Optimization of the Agilent pH-Xtra Glycolysis Assay using Agilent BioTek Gen5 Software

Step-by-Step Instructions

Gen5

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CHECK USER GUIDE FOR SAFETY WARNINGS BEFORE CONTINUING

This guide contains step-by-step instructions for the crucial steps during plate reader setup, assay optimization and data analysis on the BioTek Synergy H1 multimode plate reader. These tips help to maximize the functionality of the pH-Xtra template (AgilentXpH) for Gen5. For more detailed instructions on the assay workflow, please refer to the pH-Xtra User Guide.[1]

Order a filter cube. → Install the latest Gen5. → Add the filter cube to the Gen5 optics library. → Download AgilentpHX.prt → Edit the protocol as needed for each experiment. → Run an Agilent pH Xtra Glycolysis Assay.

Figure 1. Top level workflow for measuring the pH-Xtra Assay using Agilent BioTek Gen5 data analysis software.

1. Instrument setup

Key steps

Figure 2. Instructions on how to enter a new filter cube into the Optics Library.

1.1 Cube installation

For optimal performance of this assay on a Synergy H1, the BioTek filter cube for pH-Xtra (part number 8040588) or the combination cube for MitoXpress Xtra and pH-Xtra (part number 8040594) are recommended.

- https://www.biotek.com/products/accessories/fltr-blk-ph-xtra-glycolysis-asby/

- https://www.biotek.com/products/accessories/combination-filter-cube-for-mitoxpress-and-ph-xtra-glycolysis-assays/

Confirm that the cube values match the settings in Figure 2, then define the cube in Gen5 and on the reader.

Open the hinged door on the front of the instrument and place the filter on the filter rail. If the filter cube is oriented correctly (the nonlabeled side facing the chamber), gently slide the cube into the chamber.

To add the filter cube into the **Optics library, select System > Optics Library > Filter Cubes**. Then select **Add** and enter a name for the filter cube. This name becomes available for selection in the **Procedure** section for **Protocols and Experiments**.

1.2 Template download

Download the file "AgilentpHX.prt" from:

https://www.biotek.com/resources/sample-files/agilentphx/

The template can be loaded in the task manager in **Experiment > Create using an existing protocol or Protocols**.

Tip: Save protocols for reference in future experiments as independent files when modified. Modifications to the procedure result in current data being replaced to preserve the audit trail.

Materials required
- Agilent pH-Xtra Glycolysis Assay (part number PH-200-4)
- 96-well microplates (part number 204626-100)

1.3 Signal optimization

Signal optimization is the crucial first step to a successful experiment. A robust signal-to-blank (S:B) ratio is required for good quality data and ensures that the probe signal is stable and detectable above the background.

Agilent recommends an S:B of at least 3 for this assay, although using the instrumentation and protocol described here, much higher values can be achieved. If the S:B is low, probe concentration, settings and gain can be adjusted to improve it.

The S:B only needs to be optimized once before carrying out experiments but should be checked and adjusted if the assay medium, plate type, or assay volume change.

Wells that contain pH-Xtra without any cells are referred to as Signal Control (SC). The signal from these wells should be stable after any initial temperature equilibration and should maximally be approximately 5 to 10% of the maximum scale value (default Synergy H1 scale: 100 to 99999 RFU).

These instructions briefly describe how to test S:B using the AgilentXpH.prt template. See the pH-Xtra User Guide[1] for complete instructions and troubleshooting.

i.   In the Gen5 task manager, select **New Task > Experiments > Create using existing protocol**, then select **AgilentXpH**.

ii. Save the protocol as *Signal Optimization*.

iii. Modify the plate layout under the **protocol** tab. Place **blanks (BLK)** and **signal controls (SC)** appropriately (Figure 3).

	1	2	3	4	5	6	7	8	9	10	11	12
A	SC	SC	SC	SC								
B	BLK	BLK	BLK	BLK								
C												
D												
E												
F												
G												
H												

Figure 3. Suggested plate map for S:B optimization.

iv. Confirm that the settings in **Procedure** match Figure 4.

