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

I. 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.

II. Quest 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 Quest Rhod-4™ serial calcium detection reagents have been developed to address these limitations of Rhod-2.

The absorption and emission peaks of Quest 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 Quest Rhod-4™ can be well excited with an argon ion laser at 488 nm besides the longer wavelength 514 nm, 532 nm and 546 nm excitations. Quest Rhod-4™ emits fluorescence (at wavelengths 555 nm) that increases with increasing Ca^{2+}. Quest Rhod-4™ is determined to undergo a >200-fold increase in fluorescence upon binding Ca^{2+}. Because the range of increase in Ca^{2+} in many cells after stimulation is generally 5- to10-fold, Quest Rhod-4™ is an excellent probe to use with high sensitivity in this region. The K_d of Quest 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.

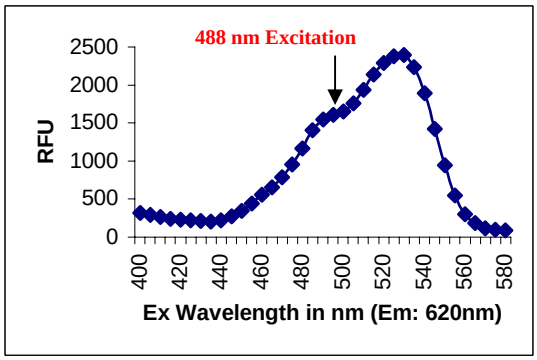

Figure 1. Excitation Spectrum of Quest Rhod-4™, sodium salt in the presence of calcium chloride.

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

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

Ca^{2+} Indicator	**Excitation**	**Emission**	**K_d of Ca^{2+}-Binding**
Quest 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.
- *Much Larger Assay Window:* 10 times better than Rhod-2 AM.
- *Enhanced Intensity:* 4 times brighter than Rhod-2 AM.
- *Faster Loading:* dye loading at room temperature.
- Versatile Packing Sizes to Meet Your Special Needs: 1 mg; 10x50 µg; 20x50 µg; HTS packages.

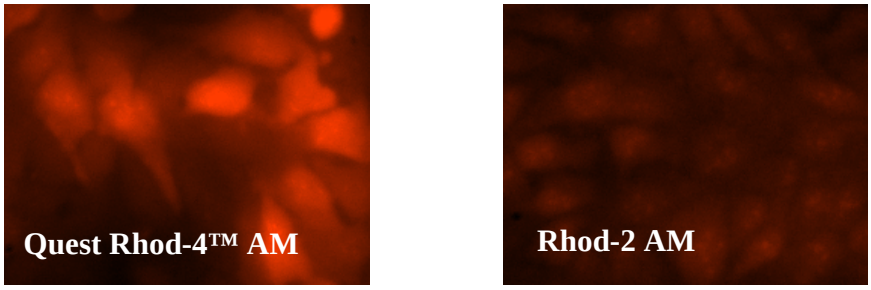

Figure 1. U2OS cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom costar plate. The growth medium was removed, and the cells were incubated with 100 µl of 4 uM Rhod-2 AM or Quest Rhod-4™ AM in HHBS at 37 °C, 5% CO_2 incubator for 1 hour. The cells were washed twice with 200 µl HHBS, then imaged with a fluorescence microscope (Olympus IX71) using TRITC channel.

III. Use of Quest Rhod-4™ AM Esters

1. Cell Loading of Quest Rhod-4™ AM Esters:

AM esters are the non-poplar 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 Quest Rhod-4™ AM esters into live cells. This protocol only provides a guideline, should be modified according to your specific needs.

a) Prepare a 2 to 5 mM stock solution of Quest Rhod-4™ AM esters in high-quality, anhydrous DMSO. The nonionic detergent Pluronic® F-127 is sometimes used to increase the aqueous solubility of Quest Rhod-4™ AM esters.
[Note: A 20% Pluronic F-127 solution can be used in replacing DMSO to prepare solutions of these calcium indicators. A variety of Pluronic F-127 solutions can be purchased from ABD Bioquest].
[Caution: long-term storage of AM esters in the presence of Pluronic F-127 is not recommended].

b) On the day of the experiment, either dissolve Quest Rhod-4™ AM solid in DMSO or thaw an aliquot of the indicator stock solution to room temperature. Prepare a working solution of 1 to 10 µM in the buffer of your choice. For most of cell lines we recommend that 4-5 uM Quest Rhod-4™ AM reagents be used. The exact concentration of indicator required for cell loading must be determined empirically. To avoid calcium buffering, toxicity and other artifacts of overloading, one should generally use the lowest probe concentration that yields sufficient signal.

c) Incubate cells with the Quest Rhod-4™ AM esters for 30 minutes to one hour at room temperature or 37 °C.
[Note: Decreasing the loading temperature might reduce the indicator compartmentalization]

d) Wash cells to remove excess probe.

