APPLICATION NOTE

Measure oxidative metabolism and glycolytic activity on the SpectraMax i3x reader

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Benefits

- **Real-time measurement of extracellular oxygen depletion and acidification in cultured cells and microorganisms**
- **Sensitive TRF detection of two simple, mix-and-measure assay workflows**
- **Faster data acquisition and analysis with preconfigured SoftMax Pro protocols**

Introduction

To survive, cells require fuel in the form of ATP to carry out most essential bioprocesses. This fuel is generated through glycolysis and mitochondrial respiration. While both produce ATP, glycolysis can function in the absence of oxygen, whereas mitochondria require oxygen for the final step in oxidative phosphorylation (OXPHOS). These pathways dynamically shift to meet energy demands of the cells while adapting to environmental stresses, substrate availability, and hypoxia. Generally, measurements of changes give more information about metabolic responses than a single point measurement (e.g. ATP endpoint). Perturbed respiration is a sensitive indicator of mitochondrial dysfunction following drug toxicity. Aberrant mitochondrial function has been implicated in a growing spectrum of diseases, from neurodegeneration to cancer.

Understanding how these pathways respond to effector compounds can provide useful insights into the overall function of cells and the underlying mechanisms that determine cell fate.

Recent advances in reagent and plate reader technology now facilitate convenient metabolic analysis on standard microtiter plates. Here, we describe the use of both the Agilent MitoXpress® Xtra Oxygen Consumption Assay and the Agilent pH-Xtra™ Glycolysis Assay on the SpectraMax® i3x Multi-Mode Microplate Reader. An enzymatic assay with glucose oxidase (GOx) and a cell-based assay were used to evaluate oxygen consumption and glycolysis using standard 96-well plates.

The MitoXpress Xtra Oxygen Consumption Assay allows direct real-time measurement of oxygen consumption. The reagent is an oxygen-sensitive, water-soluble, cell-impermeable fluorescent probe. Fluorescence emission is quenched by O_2 such that the signal is inversely proportional to the concentration of O_2 in the well. As oxygen is consumed, fluorescence increases, so that using kinetic measurements, the user can infer how active the mitochondria are by monitoring the rate of signal increase.

The pH-Xtra Glycolysis Assay allows direct real-time kinetic analysis of extracellular acidification. As glucose is processed by the glycolytic pathway, it can generate pyruvate for further oxidation in the Krebs cycle or generate lactate (lactic acid), which causes the cells to acidify the extracellular environment. This change in pH can be measured using pH-Xtra, providing insight into glycolytic activity. As with MitoXpress Xtra, pH-Xtra exhibits a positive signal response across the biological range, allowing flexible, high-throughput assessment of extracellular acidification, and is not consumed during the reaction. Rates of extracellular acidification are calculated from changes in fluorescence signal over time.

To achieve best sensitivity for MitoXpress Xtra or pH-Xtra reagents it is recommended to use a time-resolved fluorescence detection mode on a plate reader. The SpectraMax i3x reader includes absorbance, fluorescence, and luminescence detection modes. Time-resolved fluorescence detection of the MitoXpress Xtra and pH-Xtra reagents is enabled by the addition of the Time-Resolved (TRF) Detection Cartridge. The TRF detection cartridge contains a dedicated light source and emission filters to achieve a high level of performance. Both acquiring and analyzing the progression of oxygen consumption and glycolic activity is achieved by using a preconfigured protocol available at softmaxpro.org for SoftMax® Pro Software 7.0.3 or higher.

Materials

- HepG2 cells (ATCC cat. #HB-8065)
- Glucose Oxidase from *Aspergillus niger* – Type IV (Sigma-Aldrich cat. #G2133)
- Complete growth media
  - Dulbecco's Modified Eagle Medium – high glucose (DMEM, Sigma-Aldrich cat. #D5796)
  - Fetal bovine serum (Sigma-Aldrich cat. #F2442)
  - Penicillin/streptomycin (Sigma-Aldrich cat. #P4333)
- Test compounds
  - Antimycin A (Sigma-Aldrich cat. #A8674)
  - Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, Sigma-Aldrich cat.#C2920)
  - 2-Deoxy-D-glucose (2-DG, Sigma-Aldrich cat. #D8375)
  - Oligomycin A (Sigma-Aldrich cat. #75351)
- MitoXpress Xtra Oxygen Consumption Assay (Agilent Technologies cat. #MX-200-4), containing:
  - MitoXpress Xtra reagent
  - High sensitivity oil
- pH-Xtra Glycolysis Assay (Agilent Technologies cat. #PH-200-4), containing:
  - pH-Xtra reagent
  - Respiration buffer tablet
- Microplates
  - 96-well, clear-bottom, tissue-culture treated microplate (Sarstedt cat. #83.3924.300)
  - SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices, cat. #i3x) with TRF Detection Cartridge (Molecular Devices cat. #0200-7008)