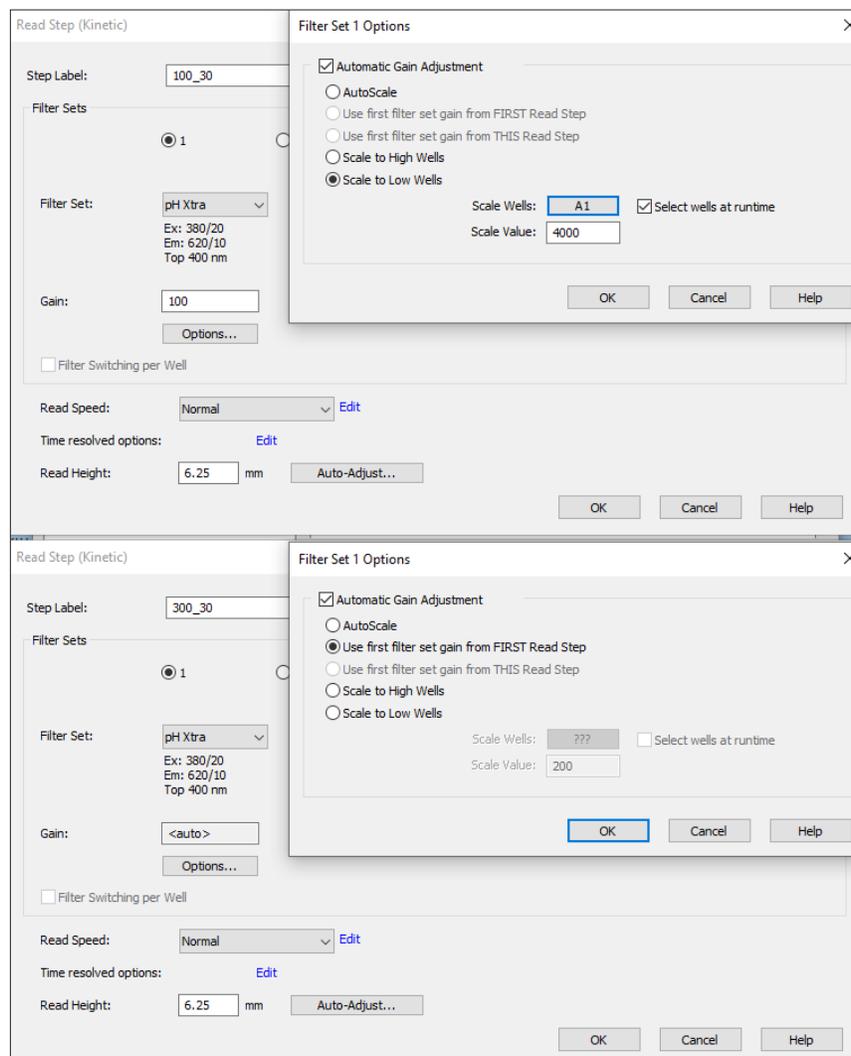**Figure 4.** Setting up automatic gain adjustment.

1.4 S:B assay

i. Prepare RB-100 as described in the User Guide[1], warm to 37 °C and adjust pH to 7.4

ii. Reconstitute pH-Xtra as described in User Guide.[1]

iii. Place the microplate on a heat block set to 37 °C.

iv. Add the following reagents to the microplate:

    a. **BLK:** 100 µL RB-100

    b. **SC:** 90 µL RB-100 + 10 µL pH-Xtra

v. Start the assay, **Read New** and allow to run for 20 to 30 minutes.

vi. (Optional) select **SC** wells for autogain.

1.5 Data reduction

Gen5 calculates the S:B and displays the values in wells A1 and B1 after the measurement. The two S:B values correspond to each of the TRF intensity read windows (delay time 1 and delay time 2) required in Lifetime mode.

Check that DS1.SC/DS1.BLK and DS2.SC/DS2.BLK are entered correctly for A1 and B1, respectively, as shown in the formula in the **Data Reduction** section.

Select **Data: S:B** from the drop-down menu. The signal should be stable following any initial temperature settling period The S:B should be calculated from the ~20-minute time point. A typical result is shown in Figure 5.

