APPLICATION NOTE

Calcium signaling with FLIPR Calcium 6 and 6-QF Assay Kits on the FlexStation 3 reader

Benefits

- **Provides largest signal window of comparable calcium kits and dyes**
- **Enables low signal screens, including endogenous, primary or stem cell targets**
- **Masking technology significantly reduces extracellular background with proprietary one-step protocol**
- **Resistance to organic ion transporters minimizes need for anion reuptake inhibitors**

Introduction

FLIPR® Calcium Assay Kits from Molecular Devices employ sensitive calcium indicators and proprietary masking dyes to enable researchers to conduct highly sensitive fluorescent screens of G-protein coupled receptors (GPCRs), ion channels, and other calcium sensitive targets. By using a novel dye formulation to further enhance the calcium flux assay signal window, assay robustness is increased, while providing greater assay protocol flexibility. As a result, the FLIPR Calcium 6 and Calcium 6-QF Calcium Kit dyes become more suitable for measuring calcium flux in 384-well plates with the medium throughput FlexStation® 3 Multi-Mode Microplate Reader using the 16 channel pipettor. Results are similar to those obtained on the FLIPR® Tetra System in high throughput mode.

Assay principle

As shown in Figure 1, the FLIPR Calcium 6 assay dye enters the cytosol of the cell. The masking technology does not enter the cell but significantly reduces background fluorescence originating from residual extracellular calcium indicator, media, and other components. The FLIPR Calcium 6-QF Assay Kit formulation contains no masking technology and delivers a new, flexible option for quench sensitive targets or multiplexing applications. Additional assay flexibility is provided with minimal to no requirement for use of probenecid in the assays. Certain cells such as Chinese Hamster Ovary (CHO) cell lines have an anion-exchange protein that requires the use of an anion reuptake inhibitor, such as probenecid, to retain commonly used calcium indicators within the cytosol. The unique FLIPR Calcium 6 Assay Kit dye formulation is more resistant to such organic anion transporters, thus less or no probenecid may be required.

Materials and methods

An assay examining the endogenous histamine receptor expressed in HeLa cells was developed using the new FLIPR Calcium 6 and Calcium 6-QF Assay Kits, and then compared to other commercially available kits. Calcium flux was measured with the FlexStation 3 reader using the 'Flex' read mode. HeLa cells kept in continual culture were plated at 5,000 cells/well in 50 µL growth media in black-wall, clear bottom 384-well microplates and then maintained overnight at 37°C, 95% humidity, and 5% CO_2. On the following day, cell plates were loaded with the appropriate calcium kit reagents following manufacturers' recommendations (including water soluble probenecid) and incubated for 60 or 120 minutes according to manufacturer protocol.

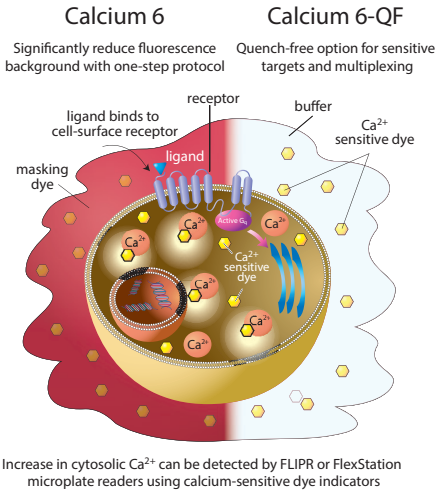


Figure 1. Assay flexibility with or without masking technology.

The cells were challenged with varying concentrations of histamine (starting at 100 µM, in 3-fold dilutions) using the FlexStation 3 reader's integrated 16-channel pipettor. Fluorescence measurements were taken for 60 seconds before, during, and after compound addition using optimized parameters (Table 1.) For the antagonist studies, pyrilamine and risperidone (starting at 30 µM, in 3-fold dilutions) were added using the FlexStation 3 reader's on-board fluidic system and allowed to equilibrate for 30 minutes. The cells were then stimulated with 15 µL/well of histamine (EC_{80} concentration) while changes in fluorescence intensity were monitored in real time.

