

Physiological Probes & Assay Kits

Calcium Indicators · Membrane Potential Assays · pH Probes

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 Cal Green™
 Calcium Blue™
 Cell Meter™
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 Fluo-8L™
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 JC-10™
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 Screen Quest™
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Calcium Green™ (Invitrogen)
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 Pluronic® (Wyandotte Chemicals)
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BIOMOL GmbH
 Waidmannstraße 35
 22769 Hamburg · Germany
 info@biomol.de · www.biomol.de
 Fon: +49 (0)40-853 260 0
 Fax: +49 (0)40-853 260 22

TOLL FREE IN GERMANY:
 Fon: 0800-246 66 51
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Custom Products and Services

Our Technologies

Amplite™ enzyme-based detection platform is optimized for measuring horseradish peroxidase (HRP), alkaline phosphates, luciferase, beta-galactosidase, lactamase, oxidase, protein kinases, protein phosphatases, phosphodiesterases, proteases, cytochrome P450, histone deacetylase (HDAC) and cell signaling molecules such as NAD/NADH, NADP/NADPH, IP₃, cAMP and cGMP etc.

Cell Explorer™ cell labeling platform is a complete set of tools for tracking live cells. This platform is also widely used for sorting mixed populations of cells.

Cell Navigator™ cell staining platform is a complete set of tools for selective labeling subcellular structures of live, fixed and dead cells.

Cell Meter™ cellular functional assay platform is a complete set of tools for functional analysis of cellular events and real time-monitoring of cell functions.

iFluor™ superior fluorescent labeling dyes are optimized for labeling proteins and nucleic acids. This group of dyes span from UV to infrared wavelength with good photostability and brightness.

mFluor™ superior fluorescent labeling dyes are optimized for flow cytometry applications.

PhosphoWorks™ detection platform is a set of tools for detection of ATP, ADP, AMP, phosphate, pyrophosphate, phosphoproteins and phosphopeptides.

Quest View™ colorimetric protease platform is a sensitive and robust tool for rapid detection of protease and glycosidase biomarkers. This technology platform has been licensed by a few diagnostic companies for developing rapid diagnostic tests.

RatioWorks™ superior cellular dyes are a sensitive and robust tool set for ratio imaging and real time monitoring of cellular functions (such as pH and ions) in live cells.

Screen Quest™ assay kits are a set of HTS-ready tools for high throughput screening of biochemical and cellular targets such as protein kinases, proteases, HDAC, cell apoptosis and cytotoxicity, GPCR, ion channels, ADME and transporters.

Tide Fluor™ and Tide Quencher™ superior labeling dyes are specially optimized for labeling nucleotides and peptides. This platform offers the best value in the industry. It is second to none in terms of performance and cost. This technology platform has been licensed by a few diagnostic companies for developing IVD diagnostic tests.

trFluor™ superior fluorescent labeling dyes are optimized for developing time-resolved fluorescence-based assays. It has been used for developing HTS assay technologies for many drug discovery targets.

Our Services

Besides the catalog products we also offer custom services to meet the distinct needs of each customer. Our current services include custom synthesis of biological detection probes, custom development of biochemical, cell-based and diagnostic assays, custom bioconjugation and custom high throughput screening of drug discovery targets.

Custom Assay Design and Development

At AAT Bioquest we not only make probes and assay kits, but also use them extensively ourselves. Scientists at AAT Bioquest are experts on assay design and have developed a wide variety of tests that range from biochemical detection to cellular functions. Our assay options include:

- Enzyme activities
- Binding assays
- Cell-based assays
- Microplate assays
- Flow cytometric analysis
- Fluorescence imaging

Custom Conjugation

AAT Bioquest offers the best and the most rapid bioconjugation service in the industry.

- Biotinylation
- Fluorescence labeling (iFluor™, mFluor™, APC, RPE and PerCP)
- Enzyme labeling (AP and HRP)
- Small molecule conjugation

Custom Screening

AAT Bioquest offers on-demand high-throughput screening and pharmacology profiling assays with multiple methodologies. Functional assays are designed, validated and customized to the needs of our pharmaceutical and biotechnology industry clients. These assays are aimed at assessing and monitoring the efficacy, tolerability and safety parameters of candidate compounds for treating and/or diagnosing cancer, infectious disease, autoimmunity and transplantation. Our screening options include:

- Full assay development for a target of your choice
- Optimization of your assay protocol for HTS
- Multiple assay platforms and detection methods
- Custom data analysis

Custom Synthesis of Fluorophores and Luminophores

AAT Bioquest is recognized by the top pharmaceutical companies and diagnostic companies as a key provider of novel fluorescent dyes and luminescent probes. Over the years we have developed and synthesized many enabling fluorescent and luminescent probes for running a variety of challenging biological detection tasks.

Calcium Detection

2

Calcium Detection

1

General Information

Calcium Detection

calcium detection at-a-glance

Detection Mode	Single Wavelength Detection	Ratiometric Detection (Dual Wavelength)
Absorption	Amplite™ Colorimetric Calcium Assay Kit	
Blue Fluorescence		BTC Fura-2 Fura-8™ Indo-1 Quin-2
FLIPR® Assay	Screen Quest™ Fluo-8® No Wash Calcium Assay Kit Screen Quest™ Rhod-4™ No Wash Calcium Assay Kit Screen Quest™ Luminometric Calcium Assay Kit	Screen Quest™ Fura-2 No Wash Calcium Assay Kit
Green Fluorescence	Cal-520™ Cal Green™-1 Fluo-3 Fluo-8®	
Orange Fluorescence	Amplite™ Fluorimetric Calcium Assay Kit Rhod-2 Rhod-4™ Rhod-5N	
Luminescence	Coelenterazines Screen Quest™ Luminometric Calcium Assay Kit	

Calcium acts as a universal second messenger in a variety of cells. Numerous functions of all types of cells are regulated by Ca²⁺, thus calcium measurement is critical for various biological investigations. Since the 1920s, scientists have attempted to measure Ca²⁺, but few were successful due to the limited availability of Ca²⁺ probes. The first reliable measurement of Ca²⁺ was 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 Indo-1, Fura-2, Fluo-3 and Rhod-2 have been the most valuable dyes for measuring Ca²⁺ with a fluorescence instrument. In recent years, AAT Bioquest has introduced the most robust calcium probes: Fluo-8® and Cal-520™, both of which enable the high throughput screening of GPCR and calcium channel drug discovery targets through the convenient calcium detection. FLIPR® and FlexStation® instruments of Molecular Devices, FDSS®/µCell of Hamamatsu and NOVOstar of BMG Technologies have further accelerated the high throughput measurement of calcium for GPCR and ion channel research.

2.1 Selection of a Calcium Indicator

Fluorescent probes that show spectral responses upon binding Ca²⁺ have enabled researchers to investigate changes in intracellular free Ca²⁺ concentrations by using fluorescence microscopy, flow cytometry, fluorescence spectroscopy and fluorescence microplate readers. Most of these fluorescent indicators are derivatives of BAPTA chelators that incorporate a PET system responsive to calcium. There are quite a few factors that need be considered when selecting a fluorescent Ca²⁺ indicator. These include:

• **Spectral Properties:** For UV excitation, Indo-1 and Fura-2 are widely used. Fura-8™ is a newly developed excitation-ratioable calcium dye. Its AM is superior to Fura-2 AM with higher signal/background ratio in cells. Fluo-8® and Cal-520™ are preferred for 488 nm excitation while Rhod-4™ and Rhod-2 are used for red emissions.

• **Measurement Mode:** Ion indicators that exhibit spectral shifts upon ion binding can be used for ratiometric measurements of Ca²⁺ concentration, which are essentially independent of uneven dye loading, cell thickness, photobleaching effects and dye leakage. Excitation and emission wavelength preferences depend on the type of instrumentation being used, as well as on sample autofluorescence and on the presence of other fluorescent or photoactivatable probes in the experiment. Indo-1, Fura-2 and our newly developed Fura-8™ are primary choices for ratiometric measurements while Fluo-3, Fluo-4, Fluo-8®, Cal-520™, Rhod-2 and Rhod-4 are predominantly used for single wavelength measurements.

• **Permeability of Ca²⁺ Indicators (salt or AM ester):** The salt forms are typically loaded into cells by microinjection, microprojectile bombardment or electroporation, or used for extracellular assays. In contrast, the cell-permeant acetoxymethyl (AM) esters can be passively loaded into cells, where they are cleaved to cell-impermeant products by intracellular esterases.

• **Dissociation Constant (K_d):** The desired indicators must have a proper K_d compatible with the Ca²⁺ concentration range of interest. The K_d values of Ca²⁺ indicators are dependent on many factors, including pH, temperature, ionic strength, viscosity, protein binding, the presence of Mg²⁺ and other ions. Consequently, K_d values for intracellular indicators are usually significantly higher than the corresponding values measured in cell-free solutions.

2.2 Single Wavelength Calcium Indicators

Among the visible light-excitabile calcium indicators, Fluo-8®, Fluo-4, Fluo-3, Rhod-2 and Rhod-4™ are most commonly used. Fluo-8® indicators are widely used in flow cytometry and confocal laser-scanning microscopy. More recently, Fluo-8® AM has been extensively used for high throughput screening GPCR targets. Fluo-8® is essentially nonfluorescent unless bound to Ca²⁺ and exhibits a quantum yield of ~0.15 in the presence of saturating Ca²⁺ and a K_d of 390 nM for Ca²⁺. Cal-520™ is by far the best 488 nm-excitabile green fluorescent calcium indicator with a significantly improved signal/background ratio and intracellular retention.

The long-wavelength Rhod-4™ is a valuable alternative Ca²⁺ indicator to the green fluorescent Fluo-8®, Fluo-4 and Fluo-3 for experiments in cells and tissues that have high levels of autofluorescence. Rhod-5N has a lower binding affinity for Ca²⁺ than any other BAPTA-based indicator (K_d = ~320 µM) and is suitable for Ca²⁺ measurements from 10 µM to 1 mM. Like the parent Rhod-2 indicator, Rhod-5N is essentially nonfluorescent in the absence of divalent cations and exhibits strong fluorescence enhancement with no spectral shift upon binding Ca²⁺. Both Fluo and Rhod indicators are available as cell-impermeant potassium salts or as cell-permeant AM esters.

Table 2.1 Classic Single Wavelength Fluorescent Calcium Indicators

Cat #	Product Name	Size	Ex (nm)	Em (nm)	K _d (nM)
20500	Cal Green™-1 (equivalent to Calcium Green™-1)	10x50 µg	506	531	190
20501	Cal Green™-1 AM (equivalent to Calcium Green™-1 AM)	10x50 µg	506	531	190
21011	Fluo-3 AM *UltraPure grade*	1 mg	506	526	390
21018	Fluo-3, pentaammonium salt	1 mg	506	526	390
21017	Fluo-3, pentapotassium salt	1 mg	506	526	390
21016	Fluo-3, pentasodium salt	1 mg	506	526	390
21064	Rhod-2 AM *UltraPure grade*	20x50 µg	549	578	570
21067	Rhod-2, tripotassium salt	1 mg	549	578	570
21068	Rhod-2, trisodium salt	1 mg	549	578	570
21070	Rhod-5N AM	1 mg	551	577	0.3 mM
21072	Rhod-5N, tripotassium salt	1 mg	551	577	0.3 mM

Fluo-8[®] Calcium Indicators

The Brightest 488 nm-Excitable Green Fluorescent Calcium Indicators

Fluo-3 and Fluo-4 were the most commonly used visible light-excitabile calcium indicators. However, Fluo-3 AM and Fluo-4 AM are only moderately fluorescent in live cells upon esterase hydrolysis, and require harsh cell loading conditions to maximize their cellular calcium responses. Fluo-8[®] dyes have been developed to improve cell loading and calcium response while maintaining the convenient Fluo-3 and Fluo-4 spectral wavelengths of maximum excitation @ ~490 nm and maximum emission @ ~520 nm. For cell loading, Fluo-8[®] AM only requires incubation at room temperature while Fluo-3 AM and Fluo-4 AM require incubation at 37 °C. In addition, Fluo-8[®] AM is 2 times brighter than Fluo-4 AM, and 4 times brighter than Fluo-3 AM in cells. AAT Bioquest offers a set of outstanding Fluo-8[®] reagents with different calcium binding affinities.

