

Development and Optimisation of a Novel Multiplex Assay for Identifying Candida Species

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Introduction

- Prompt treatment of fungal infections improves patient survival
- Identification of the causative agent of candidaemia has an important effect on the choice of antifungal
- The MycArray™ Yeast ID kit was developed to rapidly identify and distinguish between Candida species (and other yeasts)
- The kit contains probes for 18 yeasts allowing simultaneous detection of one or more species from colonies or yeast-positive blood cultures
- Validation was carried out for the five major Candida species: *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*

Methods

Assay Design

- Probe design incorporated sequences in Myconostica's patent portfolio and used a combination of approaches including BLAST searches, multiple alignments and dedicated PERL scripts
- Multiple probes were designed to capture intra-species genetic variation (Table 1)
- The assay includes 3 process controls (Table 2)

Assay Optimisation

- Assay conditions were extensively optimised by testing multiple strains of each organism using colony PCR and DNA extracted from blood culture bottles using MycXtra® (Myconostica) kit (Figure 1)
- The assay can be completed in under 4 hrs from PCR to results (Figure 2)

Assay Verification

- Optimised assay conditions were verified using at least 2 strains of each species with colony PCR
- The top 5 candida species were extensively validated (~20 strains of each) using colony PCR

Length (bases)	20 - 42
Tm (°C)	58.9 - 60.9
GC content	33.3 - 65.0 %
Discrimination	Sensitive to 2 base differences between target and probe
Number per species	<i>Candida albicans</i> 3
	<i>Candida dubliniensis</i> 2
	<i>Candida famata</i> 2
	<i>Candida glabrata</i> 3
	<i>Candida kefyr</i> 2
	<i>Candida krusei</i> 2
	<i>Candida metapsilosis</i> 1
	<i>Candida parapsilosis</i> 1
	<i>Candida parapsilosis group</i> 3
	<i>Candida pelliculosa</i> 2
	<i>Candida rugosa</i> 1
	<i>Candida tropicalis</i> 5
	<i>Candida utilis</i> 2
	<i>Cryptococcus neoformans</i> 2
	<i>Histoplasma capsulatum</i> 1
	<i>Pichia guilliermondii</i> 2
	<i>Rhodotorula mucilaginosa</i> 3
	<i>Saccharomyces cerevisiae</i> 2

Table 1: Properties of probes

Sequence	Function
Three probes for a conserved region within 5.8S	To verify that a PCR product has been added to the array.
Internal Amplification Control	To independently verify that the PCR has functioned properly
Biotin markers (biotin label printed directly onto the chip)	To verify that the final detection stage has worked.

