

Version 2a Last updated 1 March 2023

ab222945

Glycolysis Stress Test Companion Assay

For the characterization of glycolytic stress in live cells when used in combination with Glycolysis Assay [Extracellular acidification] (ab197244).

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Glycolysis Stress Test Companion Assay (ab222945) allows glycolytic characterization at user-defined glucose concentrations in live cells when used in combination with our Glycolysis Assay [Extracellular Acidification] (ab197244).

The assay uses 2-Deoxyglucose (2-DG) and oligomycin to facilitate an in-depth analysis of cellular glycolytic flux: 2-DG blocks glycolysis through competitive hexokinase inhibition allowing quantification of non-glycolytic extracellular acidification (ECA); oligomycin inhibits ATP synthase, therefore preventing aerobic ATP generation and forcing cells to increase glycolytic flux to meet ATP demand. The levels of such compensatory glycolysis achievable under test conditions can reveal underlying glycolytic perturbation not evident under basal conditions and facilitates assessment of **ATP-demand limited glycolysis capability**. Glucose oxidase is included in the kit to provide a convenient cell-free ECA assay signal control.

Glycolysis is a critical ATP generating pathway in eukaryotic cells and plays a central role in numerous pathologies including cancer, diabetes and neurodegenerative disorders. The ability to conveniently characterize the glycolytic profile of *in vitro* cell models used in these disease areas and to determine how specific interventions such as nutrition deprivation, hypoxia or drug treatment impact the profile is therefore of considerable utility.

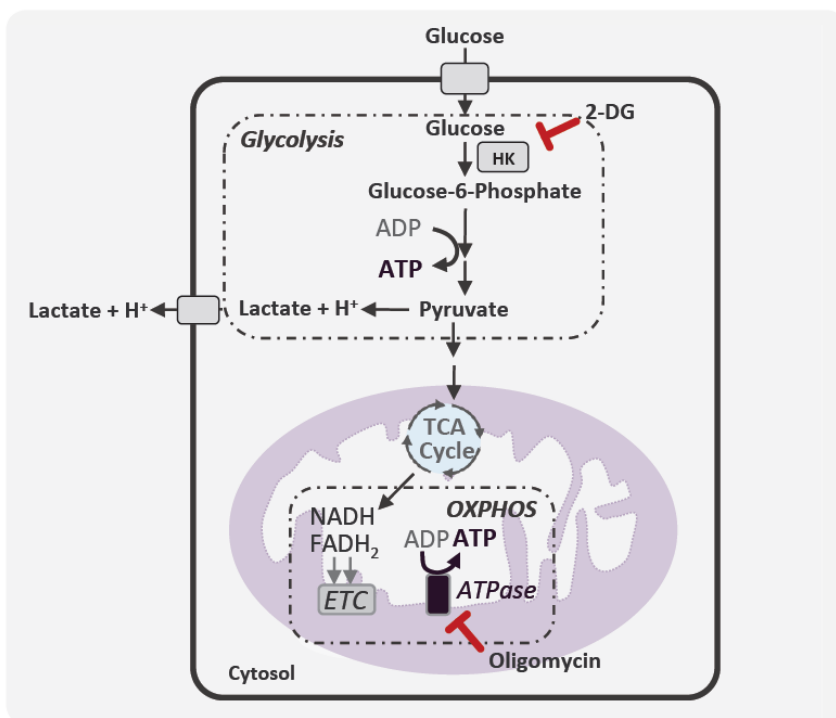


Figure 1. Schematic overview of glycolysis, oxidative phosphorylation (OXPHOS) and the specific targets of the inhibitors provided in the kit.

Glucose is taken up through specific transporters and subsequently converted to pyruvate through a series of reactions, yielding two equivalents of ATP per molecule of glucose use. Pyruvate is then reduced to lactate, eventually leading to the net production of a hydrogen ion (glycolysis). The hydrogen ions generated are exported from the cytoplasm to maintain intracellular pH, in turn, causing extracellular acidification. Pyruvate can also enter the TCA cycle, generating reducing equivalents that are used in the electron transport chain (ETC) to generate a proton gradient across the inner mitochondrial membrane, which in turn drives the generation of ATP catalyzed by F₁/F₀ ATP-synthase (OXPHOS).

