ION BIOSCIENCES

Instructions

Brilliant Intracellular pH Assay

Catalog No. 13000

Introduction

Intracellular pH (pH_i) is a fundamental regulator of numerous cellular processes, including cell growth, enzymatic activity, receptor-mediated signal transduction, ion transport, endocytosis, and cell adhesion. Dysregulated pH_i is associated with a variety of diseases, highlighting the need for reliable tools to measure dynamic pH changes in live cells.

ION Biosciences' Brilliant Intracellular pH Assay is a high-throughput, no-wash, fluorescence-based platform optimized to monitor activation of plasma membrane proton-coupled channels and transporters in response to pharmacological compounds. These include the voltage-gated proton channel (H_V1), Na^+/H^+ exchangers (SLC9), divalent metal transporters (SLC11), peptide transporters (SLC15), monocarboxylate transporters (SLC16), proton-coupled amino acid transporters (SLC36), proton-coupled sugar transporters (SLC45), and the proton-coupled folate transporter (SLC46).

The assay uses the cell-permeable dye BCECF AM, a green fluorescent pH indicator with a pK_a of ~7 and dual-excitation properties (Ex/Em ~430/535 nm and ~490/535 nm). Ratiometric measurement enables precise determination of absolute pH_i while minimizing variability from photobleaching, dye loading, or cell morphology. For high-throughput screening, BCECF can also be measured non-ratiometrically using standard fluorescein settings. The assay delivers sensitive, quantitative readouts with strong signal and broad dynamic range, compatible with 96- and 384-well plates, making it ideal for mechanistic studies, dose–response profiling, and large-scale screening campaigns.

Storage and Stability

Upon receipt, store components at the temperatures indicated on each label. Kit components are stable[1] for up to 12 months from date of shipment when stored as directed.

Table 1	**Kit Contents**	***Flex* Kit** Cat# 13000-10		***pIONeer* Kit** Cat# 13000-2		
Label	**Name**	**Size**	**Qty**	**Size**	**Qty**	**Storage**
Reagent A	BCECF AM	50 µg Vial	10	50 µg Vial	2	-20° C
Reagent B	DMSO[1]	225 µL Vial	1	Not included in pIONeer		-20° C
Reagent C	50X DySolv	4 mL Bottle	1	800 µL Vial	1	4° C
Reagent D	10X Brilliant Assay Buffer	40 mL Bottle	1	8 mL Bottle	1	4° C
Reagent E	50X TRS[2]	4 mL Bottle	1	800 µL Vial	1	4° C
Reagent F	50X Probenecid[2]	4 mL Bottle	1	800 µL Vial	1	4° C

Getting Started

Before beginning your experiment, ensure you have all the necessary reagents and materials. ION Biosciences' Brilliant Intracellular pH Assay includes everything required for the assay, except for proton-coupled channel modulators and proton-coupled channel expressing cell lines. A fluorescence plate reader is required, with excitation at 475–495 nm and emission at 515–535 nm for non-ratiometric readouts. For ratiometric readouts, a second excitation at 425–445 nm with rapid wavelength switching is necessary. Ideally, the reader should support kinetic acquisition at ~1 Hz. Examples of suitable instruments include the WaveFront Panoptic, Hamamatsu FDSS, Molecular Devices FLIPR, and Molecular Devices FlexStation.

These instructions are designed for both 96-well and 384-well microplate formats. Adjustments may be needed for other formats, such as 1536-well plates or non-adherent cells. BCECF AM should be protected from direct light and solutions containing BCECF AM should be used within 2 hours of preparation. We recommend seeding cells in 96-well or 384-well plates 24 hours prior to the assay, with 20K cells per well for 96-well plates and 10K cells per well for 384-well plates.

Laboratory Procedures

1. Add 20 µL of DMSO (**Reagent B**) to the tube containing BCECF AM (**Reagent A**).
2. Vortex until Reagent A is fully dissolved.
3. Add appropriate volume of water (**Table 2**, below) to a 15 mL centrifuge tube.
4. Add 1 mL of 10X Brilliant Assay Buffer (**Reagent D**) to the tube from **step 3**.
5. Add 200 µL of 50X DySolv (**Reagent C**) to the tube from **step 4**.
6. Add 200 µL of 50X TRS[2] (**Reagent E**) to the tube from **step 5**.
7. If desired add 200 µL of 50X Probenecid[2] (**Reagent F**) to the tube from **step 6**.
8. Add 20 µL of BCECF AM Solution from **step 2** to the tube from **step 7**.

