White Paper

Agilent
Trusted Answers

Quantifying Cellular ATP Production Rate Using Agilent Seahorse XF Technology

Authors

Natalia Romero
George Rogers
Andy Neilson
Brian P. Dranka

Agilent Technologies, Inc.,
Lexington, MA, USA

Abstract

All cellular systems have evolved to couple energetically unfavorable reactions to energetically favorable ones in order to grow, divide, move, and respond to their environment. As a result, any change in these cellular functions can be assessed by measuring energy production. However, simply measuring the *amount* of energy in the cell is insufficient because this amount is held quite constant. ATP is the most common high energy intermediate, and mammalian cells exquisitely regulate the amount of ATP present to enable the reactions mentioned above. They maintain this steady state by tightly coupling production and consumption of ATP – i.e. supply and demand are balance. Therefore, measuring the rate of ATP production enables a view into cellular function that is not provided by simply measuring the amount of ATP in the cell. In this white paper we describe a simple approach to quantifying ATP Production Rate by measuring the two major production pathways in mammalian cells: glycolysis and oxidative phosphorylation. Calculations necessary to convert the measurement of Oxygen Consumption Rate (OCR; a measure of oxidative phosphorylation) and Extracellular Acidification Rate (ECAR; a measure of glycolysis) to units of ATP Production Rate are provided and validated. Finally, an example contrasting the measurement of ATP Production Rate and ATP amount is given to demonstrate the difference in these approaches.

Introduction

Adenosine triphosphate (ATP) is the universal high-energy intermediate of living organisms and constitutes the most common cellular energy currency of intermediate metabolism. This energy is derived from the breakdown of cellular fuels which is coupled to the synthesis of ATP from ADP and inorganic phosphate. ATP is then available as a high-energy carrier to drive cell function. The free energy released from ATP hydrolysis is used to sustain various cellular functions such as anabolic synthesis, transport of molecules or ions against a gradient across membranes, cell motility, cell division, etc.

Importantly, cell systems are not in equilibrium, but they maintain a steady state sustained by a complex set of metabolic regulatory systems. One of the best regulated cellular systems is designed to maintain intracellular ATP levels under steady state conditions[1]. This allows cells to adjust to changes in ATP demand by modulating ATP production. As a result, the total intracellular ATP level is held quite constant despite wide fluctuations in the demand for ATP[2]. Thus, the measurement of ATP level or amount is used frequently to assess cell viability. Decreases in ATP amount in a sample represent losses in cell number.

In contrast, the cellular rate of ATP production is a highly informative measurement that directly reflects cellular energetic demands to accomplish cellular functions. In mammalian cells, glycolysis and mitochondrial oxidative phosphorylation (OXPHOS) pathways provide the majority of cellular ATP. Most cells can readily switch between these two pathways, thereby adapting to changes in their environment.

While OXPHOS consumes O_2, driving Oxygen Consumption Rate (OCR), both pathways contribute to the acidification of the medium. Conversion of glucose to lactic acid through glycolysis is accompanied by extrusion of one H^+ per lactate. Additionally, the mitochondrial TCA cycle which fuels the mitochondrial electronic transport chain also produces CO_2. After hydration and conversion to carbonic acid, this CO_2 acidifies the media. The sum of these reactions is the primary driver of changes in Extracellular Acidification Rate (ECAR).

Because all Seahorse XF assays measure both H^+ efflux (ECAR) and O_2 consumption (OCR) simultaneously, these measurements can be combined to assess total ATP Production Rate. In the experiments below, examples are given to demonstrate this calculation under basal conditions and after the addition of a series of metabolic inhibitors. The conversion of ECAR and OCR to ATP Production Rate units is also validated, yielding pathway-specific measurements of mitochondrial and glycolytic ATP production. Notably, this assay is label-free, kinetic, and offers the ability to intervene using a compound or treatment of interest in real time.

Approach

Total ATP Production Rate is the sum of the ATP Production Rate from glycolysis and mitochondrial oxidative phosphorylation. Using calculations previously validated for the Seahorse XF Glycolytic Rate Assay and based on known reaction stoichiometry, ECAR data can be converted to glycolytic ATP Production Rate. During oxidative phosphorylation, OCR that drives mitochondrial ATP synthesis can be calculated by addition of oligomycin (ATP synthase inhibitor). To convert ATP-coupled OCR into mitochondrial ATP Production Rate, we must know the stoichiometry of ATP phosphorylated per atoms of oxygen reduced. This value is known as the P/O ratio. An average P/O value of 2.75 was validated that accurately represents cell experimental conditions where cells oxidize a mixture of available fuels.