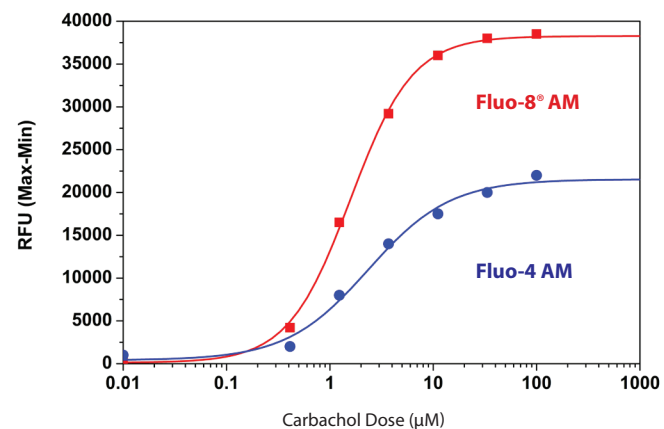


Figure 2.1. Carbachol dose responses were measured in HEK-293 cells with Fluo-8[®] AM and Fluo-4 AM. HEK-293 cells were seeded overnight at 40,000 cells/100 µL/well in a 96-well black wall/clear bottom Costar plate. The growth medium was removed, and the cells were incubated with 100 µL of dye-loading solution containing Fluo-8[®] AM or Fluo-4 AM for 1 hour at room temperature. Carbachol (25µL/well) was added by NOVOstar to achieve the final indicated concentrations. The fluorescence signals were measured at Ex/Em = 490/525 nm. The EC₅₀ of Fluo-8[®] AM is about 1.2 µM.

Key Features of Fluo-8[®] AM

- **Faster**, more readily loaded into cells than Fluo-3 AM and Fluo-4 AM. Only room temperature is required.
- **Brighter**, much brighter than Fluo-3 AM and Fluo-4 AM in cells.
- **Convenient**, almost identical spectra to those of Fluo-4 AM.

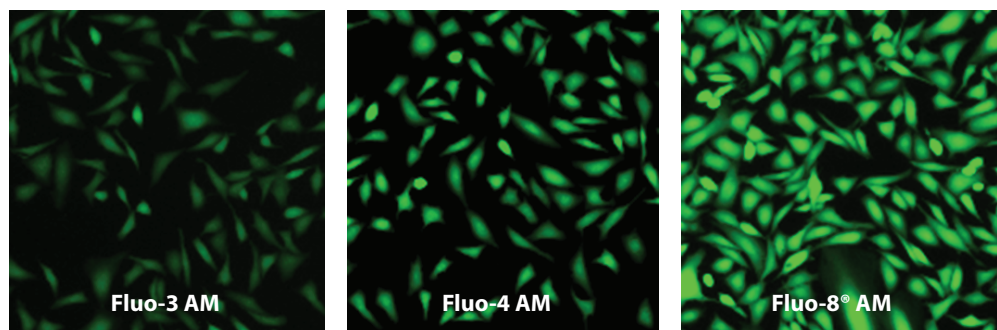


Figure 2.2. U2OS cells were seeded overnight at 40,000 cells per 100 µL per well in a Costar black wall/clear bottom 96-well plate. The growth medium was removed, and the cells were incubated with 100 µL of 4 µM Fluo-3 AM, Fluo-4 AM and Fluo-8[®] AM in HHBS at 37 °C for 1 hour. The cells were washed twice with 200 µL HHBS, then imaged with a fluorescence microscope using FITC channel.

Table 2.2 Fluo-8[®] Calcium Indicators

Cat #	Product Name	Size	Ex (nm)	Em (nm)	K _d (nM)
21080	Fluo-8 [®] AM	1 mg	494	517	389
21081	Fluo-8 [®] AM	5x50 µg	494	517	389
21088	Fluo-8 [®] , sodium salt	10x50 µg	494	517	389
21104	Fluo-8FF [™] AM	10x50 µg	494	517	10,000
21102	Fluo-8FF [™] , potassium salt	10x50 µg	494	517	10,000
21090	Fluo-8H [™] AM	1 mg	494	517	232
21095	Fluo-8H [™] , sodium salt	10x50 µg	494	517	232
21096	Fluo-8L [™] AM	1 mg	494	517	1,860
21098	Fluo-8L [™] , sodium salt	10x50 µg	494	517	1,860

Recent Citations of Fluo-8[®] Calcium Indicators

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Pay Less. Perform Better.			
Ca ²⁺ Indicator	AAT Bioquest	Sigma [†]	Invitrogen [†]
Cal-520 [™] AM	\$195/10x50 µg	not available	not available
Fluo-8 [®] AM	\$175/10x50 µg	not available	not available
Fluo-4 AM	not available	not available	\$218/10x50 µg
Fluo-3 AM	\$145/mg	\$390/mg	\$242/mg

[†]Prices are based on the websites of Sigma-Aldrich and Invitrogen as of April 2013 respectively, which may vary for different customers.

Cal-520™ Calcium Indicators

The Best 488 nm-Excitable Green Fluorescent Calcium Indicators with The Highest Signal to Background Ratio

Cal-520™ provides the most robust homogeneous fluorescence-based assay tool for detecting intracellular calcium mobilization. Cal-520™ AM is a new fluorogenic calcium-sensitive dye with a significantly improved signal to background ratio and intracellular retention compared to the existing green calcium indicators (such as Fluo-3 AM and Fluo-4 AM). The higher signal/background ratio and better intracellular retention make the Cal-520™ calcium assay a robust tool for evaluating GPCR and calcium channel targets as well as for screening their agonists and antagonists.

Our preliminary in-house research indicated that Cal-520™ AM can be readily loaded to a guinea pig heart and stays there for a few hours in the absence of probenecid. The calcium signal can be readily monitored with Cal-520™ AM while it is difficult to observe

the calcium signal under the same conditions with other green calcium dyes such as Fluo-3 AM and Fluo-4 AM.

Table 2.3 Spectral Comparison of Fluo-3, Fluo-4, Fluo-8® and Cal-520™

Dye	Ex (nm)	Em (nm)	QY*
Cal-520™	492	514	0.75
Fluo-3	506	525	0.15
Fluo-4	493	515	0.16
Fluo-8®	490	514	0.16

*QY = Fluorescence Quantum Yield in the presence of 5 mM calcium citrate.

Key Features of Cal-520™ AM

- **Better Intracellular Retention**, Cal-520™ AM is better retained in live cells than Fluo-3 AM and Fluo-4 AM.
- **Higher Sensitivity**, Cal-520™ AM has much higher signal/background ratio than Fluo-3 AM and Fluo-4 AM in cells.
- **Convenient**, Cal-520™ AM has almost identical spectra to those of Fluo-4 AM.

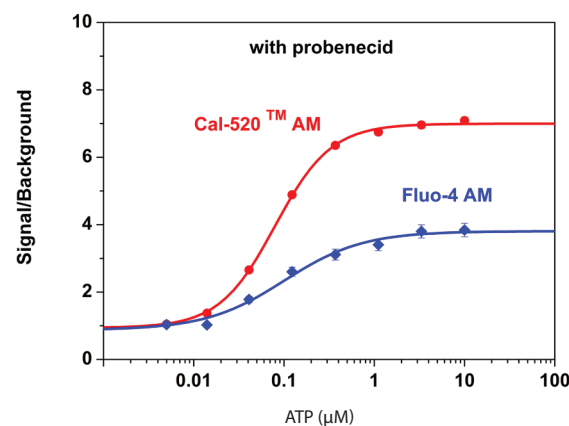


Figure 2.3. ATP-stimulated calcium responses of endogenous P2Y receptor in CHO-K1 cells incubated with Cal-520™ AM (red curve), or Fluo-4 AM (blue curve) respectively with probenecid under the same conditions. CHO-K1 cells were seeded overnight at 50,000 cells/100 µL/well in a Costar black wall/clear bottom 96-well plate. 100 µL of 5 µM Fluo-4 AM or Cal-520™ AM in HHBS with 2.5 mM probenecid was added into the cells, and the cells were incubated at 37 °C for 2 hours. ATP (50 µL/well) was added using FlexSation® to achieve the final indicated concentrations.

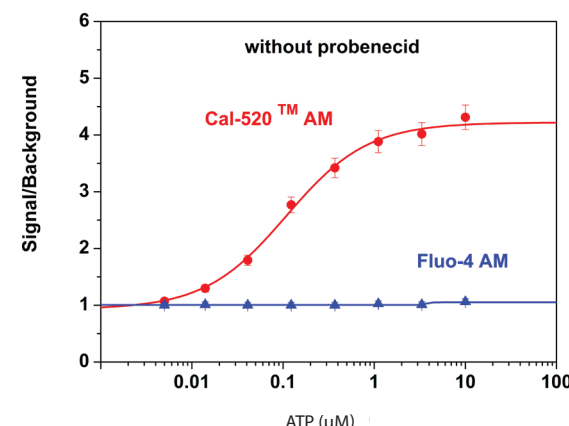


Figure 2.4. ATP-stimulated calcium responses of endogenous P2Y receptors in CHO-K1 cells incubated with Cal-520™ AM (red curve), or Fluo-4 AM (blue curve) respectively, without probenecid under the same conditions. CHO-K1 cells were seeded overnight at 50,000 cells/100 µL/well in a Costar black wall/clear bottom 96-well plate. 100 µL of 5 µM Fluo-4 AM or Cal-520™ AM in HHBS was added into the cells, and the cells were incubated at 37 °C for 2 hours. ATP (50 µL/well) was added using FlexSation® to achieve the final indicated concentrations.

Table 2.4 Fluorescent Cal Calcium Indicators

Cat #	Product Name	Size	Ex (nm)	Em (nm)	K _d (nM)
21131	Cal-520™ AM	1 mg	492	514	320
21141	Cal-520™, potassium salt	1 mg	492	514	320
21136	Cal-520™, sodium salt	1 mg	492	514	320
21142	Cal-520FF™ AM	1 mg	492	514	9,800
21144	Cal-520FF™, potassium salt	10 x 50 µg	492	514	9,800
20501	Cal Green™-1 AM (equivalent to Calcium Green™-1 AM)	10 x 50 µg	506	531	190
20500	Cal Green™-1, hexapotassium salt (equivalent to Calcium Green™-1)	10 x 50 µg	506	531	190

Rhod-4™ Calcium Indicator

The Superior Replacement to Rhod-2, Localized in Cytoplasm

Rhod-2 is the most commonly used red fluorescent calcium indicators. However, Rhod-2 AM is only moderately fluorescent in live cells upon esterase hydrolysis, and has very small cellular calcium responses. Rhod-4™ has been developed to improve the cell loading and calcium response while maintaining the spectral wavelength of Rhod-2. In CHO and HEK cells, the cellular calcium response of Rhod-4™ AM is 10 times more sensitive than that of Rhod-2 AM. Our in-house research indicated that Rhod-4™ AM can detect calcium transients in stem cell cardiomyocytes that was not detected with Rhod-2 AM under the same conditions. The higher sensitivity of Rhod-4™ AM might be due to its higher cell loading efficiency than that of Rhod-2 AM.

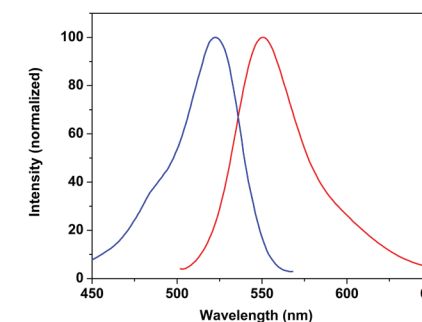


Figure 2.5. The excitation and emission spectra of Rhod-4™ in PBS buffer (pH 7.2) in the presence of 5 mM calcium chloride.

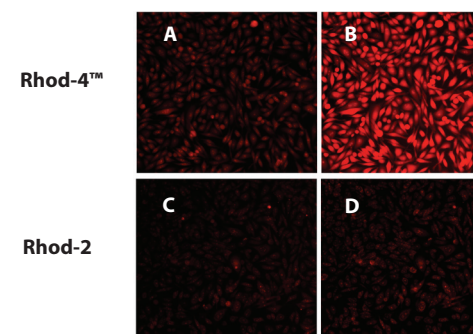


Figure 2.6. ATP-stimulated calcium responses of endogenous P2Y receptors were measured in CHO-K1 cells with Rhod-4™ AM and Rhod-2 AM. CHO-K1 cells were seeded overnight at 50,000 cells/100 µL/well in a 96-well black wall/clear bottom Costar plate. The growth medium was removed, and the cells were incubated with 100 µL of 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 cells were washed twice with 200 µL HHBS, then images before (A and C) and after (B and D) ATP treatment were taken with a fluorescence microscope (Olympus IX71) using TRITC channel.

