Application Note

Microbial Metabolism

Assessing Microbial Proliferation and Antibiotic Resistance using the MitoXpress Xtra Oxygen Consumption Assay

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Introduction

The MitoXpress Xtra Oxygen Consumption Assay facilitates microtiter-plate based analysis of microbial oxygen consumption. The 'mix & measure' procedure allows rapid and specific detection of microbial oxygen consumption, providing a simple, sensitive method to investigate microbial metabolism, screen for antimicrobial compounds, and optimize culture conditions.

The assay is based on the ability of dissolved O_2 to quench the phosphorescence of the water-soluble, oxygen sensitive MitoXpress Xtra probe. Depletion of dissolved oxygen in the sample causes an increase in MitoXpress Xtra fluorescence signal, reflecting changes in oxygen concentration within the sample.

Materials & Method

- MitoXpress Xtra Oxygen Consumption Assay, Agilent (Cat# MX-200-4)
- 96- or 384-well microtiter plates
- FLUOstar Omega fluorescence plate reader with TR-F Optical attachment, suitable filter set, pre-defined instrument protocols & data analysis templates and temperature control (BMG LABTECH)*

***NOTE:** This application is compatible with a range of alternative fluorescence plate reader models and detection modes (1. Dual-read TRF Lifetime, 2. single TRF intensity or 3. Fluorescence intensity (FI) modes).

For optimal performance, we recommend using dual-read TRF followed by single read TRF measurements as an alternative where available.

For more information on plate reader models, detection modes, instrument settings [2], pre-defined instrument protocols and data analysis templates (selected plate-reader models), see MitoXpress & pH Xtra Consumable Home Page. Basic Microbial Data Analysis Templates are available for selected plate reader models, contact cellanalysis.support@agilent.com

The assay is a simple 'mix and measure' test:

1. Microbes are dispensed into the wells of a 96 well plate in 100 µl volumes were in the appropriate culture medium.
2. 10 µl of MitoXpress Xtra probe (prepared as per User Guide [1]) was added to each well.
3. 100 µl of High Sensitivity Oil (included in MitoXpress Xtra Oxygen Consumption Assay Kit) was added to exclude ambient O_2. For 384-well plate measurement, or for easier oil dispensing, MitoXpress mineral oil (MO-200L-1) is recommended for this microbial application.
4. Time-resolved fluorescence intensity of the MitoXpress Xtra probe was measured kinetically at the required temperature (30°C / 37°C) on a FLUOstar Omega plate reader using the preconfigured instrument protocol. (Protocols can be requested from cellanalysis.support@agilent.com) or available in the Omega Instrument Guide [2] On this occasion Dual-read TRF Lifetime detection was used.
5. TR-F intensity signal (RFU or µs) were then plotted versus time as described in [1], using preconfigured OMEGA Data Analysis Template (can be requested from cellanalysis.support@agilent.com).

Results and Discussion

Analysis of bacterial growth:

The MitoXpress Xtra Assay can be used to provide a rapid assessment of bacterial growth without the need to conduct multiple sample dilutions and lengthy agar-based investigations. As bacteria in the test sample grow and respire, they deplete O_2, which is detected as an increase in MitoXpress Xtra probe signal above a baseline threshold level (Figure 1). The time required to reach this increase in probe signal (time-to-threshold) can be used to assess the number of cells in the sample (Figure 2). For the MitoXpress Xtra Assay, the threshold is chosen based on the growth characteristics of the specific microbes of interest.

Typically a signal threshold 1.4 fold the baseline MitoXpress signal level (no bacteria) is applied. The detection mode of the fluorescence plate reader should be optimized in advance to ensure robust signal acquisition.

Definition: Time to threshold reports the time required for a kinetic reaction to reach a specified OD, MitoXpress Signal (RFU or µs) (onset OD, onset RFU/µs)

This elapsed time data is useful for growth experiments where the change in sample concentration does not have an effect on the maximum signal change but changes the time required for the reaction to reach threshold / completion.