Table 2: Internal process controls

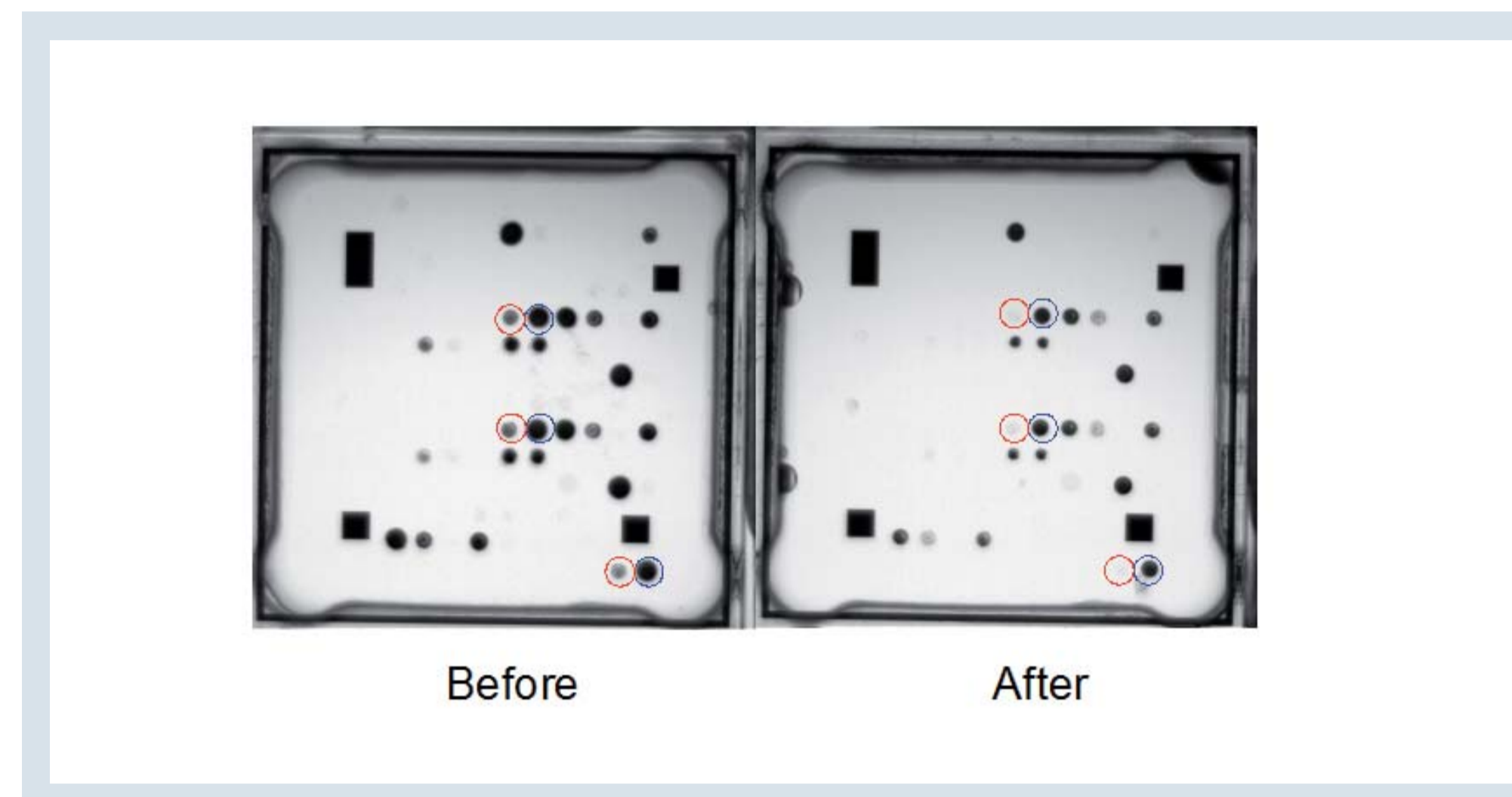


Figure 1: Assay optimisation. The chip is shown before and after optimisation of hybridisation conditions. Binding to a specific probe is ringed in blue, where as binding to a non-specific probe, which differs by two bases, is ringed in red. Probes are spotted in triplicate. Optimisation results in loss of the non-specific signal and retention of the specific signal.



