Agilent

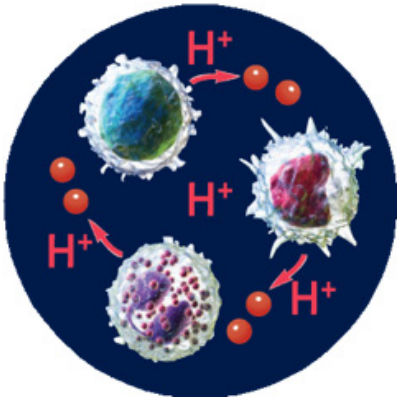
Agilent pH-Xtra

Glycolysis Assay

For the measurement of Glycolysis (Extracellular Acidification)

For use with:

- Adherent cells
- Suspension cells
- 3D cultures: tissues, spheroids
- RAFT and scaffolds



Notices

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Contents

1 General Information

Materials Supplied

Assay kit will arrive at room temperature. For best results, store as indicated below.

Cat. #	Item	96-well* Quantity/Size	Storage
PH-200-4	pH-Xtra Reagent	4 vials	+4 °C
	Respiration Buffer†	4 tablets	Room temp

* May also be used in a 384-well format, with one vial of reagent sufficient for ~200 wells

† 1mM K-phosphate, 20 mM Glucose, 70 mM NaCl, 50 mM KCl, 0.8 mM $MgSO_4$, 2.4 mM $CaCl_2$.

Storage and Stability

The pH-Xtra reagent should be stored as follows:

- Dry material between +2 to +8 °C (see Exp. Date on vial).
- Reconstituted pH-Xtra reagent can be stored in the dark between +2 to +8 °C for two days, or reconstituted in water and stored as aliquots at -20 °C for use within one month (avoid freeze thaw).

The Respiration Buffer tablet should be stored as follows:

- Dry material at room temperature (see Exp. Date on packaging)
- Reconstituted and filter sterilized product can be stored between +2 to +8 °C.

Additional Items Required

- Fluorescence plate reader, with suitable filter and plate temperature control.
- Standard clear 96-well TC$^+$ plates OR 96-well black wall clear bottom TC$^+$ plates.

Optional Items Not Supplied

- Plate block heater for plate preparation
- 0.22 µm sterilization filter, pH meter and KOH/HCl for adjustment
- Controls: Glucose Oxidase, 2-Deoxyglucose

Support

Visit our website **www.agilent.com**.

2 Description

The pH-Xtra Glycolysis Assay is an easy to use, highly flexible 96- or 384-well fluorescence-based approach for the direct, real-time, kinetic analysis of extracellular acidification rates (ECA/ECAR). As lactate production is the main contributor to this acidification, ECA measurements are a convenient and informative measure 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-Xtra 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), which, coupled with its spectral and response characteristics, make pH-Xtra the ideal choice for flexible, high-throughput assessment of ECA. This performance facilitates sensitive robust microtitre-plate based measurements, thereby 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 non-destructive and fully reversible (pH-Xtra reagent is not consumed), measurement of time courses and multiple drug treatments are possible.

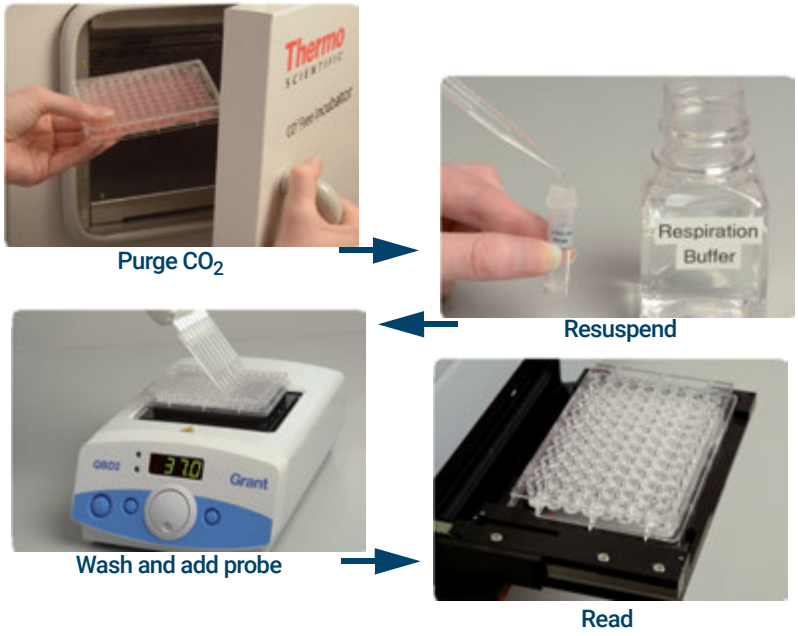

Figure 1. General workflow for preparation and use of pH-Xtra Glycolysis Assay.