If the S:B for either measurement window (see Figure 5, lower panel) is less than 3, check the gain settings. If the signal is still lower than 3, see the pH-Xtra User Guide[1] for detailed troubleshooting steps or contact the Cell Analysis Tech Support: cellanalysis.support@agilent.com.
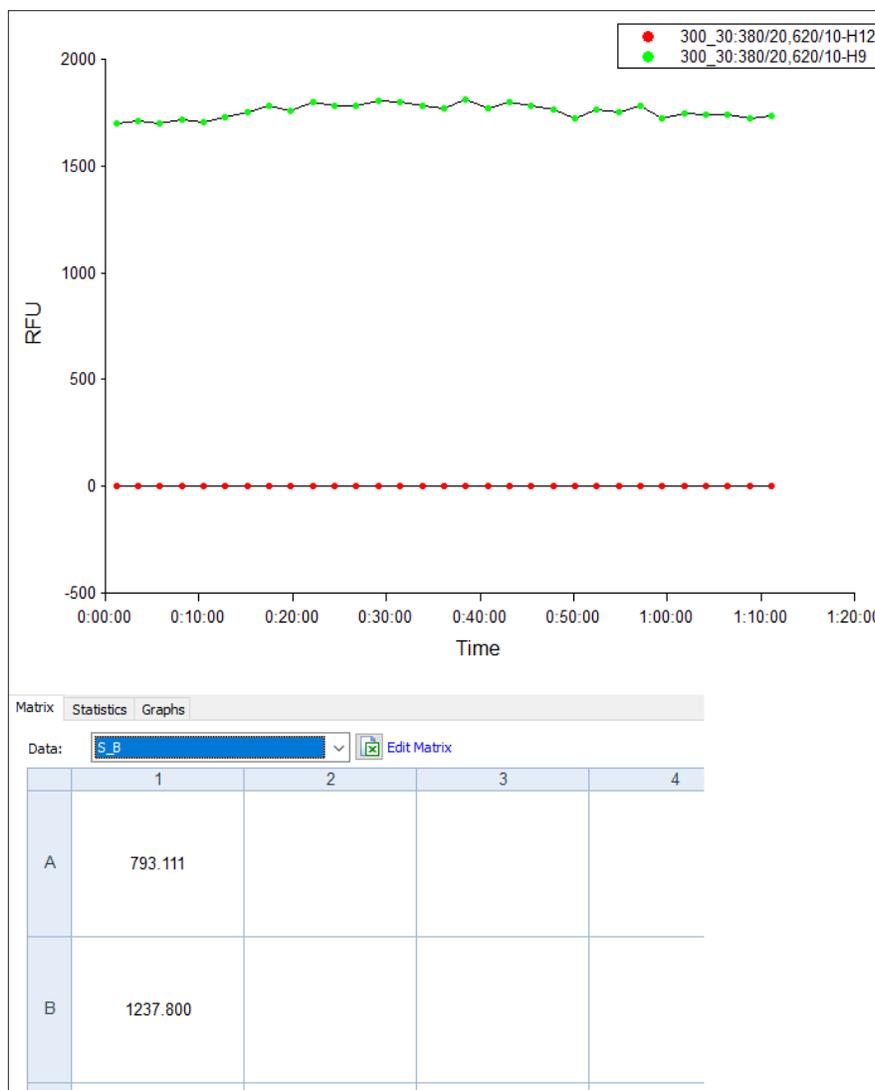
Figure 5. Signal-to-blank experiment. The signal-to-blank ratio for each TRF measurement window is displayed in the plate matrix. The value in A1 is the S:B for measurement window 1, the value in B1 is the S:B for measuremnet window 2. Both values should be >3.

2. Cell number optimization

Cell number optimization

For most adherent cells, the recommended seeding densities are between 40,000 and 80,000 cells per well for this assay. This number varies depending on the glycolytic metabolism of the cells. The exact number needs to be determined in a cell number titration step.

This step is essential, as the extracellular acidification rate is dependent on the cell density in the well. The rates are determined by calculating the slope of the linear portion of the kinetic data in Gen5. Plating too many or too few cells can lead to artifacts during data analysis.

Agilent recommends choosing a seeding density that returns a rate between 15 to 50 µs/h (µs: unit of Lifetime) for further experiments.

In this demonstration a typical cell number titration experiment using RAW264.7 cells as example.

Key steps

Additional materials
- Agilent pH-Xtra Glycolysis Assay (part number PH-200-4)
- 96-well microplates (part number 204626-100)
- Adherent cells (e.g., RAW264.7)
- Growth medium (e.g., DMEM)

2.1 Cell titration

i.   Plate cells across a range of densities (for example, 20,000 to 100,000 cells/well) in complete growth medium and allow them to attach at room temperature before placing the plate in the incubator.

ii. Culture cells overnight.

See pH-Xtra User Guide[1] for suspension cell optimization.

2.2 Template setup

i.   In the Gen5 task manager, select **New Task > Experiments > Create using existing protocol** and then select **AgilentpHX**.

ii. Save the protocol as *Cell Optimization*.

iii. Enter a plate map, select **Plate Layout**, then add a standard curve. Select **Add > Standard Curve**, then enter the values for the cell numbers (Figure 6).
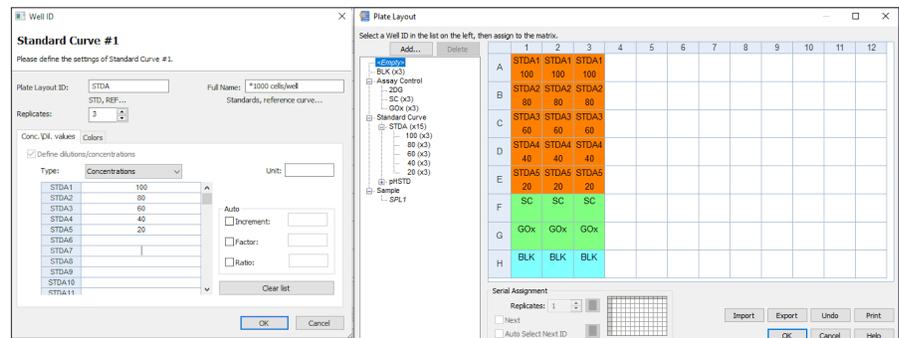

Figure 6. Adding a standard curve for cell optimization and suggested plate layout for optimization assay.

iv. Select the wells and drag to populate.