Figure 2: Summary of MycArray™ Yeast ID workflow

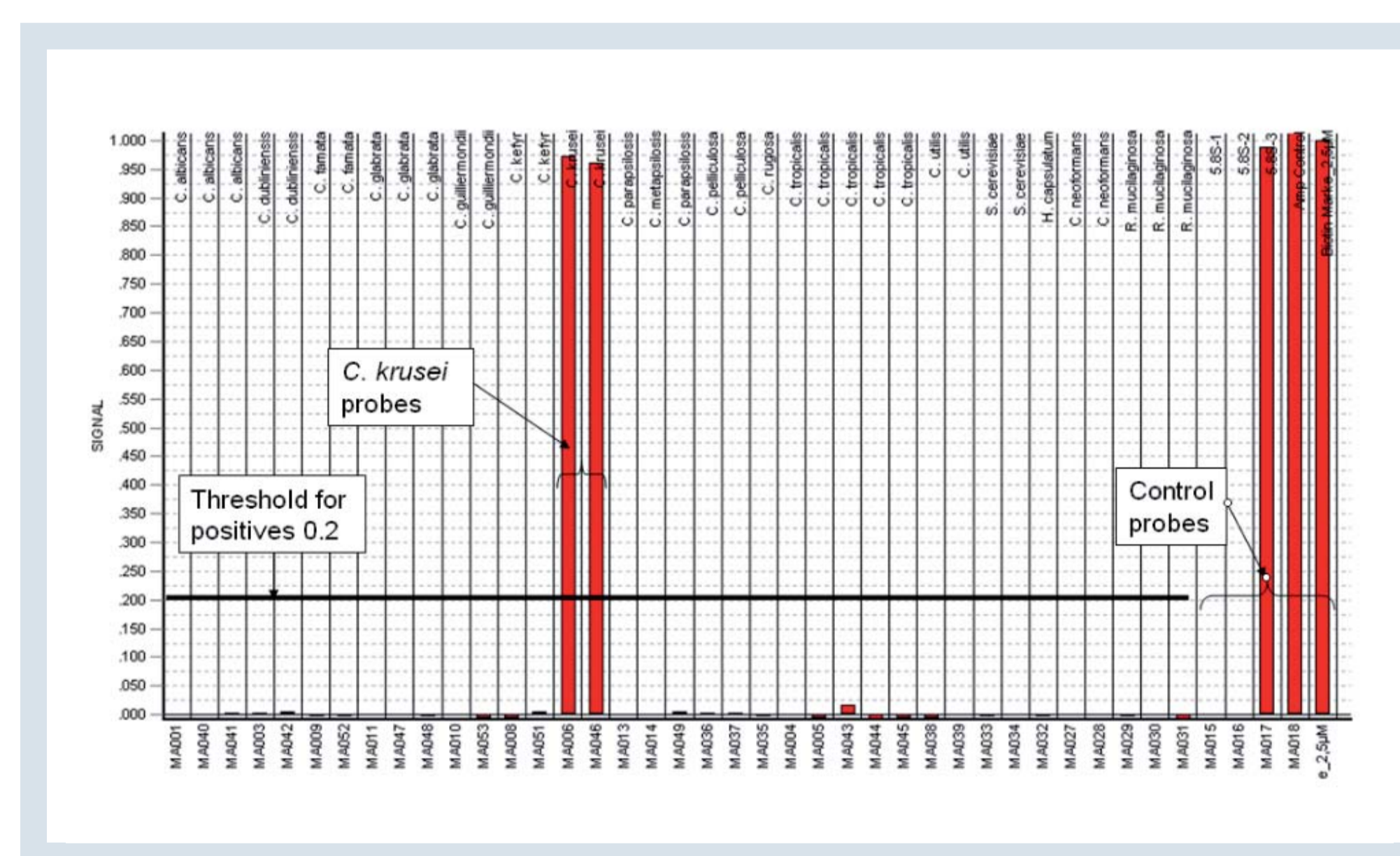


Figure 3: LoDs were defined as the lowest cell concentrations at which at least 95% of tests were positive. A typical positive result is shown for *C. krusei*.

