IN VITRO SCREENING FOR ANTIFUNGAL EFFICACY

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While serious fungal infections have historically been fairly rare, the increase in the number of susceptible immunocompromised patients has contributed to the rise in prevalence of fungal diseases. Treatment options are limited as well with only a few approved drugs currently on the market. This, coupled with the rise of antimicrobial resistance, further complicates the life of the patients with systemic fungal infections such as invasive aspergillosis or candidiasis. Moreover, the majority of patients that develop a serious systemic fungal infection often have another underlying medical condition making this population particularly vulnerable. With the growing incidence of systemic fungal infections, and the limited number of effective treatments, the development of new antifungals has become increasingly important. An essential part of this process is *in vitro* susceptibility testing, for which iFyber provides a range of assays.

The most common assays for evaluating antifungal susceptibility are the broth dilution or broth microdilution methods. Following guidelines from the Clinical & Laboratory Standards Institute (CLSI) [1-2], iFyber routinely utilizes the broth microdilution method to determine the minimum inhibitory concentrations (MICs) of antifungal agents. An important consideration for this test method is to determine the protocol to be used for assigning the MIC, which is based on the type of antifungal agent being assessed. The general guidelines provided by the CLSI are that the MIC can be assigned as either the lowest concentration that results in complete inhibition of microbial growth compared to an untreated control (Figure 1a), or the lowest concentration that results in *prominent* decrease in turbidity (i.e., 50% inhibition of growth compared to untreated controls as shown in Figure 1b). In the two examples shown in Figure 1, the MIC for amphotericin B against *Candida glabrata* is 0.25 µg/mL, while the MIC of isavuconazole is 0.125 µg/mL.

Other important aspects to consider when performing antifungal efficacy testing are growth rates of the microorganisms, which affect the assay time, the solubility of the antifungals, the stability of the antifungals (e.g., light sensitivity), assay temperature requirements, and the final product application. All of these aspects need to be taken into account when designing a screening campaign.

iFyber has also implemented the broth microdilution method to screen for synergy and antagonism of combination treatments. This applies to:

a. combinations of antifungal agents
b. antifungal-antibacterial combinations when both are needed in a formulation
c. compatibility with compounds that on their own do not elicit antimicrobial efficacy but can potentially render fungi more sensitive to the antifungal drug
d. non-active compounds that are part of the final product formulation