Calculations, derivation of constants, and assay design

ATP Production Rate Calculation

During conversion of one molecule of glucose to lactate in the glycolytic pathways, 2 molecules of each ATP, H^+, and lactate are produced (Eq. 1).

Glucose + 2 ADP + 2 Pi ➔ 2 Lactate + 2 ATP + 2 H_2O + 2 H^+ (Eq. 1)

Using the same approach previously validated for the Seahorse XF Glycolytic Rate Assay[3], glycolytic ATP Production Rate (equivalent to Glycolytic Proton Efflux Rate, glycoPER, see Eq. 1) can be calculated as:

glycoATP Production Rate (pmol ATP/min) = **glycoPER** (pmol H^+/min) (Eq. 2)

glycoPER (pmol H^+/min) = **PER** (pmol H^+/min) – **mitoPER** (pmol H^+/min) (Eq. 3)

PER (pmol H^+/min) = **ECAR** (mpH/min) **x BF** (mmol H^+/L/pH) **x Vol** $_{\text{XF microchamber}}$ (µL) **x Kvol** (Eq. 4)

mitoPER (pmol H^+/min) = **mitoOCR** (pmol O_2/min) **x CCF** (pmol H^+/pmol O_2) (Eq. 5)

mitoOCR (pmol O_2/min) = **OCR basal** (pmol O_2/min) – **OCR (Rot/AA)** (pmol O_2/min) (Eq. 6)

where BF (Eq. 4) represents buffer factor - the buffering capacity of the system (including buffer capacity of the assay media and XF assay conditions), CCF (Eq. 5) is the CO_2 Contribution Factor and has values of 0.50, 060 and 0.61 and Kvol (Eq. 4) has values of 1.1, 1.1 and 1.6 for XFp, XFe24 and XF/XFe96, respectively[3].

The rate of oxygen consumption that is coupled to ATP production during OXPHOS can be calculated as the OCR that is inhibited by addition of oligomycin (ATP synthase inhibitor) (Eq. 7).

OCR_{ATP} (pmol O_2/min) = **OCR_{basal}** (pmol O_2/min) – **OCR_{Oligo}** (pmol O_2/min) (Eq. 7)

To convert this rate of molecular oxygen consumption we need to multiply by 2 (to convert to O atoms) and by the P/O ratio, the number of molecules of ATP synthesized per atom of O reduced by an electron pair (Eq. 8):

mitoATP Production Rate (pmol ATP/min) = **OCR_{ATP}** (pmol O_2/min) * **2** (pmol O/pmol O_2) * **P/O** (pmol ATP/pmol O) (Eq. 8)

Theoretical P/O ratio values were previously estimated considering the stoichiometry of each metabolic fuel, combined with experimental results obtained using isolated mitochondria and considering F_1F_0-ATP synthase efficiency. The maximum theoretical P/O values obtained varies from 2.45 for palmitate oxidation to 2.86 for full glycogen oxidation[4]. However, under standard cell experimental conditions, cells are incubated with a mixture of different fuels (amino acids, carbohydrates, lipids) and also internal fuel stores are available for mitochondrial oxidation. In order to calculate mitochondrial ATP Production Rate under assay conditions, an estimated average P/O value should be used that represents the average P/O ratio of the different cellular fuels. This average P/O ratio is validated below.

Finally, the total cellular ATP Production Rate is the sum of the glycolytic and mitochondrial ATP Production Rates:

ATP Production Rate (pmol ATP/min) = **glycoATP Production Rate** (pmol ATP/min) + **mitoATP Production Rate** (pmol ATP/min) (Eq. 9)

Assay design

According to Eq. 1 to 6, in order to calculate glycolytic ATP Production Rate, we need to obtain basal OCR and ECAR data, and also OCR data after addition of rotenone + antimycin A (to discount CO_2 contribution to total acidification) in the presence of HEPES buffered assay media (with known and constant buffer factor – for more details see Ref. 3). To calculate mitoATP Production Rate, OCR before and after addition of oligomycin needs to be measured (Eq. 7). With these considerations, we designed the Seahorse XF Real-Time ATP Rate Assay that includes consecutive injections of oligomycin and a mixture of rotenone and antimycin A. This assay design allows for an additional injection of a test compound before assay injections enabling researchers to calculate ATP Production Rate under basal conditions and after the injection of the test compound ("induced rate") (Figure 1).