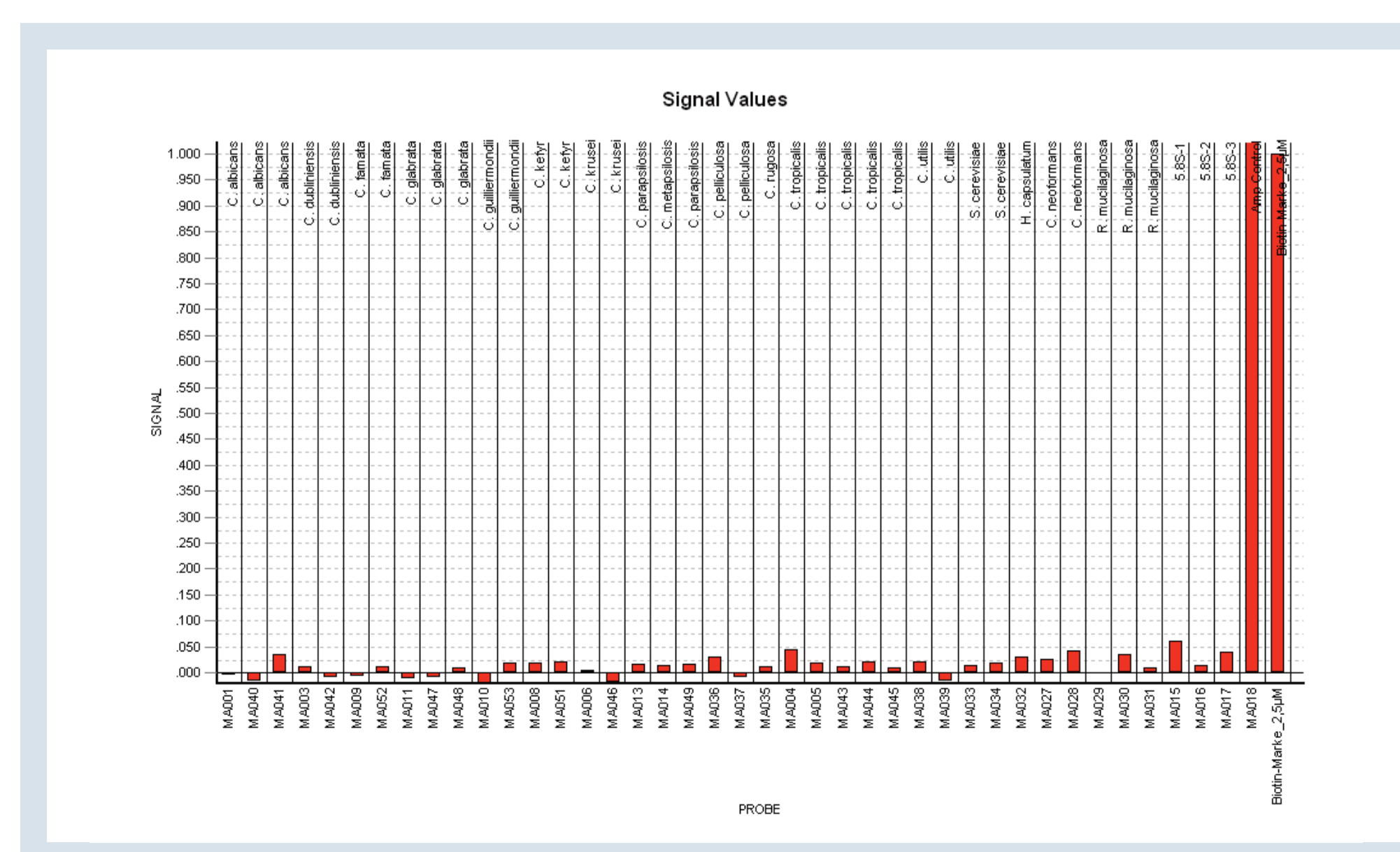


Figure 4: Human DNA gave no signal with the species-specific probes when used as a template for the PCR.

Results

- The limits of detection (LoD; cells/mL blood culture) were determined for the five main Candida species: *C. albicans* - 10⁵ cells/mL, *C. glabrata* - 10⁵ cells/mL, *C. krusei* - 10⁵ cells/mL, *C. parapsilosis* - 10⁴ cells/mL, *C. tropicalis* - 10⁵ cells/mL (Fig.3)
- Non-target organisms likely to be found in clinical samples were tested; Human, bacterial and *A. fumigatus* DNA gave no signal with the species-specific probes (Fig.4)
- All pairwise combinations of target organisms were tested at low and high cell concentrations, showing that mixed cultures could be detected with no loss of signal (Fig.5)
- Clinical samples gave strong signals (Fig.6)
- All other species represented on the chip were detected when tested using colony PCR (Fig.7).

