

# High-Throughput Method for Dual Assessment of Antifungal Activity and Growth Kinetics Using a FLUOstar Omega

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- Effect of tea tree oil on yeast growth measured on the FLUOstar Omega
- Minimal inhibitory concentration of antifungal agents determined kinetics
- Growth and inhibitory effects can be assessed in tandem

## Introduction

High throughput testing is an essential requirement for testing panels of potential antimicrobial molecules against clinically important pathogens. This is particularly important considering there is an increasing incidence of infectious diseases and dwindling arsenal of efficacious antimicrobial agents. The pharmaceutical industry, commercial laboratories and independent university researchers therefore require sensitive tools to enable them to detect antimicrobial activity from their chosen panel of molecules.

Current methodologies largely rely on endpoint broth microdilution or agar diffusion to assess antimicrobial activity. Assessing antimicrobial activity using endpoint readings is difficult, as this relies on a visual inspection of turbidity and observing well within a microtitre plate with little or no growth in comparison to the untreated control. Although the absorbance of the endpoint assay can be read spectrophotometrically, this does not provide accurate data relating to the kinetics of inhibition, which may be subtle in many instances due to slow growth rates. For example, antimicrobial activity may be influenced by the rate of growth in a particular media, e.g. Mueller Hinton broth, which again cannot be accurately assessed by an endpoint visual inspection. Therefore, an accurate interpretation of the data is not possible.

The experimental approach described within this application note aimed to determine the ability of the FLUOstar Omega to accurately quantify the inhibitory properties of the novel antimicrobial agent tea tree oil (TTO) grown against the pathogenic yeast *Candida albicans*.<sup>1-5</sup> The results are compared to growth rates when the known antimicrobial agent Amphotericin B is present.



Fig. 1: BMG LABTECH's multimode plate reader FLUOstar Omega

## Materials and Methods

- Clear polystyrene 96-well plates (Corning, NY, USA)
- Melaleuca alternifolia tea tree oil (Sigma-Aldrich, Dorset, UK)
- FLUOstar Omega (BMG LABTECH, Offenburg, Germany)
- Amphotericin B (AMB [Sigma-Aldrich, Dorset,UK])

*Candida albicans* was propagated overnight in yeast peptone dextrose (YPD) broth at 37°C on an orbital shaker. The resultant yeast suspension was washed in PBS by centrifugation and counted in a haemocytometer. The yeast cells were standardised to approximately  $1 \times 10^6$  cells per millilitre and then diluted one hundred fold into fresh YPD broth. TTO and AMB were then prepared in YPD containing 0.05% v/v Tween 80 at 0.01, 0.05, 0.1 and 0.5% for TTO and 0.0002, 0.0001, 0.00005 and 0.000025% w/v for AMB. Yeast cells were diluted into each TTO suspension to a final concentration of  $1 \times 10^4$  cells/mL, then 200  $\mu$ L volumes arrayed in quadruplicate into adjacent columns of a 96-well microtitre plate. A negative control without any reagent was included. The plate was placed inside the FLUOstar Omega with the incubator preset to 37°C. The absorbance mode was set to read at 550 nm with orbital shaking for 30 seconds prior to each read. This was programmed to take forty eight individual measurements over a 24 hour period.

## Results and Discussion

With increasing concentrations of TTO relative to the untreated control, a significant reduction of growth is observed (Figure 2).

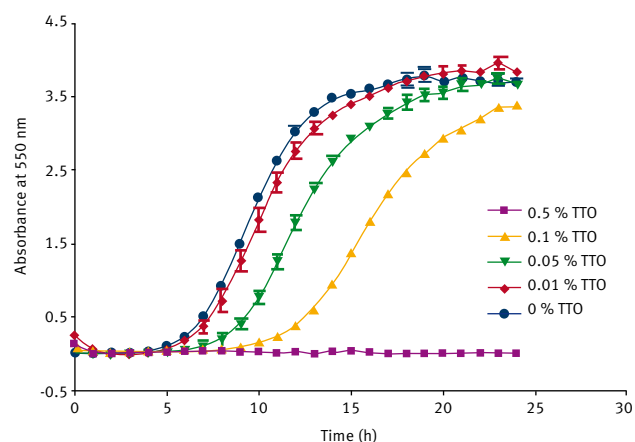
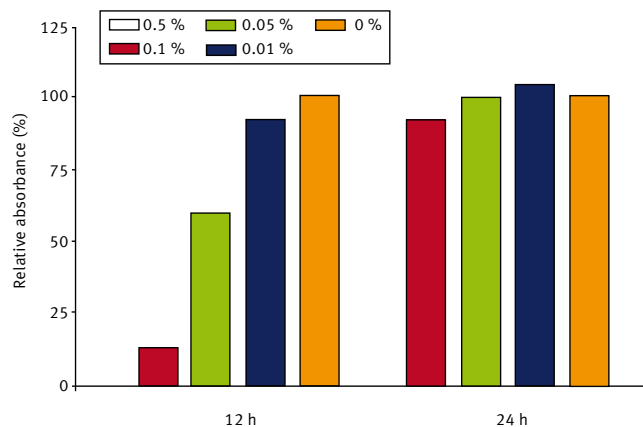


