Application Note

Glycolysis

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# Characterization of Glycolysis with a Panel of Common Cellular Models Using Agilent Seahorse XF Technology


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## Introduction

Glycolysis is one of the two major-energy producing pathways in mammalian cells. During glycolysis, the breakdown of glucose via pyruvate and ultimately to lactate results in concomitant release of two $H^+$ at physiological pH and acidification of the extracellular medium surrounding the cell.

Agilent Seahorse XF Analyzers perform simultaneous, real-time measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in live cells. OCR measurements are directly related to cellular respiration (after removing contribution from non-mitochondrial oxygen consumption by injection of mitochondrial inhibitors). However, despite the strong correlation of ECAR with glycolytic activity[i], other sources of acidification (mainly mitochondrial-derived $CO_2$[i, ii]) can limit the specificity of the measurement. Additionally, the buffer capacity of the medium and the measurement system significantly impact ECAR values. The ability to account for these factors enables a more accurate and specific measurement of glycolysis.

The **Agilent Seahorse XF Glycolytic Rate Assay** utilizes both ECAR and OCR measurements to account for $CO_2$ contribution to extracellular acidification, resulting in a Glycolytic Proton Efflux Rate (glycoPER). This metric is the rate of protons extruded to the extracellular medium during glycolysis and is equivalent to the amount of lactate produced during glycolysis[iii]. This document includes a review of the XF Glycolytic Rate Assay as well as reference values obtained for 22 common cell models.

## Assay Overview

The glycolytic rate assay is designed to accurately measure glycolysis in the presence of typical medium substrates. Basal rates of OCR and ECAR are measured first, then rotenone & antimycin A are injected to measure the amount of OCR caused by mitochondrial activity. This data is used to determine and subtract mitochondrial contribution to acidification (proton efflux rate or PER), yielding a precise measure of glycolytic proton efflux rate (glycoPER) without the need to starve cells of substrates. The injection of 2-deoxy-D-glucose (2-DG) provides visual confirmation that the acidification is predominantly due to glycolysis.

### Materials

- Reagents: XF Glycolytic Rate Assay Kit for XFe/XF analyzers (Agilent Part number 103344-100) or XFp analyzers (Agilent Part number 103346-100).
- Instrument: All data generated using a Seahorse XFe96 Analyzer. Assays can be performed on an XFp Analyzer, XF96 Analyzer, or XFe24 Analyzer with cell number adjustment.
- Cartridges and cell plates: XFe96 FluxPak (Agilent Part number 102416-100 or 102601-100) or XFe24 FluxPak (Agilent Part number 102340-100 or 102342-100) or XFp Flux Pak (Agilent Part number 103022-100)
- Cells: See Data Tables for cell seeding density and plate coatings for the cell type of interest.
- Media: XF DMEM Base Medium without Phenol Red (Agilent Part number 103335-100): Supplement with **5 mM** HEPES (Agilent Part number 103337-100), 10 mM glucose, 1 mM sodium pyruvate, 2 mM glutamine, pH 7.4 at 37ºC.

  or

- XF RPMI Medium without Phenol Red (Agilent Part number 103336-100): Supplement with **1 mM** HEPES (Agilent Part number 103337-100), 10 mM glucose, 1 mM sodium pyruvate, 2 mM glutamine, pH 7.4 at 37ºC.

### Injection Strategy

Compounds are provided in the assay kit; below indicates final concentration in the well.

- Injection 1: 0.5 µM rotenone + 0.5 µM antimycin A
- Injection 2: 50 mM 2-deoxy-D-glucose

### Notes

- For the assay to be accurate, the media must not contain phenol red.
- For detailed instructions, please consult the relevant User Guides[iv]

### Data Analysis

- All data shown is the mean of at least 3 replicate plates and was generated using Wave 2.4 and the Seahorse XF Glycolytic Rate Assay Report Generators from Agilent.
- Wave 2.4 Desktop or newer versions are required to see PER data in Wave, as well as for best results using the report generator to calculate glycoPER.
- Glycolytic Rate Assay Media must be assigned to each experimental group and background wells (or the appropriate BF in case using a different assay medium) to obtain PER data (in Wave) and glycoPER data (in Report Generator).
- PER is a post-run metric in Wave 2.4 and cannot be calculated until XF assay run is finished.
- To convert OCR and ECAR data to glycoPER data, XF assay data must be exported and analyzed using the appropriate Seahorse XF Report Generator.
  - Individual assay data can be exported directly from Wave 2.4 to XF Glycolytic Rate Assay Report Generator.
  - In case of set of replicate assays, data can be analyzed in less time using the Multi-File XF Glycolytic Rate Assay Report Generator. If analyzing a set of replicates (obtained from the same instrument using the same Assay Protocol) export each individual file to Microsoft Excel (*.xls) before analyzing using the Multi-File XF Glycolytic Rate Assay Report Generator.

