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ab197244 Glycolysis Assay [Extracellular acidification]

For the measurement of extracellular acidification [ECA/ECAR] in cell populations and 3D culture models.

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

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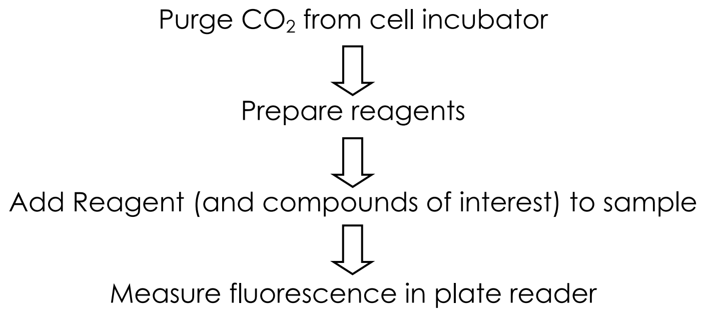
1. Overview

Glycolysis Assay [Extracellular Acidification] (ab197244) is an easy mix-and-measure, 96 or 384 well fluorescence plate reader-based approach for the analysis of extracellular acidification (ECA/ECAR). As lactate production is the main contributor to this acidification, Glycolysis Assay (ab197244) is a convenient and informative measuring tool of cellular glycolytic flux. Such measurements offer an important insight into the central role played by altered glycolytic activity in a wide array of physiological and pathophysiological processes, including cellular adaptation to hypoxia and ischemia, and the development and progression of tumorigenesis.

The pH-sensitive reagent is chemically stable and inert, water-soluble and cell impermeable. It exhibits a positive signal response (increased signal with increased acidification) across the biological range (pH 6 – 7.5). This performance, coupled with its spectral characteristics, make this kit the ideal choice for flexible, high-throughput assessment of extracellular acidification, overcoming many of the problems associated with the more cumbersome potentiometric pH approach. Rates of extracellular acidification are calculated from changes in fluorescence signal over time and, as the measurement is fully reversible, measurement of time courses and multiple drug treatments are possible.

The flexible plate reader format, allows multiparametric or multiplex combination with other products within this range. For example, this product in combination with the Extracellular O2 Consumption Assay (ab197243) or the Extracellular O2 probe (ab197242) allows the simultaneous real-time measurement of mitochondrial respiration and glycolysis and analysis of the metabolic phenotype of cells and the shift (flux) between the two pathways under pathological states.

2. Protocol Summary



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 Reagent at 4°C and Buffer tablet at RT in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

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.

Δ Note: Reconstituted reagent is stable for 1 month.

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 condition (before prep)	Storage condition (after prep)
	96 tests	4 x 96 tests		
Glycolysis Assay Reagent (lyophilized)	1 vial	4 x 1 vial	4°C	-20°C
Respiration Buffer	1 tablet	4 x 1 tablet	RT*	4°C

*Store Respiration Buffer tablet at RT in dry/dark place (desiccant recommended).

7. Materials Required, Not Supplied

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

- Microplate reader capable of measuring fluorescence, with suitable filter and plate temperature control – see Table 1 in Instrument and Measurement Settings section for suitable plate readers
- Double distilled water (ddH_2O)
- 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
- CO_2-free incubator

  For cells:
- 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.
- Refer to Instrument and Measurement Settings table (Table 1) for recommended settings for your plate reader.
- While compatible with all plate types, black border clear bottom plats give optimal signal-to-noise ratios.
- For first time users, we recommend performing a Signal Optimization Step (see Section 11).

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 Respiration Buffer:

Reconstitute Respiration Buffer tablet in 50 mL of ddH_2O. Warm to assay temperature (usually 37°C), adjust to pH 7.4 and filter sterilize using a 0.22 µm filter. Store at 4°C in the dark.

9.2 Glycolysis Assay Reagent (lyophilized):

Reconstitute Assay Reagent in 1 mL of Respiration Buffer by gently aspirating and mixing 3-4 times.

Aliquot reagent so that you have enough volume to perform the desired number of assays. Store at -20°C in the dark.

Avoid freeze/thaw. Reconstituted reagent is stable for one month.

10. Plate Reader Set-Up

10.1 Measurement Parameters

The Glycolysis Assay Reagent is a chemically stable and inert, water-soluble, cell impermeable pH-sensitive fluorophore.