Validation of P/O value of 2.75 for mitoATP Production Rate calculations

In the first series of experiments, an average P/O ratio value of 2.75 was validated for the calculation of mitoATP Production Rate. The ATP Production Rate was calculated in different cell lines under basal conditions and after the addition of a set of metabolic inhibitors that induce a metabolic switch from oxidative phosphorylation to glycolysis. In all cases, the inhibitors were used at concentrations and exposure times that were not expected to alter cell proliferation or other functions.

Therefore, we assumed that after the induced metabolic switch, total cellular ATP production should remain unaffected and the decrease in mitochondrial ATP production will be compensated with an increase in glycolytic ATP production. Decrease in total ATP production was assumed to be indicative that fuel availability was not sufficient and cellular ATP demand decreased to adjust to ATP production after the addition of the mitochondrial ATP production inhibitor. An increase in total ATP production after the inhibitors treatment indicated that the P/O value used for calculations was an underestimation of the actual value.

Figure 2 shows the results obtained when the XF Real-Time ATP Rate Assay was performed in MCF7 cells with pre-injection of vehicle, 0.35 µM oligomycin (a low concentration of this compound in these cells), UK5099 (a mitochondrial pyruvate transporter inhibitor), 2-DG (a competitive inhibitor of hexokinase), or a mixture of UK5099, etomoxir (long chain free fatty acid oxidation inhibitor) and BPTES (glutaminase inhibitor). As expected, addition of either oligomycin, UK5099 or a mix of the 3 inhibitors (UK5099, BPTES and etomoxir) inhibits mitochondrial ATP production with a compensatory increase in glycolytic ATP production without significant changes in total ATP production. Addition of 2-DG inhibits both pathways since MCF7 highly depend on glucose oxidation for mitochondrial respiration in addition to sustaining anaerobic glycolysis.

