

Extraction

[1]Williamson, 2001 MD Thesis; [2]Verweij, 2005 Med Mycol 43 S121-4; [3]Garcia *et al.*, 2002 J Clin Micro 40 1567-1568; [4]Halliday *et al.* 2005 BJH 132 478-486

Choice of Specimen

- Choice of specimen:
  - BAL
    - Linked PCR positive BAL with IA
    - Inhalation of *Aspergillus* spores
    - Colonisation
    - Invasive
  - CSF
    - Limited studies
    - Invasive
  - Serum/Plasma
    - Extensive successful studies
    - Targets Circulating DNA
  - Whole Blood
    - Extensive successful studies
    - Targets DNA, fungal fragments
    - Extended extraction procedure

Limiting Factors

Sample type

User experience

Method

Extraction Efficiency

Amplification

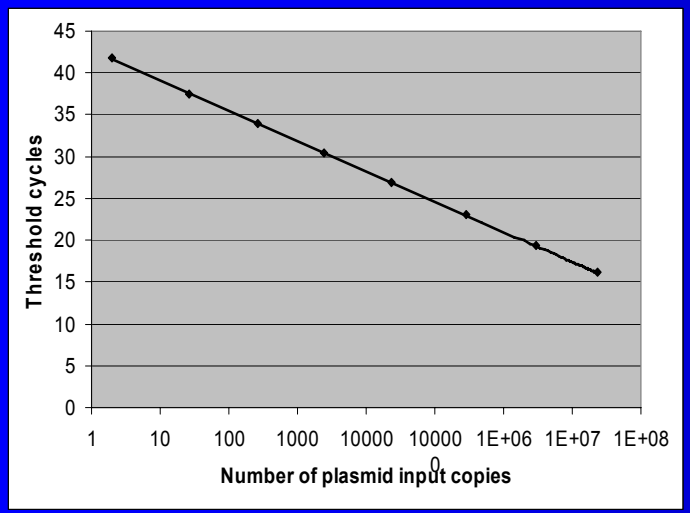
Comparison of extraction methods

- 1997[1]
  - Five commercial extractions kits
  - DNA from clinical and EDTA-blood spiked with *A. niger*
  - QIAamp Tissue kits: 1-10 cfu/ml
- 1998[2]
  - Six ‘in-house’ procedures
  - DNA extracted from *A. fumigatus* mycelial mat
  - Bead-beating
- 2002[3]
  - MagNA Pure Method and the QIAmp Tissue kit
  - DNA from culture, spiked and clinical bloods
  - MagNA Pure: Quick, sensitive with low levels of contamination

[1]Loeffler *et al.* 1997 J Clin Micro 35 3311-12; Van Burik *et al.* 1998 36 299-303;
[3]Loeffler *et al.*, 2002 J Clin Micro 40 2240-43

The Importance of efficient extraction

- In a clinical scenario IA = <1cfu/ml
  - Typical sample 2ml = <2cfu
- Targeting a single copy gene = 2 copies in 2ml
- rRNA genes = 10^2 copies/organism $\geq 2x10^2$ copies in 2ml



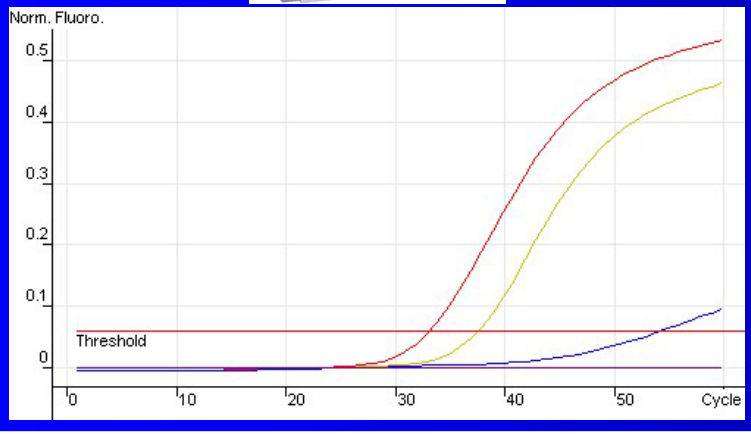
where Y = -1.5705ln(X) + 42.71[1]

