pH-Xtra™ Glycolysis Cell-Based Assay Kit

Item No. 601230

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Customer Service 800.364.9897
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1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage
601231	Glycolysis Respiration Buffer	1 vial/1 ea	-20°C
601232	pH-Xtra™ Assay Reagent	1 vial/1 ea	-20°C
601233	2-Deoxyglucose Assay Reagent	1 vial/200 µl	-20°C
701174	Oligomycin Assay Reagent	1 vial/100 µl	-20°C
600802	Cell-Based Assay Glucose Oxidase	1 vial/2 mg	-20°C

NOTE: pH-Xtra™ is a product of Luxcel Biosciences.

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

NOTE: It is recommended that gloves be worn at all times when working with isolated mitochondria and mitochondrial inhibitors.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section on page 3 and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. Fluorescent plate reader with suitable optical filters and temperature control (See Table 1 on page 9)
2. Black walled clear-bottom 96-well, tissue culture treated plates
3. Plate block heater
4. 0.22 µm Filter
5. pH Meter with acid/base for adjustment
6. Adjustable single and/or multichannel pipettes
7. A source of UltraPure water

INTRODUCTION

About This Assay

Cayman's pH-Xtra™ Glycolysis Cell-Based Assay Kit is designed to measure the rate of extracellular acidification (ECA), which is proportional to the rate of lactate production.[1] This kit combines a glucose-based respiration buffer and inhibitors along with the phosphorescent pH probe pH-Xtra™ allowing for the accurate determination of glycolytic rate in a high-throughput manner.[2]

Please read this booklet thoroughly before opening any of the vials contained in this kit.

Properties of pH-Xtra™

pH-Xtra™ is a chemically stable and inert cell-impermeable H^+-sensing fluorophore with an excitation maxima between 360-380 nm and emission peaks at 590, 615, and 690 nm. pH-Xtra™ phosphorescent Lifetime Signal (decay) increases as the pH decreases. These properties make the probe ideal for time resolved fluorescence measurements, which can offer an increased signal under conditions where background is high.

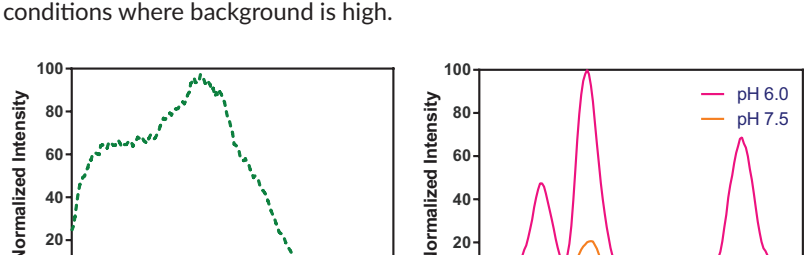

Figure 1. Excitation and emission spectrums of pH-Xtra™
Left panel: Normalized excitation spectrum of pH-Xtra™, with emission at 615 nm. Excitation maxima are observed between 360-380 nm. *Right panel:* Emission spectrum of pH-Xtra™ at pH 6.0 and 7.5.

Fluorescence Measurements

The pH-Xtra™ probe can be detected with standard fluorescence intensity or TR-F measurements, using monochromator or filter based plate-readers. TR-F measurement is advantageous in that it reduces non-specific background and increases the probe sensitivity, offering a more stable reading and wider dynamic range than measuring fluorescence intensity. Furthermore, a ratiometric TR-F measurement is used to maximize dynamic range and assay performance. To determine which measurement mode is best suited for your instrument, please refer to Table 1 on page 9, or consult your instrument manufacturer.

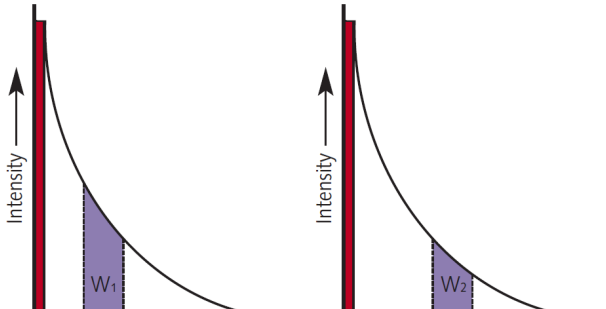

Figure 2. Illustration of ratiometric TR-F measurement

Please refer to Table 1 on page 9 for instrument specific settings.