3 Plate Reader Set-Up

Measurement Parameters

pH-Xtra reagent is a chemically stable and inert, cell impermeable H^+-sensing fluorophore.

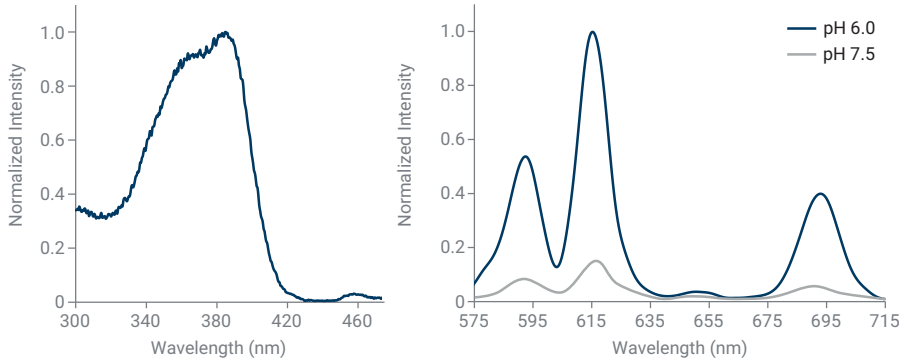

Figure 2. Excitation and Emission spectra of pH-Xtra. Left panel shows normalized excitation (Ex 340- 410 nm; Peak 360-380 nm). Right panel shows emission maxima (Em 590, 615, and 690 nm) fold increase between pH 6.0 and pH 7.5.

Instruments and Settings

Two fluorescence modalities can be optimally used with the pH-Xtra Glycolysis Assay depending on plate reader type and instrument setup, as follows:

- **Standard:** Time-resolved fluorescence measurement (TR-F), and
- **Advanced:** Dual-read Ratiometric TR-F measurement (Lifetime calculation)

NOTE

Further details, including instrument, filter selection and measurement settings can be found in "Appendix A - Instrument Settings" on page 29.

Signal Optimization - recommended for first time users

NOTE **Use a plate block heater for plate preparation and prewarm plate reader to measurement temperature.**

STEP 1: Reconstitute a Respiration Buffer tablet in 50 mL of water, warm to assay temperature (37 °C), adjust pH to approx. pH 7.4, and filter sterilize using a 0.22 µm filter. Reconstitute (transparent) contents of a pH-Xtra vial in 1 mL of distilled water, gently aspirating 3-4 times.

NOTE **Reconstituted pH-Xtra reagent can be stored in the dark between +2 to +8 °C for two days or stored as aliquots in water at -20 °C for use within one month (avoid freeze thaw).**

STEP 2: Prepare eight replicate wells of a 96-well plate, by adding 90 µL prewarmed Respiration Buffer to each well (A1- A4, B1-B4).

STEP 3: Add 10 µL reconstituted pH-Xtra reagent to four replicate wells (A1-A4) and 10 µL Respiration Buffer to the remaining replicate wells (B1-B4).

STEP 4: Read plate immediately in a fluorescence plate reader over 30 minutes (read every 2-3 minutes).

STEP 5: Examine Signal Control well (A1-A4) and Blank Control well (B1-B4) reading after 30 minutes, and calculate S:B ratio.

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

For most fluorescence TR-F plate readers, setup according to "Appendix A - Instrument Settings" on **page 29**, pH-Xtra should return a S:B ~ 3. See "Appendix B - Troubleshooting" on **page 33**.