2-Deoxyglucose (2-DG) is a competitive inhibitor of hexokinases (HK), and, by extension of, glucose-drive extracellular acidification. Oligomycin inhibits the proton flux through the F₁/F₀ ATP synthase, thereby inhibiting ATP generation via OXPHOS.

2. Protocol Summary

Day 1

Plate cells and return to culture overnight



Incubate Over Night

Day 2

Prepare Glucose-Free Respiration Buffer

Prepare Glycolysis Stress Test controls

Prepare Glycolysis Assay Reagent (ab197244)



Place cells in CO₂-free incubation for 2 hours

Wash cells twice with Glucose-free Respiration Buffer

Add Glucose-free and/or Glucose-containing Respiration Buffer
and Glycolysis Assay Reagent (ab197244)

Add control compounds at pre-optimized concentrations



Measure on fluorescence plate reader using recommended settings

Analyze kinetic data output to determine sample glycolytic
characterization

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at -20°C (Respiration Buffer tablet and glucose should be stored at RT) in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
2-Deoxyglucose (2-DG)	600 µL	-20°C	-20°C
Glucose (720 mg)	1 vial	RT	4°C
Glucose Oxidase (112.5 µg)	1 vial	-20°C	-20°C
Glucose-free Respiration Buffer	1 tablet	RT	4°C
Oligomycin (10.4 µg)	1 vial	-20°C	-20°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Glycolysis Assay [Extracellular acidification] (ab197244)
- Microplate reader capable of measuring fluorescence, with suitable filter and plate temperature control – see Instrument and Measurement Settings section on the Glycolysis Assay [Extracellular acidification] Assay (ab197244) protocol for suitable plate readers
- CO₂-free incubator
- Double distilled water (ddH₂O)
- DMSO
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- Sterile 96-well plate (black wall with clear flat bottom), or standard clear plates for cell culture
- Sterilizing filter 0.22 µm
- HCl and KOH – for adjusting pH
- Cell culture medium

8. Technical Hints

- This kit is sold based on number of tests. A “test” simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 2-Deoxyglucose (2-DG, 500 mM):

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2 Glucose (720 mg D-(+)-Glucose):

Reconstitute Glucose powder in 4 mL of ddH₂O to generate a 1 M Glucose stock solution. Filter sterilize using a 0.22 µm filter. Store at 4°C. Use Glucose stock solution within 3 weeks.

9.3 Glucose-free Respiration Buffer:

Dissolve provided table in 50 mL of ddH₂O. Warm to assay temperature (usually 37°C), adjust to pH 7.4 with HCl and/or KOH and filter sterilize using a 0.22 µm filter. Store at 4°C. Use buffer within 3 weeks.

9.4 Glucose Oxidase Signal Control (GOx, 112.5 µg):

Reconstitute Glucose Oxidase in 40 µL of ddH₂O to generate a 3 mg/mL GOx stock solution. Aliquot into three equal volumes (10 µL) and store at -20°C. Each aliquot is sufficient for 2 wells (5 µL GOx/well).

9.5 Oligomycin (10.4 µg):

Reconstitute oligomycin in 95 µL of DMSO to generate a 100 µM (100X) stock solution. Aliquot into three equal volumes (30 µL) and store at -20°C. Each aliquot is sufficient for 30 wells (1 µM final concentration).

10. Sample Preparation

- Prepare a cell titration experiment to identify a suitable cell density for a specific cell type and conditions.
- Some cell lines (such as some glioblastoma cell lines) require the addition of glutamine to achieve their full glycolytic activity. This can be determined experimentally by adding Glutamine to the Respiration Buffer (typical concentration: 2 mM).
- Cells are seeded at a density to achieve full confluence on the day of measurement. Plating density, cell type and basal metabolic rate will determine the acidification rate measured.
- If edge effects are observed, we recommend incubating the plate at RT for 30 minutes after plating before returning plate to cell culture. Alternatively, omit the outer wells of the plate.
- We recommend following the plate map suggested in figure 2 to facilitate assay procedure.