The pH-Xtra™ probe signal to background ratio (S/B) for W_2 measurement is recommended to be >10/1 to allow accurate Lifetime calculation

Subsequent Lifetime Calculation: Use the dual intensity readings and the following transformation to calculate the corresponding Lifetime (µs):

$$\text{Lifetime (µs) } [\tau] = (300\text{-}100)/\ln(W_1/W_2)$$

Where W_1 and W_2 represent window 1 and 2, respectively, for the measured intensity readings at each time point, and 300 and 100 represent the delay time of W_2 and W_1, respectively. This provides Lifetime values in µs at each measured time point for each individual sample.

Example calculation:

W_1 = 75,629 counts and W_2 = 14,654 counts

Lifetime = (300-100)/ln(75,629/14,654)

Lifetime = 121.9 µs

Lifetime Signal should be in the range ~110 to ~250 µs, increasing to >400 µs upon acidification. Lifetime values can only be calculated from samples containing pH-Xtra™. S/B should be greater than 10 for W_2. Lifetime values should not be calculated from blank wells.

Instrument	Optical Configuration	Integration 1 (Delay 1/Read Window 1) Integration 2 (Delay 2/Read Window 2)	Measurement***	Excitation/Emission
BMG Labtech:* FLUOstar Omega POLARstar Omega	Filter-based Top or bottom read	100/30 µs 300/30 µs	Ratiometric TR-F	340 ± 50 nm (TR-EX L) 615 ± 10 nm (BP-615)
BMG Labtech:* CLARIOstar	Hybrid – filter-based Top or bottom read	100/30 µs 300/30 µs	Ratiometric TR-F	340 ± 40 nm 620 ± 10 nm
BMG Labtech:* PHERAstar FS	Filter-based Top read (HTRF-module)	100/30 µs 300/30 µs	Ratiometric TR-F	337 nm 620 nm
BMG Labtech:* FLUOstar Optima/ POLARstar Optima	Filter-based Top or bottom read	100/100 µs	TR-F	340 ± 50 nm (TR-EX L) 615 ± 10 nm (BP-615)
Perkin Elmer: VICTOR series/X4, X5	Filter-based Top read	100/30 µs 300/30 µs	Ratiometric TR-F	340 ± 40 nm (D340) 615 ± 8.5 nm (D642)
Perkin Elmer: EnVision	Filter-based Top read	100/50 µs 300/50 µs	Ratiometric TR-F	340 ± 60 nm (X340) 615 ± 8.5 (M615)
Perkin Elmer: EnSpire	Monochrometer Top or bottom read	100/100 µs	TR-F	380 nm 615 nm
BioTek: Synergy H1, H4, HT, Neo, 2 Cytation 3**	Filter-based Top or bottom read	100/30 µs 300/30 µss	Ratiometric TR-F	360 ± 40 nm 620 ± 10 nm
BioTek: MX, H1m	Monochrometer Top or bottom read	100/100 µs	TR-F	380 nm 615 nm
Tecan: Infinite/Safire/Genios Pro	Filter-based/Monochrometer Top or bottom read	100/100 µs	TR-F	380 ± 20 nm 615 ± 10 nm
Mol. Devices: SpectraMax/Flexstation/ Gemini	Monochrometer-based Top or bottom read	50/250 µs	TR-F	380 nm 615 nm

Table 1. Recommended instrument and measurement settings. For more information, contact the manufacturer of your specific instrument.

*Assay specific protocols and notes are available from plate reader manufacturer

**Assay specific protocols in development (please contact: TechSupport@luxcel.com)

***Where TR-F indicated, a TR-F module must be installed

PRE-ISOLATION PREPARATION

Reagent Preparation

1. **Glycolysis Respiration Buffer - (Item No. 601231)**

   Reconstitute the Respiration Buffer tablet in 50 ml of UltraPure water and adjust the pH to 7.4 at the temperature that you wish to perform the assay. *Failure to adjust the pH can drastically effect the experimental outcome.* Please note, this is a minimally buffered solution and only small amounts of acid or base are required. Filter sterilize using a 0.22 µm sterile filter. Store at 4°C for two weeks. Any non-sterile reconstituted Respiration Buffer should be discarded.

2. **pH-Xtra™ Assay Reagent - (Item No. 601232)**

   Reconstitute the clear contents of this vial in 1 mL of UltraPure water. Gently triturate the contents 3-4 times. Store unused portions in the dark at 4°C for up to one week or store in aliquots at -20°C for up to one month (avoid freeze thaw).