	1	2	3	4
A	Respiration Buffer + pH-Xtra	Respiration Buffer + pH-Xtra	Respiration Buffer + pH-Xtra	Respiration Buffer + pH-Xtra
B	Respiration Buffer	Respiration Buffer	Respiration Buffer	Respiration Buffer

4 Performing the Glycolysis Assay

Cell Culture and Plating

NOTE

Always leave six wells per 96-well plate free from the addition of cells as Blank and Signal control wells. See "Recommended controls (optional):" on page 19 for further details.

- For Adherent cells, seed cells in a 96-well plate in 200 µL culture medium. Incubate overnight in a CO_2 incubator at 37 °C.
- For Suspension cells, seed on the day of assay in 100 µL culture medium.

NOTE

Required seeding densities are cell type dependent. We recommend performing a cell seeding density titration experiment to determine the optimal cell number per well, starting with a high cell density (typically 60,000 - 80,000 cells/well for adherent cells and ~5 x 105/well for suspension cells in a 96 well plate). See "Appendix B - Troubleshooting" on page 33 and Figure 7 on page 25 for further details.

Pre-Assay Preparation

NOTE

Where cells are cultured in a CO_2 incubator overnight, it is important to purge the media and plastic ware of CO_2 prior to conducting the pH-Xtra Glycolysis 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, approximately two hours prior to performing the Glycolysis Assay.

- Reconstitute Respiration Buffer tablet in 50 mL of water, pH adjust to approximately pH 7.4 and filter sterilize using a 0.22 µm filter. Reconstitute transparent contents of the pH-Xtra vial in 1 mL of distilled water, gently aspirating 3-4 times.

NOTE

Reconstituted pH-Xtra reagent can be stored as aliquots in water at -20 °C for use within one month (avoid freeze thaw).

- Prepare test compounds, controls and dilutions as desired.

Recommended controls (optional):

Blank control: Leave two or three wells free from the addition of cells for use as Blank control. Add in total 100 µL of fresh measurement buffer to each well as described in Step 1 and 2 in the **"Typical Assay"** on page 21. Do not add pH-Xtra reagent to these wells.

Cell-free negative control: Leave two or three wells free from the addition of cells for use as Cell-free negative control. Add 90 µL of fresh Respiration Buffer + 10 µL of reconstituted pH-Xtra reagent to each well as described in Steps 1 and 2 in the **"Typical Assay"** on page 21.

Cell-free positive control: Leave two or three wells free from the addition of cells for use as Cell-free positive controls. Add 90 µL of fresh Respiration Buffer + 10 µL of (1 mg/mL) Glucose Oxidase stock solution (in water) + 10 µL reconstituted pH-Xtra reagent to each well. If plate reader settings are correct, these wells will show a rapid increase in Fluorescence Intensity/Lifetime as sign for acidification caused by Glucose Oxidase activity.

Cell-based negative control: To two or three wells containing cells, add 10 µL of a 500 mM 2-Deoxyglucose stock solution (in water or Respiration Buffer) + 10 µL reconstituted pH-Xtra reagent as described in Steps 1 and 2 in the **"Typical Assay"** on page 21. 2-Deoxyglucose, a competitive Hexokinase inhibitor, will inhibit extracellular acidification derived from glycolysis.

NOTE **We recommend that all culture media and stock solutions to be used in the assay are prewarmed at 37 °C prior to use. Use a plate block heater for plate preparation, and prewarm the fluorescence plate reader to measurement temperature.**

Figure 3. Reconstitution of pH-Xtra

Typical Assay

To assess Extracellular Acidification (ECA) or to investigate the effect of a treatment on glycolytic flux, cells are treated immediately prior to measurement.

NOTE **We recommend the use of triplicate wells for each treatment.**

STEP 1: Remove spent culture medium from all assay wells and wash cells twice (2x), using 100 µL of Respiration Buffer per well for each wash (**Figure 4**). After removing the second wash, replace with 90 µL of fresh Respiration Buffer.

NOTE **We recommend to always use at least two wells without cells as Blank control wells (do not add pH-Xtra reagent into these wells) and at least two additional wells without cells as Cell-free positive control wells (add pH-Xtra reagent into these wells in Step 2). Also consider to include a Cell-free positive control as described in the "Recommended controls (optional):" on page 19. Add 90 µL of Respiration Buffer to these control wells also.**

STEP 2: Add 10 µL reconstituted pH-Xtra reagent to each well, except those wells for use as Blank Controls. Add 10 µL of Respiration Buffer to these Blank Control wells.

