FLIPR® Calcium 4 Assay Kit – a New Generation Quench Based, No-wash Calcium Kit: Performance Evaluation and Competitive Comparison using FLIPR TETRA™

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Introduction

Cell based calcium flux assays on FLIPR® fluorometric imaging detection systems are widely used in high throughput screening for GPCR agonists and antagonists within the pharmaceutical industry. To date, no-wash calcium flux assays provide one of the most reliable HTS methods for identifying potential drug candidates. We report here on FLIPR Calcium 4 Assay kit, a no-wash, fluorescence-based method for detecting changes in intracellular calcium concentration for use across a broad spectrum of GPCR targets.

In this study, we used FLIPR$^{TETRA}_{TM}$ to compare the performance of the FLIPR Calcium 4 Assay Kit, a soon-to-be released product, versus competitors' kits. HEK-293 cells were used to evaluate signal quality (signal intensity, signal-to-noise ratio, and background) and the addition artifact "dip" effect. FLIPR Calcium 4 assay data illustrates that this new kit has good performance vs. competitive kits. FLIPR Calcium 4 Kit is the latest development in the evolution of FLIPR Calcium assay kits to meet the increasing challenges for screening GPCR targets.

FLIPR Calcium 4 Assay Kit Features

The FLIPR Calcium 4 assay was developed with a new inert quencher that significantly reduces ligand interference, increases signal quality, minimizes the addition artifact, and works well with and without media removal before dye loading of the cells. The assay is a homogenous, mix-and-read, no-wash protocol designed to accurately and easily detect intracellular calcium flux from chemokine and small peptide targets as well as transient, endogenous, and low-expression receptors that have been difficult to assay with standard methods. Using the Bayer patented quench technology (U.S. patent # 6,420,183), the assay protocol results in fewer spontaneous calcium fluxes and unresponsive cells.

FLIPR^{TETRA} and FlexStation® microplate readers are optimal instruments for rapid detection and analysis. Throughput is increased by eliminating labor intensive wash steps, reducing preparation time for wash buffers and wash calibrations, and the assay functions at room temperature making it conducive to automation using stackers and robots.

Materials and Methods

HEK 293 cell line was used for comparison assays. To create 384-well assay plates, cells were plated at 12,500 cells per well in culture media in B-D black-wall clear-bottom 384-well poly-D-lysine coated plates (Cat# 356663, BD Life Sciences). Plates were incubated overnight at 37 °C in 5% CO_2.

FLIPR Calcium 4 Assay Kit protocol:

Wash buffer was prepared fresh daily. 10X HBSS (Cat#1406-5056, Invitrogen) was diluted in sterile water for injection (Cat# 9309, Irvine Scientific) including 20 mM HEPES (Cat# 15630-080, Invitrogen). pH was adjusted to 7.4. Calcium 4 dye loading buffer for 1 plate was prepared by dissolving contents of one FLIPR Calcium 4 Explorer Kit vial (Cat # R8142, Molecular Devices) completely with a final volume of 10 mL wash buffer. Cell plates were removed from the incubator and culture media was removed from the wells. 25 µL wash buffer and 25 µL Calcium 4 dye loading buffer were added to each well. Plates were not washed after dye addition. Plates were incubated 1 hour at 37 °C, 5% CO_2 and allowed to come to room temperature 30 minutes prior to reading on FLIPR.

***Competitor Calcium Assay Kit Protocols*:**

Instructions for preparation of competitor calcium kits were followed as listed in the respective protocol.

***Calcium mobilization assay on FLIPR^{TETRA}*:**

A 5X dose response of Carbachol (Cat# C4382, Sigma) was prepared as agonist in 384-well plates. Agonist was added on FLIPR^{TETRA} at optimized parameters. Relative Fluorescence Units (RFU) were measured for each response for approximately 2 minutes after addition. FLIPR^{TETRA} parameters are listed in Table 1. Maximum minus minimum peak height was used to calculate response. Graphs and EC_{50} concentrations were calculated using GraphPad Prism®. Z factor calculations were performed using the method from Zhang, *et.al*.

