G722

Quencher Dyes Can Alter the Pharmacokinetic Response Profiles of Living Cells in Fluorescence-based Calcium Mobilization Assays

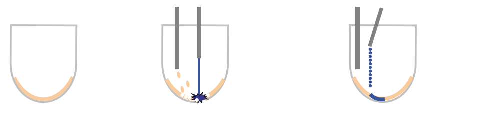
Dee Shen[1], Paul Held[2], Peter Banks[2] and Wayne F. Patton[1*]; [1]Enzo Life Sciences, Farmingdale, NY 11735 and [2]BioTek Instruments, Winooski, VT 05404

ABSTRACT

G protein-coupled receptors (GPCRs) have been the target of choice for numerous high-throughput screening (HTS) campaigns designed to search for new drug leads of potential therapeutic significance. By coupling receptors to G_q proteins, which stimulate intracellular calcium flux upon agonist binding, a functional response can readily be measured using various calcium-sensitive dyes and a fluorescence microplate reader. GPCR assays using fluorescent dyes, such as Fluo-3 AM or Fluo-4 AM, typically require both a serum-containing medium removal step and then a wash step after dye loading to reduce background fluorescence signal. These wash steps may introduce assay variation, especially when loosely adherent cells become dislodged. Consequently, dyes have been introduced for the purpose of quenching extracellular background fluorescence in calcium flux assays. These include trypan blue, brilliant black, hemoglobin and proprietary dyes available in certain commercial no-wash calcium assay kits (C. Mehlin, et al.; Biotechniques **34,** 164 (2003); D.G. Cronshaw, et al.; J Leukoc. Biol. **79,** 1369 (2006)). A systematic study of a quencher-based kit, as well as a kit employing the calcium-sensitive FluoForte™ dye, in the presence or absence of Trypan Blue dye, demonstrated exogenous addition of a quencher dye can adversely affect receptor-ligand interaction kinetics. Enhanced assay performance can be achieved by employing the FluoForte™ dye in combination with a cell loading buffer that enhances cell retention and prevents dye extrusion through plasma membrane anion transporters. Incorporating the EL406 Combination Washer Dispenser in the workflow provides fast, gentle removal of the serum-containing medium, without loss of cells. Omission of the quencher dye in the assay reduces the potential for non-specific pharmacological interferences in the workflow.

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

Using the BioTek EL406 Combination Washer Dispenser in the Workflow Provides Fast, Gentle Removal of the Serum-containing Medium, Without Loss of Poorly Adherent Cells



Cell layer on bottom of well

With some assays, the layer of cells is poorly bound to the well bottom. (Considered "non-adherent" or "loosely adherent") Strong dispense flow can disrupt these cells – poking "holes" in the layer and causing cells to be dislodged and aspirated.

Using the EL406 Select with flow rate 10 or 11, the fluid delivery is much gentler. Positioning the tubes closer to the well sides reduces the force of the flow on the cell layer, leaving it intact.

FluoForte™ Calcium Assay Mechanism

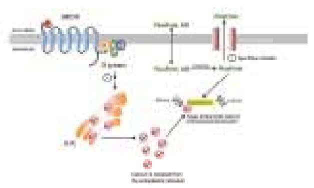


FIGURE 1: FluoForte™ dye enters cell as a membrane permeable acetoxymethyl (AM) ester. Once inside the cell, FluoForte ™ dye is hydrolyzed (cleaved) by intracellular esterases. Cell membrane impermeable, negatively charged form of Fluo-Forte ™ dye is now capable of binding with Ca^{2+}. Some cell lines 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.

FluoForte™ Reagent is brighter than Fluo-4 AM Dye

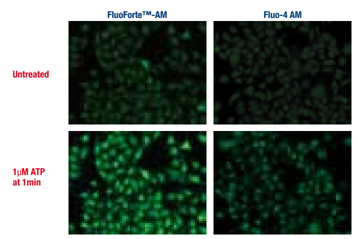


FIGURE 2: Hela cells were seeded overnight at 40,000 cells per 100 µl per well in a 96-well black wall/clear bottom microplate. The growth medium was removed, and the cells were incubated with 100 µl of 4 µM Fluo-4 AM or FluoForte™ AM dye in HHBS at 37°C in a 5% CO_2 cell culture incubator for 1 hour. 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.

FluoForte™ Reagent is Brighter than Fluo-4 AM Dye

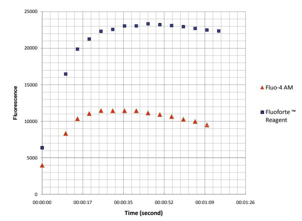


FIGURE 3: Comparison of FluoForte™ & Fluo-4 dye-based detection of intracellular calcium mobilization in CHO-K1 cells. CHO cells were seeded overnight at 40,000 cells per 100 µl per well in a 96-well black wall/clear bottom plate. The cells were incubated with 100 µl of 4 µM Fluo-4 or FluoForte™ dye. ATP (20 µl/well) was added using a BioTek two syringe pump dispenser to achieve a final concentration of 400 nM.

