Rhod-4™ Calcium Reagents and Screen Quest™ Rhod-4 NW Calcium Assay Kits

Introduction

Calcium acts as a universal second messenger in a variety of cells. The beginning of life, the act of fertilization, is regulated by Ca^{2+}. Numerous functions of all types of cells are regulated by Ca^{2+} to a greater or lesser degree. Since the 1920s, scientists have attempted to measure Ca^{2+}, but few were successful due to limited availability of Ca^{2+} probes. The first reliable measurements of Ca^{2+} were performed by Ridgway and Ashley by injecting the photoprotein aequorin into the giant muscle fiber of the barnacle. Subsequently, in the 1980s, Tsien and colleagues produced a variety of fluorescent indicators. Among them the rhodamine-based Ca^{2+} reagents (such as Rhod-2) have been the most valuable longer wavelength dye for measuring Ca^{2+} with red fluorescence.

Rhod-4™ Calcium Indicators, the Most Sensitive Red Fluorescent Calcium Probe

Although Rhod-2 has been the most popular red fluorescent Ca^{2+} indicator, its mitochondrial localization and high basal Ca^{2+} signal in cells have severely limited its cellular applications. In addition, the less optimal excitation of Rhod-2 at 488 nm makes it less robust to use with some instruments (such as FLIPR™) that have only 488 nm excitation light source. Our Rhod-4™ serial calcium detection reagents have been developed to address these limitations of Rhod-2.

The absorption and emission peaks of Rhod-4™ reagents are 530 nm and 555 nm, respectively. Although Rhod-4 has maximum absorption at 530 nm, its absorption at 488 nm is quite strong (see Figure 1). It is quite unique that Rhod-4™ can be well excited with an argon ion laser at 488 nm besides the longer wavelength excitations at 514 nm, 532 nm and 546 nm. Rhod-4™ emits fluorescence (at 555 nm) which increases with the increasing Ca^{2+}. Rhod-4™ is determined to undergo a >200-fold increase in fluorescence upon binding to Ca^{2+}. Because the range of increase in Ca^{2+} in many cells after stimulation is generally 5- to10-fold, Rhod-4™ is an excellent probe to use with high sensitivity in this region. The K_d of Rhod-4™ is estimated to be 525 nM (22 °C, pH 7.0 –7.5), but this value may be significantly influenced by pH, viscosity, and binding proteins *in vivo* conditions.

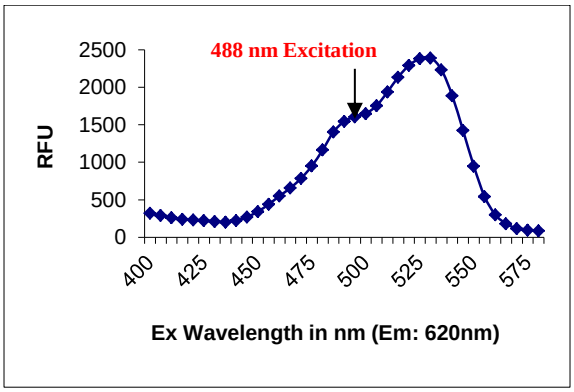

Figure 1. Excitation Spectrum of Rhod-4™, sodium salt in the presence of calcium chloride

Besides their convenient excitation wavelengths and large fluorescence enhancement by calcium, Rhod-4™ is much brighter in cells than Rhod-2 upon agonist stimulation (signal to background ratio) as shown in Figure 2. More importantly, Rhod-4™ is predominantly localized in cytosols unlike Rhod-2 that is mainly localized in mitochondria. Rhod-4™ reagents have a less temperature-dependent cell loading property, giving similar results either at room temperature or 37 °C. This characteristic makes Rhod-4™ more robust for HTS applications than Rhod-2 AM.

Table 1. Spectral and Ca^{2+}–Binding Properties of Rhod-4™ Calcium Detection Reagents

Ca^{2+} Indicator	**Excitation**	**Emission**	**K_d of Ca^{2+}-Binding**
Rhod-4™	530 nm	555 nm	525 nM

Compared to Rhod-2, our Quest Rhod-4™ calcium detection reagents have the following advantages:

- *Convenient Excitation Wavelengths:* multiple excitation options @ ~490 nm, 514 nm, 532 nm and 546 nm.

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- *Much Larger Assay Window:* 10 times larger than Rhod-2 AM.
- *Flexible Loading:* dye loading at room temperature rather than 37 °C.
- *Versatile Packing Sizes to Meet Your Special Needs*: 1 mg; 10 x 50 µg; 20 x 50 µg; HTS packages.