NOTE **If plating a full 96-well plate of assays, we recommend simplifying Steps 1 and 2 by preparing a stock solution containing the 1 mL of reconstituted pH-Xtra reagent added to 10 mL prewarmed Respiration Buffer, and using a multi-channel pipette to add 100 µL of this diluted pH-Xtra stock to each well. Add 100 µL of Respiration Buffer only (no pH-Xtra) to each Blank Control well.**

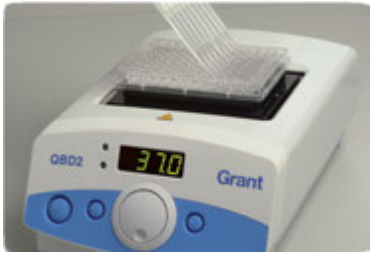

Figure 4. Adding fresh Respiration Buffer (± pH-Xtra)

STEP 3: Test compound stock or vehicle (typically 1-10 µL) may be added at this point if desired.

NOTE **We recommend keeping the volume of added compound low to minimize any potential effects of solvent vehicle.**

STEP 4: Read the plate immediately in a fluorescence plate reader, with the set-up as described in "Appendix A - Instrument Settings" on **page 29** (**Figure 5**). The plate should be measured kinetically for >120 minutes. When the measurement is completed, remove the plate and save measured data to file.

Figure 5. Reading the assay plate

5 Analysis

NOTE **We recommend that all first time users perform a Signal Optimization test, as described.**

Assessing Extracellular Acidification

Plot the Blank Control well-corrected pH-Xtra Intensity or Lifetime values versus Time (**Figure 6**). Select the linear portion of each signal profile (avoiding any initial lag or subsequent plateau), and apply linear regression to determine the slope (ECAR) and correlation coefficient for each well.

NOTE **This approach is preferable to calculating a slope from averaged profiles.**

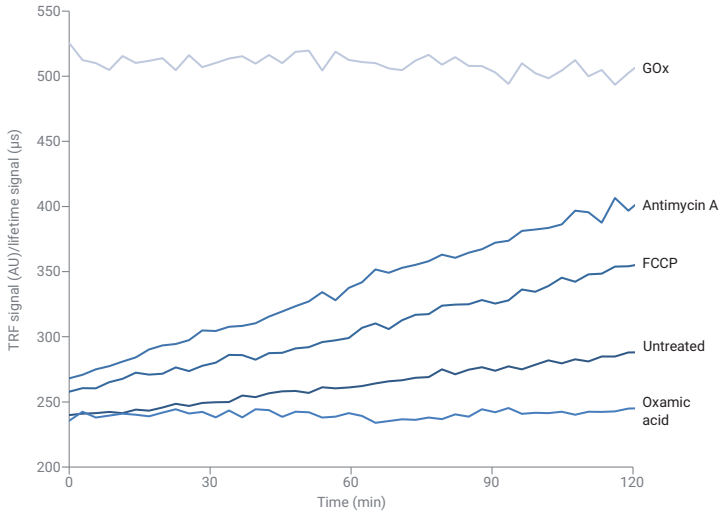

Figure 6. Typical Signal profile (TR-F arbitrary units [AU] or Lifetime [µs]) pH-Xtra for adherent cells, treated with typical control compounds, including 2-Deoxyglucose recommended as a negative control. The effect of glucose oxidase as positive signal control is illustrated schematically.

NOTE **If using FCCP, it is strongly recommended to perform a dose titration, since FCCP exhibits a bell-shaped dose response.**

Tabulate the slope values for each test sample, calculating appropriate average and standard deviation values across replicate wells. The slope obtained for the Cell-free negative control samples or for the Cell-based negative control samples can be subtracted from all test values if desired.

Protocols, data analysis templates and other technical resources are available for download through our website **www.agilent.com**.

Titration of Cell Seeding Density

To determine an optimal cell seeding density for performing the pH-Xtra Glycolysis Assay, for new cell types, seed replicate wells with a range of seeding densities (typically 0, 10,000, 20,000, 40,000, 60,000 and 80,000 cells/well). Plot the data generated as a function of intensity or Lifetime values versus time, as illustrated (**Figure 7**).