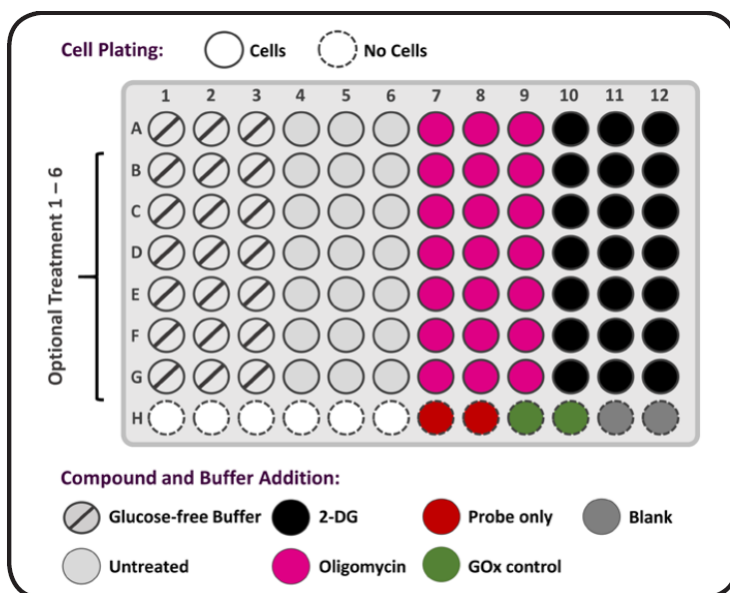


Figure 2. Recommended plate map for stress test assay with optional different treatments. Using this plate map, up to 7 different conditions can be tested simultaneously.

10.1 Cell plating:

10.1.1 Count cells and adjust to the desired plating density in culture medium – initial recommendation: $3-8 \times 10^4$ cells/well.

10.1.2 Return plate to incubator and culture overnight (~ 14 hours).

Δ Note: We recommend following plate map described in Figure 2 and leave row H with no cells.

Δ Note: If cells are cultured in CO₂ incubator overnight, you will need to place cells in a CO₂-free humidified incubator 2 hours prior to performing the assay (Step 11.2).

10.2 Compounds and assay controls set up:

General guidelines for optimal concentrations and treatment times for assay controls and test compounds:

- Oligomycin is typically used at 1 μM. However, the optimal concentration can be cell type dependent. Optimum concentrations can be determined by measuring ECA at varying oligomycin concentrations.
- Under standard measurement conditions, 50 mM 2-DG is usually sufficient to completely inhibit ECA caused by glycolysis. However, in some instances, higher concentrations may be necessary. Optimum concentrations can be determined by measuring ECA at varying 2-DG concentrations. Optional additional controls:
- Glucose Oxidase is used as positive signal control for the assay.
- Compounds are typically added immediately prior to measurement. Longer times can be used as required: in this instances, compound should be present in both culture media (during incubation) and measurement media.
- If using test compounds, dilute so that the final concentration does not exceed 0.5% (v/v) and that volume added to well does not exceed 5 μL.

11. Assay Procedure

- This assay is designed to be assay as companion kit together in combination with Glycolysis Assay [Extracellular acidification] (ab197244). The Glycolysis Assay User Manual describes instrument set-up, assay optimization, data analysis and troubleshooting. The described instrument set-up and signal optimization steps should be performed prior running a Glycolysis Stress assay.
- We recommend that you assay all controls and samples in triplicate.
- Prepare all reagents and samples as directed in the previous sections.
- Use a plate block heater for plate preparation and pre-warm plate reader to measurement temperature (typically 37°C). Ensure Respiration buffers are at the appropriate pH and pre-warmed at assay temperature (typically 37°C).