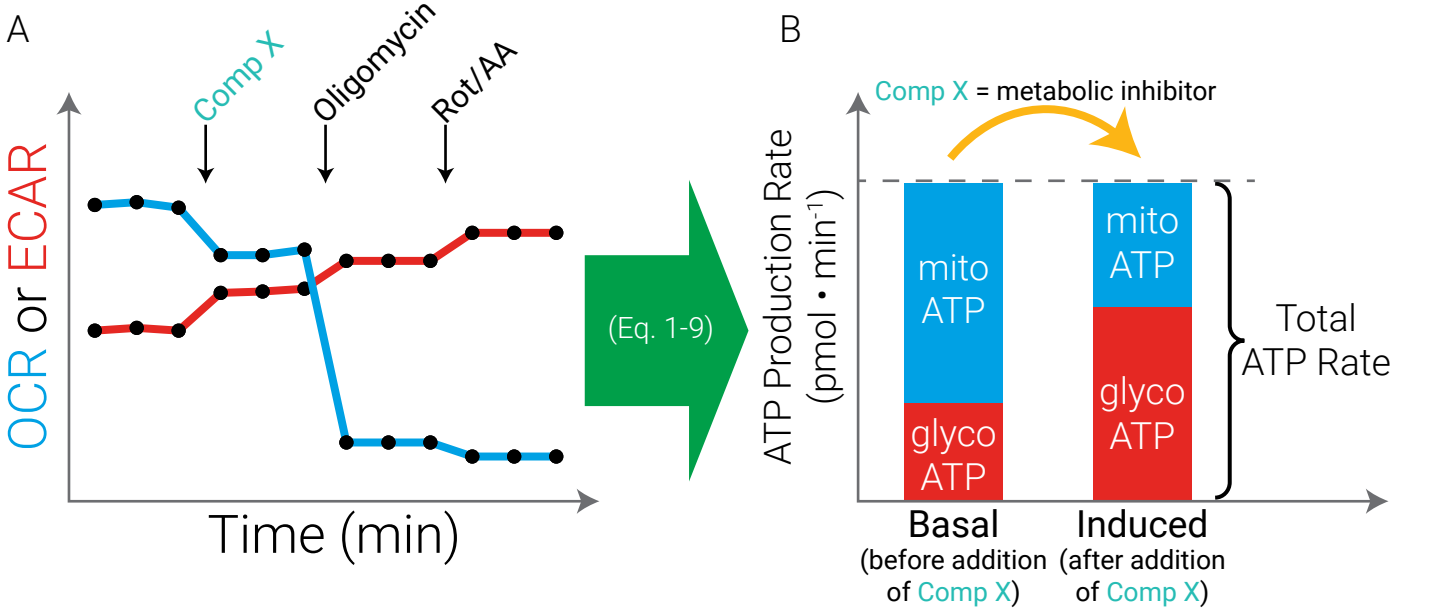

Figure 1. A) Schematic OCR and ECAR traces of an induced XF Real-Time ATP Rate Assay with injection of a metabolic inhibitor before addition of assay injections (oligomycin, rotenone + antimycin A). B) Cellular ATP Production Rates calculated for basal conditions and after addition of the test compound that induced a metabolic switch (decrease in mitoATP Production Rate and increase in glycoATP Production Rate) without significant changes in total ATP Production Rate.

The same assay was repeated using five different cell lines (A431, A549, C2C12, HepG2 and MCF7) which each have unique metabolic phenotypes. We defined Q (%) as the relative difference between ATP Production Rate after addition of the metabolic modulators compared to basal rate (Eq. 10).

$$Q\ (\%) = \frac{\text{ATP Production Rate (induced)} - \text{ATP Production Rate (basal)}}{\text{ATP Production Rate (basal)}} \quad \text{(Eq. 10)}$$

Results obtained pooling data from 3 independent experiments with each of the 5 cell lines tested is shown in Figure 3. When cells were exposed to a low dose of oligomycin, UK5099, or a mix of the fuel inhibitors UK5099, BPTES and etomoxir, a decrease in mitoATP Production Rate is observed for all cell lines accompanied by an increase in glycoATP Production Rate (Figure 3A). When the changes in total ATP production after addition of the inhibitors using a P/O value of 2.75 were analyzed, we observed that cells almost fully compensated for the decrease in mitoATP production with increase in glycolytic activity with no changes ($Q \approx 0$) or a very small decrease of ATP production ($Q < 0$) after the treatment with the metabolic inhibitors (Figure 3B). Importantly, the estimation of total ATP Production Rate never increased, which would have indicated an inaccurate value used for P/O ratio. Together, these data suggest that the average P/O ratio of 2.75 accurately estimates mitoATP Production Rate within the sensitivity range of the assay.

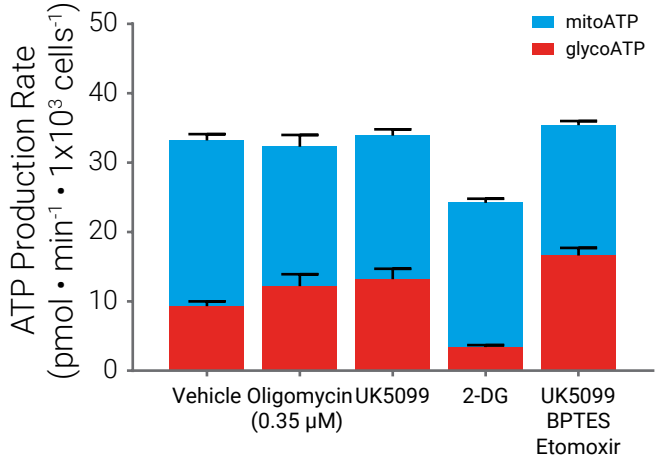

Figure 2. Seahorse XF Real-Time ATP Rate Assay in MCF7 cells treated with different metabolic inhibitors. MCF7 cells (20K/well) were incubated in assay medium (Seahorse XF DMEM Medium, pH 7.4 supplemented with 10 mM glucose, 1 mM pyruvate and 2 mM glutamine). MitoATP Production Rate and glycoATP Production Rate were calculated according to Eq. 1 to 9 from OCR and ECAR measurements, obtained using an Agilent Seahorse XFe96 Analyzer, under basal conditions and after injection of oligomycin (0.35 µM), UK5099 (2 µM), 2-DG (50 mM), or the combination of UK5099 (2 µM), BPTES (3 µM) and etomoxir (4 µM).

