Measuring Glycolysis as Extracellular Acidification

ENZ-51048 Mito-ID® Extracellular pH Sensor Probe

INTRODUCTION

Metabolic perturbations play a critical role in a variety of disease states and toxicities. Knowledge of the interplay between the two main cellular ATP generating pathways; glycolysis and oxidative phosphorylation, is therefore particularly informative when examining such perturbations. Here we describe a fluorescence based assay for the assessment of Extracellular Acidification which provides data on the rate of conversion of pyruvate to lactic acid and is therefore a convenient measure of glycolytic activity. Such assays are particularly informative when assessing alterations in glucose metabolism, detecting glycolytic inhibition and as a confirmatory analysis in the identification of mitochondrial dysfunction. The following protocol outlines how such measurements can be carried out on standard microtiter plates using the fluorescent pH-sensitive probe, Mito-ID® Extracellular pH sensor Probe and ratiometric time resolved fluorescence detection. This approach overcomes the calibration and biocompatibility issues associated with some existing probes thereby allowing conventional cell culturing and assay procedures whilst also facilitating accurate quantitative analysis. In addition, spectral compatibility with Mito-ID® extracellular O_2 sensor kit facilitates a multiplexed measurement approach providing a comprehensive metabolic assessment of test cells.

PROTOCOL

Plate Preparation and Reading

• Warm instrument to measurement temperature (typically 30°C). Prepare kinetic measurement protocol to read the plate at 2-4 min intervals over 1-2 h period using the recommended ratiometric measurement parameters (see Note 1). Recommended readers: Victor (PerkinElmer) and FLUOstar Omega (BMG Labtech).
• Adherent cells were either (A) plated at the indicated concentration in L15 medium and cultured in CO_2-free conditions, 95% humidity at 37°C overnight or (B) cultured overnight in a standard CO_2 incubator and then maintained in CO_2-free conditions for 2.5h prior to the measurement (see Note 2).
• Reconstitute pH-Xtra probe in 1 ml of Millipore water. Warm to measurement temperature.
• Wash cells with measurement buffer (see Note 3) being careful not to dislodge cells from the base of the wells. Add 150µl of the pre warmed measurement buffer to each well and place the plate on a plate heater equilibrated to 30°C.
• Using a repeater pipette, add 10µl to Mito-ID® pH probe to each well. Follow with drug addition if applicable. Oil can be added if bulk acidification data is required (see Note 4)

Measurement

Insert the microplate into the fluorescence plate reader pre-set as described in note 1 and commence reading. Plate preparation time should be kept to a minimum. When measurement cycle is completed, remove the plate from the instrument and save measured data to file.

Data Analysis

Data analysis can be performed automatically on the data analysis software of recommended readers [WorkOut, DazDaq/PerkinElmer and MARS, BMG Labtech].

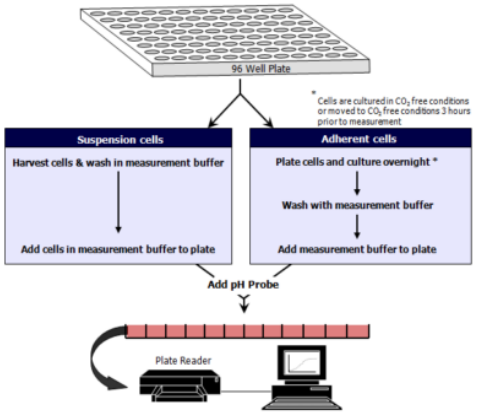

Fig. 1: Schematic diagram of Mito-ID® pH sensor probe Assay.

RESULTS AND DISCUSSION

Monitoring Cellular Respiration

The ability of the Mito-ID® pH probe assay to assess cell respiration is illustrated in Fig. 2. A typical data read out is presented in Fig 2A showing the parallel analysis of 96 individual samples. A serial dilution of HepG2 is presented in Fig 2B with increasing cell numbers causing an increased rate of acidification. This is seen as in increased rate of signal change. Profiles are highly reproducible showing %CV values of the order of <5%. Z' factor analysis (Fig 2C) assesses assay performance in terms of signal window and measurement reproducibility and shows excellent performance (Z' = ~0.7).

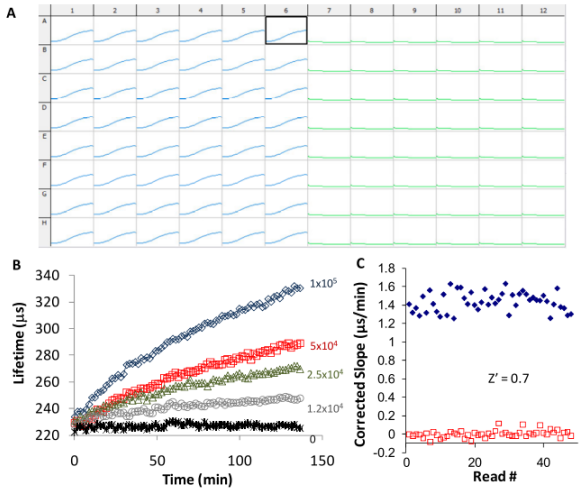

Fig. 2: A) 96 well plate based analysis of extracellular acidification (cell profiles on left, control profiles on right) . **B)** Acidification profiles for HepG2 cultured in L15 medium at the indicated seeding concentration (cells/well) **C)** Z' factor analysis HepG2 plated in L15 medium at ~1x10^5 cells/well. Data generated on FLUOstar Omega, BMG Labtech (A) and on a VictorX4, PerkinElmer (B&C).