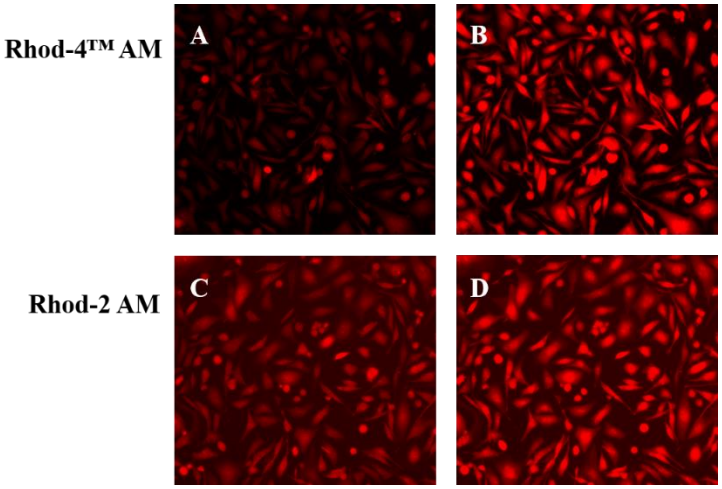

Figure 2. ATP-stimulated calcium responses of endogenous P2Y receptors were measured in CHO-K1 cells with Rhod-4™ AM (Cat# 21120) and Rhod-2 AM (Cat# 21064). CHO-K1 cells were seeded overnight at 50,000 cells/100 µL/well in a Costar 96-well black wall/clear bottom plate. The growth medium was removed, and the cells were incubated with 100 µL of dye loading solution using Rhod-4™ AM (4 µM, A and B) or Rhod-2 AM (4 µM, C and D) for 1 hour in a 37 °C, 5% CO2 incubator. The staining solution was replaced with 200 µL HHBS, then the cells were imaged before (A and C) and after (B and D) ATP treatment with a fluorescence microscope (Olympus IX71) using TRITC channel.

Use of Rhod-4™ AM Esters

1. Load Cell with Quest Rhod-4™ AM Esters:

AM esters are the non-polar esters that readily cross live cell membranes, and rapidly hydrolyzed by cellular esterases inside live cells. AM esters are widely used for loading a variety of polar fluorescent probes into live cell non-invasively. However, cautions must be excised when AM esters are used since they are susceptible to hydrolysis, particularly in solution. They should be reconstituted just before use in high-quality, anhydrous dimethylsulfoxide (DMSO). DMSO stock solutions may be stored desiccated at –20 °C and protected from light. Under these conditions, AM esters should be stable for several months.

Following is our recommended protocol for loading Rhod-4™ AM esters into live cells. This protocol only provides a guideline, and should be modified according to your specific needs.

a) Prepare a 2 to 5 mM stock solution of Rhod-4™ AM esters in high-quality, anhydrous DMSO.

b) On the day of the experiment, either dissolve Rhod-4™ AM in DMSO or thaw an aliquot of the indicator stock solution to room temperature. Prepare a working solution of 1 to 10 µM in Hanks and Hepes buffer (HHBS) or the buffer of your choice with 0.02% *Pluronic® F-127*. For most of cell lines, Rhod-4™ AM reagents with a concentration ranging from 4-5 uM are recommended. The exact concentration of the indicator required for cell loading must be determined empirically. To avoid any artifacts caused by overloading and potential dye toxicity, it is recommended to use the minimal dye concentration that can generate sufficient signal strength.
Note: The nonionic detergent Pluronic® F-127 is sometimes used to increase the aqueous solubility of Rhod-4™ AM esters. A variety of Pluronic® F-127 solutions can be purchased from AAT Bioquest.

c) If your cells containing the organic anion-transports, probenecid (1–2.5 mM) or sulfinpyrazone (0.1–0.25 mM) may be added to the cell medium to reduce leakage of the de-esterified indicators.
Note: A variety of ReadiUse™ probenecid including water soluble sodium salt and stabilized solution can be purchased from AAT Bioquest.

d) Add equal volume of the dye working solution (from Step b or c) into your cell plate.

e) Incubate the dye-loading plate at a cell incubator or room temperature for 30 minutes to one hour at room temperature or 37 °C.
Note: Decreasing the loading temperature might reduce the compartmentalization of the indictor.

f) Replace the dye working solution with HHBS or buffer of your choice (containing an anion transporter inhibitor, such as 2.5 mM probenecid, if applicable) to remove excess probes.
g) Run the experiments at Ex/Em = 540/590 nm

2. Measure Intracellular Calcium Responses: see figure 3.

To determine either the free calcium concentration of a solution or the K_d of a single-wavelength calcium indicator, the following equation is used:

$$[Ca]_{free} = K_d[F - F_{min}]/F_{max} - F]$$

Where F is the fluorescence of the indicator at experimental calcium levels, F_{min} is the fluorescence in the absence of calcium and F_{max} is the fluorescence of the calcium-saturated probe.

