Cell Meter™ Fluorimetric Intracellular pH Assay Kit

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 21180 (10 plates)	Freeze and avoid light	FLIPR, FDSS, NOVOStar, FlexStation, ViewLux, IN Cell Analyzer, ArrayScan

Introduction

Intracellular pH change are implicated in diverse physiological and pathological processes, including cell proliferation, apoptosis, fertilization, malignancy, multidrug resistance, ion transport, lysosomal storage disorders and Alzheimer's disease. The Cell Meter™ Fluorimetric Intracellular pH Assay Kit utilizes AAT Bioquest's proprietary fluorescent indicator for measuring the relative intracellular pH changes. It is a homogeneous, kinetic, live-cell fluorescent assay that utilizes either a standard procedure or acid-load procedure. The standard protocol is designed for measuring the therapeutic targets of interest with a decrease in intracellular pH upon treatment. The 'Acid-Load' procedure is designed to measure the increase of intracellular pH associated with changes in cellular metabolism due to GPCR activation or growth factor activity. With the 'Acid-Load' procedure ammonium chloride solution is added after the fluorescent pH dye is loaded into cells in a minimum volume. This 'acid-loading' step is followed by the addition of agonist in a relatively large volume (~4X) of buffer. The sudden volume change initiates an efflux of ammonia (NH_3) from the cells causing a rapid decrease in intracellular pH, and thus a decrease in fluorescence signal. The effect of agonist on the subsequent recovery of intracellular pH is measured by the relative fluorescence signal increase.

Kit Components

Components	**21180 (10 plates)**
Component A: RatioWorks™ BCFL, AM	1 vial
Component B: 10X Pluronic F127 Plus	1 bottle (10 mL)
Component C: HHBS (Hanks' buffer with 20 mM Hepes)	1 bottle (100 mL)
Component D: 50 mM ReadiUse™ probenecid	1 bottle (10 mL)

I. Assay Protocol for Standard Cell Load (One Plate)

Brief Summary

Prepare cells in growth medium→ Add equal volume of RatioWorks™ BCFL, AM dye-loading solution (100 µL/well for 96-well plate)→ Incubate at 37°C for 1 hour→ Read Fluorescence at Ex/Em= 490/535 nm with 50 µL/well compound addition (or 490/535 and 430/535 nm for ratio)

1. Prepare Cells:

1.1 For adherent cells, plate cells overnight in growth medium at 40,000 to 80,000 cells/well/100µL for 96-well or 10,000 to 20,000 cells/well/25µL for 384-well plates.

1.2 For non-adherent cells, centrifuge the cells from the culture medium and then suspend the cell pellets in growth medium at 125,000 to 250,000 cells/well/100µL for 96-well or 30,000 to 60,000 cells/well/25µL for 384-well poly-D lysine plates. Centrifuge the plates at 800 rpm for 2 minutes with break off prior to the experiments
Note: Each cell line should be evaluated on an individual basis to determine the optimal cell density.

2. Prepare RatioWorks™ BCFL, AM dye-loading solution (for 1 plate):

2.1 Thaw all the Components at room temperature before use.

2.2 Make RatioWorks™ BCFL, AM stock solution by adding 200 µL DMSO into Component A (RatioWorks™ BCFL, AM) and mixing them well.

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Note: 20 µL of reconstituted RatioWorks™ BCFL, AM is enough for 1 plate, unused RatioWorks™ BCFL, AM can be aliquoted and stored at ≤-20°C for one month if the tubes are sealed tightly, avoided light and repeated freeze-thaw cycles.

2.3 Make 1X assay buffer by adding 1 **mL** of Component B (10X Pluronic F127 Plus) into **9 mL of** Component C **(**HHBS), mix them well.
Note: For cells that require probenecid for loading (e.g. CHO cells), dilute 50 mM ReadiUse™ probenecid (Component C) at concentration of 1 to 5 mM (prefer 5 mM for CHO cells).

2.4 Make RatioWorks™ BCFL, AM dye-loading solution for one cell plate by adding 20 µL of DMSO reconstituted RatioWorks™ BCFL, AM (from Step 2.2) into 10 mL of 1X assay buffer (from Step 2.3), mixing them well. This working solution is stable for at least 2 hours at room temperature.

3. Run pH Assay

3.1 Add 100 µL/well (96-well plate) or 25 µL/well (384-well plate) RatioWorks™ BCFL, AM dye-loading solution into the cell plate (from Step 2.4).
Note: It is important to replace the growth medium with HHBS buffer (100 µL/well for 96-well plate or 25 µL/well for 384-well plate before dye-loading) if your compounds interfere with the serum.

3.2 Incubate the dye-loading plate at cell incubator for 30 minutes, and then incubate the plate at room temperature for another 30 minutes.
Note: If the assay requires 37°C, perform the experiment immediately without further room temperature incubation.

3.3 Prepare the compound plates by using HHBS or your desired buffer.

3.4 Run the pH assay by monitoring the fluorescence at Ex/Em = 490/535 nm (cut off at 515 nm) or 490/535 nm and 430/535 nm (cut off at 515 nm) for ratio measurements. The compound addition is 50 µL/well (96-well plate) or 25 µL/well (384-well plate).
Note: The assay should be complete within 3 to 5 min after compound addition, however a minimum of 8 min data collection are recommended for during assay development.

