

## Quest Fluo-8™ Calcium Reagents and Screen Quest™ Fluo-8 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  $\text{Ca}^{2+}$ . Numerous functions of all types of cells are regulated by  $\text{Ca}^{2+}$  to a greater or lesser degree. Since the 1920s, scientists have attempted to measure  $\text{Ca}^{2+}$ , but few were successful due to limited availability of  $\text{Ca}^{2+}$  probes. The first reliable measurements of  $\text{Ca}^{2+}$  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 fluorescein-based  $\text{Ca}^{2+}$  reagents (such as Fluo-3 and Fluo-4) have provided trustworthy methods for measuring  $\text{Ca}^{2+}$ . Since the development of these  $\text{Ca}^{2+}$  probes, investigations of  $\text{Ca}^{2+}$ -related intracellular phenomena have skyrocketed.

### Quest Fluo-8™ Calcium Indicators, the Most Robust and Brightest Calcium Probes

Since being introduced, Fluo-3 imaging and its analogs (such as Fluo-4) have revealed the spatial dynamics of many elementary processes in  $\text{Ca}^{2+}$  signaling. Fluo-3 and Fluo-4 have also been extensively used for flow cytometry and microplate-based (such as FLIPR™) calcium detections. However, the weak signal and harsh dye-loading conditions have limited their applications in some cellular analysis. Our Quest Fluo-8™ serial calcium detection reagents have been developed to address these limitations of Fluo-3 and Fluo-4.

The most important properties of Fluo-3 and Fluo-4 in cellular applications are their absorption spectrum compatible with excitation at 488 nm by argon-ion laser sources, and a very large fluorescence intensity increase in response to  $\text{Ca}^{2+}$  binding. These two valuable properties have been retained intact with our Quest Fluo-8™  $\text{Ca}^{2+}$  detection reagents. The absorption and emission peaks of Quest Fluo-8™ reagents are 490 nm and 514 nm, respectively. They can be well excited with an argon ion laser at 488 nm, and their emitted fluorescence (at wavelength 514 nm) increases with increasing  $\text{Ca}^{2+}$ . Quest Fluo-8™ is determined to undergo a > 200-fold increase in fluorescence upon binding to  $\text{Ca}^{2+}$ . Because the range of increase in  $\text{Ca}^{2+}$  in many cells after stimulation is generally 5- to 10-fold, Quest Fluo-8™ is an excellent probe to use with high sensitivity in this region. The  $K_d$  of Quest Fluo-8™ is estimated to be 389 nM (22 °C, pH 7.0–7.5), but this value may be significantly influenced by pH, viscosity, and binding proteins *in vivo* conditions.

Besides their convenient 488 nm excitation wavelength and large fluorescence enhancement by calcium, Quest Fluo-8™ is much brighter in cells than Fluo-3 and Fluo-4 as shown in Figure 1. In addition, Quest Fluo-8 is much more readily loaded into live cells than Fluo-3 and Fluo-4, both of which require 37 °C for optimal cell loading. Quest Fluo-8™ reagents have a less temperature-dependent cell loading property, giving similar results either at room temperature or 37 °C. This characteristic makes Quest Fluo-8™ more robust for HTS applications.

**Table 1.** Spectral and  $\text{Ca}^{2+}$ -Binding Properties of Quest Fluo-8™ Calcium Detection Reagents

$\text{Ca}^{2+}$ Indicator	Excitation	Emission	$K_d$ of $\text{Ca}^{2+}$ -Binding
Quest Fluo-8™	490 nm	514 nm	389 nM
Quest Fluo-8HT™	490 nm	514 nm	232 nM
Quest Fluo-8L™	490 nm	514 nm	1.86 $\mu\text{M}$

Compared to Fluo-3 and Fluo-4, our Quest Fluo-8™ calcium detection reagents have the following advantages:

- *Convenient Wavelengths:* maximum excitation @ ~490 nm; maximum emission @ ~514 nm.
- *Enhanced Intensity:* 2 times brighter than Fluo-4 AM; 4 times brighter than Fluo-3 AM.
- *Faster Loading:* dye loading at room temperature (rather than 37 °C that is required for Fluo-4 AM).
- *Versatile  $\text{Ca}^{2+}$ -Binding  $K_d$*  as shown in Table 1.
- *Versatile Packing Sizes to Meet Your Special Needs:* 1 mg; 10x50  $\mu\text{g}$ ; 20x50  $\mu\text{g}$ ; HTS packages.