Table 2	**Dye Loading Solution**		
Label	**Name**	**Method A**	**Method B**
Reagent A+B	BCECF AM solution in DMSO	20 µL	20 µL
Reagent C	50X DySolv	200 µL	200 µL
Reagent D	10X Brilliant Assay Buffer	1 mL	1 mL
Reagent E	50X TRS[2]	200 µL	200 µL
Reagent F	50X Probenecid[2]	200 µL	-
	Water	8.4 mL	8.6 mL
	Total	**10 mL**	**10 mL**

Procedure Continues on Next Page

Laboratory Procedures (Continued)

9. Briefly vortex the **Dye Loading Solution** from **step 8** to mix well.
10. Prepare the **Wash Solution**[3] in a 15 mL centrifuge tube by adding the appropriate amounts of water and 10X Brilliant Assay Buffer (**Reagent D**) as shown in **Table 3** below. Briefly vortex to mix well.

Table 3	Wash Solution[3] (Optional)	
Label	**Name**	**Volume**
Reagent D	10X Brilliant Assay Buffer	1 mL
	Water	9 mL
	Total	**10 mL**

11. Completely remove the cell-culture medium from the 96-well or 384-well microplate containing the cells of interest
12. Wash the cell-containing microplate once with **Wash Solution**[3]. 100 µL/well for 96-well plate and 20 µL/well for 384-well plate. Completely remove the **Wash Solution**[3] from the 96-well or 384-well plates.
13. Add the **Dye Loading Solution** from **step 9** to the cell-containing microplate. 100 µL/well for 96-well plate and 20 µL/well for a 384-well plate.
14. Incubate the cell-containing microplate with the **Dye Loading Solution** for 60 minutes at 37°C.

Note: If assaying proton-coupled channel inhibitors, continue with steps 15–17; otherwise, proceed to Step 18.

15. Prepare the **Inhibitor Solution** in a 15 mL centrifuge tube by adding the appropriate amounts of water, 10X Brilliant Assay Buffer (**Reagent D**), and 3X concentrated proton-coupled channel inhibitors as shown in **Table 4** below. Briefly vortex to mix well.
16. Add the **Inhibitor Solution** from **step 15** to the cell-containing microplate. 50 µL/well for a 96-well plate and 10 µL/well for a 384-well plate.
17. Incubate the cell-containing microplate for 15 minutes at 37°C.

Table 4	Inhibitor Solution (Optional)	
Label	**Name**	**Volume**
Reagent D	10X Brilliant Assay Buffer	1 mL
(Customer Supplied Material)	Proton-coupled Channel Inhibitors	Enough for 3X concentration at total volume
	Water	Q.S. to total volume
	Total	**10 mL**

Procedure Continues on Next Page

Laboratory Procedures (Continued)

18. Prepare the **Stimulus Solution**[4] in a 15 mL centrifuge tube by adding the appropriate amounts of water, 10X Brilliant Assay Buffer (**Reagent D**), and 4X concentrated proton-coupled channel activators as shown in **Table 5** below. Briefly vortex the **Stimulus Solution**[4] to mix well.
19. Load the **Stimulus Solution**[4] from **step 18** into a V-shaped reagent plate for assay readout. 100 µL/well for a 96-well plate and 20 µL/well for a 384-well plate.

