Enzo® Life Sciences

II P216

A COST-EFFECTIVE WORKFLOW FOR HIGH-THROUGHPUT SCREENING OF G PROTEIN-COUPLED RECEPTORS (GPCRS)

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ABSTRACT

Drugs targeting members of the GPCR super-family represent the core of modern medicine, accounting for the majority of the best-selling drugs and roughly 40% of all prescription pharmaceuticals on the market today. Cell-based assays that monitor the functional activation of GPCRs are thus considered a critical part of the drug discovery process. We describe a new fluorescent probe for drug discovery which is provided to cells as a cell membrane-permeable acetoxymethyl (AM) ester. Once within the cells, the probe is hydrolyzed by intracellular esterases, leading to the generation of a cell membrane impermeable form, in an analogous manner as commonly used Fluo-3 or Fluo-4 dyes. Intracellular calcium binding to the fluorescent probe is readily measured using a fluorescence microplate reader equipped with a dual-reagent dispenser. This fluorescence-based assay workflow is suitable for monitoring calcium mobilization across a broad spectrum of biological targets. The easy-to-use protocol is automation-friendly and can be performed in a convenient 96-well or 384-well microplate format. The described assay workflow results in low sample-to-sample variability and excellent Z' values in a miniaturized format. The ease-of-use of the kit, affordability and high performance of the instrument and minimal installation requirements make the described workflow readily accessible, even to academic research groups with modest consumables and instrumentation budgets.

INTRODUCTION

The calcium ion is an important second messenger involved in many physiological and signaling processes within cells. Fluo-3 and Fluo-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. However their relatively weak fluorescence signals have limited their application in some challenging cell lines and with certain membrane receptors.

We report upon a new calcium-sensitive fluorescent dye, referred to as FluoForte™ reagent, which provides superior performance relative to the conventional dyes employed in such assays. FluoForte™ dye is added to cells in the form of a non-fluorescent AM ester. Once inside cells, the lipophillic AM blocking groups are cleaved by non-specific cellular esterases, resulting in negatively charged fluorescent dye that is retained within the cells. The fluorescence intensity of the dye is greatly enhanced upon binding to calcium. When cells are stimulated with active screening compounds, the GPCR-mediated signals release stores of intracellular calcium, which lead to greatly increased fluorescence signals.

The Synergy Mx instrument (BioTek Instruments) is a multifunctional microplate reader with liquid handling and kinetic fluorescent reading capability. This study validates the instrument as a low-cost platform for performing cell-based, fast-kinetic assays, such as intracellular calcium mobilization measurements with FluoForte™ reagent. The instrument provides a unique pipetting system that facilitates addition of small amounts of agonist or antagonist (≤10 µL) to cells, followed by instantaneous reading. Thus the Synergy Mx instrument and FluoForte™ dye, when combined, provide a robust platform for which to measure intercellular calcium changes.

FluoForte™ Calcium Assay Mechanism

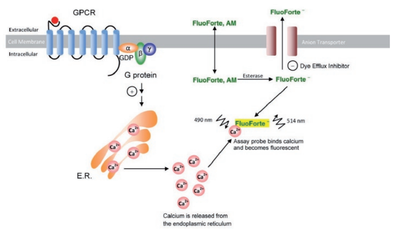
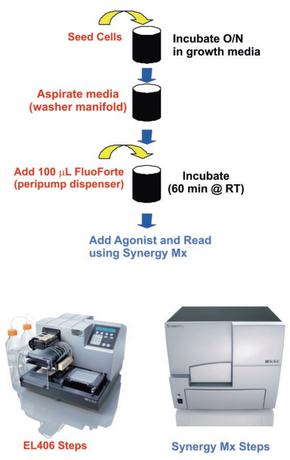


FIGURE 1: FluoForte™ dye enters cell as a membrane permeable acetoxymethyl (AM) ester. Once inside the cell, FluoForte ™ dye is hydrolyzed (cleaved) by intracellular esterases. Cell membrane impermeable, negatively charged form of Fluo-Forte ™ dye is now capable of binding with Ca^{2+}. Some cell lines express an organic anion transporter, which leads to the export of negatively charged Fluo-Forte™ dye. This can be prevented by adding Dye Efflux Inhibitor, an anion transporter inhibitor.