11.1 Prepare additional reagents:

- 11.1.1 Oligomycin 10X working stock (1 μ M end concentration): dilute 100X Oligomycin stock 1:10 in Respiration Buffer.
- 11.1.2 Glucose-containing Respiration Buffer: add Glucose Solution (Step 9.2) to Glucose-free Respiration Buffer to obtain desired glucose concentration (typically 5-20 mM). Buffer can be stored at 4°C for up to 3 weeks.

Δ Note: The user can define glucose concentration in the media to perform measurements at physiologically relevant glucose concentrations. Additionally, dependence of glucose concentration of glycolytic rate can be determined by measuring the ECA in the presence of oligomycin (ATP-limited glycolytic capacity) across a range of glucose concentrations.

- 11.1.3 Glycolysis Assay Reagent: prepare reagent as described in Glycolysis Assay [Extracellular acidification] (ab197244) user manual.

11.2 Wash cells:

Δ Note: Place cells in a CO₂-free humidified incubator 2 hours prior to performing the assay if cells were cultured in a CO₂ incubator overnight.

Δ Note: We recommend following plate map described in Figure 2 and leave row H with no cells.

11.2.1 Place the plate on a plate block heater set to assay temperature (typically 37°C) and remove spent culture media with an aspirator (be careful not to dislodge cells from the base of the wells).

11.2.2 With a multichannel or repeater pipette, add 100 µL of the pre-warmed Glucose-Free Respiration Buffer to each well.

11.2.3 Repeat wash step one more time.

11.3 Add respiration buffer to wells:

- Glucose-free control (wells A-H 1-3) = 90 µL pre-warmed Glucose-free Respiration Buffer
- Untreated sample (wells A-H 4-6) = 90 µL pre-warmed Glucose-containing Respiration Buffer
- ATP-demand limited glycolysis (wells A-G 7-9) = 90 µL pre-warmed Glucose-containing Respiration Buffer
- Non-Glycolytic ECA (wells A-G 10-12) = 90 µL pre-warmed Glucose -containing Respiration Buffer
- Probe only (wells H7 and H8) = 90 µL pre-warmed Glucose -containing Respiration Buffer
- GOx Signal control (wells H9 and H10) = 90 µL pre-warmed Glucose -containing Respiration Buffer
- Blank control (wells H11 and H12) = 90 µL pre-warmed Glucose -containing Respiration Buffer

11.3.1 Add 10 µL of Glycolysis Assay Reagent (Step 11.1.2) to each well except to Blank Controls.

11.3.2 Add 10 µL of Glucose-containing Respiration Buffer to Blank Control wells (H11 and H12).

Δ Note: If measuring a full 96-well plate, we recommend diluting reconstituted ab197244 stock 1 in 10 in the relevant measurement media and, using a multichannel pipette, to add 100 µL to each well. Add 100 µL of Glucose-containing Respiration Buffer (no ab197244 reagent) to the Blank Control wells.

11.4 Treat cells:

Add relevant treatment or compounds to cells (see Section 12 for more detailed information on how to perform assays).

- 11.4.1 Add test compound or vehicle (typically 1-5 μ L) to test wells (rows B-H, labeled "Optional Treatment 1-6" in Figure 2).
- 11.4.2 Add 10 μ L oligomycin 10X Working Stock (Step 11.1.1) to wells to measure ATP-demand limited glycolysis (wells A-H 7-9).
- 11.4.3 Add 10 μ L 2-DG Working Stock (Step 9.1) to wells to measure on-glycolytic ECA (wells A-H 10-12).
- 11.4.4 Add 10 μ L Glucose-free Respiration Buffer to Glucose-free control (wells A-H 1-3) and Probe only (wells H7 and H8).
- 11.4.5 Add 5 μ L GOx + 5 μ L Glucose Solution (Step 9.2) to GOx Signal Control (wells H9 and H10).

11.5 Measurement:

- 11.5.1 Read plate immediately in a fluorescence microplate reader as described in the user manual instructions for Glycolysis Assay (ab197244).