## Results

Table 1 shows reference OCR and PER values obtained at the indicated seeding density using the Seahorse XF Glycolytic Rate Assay.

- Proton Efflux Rate (PER) data is a definitive measurement of extracellular acidification rate and independent of assay medium, buffer factor and instrument type so it is the recommended standard method for reporting Seahorse acidification data.
- ECAR values are inversely proportional to Buffer Factor of media. Therefore, the ECAR values in the table are, in general, lower than the values obtained when using non-buffered XF Base Medium in other Seahorse XF Assays. However, despite the lower absolute values, the addition of HEPES improves the consticancy and reproducibility of ECAR data.
- All data provided is for reference only. Absolute rates and magnitude responses may vary depending on biological and experimental variable conditions such as cell passage number, cell seeding density, cell culture conditions, etc.

Table 2 summarizes the parameters obtained after analysis of set of 3 replicates per cell type using the Seahorse Multi-File XF Glycolytic Rate Assay Report Generator. The definitions of these parameters are outlined below.

- **Glycolytic Proton Efflux Rate** (glycoPER) is an accurate measurement of basal glycolytic rate and accounts for $CO_2$ contribution to extracellular acidification. This approach is the best method available for quantifying glycolysis through monitoring extracellular acidification. Studies with more than 20 cellular models demonstrate that glycoPER is equivalent to lactate production rate.
- **Compensatory glycolysis** is a measurement of the capability of the cell to compensate energy production through glycolysis after blockage of mitochondrial ATP production and is expressed as a percentage of basal glycolysis.
- **% PER from glycolysis** represents the contribution of the glycolytic pathway to total extracellular acidification. For most of the immortalized cell lines tested, the contribution of glycolysis represents between 75-90% of the total acidification observed (see Figure 1).

## Discussion

The percent PER (% PER) from glycolysis gives an indication of the amount of acidification due to glycolysis vs. other sources like mitochondrial $CO_2$. For many immortalized cell lines, glycolysis is THE major contributor to acidification (Figure 1). From a practical perspective, this means that the contribution of $CO_2$-derived acidification to the total PER (or ECAR) signal is very small. On the other hand, for some primary cell types (e.g. splenocytes or neonatal rat ventricular myocytes), contribution of $CO_2$ from respiration to extracellular acidification is significantly higher and glycolysis only represents 50-75% of the acidification observed. This highlights the importance of using glycoPER and not total acidification metrics data as a quantitative measurement of glycolysis, especially when substrates other than glucose are available.

The Seahorse XF Glycolytic Rate Assay is an optimized solution (kit, media, and report generator) that quantifies glycolysis in live cells in real time. The data presented in this application show a wide range of basal glycolytic rates and capacity among cells, demonstrating the utility of this assay in studies of metabolic phenotype and preference for glycolysis as a source of energy in cellular disease models. It also allows to compare and rank cells based on the contributions of glycolysis and mitochondria respiration to extracellular acidification, providing a view of cellular metabolic phenotype.

Key features and benefits of XF Glycolytic Rate Assay include:

- Precise- Improved reproducibility day to day by controlling buffering properties of the assay media
- Accurate- measures and subtracts mitochondrial-related acidification and uses a controlled assay media for an accurate measurement of glycolytic rate
- Reliable - validated against lactate accumulation assays
- Relevant- no cell starvation required, more flexible substrate conditions allowed

**Table 1.** Basal OCR, ECAR and PER data obtained using Seahorse XF Glycolytic Rate Assay. Data represent mean ± SEM from at least 3 replicate plates.

