The Chemistry behind the assay:

The Agilent Seahorse XF ATP Real-Time rate assay measures and quantifies the rate of ATP production from glycolytic and mitochondrial system simultaneously using label-free technology in live cells.

In mammalian cells, glycolysis and oxidative phosphorylation (OXPHOS) pathways provide the majority of cellular ATP. While OXPHOS consumes O_2, driving the oxygen consumption rate (OCR), both pathways can contribute to the acidification of the assay medium. Conversion of glucose to lactate through glycolysis is accompanied by extrusion of one H+ per lactate, while the TCA cycle that fuels ETC/OXPHOS produces CO_2, which also results in acidification of the assay medium. The sum of these reactions is the primary driver of changes in extracellular acidification (ECAR). Seahorse XF technology measures the flux of both H+ production (ECAR) and O_2 consumption (OCR), simultaneously. By obtaining these data under basal conditions and after serial addition of mitochondrial inhibitors (oligomycin and rotenone/antimycin A), total cellular ATP Production Rates and pathway-specific mitoATP and glycoATP Production rates can be measured. The series of calculations used to transform the OCR and ECAR data to ATP Production rates as described below.

Glycolytic ATP production rate calculation: During conversion of one molecule of glucose to lactate in the glycolytic pathway, 2 molecules each of ATP, H+ and lactate are produced

Glucose + 2 ADP + 2 Pi = 2 Lactate + 2 ATP + 2 H2O + 2 H+

Considering the stoichiometry of the glycolytic pathway, the rate of ATP production in the glycolytic pathway (glycoATP Production Rate) is equivalent to Glycolytic Proton Efflux Rate (glycoPER) and can be calculated using the same approach

glycoATP Production Rate (pmol ATP/min) = glycoPER (pmol H+/min)

Mitochondrial ATP production rate calculation: 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 the ATP synthase inhibitor, oligomycin:

OCR_{ATP} (pmol O_2/min) = OCR (pmol O_2/min) - OCR_{Oligo} (pmol O_2/min)

Transformation of OCR_{ATP} to the rate of mitochondrial ATP production consists of: multiplying by 2 to convert molecules of O2 to oxygen (O) atoms consumed, then multiplying the P/O ratio, the number of molecules of ADP phosphorylated to ATP per atom of O reduced by an electron pair flowing through the electron transfer chain. The Seahorse XF Real-Time ATP Rate Assay uses an average P/O value of 2.75 for these calculations that was validated to accurately represents the assay conditions for a broad panel of cells under different fuels availabilities

With these considerations, the rate of mitochondrial ATP production is calculated as:

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

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).

Experimentation:

1. The cells need to be attached to the bottom of culture plate so that the probes measuring OCR/ECAR can stand just above the cells and monitor the OCR or ECAR from the cellular microenvironment. The number of cells must be equal for any quantitative comparison. There is no problem with adherent cell lines, as we can count and seed the cells couple of hours before the assay, special care needs to be taken for suspension cell lines. For measuring OCR/ECAR in suspension cell lines, the plates need to be coated with poly-L-lysine and plate need to be centrifuged to make the cells attached to bottom.

2. As, the experimental culture medium is different from the normal growth medium (Bicarbonate free) and cells need to be grown in CO_2 free conditions for 1 hour before experiment.

3. The culture medium volume should be equal to 180 ul, to allow addition of mitochondrial inhibitors (Oligomycin, antimycin D and rotenone).

Procedure:

(a) Cells were grown in normoxia or hypoxia conditions in regular T25 flask in regular growth medium (I grew the BJAB and BJAB-KSHV cells at a concentration of 250,000 cells/ml).
(b) Day before assay, using multichannel pipette, I added 25 ul of poly-L-lysine per well of culture plate (This is the seahorse plate designed to run on Seahose machine) and left the plate in hood for 2 hours. poly-L-lysine was aspirated using multichannel pipette and the wells were washed with 200 ul sterile water. Plates were dried for 1 hour in culture hood.
(c) On the day of experiment, a small fraction of cells were isolated after mixing well and the flask was putted back in the incubator. This fraction of cells was used for counting.

(d) The cells grown under normoxic or hypoxic conditions, were counted and resuspended in recommended medium (Seahores XF medium) at a concentration of 100,000 cells/180 ul.
(e) Cells were incubated in 1%O_2 and/or CO_2 free conditions for 1 hour. The plates were immediately sealed with parafilm and centrifuges to allow all the cells to get fully attached (1500 rpm for 5 minutes) in the cell plate compatible centrifuge. The cell attachment was confirmed by looking under light microscope.
(f) Mitochondrial inhibitor addition is programmed in the machine, we just need to add these inhibitors in specified port before putting the plate in the machine.
(g) The experiment template on the machine is similar to real-time PCR machine so we can assign sample and treatment condition in the plate easily there.
(h) Dat analysis can be performed using **"wave"** software form Seahorse.

Notes:

1. Plate coating with poly-L-lysine should be done in day advance of final seeding.
2. The cartridge need to be equilibrated over night before the experiment day.
3. The Bicarbonate free XF medium need to be equilibrated at 37 degree centigrade for at least 1 hour.
4. CO_2 incubator nearly takes 20-25 minutes to reach 0% reading, so plan accordingly.
5. For me 100, 000 cells/well for BJAB and BJAB-KSHV worked well.
6. Cell counting, resuspending at particular concentration, seeding and allowing them to attach and giving 1-hour CO_2 free incubation may take time. In my case the instrument was booked from 10:00 AM. I started at 7:00 in morning and was running little late. So start early morning on the day of running the experiment or book the instrument from 11AM onward.

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Instrument/System Used

Seahorse XFe96 Analyzer