Elite™ Fluorescent Membrane Potential Dye Kit

CATALOG NUMBER: CA-M165-10

Description

cAMP is a key second messenger involved extensively in cellular signal transduction pathways associated with most of the G-protein coupled receptors (GPCRs). The activation of these GPCRs by neurotransmitters, lipids, nucleotides, peptides and hormones results in the activation or the inhibition of plasma membrane-bound adenylate cyclase through heterotrimeric G-proteins. This cAMP assay is the first in its class in providing real-time information on intracellular cAMP changes in a high-throughput format without a cell lysis step. The channel is activated by elevated intracellular levels of cAMP, resulting in ion flux (often detectable by calcium-responsive dyes) and cell membrane depolarization [which can be detected with a fluorescent membrane potential (MP) dye].

The Elite™ fluorescent membrane potential dye kit allows both end-point and kinetic measurement of intracellular cAMP changes with a FLIPR, or a fluorescence microplate reader. It is a simple homogenous assay involving only dye and compound addition steps, allowing easy implementation in a high-throughput environment.

Application

- High throughput screening of GPCR compounds using ACTOne™ GPCR stable cell lines

Features

- **Continuous**: Easily adapted to automation without a separation step
- **Convenient**: Formulated to have minimal hands-on time. No interference with magnesium
- **Non-radioactive**: No special requirement for waste disposal

Kit Components

• **Component A:** 10x Elite™ Fluorescent Membrane Potential Dye Solution	10 ml
• **Component B:** 10x Dye Dilution Buffer	11 ml
• **Component C:** Ro 20-1724 (50 mM), PDE inhibitor	120 µl

Storage

Keep in **Component A & C** in freezer (-20 °C) and avoid exposure to light; **Component B** at room temperature.

Materials Required (but not supplied)

- Dulbecco's Phosphate Buffered Saline (DPBS) (Cat# D8537, Sigma)
- Water (Cat# 320072, Sigma)
- 96 or 384-well microplates: Tissue culture microplate with black wall and clear bottom is recommended
- FlexStation (Molecular Device) or a fluorescence microplate reader

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Assay Protocol for 96-Well/or 384-Well Plate

Thaw all the kit components to room temperature before starting the experiment.

1. **Cell Preparation:**
   1.1 Harvest cells when they reach 80-90% confluence in flasks. Trypsinize cells as described in Cell Culture Protocol above. Count a portion of the cells with a hemocytometer.

   Note: *Do not let the cells reach >90% confluence. Over-confluent growth can result in a significantly reduced response to ligands, and it may take several passages for the cells to recover to optimal stage.*

   1.2 The cell number needs to be optimized for each assay. Optimal assay conditions require a confluent monolayer of cells prior to the assay. Poly-D-Lysine coated plates are recommended to improve cell attachment. Plate 70K cells/well for a 96-well plate (14K cells/well for a 384 well plate) the day before the experiment. Add 100 µl/well of cell suspension to 96-well plates (20 µl/well to 384-well plates).

   1.3 Allow cells to attach by leaving the cell plates at room temperature for 30 minutes. Transfer the plates to a cell culture incubator and grow the cells overnight.

2. **Preparation of 1x Dye-Loading Solution**:
   2.1 Remove **Component A (**10x Elite™ Fluorescent Membrane Potential Dye Solution) from -20°C, and allow it to thaw at room temperature.

   2.2 Preparation of 1x Dye Dilution Buffer: add 10 ml of **Component B** to 90 ml of deionized-distilled or reverse osmosis H_2O, mix well.

   2.3 Preparation of 1x Dye-Loading Solution (10 plates): pipette 10 ml of **Component A** (10x Elite™ Fluorescent Membrane Potential Dye Solution) to 90 ml of 1x Dye Dilution Buffer, mix well.

   2.4 Aliquot the unused 1x Dye Loading Solution to several tubes and store at -20°C. Store 1x Dye Dilution Buffer at room temperature.

   Note: *1x Dye-Loading Solution is stable for more than one month if stored at -20°C. Avoid repetitive freeze-thaw cycle.*

3. **Dye Loading:**
   3.1 Add phosphodiesterase inhibitor Ro 20-1724 (**Component C**) to a concentration of 50 µM in 1x Dye-Loading Solution (optional).

   Note: *Adding a phosphodiesterase inhibitor will increase the assay sensitivity.*

   3.2 Remove cell plates from incubator and add an equal volume of 1x Dye-Loading Solution to each well (100 µl to 100 µl culture medium/well for 96-well plates, or 20 µl to 20 µl culture medium/well for 384-well plates).

   Note: *For assays that require a serum-free environment, culture medium that contains serum should be removed prior to dye loading and replaced with an equal volume of DMEM containing 0.2 to 0.5% BSA.*

   3.3 Incubate cell plates with dye for 2 hours at room temperature in the dark.

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4. **Preparation of Compound Plates:**
   - For agonist screening: dilute compounds in DPBS at 5x final concentrations. Add the compound solution at 1/5 volume to each well (add 50 µl/well for 96-well plate with final volume of 250 µl, or 10 µl/well for 384-well plate with final volume of 50 µl).

     Note: *Adding a phosphodiesterase inhibitor will increase the assay sensitivity.*
   - For antagonist assay: dilute antagonist in DPBS at 5x final concentrations. Add the antagonist compound solution at 1/5 volume to each well (add 50 µl/well for 96-well plate with final volume of 250 µl, or 10 µl/well for 384-well plate with final volume of 50 µl). Incubate the plates at room temperature for 15 minutes prior to the addition of the specific agonists. Agonists should be prepared at 6x final concentrations in DPBS, and added to each well at 1/6 volume (add 50 µl/well for 96-well plate with final volume of 300 µl, or 10 µl/well for 384-well plate with final volume of 60 µl).