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2.3 Cell titration assay

i.   Incubate plate for 2 hours in CO_2-free incubator at 37 °C.

ii. Prepare RB-100 as described in the User Guide[1], warm to 37 °C and adjust the pH to 7.4.

iii. Reconstitute pH-Xtra as described in the User Guide.[1]

iv. Place the microplate on a heat block set to 37 °C.

v.   Carefully aspirate the medium, wash cells twice with RB-100.

vi. Add the following reagents to the microplate:

    a. **BLK:** 100 µL RB-100.

    b. **Samples + SC:** 90 µL of RB-100 and 10 µL pH-Xtra.

vii. Start the assay, select the **Read Now** icon.

2.4 Data reduction

i.   To generate a standard curve (Figure 7), select **Data Reduction > Curve analysis > Standard Curve**.

    a. Well ID – STDA

    b. X-Axis Data – Concentrations/dilutions

    c. Y-Axis Data – ECAR Mean V [Lifetime]

    d. Name the curve *Cell Seed StdCurve.*

Figure 7. Calculating optimal seeding density from the standard curve.

The cell numbers displayed as X in the **Results** section result rates between 15 to 50 µs/h. Cell seeding densities in this range generally result in rates well within the dynamic range of this assay and should be used for subsequent experiments.

To visualize the titration, select the **Graphs** plate view window and display **Cell Optimization_StdCurve plot** (Figure 8).
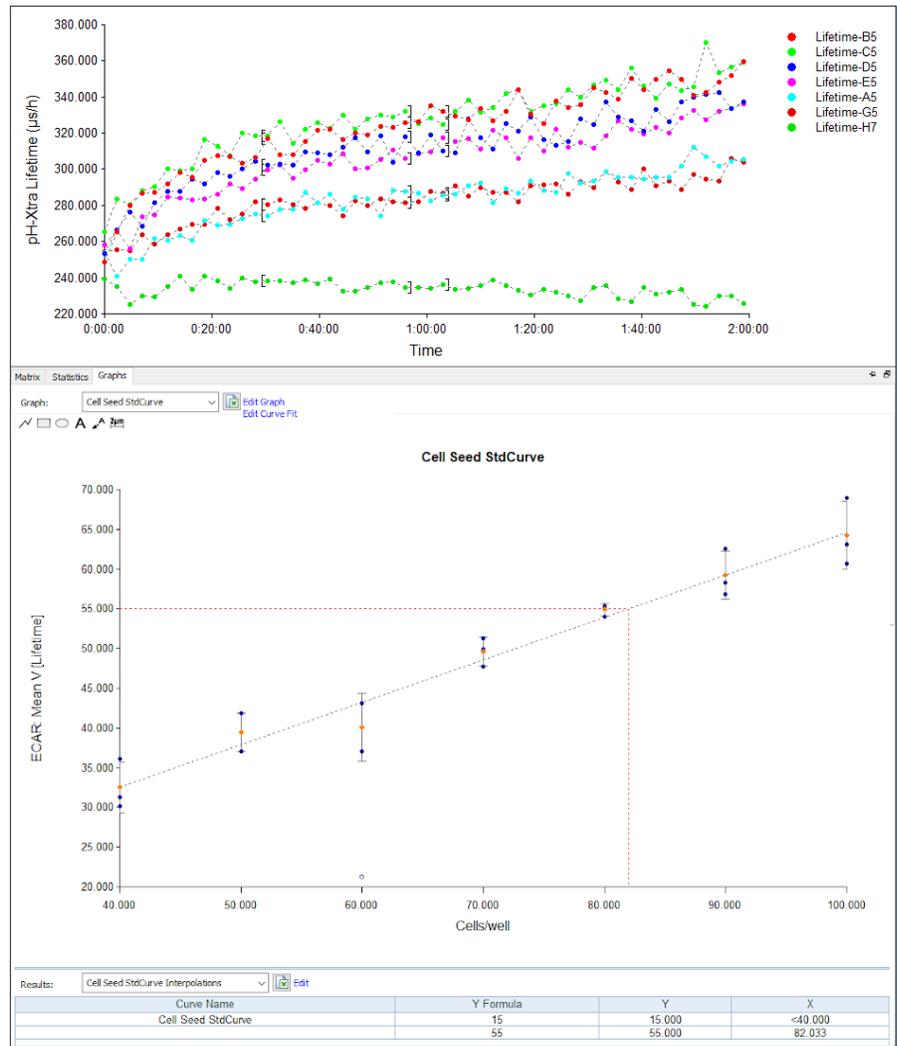

Figure 8. Signal curves for cell optimization assay and calculated rates.