Table 5	**Stimulus Solution** [4]	
Label	**Name**	**Volume**
Reagent D	10X Brilliant Assay Buffer	1 mL
(Customer Supplied Material)	Proton-coupled Channel Activators	Enough for 4X concentration at total volume
	Water	Q.S. to total volume
	Total	**10 mL**

20. Transfer the cell-containing microplate and reagent plate to a kinetic-imaging plate reader (e.g. WaveFront Panoptic, Hamamatsu FDSS, Molecular Devices FLIPR, or Molecular Devices FlexStation).
21. For non-ratiometric readouts, acquire data using an excitation wavelength of ~490 nm, emission at ~530 nm, and an acquisition frequency of ~1 Hz. For ratiometric readouts, acquire data using a dual excitation wavelength of ~430 and ~490 nm, an emission collected at ~530 nm and an acquisition frequency of ~1 Hz for each wavelength. Excitation sources commonly used for fluorescein, Fluo-4, and GFP (480–490 nm) are compatible with BCECF AM dye for non-ratiometric readouts. A dedicated BCECF or dual excitation source may be required for a ratiometric readout. See **Table 6** below for recommended instrument settings.
22. Begin baseline data acquisition for 30 seconds, then transfer the volume from the reagent plate to the cell-containing microplate. 50 µL/well for a 96-well plate and 10 µL/well for a 384-well plate. Continue data acquisition for an additional 5 minutes.

Table 6	**Recommended Instrument Settings**
Setting	**Recommendation**
Read Mode	'Bottom' read mode only
Ex/Em wavelengths[5] (Ratiometric)	~430 and 490 nm / 530 nm
Ex/Em wavelengths[5] (Non-ratiometric)	~490 nm / 530 nm
Cutoff wavelength	515 nm
Filter selection	BCECF (ratiometric analysis), FITC (non-ratiometric imaging)
Contact support@ionbiosciences.com for additional recommendations and guidance on optimizing to your application.	

Laboratory Procedures - Footnotes

[1] DMSO is hygroscopic and should be stored tightly closed at -20° C with desiccant pack to prevent the solvent from becoming wet. Wet solvent causes difficulties with dissolution of the dye. Use the DMSO within 6 months of receipt.

[2] Caution is advised when using Probenecid and/or TRS as they may have undesirable effects on assay performance for the target of interest. Probenecid may be included in the Dye Loading Solution to aid in dye retention. This may be particularly important in certain cell lines (e.g. CHO cells). TRS contains a membrane-impermeant dye useful for masking extracellular fluorescence.

[3] We recommend washing the cells with wash solution only if residual culture medium remains in the well.

[4] The timing and volume of stimulus solution addition may vary. Some experiments may include the addition of other solutions to the cell-containing microplate prior to the addition of the stimulus solution. In these cases, the volume of the stimulus solution addition should be altered to account for the additional volume of solution in the cell-containing microplate.

[5] To prevent bleed-through or spectral overlap, the Ex/Em wavelengths may need to be optimized by broadening the interval between the wavelengths. Results displayed were generated on a Wavefront Panoptic.

Data Analysis

Non-ratiometric Analysis

1. Plot the relative fluorescence units (F) over time as the raw kinetic fluorescence data (**Figure 1**, below).
2. Define the initial 30 seconds of relative fluorescence units (F) as the baseline. Average the baseline and define it as F_0.
3. Calculate the ratio of F/F_0 at each time point. Plot F/F_0 over time as the baseline-normalized kinetic fluorescence data (**Figure 2**, next page).

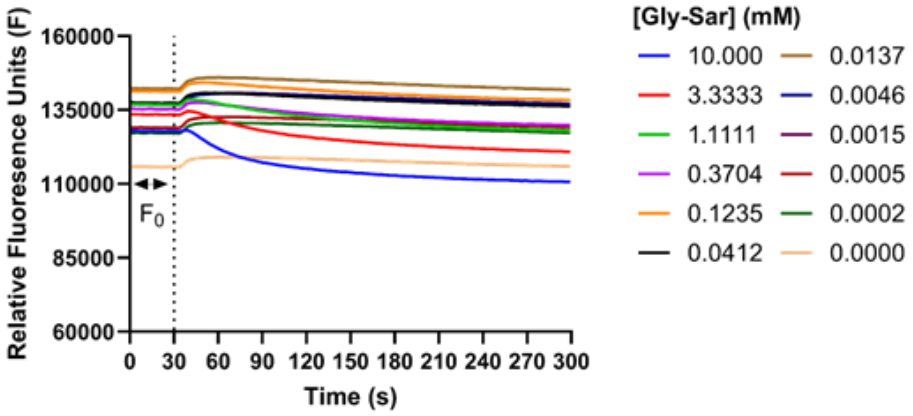

Figure 1. Non-ratiometric Raw Fluorescence Data. Raw fluorescence data of PepT1-expressing HEK293T cells collected using Wavefront Panoptic (Excitation: 477 nm, Emission: 515(30) nm). Relative Fluorescence Units (F) were collected for 30 seconds before the addition of the Stimulus Solution. After addition, the plate continued to be read for an additional 5 minutes.

