Improving Quantification of Cellular Glycolytic Rate Using Agilent Seahorse XF Technology

White Paper

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Abstract

Agilent Seahorse XF Analyzers are used to study cellular metabolism in a diverse array of research areas. Simultaneous measurement of mitochondrial oxygen consumption and glycolytic extracellular acidification rates gives researchers an integrated view of total cellular metabolic activity. Quantification of glycolytic rate is particularly important in immune cell activation and cancer metabolism as these cells dynamically upregulate glycolysis to meet biosynthetic demands. However, the potential for non-glycolytic acidification can confound the interpretation of extracellular acidification data. In mammalian cell culture, the largest contributor to non-glycolytic acidification is CO_2 produced by the TCA cycle. This White Paper describes an approach to account for this non-glycolytic CO_2-dependent acidification using the mitochondrial oxygen consumption rate measured simultaneously. A new metric – proton efflux rate (PER) – is introduced that improves the measurement of extracellular acidification by accounting for buffering and plate geometry. In addition, by measuring and subtracting CO_2-dependent acidification, the glycolytic proton efflux rate (glycoPER) can be calculated. Together, these approaches significantly improve the accuracy of glycolytic rate measurements, and provide results equivalent with cellular lactate efflux. As an example of this analysis, we demonstrate that cells treated with UK5099 (an inhibitor of pyruvate uptake into the mitochondria) increase glycoPER in a quantitative manner consistent with higher lactate efflux. Together, these data demonstrate that accounting for mitochondrial CO_2 production enables quantification of glycolytic rate in extracellular flux assays.

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Introduction

Glycolysis and Oxidative Phosphorylation (OXPHOS) are the two major energy-producing pathways in mammalian cells. Most cells are able to readily switch between these two pathways, thereby adapting to changes in their environment. To produce ATP, glucose is catabolized by the cell to form pyruvate, then converted to lactate in the cytoplasm (referred to as glycolysis), or it can be fully oxidized to CO_2 and water in the mitochondria[1]. The conversion of glucose to pyruvate, and subsequently lactate, results in a net production and extrusion of protons into the extracellular medium[2]. The extrusion of protons results in the acidification of the medium surrounding the cell.

$$\text{Glucose} \rightarrow 2\ \text{Lactate} + 2\ H^+$$

Equation 1.

Agilent Seahorse extracellular flux analysis has been successfully applied to the study of cell metabolism for more than 10 years. In particular, measurements of extracellular acidification rate (ECAR) have been used to study glycolysis based on the knowledge that the reaction in Equation 1 acidifies the cell culture medium. In general, the extrusion of lactate constitutes the primary source of extracellular acidification. However, other sources of acidification limit the specificity of this measurement. Thus, accounting for these other sources is a viable means of improving the accuracy of extracellular acidification as a measure of glycolysis.

The most significant source of confounding acidification is CO_2[3]. Specifically, CO_2 is produced during mitochondrial substrate oxidation. Mitochondrial-derived CO_2 acidifies the extracellular medium through hydration to bicarbonate in the reaction shown in Equation 2.

$$H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \ (pKa' = 6.1 \text{ at } 37\ °C)$$

Equation 2.

This paper demonstrates an approach to account for this mitochondrial CO_2 production. An assay incorporating this approach is also defined using serial injection of mitochondrial inhibitors followed by a glycolysis inhibitor. Together, these changes result in improved glycolytic rate measurements using extracellular flux analysis. The described assay provides a glycolytic rate that directly correlates with a standard lactate assay.

Approach

All Agilent Seahorse extracellular flux assays measure both ECAR and oxygen consumption rate (OCR) simultaneously. Because the production of CO_2 in the mitochondria is a function of the rate of OXPHOS, and the rate of OXPHOS is measurable as mitochondrial OCR, the amount of acidification as a consequence of mitochondrial function can be determined[3]. Subtracting this rate from the total cellular proton efflux rate (PER) yields the glycolytic proton efflux rate (glycoPER). For this approach to be quantitative, additional controls in three areas are required:

- The relationship between the total acidification and the measured extracellular acidification must be constant over the desired measurement range.
- The extracellular acidification rate must be representative of the entire well.
- The relationship between mitochondrial respiration and CO_2-specific extracellular acidification must be consistent across a wide range of cell types.