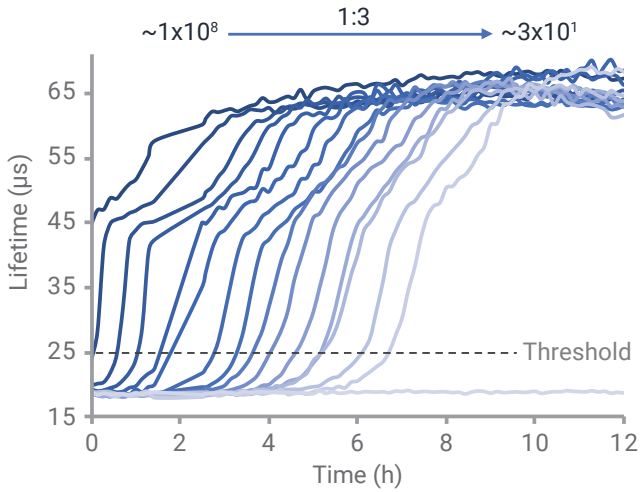

Figure 1. Oxygen-based growth curves from serial dilution of *E. coli* in enrichment broth (EB). As bacteria replicate, oxygen consumption increases. At a critical point (*Time-to-Threshold*), oxygen consumption exceeds back diffusion. This is seen as an increase in probe signal.

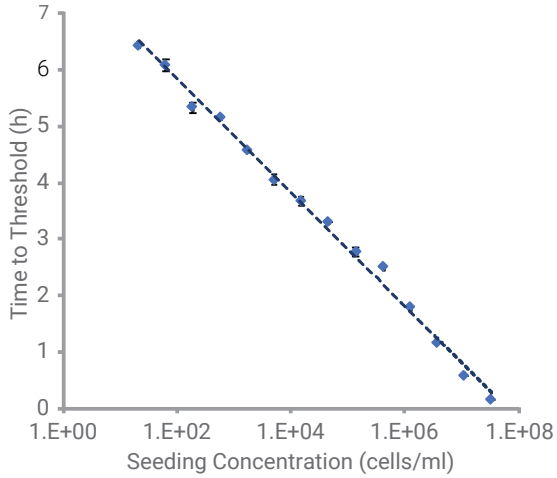

Figure 2. Correlation between *Time-To-Threshold* and seeding concentration. The time required to reach the threshold signal (24 µs) reflects the seeding concentration and is dependent on the replication rate and cellular oxygen consumption rate.

Antibiotic Treatment:

Microtiter plate-based analysis of microbial oxygen consumption allows the identification and high-throughput generation of IC_{50} and MIC values of antimicrobial compounds. Sample dose-response curves for compounds active against *S. aureus* are presented in Figure 3.

Effective compounds (Rifampicin, Streptomycin and Vancomycin) show a strong dose-dependent impact on measured time-to-threshold (*S. aureus* growth & respiration) compared to no treatment (0 µM) sample. In some cases the higher compound concentrations eliminated *S. aureus* growth / respiration completely (no onset time, flat signal curve) and hence no growth within the assay duration.

Whereas Etambutol treatment exhibited little if no impact on *S. aureus* growth / respiration at tested concentrations (signal curve onset time unchanged).

Analysis of Fungal Growth – *C. albicans*

The MitoXpress Xtra Assay is suitable to measure oxygen depletion and assess cell numbers and metabolism of yeasts (Figure 4).

Short term analysis (high cell numbers measured for ~ 2-3 h) allows the assessment of immediate effects on cell metabolism (Figure 5), while longer term analysis (lower cell numbers & extended measurement times) facilitates analysis of cell growth and metabolism (Figure 4).

Data reduction: In the case of short term analysis, the signal curves are amenable to linear regression (slope) calculation, where as for long-term analysis, the signal curves are more suited to time-to-threshold calculation.