Fig. 2: Dose dependant growth curves of *Candida albicans* in TTO. The mean of the quadruplicate data was taken and standard error bars presented. The data was imported and presented using GraphPad Prism software.

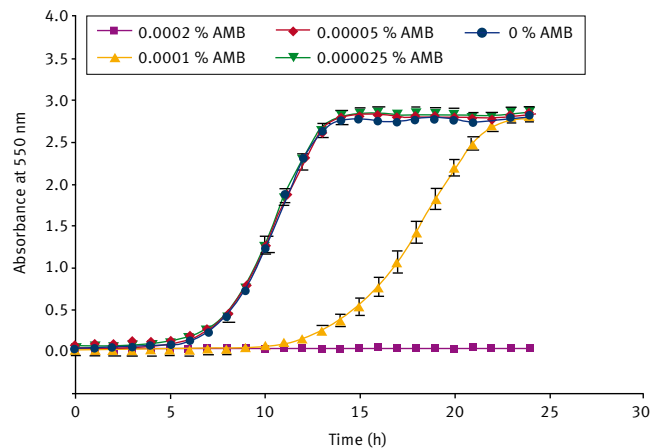
In addition, this data demonstrates dose dependant inhibitory characteristics, which would be impossible to differentiate from a 24 endpoint read. This point is clearly demonstrated when the absorbance data from the 12 h and 24 h time points are compared to the untreated control (Fig 3). After 12 h incubation, significant differences are observed between the various concentrations

tested, whereas at 24 h incubation the difference in mean absorbance are minimal.



**Fig. 3:** Relative absorbance of *Candida albicans* at 12 and 24 h in TTO. The mean of data points at 12 and 24 h from each concentration tested were calculated, and the treated cells were compared relatively to 12 and 24 h controls (100%).

Following studies on the novel antifungal TTO, the well described and potent antifungal agent AMB was added as described above at different concentrations to *Candida albicans* suspensions (Fig 4). The data showed that AMB exhibited a clear inhibitory profile at the highest concentrations (0.0002%), with a reduced growth rate at 0.0001%. However, at all other concentrations examined the growth rates were similar to AMB free cell suspensions, indicating that the concentration of AMB is critical in inducing cell membrane instability.



**Fig. 4:** Dose dependant growth curves of *Candida albicans* in AMB. The mean of the quadruplicate data was taken and standard error bars presented.

Overall these data demonstrate that the antifungal mode of action is an important determinant of the inhibitory and growth profiles exhibited by *Candida albicans*. AMB is a polyene antifungal agent that disrupts cell membranes and allows cytoplasmic components leak out causing cell death, which is induced through specific interaction with ergosterol in the cell membrane. TTO is also proposed to act on the cell membrane, but as this is comprised of a complex mixture of terpinenes, its specific mode of action has yet to be determined. It is likely to alter membrane permeability in a non-specific and concentration dependant manner, which is demonstrated from the pattern of growth kinetics obtained in the experiments described herein.

## Conclusion

In this experiment we show that minimum inhibitory concentration assays can be assessed using the FLUOstar Omega to provide a sensitive and accurate analysis of growth kinetics to demonstrate subtle dose dependant drug effects that otherwise would be missed using endpoint readings. The benefit to this method for screening is that *Candida albicans* and other pathogenic microorganisms can be tested with a range different antimicrobial agents and concentrations. Furthermore, if different fluorescent strains are available, both growth and inhibitory properties of mixed microbial populations can be assessed in tandem.

## References

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