# Evaluation of FLIPR Calcium Assays for Screening GPCR and Calcium Channel Targets

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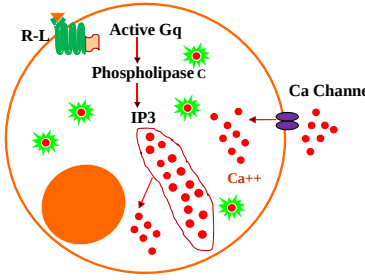
## Introduction

Quest Fluo-8™ AM, Fluo-4 Direct™ and Calcium-4™ have been compared and evaluated for several GPCR and ion channel targets. Quest Fluo-8 AM, Fluo-4 and Calcium 4 share the same assay principle. All the three fluorogenic calcium indicators are in the form of non-fluorescent AM esters. Once inside cells, the lipophilic AM blocking groups are cleaved by non-specific cellular esterases, resulting in negatively charged fluorescent dyes that stay inside cells, and their fluorescence intensities are greatly enhanced upon binding to calcium. When cells stimulated with screening compounds, the receptor signals release of intracellular calcium, which greatly increases their fluorescence signals. Three GPCRs (Muscarinic M1 receptor, purinergic P2Y receptor, and chemokine CCR2b receptor) and one calcium channel capacitative calcium entry (CCE) were assayed. In comparison with Fluo-4 Direct and Calcium 4, Quest Fluo-8 yields the brightest signal and largest assay window. Quest Fluo-8 AM has a less temperature-dependent cell loading property, giving more consistent results either at room temperature or 37°C compared to Fluo-4 Direct or Calcium-4. This characteristic makes Quest Fluo-8 AM much more robust for HTS applications.

* Quest Fluo-8™ AM, Fluo-4 Direct™ and Calcium-4™ are the trademarks of ABD Bioquest, Invitrogen and MDS. respectively

## Screen Quest™ Fluo-8 Calcium Assay




**[$Ca^{2+}$] Increase via Gq or calcium channel is measured by the fluorescence enhancement of Quest Fluo-8 AM.**

## Material and Methods

1. CHO-M1, HEK or Jurkat cells were plated at 96-well black wall/clear bottom costar plate at 37°C incubator for overnight.
2. The cells were incubated with Fluo-4 Direct, Calcium 4 or Quest Fluo-8™ AM.
3. Run calcium efflux experiments on FlexStation.

**Plating cells for overnight**

↓

**Dye loading for 1 hr at RT or 37°C**

↓

**Run calcium assay at Ex 485-490nm/Em 520-530nm**

## Results

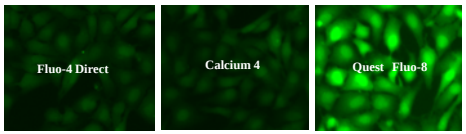



Figure1. U2OS cells were seeded overnight at 30,000 cells per 100 µL per well in a 96 well black wall/clear bottom costar plate. The cells were incubated with 100 µl of Fluo-4 Direct, Calcium 4, or Quest Fluo-8 AM at room temperature for 1 hour. The cells then imaged with a fluorescence microscope (Olympus IX71) using FITC channel.

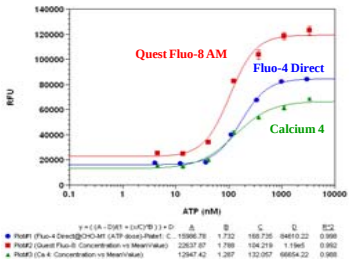



Figure 2. **ATP Dose Response in CHO-M1 cells.** CHO-K1 cells were seed edovernight in 50,000 cells per 100 µl per well in a 96-well black wall/clear bottom costar plate. The cells were incubated with 100 µl of Fluo-4 Direct, Calcium 4or Quest Fluo-8 AM for 30 min at 37°C, 5% $CO_2$ incubator, and then another 30 min at RT. ATP (50 µl/well) was added by FlexStation to achieve the final indicated concentrations. The $EC_{50}$ of ATP for Quest Fluo-8™ AM, Fluo-4 Direct and Calcium 4 are 104, 169 & 132 nM respectively.