Methods

Signal controls

To assess both signal-to-blank ratio (S:B) and signal fold increase (F0:F) a cell-free kinetic experiment using a 96-well plate was set up. The control wells were set up as follows for the MitoXpress Xtra Oxygen Consumption Assay:

- Blank (B) = media
- Signal control (S, F) = media and MitoXpress Xtra reagent
- GOx control (F0) = media and reagent deoxygenated by using 0.1 mg/mL GOx

For the pH-Xtra Glycolysis Assay the following controls were prepared:

- Blank (B) = respiration buffer
- Signal control (S, F) = respiration buffer and pH-Xtra reagent
- GOx control (F0) = respiration buffer containing both pHXtra reagent and GOx at 0.1 mg/mL.

Cell-based assay

HepG2 cells were seeded at 50,000 cells per well in a 96-well, clear-bottom, tissue-culture treated microplate. Outer perimeter wells were not used for cells but were filled with PBS to avoid edge effects. To ensure an even distribution, cells were incubated at room temperature for 15 minutes prior to overnight culture in an incubator (5% CO_2 at 95% humidity, 37°C).

For the MitoXpress Xtra Oxygen Consumption Assay, MitoXpress Xtra reagent was reconstituted in 1 mL of deionised water, then warmed to 37°C. Cells were carefully washed with complete growth media so as not to dislodge cells from the bottoms of the wells. 80 µL of the pre-warmed complete growth media were added to each well, and the plate was then placed on a plate heater to equilibrate it to 37°C. A repeater pipette was used to add 10 µL MitoXpress Xtra reagent to all wells except wells designated Blank, to which 10 µL of water was added instead. 10 µL of 10X compounds (FCCP, oligomycin, antimycin A), or water containing DMSO at a final concentration of 0.1%, were added to triplicate wells. 100 µL of HS oil was added on top of each well to limit oxygen entering the samples. The plate was then transferred to the SpectraMax i3x reader, and data acquisition was started using a preconfigured protocol in SoftMax Pro Software 7.0.3 or higher.

For the pH-Xtra Glycolysis Assay, cells were first incubated under CO_2- free conditions, 95% humidity at 37°C, for 2.5 hours prior to measurement. Meanwhile, pH-Xtra reagent was reconstituted in 1 mL of deionised water, then warmed to 37°C. Respiration buffer was made by dissolving the respiration buffer tablet in 50 mL of deionised water, then the pH was adjusted to 7.4, and the solution was filter-sterilised. Cells in the microplate were carefully washed with respiration buffer. 80 µL of pre-warmed respiration buffer was added to each well, and the plate was placed on the plate heater to equilibrate it to 37°C. A repeater pipette was used to add 10 µL pH-Xtra reagent to each well. 10 µL of 10X compounds (2-deoxyglucose, oligomycin), or water containing 0.1 % DMSO, were added to wells in triplicate.

Data acquisition and analysis

The SpectraMax i3x reader was used to acquire assay data using the TRF Detection Cartridge. The optimized acquisition parameters in TRF mode for both MitoXpress Xtra and pH-Xtra reagents are shown in Table 1. 100 pulses per read was found to be optimal for this assay (data not shown). The reader temperature was set to 37°C for at least 15 minutes prior to the measurement. The microplate was inserted into the reader, and kinetic data traces were recorded as intensity over time using SoftMax Pro Software. The kinetic read was set to read at 2- to 4-minute intervals over a 45- to 200-minute period. For each experiment, the shortest possible interval was selected.

Prior to exporting the data to Excel, the data were blank corrected in the software. The slope function in Excel was used to calculate the rates for either oxygen consumption (MitoXpress Xtra) or glycolytic activities (pH-Xtra). The slope

was determined in the linear portion of the graph to ensure that the appropriate section of the sample signal curve was interrogated.

Preconfigured protocols for use with compatible Molecular Devices plate readers, for SoftMax Pro 7.0.3 and higher, are available for download from www.softmaxpro.org. Recommended acquisition settings for the readers are listed in the protocols' Instrument Settings section. To process the data directly in the software, use the kinetic reduction to calculate the rates. The linear portion of the kinetic data trace is adjustable by manually selecting the lag and end time in the Reduction dialog.