     Note: *Antagonist incubation times should be optimized for each assay. Do not allow final concentration of DMSO in assay well to exceed 1.5%.*
5. **Membrane Potential Assay:**
   - **Kinetic assay with on-line compound addition:** Place the cell plates on a FLIPR, FlexStation or FDSS, and perform the membrane potential assay with on-line addition of test compounds in 1x DPBS to the cell plates at 50 µl/well for 96-well plates (250 µl total each well after addition) or 10 µl/well for 384-well plates (50 µl total each well after addition).

     For assay performed on a FLIPR, use the 540-590 bandpass FLIPR filter (#2) from Molecular Devices.

     For assay performed on a FlexStation, use the following wavelength parameters: Excitation/Emission = 530nm/565nm, cutoff: 550nm.

     Note: *Dispense speed and height for compound additions need to be optimized for each instrument.*
   - **Endpoint assay or kinetic assay with off-line compound addition:** a fluorescent plate reader with bottom read-head is required. Test filter settings to optimize fluorescence light collection and eliminate bleedthrough of excitation light to emission filters. For example, the following filter settings can be used with excitation = 530±20nm and emission = 590±20nm.

     Read fluorescent baseline and record the fluorescent counts prior to drug addition. Add test compounds to cell plates off-line, and then read the plates at the appropriate time after drug addition.

     Note: Dispense speed and height for compound additions need to be optimized so that the cell monolayer is not disturbed.

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Data Examples

The Elite™ Fluorescent Membrane Potential Dye Kit has been evaluated against a panel of diversified receptors and ligands to ensure that the dye kit is pharmacologically compatible with drug screening. A variety of endogenous and recombinant receptors and their cognate ligands have been tested using this kit, including ligands with pharmacophores of adenosine, biogenic amines, lipids, and peptides. Potency (EC50) of the ligands as well as maximal signal (Fluorescence Fold-change) in ACTOne assay has been determined (see table).

Receptor	Ligand	Elite™ Fluorescent Membrane Potential Dye Assay Kit	
		EC50	Fluorescence Fold-change
Adenosine A2b receptor (A2b)	NECA	560 nM	5.5
Adenylyl Cyclase	Forskolin	0.16 μM	5.1
β-adrenoceptor (BAR)	Isoproterenol	2.6 nM	5.4
Cannabinoid receptor (CNR1, CB1)	Anadamide	208 nM	4.3
Dopamine Receptor a (DRD1)	Dopamine	4.1 pM	6.7
Dopamine Receptor 2 (DRD2)	Dopamine	20 nM	3.9
Gastric Inhibitor Peptide Receptor (GIPR)	GIP	3.0 pM	6.0
Glucagon Receptor (GCGR)	Glucagon	2.5 pM	6.5
Melanocortin 4 Receptor (MC4R)	[Nle4-D-Phe7]α-MSH	4.9 nM	6.5
Parathyroid Hormone Receptor 1 (PTHR1)	PTH	7.8 pM	5.5
Prostaglandin E Receptor 4 (PTGER4, EP4)	PGE2	25.4 pM	6.3
Somatostatin Receptor 5 (SSTR5)	Somatostatin	2.4 nM	4.4
Vasoactive Intestinal Peptide Receptor 2 (VIPR2, VPAC2R)	VIP	28 pM	6.3

Troubleshooting Guide

1. Low survival rate of cells after thawing
    a. Cell vials could have thawed accidently. Store cell vials in liquid nitrogen immediately after receiving and keep frozen at all times.
    b. Leaving the vial at 37°C for too long during thawing will lower the survival rate. Place the vial at 37°C until cells are just thawed.
    c. Handle the cells gently. Don't tap the vial or pipette the cells too many times before plating the cells.
    d. Replace the medium four hours after thawing or when the cells have settled to remove DMSO.
2. Slow growth rate of cells
    a. Do not split cells before they have completely recovered from thawing and reach at least 50% confluence.
    b. Do not dilute cells excessively while splitting.
    c. Split cells before they reach 80-90% confluence.
    d. Use trypsin-EDTA solution to dissociate cells.
    e. Cells may not be able to recover to an optimal stage if trypsin-free dissociation buffer is used.
3. High baseline fluorescent signal
    a. Inspect the cell density and morphology under a microscope. High cell density or unhealthy cells can result in high baseline signal.
    b. Do not remove serum-containing medium from cell plates before dye-loading. If a serum-free environment is required, use DPBS buffer containing 0.2 to 0.5% BSA to replace medium.
4. Response to agonist is lower than expected.
    a. Check the overall health of cells.
    b. Cell density is too high or too low. Cell number titration may be necessary.
    c. Keep cells growing in medium with proper drug selection.
    d. Check settings of fluorescence readers.
5. High well-to-well variations
    a. Cells should be evenly distributed among wells. Before plating, microscopically examine the culture to be sure that they have been dissociated into single cells. Leave the cell plates at room temperature for 30 minutes prior to transferring the plates to a cell culture incubator.
    b. Check the liquid handling system for dispensing accuracy. Optimize the settings of liquid handling system so that cell monolayer is not disturbed by dye and compound addition.
    c. Check settings of fluorescence readers.
6. Response from cells after the addition of buffer containing only DMSO
    a. Keep the final DMSO concentration below 1.5%.