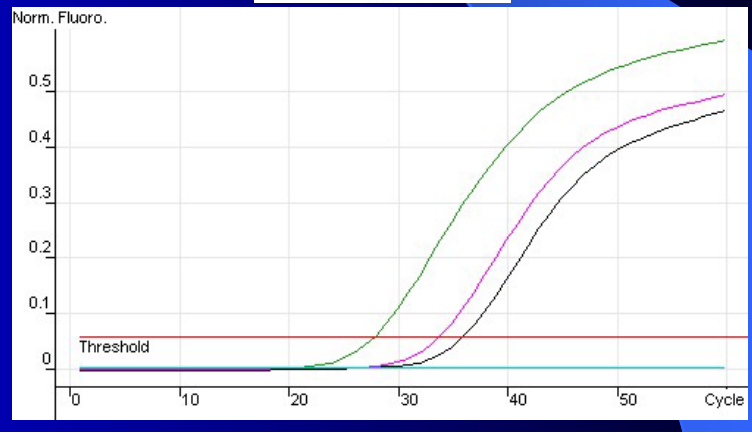
Sample	Est. copies	Result	Cp	Calc. copies
1000cfu	10^5	Pos	34.9	144
500cfu	$5x10^4$	Pos	36.4	56
100cfu	$1x10^4$	Pos	37.6	25
75cfu	$7.5x10^3$	Pos	37.8	23
50cfu	$5x10^3$	Pos	38.1	19
10cfu	$1x10^3$	Pos	38.0	20
0cfu	0	Neg	-	-

[1]White *et al.* 2006, CID 42 479-86

Comparison of automated methods

- *A. fumigatus* (1000, 100 and 20conidia)
  - Bead-beaten
  - Extracted using the MagNA Pure (Total NA) or Easy Mag





Sample	Result	Cp	Copies
1000cfu	Pos	33.3	226
100cfu	Pos	37.8	
20cfu	Pos	54.5	
0cfu	Neg	-	

Sample	Result	Cp	Copies
1000cfu	Pos	28.1	6512
100cfu	Pos	34.0	6
20cfu	Pos	36.2	
0cfu	Neg	-	

Limiting Factors

Extraction Efficiency

Antifungal therapy[1-2]