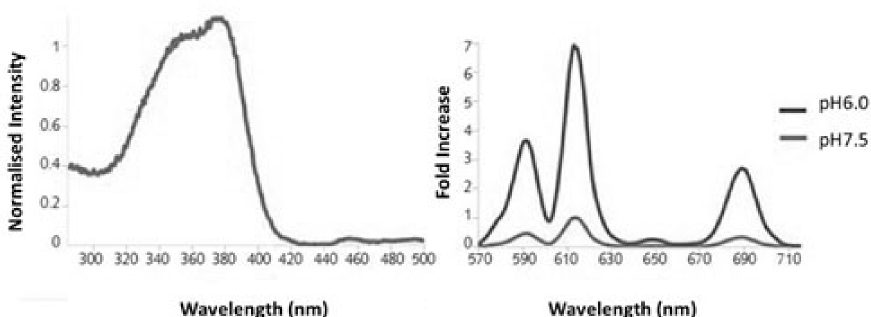


Figure 1. Excitation and emission spectra of the Glycolysis Assay Reagent. Left panel shows normalized excitation (Ex = 340-410 nm; Peak 360-380 nm). Right panel shows emission maxima fold increase (Em = 590, 615 and 690 nm) between pH 6.0 and pH 7.5.

– We strongly recommend using only fluorescence plate readers equipped with temperature control.

10.2 Fluorescence measurements

Outlined below are two fluorescence modalities that can be used with this assay, depending on the plate reader type and instrument setup.

10.2.1 Standard: TR-F Measurement

Time-resolved fluorescence (TR-F) measurement can be done in a wide range of commonly available plate readers (although users may see better performance using filter-based plate readers). Optimal delay time is ~100 µs and gate (integration) time is 100 µs.

Δ **Note:** Glycolysis reagent should return Signal to Blank ratio (S:B) ≥ 3.

10.2.2 Advanced: Dual-Read TR-F (Lifetime calculation)

Optimal performance can be achieved using dual-read TR-F in combination with subsequent ratiometric Lifetime calculation, to maximize dynamic range and to express ECA as a function of [H^+].

Δ **Note:** Glycolysis reagent should return Signal to Blank ratio (S:B) ≥ 10.

Dual-read TR-F and subsequent Lifetime calculation allows measurement of the rate of fluorescence decay of the Extracellular consumption reagent, and can provide measurements of oxygen consumption that are more stable and with a wider dynamic range than measuring signal intensity.

Optimal dual-delay and gate (integration) times:

- Integration window 1: 100 µs delay (D1), 30 µs measurement time (W1)
- Integration window 2: 300 µs delay (D2), 30 µs measurement time (W2)

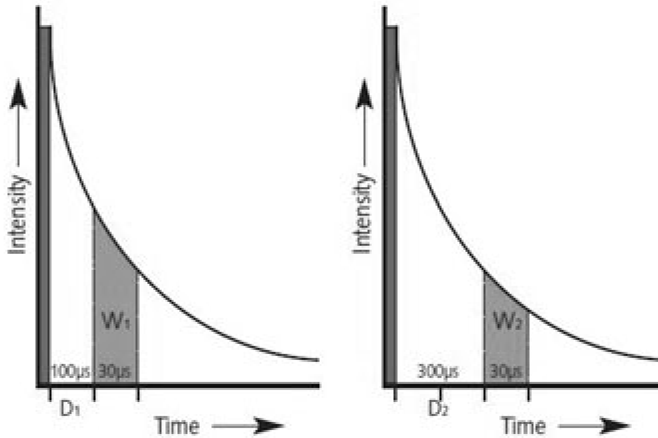


Figure 2. Illustrating dual read TR-F measurement.

Use the dual intensity readings to calculate the corresponding Lifetime (µs) using the following transformation:

Lifetime (µs) [T] = (D2-D1)/ln(W1/W2)

Where W1 and W2 represent the two (dual) measurement windows and D1 and D2 represent the delay time prior to measurement of W1 and W2 respectively. This provides Lifetime values in µs at each measured time point for each individual sample (Figure 2).

Δ Note: S:B for Integration window 2 is recommended to be ≥ 10 to allow accurate Lifetime calculation. Range of Lifetime values should be ~200 µs for cells assayed in respiration buffer at approx. pH 7.4, increasing up to > 400 µs upon acidification, and should only be calculated from samples containing reagent. Lifetime values should not be calculated from blank wells.