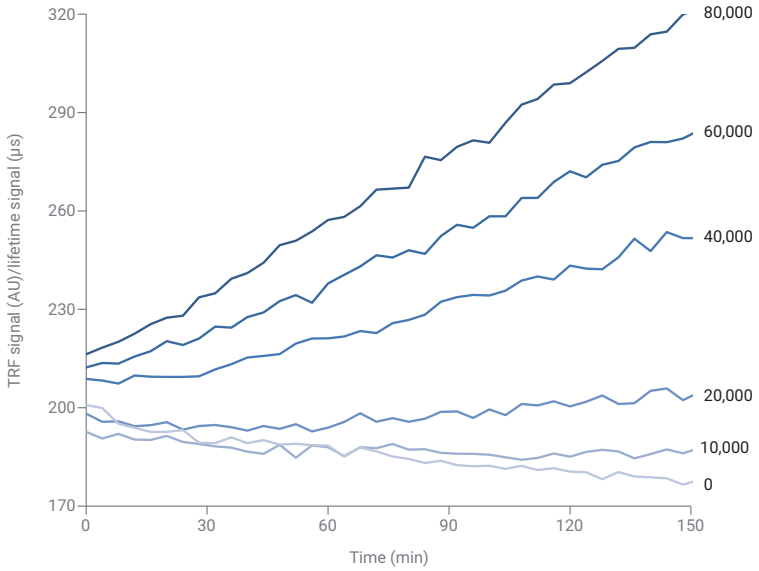

Figure 7. Extracellular Acidification profiles (ECA) are shown for A549 cells seeded between 0 and 80,000 cells/well. In this experimental example, a seeding density of 40,000 cells/well was chosen as this provides a suitable ECA response.

Measuring Altered Metabolism

Multiparametric (or multiplex) combination of pH-Xtra Glycolysis Assay together with the Agilent MitoXpress Xtra - Oxygen Consumption Assay [HS Method] (Cat No: MX-200-4) allows the simultaneous real-time measurement of glycolysis and mitochondrial respiration, and analysis of the metabolic phenotype of cells and the shift (flux) between the two pathways under pathological states (**Figure 8**).

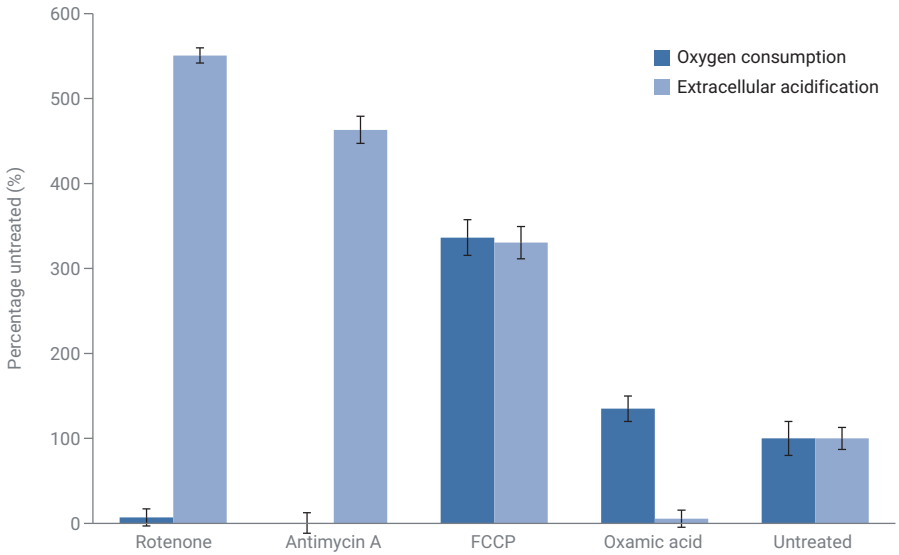

Figure 8. Measuring altered metabolism in HepG2 cells treated with a combination of compounds modulating the electron transport chain or lactate production, shown as a percentage relative to untreated control cells. Comparative measurements with MitoXpress Xtra (oxygen consumption) show the shift between glycolysis and mitochondrial respiration.