Data to support improvements in these three relationships and derivation of new constants are described in more detail below.

Calculations and derivation of constants

ECAR is a qualitative measurement of acidification in the extracellular medium that depends on cell-derived acid efflux rates. However, ECAR is significantly impacted by the buffer capacity of the medium, the buffer capacity of the measurement system, and the inherent sensitivity of the system to changes in pH throughout the well. To determine the actual PER (pmol H^+/min) of cells given an observed ECAR (mpH/min), the relationship and conversion coefficients between ECAR and PER must first be established and validated within the pH range of a standard XF assay (7.6–6.6).

Maintaining a constant response to acidification during an assay

Typically, XF assay medium is formulated to minimize buffering (for maximum ECAR sensitivity). However, the absence of any buffer system in the medium makes its inherent buffering capacity very sensitive to variations in temperature, pH, and medium composition. Variations in media components (glutamine, pyruvate, phosphate, phenol red, and so forth) can additionally have a significant impact on buffer capacity in the absence of added buffering agent. To maintain a linear relationship between actual proton efflux rate and ECAR, a modified assay medium that includes HEPES buffer (5.0 mM final concentration) was used. This addition of HEPES stabilizes the starting pH of the assay medium, and maintains a constant buffer capacity across the measurement range of the assay. The increased buffer expands the dynamic range of the assay without significantly affecting detection of low acidification rates.

During XF assays, XF pH sensor material can affect the apparent buffer capacity of the medium due to the inherent properties of acid/base indicators. To account for the buffer capacity of the intact measurement system (medium + measurement chamber w/sensor), a new parameter called buffer factor (BF) was defined. BF is a measurement of the *in situ* buffer capacity obtained in XF instruments, and accounts for both medium buffer capacity and XF assay conditions (instrument, sensor, labware, and so forth). This value is determined based on the amount of H^+ or OH^- (in mmol/L) added to the well to change the pH level by 1 unit using Agilent Seahorse instrumentation and consumables. BF can be determined in the XF instrument by titrating (via port injection) known amounts of an acid (for example, HCl or H_2SO_4) to the assay medium, and measuring the changes in pH value after each injection.

The slope of these data (Figure 1B) can be used along with the value from an assay medium control to obtain the value of BF, as calculated by Equation 3:

$$\text{BF (mmol/L/pH)} = \frac{1}{\text{slope HCl} - \text{slope assay medium}}$$

Equation 3.

Effective volume of the measurement chamber

Due to the semiclosed nature of the XF Analyzer measurement system, the physical (geometric) volume is an estimate of the effective volume. Since knowing the effective volume of the measurement chamber is critical for the determination of the actual proton efflux rate, the data must be scaled to account for the effective volume of the measurement chamber (similar in concept to the correction used in the AKOS algorithm for OCR)[4]. The volume scaling factor, Kvol, was empirically determined by comparing XF outputs to lactate production rate determined using a standard lactate assay in the presence of rotenone and antimycin A (Rot/AA) in the medium. The presence of Rot/AA suppresses CO_2 production (and resultant acidification) through mitochondrial substrate oxidation, since the TCA cycle and mitochondrial respiration are coupled processes. Glycolysis remains the primary source of medium acidification.

For a given measurement system, the volumetric scaling factor, Kvol, can be determined by Equation 4:

$$\text{Kvol} = \frac{\text{Lactate production rate (pmol/min)}}{\text{BF ((mmol/L)/pH)} \times \text{ECAR (mpH/min)} \times \text{Vol measurement chamber (µL)}}$$

Equation 4.

Kvol correction relies on the assumption that cells are evenly distributed, and that the XF pH sensor monitors a representation of the whole monolayer, so that diffusion of protons to the sensor surface does not compete with proton diffusion across the well volume.