10.3 Instrument and Measurement Settings

See Instrument and Measurement Setting table below for instrument-specific setting and filters. Readers equipped with a TR-F mode, may achieve improved performance using delay and gate time of 30 µs and 100 µs.

Table 1. Recommended Instrument and Measurement Settings

Instrument	Optical Configuration	Intg1 (D_1/W_1) Intg2 (D_2/W_2)	Optimum Mode	Ex (nm) Em (nm)
BioTek: Synergy Neo, H4, 2 Cytation 3/5	Filter-based Top or bottom read	100 / 30µs 300 / 30µs	Dual-read TR-F (Lifetime)	Ex 360±40nm Em 620±10nm
BMG Labtech: CLARIOstar	Filter-based Top or bottom read	100 / 30µs 300 / 30µs	Dual-read TR-F (Lifetime)	Ex 340 ± 50nm (TR-EX L) Em 615 ± 10nm (BP-615)
BMG Labtech: FLUOstar Omega / POLARstar Omega	Filter-based Top or bottom read	100 / 30µs 300 / 30µs	Dual-read TR-F (Lifetime)	Ex 340 ± 50nm (TR-EXL) Em 615 ± 10nm (BP-615)
BMG Labtech: PHERAstar FS	Filter-based Top read (HTRF Module)	100 / 30µs 300 / 30µs	Dual-read TR-F (Lifetime)	Ex 337 nm Em 620 nm
Perkin Elmer: VICTOR series/ X4, X5	Filter-based Top read	100 / 30µs 300 / 30µs	Dual-read TR-F (Lifetime)	Ex 340 ± 40nm (D340) Em 615 ± 8.5nm (D615)

Instrument	Optical Configuration	Intg1 (D_1/W_1) Intg2 (D_2/W_2)	Optimum Mode	Ex (nm) Em (nm)
Perkin Elmer: EnVision	Filter-based Top read	100 / 50µs 300 / 50µs	Dual-read TR-F (Lifetime)	Ex 340 ±60nm (X340) Em 615 ± 8.5nm (M615)
Tecan: Infinite M1000 Pro/ F200 Pro	Monochromator Filter-based Top or bottom read	100 / 100µs 300 / 30 µs	Dual-read TR-F (Lifetime)	Ex 380±20nm Em 615±10nm
BMG Labtech: FLUOstar Optima / POLARstar Optima	Filter-based Top or bottom read	100 / 100µs n/a	TR-F	Ex 340 ± 50nm (TR-EXL) Em 615 ± 10nm (BP-615)
Perkin Elmer: EnSpire	Monochromator Top or bottom read	100 / 100µs n/a	TR-F	Ex 380 nm Em 615 nm
Tecan: Infinite M200 Pro/ Saffire / Genios Pro	Monochromator Filter-based / Top or bottom read	100 / 100µs n/a	TR-F	Ex 380±20nm Em 615±10nm
Mol. Devices: SpectraMax / Flexstation / Gemini	Monochromator based Top or bottom read	50 /250µs n/a	TR-F	Ex 380nm Em 615nm

11. Signal Optimization

– This step is recommended for first time users.
– Use a plate block heater for plate preparation and pre-warm plate reader to measurement temperature.

11.1 Signal Optimization:

11.1.1 Prepare 8 replicate wells of a 96-well plate, by adding 90 µL pre-warmed Respiration Buffer to each well (A1-A4, B1-B4).
11.1.2 Add 10 µL reconstituted Glycolysis Assay Reagent to 4 of the replicate wells (A1-A4) and 10 µL ddH_2O, PBS or media to the remaining replicates wells (B1-B4).
11.1.3 Read plate immediately in a fluorescence plate reader over 30 minutes (read every 2-3 minutes).
11.1.4 Examine Signal Control well (A1-A4) and Blank Control well (B1-B4) readings (linear phase) and calculate Signal to Blank (S:B) ratio.

Δ Note: For dual read TR-F, calculate S:B for each measurement window.

