Cell based assays on the FLIPR® Tetra System: Comparison of a novel FLIPR® calcium assay to other fluorescence based calcium flux assays

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Abstract

Cell-based calcium flux assays on the FLIPR® Tetra system are widely used in high-throughput screening (HTS) for identification of GPCR agonists and antagonists as well as other applications such as cardiac beating assays. Here we introduce a new reagent system utilizing a novel calcium sensitive ionophore that has a larger signal window with low background compared to other kits while maintaining Z' factors. As the science of HTS has matured, the need for reagent flexibility has increased and as such we have developed calcium dye kits with two different formulations. The first combines the new calcium sensitive ionophore with the Molecular Devices proven proprietary masking technology that can be used for traditional no-wash fluorescence-based detection of changes in intracellular calcium concentration. Masking technology significantly lowers background fluorescence and increases the signal-to-noise ratio without the need to remove growth media or wash cells. We also introduce a second kit, without masking technology, for use in assays where quench could interfere with receptor biology or multiplexing assays where a second readout based upon cell luminescence or detection of a product after cell lysis is required. Comparisons are made between current calcium flux reagents and the new reagent system.

Introduction

Calcium assays from Molecular Devices employ sensitive calcium indicators and masking dyes. Two new FLIPR® Calcium 6 Assay Kits contain a new dye formulation that further enhances the calcium flux assay with an increased signal window. Kit components are mixed with buffer and incubated for approximately two hours with cells. During incubation, the indicator passes through the cell membrane and esterases in the cytoplasm cleave the AM portion of the molecule. After incubation with the dye, the cells are ready to be assayed. Once the target is activated, direct measurement of intracellular fluorescence change due to increased calcium concentration is enabled. The masking dye, in the FLIPR® Calcium 6 formulation, does not enter the cell, but significantly reduces background originating from residual extracellular fluorescence of calcium indicator, media and other components. The FLIPR® Calcium 6-QF Assay Kit formulation is a new flexible option for quench sensitive targets or multiplexing applications. Some cell lines have an anion-exchange protein that requires the use of an anion reuptake inhibitor such as probenecid to retain the commonly used calcium indicators such as FLuo-3 and Fluo-4. However, the new dye formulation for the FLIPR Calcium 6 Assay Kits is more resistant to such organic anion transporters, and thus less or no probenecid may be required for assays performed with FLIPR Calcium 6 Assay Kits. This is especially useful for evaluating targets that may be sensitive to probenecid, as well as for screening agonists and antagonists.

Materials and Methods

FLIPR Calcium 6 and Calcium 6-QF Assay Preparation

FLIPR® Calcium 6 Explorer Kit (Product #R8190, Molecular Devices, Sunnyvale, CA) includes 10 vials of 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. Competitor kits contain the same type of components. FLIPR Calcium 6-QF Explorer Kit (Product #R8192) contains 10 vials of Component A, 1 bottle Component B Buffer, and 10 vials of Component C. Using the Calcium 6 Kit, 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. Using the Calcium 6-QF kit, 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. Component C is dissolved in 36 µL DMSO and added to Components A and B. Probenecid (PBX) was added where necessary. Cell plates were removed from the incubator and 25 µL Calcium 6 Kit, Calcium 6-QF Kit, FLIPR® Calcium 5 Assay Kit, or competitor kit dye loading buffer was added to each well. Plates were *not* washed after dye addition. In the case of Calcium 6 and Calcium 6-QF Kits, Dye loaded plates were incubated TWO hours @ 37°C, 5% CO_2 and allowed to cool to room temperature 15 minutes prior to reading on the FLIPR Tetra or FlexStation® 3 instruments. Calcium 5 Kit and competitor kits were incubated following recommended protocol for 1 hour @ 37°C, 5% CO_2 prior to assay.

Calcium Mobilization Assay on the FLIPR Tetra and FlexStation 3 Systems

A 5X volume of appropriate 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. Antagonists were prepared at 5X concentration and added 15 minutes prior to addition of a 6X volume of EC_{80} concentration of 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*.

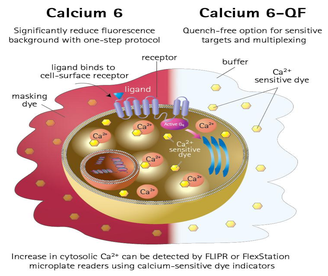
Cell Lines and Compounds

HEK-293, CHO M1WT3, and HeLa cells were all obtained from ATCC, Manassas, VA. Cryopreserved "Assay Ready" 1321N1 cells expressing Histamine H1 receptor from ECACC, Porton Down, Salisbury, UK. Histamine, Carbachol, Atropine, Pyrilamine, Diphenhydramine, and Probenecid compounds were all from Sigma Aldrich, St. Louis, MO