11.6 Calculations:

- 11.6.1 Process data as described in Section 14 of the user manual instructions for Glycolysis Assay (ab197244).

12. Typical Assays/Data

12.1 Sample Glycolytic Characterization

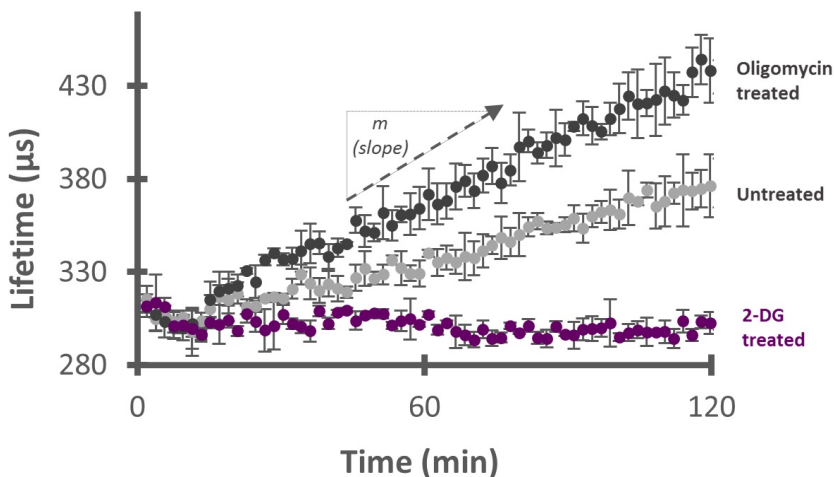


Figure 3. Extracellular acidification of A549 cells treated with 2-DG (purple) and oligomycin (black).

Basal Glycolysis: untreated cells (gray) show a steady increase in extracellular acidification caused by the export of glycolysis-derived protons. Basal glycolysis under given measurement conditions is dependent on factors such as glucose availability, demand for ATP and glycolytic intermediates, and the balance between oxidative phosphorylation and glycolytic ATP production.

Glycolytic Capacity: oligomycin treatment (black) inhibits F_1/F_0 ATPase, thereby preventing oxidative ATP generation and forcing the cells to compensate by increasing glycolytic flux to meet cellular ATP demand. The level of **Compensatory Glycolysis** can be determined by subtracting the Basal rate from the oligomycin-treated plate (Figure 4). This increase will be dependent on cellular ATP demand and the maximal glycolytic capacity under measurement conditions.

In instances where the ATP demand meets or exceeds the capacity of the cell to generate ATP glycolytically, the oligomycin-treated rate is reflective of ***Maximal Glycolytic Capacity***. In instances where ATP demand does not exceed the capacity of the cell to generate ATP glycolytically, oligomycin-treated rate reflects ***ATP-demand limited glycolysis*** and informs of cellular response to the treatment condition.

Non-glycolytic ECA: 2-DG treatment (purple) inhibits glycolysis through competitive hexokinase inhibition allowing the measurement of non-glycolytic ECA; the acidification that is independent of transformation of glucose to lactate (eg, degradation of glycogen).

After conversion of the fluorescence lifetime signal into acidification rate (refer to Glycolysis Assay (ab197244) user manual for details), the calculated slopes (m , measure for acidification rates) can be used to determine the rate of non-glycolytic ECA (2-DG treated), basal glycolysis, ATP-demand limited glycolysis and the compensatory glycolysis (Figure 4).