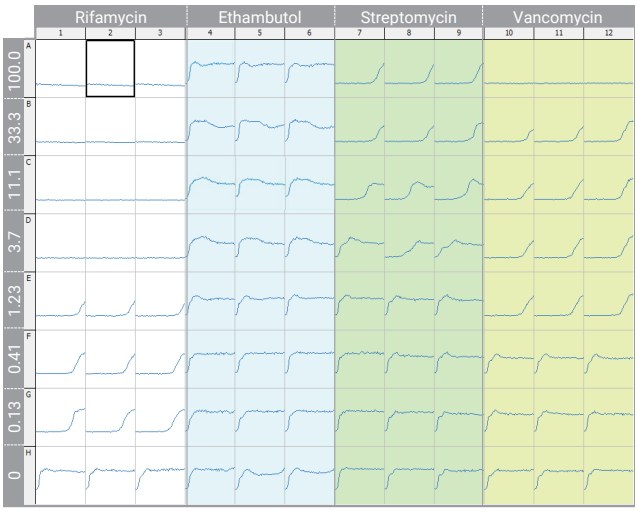

Figure 3. *S. aureus* seeded at ~1x10^7 cells/ml in EB, exposed to increasing concentrations (µM) of the indicated antibiotic (Rifampicin, Etambutol, Streptomycin, Vancomycin) and measured kinetically at 37°C. Compound dilutions applied from row A (max conc. 100 µM) downwards to row G (min. conc. 0.13 µM) in a 96-well plate.

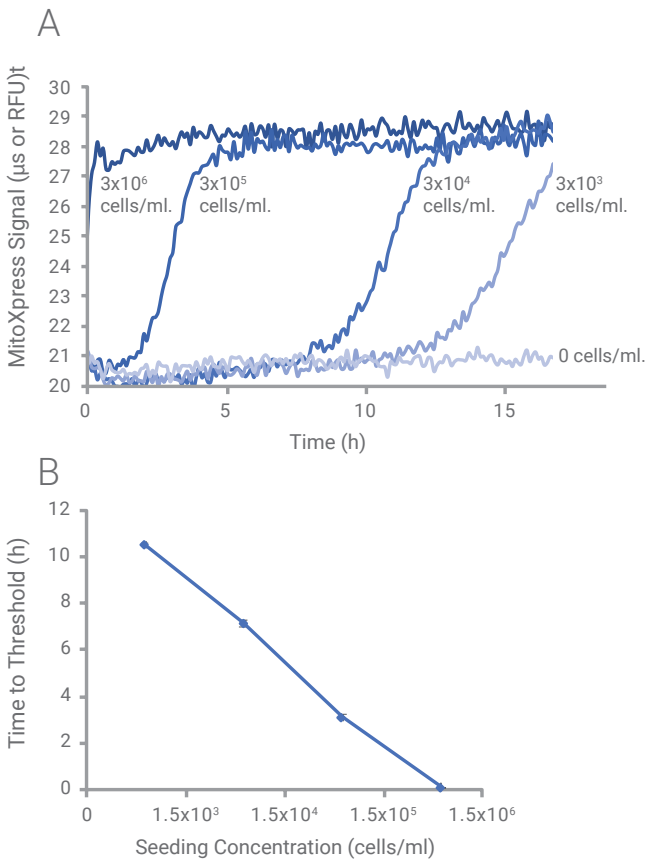

Figure 4. (A) *C. albicans* oxygen consumption profiles measured at decreasing seeding concentrations (3x10^6, 3x10^5, 3x10^4, 3x10^3 and 0 cells/ml). **(B)** Relationship between *Time-to-Threshold* and *C. albicans* seeding concentration.

Mode of Drug Action Assessment:

The electron transport chain inhibitor Antimycin (Fig. 5A) and the polyene antifungal Amphotericin B (Fig. 5B) cause immediate and dose- dependent decreases in oxygen consumption while the triazole antifungal Fluconazole (Fig. 5C) causes no appreciable decrease in oxygen consumption. These data are amenable to linear regression (slope) analysis, which is available in most plate reader data analysis software. These observations correlate with mode of drug action and demonstrate how such short-term measurements can be used to assess the specific metabolic effects of compound treatment.