Conclusions

- The MycArray Yeast ID has been optimised to identify 18 clinically relevant yeast species from agar plates and positive blood culture bottles
- The array process can be completed in 4 hrs
- The kit will be CE marked for identification of the five major Candida species by Q4 2009

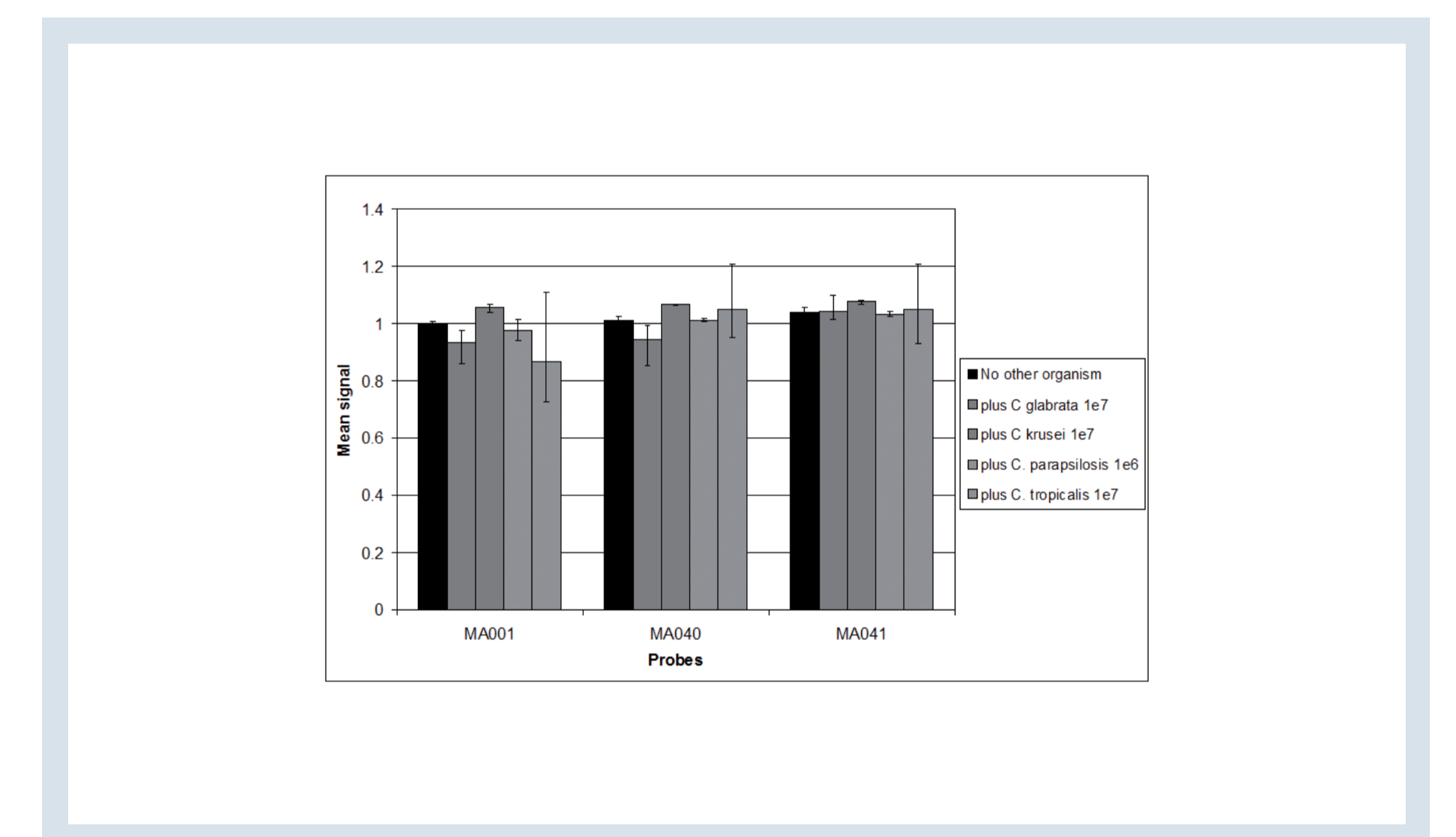


Figure 5: The effect of one organism on the presence of a second. Blood bottles were spiked with *C. albicans* cells at 106 cells/mL plus other species as indicated, then extracted and arrays carried out. Signals were unaffected by the presence of a second organism.