**Figure 1.** U2OS cells were seeded overnight at 40,000 cells/100  $\mu$ L/well in a 96-well black wall/clear bottom costar plate. The growth medium was removed, and the cells were incubated with, respectively, 100  $\mu$ L of Fluo-3 AM, Fluo-4 AM and Quest Fluo-8™ AM in HHBS at a concentration of 4  $\mu$ M in a 37 °C, 5% CO<sub>2</sub> incubator for 1 hour. The cells were washed twice with 200  $\mu$ L HHBS, then imaged with a fluorescence microscope (Olympus IX71) using FITC channel.

## Use of Quest Fluo-8™ AM Esters

### 1. Load Cells with Quest Fluo-8™ 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 Quest Fluo-8™ AM esters into live cells. This protocol only provides a guideline, and should be modified according to your specific needs.

- Prepare a 2 to 5 mM stock solution of Quest Fluo-8™ AM esters in high-quality, anhydrous DMSO. The nonionic detergent Pluronic® F-127 is sometimes used to increase the aqueous solubility of Quest Fluo-8™ 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 AAT Bioquest. The long-term storage of AM esters in the presence of Pluronic® F-127 is not recommended.*
- On the day of the experiment, either dissolve Quest Fluo-8™ in DMSO or thaw an aliquot of the indicator stock solution to room temperature. Prepare a working solution of 1 to 10  $\mu$ M in Hanks and Hepes buffer (HHBS) or the buffer of your choice. For most of cell lines, Quest Fluo-8™ reagents with a concentration ranging from 4-5  $\mu$ M 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.
- If your cells containing the organic anion-transporters, probenecid (1–2.5 mM) or sulfapyrazone (0.1–0.25 mM) may be added to the cell medium to reduce leakage of the de-esterified indicators.
- Incubate cells with the Quest Fluo-8™ AM esters for 20 minutes to one hour at room temperature or 37 °C.  
*Note: Decreasing the loading temperature might reduce the compartmentalization of the indicator.*
- Wash cells twice in HHBS or buffer of your choice (containing an anion transporter inhibitor, such as 2.5 mM probenecid, if applicable) to remove excess probes.
- Run the experiments at Ex/Em = 490/525 nm

### 2. Measure 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:

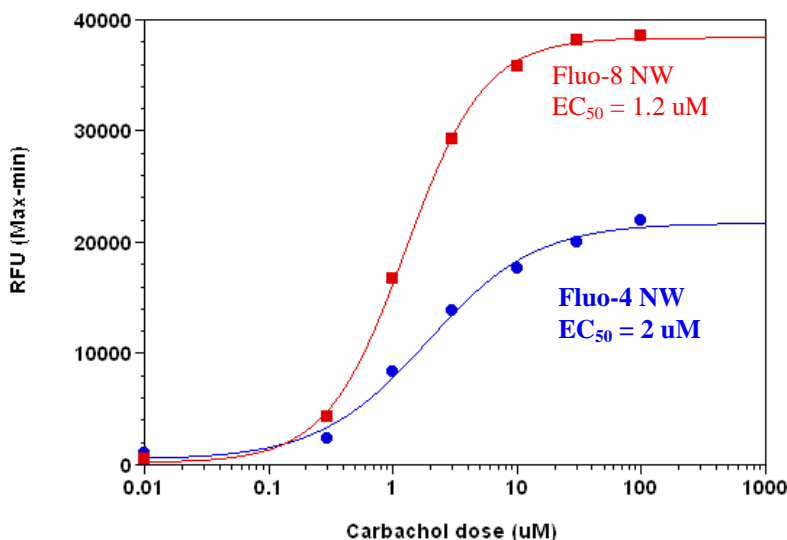
$$[Ca]_{free} = K_d[F - F_{min}]/F_{max} - F]$$

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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  levels of the extracellular medium. The  $K_d$  values of Quest Fluo-8™ reagents are listed in Table 1 for your reference.

### Use of Screen Quest™ Fluo-8 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  $\text{Ca}^{2+}$  concentration. GPCR targets that couple via Gq produce an increase in intracellular  $\text{Ca}^{2+}$  that can be measured using a combination of Quest 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™ Fluo-8 Calcium Assay Kits can be also used for characterizing calcium ion channels and screening calcium ion channel-targeted compounds.



**Figure 2.** Carbachol Dose Response was measured in HEK-293 cells with Screen Quest™ Fluo-8 NW Assay kit and Fluo-4 NW Assay Kit. HEK-293 cells were seeded overnight at 40,000 cells/100  $\mu\text{L}$ /well in a 96-well black wall/clear bottom costar plate. The growth medium was removed, and the cells were incubated with, respectively, 100  $\mu\text{L}$  of the Screen Quest™ Fluo 8-NW calcium assay kit and Fluo-4 NW kit (according to the manufacturer's instructions) for 1 hour at room temperature. Carbachol (25 $\mu\text{L}$ /well) was added by NOVOstar (BMG LabTech) to achieve the final indicated concentrations. The  $\text{EC}_{50}$  of Fluo-8 NW is about 1.2  $\mu\text{M}$ .