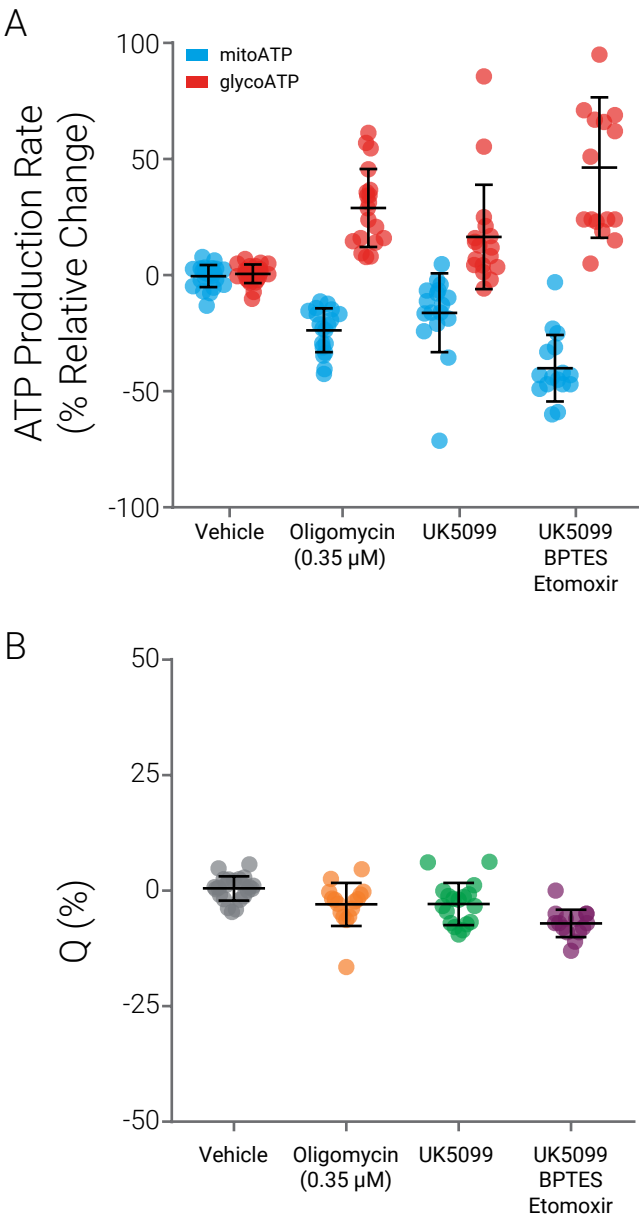

Figure 3. Seahorse XF Real-Time ATP Rate Assay was performed using A431 cells (15K/well), A549 cells (15K/well), C2C12 (12K/well), HepG2 (15K/well), MCF7 (20 K/well) in assay medium (Seahorse XF DMEM Medium, pH 7.4 supplemented with 10 mM glucose, 1 mM pyruvate and 2 mM glutamine) using a Seahorse XFe96 Analyzer. MitoATP Production Rates and glycoATP Production Rates were calculated from OCR and ECAR measurements under basal conditions and after injection of vehicle, oligomycin (0.35 µM), UK5099 (2 µM), or the combination of UK5099 (2 µM), BPTES (3 µM) and etomoxir (4 µM). (A) Relative changes of mitoATP Production Rate and glycoATP Production Rate obtained after injection of indicated compounds. The graph represents the pooled results obtained of the 5 cell lines analyzed, 3 independent experiments per cell line. (B) Relative change in total ATP Production Rate (Eq. 10) obtained after injection of indicated compounds.

Since different metabolic fuels have different P/O values, this analysis was repeated while varying the P/O values between 3.25 and 1.75 to demonstrate the impact of P/O value in the assay calculations. This sensitivity analysis was conducted using the same data set described in Figure 3. Under the different metabolic switch approaches tested, P/O values between 2.75 and 2.5 do not have a significant impact in the assay results resulting in Q ≤ 0 for all the cell lines/fuel conditions. However, when a P/O value of 2.25 or lower was used, several cell conditions result in Q values higher than 0, demonstrating that these values are underestimating P/O ratio. P/O ratios of 3 or above still provide negative Q values but they are above the max theoretical P/O ratios.

