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Product Information

Fluorometric Intracellular pH Assay Kit

Catalog Number **MAK150**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Intracellular pH changes 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 Fluorometric Intracellular pH Assay Kit utilizes a proprietary cell-permeable fluorescent indicator, BCFL-AM, for measuring relative intracellular pH changes. BCFL-AM is a superior alternative to the popular ratiometric pH probe, 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF). Unlike BCECF, which is a complex and variable mixture of isomers, BCFL-AM is a single isomer species. BCFL-AM has the same spectral responses as BCECF-AM but enhanced reproducibility. BCFL-AM exhibits pH-dependent dual excitability, pK_a ~7.0, and an excitation isosbestic point of 454 nm.

This kit can be utilized to measure decreases in intracellular pH in cells treated under various conditions using a simple standard protocol. Alternatively, an Acid-Load procedure can be used to measure the increase of intracellular pH associated with changes in cellular metabolism due to GPCR activation, growth factor activity, or other cellular stimulation. 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 (~4×) 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.

Components

The kit is sufficient for assaying ten 96 well plates.

BCFL-AM Catalog Number MAK150A	1 vl
10× Dye Solubilization Buffer Catalog Number MAK150B	10 mL
HHBS (Hank's Buffer with 20 mM HEPES) Catalog Number MAK150C	100 mL
Probenecid, 50 mM Catalog Number MAK150D	10 mL

Reagents and Equipment Required but Not Provided.

- 96 well flat-bottom plate – It is recommended to use black plates with clear bottoms for fluorometric assays.
- Fluorescence multiwell plate reader, flow cytometer, or other compatible platform.
- Ammonium chloride (Catalog Number A9434 or equivalent)

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped under ambient conditions and storage at –20 °C, protected from light, is recommended.

Preparation Instructions
Allow reagents to come to room temperature before use. Briefly centrifuge vials before opening.

BCFL-AM Reagent – Add 200 µL of DMSO to generate the BCFL-AM Reagent. Mix well by pipetting, aliquot, and store at –20 °C. Stable for 1 month when stored at –20 °C.

Sample Preparation for One Plate
Adherent cells: Plate cells overnight in growth medium at 40,000–80,000 cells/well/100 µL for a 96 well plate or 10,000–20,000 cells/well/25 µL for a 384 well plate.

Non-adherent cells: Centrifuge the cells from the culture medium and resuspend the cell pellets with culture medium in poly-D-lysine-coated plates at 125,000–250,000 cells/well/100 µL for a 96 well plate or 30,000–60,000 cells/well/25 µL for a 384 well plate. Centrifuge the plate at 800 rpm for 2 minutes with brake off prior to the experiment.

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

Procedure
All samples and standards should be run in duplicate.

Assay Reaction for Standard Cell Load (one plate)
1. Prepare the 1× Assay Buffer by adding 1 mL of 10× Dye Solubilization Buffer to 9 mL of HHBS. Mix well by pipetting.

2. Set up the Dye Loading Solution according to the scheme in Table 1.

Table 1.
Dye Loading Solution

Reagent	Volume
BCFL-AM Reagent	20 µL
1× Assay Buffer	10 mL

Notes: The Dye Loading Solution is enough for one plate. It is best used within 2 hours.

Probenecid is a nonspecific inhibitor of anion exchange transporters. In certain cell types such as HeLa and CHO, high anion exchange activity can result in dye extrusion and poor dye loading. For cells that require probenecid for loading, dilute 50 mM Probenecid to a concentration of 1–5 mM (5 mM preferred for CHO cells) in the Dye Loading Solution.

3. Add 100 µL/well (96 well plate) or 25 µL/well (384 well plate) of Dye Loading Solution into the cell plate.
   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 compounds used for cell treatment interfere with the serum in the medium.

4. Incubate the cells, protected from light, in a 5% CO_2, 37 °C incubator for 30 minutes, followed by incubation at room temperature for an additional 30 minutes.
   Note: If the assay requires 37 °C incubation, perform the experiment immediately without further room temperature incubation.

5. Prepare the compound to be tested by using HHBS or other suitable desired buffer. The compounds are added to the assay plate at 50 µL/well (96 well plate) or 25 µL/well (384 well plate).

6. Run the pH assay by measuring the fluorescence at λ_{ex} = 490/λ_{em} = 535 nm (cut off at 515 nm) or λ_{ex} = 505/λ_{em} = 535 nm, and λ_{ex} = 430/λ_{em} = 535 nm (cut off at 515 nm) for ratio measurements. The single wavelength (λ_{ex} = 490/λ_{em} = 535) can be used for most instruments. The ratio measurement will give better separation.

Note: The assay should be complete within 3–5 minutes after compound addition; however, a minimum of 8 minutes of data collection are recommended during assay development.

Assay Reaction for Acid-Load (one plate)

1. Prepare the 1× Assay Buffer by adding 1 mL of 10× Dye Solubilization Buffer to 4 mL of HHBS. Mix well by pipetting.

2. Set up the Dye Loading Solution according to the scheme in Table 1.

Table 1.
Dye Loading Solution

Reagent	**Volume**
BCFL-AM Reagent	10 μL
1× Assay Buffer	5 mL

Notes: The Dye Loading Solution is enough for one plate. It is best used within 2 hours.

Probenecid is a nonspecific inhibitor of anion exchange transporters. In certain cell types such as HeLa and CHO, high anion exchange activity can result in dye extrusion and poor dye loading. For cells that require probenecid for loading, dilute 50 mM Probenecid to a concentration of 0.5–2.5 mM (2.5 mM preferred for CHO cells) in the Dye Loading Solution.

3. Remove growth medium from the cell plate. Add 50 μL/well (96-well plate) of Dye Loading Solution to the cell plate.

4. Incubate the cells, protected from light, in a 5% CO_2, 37 °C incubator for 30 minutes, followed by incubation at room temperature for an additional 30 minutes.
   Note: If the assay requires 37 °C incubation, perform the experiment immediately without further room temperature incubation.

5. Add 5 μL of 220 mM NH_4Cl (freshly prepared in HHBS) to each well then centrifuge the plates for 5 seconds. Incubate 15 minutes at room temperature.

6. Prepare the compound to be tested by using HHBS or desired buffer. The compound addition is 200 μL/well /96 well plate

7. Run the pH assay by monitoring the fluorescence at λ_{ex} = 490/λ_{em} = 535 nm (cut off at 515 nm) or λ_{ex} = 505/λ_{em} = 535 nm, and λ_{ex} = 430/ λ_{em} = 535 nm (cut off at 515 nm) for ratio measurements. The single wavelength (λ_{ex} = 490/λ_{em} = 535) can be used for most instruments. The ratio measurement will give better separation.

Note: The assay should be complete within 3–5 minutes after compound addition; however, a minimum of 8 minutes of data collection are recommended during assay development.

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay not working	Cold assay buffer	Assay Buffer must be at room temperature
	Omission of step in procedure	Refer and follow Technical Bulletin precisely
	Plate reader at incorrect wavelength	Check filter settings of instrument
	Type of 96 well plate used	For fluorometric assays, use black plates
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Incorrect incubation times or temperatures	Refer to Technical Bulletin and verify correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly
Unanticipated results	Samples measured at incorrect wavelength	Check the equipment and filter settings

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