The dissociation constant (K_d) is a measure of the affinity of the probe for calcium. The Ca-binding and spectroscopic properties of fluorescent indicators vary quite significantly in cellular environments compared to calibration solutions. *In situ* response calibrations of intracellular indicators typically yield K_d values significantly higher than *in vitro* determinations. *In situ* calibrations are performed by exposing loaded cells to controlled Ca^{2+} buffers in the presence of ionophores such as A-23187, 4-bromo A-23187 and ionomycin. Alternatively, cell permeabilization agents such as digitonin or Triton® X-100 can be used to expose the indicator to the controlled Ca^{2+} levels of the extracellular medium. The K_d value of Quest Rhod-4™ is listed in Table 1 for your reference.

Use of Screen Quest™ Rhod-4 NW Calcium Assay Kits for HTS Applications

GPCR activation can be detected by direct measurement of the receptor mediated cAMP accumulation, or changes in intracellular Ca^{2+} concentration. GPCR targets that couple via Gq produce an increase in intracellular Ca^{2+} that can be measured using a combination of Fluo-8® reagents and a fluorescence microplate reader. The fluorescence imaging plate readers (such as, FLIPR™, FDSS or BMG NovoStar™) have a cooled CCD camera imaging system which collects the signal from each well of a microplate (both 96 and 384-well) simultaneously. These plate readers can read at sub-second intervals, which enables the kinetics of the response to be captured, and has an integrated pipettor that may be programmed for successive liquid additions. Besides their robust applications for GPCR targets, our Screen Quest™ Rhod-4 Calcium Assay Kits can be also used for characterizing calcium ion channels and screening calcium ion channel-targeted compounds.

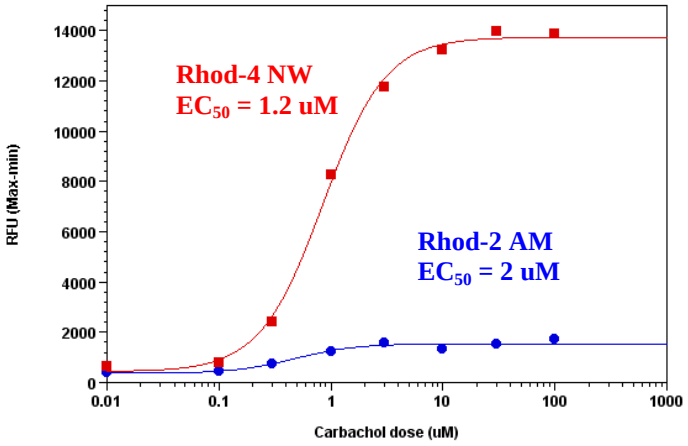

Figure 3. Carbachol Dose Response was measured in HEK-293 cells with Screen Quest™ Rhod-4 NW Assay kit and Rhod-2 AM under the same assay conditions**.** HEK-293 cells were seeded overnight at 40,000 cells/100 µL/well in a 96-well black wall/clear bottom costar plate. The growth medium was removed, and the cells were incubated with 100 µL of the Screen Quest™ Rhod-4 NW calcium assay kit or Rhod-2 AM for 1 hour at room temperature. Carbachol (25µL/well) was added by NOVOstar™ (BMG LabTech) to achieve the final

Our Screen Quest™ Rhod-4 Calcium Assay Kits have the following advantages for HTS applications:

- *Longer Wavelengths:* multiple excitations @ 488, 514, 532 &546 nm; maximum emission @ ~555 nm.
- *No Wash Required and No Quencher Interference with Your Targets.*
- *Robust Performance:* enable calcium assays that are impossible with Rhod-2 AM.
- *Larger Assay Window:* 10 times larger than Rhod-2 AM.

