A New Red Fluorescent & Robust Quest Rhod-4™ Ca^{2+} Indicator for Screening GPCR & Ca^{2+} Channel Targets

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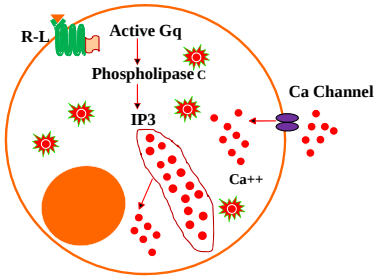
Introduction

Calcium flux assays are preferred methods in drug discovery for screening G protein coupled receptors (GPCRs). Although Rhod-2 has been the most popular red fluorescent Ca^{2+} indicator for minimizing the compounds auto-fluorescence interference. In addition, its mitochondrial localization and high basal Ca^{2+} signal in cells have severely limited its cellular applications. Finally the less optimal excitation of Rhod-2 at 488 nm makes it less robust to use with some instruments that have only 488 nm excitation light source. Our Quest Rhod-4™ serial calcium detection reagents have been developed to address these limitations of Rhod-2. It is quite unique that Quest Rhod-4™ can be well excited with an argon ion laser at 488 nm besides its 514 nm, 532 nm and 546 nm excitations. The characteristics of its predominantly cytosol localization, and long multi-wavelength excitation and >100 times fluorescence enhancement (when it forms a complex with calcium) make Quest Rhod-4™ an ideal indicator for measurement of cellular calcium especially when the library compounds have strong fluorescence and the applications require GFP. Our Quest Rhod-4™ AM is more than 4 fold brighter than Rhod-2 AM in cells. This characteristic makes the Screen Quest™ Rhod-4™ more robust for HTS applications.

Screen Quest™ Rhod-4 Calcium Assay



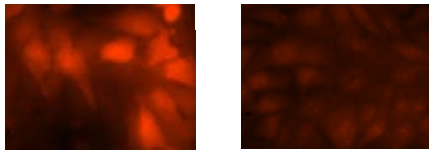
Increase in [Ca^{2+}] in cytosol via Gq or calcium channel is measured by the fluorescence enhancement of Rhod-4

Material and Methods

1. CHO-M1 or HEK cells were plated at 96-well black wall/clear bottom costar plate at 37 °C incubator for overnight.
2. Take growth medium off (if cells were plating in 0.5-1% FBS, skip this step), incubate the cells with Rhod-4 AM or Rhod-2 AM at room temperature for 1-2 h (or at 37 °C for 1 h, then at room temperature for 30 min).
3. For wash experiments: wash cells with HHBS buffer twice, then replace with HHBS buffer.
4. For No wash experiments: run the experiments directly.
5. Run calcium efflux experiments on NOVOstar (BMG Labtec) at EX 530nm/Em 570nm

Plating cells for overnight
↓
Aspirate growth medium
↓
Dye loading for 1 h at RT or 37°C
↓
Run calcium assay at Ex 530nm/Em 570nm

Results



Rhod-4 AM **Rhod-2AM**

Figure1. U2OS cells were seeded overnight at 40,000 cells per 100 µL per well in a 96 well black wall/clear bottom costar plate. The growth medium was removed, and the cells were incubated with 100 µl of 5µM Rhod-4 AM or Rhod-2 AM in HHBS at 37 °C for 1 hour. The cells were washed twice with 200 µl HHBS, then imaged with a fluorescence microscope (Olympus IX71) using FITC channel.

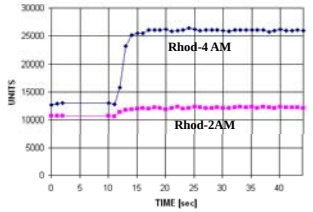


Figure 2. **Comparisons of Screen Quest™ Rhdo-4 AM and Rhod-2 AM in HEK-293 cells.** HEK-293 cells were seeded overnight in 40,000 cells per 100 µl per well in a 96-well black wall/clear bottom costar plate. The growth medium was removed, and the cells were incubated with 100 µl of 5 µM Rhod-4 AM or Rhod-2 AM in HHBS at 37 °C for 1 hr. Then wash the cells with 2 times of HHBS. 30 µM of Carbachol (50µl/well) was added by NOVOstar (BMG Labtech).