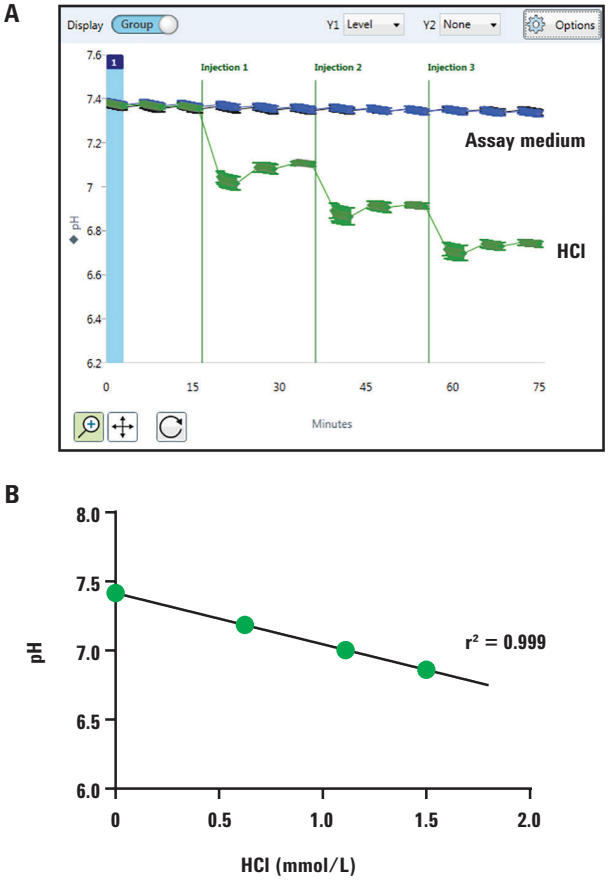

Figure 1. Calculation of the BF of assay medium. A) Measurement of pH level in assay medium after addition of known amounts of 5.0 mM HCl or assay medium using port injections in an Agilent Seahorse XFe96 analyzer. B) Calculation of BF from pH levels obtained after each injection, as shown in A.

Figure 2A shows the correlation between ECAR values (obtained in a XFe96 instrument and adjusted by buffer factor and geometric measurement chamber volume) and lactate production rate (determined using a fluorescent assay kit). The graph represents experimental points obtained using five different cell lines (A549, C2C12, HepG2, MDA-MB 231, and MCF7) to cover a wide range of ECAR values. The slope of the graph represents the scaling factor (Kvol) for XF96/XFe96 microplates. Table 1 shows Kvol values obtained using an analogous approach in XFe24 and XFp instruments. This approach is unsuitable for XF24 instruments due to the large difference between the sensor spot diameter and well bottom area, as well as the lower sensitivity of the instrument.

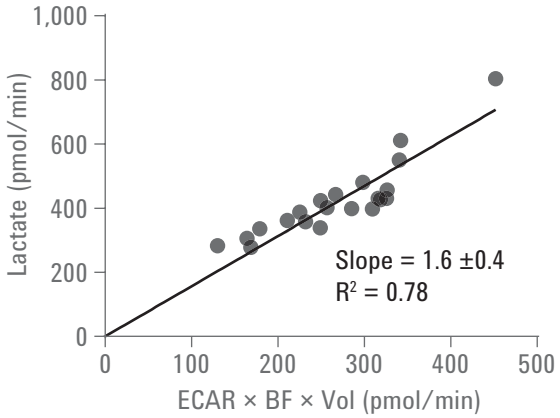

Figure 2. Correlation between XF ECAR measurements (adjusted by the BF of the assay medium and geometric measurement chamber volume) and lactate production rate measured using an enzymatic fluorescent LDH assay. Data represent multiple experimental replicates obtained using five different cell lines (A549, C2C12, HepG2, MDA-MB-231, and MCF7). For each experimental point, background ECAR/lactate obtained under identical conditions, but in the absence of glucose, was subtracted.

Table 1. Kvol values obtained for Agilent Seahorse XFp, XFe24, and XFe96/XF96 instruments using their corresponding standard miniplates/microplates.

Instrument | microplate	Vol measurement chamber (µL)	Kvol
Seahorse XFp | XFp miniplate	2.28	1.19
Seahorse XFe24 | V7 microplate	5.65	1.19
Seahorse XFe96/XF96 | V3 microplate	2.28	1.60

Once BF is calculated for the assay medium, and knowing Kvol for the corresponding XF cell culture plate, ECAR can be converted to PER (Equation 5).

PER (pmol H^+/min) = ECAR (mpH/min) × BF (mmol/L/pH) × Vol measurement chamber (µL) × Kvol

Equation 5.