The calcium indicators used for the comparison were:

- FLIPR Calcium 6 Assay Kit (Molecular Devices)
- FLIPR Calcium 6-QF Assay Kit (Molecular Devices)
- Fluo-4 Direct Calcium Assay Kit (Life Technologies)
- Fluo-4 Direct Calcium Assay Kit (Life Technologies)

FlexStation 3 reader settings

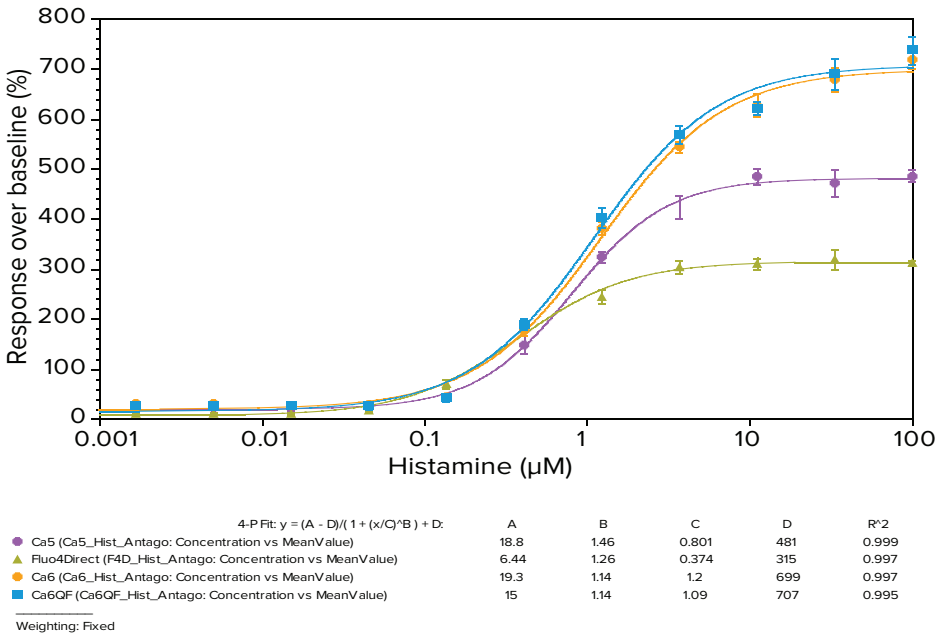
Cell plates were maintained at 37°C inside the FlexStation 3 reader. Compounds were prepared in Greiner 384-well polystyrene deep-well plates and FlexStation 3 Tips (black) were used for compound transfer. The specific parameters are indicated in Table 1.

Results and analysis

Responses were measured as peak fluorescence intensity. To enable comparison, data was normalized as % response over baseline and expressed as mean ± standard deviation with n=4. Individual sets of concentration-response data were fitted to a four-parameter curve using SoftMax® Pro Software (Figures 2-5).

Parameter	Settings
Read type	Flex
Read mode	Fluorescence, bottom read
Ex wavelength	485 nm
Em wavelength	525 nm
Cut-off	515 nm
Run time	60 sec
Interval	2.2 sec
PMT level	Medium
Compound addition	
Initial volume	50 µL
Pipette height	40 µL
Volume	1st addition: 12.5 µL antagonist
	2nd addition: 15 µL agonist
Rate	3 (12 µL/s)
Addition time point	19 sec.

Table 1. FlexStation reader settings. Optimized instrument settings for the calcium assays described are shown.