About the FLIPR Tetra System

- The flexibility of the FLIPR Tetra System makes it possible to detect a variety of assays beyond fluorescence based calcium flux and membrane potential assays
- ICCD camera provides luminescent detection for Aequorin in addition to kinetic cAMP luciferase assays such as GloSensor
- Enable the application flexibility you require with a wide range of optic LEDs from 340nm to 620 nm
- Measure changes in transient calcium signaling in beating cardiomyocytes with FLIPR Calcium 6 Assay Kit, ScreenWorks® PeakPro™ software, and the ICCD camera
- Scalable assay throughput: 96-, 384- and 1536-well plate formats, easily integrated with automation

FLIPR Calcium 6 Assay Principle



- Background fluorescence is reduced by masking technology
- New dye formulation delivers larger signal window due to enhanced retention of dye within the cell
- Anion exchange protein inhibitor sensitive targets can be run with less or no probenecid
- Calcium 6-QF formulation is a new flexible option for quench sensitive targets or multiplexing applications

Results

Optimization of Calcium 6 Assay Incubation Time

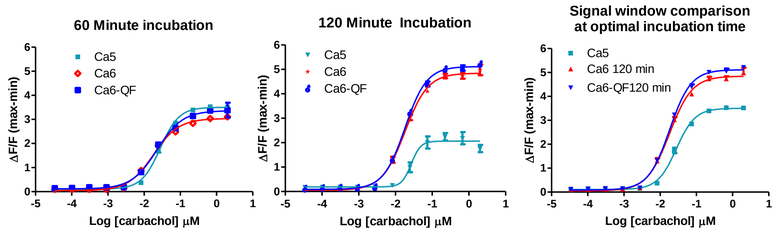
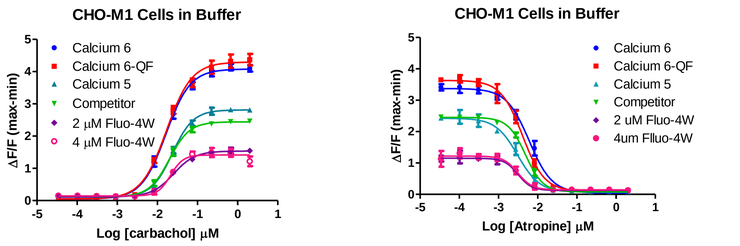


Figure 1. Optimization of assay incubation time shows that both Calcium 6 and Calcium 6-QF assays benefitted from a 2 hour incubation to achieve maximum signal window due to larger molecule size. Calcium 5 Kit and competitor dyes were incubated at their optimal incubation time of one hour. All assays were run with CHO-M1 cells in buffer. EC_{50} values were comparable to historical values (data not shown) and Z @ EC_{80} values were > 0.8.

Comparison of Calcium 6 Kits to Other Calcium Flux Assays

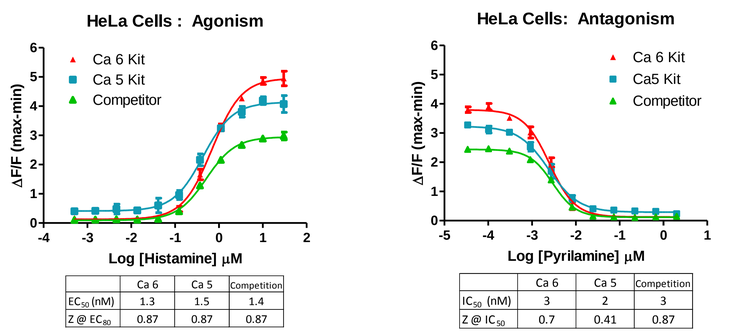


	Ca 6	Ca6-QF	Ca 5	Competition	2 uM Fluo4	4 uM Fluo-4
EC_{50} (nM)	16	17	23	20	25	21
Z @ EC_{80}	0.88	0.84	0.89	0.85	0.86	0.71

	Ca 6	Ca6-QF	Ca 5	Competition	2 uM Fluo-4	4 uM Fluo-4
IC_{50} (nM)	6	4	3	5	3	3
Z @ IC_{80}	0.77	0.76	0.7	0.83	0.53	0.1

Figure 2. Comparison of Calcium 6 kits to other calcium flux assays. Each assay was incubated at optimal time for dye loading. Fluo-4 wash assay had the smallest signal window due to greater cell manipulation and extracellular fluorescence background. Both Calcium 6 and Calcium 6-QF kits provided the highest signal windows compared to the other kits or Fluo-4 Wash. EC_{50} values were preserved across all assays and Calcium 6 Kits showed Z @ EC_{80} > 0.84 in the agonist.

Calcium 6 Kit Assay with HeLa Cells With Endogenous H1 Receptor



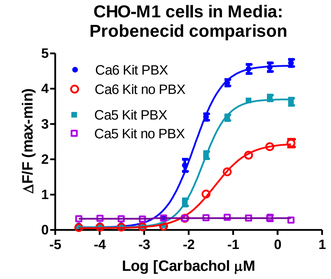
	Ca 6	Ca 5	Competition
EC_{50} (nM)	1.3	1.5	1.4
Z @ EC_{80}	0.87	0.87	0.87

	Ca 6	Ca 5	Competition
IC_{50} (nM)	3	2	3
Z @ IC_{50}	0.7	0.41	0.87

Figure 3. HeLa cells express an endogenous Histamine H1 receptor. Cells were run in 1.25 mM PBX in media. Calcium 6 Kit continues to show a higher signal window with conserved EC_{50} values compared to other kits. Antagonist IC_{50} values are also conserved and Z at EC_{80} and at IC_{50} are also strong.