Automation Workflow for FluoForte™ Assay Kit on the Synergy Mx Instrument



FluoForte™ Reagent is brighter than Fluo-4 AM Dye

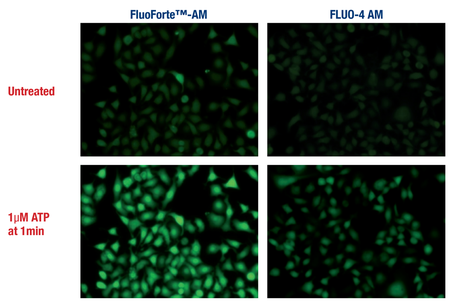


FIGURE 2: Hela cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom microplate. The growth medium was removed, and the cells were incubated with 100 µL of 4 uM Fluo-4 AM or FluoForte™ AM dye in HHBS at 37 °C in a 5% CO_2 cell culture incubator for 1 hour. The cells were washed twice with 200 µL HHBS, ATP (20 µL/well) was added to achieve a concentrations of 1 µM with dye efflux inhibitor, then immediately imaged with a fluorescence microscope (Carl Zeiss, Inc) using a FITC filter set.

The EL406 Washer Dispenser and Synergy Mx Microplate Reader's Unique Pipeting System Allow Automation of the FluoForte™ Assay

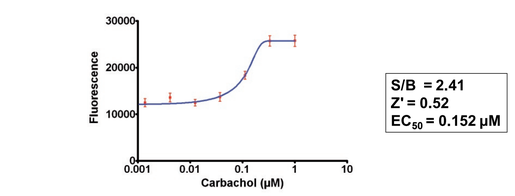
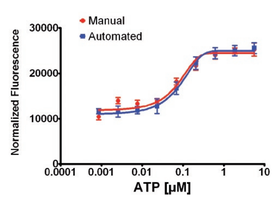


FIGURE 3: Automated Assay with carbachol dose response curves in CHO-M1 cells, expressing M3-muscarinic receptor: CHO cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom microplate. For the automated protocol, cell media was aspirated and 100 µL of working FluoForte™ reagent was added using a BioTek EL406 Washer Dispenser. The media for all 96- well plates was aspirated using the washer manifold and 100 µL of reagent was immediately added using the peristaltic pump. The plate was then allowed to incubate for 60 min at 37°C. Carbachol (20 µL/well) was subsequently added using a BioTek two syringe pump dispenser to achieve the final indicated concentrations. High Z' factor value and large assay window is observed.

The Automated Assay Provides Similar Performance as the Manual Assay



Parameter	Manual Protocol	Automated Protocol
S/B	2.528	2.561
Z'	0.57	0.61
EC_{50}	0.064 µM	0.091 µM

FIGURE 4: Comparison of manual and automated assay with ATP dose response curves in CHO-M1 cells, expressing P2Y endogenous receptors: CHO cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom microplate. For the atomated protocol, cell media was aspirated and 100 µL of working FluoForte™ reagent was added using a BioTek EL406 Combination Microplate Washer Dispenser. The media for the 96-well plates was aspirated using the washer manifold and 100 µL of reagent was immediately added using the peristaltic pump. The cells were incubated with 100 µL of 4 µM FluoForte™ reagent for 1 hour at 37°C. ATP (20 µL/well) was subsequently added using a BioTek two syringe pump dispenser to achieve the final indicated concentrations. No significant difference in EC_{50} of ATP and Z' between manual and automation assay was observed.

Cell Loading with FluoForte™ Reagent can be Performed at Room Temperature or 37°C

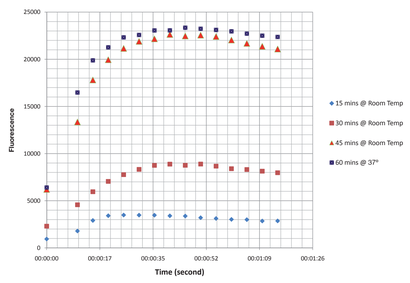


FIGURE 5: Time course study of FluoForte™ detection of intracellular calcium mobilization in CHO-K1 cells. CHO cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom plate. The cells were incubated with 100 µL of 4 µM FluoForte™ reagent for 15-45 min at room temperature or 60 min at 37°C. ATP (20 µL/well) was added using a BioTek two syringe pump dispenser to achieve a final concentration of 400 nM.

