Enabling Discovery in Life Science®

FluoForte® Calcium Assay Kit

for microplates

Instruction Manual

Cat. No. ENZ-51016	High-Throughput, 100 plates kit
Cat. No. ENZ-51017	Starter Pack, 10 plates kit

For research use only.

Rev. 1.1..3 December 2010

Notice to Purchaser

The FluoForte® Calcium Assay Kit for microplates is a member of the CELLestial® product line, reagents and assay kits comprising fluorescent molecular probes that have been extensively benchmarked for live cell analysis applications. CELLestial® reagents and kits are optimal for use in demanding cell analysis applications involving confocal microscopy, flow cytometry, microplate readers and HCS/HTS, where consistency and reproducibility are required.

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I. Introduction

The calcium ion is an important second messenger involved in many physiological and signal transduction processes within cells. Fluo-3, Fluo-4 and Calcium 4 dyes are widely used calcium ion indicators for in-cell measurement of agonist-stimulated and antagonist-inhibited calcium signaling in high-throughput screening applications.[1-4] However their relatively weak fluorescence signals have limited their application in some challenging cell lines and with certain membrane receptors.

Enzo Life Sciences' FluoForte® Calcium Assay Kit for microplates provides a homogeneous fluorescence-based assay for detecting intracellular calcium mobilization across a broad spectrum of biological targets. Relative to other commercially available dyes, FluoForte® dye yields the brightest signal and largest assay window. The kit provides a homogeneous mix-and-read, no-wash calcium mobilization assay. The homogenous cell-based assay for calcium offers fewer steps, lower variability and an easier protocol for adherent and non-adherent cell lines. In addition, it requires neither a washing step, nor exogenous addition of a quencher dye, which could adversely effect receptor-ligand interaction kinetics.[5]

II. Reagents Provided and Storage

All reagents are shipped on dry ice. Upon receipt, the kit should be stored at -20°C, protected from light. When stored properly, these reagents are stable for six months from date of receipt. **Avoid repeated freezing and thawing.**

Reagent	Quantity	
	ENZ-51017 (10 plates kit)	**ENZ-51016 (100 plates kit)**
Reagent A: FluoForte® dye, lyophilized	1 vial	10 vials
Reagent B: Dye efflux inhibitor	10 x 1 mL	10 x 10 mL
Reagent C: Hanks' buffer with 20 mM HEPES (HHBS)	1 x 100 mL	Not included

III. Additional Materials Required

- A fluorometric imaging plate reader capable of performing quantitative optical screening for cell-based kinetic assays. (Molecular Devices FLIPR, PerkinElmer CellLux, Hamamatsu FDSS system, or similar instrumentation)
- Calibrated, adjustable precision pipetters, preferably with disposable plastic tips
- Deionized water
- Anhydrous DMSO
- Serum (optional).
- Growth medium (e.g. Dulbecco's modified Eagle medium, D-MEM)
- 10X Hanks' Balanced Salt Solution, HBSS (*e.g.,* Invitrogen # 14065-056)
- 1M HEPES Buffer ((*e.g.,* Invitrogen # 15630-080)
- Assay Plates: 96- or 384-well black-wall, clear bottom plates or 1536-well low base black-wall, clear bottom plates, 1536-well lids
- Compound plates: 96-well or 384-well polypropylene plates, 1536-well polystyrene plates

IV. Safety Warnings and Precautions

- This product is for research use only and is not intended for diagnostic purposes.
- Some components of this kit may contain hazardous substances. They can be harmful if ingested or absorbed through the skin and may cause irritation to the eyes. The reagents of the kit should be treated as possible mutagens and should be handled with care and disposed of properly.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. All blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.
- To avoid photobleaching, perform all manipulations in low light environments or protected from light by other means.

V. Methods and Procedures

Brief Summary of Assay Work Flow

- *Prepare cells.*
- *Remove medium.*
- *Add FluoForte® dye-loading solution.*
- *Incubate plate for 1 hour.*
- *Add test agents.*
- *Read fluorescence.*

NOTE: *PLEASE READ THE ENTIRE PROCEDURE BEFORE STARTING. Allow all reagents to be used to warm to room temperature before proceeding. Upon thawing of solutions, gently hand-mix or vortex the reagents prior to use to ensure a homogenous solution.*

A. Cell Preparation

1. **Adherent Cells.** The day before the experiment, plate the cells overnight in growth medium using 4 x 10^4 to 8 x 10^4 cells per well at a plating volume of 100 µL per well for 96-well plates, or using 1 x 10^4 to 2 x 10^4 cells per well at a plating volume of 25 µL per well for a 384-well plates.

   After overnight incubation, remove the growth medium from the cell plates. Then proceed to section D, page 5.

   NOTE: *It is important to remove the growth medium in order to minimize background fluorescence, and compound interference with serum or culture media.*

2. **Non-adherent Cells.** On the day of the experiment, centrifuge the cells from the culture medium and then resuspend the cell pellets in FluoForte® dye-loading solution (see section B). Plate the cells using 1.25 x 10^5 to 2.5 x 10^5 cells per well at a plating volume of 100 µL per well for 96-well plates, or 3 x 10^4 to 6 x 10^4 cells per well at a plating volume of 25 µL per well for 384-well plates. Centrifuge the plates at 800 rpm for 2 minutes, **with brake off**, prior to starting the experiments. Proceed to section D, page 5.