	1	2	3	4
A	Respiration Buffer + Glycolysis Reagent	Respiration Buffer + Glycolysis Reagent	Respiration Buffer + Glycolysis Reagent	Respiration Buffer + Glycolysis Reagent
B	Respiration Buffer	Respiration Buffer	Respiration Buffer	Respiration Buffer

12. Assay Procedure

- We recommend that you assay all controls and samples in duplicate.
- Prepare all controls 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).
- The amount of signal change will be directly dependent on the rate of glycolytic flux of the cell type being measured. We recommend using a medium – to – high cell density per well as a starting point, and reducing cell numbers as required.
- Prepare test compounds for sample treatment as desired. Example of typical compounds that can be used as assay control are shown in the table below.

Typical control	Stock concentration
Glucose Oxidase (positive signal control)	1 mg/mL in ddH_2O
FCCP (ETC uncoupler) (positive control, increases ECA)	Titration recommended to establish best concentration
Oxamic acid (negative control, decreases ECA)	750 mM in ddH_2O

12.1 Pre-assay preparation – Adherent cells:

12.1.1 Seed cells in a 96-well plate at a density of 3 – 8 x 10^4 cells/well in 200 µL culture medium.

12.1.2 Incubate overnight in a CO_2 incubator at 37°C.

Δ Note: Prepare a cell titration experiment to identify a suitable cell density for a specific cell type and conditions.

Where cells are cultured in CO_2 incubator overnight, it is important to purge the media and plasticware of CO_2 prior to conducting the assay as residual CO_2 may contribute to acidification. Perform a CO_2 purge by incubating cells in a CO_2-free incubator at 37°C with 95% humidity, approx. 3 hours prior to performing the Glycolysis assay measurement.

12.2 Pre-assay preparation – Suspension cells:

12.2.1 Harvest suspension cells.

12.2.2 Wash cells in Respiration Buffer.

12.2.3 Seed cells in a 96-well plate at a density of 2.5 – 5 x 10^5 cells/well in 150 µL Respiration Buffer.

12.2.4 Proceed to plate relevant controls (Step 12.3.4).

12.3 Plate loading:

12.3.1 Remove spent culture media from all assay wells and wash cells with 100 µL of Respiration Buffer.

12.3.2 Repeat washing step.

12.3.3 Add 150 µL of Respiration Buffer to all wells containing cells.

12.3.4 Blank controls (we suggest using wells H11 and H12): add 150 µL of Respiration Buffer.

12.3.5 Optional positive control: add 10 µL of glucose oxidase solution (1 mg/mL in ddH_2O) to wells containing 150 µL Respiration Buffer only.

12.3.6 Optional negative control: add 10 µL of oxamic acid solution (750 mM in ddH_2O) to wells containing cells.

12.4 Assay set up:

12.4.1 Add 10 µL reconstituted Glycolysis Assay Reagent to each sample well and positive/negative control wells.

12.4.2 Add 10 µL of Respiration Buffer to blank control wells.

12.4.3 Add 1 – 10 µL test compound (vehicle control and/or stock) to the wells.

Δ Note: We recommend keeping the volume of added compound low to minimize any potential effects of solvent vehicle.

Δ Note: Plate preparation time should be kept to a minimum.

12.5 Measurement:

12.5.1 Insert the prepared plate into a fluorescence plate reader pre-set to the measurement temperature (typically 37°C).

12.5.2 Measure Glycolysis assay signal at 1.5 min intervals for ≥ 120 minutes using excitation and emission wavelengths of Ex/Em = 380/615 nm respectively (see Table 1 for Instrument and Measurement Setting table for instrument specific information).

13. Assay Procedure for 384 well plate

This kit provides enough reagent to perform 200 tests in 384-wp format (half plate).
Follow the same recommendations outlined in Section 12.

13.1 Pre-assay preparation – Adherent cells:

13.1.1 Seed cells in a 384-wp at a density of 2 – 4 x 10^4 cells/well in 100 µL culture medium.
13.1.2 Incubate overnight in a CO_2 incubator at 37°C.

Δ **Note:** Prepare a cell titration experiment to identify a suitable cell density for a specific cell type and conditions.

Δ **Note:** Where cells are cultured in CO_2 incubator overnight, it is important to purge the media and plasticware of CO_2 prior to conducting the assay as residual CO_2 may contribute to acidification. Perform a CO_2 purge by incubating cells in a CO_2-free incubator at 37°C with 95% humidity, approx. 3 hours prior to performing the Glycolysis assay measurement.

13.2 Pre-assay preparation – Suspension cells:

13.2.1 Harvest suspension cells.
13.2.2 Wash cells in Respiration Buffer.
13.2.3 Seed cells in a 384-wp at a density of 1.2 – 2.5 x 10^5 cells/well in 75 µL Respiration Buffer.
13.2.4 Proceed to plate relevant controls (Step 13.3.4).