2. Measuring Intracellular Calcium Responses

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.

IV. 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 Quest Rhod-4™ reagents and a fluorescence microplate reader. The fluorescence imaging plate readers (such as, FLIPR™, FDSS or BMG NovoStar™) has 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. Beside 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.

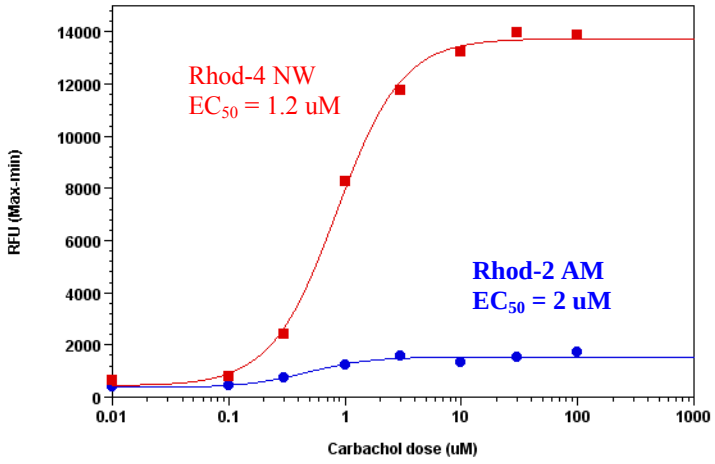

Figure 2. **Carbachol Dose Response in HEK-293 cells measured with Screen Quest™ Rhod-4 NW Assay kit and Rhod-2 AM under the same assay conditions.** HEK-293 cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom costar plate. The growth medium was removed, and the cells were incubated with 100 µl of 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 indicated concentrations. The EC_{50} of Rhod-4 NW is about 1.2 uM. The excitatin and emmision were 530nm and 570nm respectively.

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.
- *Strongest Signal Intensity:* 4 times brighter than Rhod-2 AM.
- *Larger Assay Window:* 10 times better than Rhod-2 AM.

V. 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. Unfortunately, however, there is no one best technique/method with which one can measure Ca^{2+}. Although each method for analyzing Ca^{2+}activity has certain advantages over the others, each also suffers drawbacks. With the outstanding properties described above, we believe that Quest 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 in coupling with the rapid advance in fluorescence instrumentation.

As might have been predicted, the interests of many researchers shifted from Ca^{2+} analysis at the cellular level to that of 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 and NOVOStar™) in 2000s, measurement of intracellular Ca^{2+} has accelerated significantly. Confocal laser scanning microscopy, and more recently multiphoton microscopy, allows the precise spatial and temporal analysis of intracellular Ca^{2+} activity at the subcellular level.

VI. Product List

Cat#	Product Name	Unit Size
21120	Quest Rhod-4™, AM	1 mg
21121	Quest Rhod-4™, AM	5x50 ug
21122	Quest Rhod-4™, AM	10x50 ug
21123	Quest Rhod-4™, AM	20x50 µg
21128	Quest Rhod-4™, sodium salt	5x50 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

VII. References

1. Martin VV, Beierlein M, Morgan JL, Rothe A, Gee KR. (2004) Novel fluo-4 analogs for fluorescent calcium measurements. Cell Calcium, 36, 509.
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3. Su ZL, Li N, Sun YR, Yang J, Wang IM, Jiang SC. (1998) [Monitoring calcium in outer hair cells with confocal microscopy and fluorescence ratios of fluo-3 and fura-red]. Shi Yan Sheng Wu Xue Bao, 31, 323.
4. Perez-Terzic C, Stehno-Bittel L, Clapham DE. (1997) Nucleoplasmic and cytoplasmic differences in the fluorescence properties of the calcium indicator Fluo-3. Cell Calcium, 21, 275.
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6. Greimers R, Trebak M, Moutschen M, Jacobs N, Boniver J. (1996) Improved four-color flow cytometry method using fluo-3 and triple immunofluorescence for analysis of intracellular calcium ion ([Ca2+]i) fluxes among mouse lymph node B- and T-lymphocyte subsets. Cytometry, 23, 205.
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