3. **2-Deoxyglucose Assay Reagent - (Item No. 601233)**

   This vial contains a 1.7 M solution of 2-deoxyglucose in water. It is ready to use as supplied. Keep on ice until use. Store unused portions at -20°C.

4. **Oligomycin Assay Reagent - (Item No. 701174)**

   This vial contains a 1 mg/ml solution of oligomycin in ethanol. Wear personal protective equipment when handling mitochondrial inhibitors. Keep on ice until use. Store unused portions at -20°C.

5. **Cell-Based Assay Glucose Oxidase - (Item No. 600802)**

   Reconstitute the contents of this vial with 0.2 ml of UltraPure water. Aliquot and store unused portions at -20°C for up to one month (avoid freeze thaw).

ASSAY PROTOCOL

Instrumentation

Typical Instrument Set Up

NOTE: Instrument settings will vary between manufacturers. Please refer to Table 1 on page 9.

1. Set the plate reader temperature control to 37°C.
2. Refer to Table 1 to determine the optimal wavelengths, delay and measurement times for your specific plate reader.
3. Gain should be optimized so that signal at pH 7.4 is 20% of the maximum detectable signal.

Instrument Signal Optimization

To optimize the signal, the following steps should be performed. A Signal to Blank (S/B) ratio >2 is required.

1. In a spare black, clear bottom 96-well tissue culture-treated plate, add 150 µl of Respiration Buffer to eight wells.
2. Add 10 µl of reconstituted Respiration Buffer to four of these wells. These will be your blank wells.
3. Add 10 µl of reconstituted pH-Xtra™ reagent to the remaining four wells. These will be your signal wells.
4. Measure this plate immediately using the instrument setup above, for a short 30 minute kinetic test.
5. If required, adjust the instrument parameters of interest to increase/decrease the measurement sensitivity to achieve maximum S/B ratio. The following options may be helpful:

   - Increase gain (or PMT) settings or flash energy
   - Adjust TR-F focal height
   - Repeat as a top or bottom read (plate reader dependent)
   - Increase volume of of pH-Xtra™ from 10 µl to 15 µl
   - Contact instrument supplier for further options

Pre-Assay Preparation

Seed cells: When seeding cells, do not add cells to wells G11, G12, H11, and H12. These wells will be used as a controls for cell independent acidification (G11, G12) and background subtraction (H11, H12).

Adherent cells: Seed cells in a black, clear-bottom 96-well plate that is suitable for tissue culture. Culture cells in 200 µl of culture medium for a minimum of 14 hours, in a 37°C incubator with 5% CO_2. We recommend performing a cell seeding titration to determine the optimal seeding density for new cell types when using pH-Xtra™.

Suspension cells: Seed on the day of the assay in 150 µl of culture medium in a black, clear-bottom 96-well plate that is suitable for tissue culture. We recommend performing a cell seeding titration to determine optimal seeding density for new cell types when using pH-Xtra™.

Remove CO_2: The presence of excess CO_2, may interfere with the assay. To remove from cell culture plates and culture media, incubate plates containing cells and culture media in a non-CO_2 incubator at 37°C, for three hours. Ensure that the incubator is humidified to prevent evaporation of the cell culture media. *NOTE: A color change is expected if phenol red is present in the culture media.*

Prepare test compounds

Control compounds: Oligomycin (increases glycolytic rate by inhibiting ATP synthase) and 2-deoxyglucose (competitive inhibitor of glycolysis) are provided as controls. Oligomycin should be diluted in Respiration Buffer so that the final solvent concentration is <1%. We recommend performing a titration to determine the optimal concentration of inhibitor to use as their effects will vary with cell type.

Test compounds: Test compounds should be diluted so the final solvent concentration is <0.5%.

Pre-warm equipment: Before starting the assay, pre-warm your instrument and a block heater capable of holding a 96-well cell culture plate to 37°C. Ensure that plate reader has been set up using the optimization protocol and plate reader specific settings found in Table 1 on page 9.

Typical assay duration is 1-3 hours. Set up the plate reader to measure at 3-5 minute intervals for 180 minutes.

Performing the Assay

1. Remove the cell culture plate from the non-CO_2 incubator and place on the pre-warmed block heater. *All subsequent steps take place on the block heater unless noted.*
2. Remove cell culture media from each well:

   Adherent cells: Carefully remove culture media being careful not to disturb the cell monolayer.

   Suspension Cells: Centrifuge the plate at 300 × g for five minutes at room temperature to pellet cells. Then remove cell culture media from each well.
3. Add 100 µl of pre-warmed Respiration Buffer to each well:

   Adherent cells: After the addition of pre-warmed Respiration Buffer, carefully remove Respiration Buffer and add another 100 µl of pre-warmed Respiration Buffer.

