Cell-based calcium flux 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 other advantages. 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 binding or multiplexing assays where a second control 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 enters the cytoplasm to 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 assays 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 Fluoromycin

Results

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 or no probenecid
• Calcium 6-QF formulation is a new flexible option for quench sensitive targets or multiplexing applications

Materials and Methods
FLIPR® Calcium 6 and Calcium 6-QF Assay Preparation
FLIPR® Calcium 6 Explorer Kit (Product #RB1190, 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. Each kit contains the same type of components. FLIPR® Calcium 6-QF Explorer Kit (Product #RB1192) contains 10 vials of Component A, 2 bottle Component B Buffers, 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 dye 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, 10 vials of Component C and 10 ml of Component B dye 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, 10 vials of Component C and 10 ml of Component B dye 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, 10 vials of Component C and 10 ml of Component B dye loading buffer.

Calcium Mobilization Assay on the FLIPR® Tetra and FlexStation® 3 Systems
A 5x volume of 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 3.5x volume of buffer. Calcium Mobilization Assay Reagents and Fluorescence Units (RFU) were measured for each response for signal maximum minus minimum during approximately 90 seconds after addition. Graphs and ECF<sub>50</sub> concentrations were calculated using GraphPad Prism. 2-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, Pochton Down, Salisbury, Wiltshire, UK. Histamine, Carbachol, Acrylamide, Phthalimide, 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 excitation 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 GoSensor.
• 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<sup>®</sup> ProPeak™ software, and the ICCD camera.
• Scalable assay throughput: 96-, 384- and 1536-well plate formats, easily integrated with automation

FLIPR® Calcium 6 Assay Kit

Comparison of Calcium 6 KITs to Other Calcium Flux Assays

CHO-M1 Cells in Buffer

- Calcium 6
- Calcium 6-QF
- Carbachol
- Ca<sub>5</sub> Kit
- Ca<sub>6</sub> Kit
- Ca<sub>6</sub> Kit (Comp)
- Ca<sub>6</sub> Kit (QF)
- Ca<sub>6</sub> Kit (QF 10mM)

FLIPR® Calcium 6 Assay Kit

Calcium 6 Assay Enables Study of Probenecid Sensitive Targets

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 Fluoromycin and Fluo-4. Due to its larger molecular size, it is possible to run a calcium flux assay with or without probenecid addition. Fig 4 shows that Calcium 6 QF demonstrates nearly no calcium response in the presence of probenecid concentration or no probenecid. The signal is smaller, but for sensitive targets, running without probenecid maintains Z factors at EC<sub>50</sub> = 0.85 and conserves the EC<sub>50</sub> value compared to an assay with probenecid.

Figure 5. The FLIPR® Tetra System and Calcium 6 Kit dye can be used to monitor changes in intracellular calcium associated with cardiac/contractions. The FLIPR® Tetra system reads all wells in parallel, allowing comparison of absolute beat rates. Peak calcium fluxes with absolute beat rates were found to be very similar to data 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

Calcium 6 kit Fluo-4 dye can be used to monitor changes in intracellular calcium associated with Cardiac contraction. The Calcium 6 Kit dye is well suited for high throughput screening of compound libraries. Peak calcium fluxes with absolute beat rates were found to be very similar to data 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.

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