Parameter	pH-Xtra Glycolysis Assay	MitoXpress Xtra Oxygen Consumption Assay
Optical configuration	TRF-EUSA	
Read type	Kinetic	
Wavelength (automatically set for the detection cartridge)	Ex: 370 nm Em: 616 nm	Ex: 370 nm Em: 642 nm
Plate type	96-well standard clrbtm	
PMT and optics	Integration time: 0.1 ms Excitation time: 0.05 ms Number of pulses: 100 Measurement delay: 0.1 ms Read height: 2.30 mm	Integration time: 0.1 ms Excitation time: 0.05 ms Number of pulses: 100 Measurement delay: 0.03 ms Read height: 2.30 mm
Timing	Total run time: 1 to 2 hours Interval: 2 to 4 minutes	

Table 1. Optimized settings for MitoXpress Xtra and pH-Xtra assays on the SpectraMax i3x reader.

Results

MitoXpress Xtra Oxygen Consumption Assay

To assess the optimal instrument setup, both signal-to-blank ratio (S:B) and signal fold increase (F0:F) were calculated using a cell-free kinetic experiment. The SpectraMax i3x reader achieved a S:B greater than 10 as shown in Figure 1. To determine the signal fold increase, a GOx control (F0) was measured, which was deoxygenated by using GOx. This GOx control showed approximately 3-fold increase in signal compared to the oxygenated signal control (F), indicating that the assay was set up correctly and was measuring oxygen depletion.

A cell-based assay was used to evaluate the compatibility of the SpectraMax i3x reader with the MitoXpress Assay (Figure 2). Cells were treated with two compounds that affect oxygen consumption, oligomycin and FCCP. Untreated cells showed an increase in MitoXpress Xtra signal as oxygen was depleted in the media. Treatment with oligomycin, which decreases oxygen consumption via inhibition of ATP synthase, resulted in a lower oxygen consumption than untreated cells (Figure 2, oligomycin). FCCP increases oxygen consumption by uncoupling respiratory complexes from ATP synthase (Figure 2, FCCP), and the rate was therefore significantly higher for FCCP-treated cells than for untreated cells.

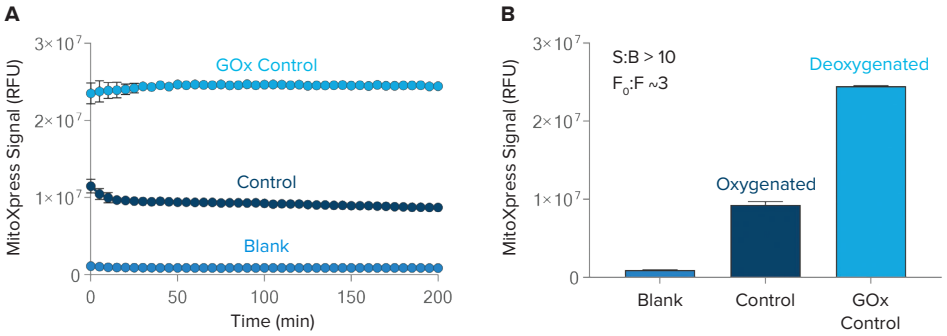

Figure 1. Determination of signal to blank and signal fold increase. (A) Signal traces were plotted for blank and control wells. The mean values of data traces were calculated from the 15th to 20th data points to avoid initial temperature equilibration and were plotted in **(B)**. Signal-to-blank ratio (S:B), as well as signal fold increase (F0:F), were calculated (n = 2, ± SD).

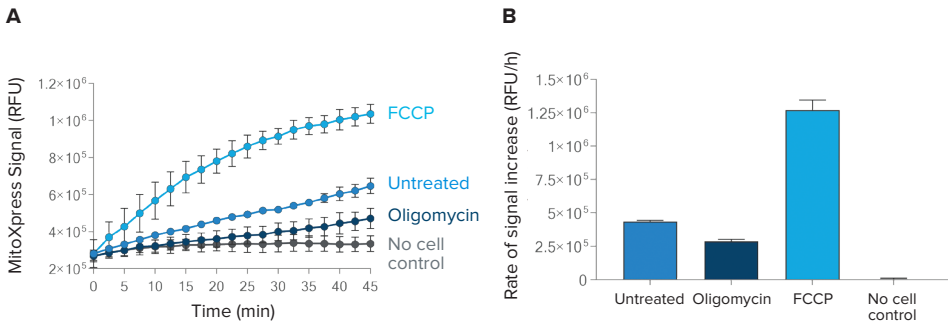

Figure 2. Cell-based oxygen consumption assay using MitoXpress Xtra. (A) Signal profiles of MitoXpress Xtra reagent for HepG2 cells. Cells were treated with oligomycin and FCCP. **(B)** Rates were calculated by taking the slope of the linear portion of kinetic traces (n = 3, ± SD).

pH-Xtra Glycolysis Assay

As with the MitoXpress Xtra Assay, signal-to-blank ratios were determined for the pH-Xtra Glycolysis Assay to ensure optimal reader settings. Control wells generated a S:B ratio greater than 100 (Figure 3). The signal control represented the starting pH of the respiration buffer. As with MitoXpress Xtra, the signal fold increase was determined using a GOx control. This control generates H+ ions by acidifying the buffer as it reacts with glucose (Figure 3). The signal fold ratio showed a significant signal increase (i.e. ratio >3), indicating that the assay was set up correctly and was measuring acidification.