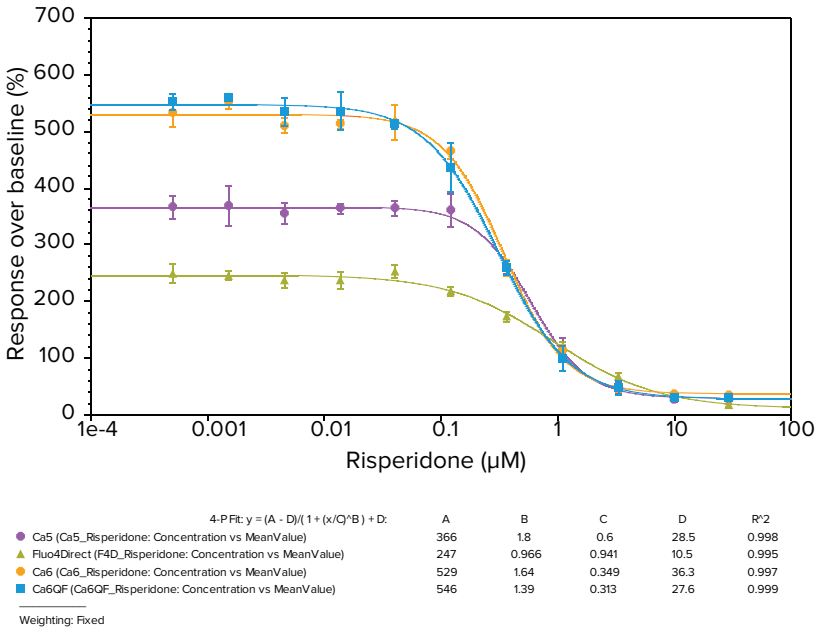
Histamine	Calcium 6	Calcium 6-QF	Calcium 5	Fluo-4 Direct
EC_{50} µM	1.2	1.1	0.8	0.37
Z@ EC_{80}	0.87	0.92	0.87	0.82

Figure 2. FLIPR Calcium 6 and 6-QF Assay Kits provide the largest signal window. Histamine H1 is an endogenous receptor in HeLa cells. Comparing FLIPR Calcium 6 and 6-QF to other dyes shows that both had the largest signal window. EC_{80} values were within one-half log and Z factors at EC_{80} were comparable.

Conclusion

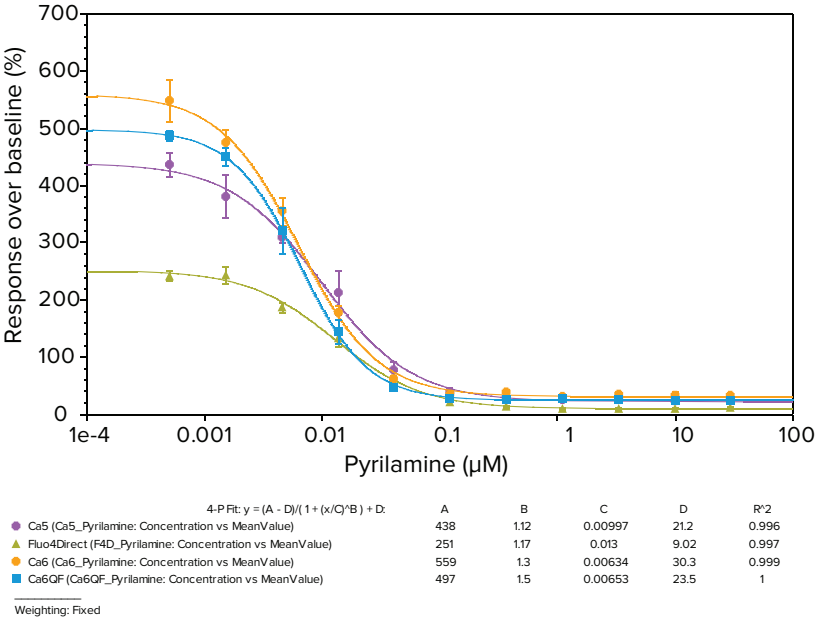
FLIPR Calcium 6 and Calcium 6-QF Assay Kits offer flexible calcium flux assays including medium throughput options such as the FlexStation 3 reader while providing reliable pharmacology, a superior signal window, and high quality assay performance. Having a greater signal window is important because many of today's assays present challenges not seen with standard agonist or antagonist assays using over-expressed receptors. Use of cell lines with endogenous receptors, lower expression of receptors, frozen cells, primary cells, or stem cells may produce lower signal windows. In addition, a larger signal window is an advantage when performing assays to identify allosteric modulators.