13.3 Plate loading:

13.3.1 Remove spent culture media from all assay wells and wash cells with 50 µL of Respiration Buffer.
13.3.2 Repeat washing step.
13.3.3 Add 75 µL of Respiration Buffer to all wells containing cells.
13.3.4 Blank controls (we suggest using wells H11 and H12): add 75 µL of Respiration Buffer.
13.3.5 Optional positive control: add 5 µL of glucose oxidase solution (1 mg/mL in ddH_2O) to wells containing 75 µL Respiration Buffer only.
13.3.6 Optional negative control: add 5 µL of oxamic acid solution (750 mM in ddH_2O) to wells containing cells.

13.4 Assay set up:

13.4.1 Add 5 µL reconstituted Glycolysis Assay Reagent to each sample well and positive/negative control wells.

13.4.2 Add 5 µL of Respiration Buffer to blank control wells.

13.4.3 Add 1 – 5 µL test compound (vehicle control and/or stock) to the wells.

Δ **Note:** we recommend keeping the volume of added compound low to minimize any potential effects of solvent vehicle.

Δ **Note:** plate preparation time should be kept to a minimum.

13.5 Measurement:

13.5.1 Insert the prepared plate into a fluorescence plate reader pre-set to the measurement temperature (typically 37°C).

Measure Glycolysis assay signal at 1.5 min intervals for ≥ 120 minutes using excitation and emission wavelengths of Ex/Em = 380/615 nm respectively (see Table 1 for Instrument and Measurement Setting table for instrument specific information).

14. Calculations

- Plot the Blank control well-corrected Glycolysis Assay Intensity or Lifetime values versus Time (min).
- Select the linear proportion of the signal profile (avoiding any initial lag of subsequent plateau) and apply linear regression to determine the slope (ECA) and correlation coefficient for each well.

Δ **Note:** this approach is preferable to calculating a slope from averaged profiles.

- Tabulate the slope values for each test sample, calculating appropriate average and standard deviation values across replicate wells. If optional Signal Control wells are included, the slope obtained for the Signal Control (sample without cells) should be subtracted from all test values.

15. Typical data

Data provided for **demonstration purposes** only.

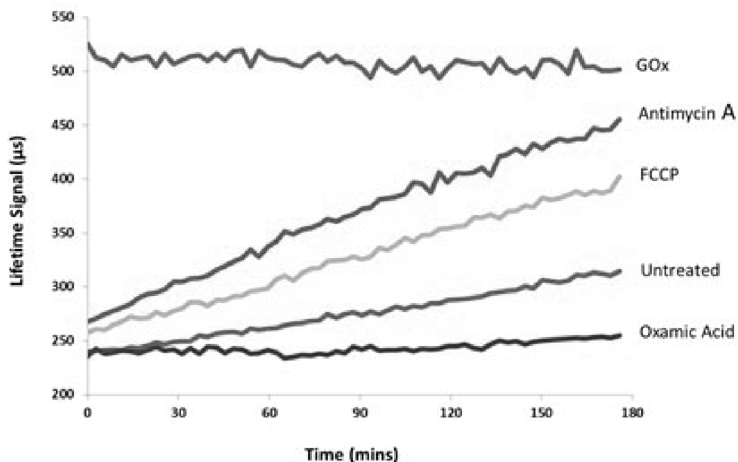


Figure 3. Typical Lifetime profile of Glycolysis Assay for adherent cells (HepG2), treated with modulator compounds, including oxamic acid (recommended negative control as inhibits conversion of pyruvate to lactate). Effect of glucose oxidase (GOx) as positive signal control is illustrated schematically.

16. Assay Throughput and Performance

Figure 4A illustrates a typical read out of showing the parallel analysis of 96 individual samples. A serial dilution of HepG2 is presented in Figure 4B, with increasing numbers cell numbers causing an increased rate of acidification. This is seen as an increased rate of signal change.