   NOTE: *Each cell line should be evaluated on an individual basis to determine the optimal cell density for the intracellular calcium mobilization assay.*

B. PREPARATION OF FLUOFORTE® DYE-LOADING SOLUTION USING ENZ-51017 (10 plates kit)

The following procedure is for preparation of FluoForte® dye-loading solution **for use in 1 plate**. Before starting, equilibrate Reagent A (FluoForte™ dye), a vial of Reagent B and Reagent C (HBSS) to room temperature.

1. **Reagent A Stock Solution.** Add 100 µL DMSO to the vial containing **Reagent A**. Mix well.

   NOTE: *10 µl of the reconstituted Reagent A is enough for 1 plate. The remaining unused, reconstituted Reagent A can be aliquoted and stored at ≤ -20ºC for at least one month if stored properly. The tubes (preferably amber vials) should be capped tightly. Avoid exposure to light and repeated freeze-thaw cycles.*

2. **1X Assay Buffer.** Mix well 9 mL of **Reagent C** (HHBS) with the contents of 1 vial (1 mL) of **Reagent B**.

   NOTE: *10 mL of 1X Assay Buffer is sufficient for one plate. Unused buffer may be stored at ≤ -20°C up to 1 month . Avoid exposure to light and repeated freeze-thaw cycles.*

3. **FluoForte® Dye-Loading Solution.** Add 10 µL of Reagent A Stock Solution (from step B-1) to 10 mL of 1X Assay Buffer (from step B-2). Mix well. This working solution is stable for at least **2 hours** at room temperature.

C. PREPARATION OF FLUOFORTE® DYE-LOADING SOLUTION USING ENZ-51016 (100 plates kit)

The following procedure is for preparation of FluoForte® dye-loading solution **for use in 10 plates**. Before starting, equilibrate Reagent A (FluoForte™ dye) and a vial of Reagent B to room temperature.

1. **Hanks' Buffer with 20 mM HEPES (HHBS).** Prepare 100 mL of HHBS by mixing the following:

   10 mL 10X Hanks' Balanced Salt Solution (HBSS)

   2 mL HEPES Buffer

   88 mL Deionized water

2. **1X Assay Buffer.** Mix well 90 mL of **HHBS** (from step C-1) with the contents of 1 vial (10 mL) of **Reagent B**.

3. **FluoForte® Dye-Loading Solution.**

   a. Dissolve the contents of 1 vial of **Reagent A** in 100 µL of DMSO. Mix well.

   b. Add 100 µL of **Reagent A** solution to 100 mL of 1X Assay Buffer (from step C-2). Mix well. This working solution is stable for at least **2 hours** at room temperature.

D. Calcium Mobilization Assay

1. Obtain prepared cell plates (see section A).
2. Add FluoForte™ Dye-Loading Solution to each well (100 µL/well (for 96-well plates) or 25 µL/well for 384-well plates).
3. Incubate the cell plates for 1 hour at room temperature, or incubate about 45 minutes at 37°C then incubate for 15 minutes at room temperature prior to assay.

   NOTE: *The incubation time should be optimized for each cell line. The incubation time should be limited to 1~2 hours.* ***DO NOT*** *wash the cells after dye loading.*

4. Prepare the compound plates by dissolving the compound in the buffer of choice. The FluoForte™ Calcium Assay is optimized for an agonist addition at one-fifth of the final volume.
5. Run the calcium flux assay by monitoring the fluorescence at Ex=490 nm/Em=525 nm with a fluorometric imaging plate reader.

   NOTE: *Faster addition speeds can lead to better mixing of compounds and lower signal variance across the plate. Make sure to follow the recommended experimental setup parameters provided by the instrument manufacturer before reading the plate. It is also important to run the signal test before the experiment. Different instruments have their own intensity range. Adjust the signal test intensity to the level of 10% to 15% of the maximum instrument intensity counts. For example, the maximum fluorescence intensity count for FLIPR-384 is 65,000, so the instrument settings should be adjusted to have its signal test intensity around 7,000 to 10,000.*

VI. Expected Results

In a side-by-side comparison of FluoForte®, Fluo-4 Direct assay and Calcium 4 assay, CHO M1 cells were stimulated with 200 nM of ATP. FluoForte™ yields the brightest signal and largest assay window. (shown in Figure 1). This facilitates measurements of challenging cell lines and receptors.

Dose responses for ATP in CHO M1 cells gave similar EC_{50} for all the assays (shown in Figure 2). This demonstrates consistent pharmacology among the assays. However, relative fluorescence units (RFU) of FluoForte™ are much higher than Fluo 4 and Calcium 4.

Overall FluoForte® Calcium Assay kit provides an optimized assay method for monitoring G-protein-coupled receptors (GPCRs) and calcium channels.[6] Its ability to generate very strong signal enables researchers to perform calcium mobilization assays with a wide range of receptor and calcium channel targets.