| Cell Type | Seeding Density (cells/well) | basal OCR (pmol/min) | Basal ECAR mpH/min) @ BF = 2.5 | Basal PER (pmol/min) |
|---|---|---|---|---|
| 3T3L1[1] | 15K | 58 ± 5 | 11 ± 1 | 95 ± 7 |
| A431 | 10K | 88 ± 12 | 82 ± 8 | 599 ± 73 |
| A549 | 15K | 95 ± 7 | 33 ± 1 | 243 ± 10 |
| Bovine Aortic Endothelial Cells[1] | 30K | 215 ± 19 | 31 ± 4 | 224 ± 32 |
| BT474 | 30K | 185 ± 7 | 20 ± 1 | 195 ± 43 |
| C2C12 | 12K | 134 ± 3 | 60 ± 1 | 441 ± 9 |
| Human CD4+ T cells activated with antiCD3/CD28 beads[2] | 200K | 32 ± 1 | 15 ± 1 | 81 ± 5 |
| Human CD4+ T cells naïve[2] | 200K | 23 ± 1 | 5 ± 1 | 27 ± 2 |
| H460 | 15K | 105 ± 11 | 49 ± 4 | 364 ± 41 |
| H9C2 | 10K | 43 ± 6 | 6 ± 1 | 46 ± 7 |
| HCT116 | 10K | 62 ± 1 | 28 ± 3 | 206 ± 28 |
| HeLa | 15K | 68 ± 7 | 25 ± 2 | 183 ± 16 |
| HepG2 | 20K | 120 ± 17 | 36 ± 5 | 265 ± 42 |
| HUVEC[1] | 15K | 39 ± 6 | 13 ± 1 | 96 ± 3 |
| Jurkat[3] | 100K | 78 ± 4 | 50 ± 1 | 363 ± 12 |
| MB-MDA-231 | 30K | 52 ± 11 | 20 ± 1 | 146 ± 13 |
| MCF10a | 12K | 164 ± 27 | 32 ± 4 | 268 ± 36 |
| MCF7 | 20K | 129 ± 13 | 35 ± 2 | 257 ± 19 |
| Neonatal Rat Ventricular Myocytes | 70K | 158 ± 15 | 22 ± 2 | 152 ± 18 |
| PC12[3] | 80K | 178 ± 10 | 38 ± 1 | 278 ± 18 |
| Rat Cortical Neurons[5] | 32K | 264 ± 37 | 59 ± 4 | 451 ± 33 |
| Raw264.7 | 25K | 247 ± 5 | 84 ± 6 | 598 ± 35 |
| Mouse Splenocytes | 150K | 58 ± 10 | 9 ± 3 | 64 ± 17 |

[1]Coated with Gelatin (0.2%)

[2]Coated with Cell-Tak (Corning) according to manufacture recommendations.

[3]Coated with Poly-D-Lysine

[4]Coated with Fibronectin 0.02%/Gelatin 0.05%. Cells were grown 2 days in the presence of 200 µM BrdU before the assay.

[5]Coated with Poly-D-Lysine (30 µg/mL)/Laminin (2 µg/mL). Cells were assayed 14 days Post differentiation with NSF-1 (Neural Survival Factor-1).

**Table 2.** Glycolytic Activity obtained using Seahorse XF Glycolytic Rate Assay. Data shown are mean ± SEM of at least 3 replicate plates.

| Cell Type | Seeding Density (cells/well) | Basal glycoPER (pmol/min) | Compensatory glycolysis (%) |
|---|---|---|---|
| 3T3L1[1] | 15K | 70 ± 8 | 267 ± 12 |
| A431 | 10K | 558 ± 69 | 154 ± 14 |
| A549 | 15K | 203 ± 9 | 201 ± 11 |
| Bovine Aortic Endothelial Cells[1] | 30K | 121 ± 21 | 510 ± 60 |
| BT474 | 30K | 71 ± 1 | 369 ± 7 |
| C2C12 | 12K | 380 ± 9 | 179 ± 5 |
| Human CD4+ T cells activated with antiCD3/CD28 beads[2] | 200K | 70 ± 5 | 163 ± 24 |
| Human CD4+ T cells naïve[2] | 200K | 19 ± 2 | 167 ± 8 |
| H460 | 15K | 319 ± 36 | 198 ± 22 |
| H9C2 | 10K | 27 ± 8 | 171 ± 31 |
| HCT116 | 10K | 179 ± 28 | 216 ± 11 |
| HeLa | 15K | 152 ± 16 | 221 ± 15 |
| HepG2 | 20K | 210 ± 33 | 203 ± 28 |
| HUVEC[1] | 15K | 83 ± 5 | 164 ± 16 |
| Jurkat[3] | 100K | 327 ± 12 | 156 ± 9 |
| MB-MDA-231 | 30K | 123 ± 12 | 234 ± 16 |
| MCF10a | 12K | 187 ± 21 | 312 ± 72 |
| MCF7 | 20K | 194 ± 13 | 251 ± 11 |
| Neonatal Rat Ventricular Myocytes | 70K | 88 ± 10 | 337 ± 43 |
| PC12[3] | 80K | 197 ± 19 | 398 ± 25 |
| Rat Cortical Neurons[5] | 32K | 328 ± 29 | 180 ± 31 |
| Raw264.7 | 25K | 477 ± 33 | 239 ± 14 |
| Mouse Splenocytes | 150K | 36 ± 10 | 208 ± 33 |

