

Cal-520™ Calcium Reagents and Cal-520™ No Wash 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 fluorescein-based Ca^{2+} reagents (such as Fluo-3 and Fluo-4) have provided trustworthy methods for measuring Ca^{2+} . Since the development of these Ca^{2+} probes, investigations of Ca^{2+} -related intracellular phenomena have skyrocketed.

Cal-520™ Calcium Indicators, the Most Robust 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 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 colorless Cal-520™ 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 Ca^{2+} binding. These two valuable properties have been retained intact with our Cal-520™ Ca^{2+} detection reagents. The absorption and emission peaks of Cal-520™ reagents are 492 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 Ca^{2+} . Cal-520™ is determined to undergo a > 100-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- to 10-fold, Cal-520™ is an excellent probe to use with high sensitivity in this region. The K_d of Quest Cal-520™ is estimated to be 320 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, Cal-520™ has much better cell retention ability in addition to its significantly higher signal to background ratio compare to all other existing calcium indicators. It requires much less organic-anion transporter inhibitors (such as probenecid) present in the assay system. Organic-anion transporter inhibitors are often toxic to cells and also interfere with the activities of bioactive compounds to be screened. This characteristic makes Cal-520™ calcium assay a more robust tool for evaluating GPCR and calcium channel targets as well as for screening their agonists and antagonists.

Table 1. Spectral and Ca^{2+} -Binding Properties of Cal-520™ Calcium Detection Reagent

Ca^{2+} Indicator	Excitation	Emission	K_d of Ca^{2+} -Binding
Cal-520™	492 nm	514 nm	320 nM

Compared to Fluo-3 and Fluo-4, our Cal-520™ calcium detection reagents have the following advantages:

- **Organic anion transporter resistance:** Enable Ca^{2+} assays with probenecid-interference GPCRs and Ca^{2+} channels.
- **Robust:** Significantly higher S/N ratio than those of Fluo-4 AM and any other commercially available fluorescent Ca^{2+} assays.
- **Convenient: Essentially identical spectra to Fluo-3, Fluo-4, and Fluo-8™** (maximum excitation @ ~492 nm; maximum emission @ ~514 nm).
- **Versatile Packing Sizes to Meet Your Special Needs:** 1 mg; 10x50 µg; and HTS packages.

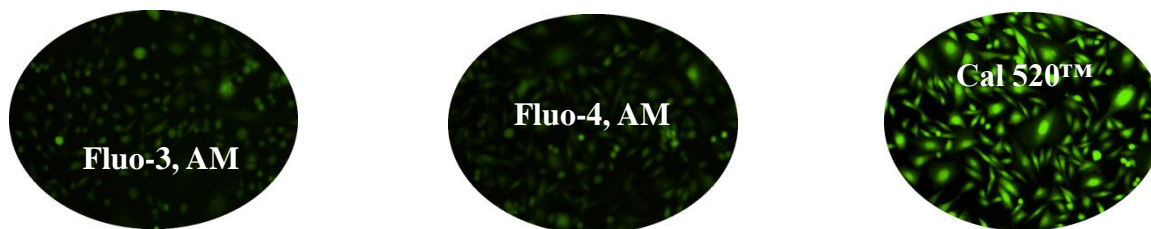


Figure1. **Response of endogenous P2Y receptor to ATP in CHO-M1 cells without Probenecid.** CHO-M1 cells were seeded overnight at 40,000 cells per 100 μ L per well in a 96-well black wall/clear bottom costar plate. 100 μ L of 4 μ M Fluo-3 AM, Fluo-4 AM or Cal 520™ AM in HHBS were added into the wells, and the cells were incubated at 37 °C for 2 hour. The dye loading medium were replaced with 100 μ L HHBS, 50 μ L of 300 μ M ATP were added, and then imaged with a fluorescence microscope (Olympus IX71) using FITC channel.

Use of Quest Cal-520™ AM Esters

1. Load Cells with Cal-520™ 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 Cal-520™ 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 Cal-520™ AM esters in high-quality, anhydrous DMSO.
- On the day of the experiment, either dissolve Cal-520™ in DMSO or thaw an aliquot of the indicator stock solution to room temperature. Prepare a Cal-520™ dye working solution of 5 to 10 μ M in Hanks and Hepes buffer (HHBS) or the buffer of your choice with 0.02% *Pluronic® F-127*. The exact concentration of the indicator required for cell loading must be determined empirically.

Note: The nonionic detergent Pluronic® F-127 is sometimes used to increase the aqueous solubility of Cal-520™ AM esters. A variety of Pluronic® F-127 solutions can be purchased from AAT Bioquest.