Alternatively, rate data from the **ECAR Mean V** table in **Plate View** can be plotted manually.

3. Glycolysis assay

Glycolysis assay

The pH-Xtra Glycolysis assay is a tailored solution for assessing extracellular acidification using a BioTek plate reader. Combined with the Agilent Glycolysis Stress Test kit, several parameters that reflect glycolysis can be assessed, including basal glycolytic rates, glycolytic capacity and nonglycolytic acidification. These parameters are established indicators for investigating functional metabolic differences among cell types and the outcome of genetic or drug interventions.

Provided here is a step-by-step guide for testing glycolytic rates from assay setup to data generation.

Key steps

Additional materials

- Agilent Seahorse XFp Glycolysis Stress Test kit (part number 103017-100)

- 96-well microplates (part number 204626-100)

- Adherent cells (e.g., RAW264.7)

- Growth medium (e.g., DMEM)

3.1: Plate preparation

i. Plate cells at the density determined in the previous section (here, 70,000 cells/well) in growth medium, and allow cells to attach at room temperature before placing the plate in the incubator.

ii. Culture cells overnight.

3.2: Template setup

i. Select **New Task > Experiments > Create using existing protocol**, then select **AgilentpHX** in the Gen5 task manager.

ii. Save the protocol as *Glycolysis Assay*.

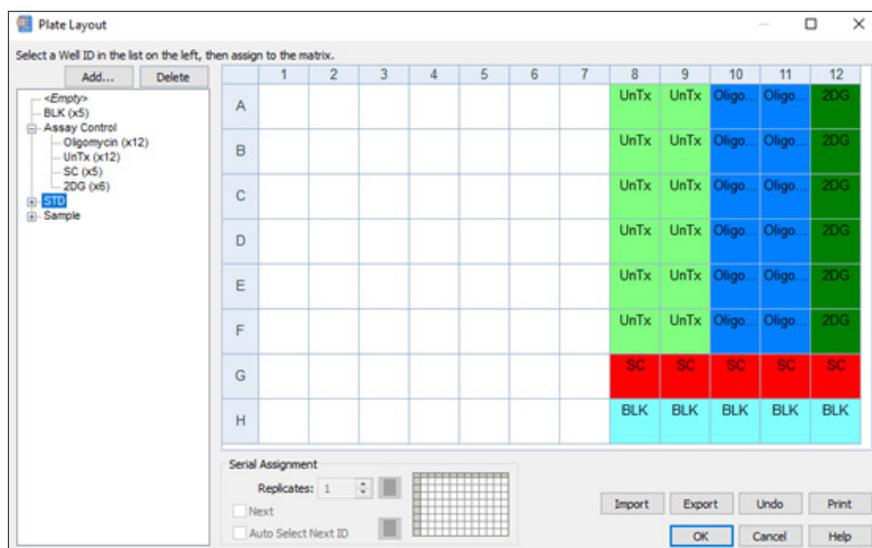iii. Set a plate map, select **Plate Layout**, and place blank and signal control in the correct locations.

Figure 9. Sample plate layout for glycolysis assay.

iv. Add new groups, select **Assay Control**, then name each sample and place them appropriately (see Figure 8). Alternatively, enter each group as a sample, for example, SPL1-4, then label samples using **Plate > Sample ID**.

3.3: Assay preparation

i.   Incubate plate for 2 hours in a CO_2-free incubator at 37 °C.

ii.   Prepare RB-100 as described in User Guide[1], warm to 37 °C and adjust pH to 7.4.

iii. Reconstitute pH-Xtra as described in the User Guide.[1]

iv.   Prepare the compound stocks (see Table 1).

Table 1. Recommended concentrations for test compounds.

Compound	Reconstitution of vial Volume of RB-100 (µL)	Dilution 10X Stocks Stock : RB-100 (µL)	10x Stock Conc.	Final Conc.
Oligomycin	288	100:400	10 µM	1 µM
2-DG	300	–	500 mM	50 mM

v.   Place the microplate on a heat block set to 37 °C.

vi.   Carefully aspirate the medium, wash cells twice with RB-100, and add RB-100 containing pH-Xtra.

vii. Add the following to each group:

    a.   **Blank wells:** 100 µL RB-100

    b.   **Signal control (SC):** 90 µL RB-100 + 10 µL pH-Xtra

    c.   **Compound groups:** 80 µL RB-100 + 10 µL pH-Xtra and 10 µL 10x compound stocks/vehicle control

3.4 Glycolysis assay

i.   Place the plate into the Synergy H1.

ii.   Select **Procedure** and confirm that the gain settings are correct or perform autogain. Ensure the gain for both measurement windows is identical.

iii. Select **Read Now**.