Table 2.5 Visible Light-Excitable Orange Fluorescent Calcium Indicators

Cat #	Product Name	Size	Ex (nm)	Em (nm)	K _d (nM)
21064	Rhod-2 AM *UltraPure grade"	20 x 50 µg	549	578	570
21067	Rhod-2, tripotassium salt	1 mg	549	578	570
21068	Rhod-2, trisodium salt	1 mg	549	578	570
21120	Rhod-4™ AM	1 mg	524	551	451
21128	Rhod-4™, sodium salt	5 x 50 µg	524	551	451
21070	Rhod-5N, AM	1 mg	551	577	0.3 mM
21072	Rhod-5N, tripotassium salt	1 mg	551	577	0.3 mM

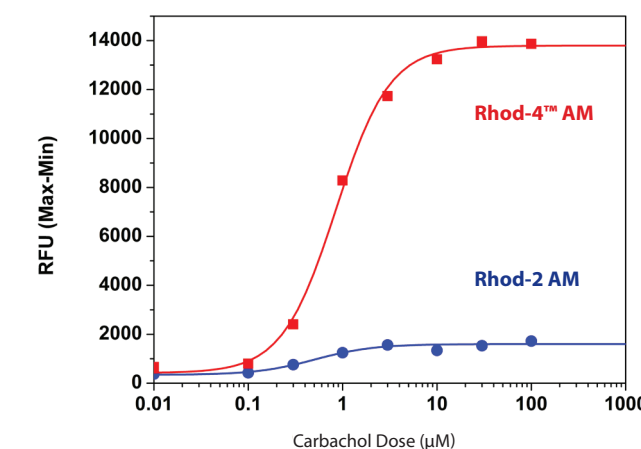


Figure 2.7. Carbachol dose responses were measured in HEK-293 cells with Rhod-4™ AM and Rhod-2 AM. HEK-293 cells were seeded overnight at 40,000 cells/100 µL/well in a Costar black wall/clear bottom 96-well plate. The growth medium was removed, and the cells were incubated with 100 µL Rhod-4™ AM dye loading solution, or 100 µL Rhod-2 AM dye loading solution (5 µM) for 1 hour at room temperature. Carbachol (25 µL/well) was added by NOVOstar (BMG Labtech) to achieve the final indicated concentrations. The EC₅₀ of carbachol with Rhod-4™ AM is about 0.8 µM.

Recent Citations of Rhod-4™ Calcium Indicator

Alice P. Liou, Yoshitatsu Sei, Xilin Zhao, Jianying Feng, Xiping Lu, Craig Thomas, Susanne Pechhold, Helen E. Raybould, and Stephen A. Wank. The extracellular calcium-sensing receptor is required for cholecystokinin secretion in response to L-phenylalanine in acutely isolated intestinal I cells. *Am J Physiol Gastrointest Liver Physiol.* 2011, 300, G538 - G546.

Zhiheng Jia, Virginijus Valiunas, Zongju Lu, Harold Bien, Huilin Liu, Hong-Zhang Wang, Barbara Rosati, Peter R. Brink, Ira S. Cohen and Emilia Entcheva, Stimulating. Cardiac Muscle by Light, Cardiac Optogenetics by Cell Delivery. *Circ Arrhythm Electrophysiol.* 2011, 4, 753 - 760.

2.3 Ratiometric Calcium Indicators

BTC

Among the ratiometric calcium indicators, Fura-2 and Indo-1 are most commonly used. BTC is another excitation-ratioable calcium indicator. However, BTC can only be used for high calcium level detection due to its low affinity to calcium. In recent years, BTC has been increasingly used for monitoring potassium channels since BTC demonstrated an excellent fluorescence enhancement response upon binding thallium ion that selectively passes through potassium channels.

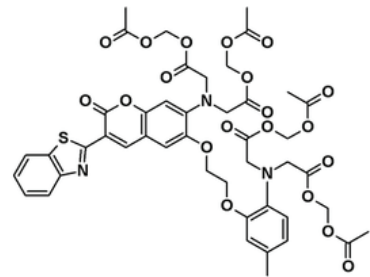


Figure 2.8. The chemical structure of BTC AM

Fura-2

Fura-2 is a ratiometric fluorescent dye which binds free intracellular calcium. It was the first widely-used dye for calcium imaging, and remains very popular. Fura-2 is excited at 340 nm and 380 nm, and the ratio of the emissions at those wavelengths is directly correlated to the amount of intracellular calcium. Regardless of the presence of calcium, Fura-2 emits at 510 nm. The use of the ratio automatically cancels out confounding variables, such as variable dye concentration and cell numbers, making Fura-2 one of the most appreciated tools to quantify calcium levels. Fura-2 is preferred for ratio-imaging microscopy, in which it is more practical to change excitation wavelengths than emission wavelengths.

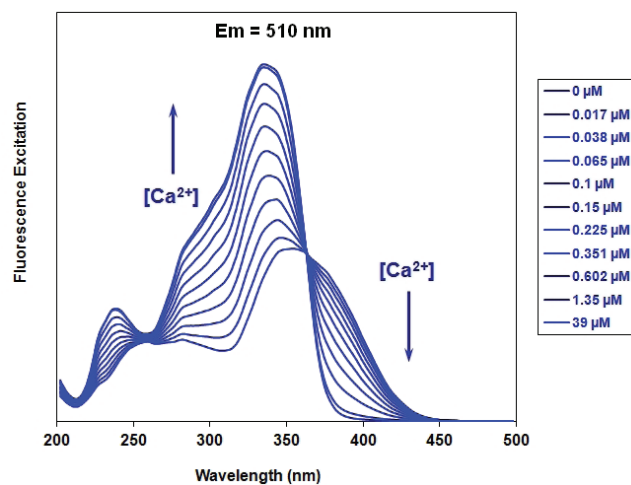


Figure 2.9. Fluorescence excitation spectra of Fura-2 in solutions containing 0 to 39 μM free Ca²⁺

Fura-8™

Although Fura-2 has been widely used as the preferred excitation-ratioable calcium indicator, it has certain limitations, e.g., lower sensitivity compared to the single wavelength calcium dyes such as Fluo-8® and Cal-520™. AAT Bioquest has recently developed Fura-8™ to improve the calcium response of Fura-2. As demonstrated in Figures 2.10 and 2.11, Fura-8™ AM is more sensitive to calcium than Fura-2 AM. In addition, Fura-8™ has its emission shifted to longer wavelength (Em = 525 nm). Fura-8™ might be also used for the flow cytometric analysis of calcium in cells due to its excellent excitation at 405 nm of violet laser.

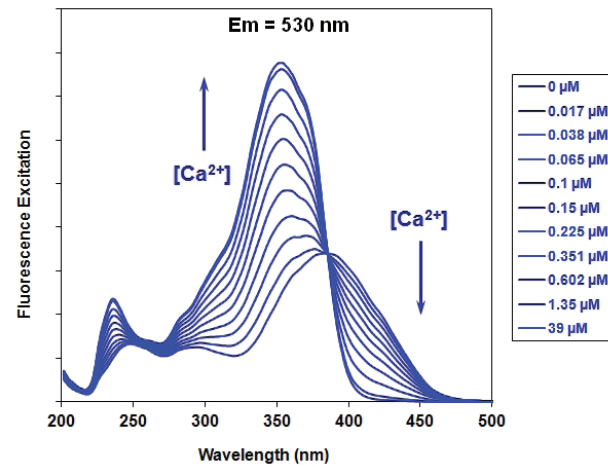


Figure 2.10. Fluorescence excitation spectra of Fura-8™ in solutions containing 0 to 39 μM free Ca²⁺

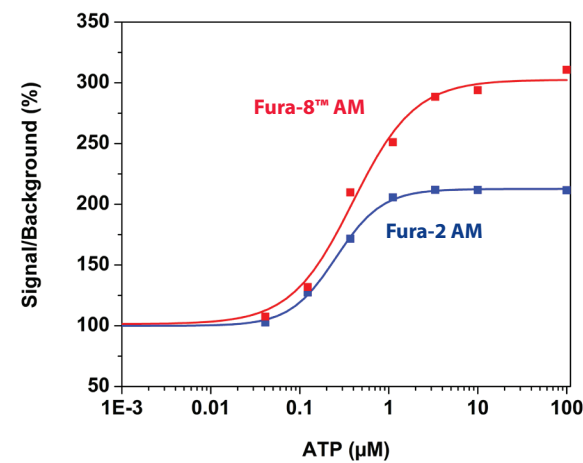


Figure 2.11. ATP dose responses in CHO-K1 cells measured with Fura-2 AM and Fura-8™ AM respectively. CHO-K1 cells were seeded overnight at 40,000 cells/100 μL/well in a Costar black wall/clear bottom 96-well plate. The cells were incubated with Fura-2 AM or Fura-8™ AM calcium assay dye-loading solution for 1 hour at room temperature. ATP (50 μL/well) was added by FlexStation®.

Key Features of Fura-8™

- Fura-8™ responds to calcium the same way as Fura-2 does
- Red-shifted dual excitation wavelengths (354 nm/415 nm)
- Better excited at 405 nm for flow cytometric applications
- Compatible with common filter sets.
- Higher signal/background ratio than that of Fura-2

Indo-1

In contrast to Fura-2, Fura-8™ and BTC, Indo-1 is the preferred emission-ratioable dye for flow cytometry, where it is more practical to use a single laser for excitation (usually the 351–364 nm spectral lines of the argon-ion laser). The emission maximum of Indo-1 shifts from ~475 nm in Ca²⁺-free medium to ~400 nm when the dye is saturated with Ca²⁺ (see Figure 2.12). While Indo-1 is not cell permeant, its pentaacetoxymethyl ester, Indo-1 AM, enters the cell where it is cleaved by intracellular esterases to give Indo-1.

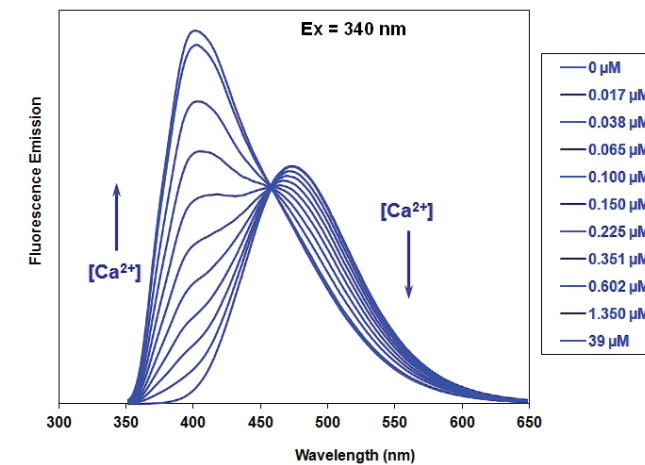


Figure 2.12. Fluorescence emission spectra of Indo-1 in solutions containing 0 to 39 μM free Ca²⁺.

Table 2.6 Ratiometric Fluorescent Calcium Indicators

Cat #	Product Name	Size	Zero Calcium		High Calcium		K _d (nM)
			Ex (nm)	Em (nm)	Ex (nm)	Em (nm)	
21054	BTC AM	1 mg	464	533	401	529	7,000
21053	BTC, tetrapotassium salt	1 mg	464	533	401	529	7,000
21021	Fura-2 AM *UltraPure grade*	1 mg	363	512	335	505	145
21025	Fura-2, pentapotassium salt	1 mg	363	512	335	505	145
21026	Fura-2, pentasodium salt	1 mg	363	512	335	505	145
21055	Fura-8™ AM	1 mg	386	532	354	524	260
21056	Fura-8™ AM	10x50 μg	386	532	354	524	260
21057	Fura-8™, potassium salt	1 mg	386	532	354	524	260
21058	Fura-8™, sodium salt	1 mg	386	532	354	524	260
21032	Indo-1 AM *UltraPure grade*	1 mg	346	475	330	401	230
21040	Indo-1, pentapotassium salt	1 mg	346	475	330	401	230
21044	Indo-1, pentasodium salt	1 mg	346	475	330	401	230
21050	Quin-2 AM	1 mg	353	495	333	495	60
21052	Quin-2, tetrapotassium salt	5 mg	353	495	333	495	60

Ratio Imaging: Research activities are increasingly focusing on the identification and the spatial and temporal distribution of e.g. local “hot spots” for dynamic changes in ion concentration, voltage or pH in a cell or a cellular network. Such “hot spots” are often localized in specialized parts of a cell or in certain cells in a cellular network. Additionally, these areas often have different properties compared to the rest of the specimen in terms of cell metabolism or structure. Conventional fluorophores used to investigate dynamic physiological states change their emission intensity upon ion binding, pH change or voltage change (e.g. fluo-8® has increased emission upon calcium binding). However, these markers do not take into account that differences in structure, diameter or marker uptake/expression can cause changes in the quantity of emitted light that are not in correlation with the actual ion concentration, voltage or pH. To quantitatively and comparably detect the changes in cellular structures or different cells, a method insensitive to structure diameter and fluorophore concentration is needed. Ratiometric imaging offers the opportunity to reproducibly measure absolute intracellular ion, voltage and pH levels/changes with respect to cell diameter, fluorophore concentration and optical properties of the imaging setup. However, ratiometric imaging depends on a fast change of excitation wavelength or the detected wavelength, a strong light source, excellent transmission of optical components and fast signal detection. The recent development of ultrafast filter wheels, UV-light optimized objectives, highly sensitive fluorophores and new CCD cameras allows affordable quantitative high speed live cell imaging in high spatial resolution.