Compared to other commercial calcium assay kits that either based on Fluo-3 or Fluo-4, our Screen Quest™ Calcium Assay Kits have the following advantages for HTS applications:

- *Broad Applications:* work with both GPCR and calcium channel targets.
- *Convenient Spectral Wavelengths:* maximum excitation @ ~490 nm; maximum emission @ ~514 nm.
- *Flexible Dye Loading:* dye loading at room temperature (rather than 37 °C required for Fluo-4 AM).
- *No Wash Required and No Quencher Interference with Your Targets.*
- *Robust Performance:* enable calcium assays that are impossible with Fluo-4 AM or Fluo-3 AM.
- *Strongest Signal Intensity:* 2 times brighter than that of Fluo-4 AM; 4 times brighter than that of Fluo-3 AM.

### Conclusions

Because of the importance of  $\text{Ca}^{2+}$  in biology, numerous techniques/methods for analyzing the mechanisms of cellular and/or subcellular  $\text{Ca}^{2+}$  activity have been established. Although each method for analyzing  $\text{Ca}^{2+}$  activity has

certain advantages over the others, each also suffers from drawbacks. With the outstanding properties described above, we believe that Quest Fluo-8™ calcium detection reagents and Screen Quest™ Fluo-8NW Calcium Assay Kits provide new powerful tools for intracellular calcium analysis and monitor in a variety of biological systems.

As might have been predicted, the interests of many researchers in Ca<sup>2+</sup> analysis shifted from the cellular level to the subcellular level. It has been found that Ca<sup>2+</sup> is not even distributed throughout the whole cell and that intracellular heterogeneity of Ca<sup>2+</sup> (such as Ca<sup>2+</sup> waves and Ca<sup>2+</sup> 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 in 2000s (such as FLIPR, FDSS and NOVOSTar dedicated for intracellular Ca<sup>2+</sup> detections), the measurement of intracellular Ca<sup>2+</sup> has accelerated significantly. Confocal laser scanning microscopy and more recently multiphoton microscopy allow the precise spatial and temporal analysis of intracellular Ca<sup>2+</sup> signaling at the subcellular level in addition to the measurement of its concentration.

## Product List

**Table 2** Quest Fluo-8™ Product list

Cat. #	Product Name	Unit Size
21080	Quest Fluo-8™, AM *Cell-permeable*	1 mg
21081	Quest Fluo-8™, AM *Cell-permeable*	5x50 µg
21082	Quest Fluo-8™, AM *Cell-permeable*	10x50 µg
21083	Quest Fluo-8™, AM *Cell-permeable*	20x50 µg
21088	Quest Fluo-8™, sodium salt	10x50 µg
21090	Quest Fluo-8H™, AM *Cell-permeable*	1 mg
21091	Quest Fluo-8H™, AM *Cell-permeable*	10x50 µg
21095	Quest Fluo-8H™, sodium salt	10x50 µg
21096	Quest Fluo-8L™, AM *Cell-permeable*	1 mg
21097	Quest Fluo-8L™, AM *Cell-permeable*	10x50 µg
21098	Quest Fluo-8L™, sodium salt	10x50 µg
36307	Screen Quest™ Fluo-8 NW Calcium Assay Kit *Medium Removal*	1 Plate
36308	Screen Quest™ Fluo-8 NW Calcium Assay Kit *Medium Removal*	10 Plates
36309	Screen Quest™ Fluo-8 NW Calcium Assay Kit *Medium Removal*	100 Plates
36314	Screen Quest™ Fluo-8 NW Calcium Assay Kit *1% FBS Growth Medium*	1 Plate
36315	Screen Quest™ Fluo-8 NW Calcium Assay Kit *1% FBS Growth Medium*	10 Plates
36316	Screen Quest™ Fluo-8 NW Calcium Assay Kit *1% FBS Growth Medium*	100 Plates

## 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.
2. do Ceu Monteiro M, Sansonetty F, Goncalves MJ, O'Connor JE. (1999) Flow cytometric kinetic assay of calcium mobilization in whole blood platelets using Fluo-3 and CD41. *Cytometry*, 35, 302.
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.
5. Tretyn A, Kado RT, Kendrick RE. (1997) Loading and localization of Fluo-3 and Fluo-3/AM calcium indicators in sinapis alba root tissue. *Folia Histochem Cytobiol*, 35, 41.
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 ([Ca<sup>2+</sup>]<sub>i</sub>) fluxes among mouse lymph node B- and T-lymphocyte subsets. *Cytometry*, 23, 205.