3.5 Data reduction and results

The Gen5 data reduction template displays the kinetic lifetime data and automatically calculates the rates of signal change due to extracellular acidification by linear regression.

By selecting wells in **Curves [Lifetime]**, signal curves can be displayed as shown in Figure 10. The trendline for the rate calculation is also displayed in this view. The rate must be only calculated over the linear range of the signal curve, excluding any initial drop in Lifetime (usually due to temperature equilibration) or plateaus. See the "Tips and Tricks" section in this document or refer to the User Guide[1] on guidance on how to change the automatically chosen range if needed.
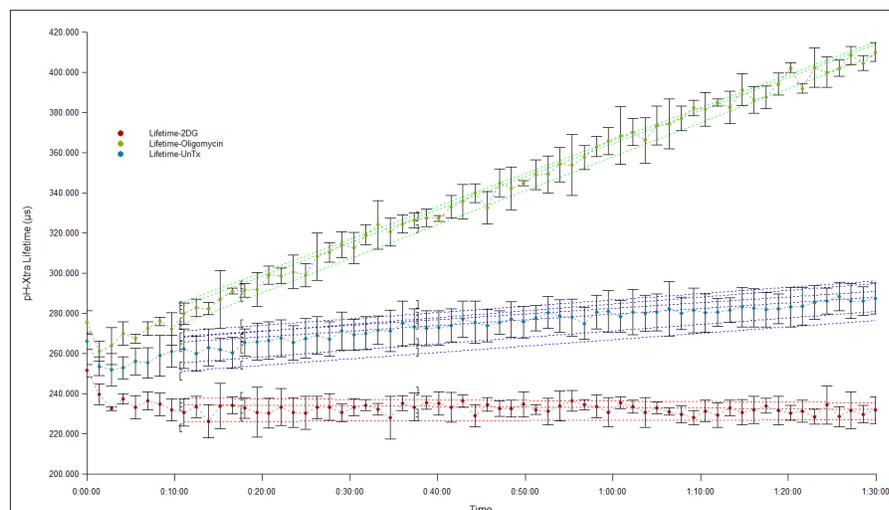

Figure 10. pH-Xtra Glycolysis data of RAW264.7 cells after treatment with glycolysis modulators. The data are shown in lifetime scale. Oligomycin treatment causes increased glycolysis, visible as increased change in signal intensity over time relative to untreated or 2-DG treated cells.

Converting pH-Xtra Lifetime data to the analyte scale allows for quantitative analysis of extracellular acidification rates. The pH-Xtra Gen5 template contains a default calibration function to automatically convert the lifetime data to the pH and [H$^+$] ion scale and therefore does not require to include a calibration experiment for each experiment. The corresponding signal curves can be displayed in the same way as the Lifetime data (Figure 11).
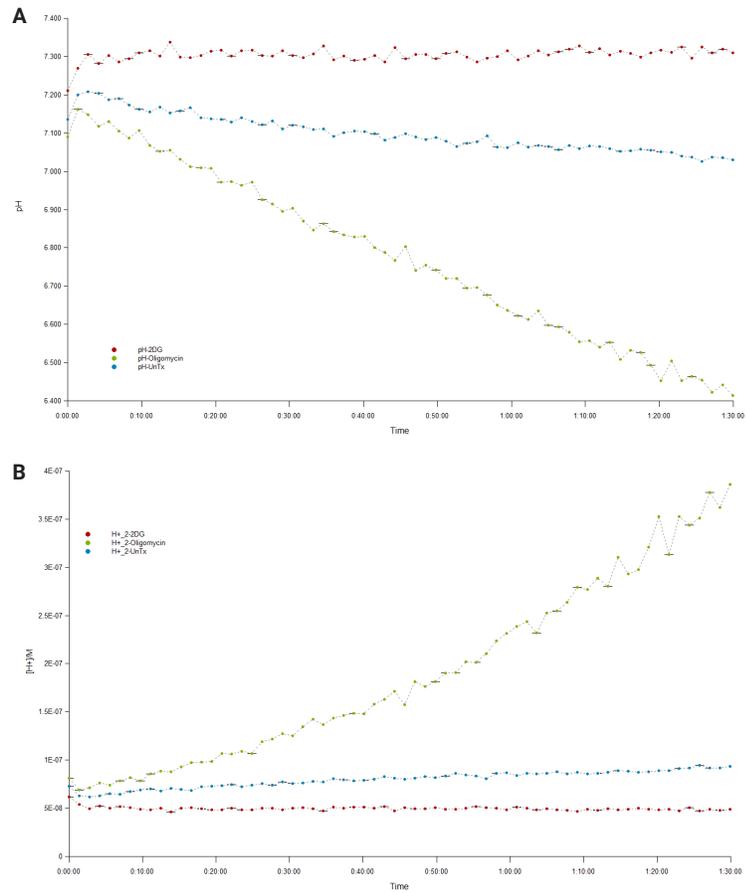
Figure 11. pH-Xtra data from RAW 264.7 cells treated with modulators of glycolysis in (A) pH- and (B) [H⁺]-scale.