II. Assay Protocol for Acid-Load (One 96-well Plate)

Brief Summary

Prepare cells in growth medium→ Remove the growth medium → Add 50 µL/well /96-well plate of RatioWorks™ BCFL, AM dye-loading solution → Incubate at 37°C for 1 hour → Add 5 µL/well of 220 mM NH_4Cl → Incubate at RT for 15 minutes → Read Fluorescence at Ex/Em= 490/535 nm with 200 µL/well compound addition (or 490/525 and 440/535 nm for ratio)

1. Prepare Cells:

1.1 For adherent cells, plate cells overnight in growth medium at 40,000 to 80,000 cells/well/100µL for 96-well plates.

1.2 For non-adherent cells, centrifuge the cells from the culture medium and then suspend the cell pellets in growth medium at 125,000 to 250,000 cells/well/100µL for 96-well poly-D lysine plates. Centrifuge the plates at 800 rpm for 2 minutes with break off prior to the experiments

Note: Each cell line should be evaluated on an individual basis to determine the optimal cell density.

2. Prepare RatioWorks™ BCFL, AM dye-loading solution (for 1 plate):

2.1 Thaw all the Components at room temperature before use.

2.2 Make RatioWorks™ BCFL, AM stock solution by adding 200 µL DMSO into Component A (RatioWorks™ BCFL, AM) and mixing them well.
Note: 10 µL of reconstituted RatioWorks™ BCFL, AM is enough for 1 plate, unused RatioWorks™ BCFL, AM can be aliquoted and stored at ≤-20°C for one month if the tubes are sealed tightly, avoided light and repeated freeze-thaw cycles.

2.3 Make 1X assay buffer by adding 1 **mL** of Component B (10X Pluronic F127 Plus) into 4 **mL of** Component C **(**HHBS), mix them well.
Note: For cells that require probenecid for loading (e.g. CHO cells), dilute 50 mM ReadiUse™ probenecid (Component C) at concentration of 0.5 to 2.5 mM (prefer 2.5 mM for CHO cells).

2.4 Make RatioWorks™ BCFL, AM dye-loading solution for one cell plate by adding 10 µL of DMSO reconstituted RatioWorks™ BCFL, AM

(from Step 2.2) into 5 mL of 1X assay buffer (from Step 2.3), mixing them well. This working solution is stable for at least 2 hours at room temperature.

3. Run Calcium Assay

3.1 Remove the growth medium from the cell plate.

3.2 Add 50 µL/well/96-well plate RatioWorks™ BCFL, AM dye-loading solution into the cell plate (from Step 2.4).

3.3 Incubate the dye-loading plate at cell incubator for 30 minutes, and then incubate the plate at room temperature for another 30 minutes.
Note: If the assay requires 37°C, perform the experiment immediately without further room temperature incubation.

3.4 Add 5 µL of 220 mM NH_4Cl and then centrifuge the plates for 5 seconds, and Incubate 15 minutes at room temperature.
Note: NH_4Cl solution should be prepared freshly in HHBS (Component C).

3.5 Prepare the compound plates by using HHBS or your desired buffer.

3.6 Run the calcium flux assay by monitoring the fluorescence at Ex/Em = 490/535 nm (cut off at 515 nm) or 490/535 nm and 430/535 nm (cut off at 515 nm) for ratio measurements. The compound addition is 200 µL/well /96-well plate.
Note: The assay should be complete within 3 to 5 min after compound addition, however a minimum of 8 min data collection are recommended for during assay development.

Data Analysis

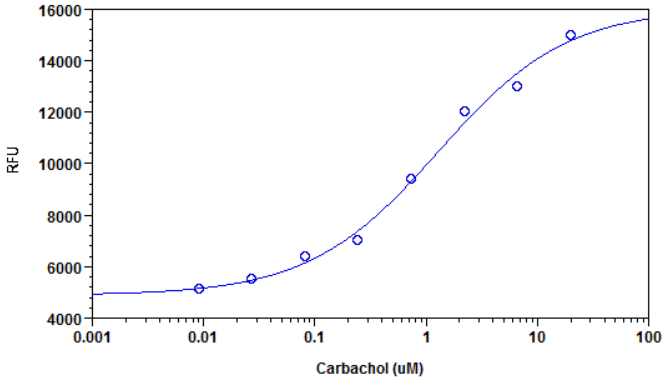

Figure1. Carbachol dose response in CHO-M1 cell. CHO-M1cells were seeded overnight in 60,000 cells per 100 µL per well in a 96-well black wall/clear bottom costar plate. The growth medium was replaced with 50 µL/well of RatioWorks™ BCFL, AM dye-loading solution for 37°C for 1 hour, follow by 15 minutes incubation with 5 µL/well of 220 mM NH_4Cl. Carbachol (200µL/well) was added by FlexStation (Molecular Devices) to achieve the final indicated concentrations.

Warning: This kit is only sold for the end users. It is covered by a pending patent. Neither resale nor transfer to a third party is allowed without written permission from AATBioquest®. Chemical analysis of kit components is strictly prohibited. Please call 408-733-1055 or e-mail us at info@aatbio.com if you have any questions.

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