2.4 FLIPR® Calcium Assays

Calcium flux assays are preferred methods in drug discovery for screening G protein coupled receptors (GPCRs). Cells expressing a GPCR of interest that signals through calcium are pre-loaded with our proprietary Fluo-8® AM or Rhod-4™ AM which can cross cell membrane. Screen Quest™ Calcium Assay Kits provide an optimized assay method for monitoring GPCRs and calcium channels. The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation.

Fluo-8® AM is the brightest calcium indicator available for HTS screening. The characteristics of the convenient 488 nm excitation, high sensitivity, and 100-250 times fluorescence increases (when it forms complexes with calcium) make Fluo-8® AM an ideal indicator for the measurement of intracellular calcium.

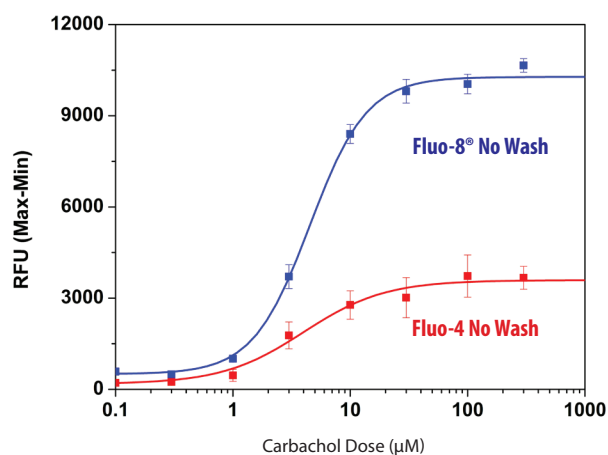


Figure 2.13. Carbachol dose responses were measured in HEK-293 cells with Screen Quest™ Fluo-8® No Wash Calcium Assay Kit and Fluo-4 No Wash Calcium Assay Kit. HEK-293 cells were seeded overnight at 40,000 cells/100 µL/well in a Costar black wall/clear bottom 96-well plate. The cells were incubated with 100 µL of dye-loading solution using Screen Quest™ Fluo-8® No Wash Calcium Assay Kit or Fluo-4 No Wash Kit (according to the manufacturer's instructions) for 1 hour at room temperature. Carbachol (50 µL/well) was added by NOVOstar (BMG Labtech) to achieve the final indicated concentrations.

Table 2.7 Screen Quest™ FLIPR® Calcium Assay Kits

Cat #	Product Name	Size	Ex (nm)	Em (nm)
36315	Screen Quest™ Fluo-8® No Wash Calcium Assay Kit	10 plates	490	525
36316	Screen Quest™ Fluo-8® No Wash Calcium Assay Kit	100 plates	490	525
36308	Screen Quest™ Fluo-8® No Wash Calcium Assay Kit *Medium Removal*	10 plates	490	525
36309	Screen Quest™ Fluo-8® No Wash Calcium Assay Kit *Medium Removal*	100 plates	490	525
36320	Screen Quest™ Fura-2 No Wash Calcium Assay Kit	10 plates	340/380	510
36321	Screen Quest™ Fura-2 No Wash Calcium Assay Kit	100 plates	340/380	510
36334	Screen Quest™ Rhod-4™ No Wash Calcium Assay Kit	10 plates	530	590
36335	Screen Quest™ Rhod-4™ No Wash Calcium Assay Kit	100 plates	530	590
36331	Screen Quest™ Rhod-4™ No Wash Calcium Assay Kit *Medium Removal*	10 plates	530	590
36332	Screen Quest™ Rhod-4™ No Wash Calcium Assay Kit *Medium Removal*	100 plates	530	590

Rhod-4™ AM is the brightest red calcium indicator available for HTS screening. Once inside the cell, the lipophilic blocking groups of Rhod-4™ AM are cleaved by non-specific cell esterase, resulting in a negatively charged fluorescent dye that stays inside cells, and its fluorescence is greatly enhanced upon binding to calcium. When cells stimulated with screening compounds, the receptor signals the release of intracellular calcium, which greatly increases the fluorescence of Rhod-4™. The characteristics of its long wavelength, high sensitivity, and >250 times fluorescence increases (when it forms complexes with calcium) make Rhod-4™ AM an ideal indicator for the measurement of intracellular calcium.

Our Fura-2 calcium kit provides the only ratiometric FLIPR® calcium assay commercially available for screening GPCRs and calcium channel targets. The Fura-2 kit uses excitation ratio of 340/380 nm, monitoring emission at 510 nm.

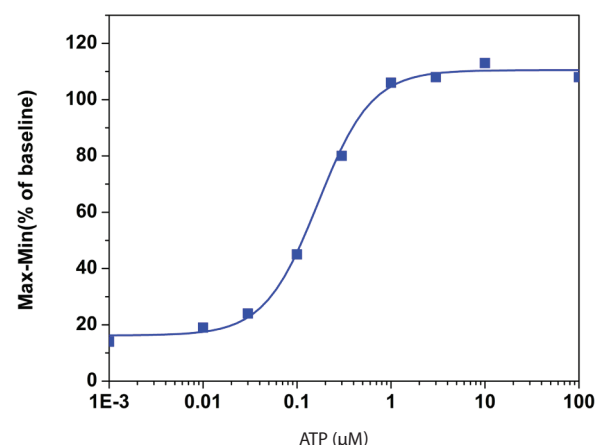


Figure 2.14. ATP dose responses were measured in CHO cells with Screen Quest™ Fura-2 No Wash Calcium Assay Kit. CHO-K1 cells were seeded overnight at 40,000 cells/100 µL/well in a Costar black wall/clear bottom 96-well plate. The cells were incubated with 100 µL of Screen Quest™ Fura-2 No Wash Calcium Assay Kit for 1 hour at room temperature. ATP (50 µL/well) was added by FlexStation® (Molecular Devices) to achieve the final indicated concentrations.

2.5 Luminescent Calcium Detection: Coelenterazine and Its Synthetic Analogs

The aequorin complex comprises a 22,000-dalton apoaequorin protein, molecular oxygen and the luminophore coelenterazine. When three Ca²⁺ ions bind to this complex, coelenterazine is oxidized to coelenteramide, with a concomitant release of carbon dioxide and blue light. The approximately third-power dependence of aequorin's bioluminescence on Ca²⁺ concentration allows the measurement of Ca²⁺ concentrations with a broad detection range from ~0.1 µM to >100 µM. Unlike fluorescent Ca²⁺ indicators, Ca²⁺-bound aequorin can be detected without illuminating the sample, thereby eliminating the interference from autofluorescence.

AAT Bioquest offers coelenterazine and several synthetic coelenterazine analogs for reconstituting aequorin in cells that have been transfected with apoaequorin cDNA. In addition to native coelenterazine, we also offer a few derivatives of coelenterazine that confer different Ca²⁺ affinities and spectral properties on the aequorin complex. Recombinant apoaequorin reconstituted with coelenterazine hcp is reported to have the best luminescence overall, with both a high quantum yield and a fast response time. However, intracellular reconstitution of aequorin from coelenterazine analogs can be relatively slow. Aequorins containing the cp, f or h form of coelenterazine exhibit 10–20 times stronger luminescence than that of apoaequorin reconstituted with native coelenterazine. Coelenterazine h has been predominantly used in HTS screening assay for GPCRs.

Besides the standalone coelenterazine products, AAT Bioquest offers a luminescent calcium assay kit. The kit uses a highly calcium-sensitive and membrane-permeable coelenterazine analog as a calcium indicator for the cells transfected with apoaequorin gene. Our coelenterazine-based kit is much more sensitive than the fluorescence-based calcium assay kits (such as Fluo-4, Fluo-3, Calcium-3 and Calcium-4). This kit provides an optimized assay method for monitoring GPCRs and calcium channels. The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation.

Table 2.8 Luminescent Calcium Indicators*

Cat #	Product Name	Size	Em (nm)	RL	HRT (ms)
21150	Coelenterazine *UltraPure grade*	250 µg	466	1	6-30
21151	Coelenterazine cp *UltraPure grade*	250 µg	442	28	2-5
21152	Coelenterazine f *UltraPure grade*	250 µg	472	20	6-30
21153	Coelenterazine h *UltraPure grade*	250 µg	466	16	6-30
21154	Coelenterazine hcp *UltraPure grade*	250 µg	445	500	2-5
21155	Coelenterazine n *UltraPure grade*	250 µg	468	0.15	6-30
36305	Screen Quest™ Luminometric Calcium Assay Kit	10 plates	466	16	6-30

* Notes: a). RL = relative luminescence; HRT = half rise time in milli seconds; b). Data from O. Shimomura, et al. (1993). The relative rate of aequorin regeneration from apoaequorin and coelenterazine analogues. Biochem J 296 (Pt 3), 549-51.

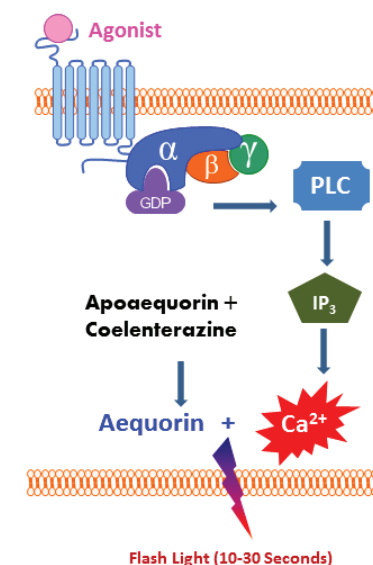


Figure 2.15. The aequorin-based calcium assay principle. Coelenterazine h is the preferred luminophore used in the luminescence-based calcium assays.

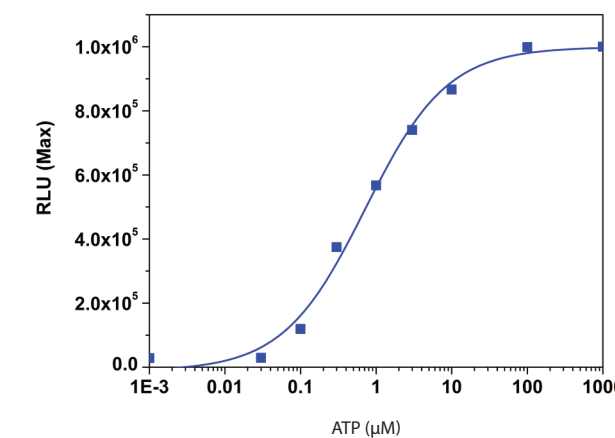


Figure 2.16. ATP dose responses on CHO-aeg cells. CHO cells stably transfected with apoaequorin were seeded overnight at 50,000 cells/100 µL/well in a Costar white wall/clear bottom 96-well plate. The growth medium was removed and the cells were incubated with 100 µL of dye-loading solution using the Screen Quest™ Luminometric Calcium Assay Kit for 3 hours at room temperature and protected from light. ATP (25 µL/well) was added by NOVOstar (BMG Labtech) to achieve the final indicated concentrations. The EC₅₀ of ATP is about 0.8 µM.

2.6 Measurement of Calcium *In Vitro*

Calcium is essential for all living organisms, particularly in cell physiology, where the movement of calcium ion into and out of the cytoplasm functions as a signal for many cellular processes. Calcium also plays an important role in mediating the constriction and relaxation of blood vessels, nerve impulse transmission, muscle contraction, and hormone secretion. The serum level of calcium is closely regulated (9 to 10.5 mg/dL) in the human body. Both hypocalcemia and hypercalcemia are serious medical disorders.

Amplite™ Colorimetric Calcium Assay

Amplite™ Colorimetric Calcium Quantitation Kit provides a simple method for detecting calcium in physiology solutions. The kit uses Calcium Blue™ as the chromogenic calcium indicator. Its absorbance changes in response to calcium binding. The absorbance signal can be easily read by an absorbance microplate reader at 600 or 650 nm. The kit can be performed in a convenient 96-well or 384-well microtiter-plate format within 5 minutes and easily adapted to automation without a separation step. With Amplite™ Colorimetric Calcium Quantitation Kit, the calcium detection linear range is from 0.1 to 7.5 nmoles in 100 µL final test volume (2.5 to 150 µM calcium).

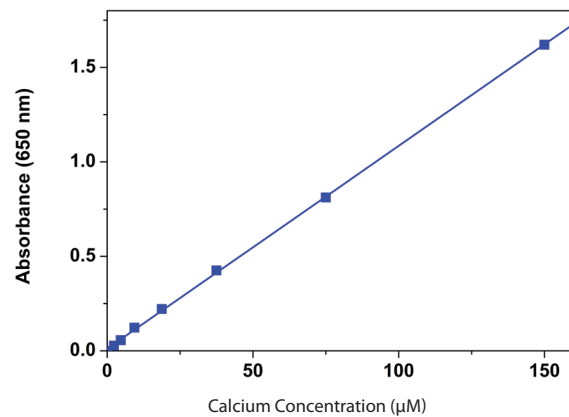


Figure 2.17. Calcium dose responses were measured in a 96-well white wall/clear bottom plate with Amplite™ Colorimetric Calcium Quantitation Kit. As low as ~ 2.5 µM Ca²⁺ was detected with 5 minutes incubation (n=3).