Select **ECAR: Mean V** in the **Plate View Matrix** to display the rates for each well. These values can be exported or pasted into graphing software for further analysis and visualization, for example as bar charts. Figure 12 shows a typical bar chart representation of ECAR data on Lifetime. When glycolysis is increased (here: Oligomycin treatment), rates are higher than in vehicle-treated wells, while reduced glycolysis (here: 2-DG treatment) results in lower rates.
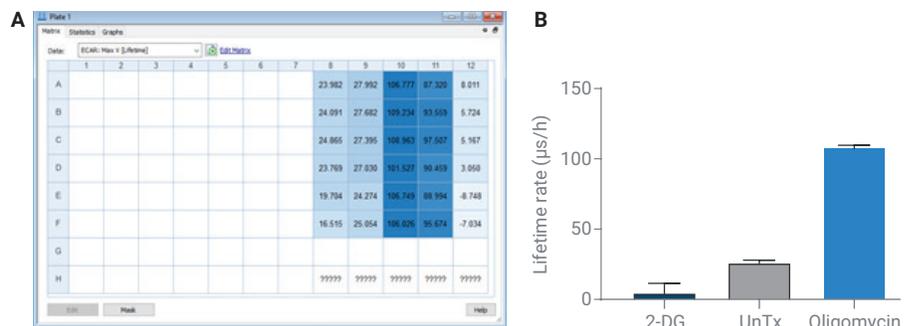

Figure 12. (A) Rate of signal change (from lifetime data) calculated in Gen5; and (B) representation as bar chart after export from Agilent BioTek Gen5 into graphing software.

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4. Reference

1. pH-Xtra Glycolysis Assay User Guide: https://www.agilent.com/cs/library/usermanuals/public/5991-9329EN_pH_Xtra_User_Guide.pdf

5. Appendix

Tricks and tips

For easier analysis, open and pin multiple plate views to compare the layout with rate data. Use the multiple window button in the top-right corner of the Plate Matrix to do this. This enables to quickly reference plate maps or any combination of data outputs.

Mean V range adjustments

When using Mean V for rate calculation, choose a range as close to the linear portion of the data as possible (Figure 13).

Only choose time ranges after the initial temperature equilibration (if present, an initial decrease in signal) and before the signal plateaus.

If the tread line is off, adjust the time range for the calculation in the **Data Reduction** window under **Kinetic Analysis**, as shown in Figure 14.
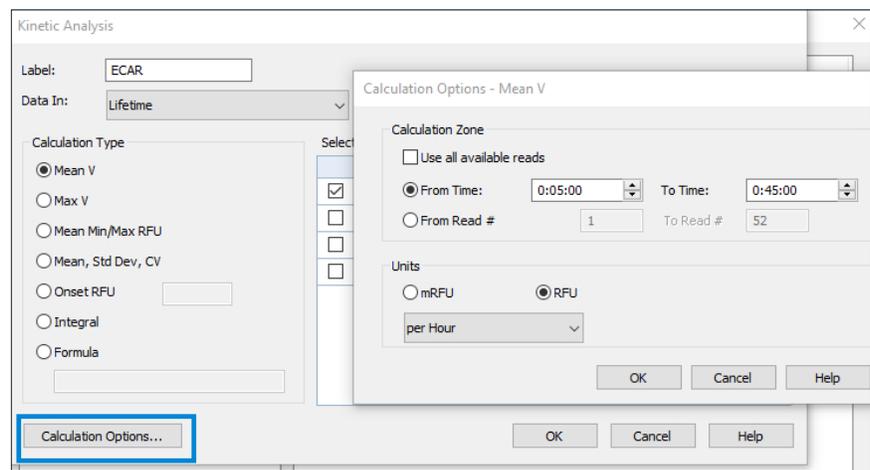

