Cell Based Assays on the FLIPR$^{TETRA®}$ System: Comparison of the FLIPR® Calcium 5 Assay Kit to Other Fluorescence Based Calcium Flux Assays

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Abstract

Cell-based calcium flux assays on FLIPR® fluorometric imaging plate readers are widely used in high throughput screening for identification of GPCR agonists and antagonists within the pharmaceutical industry. Masking technology enables no-wash fluorescence-based detection of changes in intracellular calcium concentration, and remains one of the most popular HTS methods for identifying potential drug candidates. Masking technology significantly lowers background fluorescence and increases the signal-to-noise ratio without the need to wash cells. In this study, we used the FLIPR$^{TETRA®}$ instrument to compare the performance of FLIPR® Calcium 4 and Calcium 5 Assay Kits to a competitor kit. Several endogenous GPCRs as well as transfected Muscarinic M1 receptor were studied in four cell lines. This evaluation demonstrates that the superior performing FLIPR® Calcium 5 Assay Kit provides a larger signal window, increased signal to noise ratio, consistent pharmacology, and equivalent or improved Z factors.

Introduction

The FLIPR® Calcium 5 Assay Kit contains a new, superior performance calcium sensitive indicator that does not require media removal and utilizes the same proven quench technology as the FLIPR® Calcium 4 Assay Kit. This masking dye technology is licensed to Molecular Devices, Inc. from Bayer AG (patent nos. US 6,420,183, EP 0906572). The masking technology combined with the novel indicator significantly lowers background fluorescence and improves signal-to-noise without washing. Throughput is increased by eliminating labor intensive wash steps, reducing preparation time for wash buffers and wash calibrations. After incubation, the assay functions at room temperature making it applicable to automation using stackers and robots.

FLIPR® Calcium 5 Assay Kit Response to Intracellular Calcium Mobilization

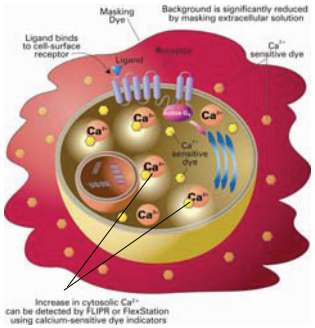


Figure 1. During cellular response to GPCR signaling, fluorescent signal increases as released intracellular calcium molecules bind to the Calcium 5 Assay Kit indicator. Background signal is decreased by extracellular masking technology.

Materials and Methods

Cell lines, receptors, and ligands:

M1WT3 CHO cells (CHO M1) (ATCC Cat# CRL 1985) were plated at 10,000 cells per well in culture media in black-wall clear-bottom 384-well TC coated plates (Corning Cat# 3712). Carbachol (Sigma-Aldrich Cat# C4382)was used to stimulate the Muscarininc M1 receptor and Atropine (Sigma-Aldrich Cat# A0132) was tested as antagonist.

HeLa cells (ATCC Cat# CCL2) were plated at 10,000 cells per well as listed above. Histamine (Sigma-Aldrich Cat# H7125) was used to stimulate the endogenous Histamine H1 agonist and Pyrilamine maleate (Sigma-Aldrich Cat# P5514) was tested as antagonist.
Dye for CHO M1 and HeLa cell lines was formulated in each case with 2.5 mM final concentration of water soluble probenecid (Invitrogen Cat# P36400). All cell plates were incubated overnight at 37°C in 5% CO_2.

HEK 293 cells (ATCC Cat# CRL 1573) cells were plated at 15,000 cells per well in culture media in black-wall clear-bottom 384-well poly-D-lysine coated plates (Cat# 356663, BD Life Sciences). Carbachol was used to stimulate the endogenous Muscarinic M3 receptor. Atropine was tested as the antagonist.

Jurkat immortalized T-cells (ATCC Cat# TIB-151) were plated at 75,000/well in 50 µL 1X dye loading media containing 0.1% BSA and centrifuged to insure that they were on the bottom of the well. Cells were incubated at 37 °C in 5% CO_2 for one hour and allowed to come to room temperature prior to the assay on the FLIPR^{TETRA} instrument.

Adenosine 5′ triphosphate (Sigma-Aldrich Cat# A2383) was used to stimulate the endogenous P2Y receptor in all cell lines except Jurkat.

FLIPR® Calcium 4 and Calcium 5 Assay Kit Protocol:

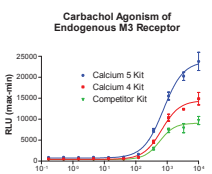
FLIPR® Calcium 5 Explorer Kit (Molecular Devices, Inc.) includes 10 vials of dye (Component A) and 1 bottle of Hanks Balanced Salt Solution (HBSS) and 20 mM HEPES adjusted to pH 7.4 (Component B) sufficient for 1 plate each. Bulk kits contain 10 vials of dye sufficient for 10 plates each. Competitor kits contain the same type of components

Dye loading buffer for 1 plate was prepared by dissolving contents of one vial of Component A completely with a final volume of 10 mL Component B loading buffer. Cell plates were removed from the incubator and 25 µL Calcium 4 Kit, Calcium 5 Kit, or competitor kit dye loading buffer was added to each well. Plates were *not* washed after dye addition. Dye loaded plates were incubated 45 minutes at 37° C, 5% CO_2 and allowed to come to room temperature 15 minutes prior to reading on the FLIPR$^{TETRA®}$ instrument.