Data Analysis (Continued)

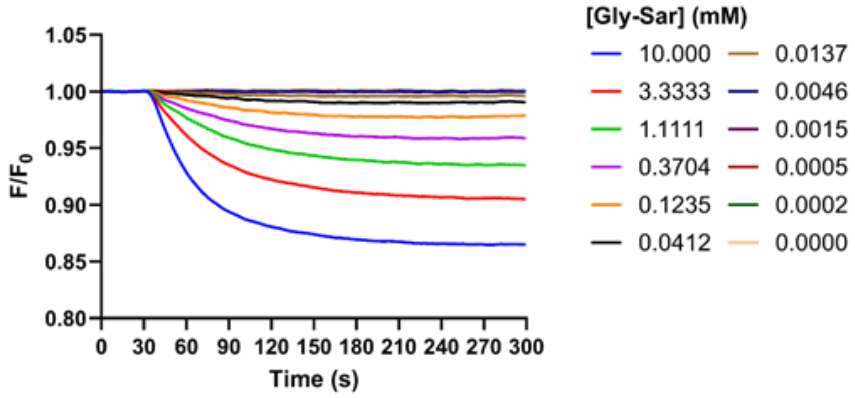

Figure 2. Baseline-Normalized Fluorescence Data. Based on the raw fluorescence data from figure 1, average the Relative Fluorescence Units (F) of the initial 30 seconds before stimulus addition as the baseline (F_0). Calculate the ratio of Relative Fluorescence Units (F) over the baseline (F_0) at each time point as F/F_0. Plot F/F_0 over time as the Baseline-Normalized Fluorescence Data.

Ratiometric Analysis

4. Divide the relative fluorescence units from the ~490 nm excitation wavelength ($F_{\sim490}$) by the relative fluorescence units from the ~430 nm excitation wavelength ($F_{\sim430}$) to obtain the ratiometric signal ($F_{\sim490/430}$). Plot $F_{\sim490/430}$ over time as the ratiometric fluorescence data (**Figure 3**, below).
5. Generate a calibration curve by plotting $F_{\sim490/430}$ against solutions of known pH. We recommend using AAT Bioquest's Spexyte™ Intracellular pH Calibration Buffer Kit for this step (**Figure 4**, next page).
6. Use GraphPad® Prism (or another similarly capable graphing and statistical analysis software) to interpolate the ratiometric fluorescence data ($F_{\sim490/430}$) into intracellular pH (pH_i) using the calibration curve. Plot pH_i over time as the intracellular pH data (**Figure 4**, next page).