Results (continued)

Calcium 6 Assay Enables Study of Probenecid Sensitive Targets

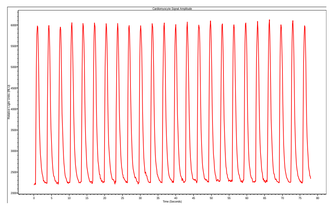


	Ca6 + PBX	Ca6 - PBX	Ca5 + PBX	Ca5 - PBX
EC_{50} (nM)	13	39	14	ND
Z @ EC_{80}	0.9	0.86	0.92	ND

Figure 4. CHO-M1 cells contain an anion-exchange protein that typically requires the use of an anion reuptake inhibitor such as probenecid to retain the commonly used calcium indicators such as Fluo-3 and Fluo-4. Due to its larger molecular size, it is possible to run a calcium flux assay with FLIPR Calcium 6 Kit with a lower concentration or no probenecid. The signal is smaller, but for sensitive targets, running without probenecid maintains Z factors at EC_{80} > 0.86 and conserves the EC_{50} value compared to an assay with probenecid.

Transient Calcium Signaling in Beating Cardiomyocytes

Figure 5. The FLIPR Tetra system and Calcium 6 Kit dye can be used to monitor changes in intracellular Ca^{2+} fluxes associated with cardiomyocyte contractions. The FLIPR Tetra system reads all well simultaneously thus reducing variability. The absolute beat rates were found to be very similar to that measured by imaging methods. Temporal response curves for analysis and visualization of beating can be acquired in ~ 2 min per plate making this assay suitable for high throughput screening of compound libraries.



Calcium 6 Assay Kit on FlexStation 3 Instrument

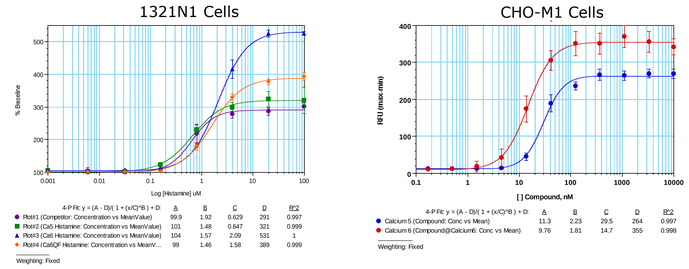
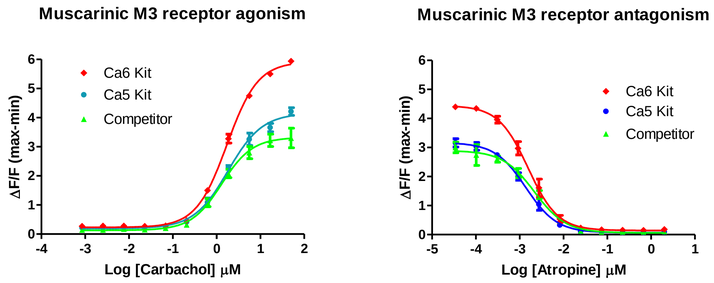


Figure 7. Calcium 6 Kits can be used with the Molecular Devices FlexStation 3 instrument. In figure A. 1321N1 frozen cells express an endogenous Histamine 1 receptor. Comparison to Calcium 5 kit and competitor shows that Calcium 6 with quench gives the highest signal window. SoftMax® Pro software calculated EC_{50} values are within a half log. In figure B, CHO-M1 Cells were stimulated with Carbachol. The assay was run at 37° C. Again, Calcium 6 showed a larger signal window and the EC_{50} values were within one half log.

HEK 293 Cells Expressing Endogenous M3 Target



	Calcium 6	Calcium 5	Competitor
EC_{50} (uM)	1.7	1.8	1.3
Z @ EC_{80}	0.93	0.76	0.74

	Calcium 6	Calcium 5	Competitor
IC_{50} (nM)	2	1	3
Z @ IC_{50}	0.74	0..69	0..55

Figure 8. HEK 293 cells have an endogenous Muscarinic M3 receptor that shows low expression. This is representative of other targets that benefit from a larger signal window. Calcium 6 Kit provides a larger signal window. The assay was run in buffer. EC_{50} and IC_{50} values are conserved and the Z factors are 0.93 for agonism and 0.74 antagonism, the best in each group.

Conclusions

- Largest signal window of comparison calcium kits and dyes
- Enable low signal screens, including endogenous, primary or stem cell targets
- Masking technology significantly reduces extracellular background with proprietary one-step protocol
- Quench-free option permits flexibility with calcium flux and other assay multiplexing to better characterize signal pathways
- Understand target behavior in cells with an anion exchange protein transporter using probenecid optional protocol

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