Amplite™ Fluorimetric Calcium Assay

Amplite™ Fluorimetric Calcium Quantitation Kit provides a simple method for detecting calcium in physiology solutions by using our proprietary red fluorescence probe. The fluorescence signal can be easily read with a fluorescence microplate reader at Ex/Em = 540/590 nm. The kit can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation. The assay can be completed within 30 minutes. With Amplite™ Fluorimetric Calcium Quantitation Kit, we have detected as little as 0.03 mM calcium. The kit has a broad dynamic range (30 µM to 10 mM). If more sensitive calcium detection is required, we recommend that Fluo-8® or Fluo-3 be used instead. Both Fluo-8® and Fluo-3 can be used for determining calcium in nM range.

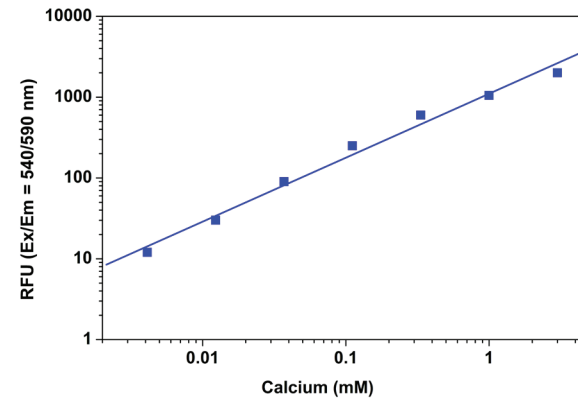


Figure 2.18. Calcium dose responses were measured in a 96-well black solid plate with Amplite™ Fluorimetric Calcium Quantitation Kit. As low as 0.03 mM calcium can be detected with 5 minutes incubation (n=3).

Table 2.9 *In Vitro* Calcium Assay Kits

Cat #	Product Name	Size
36361	Amplite™ Colorimetric Calcium Quantitation Kit	1 kit
36360	Amplite™ Fluorimetric Calcium Quantitation Kit	1 kit

2.7 Non-Fluorescent Reagents for Calcium Detection

Intracellular calibration of Ca²⁺ indicators may be achieved either by manipulating Ca²⁺ levels inside cells using an ionophore or by releasing the indicator into the surrounding medium of known Ca²⁺ concentration via detergent lysis of the cells. Besides the fluorescent and luminescent calcium detection reagents, we also offer several non-fluorescent compounds for measuring and manipulating intracellular and extracellular Ca²⁺.

Table 2.10 Non-Fluorescent Calcium Detection Reagents

Cat #	Product Name	Size
21001	BAPTA AM	25 mg
21002	BAPTA AM *UltraPure grade*	25 mg
21003	BAPTA, tetrapotassium salt	100 mg
21004	BAPTA, tetrasodium salt	100 mg
21005	EGTA AM	10 mg
21006	EGTA AM *10 mM DMSO solution*	1 mL
21008	EGTA, tetrasodium salt *10 mM aqueous solution*	10 mL
21007	EGTA, tetrasodium salt *UltraPure grade*	1 g
20053	Pluronic® F-127 *10% solution in water*	10 mL
20052	Pluronic® F-127 *20% solution in DMSO*	10 mL
20050	Pluronic® F-127 *Cell culture tested*	10 g
20060	Probenecid *Cell culture tested*	10x72 mg
20061	Probenecid *Water-soluble*	10x77 mg

Zinc Ion Detection

zinc ion detection at-a-glance

Detection Mode	Cell-Impermeant	Cell-Permeant
Absorption	Amplite™ Colorimetric Zinc Assay Kit	
Blue Fluorescence	Zinquin	TSQ Zinquin AM Ester Zinquin Ethyl Ester
Green Fluorescence	Amplite™ Fluorimetric Zinc Assay Kit Metal Fluor™ Zn 520	Metal Fluor™ Zn 520 AM

Zinc is the second most abundant transition metal in living organisms after iron. While most Zn²⁺ in the brain is tightly bound, such that free Zn²⁺ levels extracellularly and intracellularly are likely to be picomolar, a subset of glutamatergic neurons possess weakly bound zinc in presynaptic boutons which is released at micromolar levels in response to a variety of stimuli. The intracellular concentration of free Zn²⁺ is extremely low in most cells (<1 nM), with the remainder being bound to proteins or nucleic acids.

Mounting evidence indicates that zinc has multiple roles in cell biology, as a part of metalloenzyme catalytic sites, as a structural component of gene regulatory proteins, and as a free signal ion, particularly in the cortex of the brain. It is of particular importance in the regulation of gene expression, as Zn²⁺ binding proteins account for nearly 50% of the transcription regulatory proteins in the human genome. Zn²⁺ is also functionally active in pancreatic insulin secretion and is a contributory factor in neurological disorders including epilepsy and Alzheimer's disease. Free Zn²⁺ is released from metalloprotein complexes during oxidative stress.

The key to the further progress in understanding the multiple roles of zinc is the availability of fluorescent Zn²⁺ indicator systems that permits quantitative determination and imaging of zinc fluxes and levels over a broad concentration range both intracellularly and extracellularly using fluorescence microscopy and flow cytometry. AAT Bioquest offers the classic TSQ, zinquin, as well as our newly developed sensitive Metal Fluor™ Zn 520 to help elucidate the role of Zn²⁺ release and the localization of free or chelatable Zn²⁺ in cells.

3.1 Amplite™ Colorimetric Zinc Ion Assay

Zinc is an essential trace mineral element that plays an important role in a number of biological processes. It is an essential factor required for many enzymes, protein structures, and control of genetic expression. Zinc status also affects basic processes of cell division, growth, differentiation, development and aging. Clinical signs of zinc deficiency include acrodermatitis, low immunity, diarrhea, poor healing, stunting, hypogonadism, fetal growth failure, teratology and abortion. Simple, direct and automation-ready procedures for measuring are highly desirable in research and drug discovery.

AAT Bioquest's Amplite™ Colorimetric Zinc Ion Quantitation Kit provides a simple method for detecting zinc concentration in biological samples using our proprietary Zin-620™, in which zinc binds to the probe with the enhanced absorption around 620 nm. The zinc probe exhibits a large increase in 620 nm absorption in response to Zn²⁺ (>100 folds). Our kit formulation has enhanced Zn²⁺-specificity with little responses to other metals, e.g., Ca²⁺ and Mg²⁺. The assay can be used with biological samples such as serum, plasma, and urine with detection sensitivity at 1 μM.

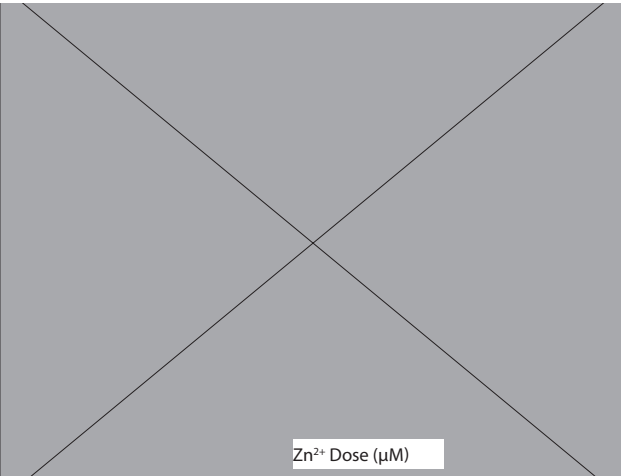


Figure 3.1. Zinc dose responses were measured in a 96-well clear bottom plate with Amplite™ Colorimetric Zinc Ion Quantitation Kit. As low as 2 μM Zn²⁺ can be detected with 30 minutes incubation (n=3).

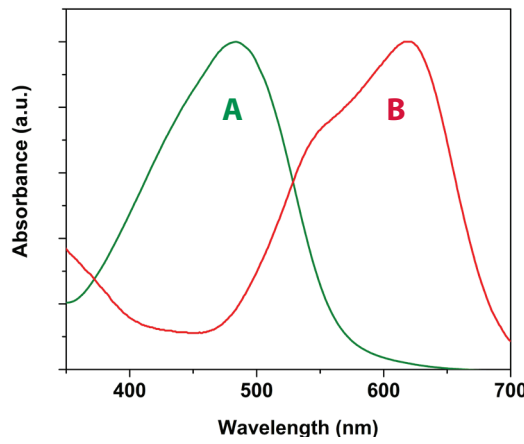


Figure 3.2. The absorption spectra of Zin-620™ in the absence of zinc ion (A) and in the presence of zinc ion (B, 1 mM).

3.2 Amplite™ Fluorimetric Zinc Ion Assay

AAT Bioquest's Amplite™ Fluorimetric Zinc Ion Quantitation Kit provides a simple method for detecting zinc concentration in biological samples using our proprietary Metal Fluor™ Zn 520, in which zinc binds to the probe with enhanced fluorescence at Ex/Em= 485/525 nm. The zinc probe exhibits a large increase in fluorescence in response to Zn²⁺ (greater than 200~300 folds). It has high Zn²⁺-specificity with little responses to other metals, including Ca²⁺, Mg²⁺, Mn²⁺, and Cu²⁺. The assay can be used with biological samples such as serum, plasma, and urine with detection sensitivity at 0.2 μM (13 ng/mL).

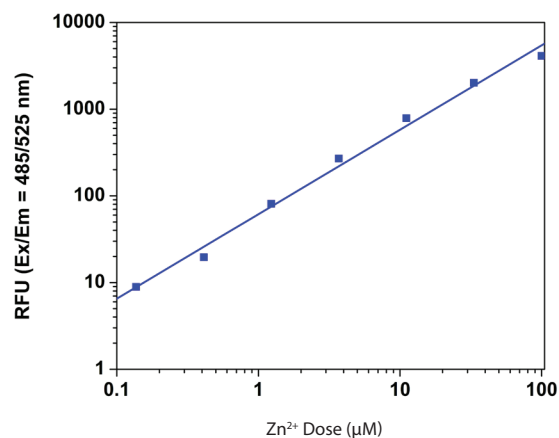


Figure 3.3. Zinc dose responses were measured in a 96-well black plate with the Amplite™ Fluorimetric Zinc Ion Quantitation Kit. As low as ~0.2 µM Zn²⁺ can be detected with 5 minutes incubation (n=3).

3.3 Metal Fluor™ Zn 520

Metal Fluor™ Zn 520 indicator is designed for the detection of Zn²⁺ at higher concentrations that are present in synaptic vesicles and released in response to electrical stimulation or excitotoxic agonists. It is designed for the detection of Zn²⁺ in the 0.1 - 100 µM range with minimal Ca²⁺ interference. Metal Fluor™ Zn 520 is suitable for the detection of Zn²⁺ concentrations in the 0.2 - 100 µM range. Its cell permeant AM-ester is useful for detecting high intracellular Zn²⁺ levels and small Zn²⁺ concentration changes.

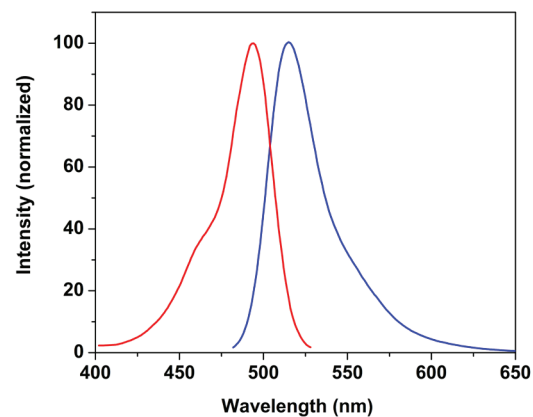


Figure 3.4. Fluorescence excitation and emission spectra of Metal Fluor™ Zn 520 in the presence of 1 mM Zn²⁺.

3.4 TSQ

TSQ is selective for Zn²⁺ in the presence of physiological concentrations of Ca²⁺ and Mg²⁺ ions. TSQ-based assays for Zn²⁺ in seawater and other biological systems exhibit a detection limit of ~0.1 nM. The complex of TSQ with free Zn²⁺ apparently has a stoichiometry of 2:1/TSQ-Zn. TSQ can be used to localize Zn²⁺ pools in the central nervous system, and to identify a broad distribution of Zn²⁺ in neonatal mice, particularly associated with rapidly proliferating tissues, such as skin and gastrointestinal epithelium. TSQ has also been used to detect Zn²⁺ translocation from presynaptic nerve terminals into postsynaptic nerve terminals when blood flow is constricted in the brain during ischemic events. TSQ is a selective nontoxic stain

for pancreatic islet cells, which have a high content of Zn²⁺, and may be useful for their flow cytometric isolation.