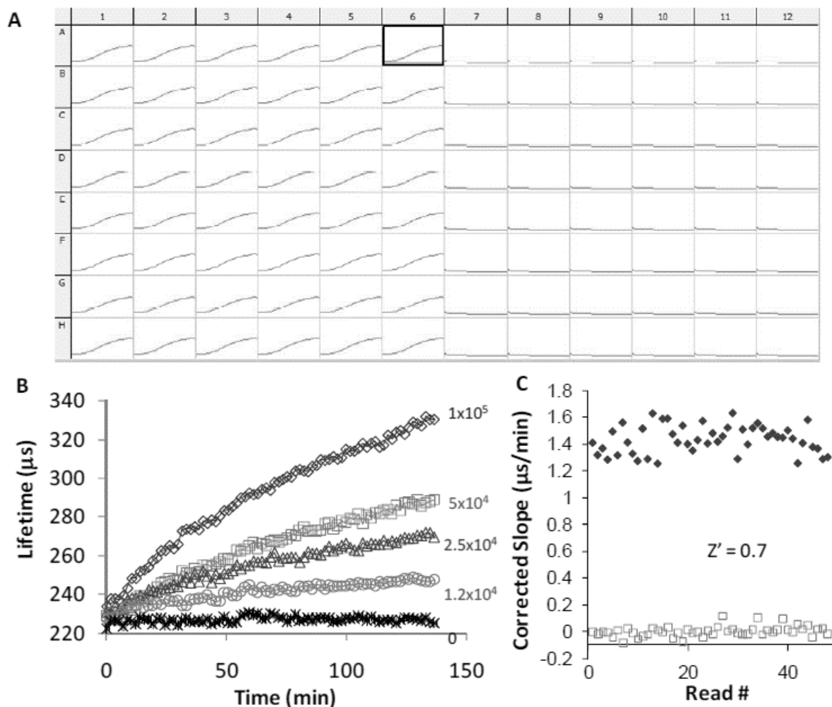


Figure 4. Analysis of cellular respiration of HepG2 cells. (A) 96-wp based analysis of extracellular acidification (cell profiles on left, control prolifes on right). (B) Acidification profiles for HepG2 cultured in L15 medium at the indicated seeding concentration. (C) Z' factor analysis for HepG2 plated in L15 medium at ~ 10^5 cells/well. Data generated on FLUOstar Omega (A) and on a Victor X4, PerkinElmer (B & C).

The performance of the assay is highlighted in Figure 4C, with a coefficient of variance (%CV) below 5%. Z' factor analysis assesses assay performance in terms of signal window and measurement reproducibility and shows excellent performance (Z' = ~0.7).

17. Additional Assays/Data

17.1 Titration of cell density

To determine an optimal cell seeding density for performing this assay with new cell types, seed cells (in replicate) with a range of seeding densities (typically 0, 10^4, 2 x 10^4, 4 x 10^4, 6 x 10^4, 8 x 10^4 cells/well).

Plot the date generated as a function of intensity or Lifetime values versus time, as illustrated in Figure 4.

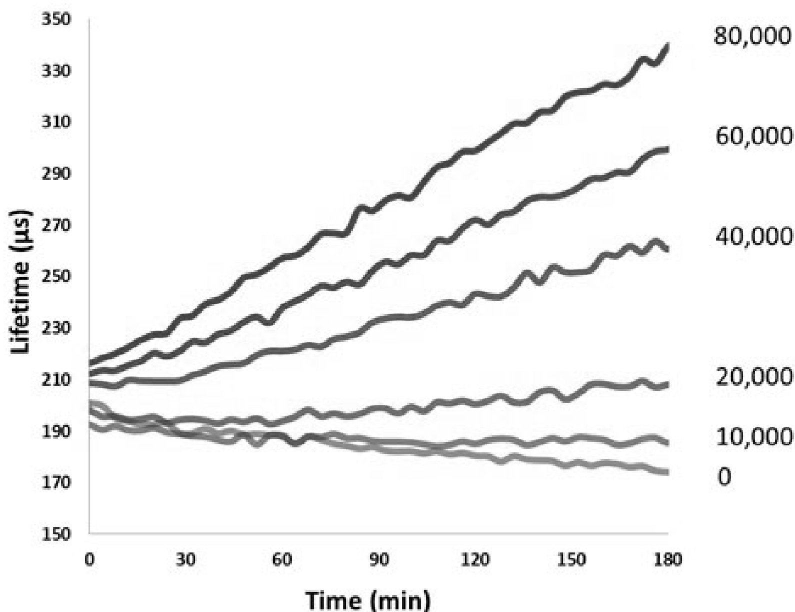


Figure 5. Extracellular acidification rate (ECA) profiles are shown for A549 cells seeded at 0, 10^4, 2 x 10^4, 4 x 10^4, 6 x 10^4, 8 x 10^4 cells/well. In this experimental example, a seeding density of 40,000 cells/well was chosen for further study as it provided a suitable between ECA response and cell availability.