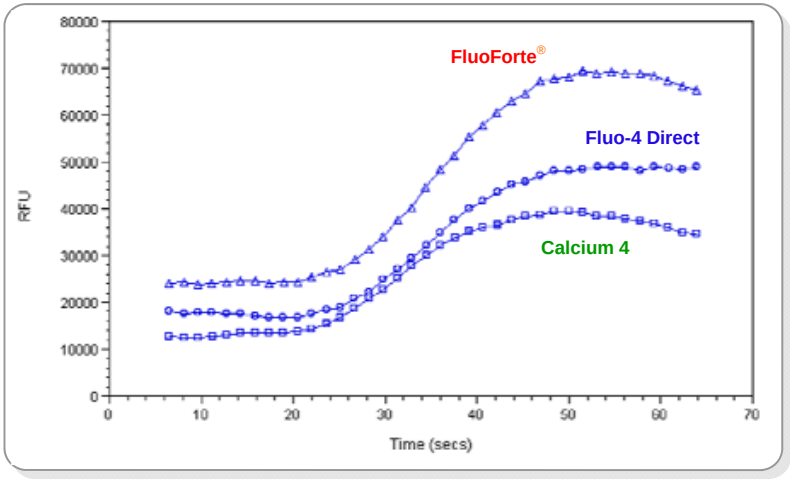

Figure 1: Comparisons of FluoForte® Fluo-4 Direct, and Calcium 4 detection of intracellular calcium mobilization in CHO-M1 cells. CHO cells were seeded overnight in 40,000 cells per 100 µL per well in a 96-well black wall, clear bottom costar plate. The cells were incubated with 100 µL of Life Technologies' Fluo-4 Direct kit, Molecular Devices' Calcium 4 kit (both based on manufactures' protocol) or Enzo's FluoForte™ kit. ATP (20 µL/well) was added by FlexStation to achieve concentrations of 200 nM.

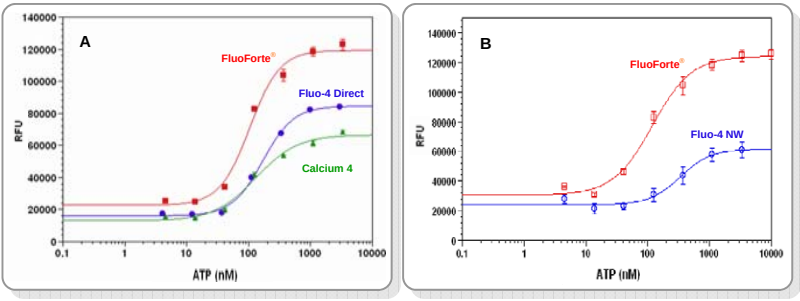

Figure 2: ATP Dose Response Curves in CHO-M1 cells. CHO cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom microplate. **Panel A:** The cells were incubated with 100 µL of Life Technologies' Fluo-4 Direct kit, Molecular Devices' Calcium 4 kit (both based upon manufacturer's protocol) or Enzo's FluoForte® dye. **Panel B:** The cells were incubated with 100 µL of Enzo's FluoForte® Calcium assay kit, or with Life Technologies' Fluo-4 NW kit (based upon manufacturer's protocol).

ATP (20 µL/well) was added by FlexStation to achieve the final indicated concentrations. No significant difference in EC_{50} of ATP for FluoForte®, Fluo-4 Direct and Calcium 4 was observed (shown in panel A), and also comparable EC_{50} values were observed between FluoForte® and Fluo-4 NW (shown in panel B). In all cases, FluoForte® generated the highest intensity signal.

VII. References

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5. Zheng, W. et al. (2004) High throughput assay technologies for ion channel drug discovery. Assay Drug. Dev. Technol. 5, 543–552.
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VIII. Troubleshooting Guide

Problem	Potential Cause	Suggestion
High baseline fluo-rescence	Contributions to baseline fluorescence by growth medium and organic anion transport	Remove the medium before adding the indicator dye to the wells. Use the dye solution within 2 hours at room tem-perature.
Untreated cells have calcium response	Inconsistent DMSO concentration	Make sure that the buffer used for the negative control wells have the same final concentration of DMSO as those in the test compounds.
Response is smaller than expected	Agonists and antago-nists may stick to the pipette tips. Experimen-tal setup parameters and dye loading time are not optimized.	Use 0.1% of BSA in all compound buffer diluents. Fast addition speeds are recommended to ensure better mixing of compounds and improved cell response. Dye loading typically takes between 30 minutes and one hour. Optimizing the conditions for each cell line is recommended.
Well to well variation observed	Incorrect dispenser and experimental setup pa-rameter used.	Use instrument manufacturer's recom-mended dispenser and setup parame-ters (*i.e.,* volume, height and speed of dispensing) for compound addition.
Fluorescence drop upon compound addition	Dislodging the cells during addition	Decrease the rate of addition or seed fewer cells in the wells to avoid this problem.

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