FluoForte™ Reagent is Brighter than Fluo-4 AM Dye

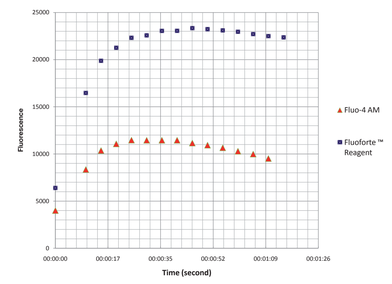
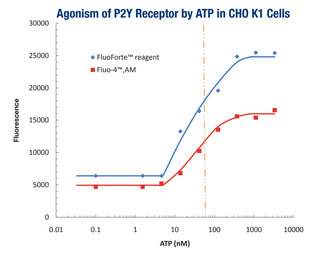


FIGURE 6: Comparison of FluoForte™ & Fluo-4 dye-based detection of intracellular calcium mobilization in CHO-K1 cells. CHO cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom plate. The cells were incubated with 100 µL of 4 µM Fluo-4 or FluoForte™ dye. ATP (20 µL/well) was added using a BioTek two syringe pump dispenser to achieve a final concentration of 400 nM.

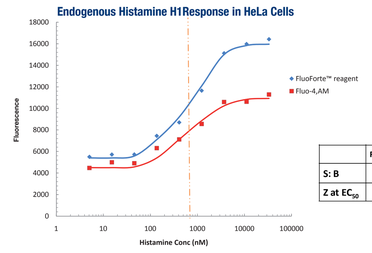
FluoForte™ Reagent Provides Similar EC_{50} Values, Plus Brighter Signal & More Robust Assay Performance than Fluo-4 Dye



	FluoForte™reagent	Fluo4 -AM
S: B	3.97	3.52
Z at EC_{50}	0.74	0.51

FIGURE 7: ATP dose response curves in CHO-K1 cells, expressing P2Y endogenous receptors: CHO cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom microplate. The cells were incubated with 100 µL of 4 µM FluoForte™ reagent or Fluo-4-AM dye for 1 hour at 37°C. ATP (20 µL/well) was added using a BioTek two syringe pump dispenser to achieve the final indicated concentrations. No significant difference in EC_{50} of ATP for FluoForte™ and Fluo-4 AM dyes was observed. FluoForte™ reagent generated much higher intensity signal, higher Z' factor value and larger assay window.

FluoForte™ Reagent Provides Similar EC_{50} Values, Plus Brighter Signal & More Robust Assay Performance than Fluo-4 Dye



	FluoForte™ reagent	Fluo4-AM
S: B	2.98	2.52
Z at EC_{50}	0.68	0.21

FIGURE 8: Histamine dose response curves in Hela cells, expressing Histamine H1 receptor receptors: Hela cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom microplate. (A) The cells were incubated with 100 µL of 4 µM FluoForte™ reagent or Fluo4-AM dye for 1 hour at 37°C. Histamine (20 µL/well) was added using a BioTek two syringe pump dispenser to achieve the final indicated concentrations. No significant difference in EC_{50} of Histamine for FluoForte™ Fluo-4 AM dye was observed. FluoForte™ reagent generated higher intensity signal and a larger assay window.

CONCLUSIONS

The BioTek Synergy Mx microplate reader provides a cost effective system to perform fast kinetic, cell-based assays, such as the FluoForte™ calcium mobilization assay. The system's unique pipeting system allows for instantaneous reading upon agonist stimulation.

When the FluoForte™ reagent is employed on the BioTek platform, analysis of both G protein-coupled receptor and calcium ion channel targets is readily accomplished. The easy-to-use protocol does not require a wash step or the addition of a quencher dye, which could potentially modify pharmacological parameters. FluoForte™ dye can be loaded at 37°C or room temperature, which makes it amenable to high throughput screening applications in drug discovery. As demonstrated, removal of culture medium can be performed using the EL406. Combination Microplate Washer Dispenser without compromising assay quality.

The Synergy Mx instrument, in combination with the FluoForte™ Calcium Assay Kit, offers researchers an integrated instrument-reagent combination that provides high-performance results at an affordable price.

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