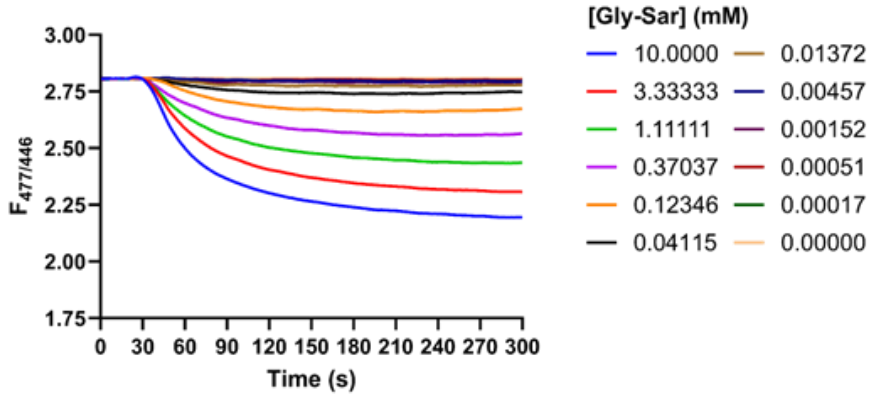

Figure 3. Ratiometric Fluorescence Data. Ratiometric fluorescence data of PepT1-expressing HEK293T cells collected using Wavefront Panoptic (Excitation: 446, 477 nm, Emission: 515(30) nm). Relative fluorescence units were collected at the 477 nm excitation wavelength (F_{477}) and at the 446 nm excitation wavelength (F_{446}) were collected for 30 seconds before the addition of the Stimulus Solution. After addition, the plate continued to be read for an additional 5 minutes. Calculate the ratio of F_{477} over F_{446} at each time point as $F_{477/446.}$ Plot $F_{477/446}$ over time as the Ratiometric Fluorescence Data.

Data Analysis (Continued)

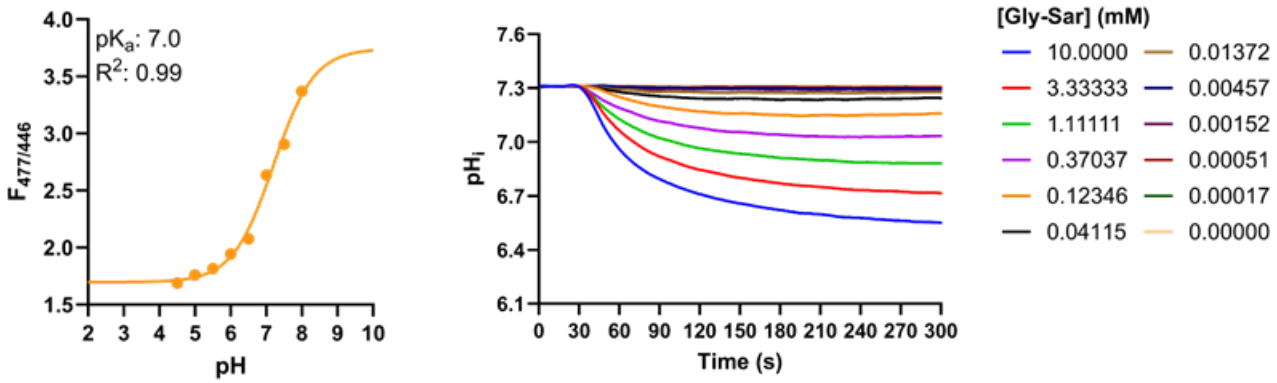

Figure 4. Intracellular pH Data. Ratiometric fluorescence data of HEK293T cells collected using Wavefront Panoptic (Excitation: 446, 477 nm, Emission: 515(30) nm). A calibration curve was generated using AAT Bioquests' Intracellular pH Calibration Kit by plotting the collected ratiometric fluorescence data of BCECF against solutions of known pH. Based on the ratiometric fluorescence data from figure 3, the $F_{477/446}$ was interpolated into intracellular pH (pH_i) values using the BCECF calibration curve. The pH_i was plotted over time as the Intracellular pH Data.

Kinetic Reduction and Concentration-Response Analysis

7. Use GraphPad® Prism (or another similarly capable graphing and statistical analysis software) to apply an Area Under Curve (AUC) analysis on the baseline-normalized fluorescence data (non-ratiometric) or the intracellular pH data (ratiometric) by measuring the area between the curve and the baseline. Plot the AUC against compound concentration to make a concentration response curve (**Figure 5**, below).
8. Using GraphPad® Prism, run the non-linear regression "agonist vs response" to calculate the half-maximal effective concentration (EC_{50}). Alternatively, run the non-linear regression "inhibitor vs response" to calculate the half-maximal inhibitory concentration (IC_{50}) (**Figure 6**, next page).