In typical cell culture media, the fuel pathways that are most typically engaged yield a P/O range that is actually quite narrow (e.g. glycogen is 2.86 and palmitate is 2.45[4]). As a result, the expected impact of real-world variations in P/O ratio on the calculations described is low. Nevertheless, consideration is given here that cells respiring on a single substrate may have subtle differences in mitoATP Production Rate. Based on the data presented in Figure 4, it is expected that these differences are within the sensitivity limit of the XF Analyzer.

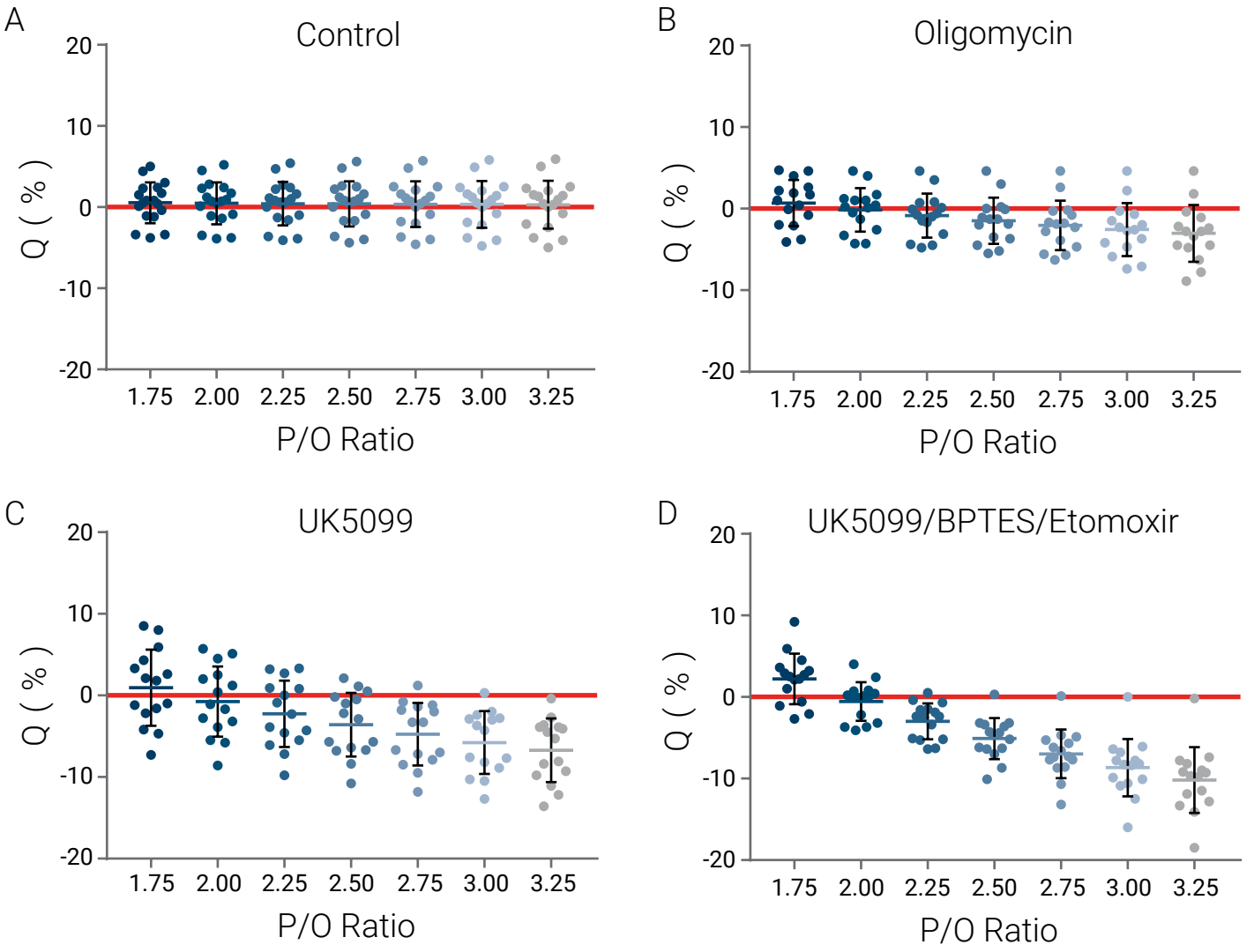

Figure 4. Sensitivity analysis of the impact of P/O ratio value in ATP Production Rate calculations. Re-analysis of data of Figure 3B using different P/O ratios values in Eq. 8 to calculate mitoATP Production Rates when cells were treated with vehicle (A), oligomycin (0.35 µM) (B), UK5099 (2 µM) (C), or the combination of UK5099 (2 µM), BPTES (3 µM) and etomoxir (4 µM) (D).