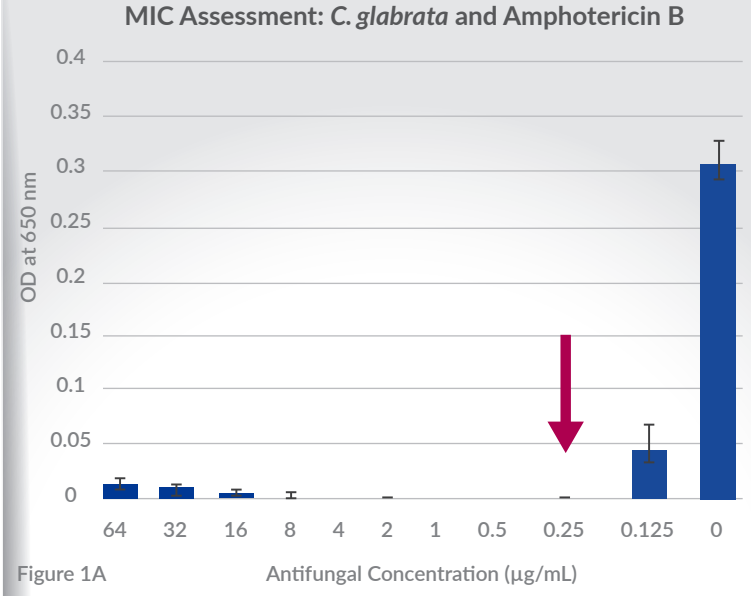

Figure 1A

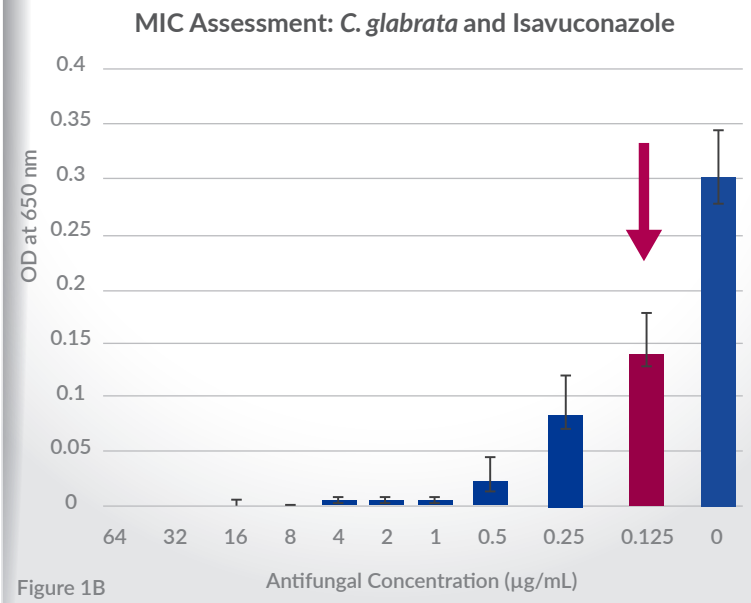

Figure 1B

Figure 1: Examples of MIC assay readouts for the evaluation of antifungal efficacy against *Candida glabrata*. Bars representing actual MIC values are highlighted in red. Note: The CLSI standard for amphotericin B (a) is no turbidity while the standard for isavuconazole (b) is a 50% decrease in turbidity compared to an untreated control.

IN VITRO SCREENING FOR ANTIFUNGAL EFFICACY *cont.*

This assay allows for rapid identification of synergistic or additive effects for combinations of treatments, as shown in the example experimental set-up in Figure 2. Here, the checkerboard assay format is a 96-well plate set-up that allows for the assessment of 64 unique combinations of concentrations of two compounds. The assay can be adapted to evaluate the effects of > 2 compounds as well.

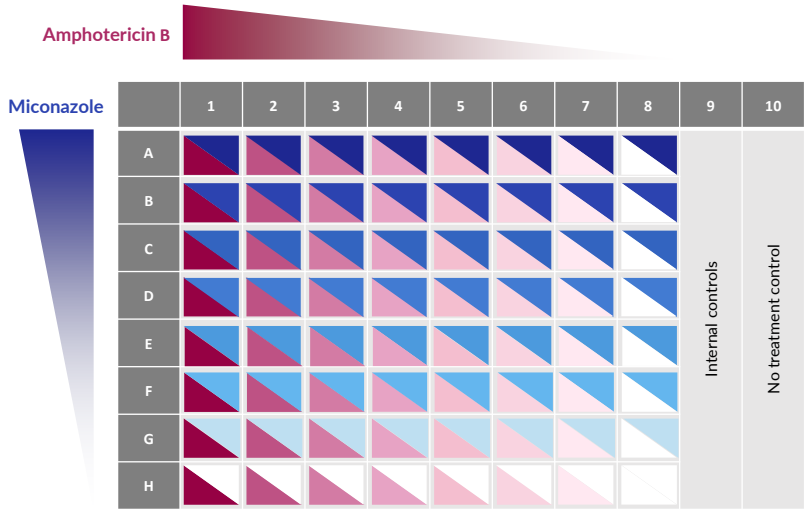

Figure 2: Example of a checkerboard assay set-up. This assay specifically looked at combinations of amphotericin B and miconazole. Amphotericin B concentration was varied by column while miconazole concentration varied by row. Note that well H8 contained no antifungal agent.