3.5 Zinquin

Zinquin ethyl ester and AM ester are lipophilic, zinc-sensitive and cell-permeant fluorescent probes. They are retained in living cells because the AM and ethyl ester are cleaved by cytosolic esterase to give zinquin that carries a negative charge, preventing its efflux across the plasma membrane. Zinquin fluorescent probes may be loaded into cells using the culture medium containing 5-40 µM zinquin AM or ethyl ester in PBS with calcium and magnesium (or in culture medium). Cells are normally incubated with the zinquin AM or ethyl ester for 15-30 minutes at 37 °C. Exact loading concentration, time and temperature depend on the purpose of the experiment and cell type, and so will need to be optimized experimentally. Cells are washed in PBS with culture medium to remove the extracellular remaining dye and observed under microscope, FACS or fluorescence spectrophotometer.

Table 3.1 Zinc Ion Indicators and Assay Kits

Cat #	Product Name	Size	Ex (nm)	Em (nm)
19001	Amplite™ Colorimetric Zinc Ion Quantitation Kit	200 tests	620	N/A
19000	Amplite™ Fluorimetric Zinc Ion Quantitation Kit	200 tests	492	514
21263	Metal Fluor™ Zn 520 AM	1 mg	490	514
21262	Metal Fluor™ Zn 520, potassium salt	1 mg	490	514
21254	TSQ	5 mg	344	385
21260	Zinquin "UltraPure grade"	5 mg	360	480
21261	Zinquin AM "UltraPure grade"	5 mg	360	480
21253	Zinquin Ethyl Ester "UltraPure grade"	5 mg	360	480

3.6 References

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Intracellular pH Probes

intracellular pH probes at-a-glance

Detection Mode	Single Wavelength Detection	Ratiometric Detection (Dual Wavelength)
Blue Fluorescence		PDMPPO
Green Fluorescence	Protonex™ Green	BCECF BCFL
Red Fluorescence	Protonex™ Red	

Fluorescent dyes provide the increased sensitivity required for optical pH measurements inside live cells. They also offer much greater spatial sampling capability when compared with microelectrode techniques. These advantages have spurred the development of improved fluorescent dyes that can sense pH changes within physiological ranges. To quantitatively measure pH, it is essential to match the indicator's pK_a to the pH of the experimental system. Intracellular pH is generally between ~6.8 and 7.4 in the cytosol and ~4.5 and 6.0 in acidic organelles such as lysosomes. AAT Bioquest offers a variety of fluorescent pH indicators, pH indicator conjugates and other reagents for pH measurements in biological systems. RationWorks™ are optimized for measuring intracellular pH accurately by the ratio of two emissions or two excitations. Protonex™ pH dyes are developed to monitor acidic organelles and endocytosis.

4.1 Near Neutral pH Measurement

BCECF

Intracellular pH plays an important modulating role in many cellular events, including cell growth, calcium regulation, enzymatic activity, receptor-mediated signal transduction, ion transport, endocytosis, chemotaxis, cell adhesion, and other cellular processes. pH-sensitive fluorescent dyes have been widely applied to monitor changes in intracellular pH in recent years. Imaging techniques that use fluorescent pH indicators also allow researchers to investigate these processes with much greater spatial resolution and sampling density that can be achieved using other technologies such as microelectrode. Among them, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) is the most popular pH probe since it can be used to monitor cellular pH ratiometrically. However, all the commercial BCECF AM is a complex mixture of six isomers with different ratios from batch to batch, complicating the BCECF applications.

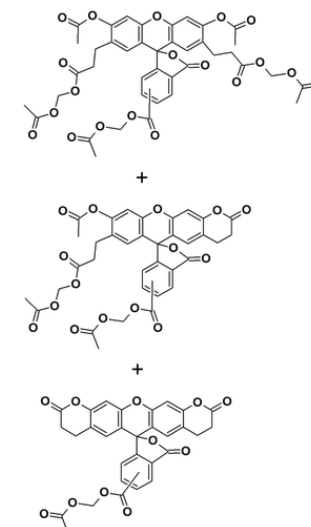


Figure 4.1. Structures of BCECF AM (the complicated mixture of six components).

BCFL AM, a superior replacement to BCECF AM

BCFL AM is developed to overcome the isomer difficulty associated with BCECF AM. As BCECF AM, BCFL AM exhibits pH-dependent dual excitations, essentially identical to those of BCECF AM. It has a pK_a of ~7.0, identical to BCECF AM too. As with BCECF AM, the dual excitation spectrum of BCFL AM with an isosbestic point at 454 nm should make BCFL AM a good excitation-ratiometric pH indicator. BCFL ratiometric imaging makes intracellular pH determination essentially independent of several variable factors, including dye concentration, path length, cellular leakage and photobleaching rate. BCFL AM is a single isomer, making the pH measurement much more reproducible than BCECF AM, which is consisted of quite a few different isomers.

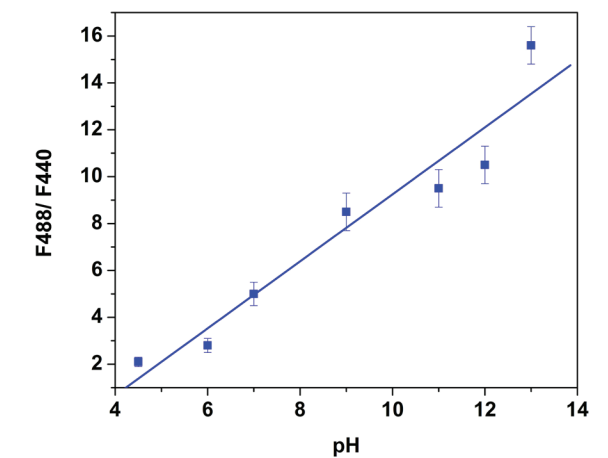


Figure 4.2. The fluorescence excitation ratios of BCFL at 488 nm and 440 nm were measured with 530 nm emission at pH 4.5, 6, 7, 9, 11, 12 and 13 using standard buffer solutions. The higher the pH, the higher the fluorescence signal with longer excitation wavelength (488 nm), and the lower the fluorescence signal with shorter excitation wavelength (440 nm).

Table 4.1 Fluorescent pH Probes for Near -Neutral pH Environments

Cat #	Product Name	Size	pK_a	Ex (nm)	Em (nm)
21201	BCECF Acid	1 mg	7.0	503	528
21202	BCECF AM	1 mg	7.0	503	528
21203	BCECF AM *UltraPure grade*	20x50 µg	7.0	505	520
21180	Cell Meter™ Fluorimetric Intracellular pH Assay Kit	500 tests	N/A	503	528
21189	RatioWorks™ BCFL Acid *Superior Replacement to BCECF*	1 mg	7.0	503	528
21190	RatioWorks™ BCFL AM *Superior Replacement to BCECF AM*	1 mg	7.0	503	528
21191	RatioWorks™ BCFL SE *Superior Replacement to BCECF SE*	1 mg	7.0	503	528

4.2 Acidic pH Measurement and Endocytosis

PDMPO, an unique dual excitation and dual-emission ratio-metric pH indicator

The existing pH probes are unsuitable to study acidic organelles such as lysosomes, endosomes, spermatozoa and acrosomes because their fluorescence is significantly reduced at lower pH. In addition, most of the existing pH probes (such as BCECF and SNARF) are not selectively localized in acidic organelles. The growing potential of ratio imaging is significantly limited by the lack of appropriate fluorescent probes for acidic organelles although ratio imaging has received intensive attention in the past few decades. PDMPO [2-(4-pyridyl)-5-((4-(2-dimethylaminoethylaminocarboxymethyl)methoxy)phenyl)oxazole] is characterized as an acidotropic dual-excitation and dual-emission pH probe. It emits intense yellow fluorescence at lower pH and gives intense blue fluorescence at higher pH. This unique pH-dependent fluorescence makes PDMPO an ideal pH probe for acidic organelles with $pK_a = 4.47$. PDMPO selectively labels acidic organelles (such as lysosomes) of live cells and the two distinct emission peaks can be used to monitor the pH fluctuations of live cells in ratio measurements. Additionally, the very large Stokes shift and excellent photostability of PDMPO make it an excellent fluorescent acidotropic reagent for fluorescence imaging. The unique fluorescence properties of PDMPO might give researchers a new tool with which to study the acidic organelles of live cells. PDMPO can be well excited by the violet laser at 405 nm for flow cytometric applications.

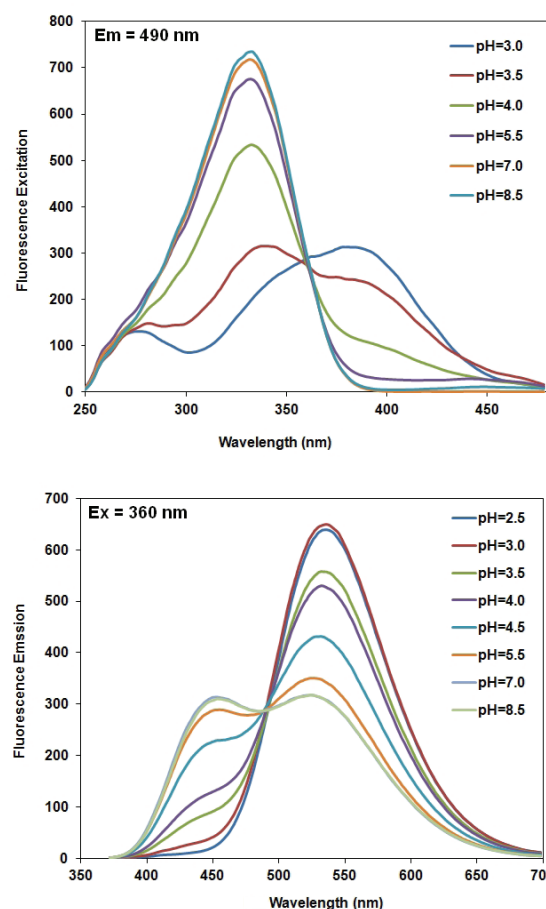


Figure 4.3. pH titration of PDMPO. Top: Excitation; Bottom: Emission.

Protonex™ Green, an unique pH probe for monitoring acidic organelles

Protonex™ Green dye demonstrates pH-dependent fluorescence. Unlike most of the existing fluorescent dyes that are more fluorescent at higher pH, acidic conditions enhance the fluorescence of Protonex™ Green dye. The fluorescence of Protonex™ Green dye increases as pH decreases from neutral to acidic. The lack of fluorescence outside the cell eliminates the wash steps. Protonex™ Green dye provides a powerful tool to monitor acidic cell compartments such as endosomes and lysosomes. Protonex™ Green dye is non-fluorescent outside the cells, but fluoresces brightly green in acidic compartments (such as phagosomes, lysosomes and endosomes). It enables the specific detection of cellular acidic compartments with reduced signal variability and improved accuracy for imaging or flow applications. Protonex™ Green has the spectral properties similar to those of FITC, making the common filter set of FITC readily available to the assays of Protonex™ Green.

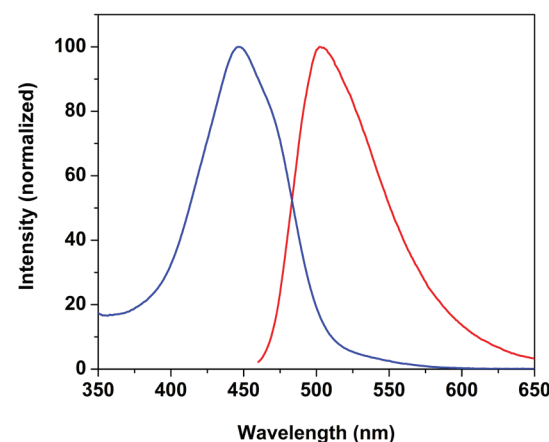


Figure 4.4. Excitation and emission spectra of Protonex™ Green at pH 4.0.

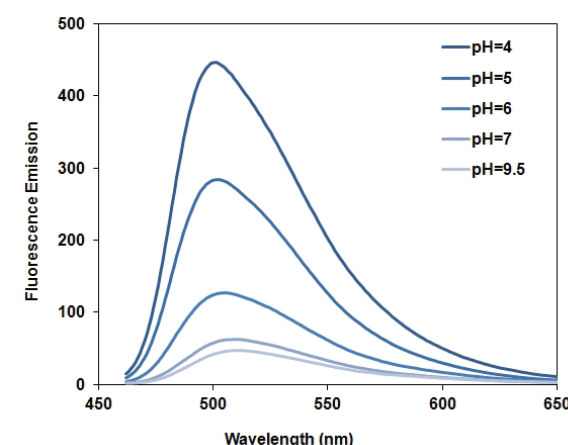


Figure 4.5. pH titration of Protonex™ Green (excited at 450 nm).