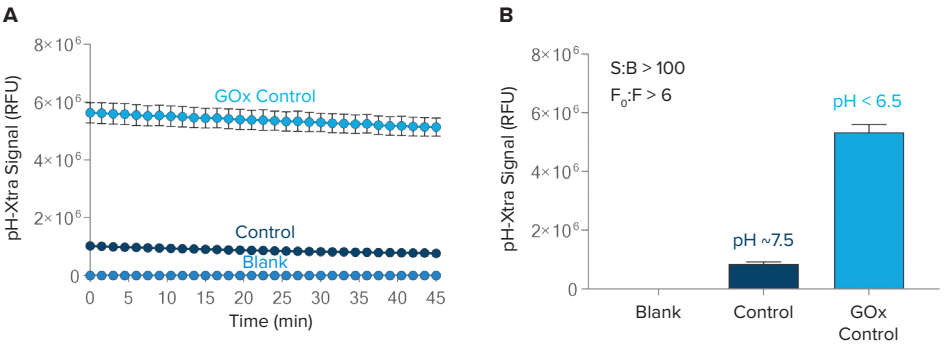

Figure 3. Signal-to-blank and maximum intensity calculations for pH-Xtra. Signal traces **(A)** were plotted for control wells as well as blank. The mean of all data traces was calculated from the 15th to 20th data points and plotted in **(B)**. Signal-to-blank ratio (S:B), as well as signal fold increase (F0:F) were calculated (n = 2, ± SD).

The ability of the pH-Xtra Glycolysis Assay to assess extracellular acidification is illustrated in Figure 4. Cells were treated with two compounds, oligomycin and 2-DG, that modulate extracellular acidification. Oligomycin treatment increased pH-Xtra signal (Figure 4, oligomycin) by inhibiting mitochondria ATP production, causing cells to account for loss of ATP by increasing glycolytic ATP production. 2-DG treatment reduced the rate of signal change by competitively inhibiting glucose phosphorylation, resulting in decreased lactate production by glycolysis (Figure 4, 2-DG).

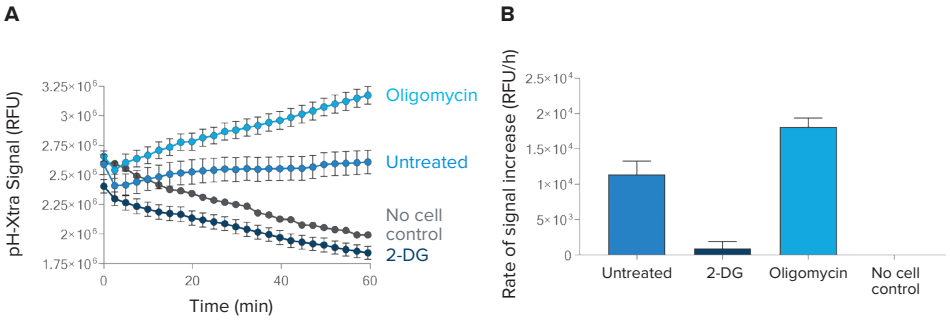

Figure 4. Cell-based pH-Xtra Glycolysis Assay. (A) TRF signal profiles for HepG2 cells using pH-Xtra Glycolysis Assay. Cells were treated with oligomycin or 2-DG. **(B)** Rates were calculated by taking the slope of the linear portion of kinetic traces. Rates were corrected to No Cell Control (n = 3, ± SD).

Conclusion

The SpectraMax i3x reader performed well above the signal performance criteria set for the assays by Agilent Technologies and was successfully used to measure cell-based assays for the analyses of mitochondrial function and glycolytic flux. Preconfigured SoftMax Pro Software protocols for Molecular Devices plate readers are available for MitoXpress Xtra and pH-Xtra to test the signal performance as well as acquire and analyze assay data. Rates can be generated either in Excel or directly from the signal profile in SoftMax Pro Software and can be further reduced for graphing within the software or easily exported for external graphing software.

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