The FLIPR Calcium 6-QF Assay Kit option without quench provides new assay flexibility to enable assays for studying quench sensitive targets. Lastly, the ability to study target behavior in cell lines such as CHO cells in the absence of an anion reuptake inhibitor can be beneficial as some receptors and ion channels may be probenecid sensitive and its use may alter the natural biological mechanisms. The larger signal window provided by FLIPR Calcium 6 Kits delivers a robust assay for compound screening and optimization utilizing these challenging targets and cell lines.



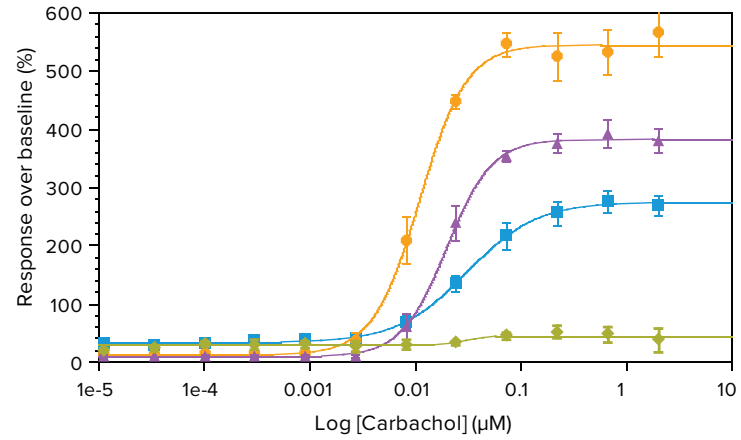
Pyrilamine	Calcium 6	Calcium 6-QF	Calcium 5	Fluo-4 Direct
IC_{50} µM	0.35	0.31	0.6	0.94
Z@ IC_{50}	0.77	0.83	0.81	0.67

Figure 3. Risperidone antagonist response to histamine challenge in HeLa cells. Risperidone is an antipsychotic drug used to treat schizophrenia. It is a dopamine antagonist that also has antihistamine properties. The signal from both Calcium 6 and 6-QF dyes provides the largest window for the antagonist assay. IC_{50} values are within one-half log of each other and Calcium 6-QF has the highest Z factor at IC_{50} concentration.



Pyrilamine	Calcium 6	Calcium 6-QF	Calcium 5	Fluo-4 Direct
EC_{50} µM	0.006	0.007	0.01	0.013
Z@ IC_{50}	0.76	0.58	0.36	0.6

Figure 4. Pyrilamine antagonist response to histamine challenge in HeLa cells. Pyrilamine is a first generation Histamine H1 antagonist. Because of the larger signal window provided by the FLIPR Calcium 6 Assay Kit, the Z factor at IC_{50} is the largest. In addition, the Calcium 6-QF kit also provides a robust assay that does not require washing when quench sensitive targets are to be studied.



4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
Ca6 - PBX (Ca6 - PBX: Conc vs MeanValue)	32.7	1.31	0.031	274	0.999
Ca6 +PBX (Ca6 + PBX: Conc. vs MeanValue)	11	1.96	0.011	545	0.998
F4D + PBX (F4D + PBX: Conc. vs MeanValue)	8.18	2.03	0.0199	382	0.999
F4D - PBX (F4D - PBX: Conc. vs MeanValue)	27.4	3.35	0.0312	45.7	0.823

Weighting: Fixed

Carbachol	Calcium 6	Calcium 6 without Probenecid	Fluo -4 Direct	Fluo-4 Direct without probenecid
EC_{50} µM	0.031	0.011	0.031	0.02
Z@ EC_{80}	0.92	0.52	0.57	-1.31

Figure 5. FLIPR Calcium 6 assay dye does not require use of anion reuptake inhibitors. Assay performed with CHO-M1 cells and Calcium 6 dye demonstrates a response to carbachol without the need for incubation with probenecid. The corresponding assay with Fluo-4 Direct shows virtually no signal. Calcium 6 dye is an important new development for understanding targets that may be sensitive to anion reuptake inhibitors.

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