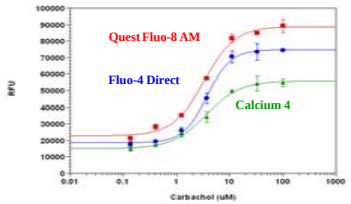



Figure 3. **Carbachol Dose Response in HEK-293.** HEK-293 cells were seeded overnight in 40,000 cells per 100 µl per well in a 96-well black wall/clear bottom costar plate. The cells were incubated with 100 µl of Fluo-4 Direct, Calcium 4or Quest Fluo-8 AM for 30 min at 37 °C, 5% $CO_2$ incubator, and then another 30 min at RT. Carbachol 50 µl/well) was added by FlexStation to achieve the final indicated concentrations. The $EC_{50}$ is about 2 µM which is similar as reported.

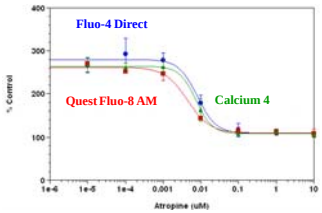



Figure 4. **Aropine Dose Response in HEK-293.** HEK-293 cells were seeded overnight in 40,000 cells per 100 µl per well in a 96-well black wall/clear bottom costar plate. The cells were incubated with 100 µl of Fluo-4 Direct, Calcium 4or Quest Fluo-8 AM for 45 min at 37°C, 5% $CO_2$ incubator, and then atropine (10 µl/well) was added for 15 min incubation at RT to achieve the final indicated concentrations. 50 µl/well of carbachol at 15 µM (5X, so final in well concentration is 3 µM) was added by FlexStation. The $IC_{50}$s of atropine for Quest Fluo-8 AM , Fluo-4 Direct and Calcium 4 are 4 nm, 7 nM, and 8 nM respectively.

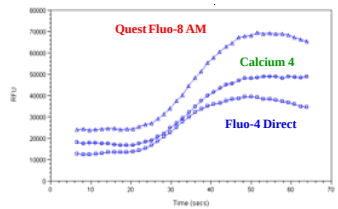



Figure 5. **Comparisons of Quest Fluo-8 AM, Fluo-4 Direct, and Calcium 4 on MCP-1 induced Ca response in CHO-CCR2 cells.** CHO-K1cells transiently transfected with Gα16 and CCR2 genes were seed edovernight in 50,000 cells per 100 µl per well in a 96-well black wall/clear bottom costar plate. The cells were incubated with 100 µl of Fluo-4 Direct, Calcium 4or Quest Fluo-8 for 30 min at 37°C, 5% $CO_2$ incubator. and then another 30 min at RT. 50 µl/well of MCP-1 at 50 nM (5X, so final in well concentration is 10 nM) was added by FlexStation.

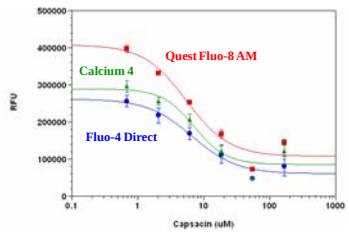



Figure 6. **Comparisons of Screen Quest Fluo-8 AM, Fluo-4 Direct, and Calcium 4 on Concanavalin A induced clacium entry (capacitative calcium entry (CCE)) into JurKat cells.** Jurkat cells were suspended at 2X$10^6$ cells per ml in calcium-free HHBS buffer, cells were incubated with equal volume of Fluo-4 Direct, Calcium 4or Quest Fluo-8 AM in HHBS buffer at 2X$10^5$ cells/well/100 µL at a 96-well black wall/clear bottom costar plate for 1 hr at 37 °C, 5% $CO_2$ incubator. At the end of the 10 min incubation, 10 ul/well of channel opener concanavalin A at 3 ug/ml (final in well concentration) with or without channel inhibitor capsacin were added into the cells to achieve the final indicated concentrations. 50µL/well of HBSS with additional 24 mM (5X) calcium (so final in well concentration of calcium is 5 mM) was added by FlexStation (Molecular Devices) .

## Summary

**Quest Fluo-8 AM provides an optimized assay method for monitoring GPCRs and calcium channels. Its ability to generate strong calcium signal from the weak receptors and difficult cell lines enables researchers to have multiple assay chemistries for different GPCR receptor and calcium channel targets. Quest Fluo-8 technology has the following benefits and features:**

- ***Brighter***
  - ❑ Enable $Ca^{2+}$ assays with weak cell lines & receptors
- ***1536 Well-Friendly***
  - ❑ The best $Ca^{2+}$ indicator for 1536-well $Ca^{2+}$ assays
- ***Large Assay Window***
  - ❑ Large Z' factor for challenging cell lines & receptors.
- ***Robust &Less Temperature-Sensitive***
  - ❑ 37 °C & RT loadings generate similar results

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