Calibration of pH-XTRA Glycolysis Assay to a pH/[H^+] Scale

It is possible to express Extracellular Acidification (ECA) as a function of pH/[H^+] versus time. This is achieved by first creating a calibration standard curve, by measuring Lifetime values (selecting stabilized readings over a 30 minute read), from a range of pH-buffered standards at the appropriate assay temperature (**Figure 9**). Select the linear portion of the standard curve and apply linear regression to determine the calibration function (See Hynes *et al.*, 2009).

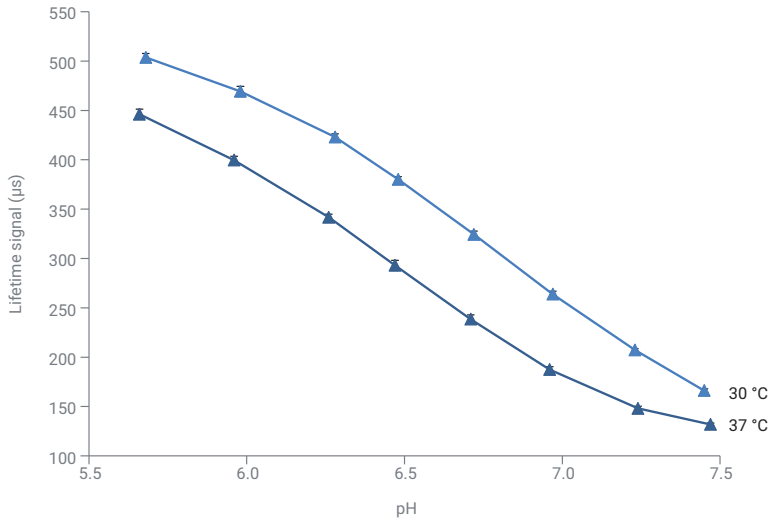

Figure 9. pH-Xtra reagent calibration in Lifetime scale, at 30 °C and 37 °C using pH-buffered PBS, at increments of 0.2 across a pH range 6.0 - 7.5.

5 Analysis

Calibration of pH-XTRA Glycolysis Assay to a pH/[H+] Scale

Appendix A - Instrument Settings

Two fluorescence modalities can be optimally used with the pH-Xtra Glycolysis Assay, depending on plate reader type and instrument setup.

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

Standard: Single TR-F Measurement

Measurement using time-resolved fluorescence (TR-F) provides flexibility to use a wide range of commonly available plate readers[1]. TR-F measurement reduces nonspecific background and increases probe sensitivity. Optimal delay time is ~100 µs and gate (integration) time is 100 µs.

NOTE **pH-Xtra should return a S:B ≥ 3.**

Advanced: Dual-Read TR-F (Lifetime)

Optimal performance is achieved using dual-read TR-F in combination with subsequent ratiometric Lifetime calculation, to maximize dynamic range (**Figure 10**) and to express ECA as a function of $[H^+]$.

NOTE **pH-Xtra should return a S:B ≥ 10.**

Optimal dual-delay and gate (integration) times:

- Integration window 1: 100 µs delay (D_1), 30 µs measurement time (W_1)
- Integration window 2: 300 µs delay (D_2), 30 µs measurement time (W_2)

1 Users may see better performance using filter-based plate readers.

Dual-Read TR-F and Lifetime Illustrated

Dual-read TR-F and subsequent Lifetime calculation allows measurement of the rate of fluorescence decay of the pH-Xtra reagent and can provide measurements of extracellular acidification that are more stable and with a wider dynamic range than measuring Signal Intensity or standard TR-F.

NOTE

S:B for Integration window 2 is recommended to be ≥ 10 to allow accurate Lifetime calculation.

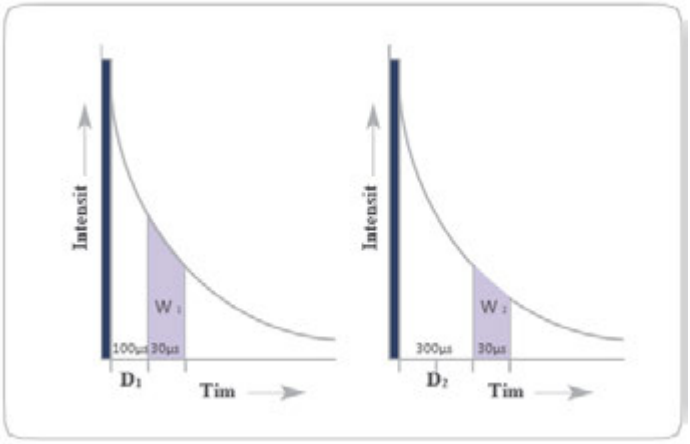

Figure 10. Illustrating dual-read TR-F measurement.