FluoForte™ Reagent Displays Less Temperature-dependency for Cell Loading

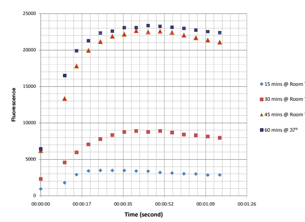
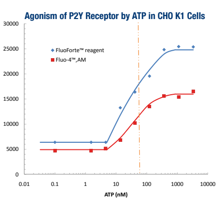


FIGURE 4: Time course study of FluoForte™ detection of intracellular calcium mobilization in CHO-K1 cells. CHO cells were seeded overnight at 40,000 cells per 100 µl per well in a 96-well black wall/clear bottom plate. The cells were incubated with 100 µl of 4 µM FluoForte™ reagent for 15-45 min at room 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.

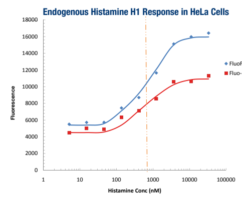
FluoForte™ Reagent Provides Similar EC_{50} Values, but Brighter Signal & More Robust Assay Performance than Fluo-4 Dye



	FluoForte™reagent	Fluo4 -AM
S: B	3.97	3.52
Z at EC_{50}	0.74	0.51
EC_{50}	41.6±7.2 (nM)	43.7±4.2 (nM)

FIGURE 5: 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.

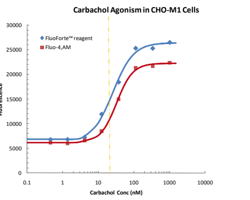
FluoForte™ Reagent Provides Similar EC_{50} Values, but Brighter Signal & More Robust Assay Performance than Fluo-4 Dye



	FluoForte™ reagent	Fluo4-AM
S: B	2.98	2.52
Z at EC_{50}	0.68	0.21
EC_{50}	945.7±134 (nM)	699.2±91.5 (nM)

FIGURE 6: Histamine dose response curves in Hela cells, expressing Histamine H1 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.

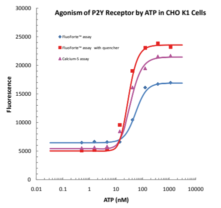
FluoForte™ Reagent Provides Similar EC_{50} Values, but Brighter Signal & More Robust Assay Performance than Fluo-4 Dye



	FluoForte™reagent	Fluo4-AM
S: B	3.91	3.38
Z at EC_{50}	0.75	0.61
EC_{50}	26.1±2.7 (nM)	32.7±1.6 (nM)

FIGURE 7: 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. The cells were incubated with 100 µl of 4 µM FluoForte™ reagent or Fluo4-AM dye for 1 hour at 37°C. Carbachol (20 µl/well) was added using a BioTek two syringe pump dispenser to achieve the final indicated concentrations. No significant difference in EC50 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.

Exogenous Addition of a Quencher Dye Adversely Affects Receptor-ligand Interaction

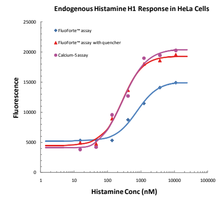


	FluoForte™ assay	FluoForte™ assay with quencher	Calcium-5 Assay (contains quencher)
EC_{50}	48.8±4.2 (nM)	23.3±2.9 (nM)	28.6±2.3 (nM)

~ 190% decrease in EC_{50} value due to quencher.

FIGURE 8: Exogenous addition of a quencher dyes affect ATP pharmacokinetic response profiles 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 from Enzo kit in the presence or absence of trypan blue or Calcium-5 kit (a quencher-based kit), 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. Quencher dyes alter the pharmacokinetic response profiles.

Exogenous Addition of a Quencher Dye Adversely Affects Receptor-ligand Interaction



	FluoForte™ assay	FluoForte™ assay with quencher	Calcium-5 Assay (contains quencher)
EC_{50}	755.9±43.7 (nM)	277.2±31.4 (nM)	283.2±61.5 (nM)

~ 270% decrease in EC_{50} value due to quencher.

FIGURE 9: Exogenous addition of a quencher dye affect Histamine pharmacokinetic response profiles 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. The cells were incubated with 100 µl of 4 µM FluoForte™ reagent from Enzo kit in the presence or absence of trypan blue or Calcium-5 kit (a quencher-based kit), 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. Quencher dyes alter the pharmacokinetic response profiles.

CONCLUSION

FluoForte™ reagent is readily employed in a homogeneous assay that is suitable for analyzing both G protein-coupled receptor and calcium ion channel targets. FluoForte™ dye can be loaded at 37°C or room temperature, which makes it amenable to high throughput screening applications in drug discovery.

FluoForte™ reagent has the following benefits and features:

a) Significantly brighter fluorescence intensity than Fluo-4 AM dye.

b) Provides comparable EC_{50} values as obtained using Fluo-4 AM dye.

c) Larger assay window and higher Z' factor values, allowing measurements of challenging cell lines and receptors.

d) Permits loading of dye in live cells at either room temperature or 37°C.

Quencher dyes are employed in calcium mobilization assays in order to avoid removal of serum-containing medium. However, as demonstrated, quencher dyes significantly alter the pharmacokinetic response profiles of living cells in these assays. The BioTek Synergy Mx microplate reader enables removal of culture medium without compromising assay quality.

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 pipetting system allows for instantaneous reading upon agonist stimulation.

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.