An example assay readout is illustrated in Figure 3 and shows the evaluation of synergy between two antifungal agents, amphotericin B and miconazole, using a range of 8 concentrations for each compound, and includes controls.

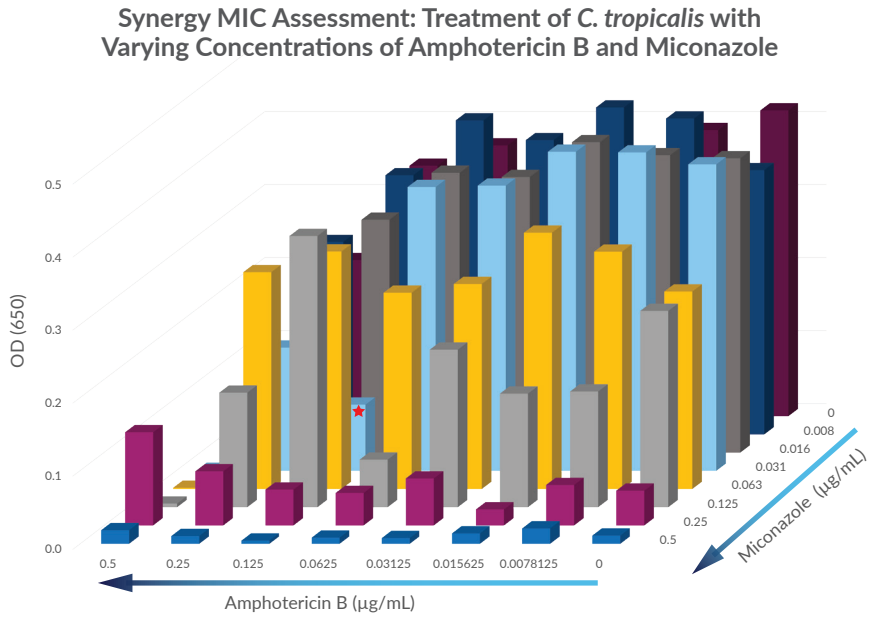

Figure 3: Results from a checkerboard study evaluating varying concentrations of amphotericin B and miconazole against *Candida tropicalis*. ★Star denotes 0.125 µg/mL amphotericin B and 0.031 µg/mL miconazole, the combination of treatments which shows synergy.

A key output of these antifungal assays is the Fractional Inhibitory Concentration Index (FICI), which is used to quantify the effects of different treatments and establish potential synergistic (desired) or antagonistic (undesired) effects. Importantly, this analysis differentiates synergistic effects from simple additive effects of the two different antifungal agents. The FICI is calculated as follows:

$$FICI = \frac{MIC(A \text{ in } AB)}{MIC(A)} + \frac{MIC(B \text{ in } AB)}{MIC(B)}$$

MIC (A in AB)	MIC of compound A when A and B are used in combination
MIC (A)	MIC of compound A when A is used alone
MIC (B in AB)	MIC of compound B when A and B are used in combination
MIC (B)	MIC of compound B when B is used alone

FICI VALUE	DESIGNATION
<0.5	Synergy
>4	Antagonism
0.5<FICI<4	Additive effect

iFyber has implemented a 96-well plate-based screening format to evaluate synergistic and antagonistic effects of combination treatments (2 or more components) against a number of pathogenic species of yeast. The assay is also amenable to evaluating specific conditions relevant to the application of the treatment or product, such as temperature and treatment time (several days). The high-throughput nature of the screening allows for simultaneous assessment of more than 60 conditions within a single plate and allows for quick down-selection of relevant treatment combinations.

References:
1. CLSI (2008a) Reference method for broth dilution antifungal susceptibility testing of yeasts; 3rd edition; CLSI document M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA.

2. CLSI (2020) Performance Standards for Antifungal Susceptibility Testing of Yeasts, 2nd edition; CLSI document M60-ED2. Clinical and Laboratory Standards Institute, Wayne, PA.