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

Lifetime (µs)[**τ**] = $(D_2\text{-}D_1)/\ln(I_{W1}/I_{W2})$

Where I_{W1} and I_{W2} represent the two (dual) measurement windows and D_1 and D_2 represent the delay time prior to measurement of W_1 and W_2 respectively. This provides Lifetime values in microsecond units (µs) at each measured time point for each individual sample (**Figure 10**).

NOTE

Lifetime values should be in the range ~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 pH-Xtra reagent. Lifetime values should not be calculated from blank wells.

Table 1 Recommended Instrument and Measurement Settings

Instrument	Optical configuration	Integ 1 (D1 / W1) Integ 2 (D2 / W2)	Optimum mode	Ex (nm) Em (nm)
BioTek: Cytation 1, 3 or 5 Synergy H1, H4, Neo or Neo2	Filter-based Top or bottom read	100 / 30 µs 300 / 30 µs	Dual read TR-F (Lifetime)	Ex 360 ± 40 nm Em 620 ± 10 nm
BMG Labtech: CLARIOstar	Filter-based Top or bottom read	100 / 30 µs 300 / 30 µs	Dual-read TR-F (Lifetime)	Ex 340 ± 50 nm (TR-EX) Em 615 ± 18 nm (BP-615)
FLUOstar Omega POLARstar Omega	Filter-based Top or bottom read	100 / 30 µs 300 / 30 µs	Dual-read TR-F (Lifetime)	Ex 340 ± 50 nm (TR-EX) Em 615 ± 18 nm (BP-615)
PHERAstar FSX	Filter-based Top read	100 / 30 µs 300 / 30 µs	Dual-read TR-F (Lifetime)	EX 337 nm Em 620 nm (HTRF Module)
Tecan: Spark (10M/20M)	Filter-based	100 / 30 µs 300 / 30 µs	Dual read TR-F (Lifetime)	Ex 380 ± 20 nm Em 615 ± 20 nm
	Fusion optics	100 / 30 µs 300 / 30 µs	Dual read TR-F (Lifetime)	Ex 380 ± 20 nm (Monochromator) Em 615 ± 20 nm (Filter)
Infinite F200Pro Infinite F Plex Infinite F Nano+	Filter-based Top or bottom read	100 / 30 µs 300 / 30 µs	Dual read TR-F (Lifetime)	Ex 380 ± 20 nm Em 615 ± 10 nm
Perkin Elmer: VICTOR series, X4 or X5	Filter-based Top read	100 / 30 µs 300 / 30 µs	Dual read TR-F (Lifetime)	Ex 340 ± 40 nm (D340) Em 615 ± 8.5 nm (D615)
EnVision	Filter-based Top read	100 / 30 µs 300 / 30 µs	Dual read TR-F (Lifetime)	Ex 340 ± 60 nm (X340) Em 615 ± 8.5 nm (M615)
Molecular Devices: SpectraMax i3x, i3 SpectraMax Paradigm	Filter-based Top or bottom read (bottom read preferred where available)	100 / 100 µs n/a	TR-F	Ex 370 nm Em 616 ± 10 nm (TRF-EuSa Filter Cartridge)
SpectraMax M series SpectraMax Flexstation	Monochromator- based Top or bottom read	100 / 100 µs n/a	TR-F	Ex 380 ± 9 nm Em 615 ± 15 nm
SpectraMax GeminiXPS SpectraMax GeminiEM	Monochromator- based Top or bottom read	100 / 100 µs n/a	TR-F	Ex 380 ± 9 nm Em 615 ± 9 nm

Table 1 Recommended Instrument and Measurement Settings (continued)