Calcium Mobilization Assay on FLIPR$^{TETRA®}$:

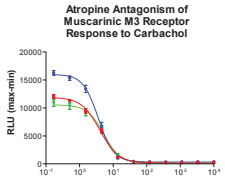
A 5X volume of CRC ligand was prepared in HBSS buffer + 20 mM HEPES in 384-well polypropylene plates. Agonist was added during detection on the FLIPR$^{TETRA®}$ instrument at optimized parameters. Antagonist was prepared at 5X concentration and added 15 minutes prior to addition of a 6X volume of EC_{80} concentration of challenge agonist. Relative Fluorescence Units (RFU) were measured for each response for signal maximum minus minimum during approximately 90 seconds after addition. Graphs and EC_{50}/IC_{50} concentrations were calculated using GraphPad Prism. Z-factor calculations were performed using the method described by Zhang, *et.al*.

Calcium Mobilization in HEK-293 Cells



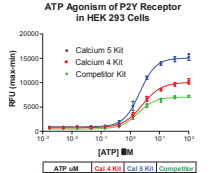
Carbachol nM	Cal 4 Kit	Cal 5 Kit	Competitor
EC_{50}	667.1	768.8	576.3
Z at EC_{80}	0.73	0.79	0.76

Figure 2A



Atropine nM	Cal 4 Kit	Cal 5 Kit	Competitor
IC_{50}	4.05	3.60	5.40
Z at IC_{80}	0.83	0.84	0.84

Figure 2B

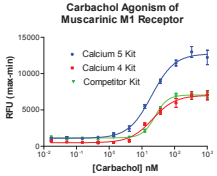


ATP uM	Cal 4 Kit	Cal 5 Kit	Competitor
EC_{50}	2.66	2.11	1.89
Z at EC_{80}	0.67	0.88	0.78

Figure 2C

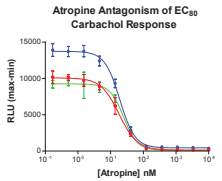
Figure 2. FLIPR® Calcium 5 Kit shows increased performance over Calcium 4 Kit and the competitor kit in calcium mobilization assays on endogenous GPCR receptors in HEK 293 cells. All Calcium 5 assays exhibit equivalent or higher Z factors at EC_{80} concentrations. A) Carbachol agonism of Muscarinic M3 receptor. B) Atropine antagonism of Carbachol response (EC_{80}= 1000 nM). C) ATP agonism of P2Y receptor.

Calcium Mobilization in CHO M1 Cells



Carbachol nM	Cal 4 Kit	Cal 5 Kit	Competitor
EC_{50}	21.4	19.65	25.19
Z at EC_{80}	0.59	0.74	0.8

Figure 3A



Atropine nM	Cal 4 Kit	Cal 5 Kit	Competitor
IC_{50}	18.2	19.13	24.99
Z at IC_{80}	0.8	0.77	0.77

Figure 3B

Figure 3. FLIPR® Calcium 5 Kit shows superior performance in calcium mobilization assays in CHO M1 Cells with good Z factors at EC_{80} concentrations. Calcium 4 Kit and the competitor kit exhibit equivalent performance. A) Carbachol agonism of Muscarinic M1 receptor. B) Atropine antagonism of Carbachol (EC_{80}= 70 nM) response.

Calcium Mobilization in Jurkat Suspension Cells

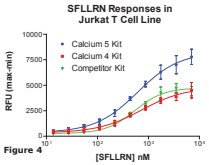
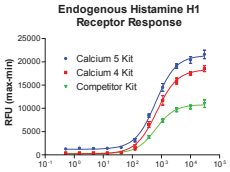


Figure 4

Figure 4. Jurkat T cell line response to synthetic SFLLRN, derived by cleavage of the thrombin receptor between Arg^{41} and Ser^{42} which unmasks a new amino-terminal sequence that functions as a ligand for the thrombin receptor[1].

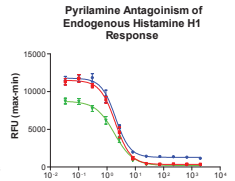
SFLLRN nM	Cal 4 Kit	Cal 5 Kit	Competitor
EC_{50}	732	609	541
Z at EC_{15}	0.62	0.76	0.72

Calcium Mobilization in HeLa Cells



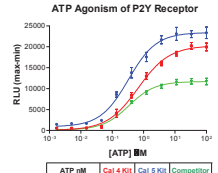
Histamine nM	Cal 4 Kit	Cal 5 Kit	Competitor
EC_{50}	686.3	636.6	637.5
Z at EC_{80}	0.72	0.79	0.85

Figure 5A



Pyrilamine nM	Cal 4 Kit	Cal 5 Kit	Competitor
IC_{50}	1.965	2.02	1.83
Z at IC_{80}	0.82	0.67	0.73

Figure 5B



ATP nM	Cal 4 Kit	Cal 5 Kit	Competitor
EC_{50}	0.67	0.33	0.34
Z at EC_{80}	0.74	0.68	0.84

Figure 5C

Figure 5. FLIPR® Calcium 5 Assay Kit shows greater signal performance in calcium mobilization assays in HeLa Cells with good Z factors at EC_{80} concentrations A) Histamine agonism of endogenous Histamine H1 receptor. B) Pyrilamine antagonism of Histamine (EC_{80}= 1700 nM) response. C) ATP agonism of P2Y receptor.

Summary

The high performance FLIPR® Calcium 5 Assay Kit consistently delivers increased signal window compared to the FLIPR® Calcium 4 and competitor kits. The combination of new dye technology and proven quench technology combine to enable high throughput screening of a variety of receptors and targets. This evaluation shows that the novel FLIPR® Calcium 5 Assay Kit provides a larger signal window, increased signal to noise ratio, consistent pharmacology, and equivalent or improved Z factors.

Reference

Mari, et. al., Thrombin and trypsin-induced Ca^{2+} mobilization in human T cell lines through interaction with different protease-activated receptors, FASEB Journal, February 1996, (10) 309-316.

Molecular Devices