   Suspension cells: Re-suspend the pellet in the added pre-warmed Respiration Buffer and centrifuge plate at 300 × g for five minutes at room temperature to pellet cells. After centrifugation, remove Respiration Buffer from each well and add another 100 µl of pre-warmed Respiration Buffer and repeat centrifugation.
4. After final centrifugation remove Respiration Buffer and add 150 µl of pre-warmed Respiration Buffer to each well.

   Suspension cells: Re-suspend the pellet in pre-warmed Respiration Buffer.
5. Add 10 µl of reconstituted pH-Xtra™ Assay Reagent to all but two wells (H11, H12). Wells H11 and H12 are left empty to be used as background controls.
6. Add 10 µl of the control compounds to corresponding wells: Add controls as prepared in step 3 of pre-assay preparation. As a control for cell independent acidification, 10 µl of the Glucose Oxidase solution, prepared on page 11, should be added to wells G11 and G12, which contain pH-Xtra™ but not cells.
7. Place the plate in the pre-warmed fluorescent plate reader: Read plate based on the parameters obtain in the typical instrument setup and instrument optimization settings sections. For a typical assay, measure the plate kinetically, taking one measurement every 3-5 minutes for a total of 160 minutes.

Calculations

The following calculation can be used to calculate ratiometric Lifetime if reading in ratiometric TR-F mode.

Lifetime (µs) = duration of W_2 (300 µs) – duration of W_1 (100 µs)/ln (fluorescence (RFU) of W_1/fluorescence (RFU) of W_2)

For an example, see subsequent Lifetime calculation and Figure 2 on pages 7 and 8.

Performance Characteristics

Sample Data

The data shown below are examples, of data obtained with this kit. Your results will not be identical to these. Do not use these data to directly compare your samples as your results may vary substantially.

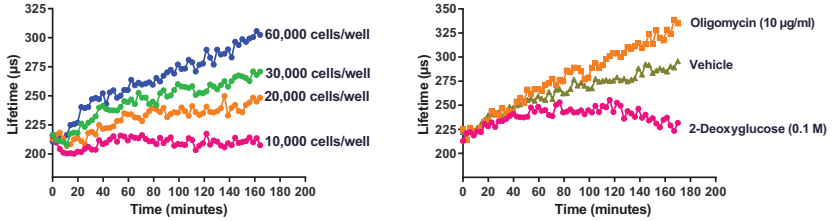

Figure 3. Sample data.
Left panel: A cell density experiment showing the increase in extracellular acidification with an increasing number of HepG2 cells. *Right panel:* The response of HepG2 cells to control compounds included in this kit.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
No signal or noisy signal detected	A. Plate reader not optimized B. Cell density too low	A. Ensure that proper steps were taken to optimize plate reader. For questions contact the manufacturer of your plate reader B. Check change in Lifetime/ fluorescence of pH-Xtra™ in wells containing Glucose Oxidase control. If change is observed, cell density may be too low. If no change is observed, contact techserv@caymanchem.com C. Perform a cell density titration to ensure optimal cell density has been achieved
Drift in signal within the first 20 minutes of the assay	A. Media and plate reader are not equilibrated to temperature B. Plate reader has poor temperature control	A. Ensure the media and plate reader have been pre-warmed to desired assay temperature B. Use only inner wells of the 96-well plate. Add 170 µl of Respiration Buffer to exterior wells to act as temperature buffer C. Lower the assay temperature to the ambient temperatire. This will eliminate signal drift, but may alter rate of acidification
Lifetime values >300 µs reported on initial reads	A. Respiration Buffer pH is too low B. Cell seeding density is too high	A. Ensure that the pH of the Respiration Buffer has been adjust to ~7.4 and equilibrated to the temperature that you wish to perform the assay B. Perform a cell density titration to ensure optimal cell density has been achieved

References

1. Hynes, J., O'Riordan, T.C., Zhdanov, A.V., *et al. In vitro* analysis of cell metabolism using a long-decay pH-sensitive lanthanide probe and extracellular acidification assay. *Anal. Biochem.* **390(1)**, 21-28 (2009).
2. Hynes, J., Natoli, E., Jr., and Will, Y. Fluorescent pH and oxygen probes of the assessment of mitochondrial toxicity in isolated mitochondria and whole cells. *Curr. Protoc. Toxicol.* 2.16.1-2.16.22 (2009).

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.