Inhibitors

Amplification

Oligonucleotide design

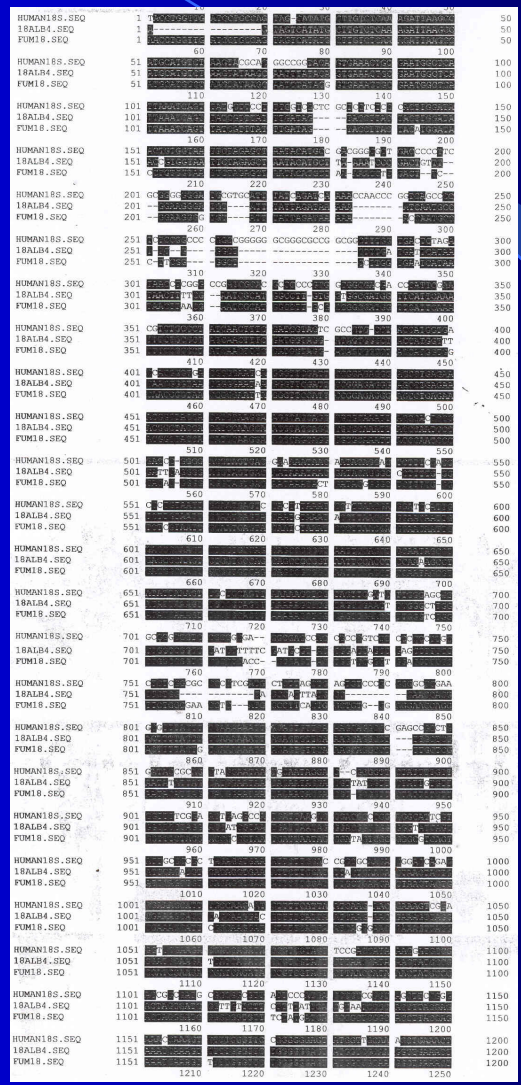
PCR platform

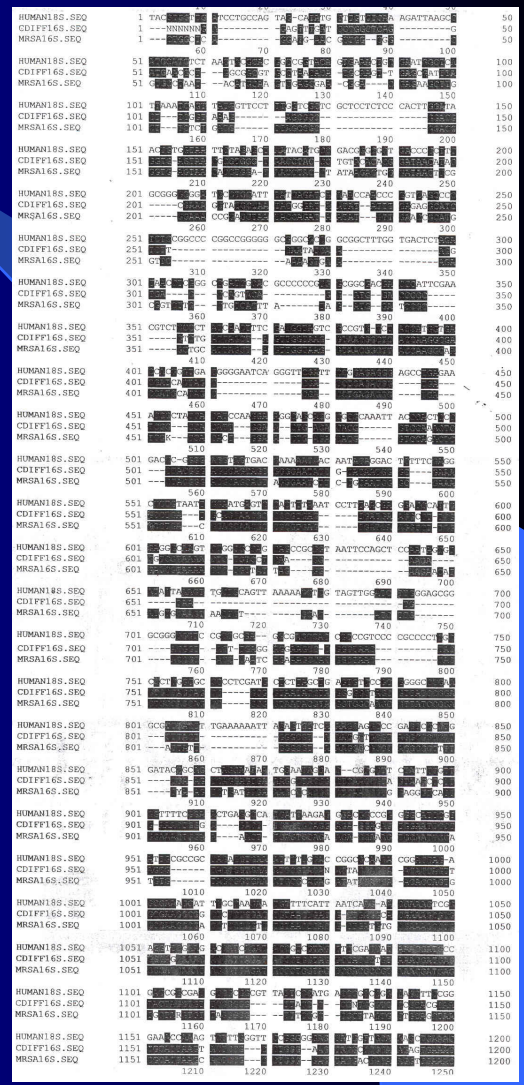
Result interpretation

1Halliday *et al*. 2005 BJH 132 478-486; [2]Buchheidt *et al*. 2004 BJH 196-202

Oligonucleotide design

- rRNA operon
  - 18S rRNA gene
  - Panfungal primers
  - Genus sp. probe
- Block-based/Sybr Green
  - False positives
- Probe based assay
  - False negatives