17.2 Cellular energy flux analysis

Multiparametric (or multiplex) combination of Glycolysis Assay together with Extracellular O_2 Consumption Assay (ab197243) allows the simultaneous real-time measurement of mitochondrial respiration and glycolysis, leading to the analysis of the metabolic phenotype of cells and the shift (flux) between the two pathways under pathological states.

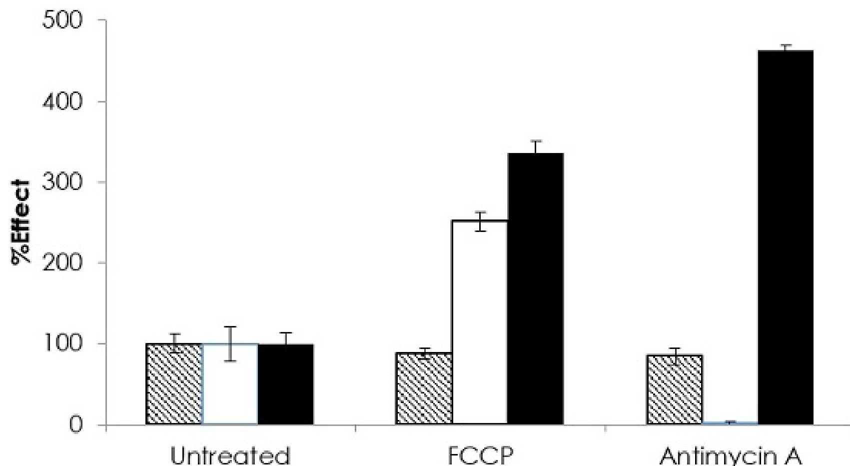


Figure 6. Cellular Energy Flux for HepG2 cells (seeded at 65,000 per well), treated with a combination of drug compounds modulating the ETC (Antimycin A [1 µM] and FCCP [2.5 µM]), shown as a percentage relative to untreated control cells. Comparative measurements were taken with Extracellular Oxygen Consumption Assay (ab197243) (white column) and Glycolysis Assay [Extracellular acidification] (ab197244) (black column) show the shift between mitochondrial respiration and glycolysis and the cellular control of energy (ATP; measured 1h post-treatment using Luminescent ATP Detection Assay kit (ab113849) (striped column)).

18. FAQ

Q. What can I do if Signal to Blank (S:B) ratio is not as high as expected?

A. Glycolysis Reagent should return a S:B ratio ≥3.

The following options may be helpful to improve S:B if the ratio is not as high as expected:

- Increase Gain (PMT) setting or flash energy
- Adjust TR-F focal height
- Increase length of integration time, the same for both delay windows.
- Repeat as top or bottom-read, respectively.
- Increase volume of Glycolysis Reagent (15µl)
- Contact Instrument Supplier for further options

Q. What do I do if I cannot detect any signal in wells containing cells and Glycolysis Reagent (or I can detect a signal but the slope rate appears very low)?

A. There are few things you should check:

- Check you have the correct instrument settings.
- Perform Signal Optimization.
- Include GOx control (max signal).
- Increase cell density.
- Check pH of pre-warmed Respiration Buffer and correct as necessary, as pH can drift over time.

Q. What do I do if I can detect a signal in wells containing cells and Glycolysis Reagent, but the slope (rate) falls initially or is variable from well to well?

A. There are few things you should check:

- Check cell seeding and pipetting consistency.
- Increase cell density.
- Ensure plate, instrument and all culture media and stock solutions are pre-warmed at 37°C prior to use.
- Reduce plate preparation times.

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

A. The Respiration Buffer provided in the kit has been optimized for the assay. It contains 1 mM Potassium Phosphate, 20 mM Glucose, 70 mM NaCl, 50 mM KCl, 0.8 mM $MgSO_4$, 2.4 mM $CaCl_2$.

Alternative media and supplements may be used as required (such as unbuffered DMEM), as long as care is taken to ensure a minimal buffering capacity.

19. Notes

Technical Support

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www.abcam.co.jp/contactus (Japan)