Instrument	Optical configuration	Integ 1 (D1 / W1) Integ 2 (D2 / W2)	Optimum mode	Ex (nm) Em (nm)
SpectraMax iD5	Filter-based Top or bottom read (bottom read preferred where available)	100 / 100 µs n/a	TR-F	Ex 350 ± 60 nm Em 616 ± 10 nm
	Monochromator-based Top or bottom read (bottom read preferred where available)	100 / 100 µs n/a	TR-F	Ex 380 ± 15 nm Em 615 ± 25 nm
BMG Labtech: FLUOstar Optima POLARstar Optima	Filter-based Top or bottom read	100 / 100 µs n/a	TR-F	Ex 340 ± 50 nm (TR-EX L) Em 615 ± 18 nm (BP-615)
Tecan: Infinite M1000Pro Infinite M200Pro Infinite M Plex Infinite M Nano^{+} Safire	Monochromator-based Top or bottom read	100 / 100 µs n/a	TR-F	Ex 380 ± 15 nm Em 615 ± 20 nm
Genios Pro	Filter-based Top or bottom read	100 / 100 µs n/a	TR-F	Ex 380 ± 9 nm Em 615 ± 20 nm

NOTE **For up-to-date instrument settings, downloadable protocols and data-analysis tools, please visit www.agilent.com.**

Appendix B - Troubleshooting

Extensive literature, including Protocols, Application Notes, Videos, Publications and email technical support is also available through our website **www.agilent.com**.

General Notes and Recommendations

Storage and Stability: On receipt the pH-Xtra reagent should be stored between +2 to +8 °C (see Exp. Date on vial). Reconstituted pH-Xtra reagent can be stored in the dark between +2 to +8 °C for two days or stored as aliquots in water at -20 °C for use within one month (avoid freeze thaw).

NOTE

pH-Xtra reagent diluted in Respiration Buffer/media should be used on the same day.

Respiration Buffer: Kit contains a single Respiration Buffer tablet sufficient for 50 mL 1x stock, containing 1 mM K-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), so long as care is taken to ensure a minimal buffering capacity.

Plate Reader: A fluorescence plate reader capable of measuring excitation between 360 nm and 390 nm (see **Figure 2** on page 14) and emission at 615 nm and having plate temperature control is required. We strongly recommend using TR-F measurement.

Temperature: We recommend the use of a plate block heater for plate preparation, to maintain a temperature of 37 °C. Prewarm the fluorescence plate reader to measurement temperature and ensure that all culture media and stock solutions to be used in the assay are prewarmed at 37 °C prior to use.

Signal Optimization and Use of Controls: We recommend performing a signal optimization check, especially for first time users, and inclusion of blank and optional additional control wells as described.

General Assay Set-Up, Pipetting and Aspirating: Prepare your assay, materials and work space in advance. Take care not to disrupt the cell monolayer (adherent cells) during pipetting and aspirating. Work rapidly once the pH-Xtra reagent has been added, to reduce the potential for assay variability. Recheck pH of Respiration Buffer prior to use.

Cell Type and Cell Density: Since the pH-Xtra reagent measures Extracellular Acidification, 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 (See **Figure 7** on page 25).

Signal to Blank (S:B) Optimization

For most fluorescence plate readers, set up according to "Appendix A - Instrument Settings" on **page 29**, pH- Xtra 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:

1. Increase Gain (PMT) setting or flash energy/number.
2. Adjust TR-F focal height.
3. Increase length of integration time, the same for both delay windows.
4. Use top reading if available.
5. Increase volume of pH-Xtra (15 µL/well)
6. Contact Instrument Supplier or **cellanalysis.support@agilent.com** for further options.

Frequently Asked Questions

Q: What do I do if I cannot detect any signal in wells containing cells and pH-Xtra (or I can detect a signal but the slope (rate) appears very low)?

A: Check correct Instrument Settings (“Appendix A - Instrument Settings” on **page 29**)- Perform Signal Optimization - Include Signal Controls as described above - Increase cell density. Check pH of prewarmed Respiration Buffer and correct as necessary, as pH can drift over time. If tested and not resolved, contact **www.agilent.com**.

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

A: Check cell seeding and pipetting consistency, increase cell density, ensure plate, instrument and all culture media and stock solutions are prewarmed at 37 °C prior to use, reduce plate preparation times. Reduce assay volume to 60 µL.

NOTE

Some plate readers have inconsistent temperature control. If you suspect this to be the case, consider reducing assay and equilibration temperatures to 30 °C and avoiding outer wells of microtiter plate. If tested and not resolved, contact www.agilent.com.

REFERENCES

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