Equation 5 quantifies the total acid extruded by cells in the well. To determine the rate of glycolysis-specific proton efflux, the contribution of CO_2 to total acidification (PER) must be subtracted.

Accounting for CO_2 contribution to PER

There are two main sources of extracellular protons in live cells: glycolysis and CO_2 generated via mitochondrial TCA activity. Thus, the total proton efflux rate can generally be expressed as the sum of these two contributors, as in Equation 6:

PER (total) = glycoPER + mitoPER

Equation 6.

As mentioned above, apart from glycolysis, cells will use catabolic substrates present in the medium and intracellular stores (glucose, glutamine, pyruvate, fatty acids) through aerobic metabolism to produce ATP with concomitant O_2 consumption and CO_2 production. Formation of CO_2 in an aqueous environment results in its hydration and dissociation into bicarbonate and H^+ (Equation 2), which contributes, to some extent, to extracellular acidification.

Since oxidative phosphorylation and TCA cycle are strongly coupled processes in the mitochondria, a relationship between mitochondrial PER and mitochondrial OCR can be determined (Equation 7), where CCF (CO_2 contribution factor) represents the empirically calculated H^+/O_2 ratio.

$$CCF = \frac{\text{mitoPER}}{\text{mitoOCR}}$$

Equation 7.

To determine CCF, a series of Cell Mito Stress Tests were performed on 20 cell types using a modified assay medium (including 5.0 mM HEPES) in the absence of glucose. Under these assay conditions (and assuming that cells do not have significant stores of glucose), glycolytic-dependent acidification was completely suppressed and CO_2 hydration/dissociation was the main factor contributing to extracellular acidification. Injection of Rot/AA under these conditions will block mitochondrial respiration and, thus, mitochondrial-derived acidification.

From OCR and ECAR measurements of XF Cell Mito Stress Test in the absence of glucose (Figure 3A and 3B), the contribution of mitochondria to oxygen consumption and proton production can be calculated as:

$$mitoPER = PER_{basal} - PER_{Rot/AA}$$

Equation 8.

$$mitoOCR = OCR_{basal} - OCR_{Rot/AA}$$

Equation 9.

Using Equation 7, CCF is calculated as the ratio between mitoPER/mitoOCR.

Using 20 different cell lines, it was determined that a single CO_2 contribution factor can be used among most cell types to correlate mitochondrial respiration with CO_2 contribution to acidification. The H^+/O_2 ratio has theoretical values between 0.67 and 1 depending on the oxidized substrate[3]. The observed contribution of CO_2 to extracellular acidification was less than the predicted amount by substrate oxidation stoichiometry (Table 2). Reasons for this could include:

- CO_2 not escaping the intracellular compartment due to dissociation or reaction with other cellular components
- A minor fraction of NADH produced in the cytosol can be transported to the mitochondria resulting in O_2 consumption that is not associated with TCA cycle/CO_2 production
- A small cellular CO_2 fraction is consumed through carboxylation reactions

Table 2. CO_2 contribution factor (CCF) determined using Agilent Seahorse XFp, XFe24, and XF96/XFe96 instruments with corresponding standard microplates.

