

APPLICATION NOTE

Enhance your calcium screens with FLIPR Calcium 6 Assay Kits

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. 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 shown in Figure 1, the FLIPR Calcium 6 assay 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 (also shown in Figure 1) 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. However, 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.

FLIPR Calcium 6 and Calcium 6-QF assay preparation

FLIPR Calcium 6 (Product #R8190) and Calcium 6-QF (Product #R8192) Explorer Kits were used in this study. Both dyes were made up in Hank's Balanced Salt Solution (HBSS) with 20 mM HEPES. Probenecid (PBX) was added where necessary to inhibit dye leaking out of cells. Adherent CHO M1 cells plated the night before, in 384-well black and clear cell plates were removed from the incubator, then 25 μ L FLIPR Calcium 6, Calcium 6-QF or Calcium 5 Assay Kit dye, or competitor kit dye loading buffer was added to wells containing 25 μ L assay buffer or media.

Benefits

- Largest signal window to screen low response targets and cells
- Robust EC₃₀ signal window to monitor allosteric modulation
- Enables screening of quench-sensitive targets
- Multiplex calcium flux with luminescent assay technologies
- Resistant to organic anion transporters

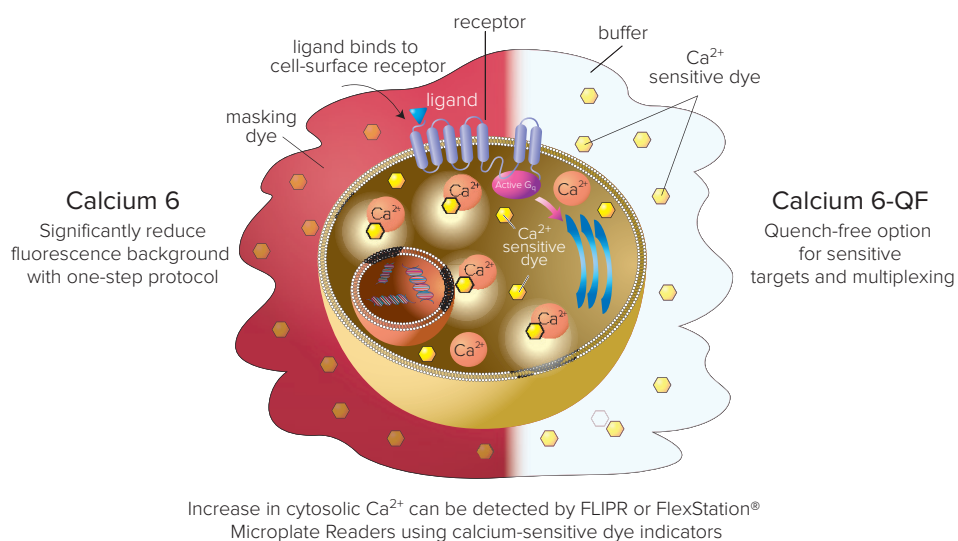


Figure 1. Assay flexibility with or without masking technology.

In the case of FLIPR Calcium 6 and Calcium 6-QF Kits, dye loaded plates were incubated two hours at 37°C, 5% CO₂. FLIPR Calcium 5 Assay Kit and competitor kits were incubated following their recommended protocols for one hour at 37°C, 5% CO₂ prior to assay. All plates were removed from the incubator and allowed to cool to room temperature 10 minutes prior to reading on either the FLIPR® Tetra System or FlexStation® 3 Multi-Mode Microplate Reader.

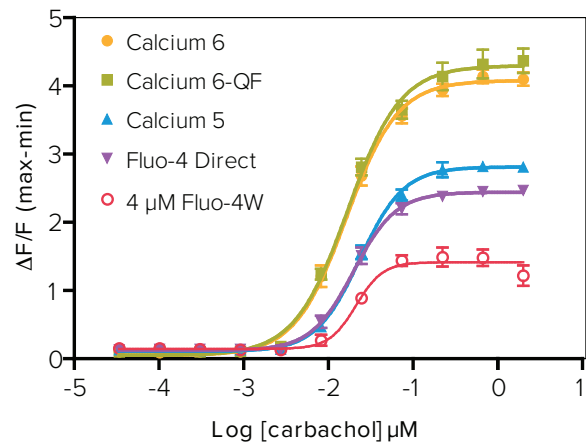
Plates with any of the FLIPR Calcium Assay Kits or Fluo-4 Direct Kit did not require a wash after dye loading. The plate with only Fluo-4 dye was washed three times with HBSS assay buffer.

Calcium mobilization assay on the FLIPR Tetra System and FlexStation 3 reader

A 5X concentration 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 System or FlexStation 3 reader at optimized parameters. Signal, measured in Relative Fluorescence Units (RFU) was detected in each well for approximately 90 seconds, including during and post addition. The output calculated was signal window ($\Delta f/f$) maximum minus minimum. Data for graphs and EC₅₀/IC₅₀ concentrations was exported from ScreenWorks® or SoftMax® Pro Software, then calculated using GraphPad Prism. Z factor calculations were performed using the method described by Zhang, et. al.