Use of Rhod-4™ Salts

Calcium calibration can be carried out by measuring the fluorescence intensity of the salt form (25 to 50 µM in fluorescence microplate readers) of the indicators in solutions with precisely known free Ca^{2+} concentrations. Calibration solutions can be used based on 30 mM MOPS EGTA Ca^{2+} buffer. In general, water contains trace

amount of calcium ion. It is highly recommended to use 30 mM MOPS + 100 mM KCl, pH 7.2 as buffer system. One can simply make a 0 and 39 µM calcium stock solutions as listed below, and these 2 solutions are used to make a serial solution of different Ca^{2+} concentrations

A. 0 µM calcium: 30 mM MOPS + 100 mM KCl, pH 7.2 buffer + 10 mM EGTA

B. 39 µM calcium: 30 mM MOPS + 100 mM KCl, pH 7.2 buffer + 10 mM EGTA + 10 mM $CaCl_2$

To determine either the free calcium concentration of a solution or the K_d of a single-wavelength calcium indicator, the following equation is used:

$$[Ca]_{free} = K_d[F - F_{min}]/F_{max} - F]$$

Where F is the fluorescence intensity of the indicator at a specific experimental calcium level, F_{min} is the fluorescence intensity in the absence of calcium and F_{max} is the fluorescence intensity of the calcium-saturated probe.

The dissociation constant (K_d) is a measure of the affinity of the probe for calcium. The calcium-binding and spectroscopic properties of fluorescent indicators vary quite significantly in cellular environments compared to calibration solutions. *In situ* response calibrations of intracellular indicators typically yield K_d values significantly higher than *in vitro* determinations. *In situ* calibrations are performed by exposing loaded cells to controlled Ca^{2+} buffers in the presence of ionophores such as A-23187, 4-bromo A-23187 and ionomycin. Alternatively, cell permeabilization agents such as digitonin or Triton® X-100 can be used to expose the indicator to the controlled Ca^{2+} levels of the extracellular medium. The K_d values of Fluo-8® reagents are listed in Table 1 for your reference.

Conclusions

Because of the importance of Ca^{2+} in biology, numerous techniques/methods for analyzing the mechanisms of cellular and/or subcellular Ca^{2+} activity have been established. Although each method for analyzing Ca^{2+} activity has certain advantages over the others, each also suffers from drawbacks. With the outstanding properties described above, we believe that Rhod-4™ calcium detection reagents and Screen Quest™ Rhod-4NW Calcium Assay Kits provide new powerful tools for intracellular calcium analysis and monitoring in a variety of biological systems.

As might have been predicted, the interests of many researchers in Ca^{2+} analysis shifted from the cellular level to the subcellular level. It has been found that $Ca^{2+}$is not even distributed throughout the whole cell and that intracellular heterogeneity of Ca^{2+} (such as Ca^{2+} waves and Ca^{2+} sparks) is observed in a variety of cells (e.g., oocyte, heart muscle cell, hepatocyte, and exocrine cell). With the advent of the confocal laser scanning microscope (CLSM) in the 1980s and advanced microplate readers dedicated for intracellular calcium detection (such as FLIPR™, FDSS, FlexStation, and NOVOStar™) in 2000s, the measurement of intracellular Ca^{2+} has accelerated significantly. Confocal laser scanning microscopy and more recently multiphoton microscopy allow the precise spatial and temporal analysis of intracellular Ca^{2+} signaling at the subcellular level.

Product List

Table 2 Quest Rhod-4™ Product list

Cat. #	Product Name	Unit Size
21120	Rhod-4™, AM	1 mg
21121	Rhod-4™, AM	5 x 50 ug
21122	Rhod-4™, AM	10 x 50 ug
21123	Rhod-4™, AM	20 x 50 µg
21118	Rhod-4™, sodium salt	1 mg
21119	Rhod-4™, potassium salt	1 mg
21128	Rhod-4™, sodium salt	5 x 50 µg
21129	Rhod-4™, potassium salt	5 x 50 µg
36330	Screen Quest™ Rhod-4 NW Calcium Assay Kit *Medium Removal*	1 Plate
36331	Screen Quest™ Rhod-4 NW Calcium Assay Kit *Medium Removal*	10 Plates
36332	Screen Quest™ Rhod-4 NW Calcium Assay Kit *Medium Removal*	100 Plates
36333	Screen Quest™ Rhod-4 NW Calcium Assay Kit *1% FBS Growth Medium*	1 Plate
36334	Screen Quest™ Rhod-4 NW Calcium Assay Kit *1% FBS Growth Medium *	10 Plates
36335	Screen Quest™ Rhod-4 NW Calcium Assay Kit *1% FBS Growth Medium*	100 Plates

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