- If your cells (such as CHO cells) containing the organic anion-transporters, probenecid (0.5–1 mM) may be added to the cell medium to reduce leakage of the de-esterified indicators

Note: A variety of ReadUse™ probenecid including water soluble sodium salt and stabilized solution can be purchased from AAT Bioquest.

- Incubate the dye-loading plate at a cell incubator for 60 to 90 minutes, and then incubate the plate at room temperature for another 30 minutes.

Note: Incubate the dye longer than 2 hours gives better signal intensity for some cell lines.

- Replace the Cal-520™ dye working solution with HHBS or buffer of your choice (containing an anion transporter inhibitor, such as 1 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:

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

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 values of Cal-520™ reagents are listed in Table 1 for your reference.

Use of Cal-520™ No Wash 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 Cal-520™ 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 Cal-520™ Calcium Assay Kits can be also used for characterizing calcium ion channels and screening calcium ion channel-targeted compounds.

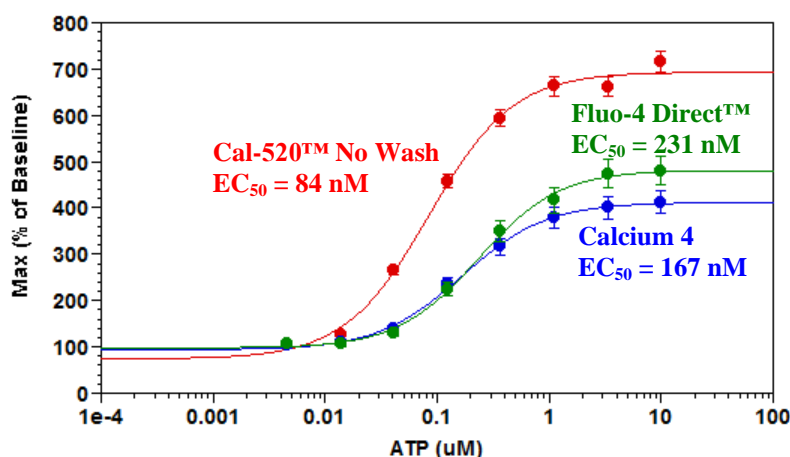


Figure 2. ATP dose response comparison in CHO-K1 cells measured with Cal-520™ No Wash, FLIPR Calcium 4 or Fluo-4 Direct™ Calcium Assay Kit. CHO-K1 cells were seeded overnight in 60,000 cells per 100 μL per well in a 96-well black wall/clear bottom costar plate. The cells were incubated with 100 μL of the Cal-520™ No Wash Calcium Assay Kit, FLIPR Calcium 4 Kit or Fluo-4 Direct™ Calcium Assay Kit (According to the Manufacturer's instructions) for 1.5 hours at 37°C. ATP (50 μL /well) was added by FlexStation (Molecular Devices) to achieve the final indicated concentrations.

Compared to other commercial calcium assay kits that either based on Fluo-3 or Fluo-4, our Cal-520™ 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.
- **No Wash Required and No or Less Probenecid Interference with Your Targets:** Enable Ca^{2+} assays with probenecid-sensitive GPCRs and Ca^{2+} channels.
- **Robust Performance:** Significantly higher S/N ratio than those of Fluo-4 AM and any other commercially available fluorescent Ca^{2+} assays, enabling calcium assays that are impossible with Fluo-4 AM or Fluo-3 AM.

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 Cal-520™ calcium detection reagents and Cal-520™ 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^{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 in 2000s (such as FLIPR, FDSS and NOVOSTar dedicated for intracellular Ca^{2+} detections), 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 in addition to the measurement of its concentration.

Product List

Table 2 Cal-520™ Product list

Cat. #	Product Name	Unit Size
21130	Cal-520™, AM *Cell-permeable*	10x50 µg
21131	Cal-520™, AM *Cell-permeable*	1 mg
21135	Cal-520™, sodium salt	10x50 µg
21136	Cal-520™, sodium salt	1 mg
21140	Cal-520™, potassium salt	10x50 µg
21141	Cal-520™, potassium salt	1 mg
36336	Cal-520™ Medium Removal Calcium Assay Kit *Optimized for Difficult Cell Lines*	10 Plates
36337	Cal-520™ Medium Removal Calcium Assay Kit *Optimized for Difficult Cell Lines*	100 Plates
36338	Cal-520™ No-Wash Calcium Assay Kit *Optimized for Difficult Cell Lines*	10 Plates
36339	Cal-520™ No-Wash Calcium Assay Kit *Optimized for Difficult Cell Lines*	100 Plates
36400	Cal-520™ PBX Calcium Assay Kit *Optimized for Probenecid-Sensitive Cell Lines*	10 Plates
36401	Cal-520™ PBX Calcium Assay Kit *Optimized for Probenecid-Sensitive Cell Lines*	100 Plates

References

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