Instrument	CCF
Seahorse XFp	0.50
Seahorse XFe24	0.60
Seahorse XFe96/XF96	0.61

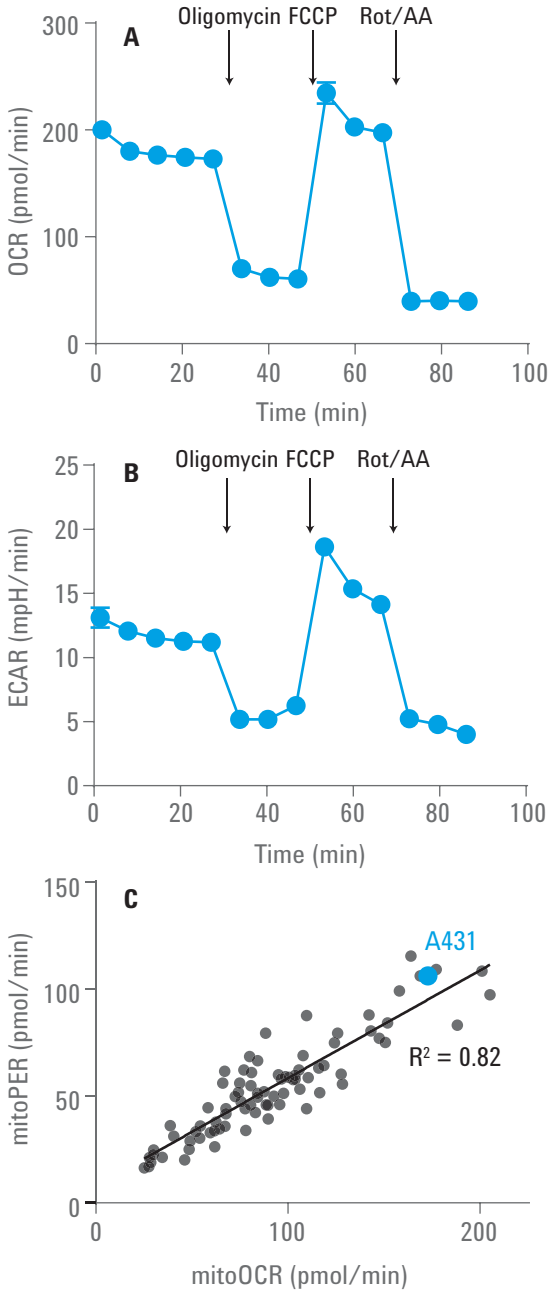

Figure 3. Seahorse XF Cell Mito Stress Test in the absence of glucose in the assay medium. A431 cells (30K/well) were starved of glucose for 1 hour in assay medium (XF Base Medium without phenol red, 5.0 mM HEPES, 2 mM glutamine, 1 mM pyruvate, pH 7.4), then OCR (A) and ECAR (B) were measured to calculate CCF. C) Correlation between mitoOCR and mitoPER measurements obtained from 20 different cell types under assay conditions described in A and B. (●) corresponds to the experimental point obtained for A431 cells.

CCF was also calculated after varying the substrate composition of the medium or adding combinations of inhibitors of the principal fuel pathways such as UK5099 (mitochondrial pyruvate uptake inhibitor), BPTES (glutaminase inhibitor), or etomoxir (long chain fatty acid mitochondrial-uptake inhibitor). CCF values were determined not be significantly affected by the nature of the oxidized fuels. Moreover, when experiments were performed using Beta-TC6 pancreatic cells, that have minimal expression of LDH, and only including glucose (10 mM) as fuel in the assay medium, the obtained CCF value was not significantly different from the one obtained in the absence of glucose, but in the presence of pyruvate and glutamine. This validates that CCF values obtained in the absence of glucose are sufficient to estimate the CO_2 contribution to extracellular acidification under described assay conditions.

Once the CCF and mitoPER are determined, the glycolytic proton efflux rate can be calculated as:

GlycoPER (pmol/min) = PER (pmol/min) – mitoPER (pmol/min)

Equation 10.

PER and mitoPER are calculated according to Equations 5 and 7, respectively.

Application

Based on the previous calculations, we designed a novel XF assay to measure glycolytic rate, and we applied it to two cell lines: a highly glycolytic cancer cell line (A431, human squamous carcinoma) and a highly oxidative primary cell culture (BAEC, bovine aortic endothelial cells) comparing the obtained results with extracellular lactate measurements, the current standard method for glycolysis analysis.

The assay was performed in XF Base Medium without phenol red, 5.0 mM HEPES, 10 mM glucose, 2 mM glutamine, and 1 mM pyruvate, pH 7.4 at 37 °C with 1 hour of pre-incubation of cells in assay medium in a non-CO_2 incubator. The BF of the measurement system was calculated by sequential injection of 5.0 mM HCl solution, and recording changes in pH level using the XF instrument, as described in Figure 1.

The calculations described above require specific compounds to be added during an assay. The Seahorse XF Glycolytic Rate Assay thus uses a sequential injection design to add the necessary inhibitors. After measuring the baseline OCR and ECAR, the addition of rotenone/antimycin A allows for the calculation of mitochondrial OCR (and therefore CO_2 contribution to PER). The addition of Rot/AA also shuts down mitochondrial ATP production, forcing the cells to compensate by increasing glycolysis to meet energetic demands as possible, referred to as compensatory glycolysis. Finally, an injection of 2-DG, a competitive inhibitor of the glycolytic pathway, was used as a qualitative control to demonstrate that acidification by other cellular processes apart from CO_2 were not significantly contributing to basal extracellular acidification.