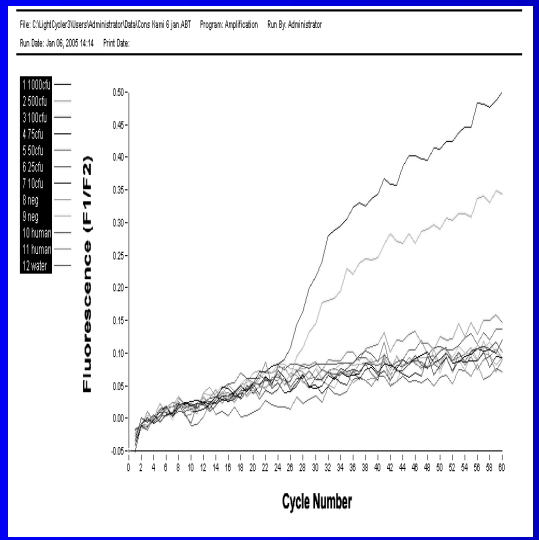




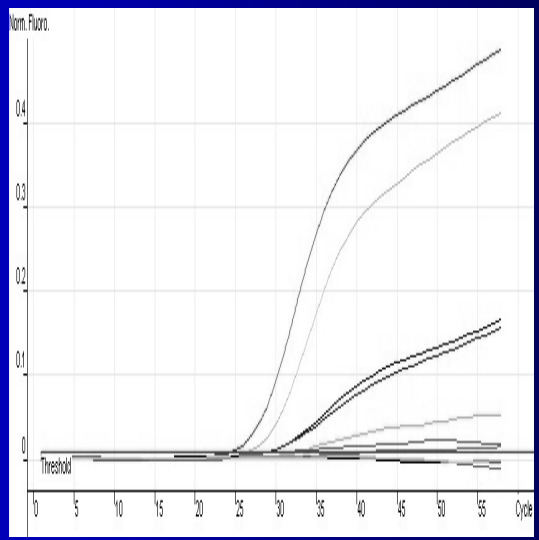
PCR platforms

- UK Fungal PCR Consensus Group
  - Evaluated *Aspergillus* PCR methods[1]
  - TaqMan Assay[2]
  - Most centres used Light Cyclers
  - Evaluate the method on other platforms
    - DNA extracted from spiked whole blood