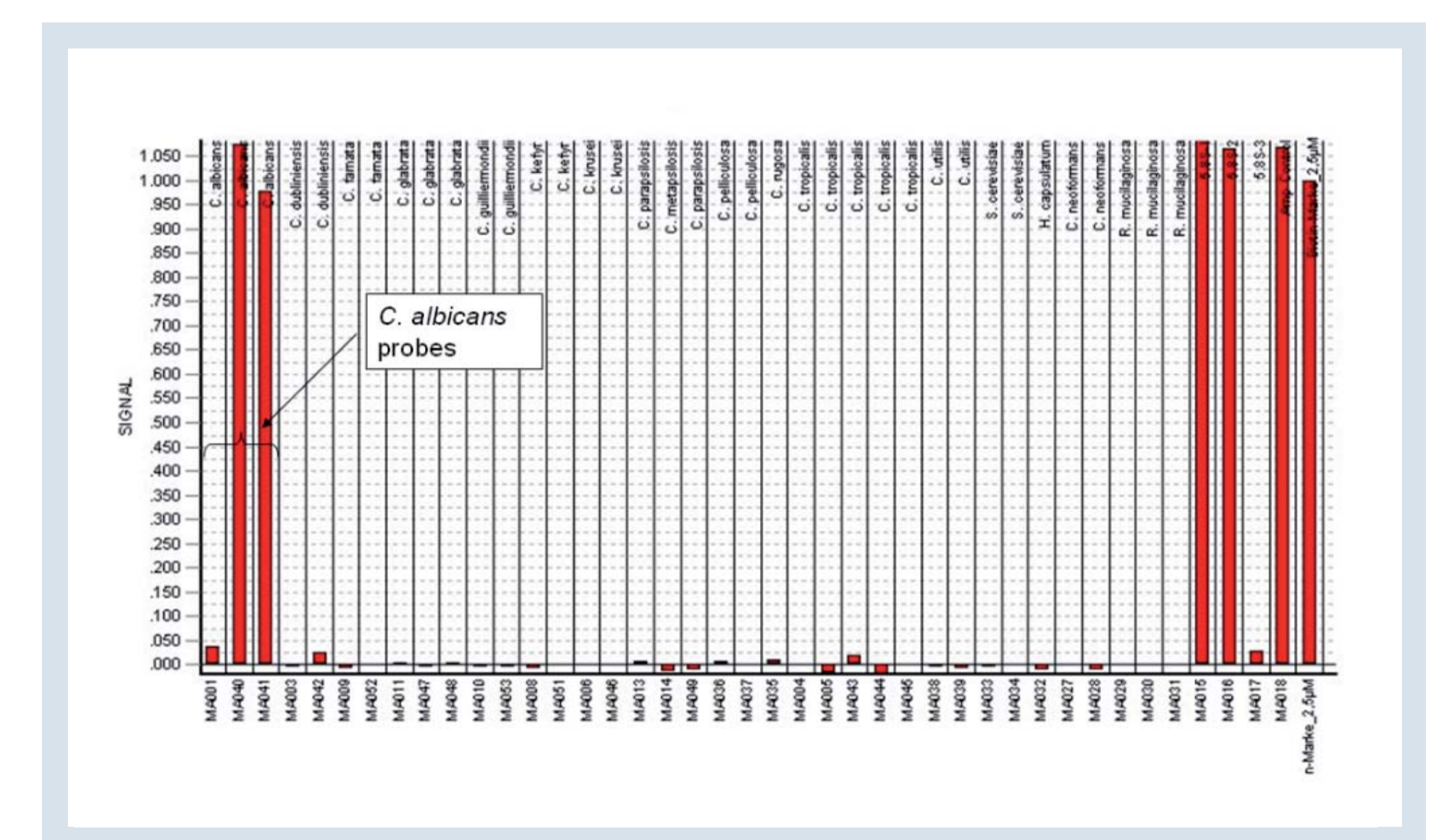


Figure 6: A clinical blood culture sample which was yeast-positive was extracted and used as input for the kit. The yeast was identified as *C. albicans*.