Results

Comparison of FLIPR Calcium 6 Assay Kits to other calcium indicators was measured using agonism of the muscarinic receptor on CHO M1WT3 cells from ATCC (Figure 2). Each assay was incubated at optimal time for dye loading according to manufacturer instructions. Fluo-4 wash (Fluo-4W) assay had the smallest window due to greater cell manipulation and extracellular fluorescence background. Both FLIPR Calcium 6 and Calcium 6-QF Kits showed the highest signal window of $\Delta f/f = 4$ and 4.2 respectively. EC₅₀ values were preserved in all cases and the FLIPR Calcium 6 Kit showed Z @ EC₈₀ = 0.88 in the agonist assay.



	Ca 6	Ca 6-QF	Ca 5	Fluo-4 Direct	4 uM Fluo-4W
EC ₅₀ (nM)	16	17	23	20	21
Z @ EC ₈₀	0.88	0.84	0.89	0.84	0.71
Signal window	4	4.2	2.7	2.3	1.4

Figure 2. FLIPR Calcium 6 Assay Kits provide the largest assay window.

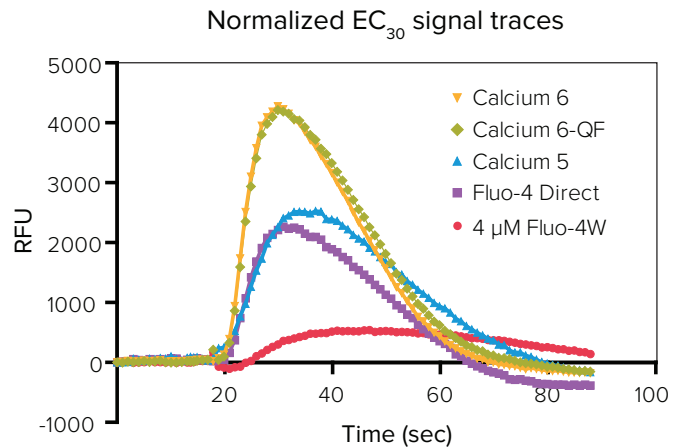


Figure 3. FLIPR Calcium 6 Assay Kit signal response at EC₃₀ enables larger screening windows for allosteric modulator assay.

Screening for allosteric modulators requires the use of an EC₃₀ ligand dose. It is essential to have a large signal window at this low ligand concentration. CHO M1WT3 cells were stimulated with an EC₃₀ concentration of carbachol (Figure 3). Signal intensity from both FLIPR Calcium 6 Assay Kits is twice that of the competitor kit.

FLIPR Calcium 6 Assay Kits can be used with a broad range of cell lines. In Figure 4, “assay ready” 1321N1 frozen cells, expressing endogenous Histamine 1 receptor (from ECACC, Porton Down, Salisbury, UK), were assayed on the FlexStation 3 reader. Comparison of histamine receptor response to increasing concentrations of histamine using FLIPR Calcium 5 Assay Kit, Fluo-4 Direct Kit, FLIPR Calcium 6 Assay Kit and FLIPR Calcium 6-QF Assay Kit demonstrates that the FLIPR Calcium 6 Assay Kit gives the highest signal window. SoftMax Pro Software calculated EC₅₀ values are within a half log of expected values.

CHO M1 cells require the use of the anion reuptake inhibitor probenecid to retain commonly used calcium indicators such as Fluo-3 and Fluo-4. The FLIPR Calcium 5 Assay Kit dye is also expelled from the cell and no response is seen without probenecid. The FLIPR Calcium 6 Assay Kit fluorophore is designed to be better retained within the cell. Figure 5 illustrates that the FLIPR Calcium 6 Assay Kit with no probenecid produces a smaller signal, however the assay still demonstrates a signal window of greater than two-fold and a Z factor at EC₈₀ = 0.86. The EC₅₀ value was within one-half log.

Conclusion

FLIPR Calcium 6 and Calcium 6-QF Assay Kits offer flexible calcium flux assay options while providing reliable pharmacology, a superior signal window, and high quality assay performance required for screening. Having a greater signal window is important because many of today’s assays present challenges not seen with standard agonism/antagonism 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 overall

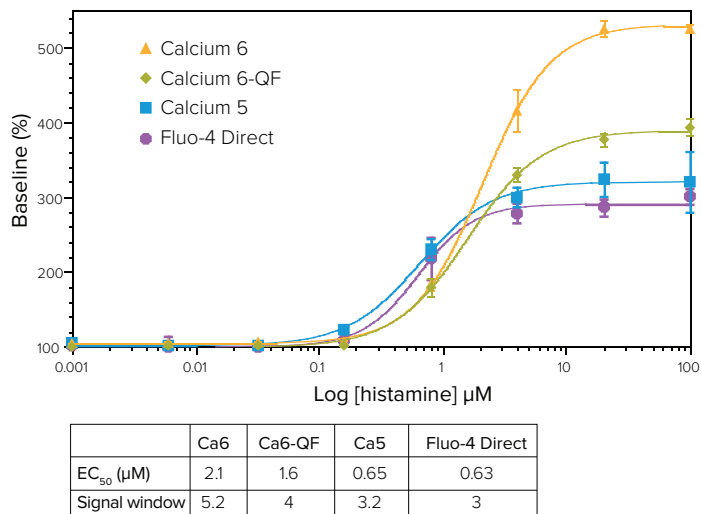


Figure 4. Assay-ready frozen cells assayed on the FlexStation 3 reader.

