GeneCopoeia™
Expressway to Discovery

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Product Information

BCECF and BCECF, AM

Catalog Number	Product Name	Unit Size
C027	BCECF	5 mg
C028	BCECF, AM	1 mg
C029	BCFCF, AM	500 µL

Storage upon receipt:
- -20°C
- Protect from light

Product Description

BCECF (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxy fluorescein), introduced by Roger Tsien and co-workers in 1982, is the most widely used fluorescent indicator for intracellular pH. Several characteristics contribute to this widespread utility:

The pKa of 7.0 is ideally matched to the normal range of cytoplasmic pH (~6.8–7.4).

The fluorescence excitation profile is pH-dependent, allowing the implementation of ratiometric measurement techniques.

The absorption maximum of the base form of BCECF is very close to the 488-nm argonion laser line, making it ideally suited for flow cytometry and confocal microscopy applications.

BCECF has 4–5 negative charges at pH 7–8, aiding intracellular retention.

The acetoxymethyl (AM) ester derivative is membrane-permeant, allowing noninvasive bulk loading of cell suspensions.

BCECF AM is nonfluorescent. Its conversion to fluorescent BCECF via the action of intracellular esterases can be used as an indicator of cell viability.

Modification of carboxylic acids with AM ester groups results in an uncharged molecule that can permeate cell membranes. Once inside the cell, the lipophilic blocking groups are cleaved by nonspecific esterases, resulting in a charged form that leaks out of cells far more slowly than its parent compound.

Although applications in mammalian cells are predominant, BCECF has also been employed for pH measurements in perfused tissues, intercellular spaces, plant cells, bacteria and yeast. BCECF AM has also been employed in assays for various functional properties of cells including viability and cytotoxicity, apoptosis, adhesion, multidrug resistance and chemotaxis.

Guidelines for Use

Preparing BCECF AM Stock Solutions

Stock solutions of **BCECF AM** should be reconstituted only as required and should be prepared by dissolving the solid material in high-quality *anhydrous* DMSO at 1–10 mM. DMSO stock solutions should be stored desiccated at ≤–20°C, and preferably used within a short period of time for one series of experiments. Stock solutions of BCECF AM should be stable for at least 6 months if prepared and stored as directed above. Solutions exhibiting strong fluorescence and coloration (indicated by absorbance at >400 nm) probably contain a significant amount of hydrolyzed material and should be discarded. Dilute working solutions in aqueous media should be used immediately and should not be stored. BCECF AM is normally colorless and nonfluorescent (although faint color andfluorescence are tolerable).

Loading BCECF AM in Mammalian Cells

Most mammalian cells can be loaded without permeabilization by incubation with dilute aqueous dispersions of cell-permeant BCECF AM. Once within the cell, nonspecific esterases hydrolyze the nonfluorescent AM ester precursor, yielding the fluorescent, pH-sensitive indicator. The low leakage rate of the polyanionic indicator and the small intracellular volume results in the final intracellular concentration being much higher than the external incubation concentration. In general, BCECF loaded via the AM ester method appears less susceptible to intracellular compartmentalization than calcium indicators such as fura-2. A variety of physical evidence has been assembled indicating that BCECF remains free and dissociated within the cytoplasm. The following protocol is recommended as an approximate guide to the loading conditions for BCECF AM:

1. Prepare viable cells in suspension (~10^6 cells/mL).

Note: Conditions for loading adherent cells are similar to those given for cells in suspension.

Adherent cells in culture dishes may be immersed in AM ester loading solution in physiological saline (step 2). To load cells grown on coverslips, simply transfer the coverslips to a dish containing BCECF AM loading medium after gently pouring off the maintenance medium. Adherent cultures do not need to be lifted for loading.

2. Dilute an aliquot of 1 mM AM ester stock solution 100- to 500-fold into a physiological saline buffer such as Hanks' buffered salt solution (HBSS). In general, the minimum concentration of AM ester necessary to obtain an adequate signal should be used (incubation concentrations as low as 0.1 µM may be sufficient) to minimize accumulation of the by-products of AM ester hydrolysis (formaldehyde and acetic acid) and to help facilitate a more complete hydrolysis. The loading medium should be free of amino acids or buffers containing primary or secondary amines, since aliphatic amines may cleave the AM esters and prevent loading, and to keep extracellular hydrolysis of the AM ester to a minimum. The presence of serum, which may contain endogenous esterase activity, should also be avoided until after loading is complete.

3. Add one volume of aqueous AM ester dispersion to one volume of cell suspension. Incubate for 15–60 minutes at 4°C to 37°C.

4. Wash the cells twice with fresh culture medium.

Application to Tissue Samples

Generally similar BCECF AM loading protocols to that described in *Loading BCECF AM in Mammalian Cells* have been successfully applied to a variety of tissue samples including rat arteries; rat salivary glands and pancreas; rabbit kidney collecting tubules and rabbit gastric glands. In most of these experiments, the tissue sample is mounted in a perfusion chamber and BCECF AM (~1–5 µM) is added to the perfusate for 5–60 minutes, followed by extensive washing with unmodified perfusate. For pH measurements in intercellular spaces, loading can be performed by direct

injection of a brief pulse of 0.2–0.5 mM BCECF acid. BCECF acid (50–100 µM) can be loaded into isolated cells or tissue slices by diffusion from a patch pipette for correlated fluorescence imaging and electrophysiological recording.