Roche LightCycler



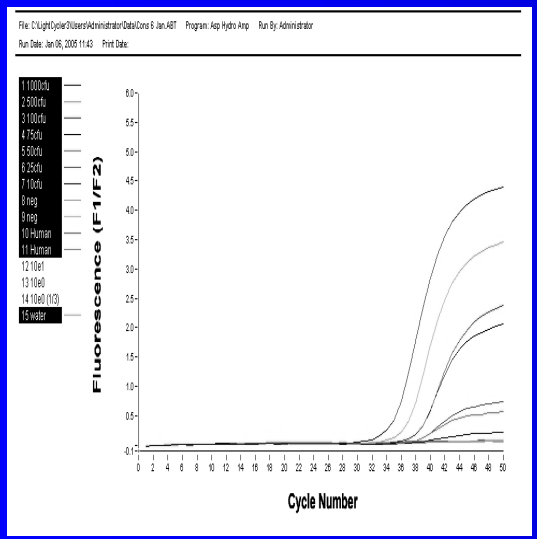
Corbett Rotor-Gene



[1]White *et al*. In press; [2]Kami *et al*. 200, CID 31 1504-12

PCR platforms

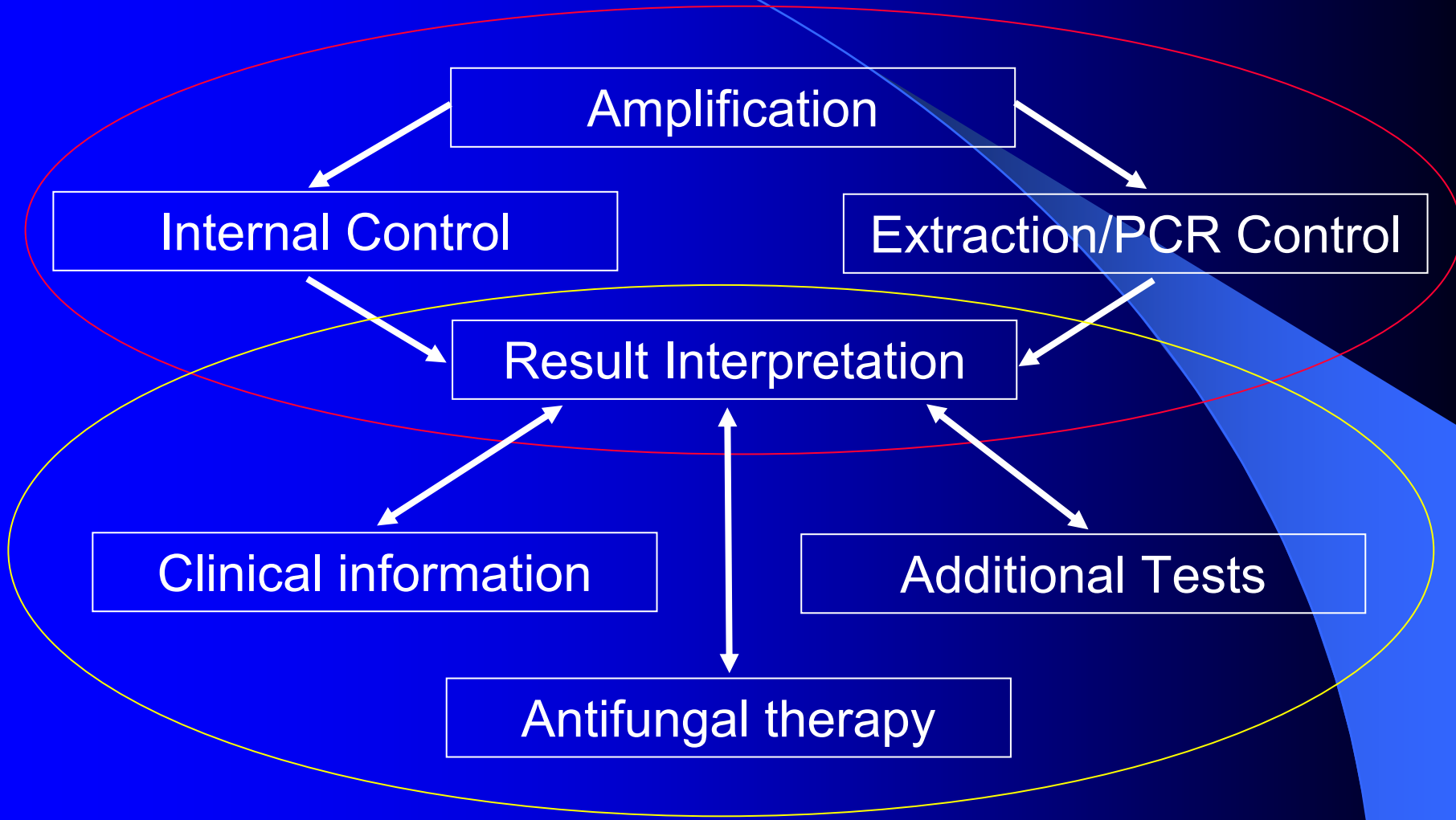
- UK Fungal PCR Consensus Group
  - Light Cycler Assay[1]



Sensitivity(%)			
Sample	**Light Cycler**	**Rotorgene**	**Taqman**
100cfu	**100**	**100**	**100**
75cfu	**86**	**100**	**100**
50cfu	**86**	**100**	**100**
25cfu	**29**	**100**	**100**
10cfu	**57**	**67**	**100**

[1]White *et al*. 2006, CID 42 479-86;

Limiting Factors



Result Interpretation

- Differentiation of false positive result from a true positive[1]
  - PCR enhanced sensitivity?
  - Early indicator of IFI?
  - Sub-clinical infection or colonisation?
- PCR positive patients[2]
  - DNA concentrations were very low
  - Below 100% reproducible limits
  - Positive sample: 1/2 replicates Cp <43
  - >DNA concentrations = more probable diagnosis of IA
  - GM ELISA + PCR positive = poor prognosis
- Result interpretation guidelines[3]

[1]Jordanides *et al.* 2005 BMT 35 389-95; [2]Millon *et al.* 2005 J Clin Micro 43 5097-101; [3]Halliday *et al.* 2005 BJH 132 478-86

Conclusions

- PCR has shown its usefulness in a number of studies
- It is an adjunctive test
  - ELISA and CT
  - Neutropenic Care Pathway
- Lack of a consensus method
  - Sample type
  - Extraction
  - Amplification
  - Interpretation
- UK Fungal PCR Consensus Group
  - Expand?
- EORTC accepted method