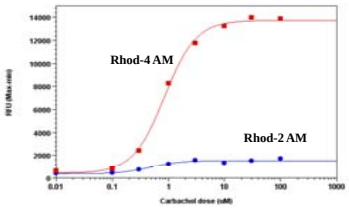


Figure 3. **Carbachol Dose Response in HEK-293 cells.** HEK-293 cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom plate. The growth medium was removed, and the cells were incubated with 100 µl of the Screen Quest™ Rhod-4 NW Calcium assay kit, or 5 µM Rhod-2 AM at 37°C, 5% CO_2 incubator for 1 hour. Carbachol (25µl/well) was added by NOVOstar to achieve the final indicated concentrations. The EC_{50} is 0.8 µM which is similar as reported.

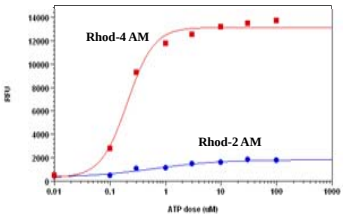


Figure 4. **ATP Dose Response in CHO-K1 Cells.** CHO-K1 cells were seeded overnight at 60,000 cells /100 µl/well in a 96-well black wall/clear bottom costar plate. The growth medium was removed and the cells were incubated with 100 µl of the Screen Quest™ Rhod-4 NW Calcium assay kit, or 5 µM Rhod-2 AM at 37°C, 5% CO_2 incubator for 1 hour. Carbachol (50µl/well) was added by NOVOstar to achieve the final indicated concentrations. The EC_{50} is 0.6 µM which is similar as reported.

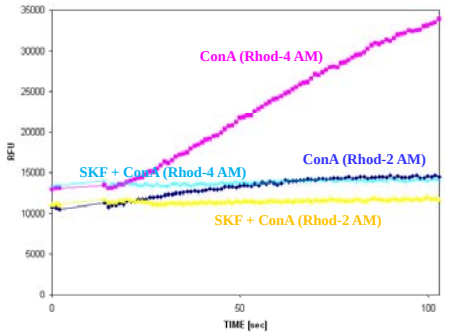


Figure 5. **Comparisons of Screen Quest™ Rhod-4 NW Calcium Assay Kit, Rhod-2 AM on Concanavalin A induced Ca entry (capacitative calcium entry (CCE)) into JurKat cells.** Jurkat cells were suspended at $4X10^6$ cells per ml in calcium-free HHBS buffer, cells were incubated with equal volume of Rhod-4 NW or Rhod-2 AM in calcium-free HHBS buffer at $2X10^5$ 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, the channel opener Concanavalin A at 1 ug/ml with or without the channel inhibitor SKF96365 at 30 µM were added into the cells. 50 µL/well of HBSS with additional 24 mM (5X) Calcium (so final in well concentration of calcium is 5 mM) was added by NOVOstar (BMG Labtech).

Summary

The Screen Quest™ Rhod-4 AM is optimized for a broad range of instruments to give maximum performance with GPCR and calcium channel targets. The Screen Quest™ Rhod-4 AM Calcium reagents have the following benefits:

- ✓ **Cytosol localization: Quest™ Rhod-4 AM is predominantly localized in cytosol while Rhod-2 AM is localized in mitochondira;**
- ✓ **Multiple excitation options @ ~490 nm, 514 nm, 532 nm and 546 nm;**
- ✓ **Much larger Assay Window: 10 times better than Rhod-2 AM ;**
- ✓ **More Signals: 4 times brighter than Rhod-2 AM at Ex 530/Em 570.**

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