Conclusion

The **MitoXpress Xtra Oxygen Consumption Assay** facilitates simple and convenient probing of microbial oxygen consumption and can be applied to the analysis of bacteria and yeast. The metabolism and growth implications of treatments such as drug exposure, genetic manipulation or altered culture conditions can be easily accessed. The assay can detect antimicrobial activity and provides the throughput and resolution necessary for screening and generating IC_{50} or MIC data.

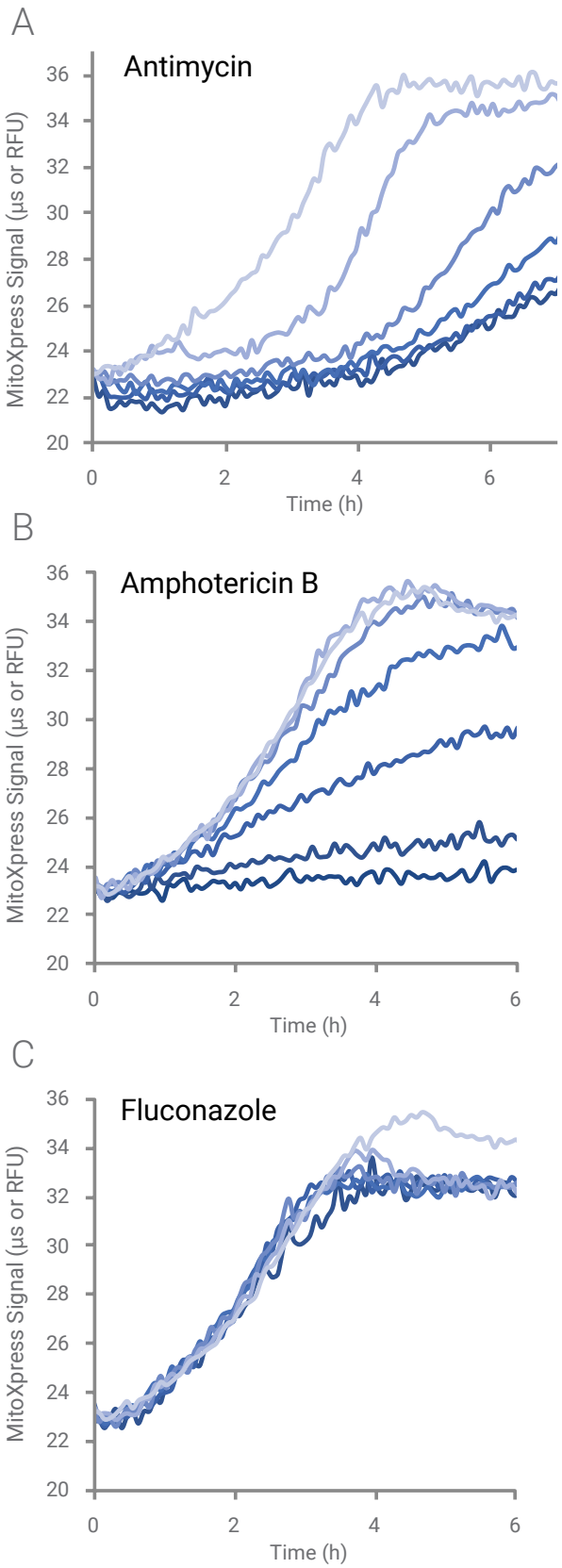

Figure 5. Oxygen consumption profiles from *C.albicans* (~3x10^5 cells/ml) treated with increasing concentrations Antimycin (● 0, ● 0.4, ● 1.1, ● 3.3, ● 10 and ● 30 µM), Amphotericin B (● 0, ● 0.07, ● 0.2, ● 0.6, ● 1.8, ● 5.3 and ● 6 µg/ml) and Fluconazole (● 0, ● 0.8, ● 2.5, ● 7.2, ● 21.7 and ● 65 µg/ml) in RPMI.

[1] MitoXpress Xtra Oxygen Consumption Assay User Guide, available on www.agilent.com
[2] Link to MitoXpress Compatibility Chart

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