Table 1. FLIPR^{TETRA} Assay Parameters

Parameter	Setting
Excitation Wavelength	470-495 nm
LED Intensity	50%
Emission Wavelength	515-575 nm
Camera gain	50
Exposure Length	0.4 Sec
Read Interval	1 Sec
Dispense Volume	12.5 µL
Dispense Height	35µL
Dispense Speed	30 µL/sec

Results

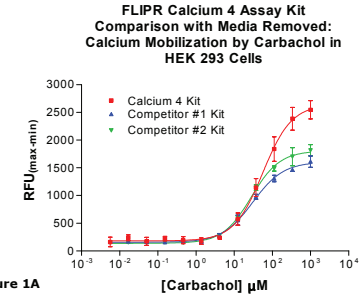

Figure 1A

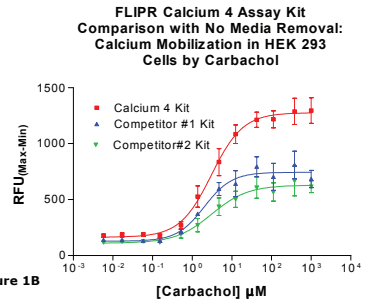

Figure 1B

Figure 1. Comparison of calcium mobilization by carbachol in HEK-293 cells. A: Culture media is removed from the wells prior to dye loading. B: Culture media is not removed prior to dye loading.

Table 2. Comparison of Z-factors and Signal to Background In Assays with Media Removed/not Removed Prior to Dye Loading

FLIPR^{TETRA} HEK 293 cells +	Media Removed		Media NOT Removed	
Carbachol	Before Dye Loading		Before Dye Loading	
Kit	EC_{50}	Z at EC_{80}	EC_{50}	Z at EC_{80}
Calcium 4 Kit	56	0.6	3.1	0.68
Competitor #1 Kit	30.8	0.75	2.2	0.62
Competitor #2 Kit	37.9	0.81	3.1	0.32

Figures 1A and 1B show comparable EC_{50} concentrations between Calcium 4 Assay Kit and competitor kits that are overall slightly higher with the media removed. Summarized in Table 2, Calcium 4 Assay Kit produces Z-factors above 0.5 with the media removed as well as without removal. This is not the case for all competitor kits. Calcium 4 Assay Kit provides the option to reduce perturbation of the cell layer by not removing media from the well yet still achieves assay performance. As seen in Figure 2A, Calcium 4 Assay Kit with media removed provides higher signal to background than without removal. Minimization of the dip in signal upon ligand addition is consistent with media removal in all kits. Evidenced by reduction in error bars, there is less variation in the signal when the media is removed in all cases.

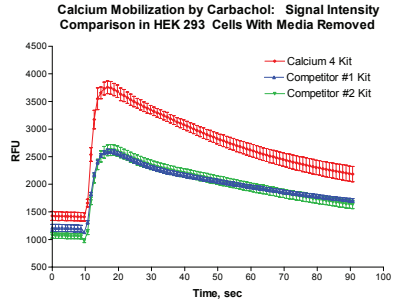

Figure 2A

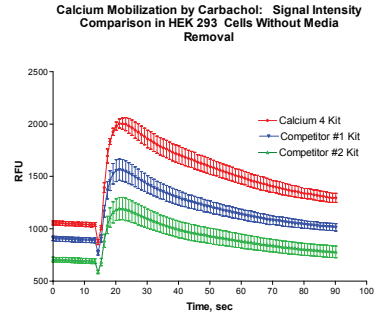

Figure 2B

Figure 2. Comparison of calcium mobilization by carbachol in HEK-293 cells. A: Signal intensity increases when media is removed. B: When culture media is not removed prior to dye loading, signal diminishes as well as an increase in the dip upon addition.

Conclusion

FLIPR Calcium 4 Assay Kit, with a new inert proprietary patented quench technology, is shown to give consistent results with and without media removal before dye loading. Not removing media makes it possible to use loosely adherent cells while still achieving acceptable Z-factors and signal to background ratio. In general, signal intensity is higher than competitor kits. With less signal dip upon addition of ligand it is possible to achieve more consistent data with FLIPR Calcium 4 Assay Kit.

Reference

J. Zhang, *et. al.* A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays, Journal of Biomolecular Screening, Vol. 4, (2)60-73, (1999)

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