As a proof of concept, the Seahorse XF Glycolytic Rate Assay was performed in A431 and BAEC cells under basal conditions or with the addition of the mitochondrial pyruvate carrier inhibitor UK5099 (2 µm). UK5099 inhibits mitochondrial pyruvate oxidation, and could result in increased glucose-derived pyruvate being converted to lactate[5-7]. Figure 4 shows OCR and ECAR profiles of the Glycolytic Rate Assay for both cell lines showing that injection of UK5099 significantly decreases OCR, as expected in both cell lines, and increases ECAR.

OCR and ECAR data were used to calculate glycoPER for both cell lines under control conditions, or after addition of UK5099 using Equation 10 (Figure 5A and 5C). As expected, blockage of mitochondrial pyruvate utilization with UK5099 increased glycolytic rate in both cell lines. In the case of A431 cells, a known highly glycolytic cancer cell line, contribution of glycolysis to total acidification was 92 %, indicating that most acidification observed was due to glycolysis. However, glycolysis only comprised 53 % of the total acidification in BAEC, highlighting the importance of accounting for CO_2 contribution to obtain an accurate measurement of glycolytic rate.

To further support the benefit of accounting for CO_2 contribution, a fluorimetric lactate assay was performed to measure lactate accumulation in the extracellular medium. By accumulating lactate over the same time period as an XF assay, the correlation between XF glycolytic rates and an additional assay for glycolysis was obtained. Figures 5B and 5D show that the basal glycolytic rate measured in XF tightly correlates with lactate production rate. These data indicate that the parameters used for XF glycolytic rate calculation accurately account for other sources of extracellular acidification and sensor/measurement chamber properties. Notably, the qualitative conclusion from the data in Figures 4 and 5 was the same (UK5099 increases glycolytic rate), however the quantitative accuracy was markedly improved by expressing the data as glycoPER.