Application

Two of the main basal ATP consuming pathways in mammalian cells are the membrane transporter Na^+/K^+ ATPase, which is responsible for maintaining ionic gradients across the plasma membrane, and protein synthesis especially in proliferating cells in culture. Based on the previous calculations and assay design, we applied the XF Real-Time ATP Rate Assay to measure ATP Production Rate in A549 and C2C12 cells. Both cell lines were treated with two compounds that affect these key cellular activities and compared with the results obtained for total ATP level measured using a commercially available luminescence kit.

The XF assay was performed in Seahorse XF DMEM Medium, pH 7.4 supplemented with 10 mM glucose, 1 mM pyruvate, 2 mM glutamine at 37°C. To test the effect of modulation of the described cell activities on ATP Production Rate, cells were treated with monensin, a known monovalent cation ionophore that raises cytosolic Na^+ concentration causing an increase in the Na^+/K^+ ATPase activity, and cycloheximide an inhibitor of protein synthesis, followed by sequential injection of the assay compounds oligomycin and rotenone/antimycin A (the injection scheme to determine ATP Production Rate). OCR and ECAR data were collected and used to calculate ATP Production Rate under control conditions or after the addition of monensin or cycloheximide.

As shown in Figure 5, the expected increase in total ATP production was observed in both A549 and C2C12 cells after treatment with monensin, reflecting the increase in ATP production to meet the increased ATP demand. In contrast, treatment with cycloheximide induces an acute inhibition of ATP demand for protein biosynthesis resulting in a decrease in total ATP production. To further support the benefits of using the XF Real-Time ATP Rate Assay to study changes in cell bioenergetics related to cell function, we measured intracellular ATP levels in identical samples treated with monensin or cycloheximide for 60 min at identical concentrations. To measure ATP level, we used a commercially available luminescent kit that uses luciferase to generate a stable luminescent signal that is proportional to ATP concentration in cell lysates. As shown in Figure 5, no significant changes in intracellular ATP levels were detected under the studied conditions supporting the notion that intracellular ATP levels are in steady state and don't reflect physiological changes in cell bioenergetics.