Protonex™ Red, an excellent replacement to pHrodo™

Protonex™ Red dye provides a powerful tool to monitor acidic cell compartments such as endosomes and lysosomes. Protonex™ Red dye is non-fluorescent outside the cells, but fluoresces brightly red in acidic compartments (such as phagosomes, lysosomes and endosomes). The fluorescence of Protonex™ Red dye dramatically increases as pH decreases from neutral to acidic. The lack of fluorescence outside the cell eliminates the wash steps. Protonex™ Red enables the specific detection of cellular acidic compartments with reduced signal variability and improved accuracy for imaging or flow cytometry applications. It can be also used for multiplexing cellular functional analysis with green fluorescent dyes such as GFP, Fluo-8®, calcein, or FITC-labeled antibodies. Protonex™ Red has the spectral properties similar to those of Texas Red®, making the common filter set of Texas Red® readily available to the assays of Protonex™ Red.

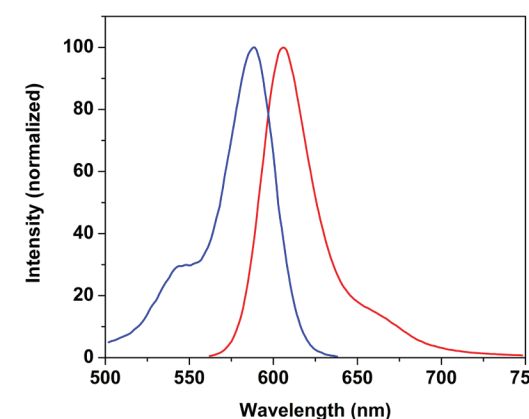


Figure 4.6. Excitation and emission spectra of Protonex™ Red at pH 4.0.

Table 4.2 Fluorescent pH Probes for Acidic pH Environments

Cat #	Product Name	Size	Ex (nm)	Em (nm)
21215	Protonex™ Green 500	1 mg	443	505
21207	Protonex™ Red 600	1 mg	575	597
21204	RatioWorks™ PDMPO	1 mg	405	550

4.3 Reactive pH Indicators and Their Dextran Conjugates

The dextran conjugates of pH probes have high water solubility, therefore must be loaded into cells by microinjection, whole-cell patch-clamping, endocytosis or liposome fusion. Once loaded, dextrans are retained in viable cells for long periods and will not pass through gap junctions. Compared to the cell-permeant acetoxymethyl (AM) ester derivatives, the pH dye dextran conjugates are less likely compartmentalized in cells.

BCECF and BCFL Dextran Conjugates for Measuring Near-Neutral pH

AAT Bioquest's BCECF and BCFL dextrans are important dual-excitation pH indicator conjugates for pH measurements near

pH 7.0. BCECF and BCFL dextran-labeled cells have been shown to produce much more stable fluorescent signals, reduced probe compartmentalization and greater resistance to light-induced damage when compared with BCECF and BCFL AM-labeled cells. The BCECF and BCFL dextran conjugates have been used to monitor intracellular pH increases during developmental processes and to measure pH in submucosal gland secretions from human lung tissues. Compared to BCECF dextran conjugates, BCFL dextran conjugates give much more reproducible results due to the minimal quality variation from batch to batch. BCECF is a mixed population of different isomers that have varied isomer ratio from lot to lot while BCFL is a single isomer although their pH profiles are almost identical. We recommend you to use BCFL for the assays that require low deviation.

Protonex™ Dextran Conjugates for Monitoring Endocytosis

Our Protonex™ dextran conjugates are excellent alternatives to other fluorescent dextran conjugates (e.g., BCECF and pHrodo™) for live-cell imaging of endocytosis. Protonex™ dextrans possess a pH-sensitive fluorescence emission that increases in intensity with increasing acidity. Protonex™ dextran is essentially nonfluorescent in the extracellular environment; however, upon internalization, the acidic environment of the endosomes generates a bright fluorescent signal from the Protonex™ dextran conjugates. The minimal fluorescent signal from the Protonex™ dextran conjugate at neutral pH prevents the detection of noninternalized and nonspecifically bound conjugates and eliminates the extra wash steps, thus providing a simple fluorescent assay for endocytic activity. AAT Bioquest offers a few Protonex™ dextran conjugates with different wavelengths for multicolor cell analysis (e.g., with GFP cell lines). They can be used to study or monitor endocytosis on a variety of platforms including fluorescence microscopy, flow cytometry and high-content imaging.

RatioWorks™ PDMPO Dextran Conjugates

Although BCECF and BCFL dextrans are useful for detecting translocation into compartments that have an acidic pH; their relative insensitivity to fluorescence change below pH ~6 limits quantitative pH estimation. The lower pK_a values of the PDMPO dextran conjugate make it a more suitable indicator for estimating the pH of relatively acidic lysosomal environments. Moreover, the shift in its excitation and emission spectra in acidic media permits ratiometric pH measurements.

Our PDMPO dextran conjugates can be used to quickly and accurately estimate the pH of lysosomes. As the labeled dextran is taken up by the cells and moves through the endocytic pathway, the PDMPO fluorescence changes from blue fluorescent in the near-neutral endosomes to yellow fluorescent in the acidic lysosomes. The greatest change in fluorescence emission occurs near the pK_a of the dye at pH ~4.2. The pH in lysosomes can be measured with PDMPO dextrans using fluorescence microscopy or flow cytometry.

Reactive pH Probes for Preparing pH-Sensitive Bioconjugates

The amine-reactive succinimidyl ester form of BCFL, Protonex™ and PDMPO pH probes provide access to a wide variety of user-defined

bioconjugates. Protonex™ dyes are extremely sensitive to its local environment, therefore the pH response of each bioconjugate must be individually determined. Protonex™ succinimidyl esters have been used to label dexamethasone-treated thymocytes for flow cytometric analysis of phagocytosis by splenic or peritoneal macrophages and for live-cell confocal imaging of antigen transfer from human B lymphocytes to macrophages.

Table 4.3 Reactive Fluorescent pH Probes and Their Dextran Conjugate

Cat #	Product Name	Size	Ex (nm)	Em (nm)
21211	RatioWorks™ PDMPO Dextran	1 mg	405	540
21210	RatioWorks™ PDMPO, SE	1 mg	405	550
21217	Protonex™ Green 500 Dextran	1 mg	443	505
21216	Protonex™ Green 500, SE	1 mg	443	505
21209	Protonex™ Red 600-Latex Bead Conjugate	1 mL	575	597
21208	Protonex™ Red 600, SE	1 mg	575	597

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Anion Detection

anion detection at-a-glance*

Anion	Colorimetric Detection	Fluorimetric Detection
Chloride	36350	21250, 21252, 21255, 21259
Phosphate	21600, 21659, 21665	21660
Pyrophosphate		21611

* products listed by catalog number

5.1 Chloride Detection

Cystic fibrosis transmembrane conductance regulator (CFTR) functions as a cAMP-activated ATP-gated anion channel, increasing the conductance for certain anions (e.g. Cl⁻) to flow down their electrochemical gradient. ATP-driven conformational changes in CFTR open and close a gate to allow transmembrane flow of anions down their electrochemical gradient. The measurement of intracellular chloride concentrations and the study of chloride channels have been stimulated by the discovery that cystic fibrosis is caused by mutations in a gene encoding CFTR. Chloride permeability assays are used to detect the activities of the CFTR and other anion transporters. A number of chronic disease states such as cystic fibrosis and Bartter's syndrome are due to defects in chloride channel functions.

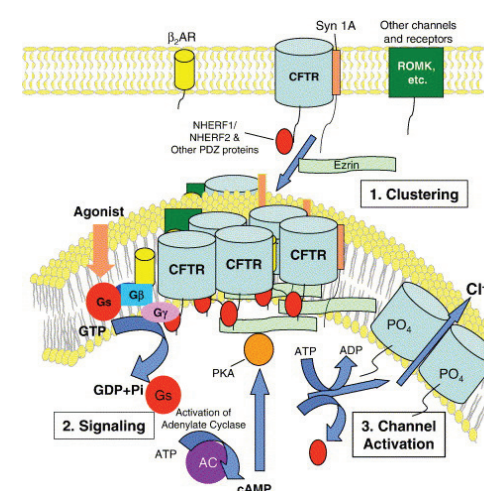


Figure 5.1. Cystic fibrosis transmembrane conductance regulator (CFTR) is a protein that is encoded by the CFTR gene. CFTR is an ABC transporter-class ion channel that transports chloride and thiocyanate ions across epithelial cell membranes. Mutations of the CFTR gene affect functioning of the chloride ion channels in these cell membranes, leading to cystic fibrosis and congenital absence of the vas deferens. The gene that encodes the CFTR protein is found on the human chromosome 7, on the long arm at position q31.2 from base pair 116,907,253 to base pair 117,095,955. CFTR orthologs have also been identified in all mammals for which complete genome data are available. The CFTR gene has been used in animals as a nuclear DNA phylogenetic marker. Large genomic sequences of this gene have been used to explore the phylogeny of the major groups of mammals, and confirmed the grouping of placental orders into four major clades: Xenarthra, Afrotheria, Laurasiatheria, and Euarchonta plus Glires.

Quinolinium-Based Fluorescent Chloride Indicators

Most of the existing fluorescent chloride indicators are derived from quinolinium, including MEQ, MQAE and SPQ. All of these indicators detect chloride via diffusion-limited collisional quenching. This detection mechanism is different from that of fluorescent indicators for Ca²⁺ and Zn²⁺. It involves a transient interaction

between the excited state of the fluorophore and a halide ion – no ground-state complex is formed. Quenching of quinolinium dyes by other halides, such as bromide and iodide, and other anions, such as thiocyanate, is more efficient than chloride quenching. Fortunately, physiological concentrations of non-chloride ions do not significantly affect the fluorescence of SPQ and other methoxyquinolinium-based chloride indicators.

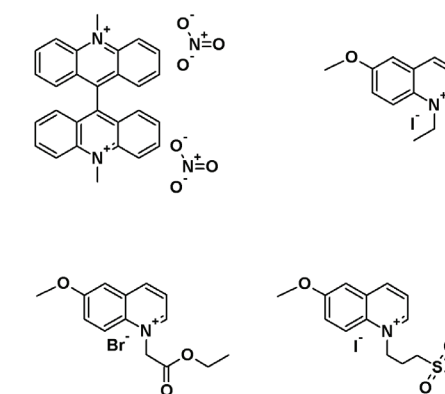


Figure 5.2. The chemical structures of fluorescent chloride indicators: lucigenin (top left), MEQ (top right), MQAE (bottom left) and SPQ (bottom right).

Fluorescence of the quinolinium dyes is quite sensitive to solution viscosity and volume since the chloride-dependent fluorescence quenching is a diffusional process. The efficiency of collisional quenching is characterized by the Stern–Volmer constant (K_{SV}) — the reciprocal of the ion concentration that produces 50% of maximum quenching. In these assays, SPQ- or MQAE-loaded cells are successively perfused with chloride-containing extracellular medium followed by medium in which the chloride content is replaced by nitrate.

SPQ is currently in widespread use for detecting CFTR activity using the chloride/nitrate exchange technique. SPQ has also been employed to investigate chloride fluxes through several other transporters such as the GABA receptor. MQAE has greater sensitivity to chloride and a higher fluorescence quantum yield than SPQ, and consequently MQAE is currently the more widely used of the two indicators. The ester group of MQAE may slowly hydrolyze inside cells, resulting in a change in its fluorescence response. MQAE has been used in a fluorescence microplate assay that has potential for screening compounds that modify chloride channel activity.

Iodide Blue™-Based Chloride Channel Assay

Chloride channels have a variety of important physiological and cellular functions that include regulation of pH, volume homeostasis, organic solute transport, cell migration, cell proliferation and

differentiation. Chloride channels represent valuable drug targets. However, the existing technologies for screening chloride channel modulators are a compromise between throughput, sensitivity and physiological relevance. Screen Quest™ Chloride Channel Assay Kit provides an optimized assay method for monitoring chloride channels. The assay uses our proprietary iodide indicator (Iodide Blue™) for measuring iodide concentration, and as low as 30 nM of iodide was detected. The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation.

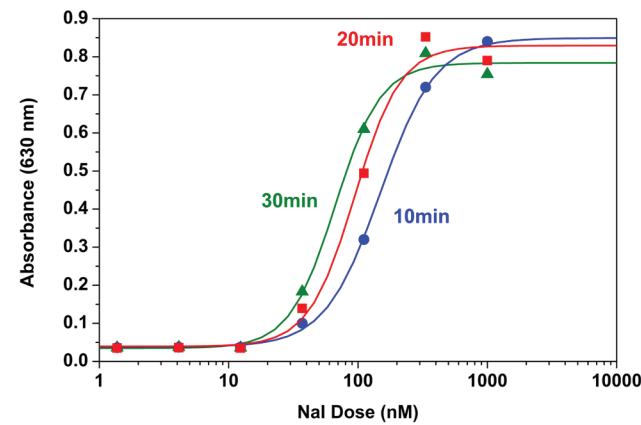


Figure 5.3. Nal dose responses were measured with Screen Quest™ Colorimetric Chloride Channel Assay Kit in a 96-well black wall/clear bottom plate. As low as 100 nM of Nal was detected with 10 minutes incubation (n=3).