Loading in Other Cell Types

Bacteria: Both gram-positive and gram-negative bacteria have been loaded with BCECF by subjecting a dense cell suspension to a brief acid shock (50 mM HCl for 5 minutes) in the presence of 0.5 mM BCECF acid. The indicator appears to be well retained in *Lactococcus lactis* incubated on ice; however energization with lactose stimulates rapid efflux.

Yeast and Fungi: Attempts to load the yeast *Saccharomyces cerevisiae* by incubation with 10 µM BCECF AM do not yield particularly good uptake of the indicator, apparently because of inefficient AM ester hydrolysis by the yeast intracellular es-terases. Loading of the fungus *Neurospora crassa* with BCECF AM reportedly results in accumulation of the indicator in vacuoles instead of an even cytoplasmic distribution.

Plants: Loading of suspended protoplasts (2×10^6/mL) from the crabgrass *Digitaria sanguinalis* by incubation with low concentrations of BCECF AM (10 nM) produces an even cytosolic distribution of the indicator. Higher incubation concentrations (3 µM) used to load maize root hair cells result in vacuolar accumulation.

Intracellular pH Calibration

The pH-dependent spectral shifts exhibited by BCECF allow calibration of the pH response in terms of the **ratio** of fluorescence intensities measured at two different excitation wavelengths (equation 1). A number of fluorescence measurement artifacts are eliminated in formulation of the ratio, including photobleaching, leakage and nonuniform loading of the indicator, cell thickness, and instrument stability.

$$\left[H^+ \right] = K_a \frac{(R-R_A)}{(R_B-R)} X \frac{F_{A(\lambda 2)}}{F_{B(\lambda 2)}} \quad \textbf{(1)}$$

where R is the ratio $F_{(\lambda 1)}/F_{(\lambda 2)}$ of fluorescence intensities (F) measured at two wavelengths λ1 and λ2 and the subscripts A and B represent the limiting values at the acidic and basic endpoints of the titration, respectively. Note that background fluorescence corrections should be subtracted **before** calculation of R. A typical BCECF calibration would use a dual-excitation ratio with λ1 = 490 nm and λ2 = 440 nm and fixed emission at 535 nm. Dual-excitation filter sets for fluorescence microscopy applications are available from

Omega Optical Inc. (www.omegafilters.com, set XF16) and Chroma Technology Corp. (www.chroma.com, set 71001).

For confocal microscopy, helium–cadmium laser output at 442 nm has been used for excitation in combination with the 488 nm argon-ion laser line. Note that selection of λ2 at the pH-independent isosbestic point (~439 nm for BCECF) eliminates the normalization factor FA(λ2)/FB(λ2) from equation (1). Although the emission spectral profile of BCECF is much less pH-dependent than that of the excitation spectrum, 525/640 nm fluorescence emission ratios

(excited at 488 nm) are occasionally used for flow cytometric pH measurements.

The logarithmic form of the equation (1) is:

$$pH = pK_a - \log \frac{(R-R_A)}{(R_B-R)} X \frac{F_{A(\lambda 2)}}{F_{B(\lambda 2)}} \quad \textbf{(2)}$$

In this form, the data should yield a linear plot with a slope of 1 and an intercept equal to the pKa. Calibrating the fluorescence response of BCECF to pH-controlled buffers *in vitro* yields a pKa of 7.0. However, since the response may be somewhat different when the dye is loaded in cells, *in situ* calibration is generally advisable for each experimental system. *In situ* calibration can be performed by using the ionophore nigericin at a concentration of 10–50 µM in the presence of 100–150 mM potassium to equilibrate the intracellular pH with the controlled extracellular medium. A number of alternative *in situ* calibration methods for BCECF have been described in the literature.

Cell Function Assays

Cytotoxicity assays using BCECF AM are based on the generation of fluorescence by intracellular esterase action and its retention correlated with membrane integrity as indicators of cell viability. Cells are loaded with BCECF AM following protocols similar to that described in *Loading in Mammalian Cells*, and cytotoxicity is expressed in terms of either the fluorescence remaining associated with the cells or the fluorescence released into the supernatant, relative to the initial cellular fluorescence prior to the cytotoxic treatment. Cytotoxicity assays using BCECF are suitable for implementation on high-throughput fluorescence microplate readers and provide rapid and convenient alternatives to ^{51}Cr release assays. Cell adhesion assays using BCECF AM employ similar principles. In a typical example, CHO cells were seeded in microplate wells, to which were then added murine R1.1 cells loaded by incubation with 2 µM BCECF AM at 37°C for 30 minutes. After incubation for 20 minutes, the plates were washed and the number of R1.1 cells bound was determined by proportionality to the remaining BCECF fluorescence measured using a fluorescence microplate reader.