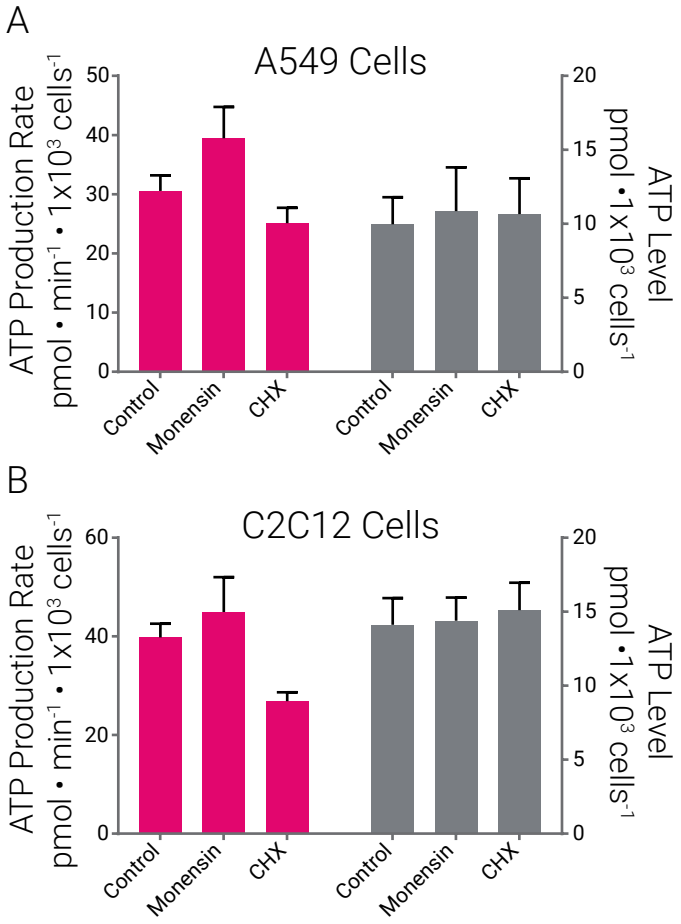

Figure 5. Seahorse XF Real-Time ATP Rate Assay was performed in A549 cells (15K/well) (A) or C2C12 cells (12K/well) (B) under control conditions or after 60 min of exposure to monensin (200 µM) or cycloheximide (10 µM) in assay medium (Seahorse XF DMEM Medium, pH 7.4 supplemented with 10 mM glucose, 1 mM pyruvate and 2 mM glutamine) using a Seahorse XFe96 Analyzer. Intracellular ATP levels were measured under identical conditions using CellTiter-Glo® Luminescent Cell Viability Assay (Promega).

Conclusions and a view of the future

The need for improved tools to study cellular function is clear and is reflected in the increasing number of cell-based assays techniques developed over the last decade. Cell energy metabolism is a fundamental driver of cell phenotype and function. However, very few tools are available to study changes in cell bioenergetics in real time. This white paper presents a new quantitative method to measure the rate of ATP production from glycolysis and mitochondria simultaneously using label-free technology in live cells, in real time.

Using this assay, both pathways can be monitored over time to observe how cells respond to total energy demand, i.e. the Total ATP Production Rate. Furthermore, because both pathways are measured simultaneously, the relative poise between these pathways can also be quantified. This is termed the Metabolic Index and may now be used to ascertain the energy source cells call upon to meet energy needs. For example, there is now early evidence that migrating cells preferentially use glucose to fuel podocyte formation[5]. Additionally, activation of immune cells is now known to require a tightly coordinated increase in both glycolytic and mitochondrial function[6,7]. Future research may explore the relative poise of metabolism in the control of stem cell differentiation[8], cancer cell line proliferation[9] and other metabolic diseases. Regardless of the model used, real-time kinetic quantification of ATP Production Rate delivers a dynamic picture of cellular bioenergetics, providing unique insight into cellular phenotype and function.

References

1. Carling, D.; Thornton, C.; Woods, A.; Sanders, M. J. AMP-activated protein kinase: new regulation, new roles? *Biochem J* **2012**, *445* (1), 11.
2. Hardie, D. G.; Ross, F. A.; Hawley, S. A. AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Biol* **2012**, *13* (4), 251.
3. Romero, N.; Swain, P.; Neilson, A.; Dranka, B. P. White paper: Improving Quantification of Cellular Glycolytic Rate Using Agilent Seahorse XF Technology. http://seahorseinfo.agilent.com/acton/fs/blocks/showLandingPage/a/10967/p/p-00ca/t/page/fm/1.
4. Mookerjee, S. A.; Gerencser, A. A.; Nicholls, D. G.; Brand, M. D. Quantifying intracellular rates of glycolytic and oxidative ATP production and consumption using extracellular flux measurements. *J Biol Chem* **2017**, *292* (17), 7189.
5. Ozawa, S.; Ueda, S.; Imamura, H.; Mori, K.; Asanuma, K.; Yanagita, M.; Nakagawa, T. Glycolysis, but not Mitochondria, responsible for intracellular ATP distribution in cortical area of podocytes. *Sci Rep* **2015**, *5*, 18575.
6. Pearce, E. J.; Pearce, E. L. Immunometabolism in 2017: Driving immunity: all roads lead to metabolism. *Nat Rev Immunol* **2018**, *18* (2), 81.
7. Mills, E. L.; Kelly, B.; O'Neill, L. A. J. Mitochondria are the powerhouses of immunity. *Nat Immunol* **2017**, *18* (5), 488.
8. Folmes, C. D.; Ma, H.; Mitalipov, S.; Terzic, A. Mitochondria in pluripotent stem cells: stemness regulators and disease targets. *Curr Opin Genet Dev* **2016**, *38*, 1.
9. Wolpaw, A. J.; Dang, C. V. Exploiting Metabolic Vulnerabilities of Cancer with Precision and Accuracy. *Trends Cell Biol* **2018**, *28* (3), 201

www.agilent.com/chem/discoverxf

Printed in the USA, April 12, 2018
5991-9303EN

Agilent
Trusted Answers