Monitoring Glycolytic Inhibition

Treatment with 2DG results in reduced glucose uptake due to competitive inhibition of glucose transport. Treated cells show dramatic, immediate and dose dependant decreases in rates of extracellular acidification (Fig 3A). Oxamic acid inhibits the conversion of pyruvate to lactate blocking the main process contributing to extracellular acidification. Treated cells show dramatic, immediate and dose dependant decreases in rates of extracellular acidification (Fig 3B). Acidification measurements are performed in unsealed conditions to ensure that the acidification observed is due almost entirely to lactate conversion (see Note 4).

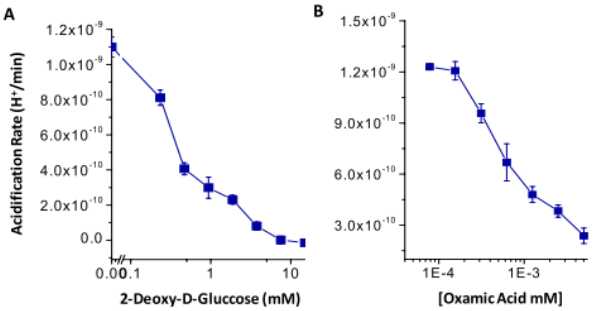

Fig 3: Effect of increasing concentrations of 2DG (A) and Oxamate (B) on measured HepG2 acidification. Data are mean ± SD, n=4.

Monitoring Drug-Induced Mitochondrial Dysfunction
Mitochondrial toxins such as electron transport chain inhibitors prevent or restrict aerobic ATP generation. In many cell systems this leads to an increase in glycolytic flux to supply cellular ATP. This increased acidification can therefore be used as a confirmatory parameter when investigating drug-induced mitochondrial dysfunction. These rates are typically compared to untreated cells to demine the direction and magnitude of the effect observed. This assay can be multiplexed with the Mito-ID® O_2 sensor kit to assess both Extracellular Acidification and Oxygen Consumption on the same plate.

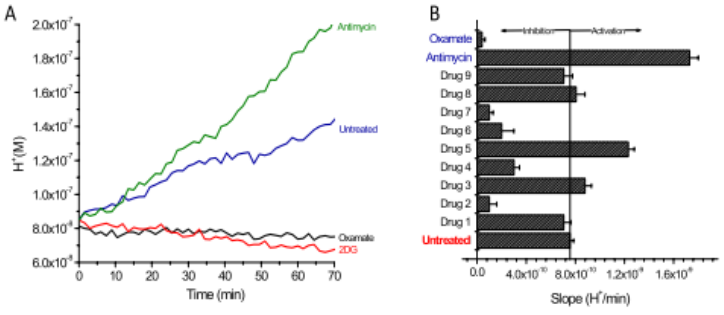

Fig 4: A) Acidification profiles of HepG2 cells illustrating the effect of compound treatment. **B)** Analysis of acidification rates allows the effect of drug treatment to be conveniently assessed

TECHNICAL NOTES

Note 1: Measurements set-up on recommended readers (Victor, PerkinElmer and FLUOstar Omega, BMG Labtech) is performed as follows: Ex/Em 340/642nm (Victor) 380/615nm (Omega), using delay times of 100µs and 300µs and measurement time of 30µs

Note 2: When culturing cells in a standard 5% CO_2 environment the polystyrene body of a standard microtitre plate soaks up CO_2. If a plate is moved immediately from 5% CO_2 to the measurement chamber this CO_2 will diffuse into the sample causing significant acidification. To circumvent this, cells may be cultured in CO_2-free conditions in specially formulated media such as L-15, or cultured in standard media and moved to a CO_2–free environment 2h prior to measurement

Note 3: Measurement Buffer 1: 1mM K-phosphate, 20mM Glucose, 0.07M NaCl and 0.05M KCl, 0.8mM, $MgSO_4$, 2.4mM $CaCl_2$, pH 7.4. Measurement Buffer 2) DMEM Base, 1.85g/l NaCl, 10ml 100x GlutaMax, 10ml 100mM Sodium Pyruvate, 15 mg Phenol Red, 25mM glucose.

Note 4: Acidification in unsealed samples is due almost entirely to lactate conversion while, in sealed samples there is a strong contribution from carbonic acid due to trapped CO_2 reflecting Krebs cycle activity.

REFERENCES

1. Hynes J. et al., Anal. Biochem. 2009; 390, 21–28.
2. Hynes J et al, Current Protocols in Toxicology.2.16, May 2009

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