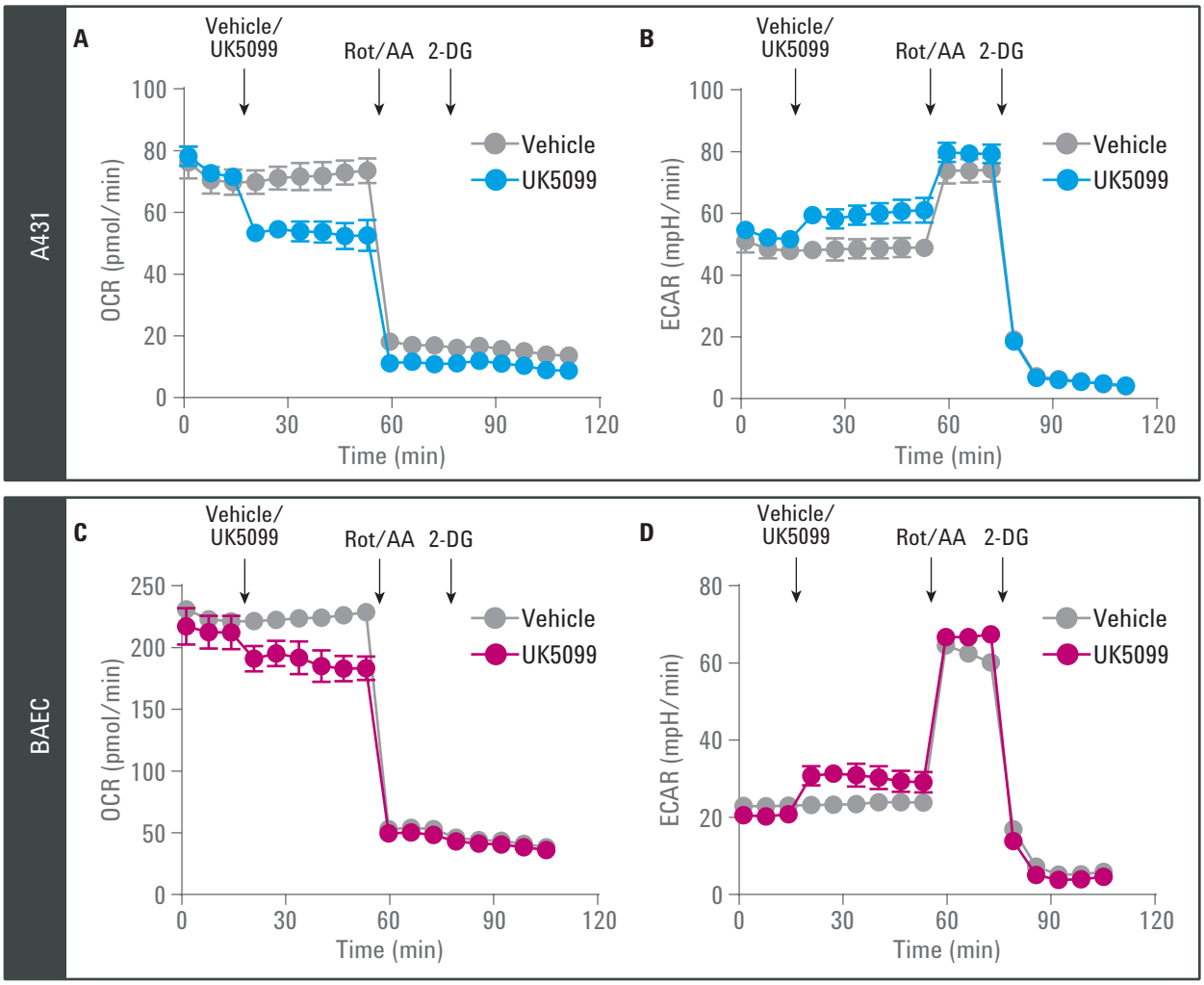

Figure 4. OCR and ECAR profiles of Glycolytic Rate Assay in A431 (A, B) and BAEC cells (C, D) under basal conditions (vehicle) or after *in situ* injection of UK5099 (2 µM).