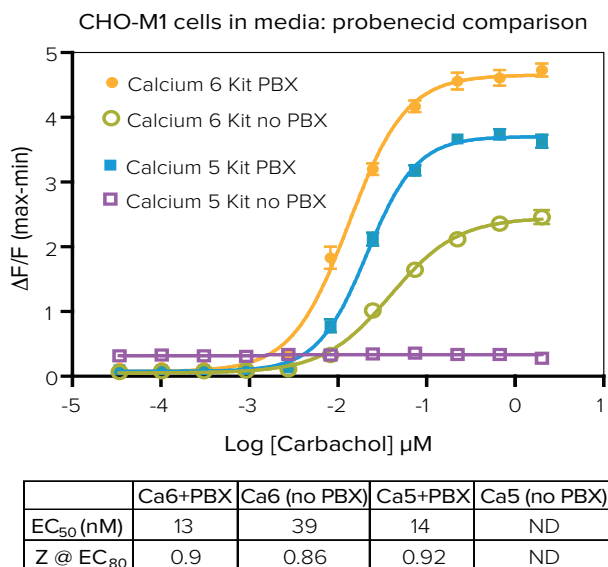


Figure 5. Probenecid sensitive assay.

response. 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. 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 performing multiplexed assays or for studying quench sensitive targets. Lastly, the ability to

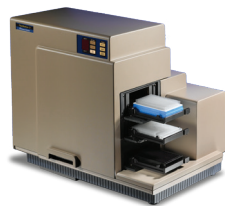
study target behavior in cell lines such as CHO cells in the absence of an anion reuptake inhibitor can be beneficial as some receptors may be probenecid sensitive and its use may alter the natural biology mechanisms.

Reagent	Description	Part number
FLIPR® Calcium 6 Assay Sample Kit	(2) vials of component A* (1) bottle of dilution buffer (Component B) * Each reagent vial (Component A) is sufficient for 1 plate (96-, 384-, 1536-well). Each kit is sufficient for 2 plates.	R6133
FLIPR® Calcium 6-QF Assay Sample Kit	(2) vials of component A* (1) bottle of dilution buffer (Component B) (2) vials of component C * Each reagent vial (Component A, C) is sufficient for 1 plate (96-, 384-, 1536-well). Each kit is sufficient for 2 plates.	R6134
FLIPR® Calcium 6 Assay Evaluation Kit	(3) vials of component A* for Calcium 6 (3) vials of component A for Calcium 6-QF (1) bottle of dilution buffer (Component B) (3) vials of component C * Each reagent vial (Component A, C) is sufficient for 1 plate (96-, 384-, 1536-well). Each kit is sufficient for 3 Calcium 6 assay plates and 3 Calcium 6-QF assay plates.	R8194
FLIPR® Calcium 6 Assay Explorer Kit	(10) vials of component A* (1) bottle of dilution buffer (Component B) * Each reagent vial (Component A) is sufficient for 1 plate (96-, 384-, 1536-well). Each kit is sufficient for 10 plates.	R8190
FLIPR® Calcium 6 Assay Bulk Kit	(10) vials of component A* * Each reagent vial (Component A) is sufficient for 10 plates (96-, 384-, 1536-well). Each kit is sufficient for 100 plates.	R8191
FLIPR® Calcium 6 Assay Express Kit	(2) vials of component A* * Each reagent vial (Component A) is sufficient for 50 plates (96-, 384-, 1536-well). Each kit is sufficient for 100 plates.	R8195
FLIPR® Calcium 6-QF Assay Explorer Kit	(10) vials of component A* (1) bottle of dilution buffer (Component B) (10) vials of component C * Each reagent vial (Component A, C) is sufficient for 1 plate (96-, 384-, 1536-well). Each kit is sufficient for 10 plates.	R8192
FLIPR® Calcium 6-QF Assay Bulk Kit	(10) vials of component A* (1) bottle of dilution buffer (Component B) (10) vials of component C * Each reagent vial (Component A, C) is sufficient for 10 plates (96-, 384-, 1536-well). Each kit is sufficient for 100 plates.	R8193
FLIPR® Calcium 6-QF Assay Express Kit	(2) vials of component A* (1) bottle of dilution buffer (Component B) (2) vials of component C * Each reagent vial (Component A, C) is sufficient for 50 plates (96-, 384-, 1536-well). Each kit is sufficient for 100 plates.	R8196

Compatible with these Molecular Devices® systems



FLIPR Tetra System



FlexStation 3 Multi-Mode
Microplate Reader

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