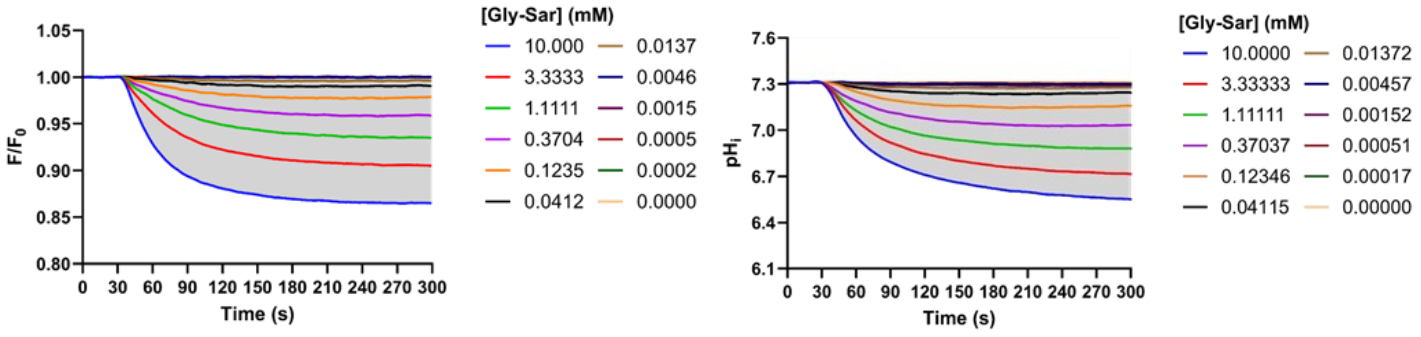

Figure 5. Area Under Curve (AUC) Calculation. Based on the Baseline-Normalized Fluorescence Data from figure 2 (non-ratiometric) or the Intracellular pH Data from figure 4 (ratiometric), calculate the area between the curve and the baseline and define it as the AUC.

Data Analysis (Continued)

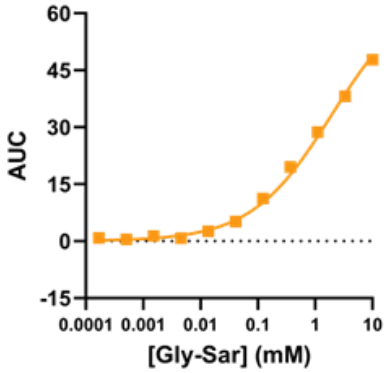

Figure 6. EC_{50} Calculation. Plot the AUC against the compound concentrations and run the non-linear regression "agonist vs response" to calculate an EC_{50}. Alternatively, run the non-linear regression "inhibitor vs response" in GraphPad® Prism to calculate an IC_{50}.