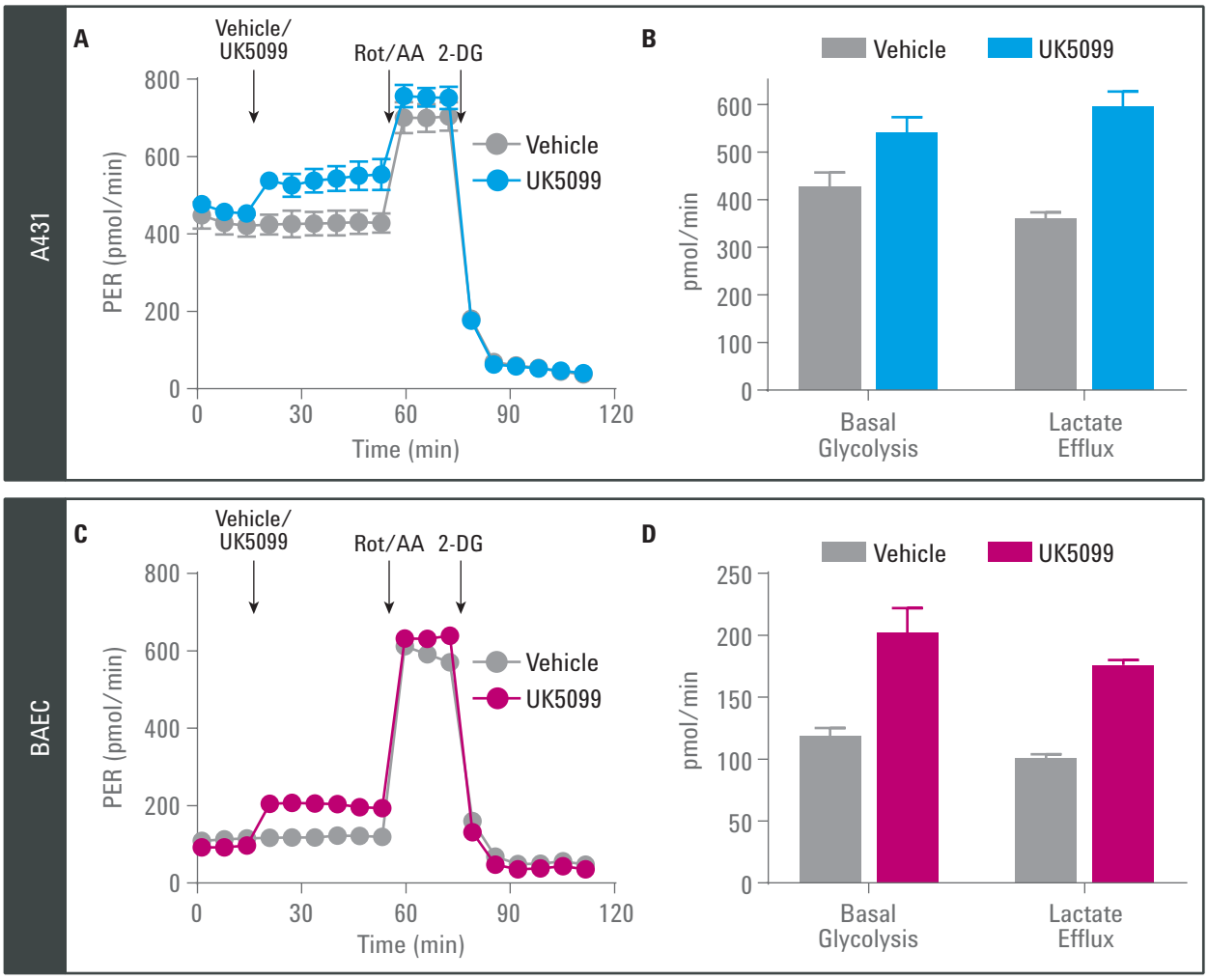

Figure 5. GlycoPER profile of (A) A431 or (C) BAEC cells under control condition (vehicle) or treated with mitochondrial pyruvate transporter inhibitor UK5099 (UK5099). (B) and (D): Comparison of Glycolytic Rates of A431 cells or BAEC, respectively obtained by Extracellular Flux Analysis (Basal Glycolysis) or measuring Lactate Accumulation (Lactate Efflux) in the extracellular medium.

Conclusions

The need for improved real-time measures of glycolysis is apparent from the increase in glycolysis-related publications in the literature, especially as related to cancer and immunology research. This White Paper presents an improved analytical method for assessing glycolytic rate in cultured cells. More than 20 cell lines representing a variety of disease models were used to empirically determine values for the CO_2 contribution to extracellular acidification. This, combined with improved knowledge and control of assay medium buffering and measurement system allows for accurate assessment of cellular glycolytic rate. These findings, when combined with the assay described in Figure 5, provide an easy measure of a glycolytic rate that is directly comparable to lactate efflux.

References

1. Lunt, S. Y.; Vander Heiden, M. G. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu. Rev. Cell Dev. Biol.* **2011**, *27*, 441-64.

2. Divakaruni, A. S.; *et al.* Analysis and Interpretation of Microplate-Based Oxygen Consumption and pH Data, in *Methods in Enzymology*; N.M. Anne, C.C. David, Eds.; Academic Press: 2014; 309-354.

3. Mookerjee, S. A.; *et al.* The contributions of respiration and glycolysis to extracellular acid production. *Biochim. Biophys. Acta* **2015**, *1847*, 171-81.

4. Gerencser, A. A.; *et al.* Quantitative microplate-based respirometry with correction for oxygen diffusion. *Anal. Chem.* **2009**, *81*, 6868-78.

5. Bricker, D. K.; *et al.* A mitochondrial pyruvate carrier required for pyruvate uptake in yeast, Drosophila, and humans. *Science* **2012**, *337*, 96-100.

6. Halestrap, A. P.; Denton, R. M. The specificity and metabolic implications of the inhibition of pyruvate transport in isolated mitochondria and intact tissue preparations by alpha-Cyano-4-hydroxycinnamate and related compounds. *Biochem. J.* **1975**, *148*, 97-106.

7. Zhong, Y.; *et al.* Application of mitochondrial pyruvate carrier blocker UK5099 creates metabolic reprogram and greater stem-like properties in LnCap prostate cancer cells in vitro. *Oncotarget* **2015**, *6*, 37758-69.

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Published in the USA, March 1, 2017
5991-7894EN

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