Non-glycolytic ECA = m_{2-DG}

Basal Glycolysis = $m_{untreated} - m_{2-DG}$

ATP-demand limited glycolysis = $m_{Oligomycin}$

Compensatory Glycolysis = $m_{oligomycin} - m_{untreated}$

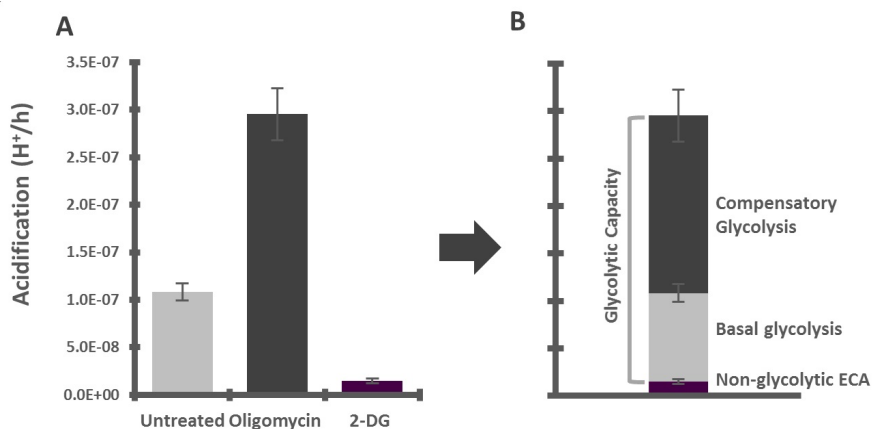


Figure 4. Glycolytic characterization of A549 cells.

12.2 Increasing ATP demand

As described in the previous section, the glycolytic rate observed upon oligomycin treatment is dependent on ATP demand and maximal glycolytic capacity under measurement conditions. To determine if oligomycin-treated glycolytic capacity is ATP-demand limited, cells can be pharmacologically challenged to increase ATP demand. In instances where increase ATP demand causes no further increase in measured ECA, this oligomycin-treated rate can be termed **Maximal Glycolytic Capacity**. Increased ECA in response to increased ATP demand illustrates that oligomycin-treated rates are ATP-limited.

Increased ATP demand can be induced by treatment with the ionophore monensin, which facilitates the electroneutral passage of monovalent cations across lipid membranes, thereby increasing the activity of the Na^+/K^+ -ATPase and by extension increasing ATP demand. Where cells have the capacity, compensatory glycolysis will increase to meet the increased ATP demand and additional detail on the glycolytic capacity of the cells under study can be obtained.

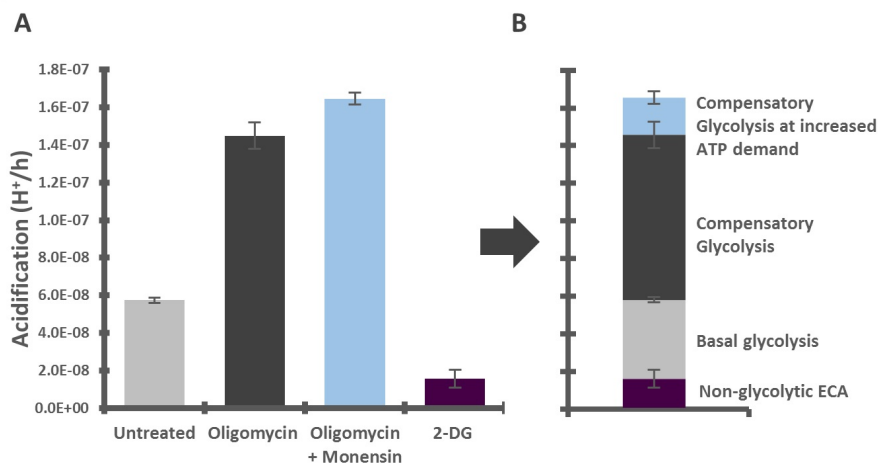


Figure 5. Increased compensatory glycolysis upon increased ATP demand in monensin-treated HepG2 cells.

13.FAQs

Q. Can I use other buffer than the Respiration Buffer provided in the kit?

A. The Respiration Buffer provided has been optimized for the assay. It contains 1 mM Potassium phosphate, 70 mM NaCl, 50 mM KCl, 0.8 mM MgSO₄, 2.4 mM CaCl₂.

Alternative media such as unbuffered DMEM can also be used for the assay, as long as care is taken to ensure a minimal buffering capacity.

14. Notes

Technical Support

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