Example Results

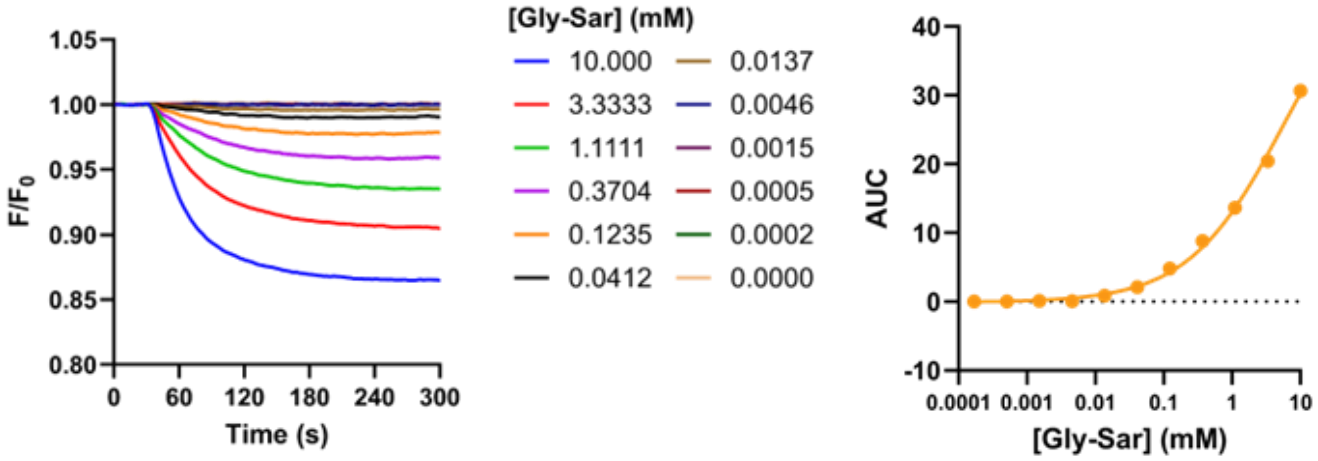

Figure 7. Non-ratiometric Gly-Sar dose response in PepT1-Expressing HEK293 Cells. Baseline-normalized fluorescence data (non-ratiometric) collected using Wavefront Panoptic (Excitation: 477 nm, Emission: 515(30) nm). Gly-Sar, a substrate of the proton-coupled peptide transporter 1 (PepT1), was added at 30 seconds. The AUC was calculated between the curve and the baseline at y=1. Error bars indicate the SEM (n = 3). Increasing concentrations of Gly-Sar caused a dose dependent decrease in fluorescence. An EC_{50} could not be determined because PepT1, a low-affinity, high-capacity proton-coupled transporter, could not be saturated at pH 7.3.

Example Results (Continued)

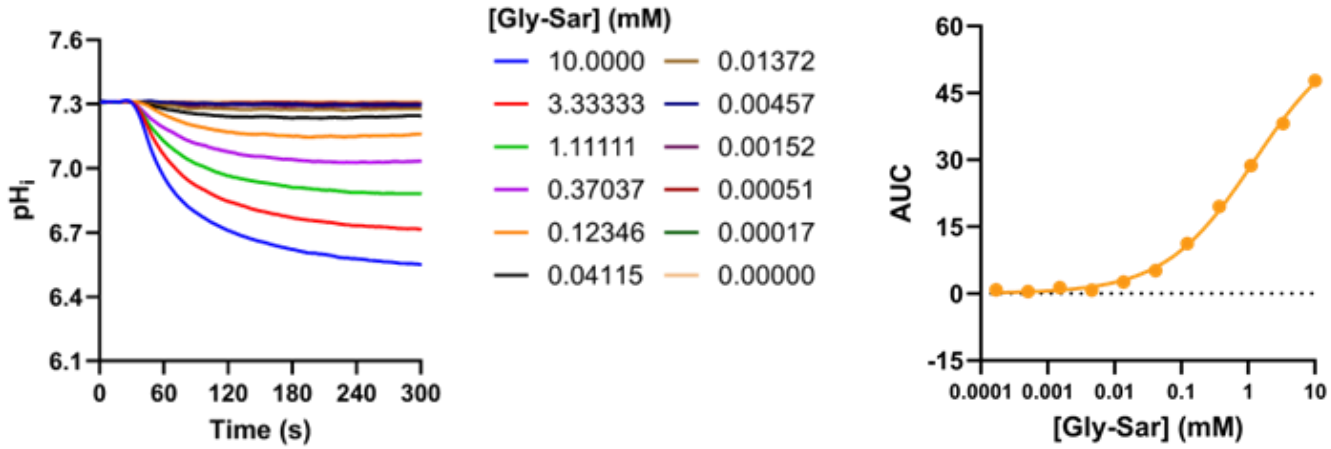

Figure 8. Ratiometric Gly-Sar dose response in PepT1-Expressing HEK293 Cells. Intracellular pH data (ratiometric) collected using Wavefront Panoptic (Excitation: 446, 477 nm, Emission: 515(30) nm). Gly-Sar, a substrate of the proton-coupled peptide transporter 1 (PepT1), was added at 30 seconds. The AUC was calculated between the curve and the baseline. Error bars indicate the SEM (n = 3). Increasing concentrations of Gly-Sar caused a dose dependent decrease in intracellular pH. An EC_{50} could not be determined because PepT1, a low-affinity, high-capacity proton-coupled transporter, could not be saturated at pH 7.3.

Additional Information

Additional dye indicator and buffer reagents can be purchased either directly from our website or by contacting our Sales Department. Custom and bulk sizes are also available. Contact Sales for more information.

Table 7	Additional Reagents	Available Sizes	
Kit Label	**Name**	**Size**	**Catalog #**
Reagent A	BCECF AM	1 mg x 1 Vial 50 µg x 20 Vials	4011B 4011E
Reagent C	50X DySolv	20 mL Bottle	7501A
Reagent D	10X Brilliant Assay Buffer	10 mL Bottle	7010X
Reagent E	50X TRS	20 mL Bottle	7060A
Reagent F	50X Probenecid	20 mL Bottle	7300P-50