[1]Coated with Gelatin (0.2%)

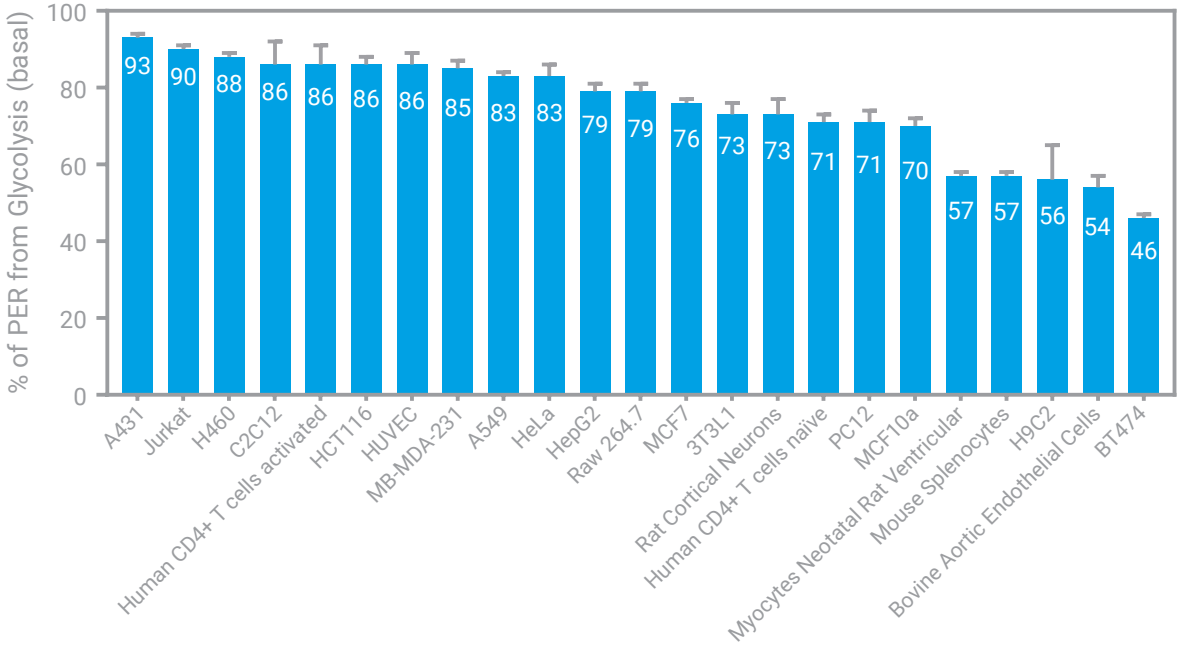
[2]Coated with Cell-Tak (Corning) according to manufacture recommendations.

[3]Coated with Poly-D-Lysine

[4]Coated with Fibronectin 0.02%/Gelatin 0.05%. Cells were grown 2 days in the presence of 200 µM BrdU before the assay.

[5]Coated with Poly-D-Lysine (30 µg/mL)/Laminin (2 µg/mL). Cells were assayed 14 days Post differentiation with NSF-1 (Neural Survival Factor-1).

**Figure 1.** Ranking of percent of PER from glycolysis in basal state among the 22 cell models tested.



**Footnotes/References**


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

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

iii. Romero, N et al. Quantifying cellular glycolytic rate using $CO_2$-corrected extracellular acidification. Agilent document number: 5991-7894EN-D4

iv. Agilent Seahorse XF/XFp GLycolytic Rate Assay Kit User Guides (Document number: 103344-400 and 103346-400)

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