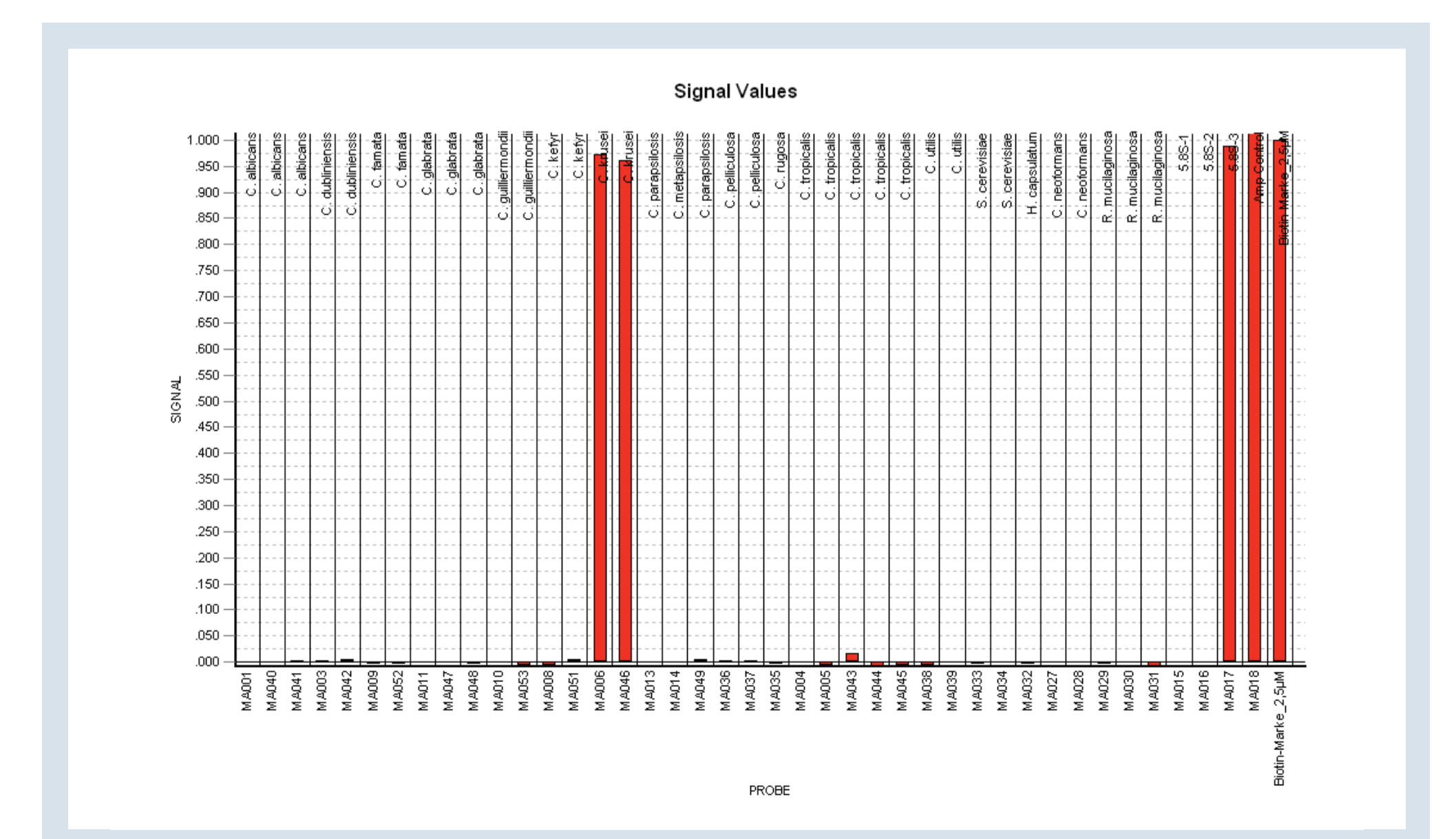


Figure 7: The Array kit detected *Cryptococcus gattii* from colony PCRs.