Figure 14. Amount of data points the Max V calculated over can influence the rate. Adjust points to ensure Max V follows the linear portion of the curve.
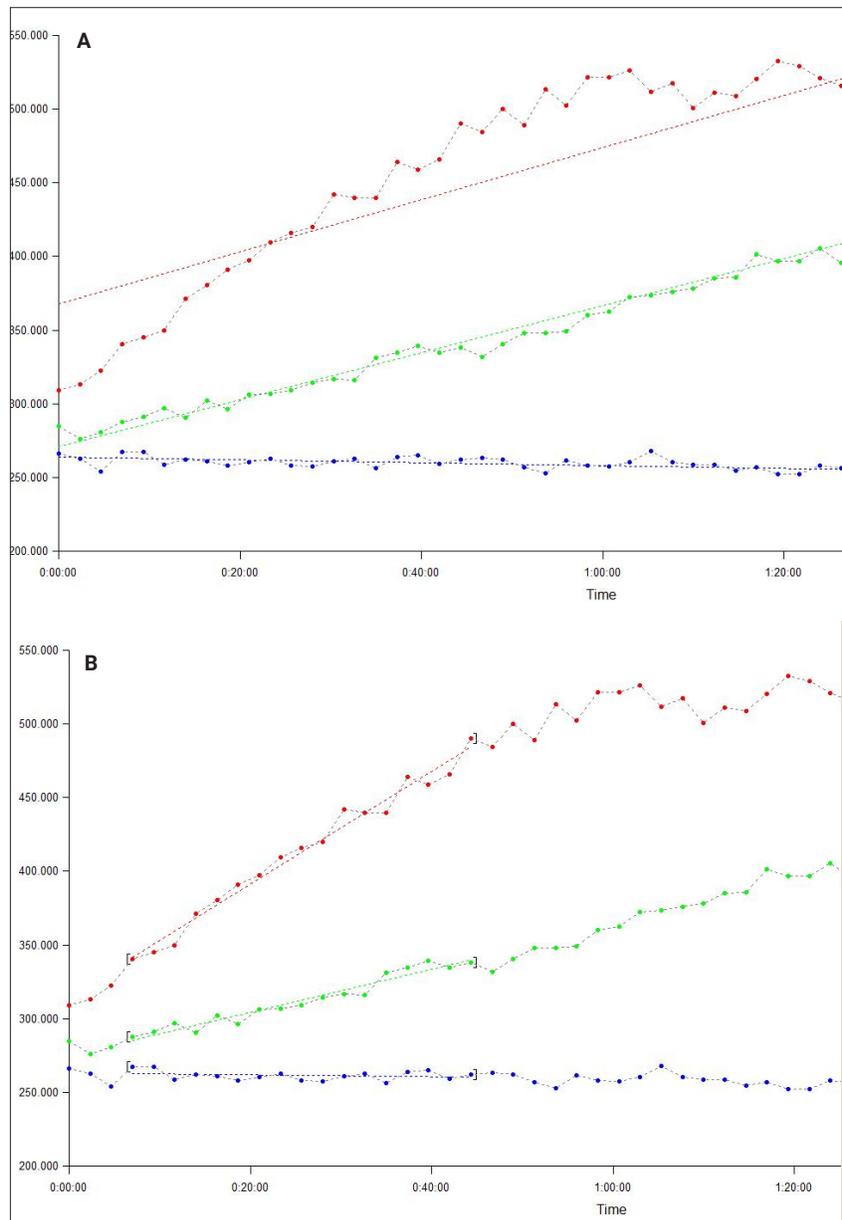
Figure 13. (A) Range of red graph not well aligned. Slope underestimated. (B) Range well aligned over linear portion of data.

Data reduction quick reference

Data Out	Description	Data In	Formula	Well
S:B	Transformation	DS1 DS2	A1: DS1.SC/DS1.BLK DS2.SC/DS2.BLK	A1 (Read 20) B1 (Read 20)
Blk100_30 Blk300_30	Blank Transformation	100_30:380/20,620/10 100_30:380/20,620/10	X-BKL	Use single formula for all wells
Lifetime	Transformation	DS1: Blk100_30 DS2: Blk300_30	200/(LN(DS1/DS2))	Use single formula for all wells
ECAR: Mean V [Lifetime]	Kinetic Analysis	Lifetime	Calculation type: Mean V Calculation options: From 5 to 45 minutes Units: RFU per Hour	
Lifetime (BG corr)	Transformation	ECAR: Mean V [Lifetime]	X-SC	Use single formula for all wells
pH	Transformation	Lifetime (BG corr)	(1687.2-x)/199.12	Use single formula for all wells
[H+]	Transformation	pH	POW(10;-X)	Use single formula for all wells
Cell Seed StdCurve	Standard curve	Well ID: STDA X-axis: Concentration/Dilutions Y-Axis: ECAR: Mean V [Lifetime]	Curve fit: Point-to-point Interpolations: Y value Formula: (1) 15; (2) 50	

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