Table 5.1 Chloride Detection Reagents and Assay Kit

Cat #	Product Name	Size	$K_{1/2}$ (M ⁻¹) (in solution)	$K_{1/2}$ (M ⁻¹) (in cell)	Ex (nm)	Em (nm)
21259	Lucigenin	10 mg	390		455	505
21250	MEQ	100 mg	145	19	344	442
21255	MQAE	100 mg	200	25-28	350	460
36350	Screen Quest™ Colorimetric Chloride Channel Assay Kit	10 plates	N/A	N/A	630	N/A
21252	SPQ	25 mg	118	12	344	433

References for Chloride Detection

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5.2 Phosphate (Pi) Detection

Phosphate (Pi) is one of the most important inorganic ions in biological systems. It functions in a variety of roles. One of the most important roles is as a molecular switch, turning enzyme activity on and off through the mediation of the various protein kinases and phosphatases in biological systems. Numerous enzymes of therapeutic relevance produce phosphate directly or through coupled reactions. These potential drug development targets include lipid and protein phosphatases, ATPases, GTPases, prenyltransferases and phosphodiesterases. Phosphate is also of great importance in mineralization processes and is a primary stimulus of algal blooms frequently found in bodies of fresh water, due to run-off from areas of high fertilizer use. The importance of phosphate in drug discovery and other fields makes high quality phosphate assays indispensable.

MESG-Based Colorimetric Phosphate Assay

In the presence of inorganic phosphate, MESG is converted to 2-amino-6-mercapto-7-methylpurine by purine nucleoside phosphorylase (PNP) with a red shift of absorption wavelength. This feature has been used to quantify phosphate spectrophotometrically. The enzymatic removal of the ribose moiety from MESG results in a shift in the wavelength of maximum absorbance (Imax) from 330 nm to 360 nm. Because conversion of MESG requires inorganic phosphate, the increase in absorbance at 360 nm can be used to measure phosphate concentration. When the PNP

enzyme and MESG substrate are in excess relative to phosphate, the increase in absorbance at 360 nm is quantitative for inorganic phosphate. Assuming there is no preexisting phosphate, any increase in the absorbance at 360 nm must be the result of Pi liberation from ATP hydrolysis.

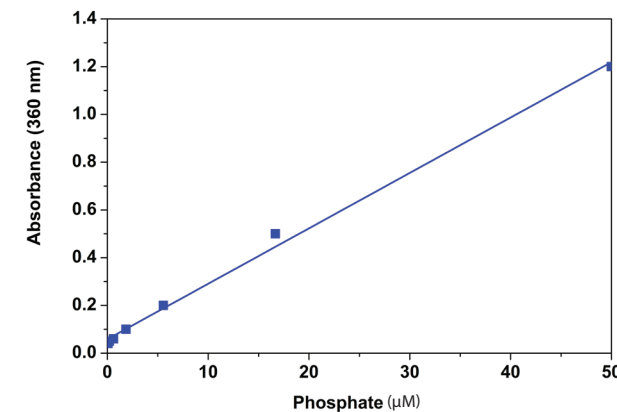


Figure 5.4. Phosphate dose responses were measured with the PhosphoWorks™ Colorimetric MESG Phosphate Assay Kit in a 96-well UV plate. As low as 0.2 μM phosphate can be detected with 30 minutes incubation.

Malachite Green-Based Colorimetric Phosphate Assay

Our PhosphoWorks™ Phosphate Assay Kit has been developed for measuring the activity of any Pi-generating enzyme through the complexation of malachite green with phosphate under acidic conditions. The measurement of Pi is based on the change in absorbance of the malachite green derivative in the presence of molybdate. This assay kit is formulated to give sensitive detection of Pi, providing an alternative to hazardous radioactive methods and other less sensitive colorimetric assays. Unlike other malachite dye formulations, this kit gives a completely stable end-point signal.

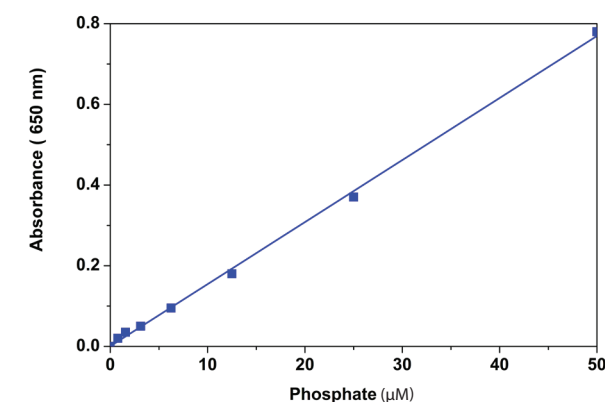


Figure 5.5. Phosphate dose responses were measured with the PhosphoWorks™ Colorimetric Phosphate Assay Kit in a 96-well clear plate. As low as 0.1 μM phosphate can be detected with 10 minutes incubation.

Fluorimetric Phosphate Assay

Detection of many phosphoester-metabolizing enzymes is difficult because suitable substrates are not available. It usually has been

necessary to determine inorganic phosphate release using tedious colorimetric assays or radioisotope-based methods. We have developed the PhosphoWorks™ Fluorimetric Phosphate Assay Kit for measuring the activity of any Pi-generating enzyme using our red fluorescent phosphate sensor. The measurement of Pi is based on the change in the absorbance and fluorescence of the phosphate sensor. The assay is shown to quantitate phosphate in solution at concentrations as low as 0.1 μM. It can be used to measure the kinetics of phosphate release from phosphatases (such as GTPases and ATPases) by coupling the two enzymatic reactions. The kit provides sensitive detection of Pi, an alternative to hazardous radioactive methods and other less sensitive colorimetric assays. It comes with all the essential reagents including phosphate sensor, phosphate standards and assay buffer. The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation with no separation steps required.

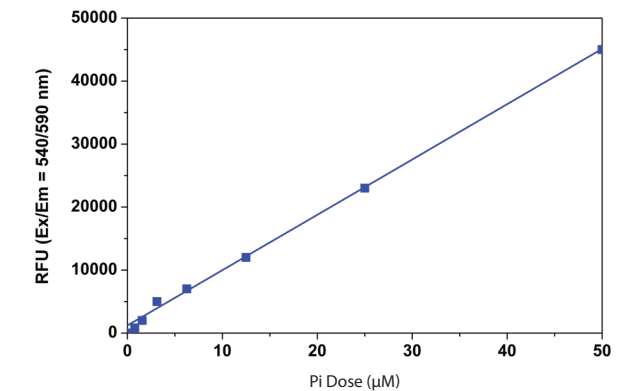


Figure 5.6. Phosphate dose responses were measured with PhosphoWorks™ Fluorimetric Phosphate Assay Kit in a 96-well black plate. As low as 0.1 μM phosphate can be detected with 1 hour incubation.

Table 5.2 Phosphate Detection Reagent and Assay Kits

Cat #	Product Name	Size	Ex (nm)	Em (nm)
21600	MESG *Phosphate assay reagent*	5 mg	330	N/A
21659	PhosphoWorks™ Colorimetric MESG Phosphate Assay Kit *UV absorption*	1 kit	360	N/A
21665	PhosphoWorks™ Colorimetric Phosphate Assay Kit *Blue Color*	1 kit	650	N/A
21660	PhosphoWorks™ Fluorimetric Phosphate Assay Kit *Red Fluorescence*	1 kit	571	585

References for Phosphate Detection

Cowan CM, Zhang X, James AW, Kim TM, Sun N, Wu B, Ting K, Soo C. (2012) NELL-1 increases pre-osteoblast mineralization using both phosphate transporter Pit1 and Pit2. *Biochem Biophys Res Commun*, 422, 351.

Kassai M, Teopipithaporn R, Grant KB. (2011) Hydrolysis of phosphatidylcholine by cerium(IV) releases significant amounts of choline and inorganic phosphate at lysosomal pH. *J Inorg Biochem*, 105, 215.

Microassay of phosphate provides a general method for measuring

Pegan SD, Tian Y, Sershon V, Mesecar AD. (2010) A universal, fully automated high throughput screening assay for pyrophosphate and phosphate release from enzymatic reactions. *Comb Chem High Throughput Screen*, 13, 27.

Pena-Pereira F, Cabaleiro N, de la Calle I, Costas M, Gil S, Lavilla I, Bendicho C. (2011) Directly suspended droplet microextraction in combination with microvolume UV-vis spectrophotometry for determination of phosphate. *Talanta*, 85, 1100.

Rejman D, Panova N, Klener P, Maswabi B, Pohl R, Rosenberg I. (2012) N-phosphonocarbonylpyrrolidine derivatives of guanine: a new class of bi-substrate inhibitors of human purine nucleoside phosphorylase. *J Med Chem*, 55, 1612.

Uemura T, Ogusu T, Takeuchi M, Tanaka H. (2010) Spectrophotometric determination of trace phosphate ions by amplitude-modulated flow analysis coupled with malachite green method. *Anal Sci*, 26, 797.

5.3 Pyrophosphate (PPi) Assay

Pyrophosphate (PPi) is produced by a number of biochemical reactions, such as ATP hydrolysis, DNA and RNA polymerizations, cyclic AMP formation by the enzyme adenylate cyclase and the enzymatic activation of fatty acids to form the coenzyme A esters. Our PhosphoWorks™ Pyrophosphate Assay Kit provides the most robust spectrophotometric method for measuring pyrophosphate. It uses our proprietary fluorogenic pyrophosphate sensor that has the fluorescence intensity proportionally dependent upon the concentration of pyrophosphate. The assay is much easier than the enzyme-coupling pyrophosphate methods that require at least two enzymes for the pyrophosphate detections. PhosphoWorks™ Pyrophosphate Assay Kit provides all the essential components for assaying pyrophosphate. It has been successfully used in high throughput screening (HTS). Please inquire special HTS bulk package discount for the screening of >10,000 assays.

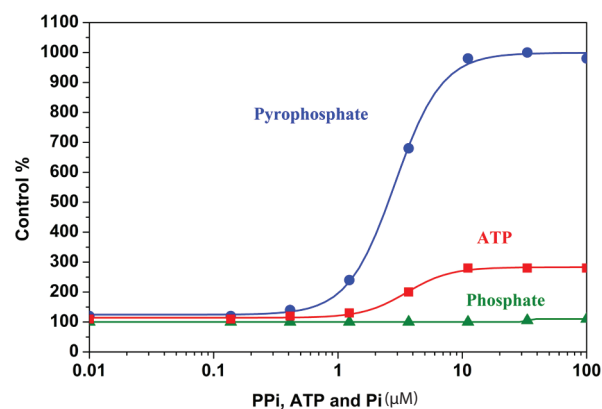


Figure 5.7. Pyrophosphate and phosphate dose responses were measured with the PhosphoWorks™ Fluorimetric Pyrophosphate Assay Kit in a 96-well black solid plate. As low as 1 μM (100 picmoles/well) pyrophosphate can be detected with 10 minutes incubation.

Table 5.3 Pyrophosphate Detection Assay Kit

Cat #	Product Name	Size	Ex (nm)	Em (nm)
21611	PhosphoWorks™ Fluorimetric Pyrophosphate Assay Kit *Blue Fluorescence*	1 kit	316	456

References for Pyrophosphate (PPi) Detection

Bruzzese FJ, Tsu CA, Ma J, Loke HK, Wu D, Li Z, Tayber O, Dick LR. (2009) Development of a charcoal paper adenosine triphosphate:pyrophosphate exchange assay: kinetic characterization of NEDD8 activating enzyme. *Anal Biochem*, 394, 24.

Inokoshi J, Nakamura Y, Hongbin Z, Uchida R, Nonaka K, Masuma R, Tomoda H. (2013) Spirohexalines, new inhibitors of bacterial undecaprenyl pyrophosphate synthase, produced by *Penicillium brasilianum* FKI-3368. *J Antibiot (Tokyo)*, 66, 37.

Lee HJ, Ho MR, Tseng CS, Hsu CY, Huang MS, Peng HL, Chang HY. (2011) Exponential ATP amplification through simultaneous regeneration from AMP and pyrophosphate for luminescence detection of bacteria. *Anal Biochem*, 418, 19.

Liu Y, Schanze KS. (2008) Conjugated polyelectrolyte-based real-time fluorescence assay for alkaline phosphatase with pyrophosphate as substrate. *Anal Chem*, 80, 8605.

Munch-Petersen B. (2009) Reversible tetramerization of human TK1 to the high catalytic efficient form is induced by pyrophosphate, in addition to triphosphates, or high enzyme concentration. *FEBS J*, 276, 571.

Phelan VV, Du Y, McLean JA, Bachmann BO. (2009) Adenylation enzyme characterization using gamma-(18)O(4)-ATP pyrophosphate exchange. *Chem Biol*, 16, 473.

Suarez AS, Stefan A, Lemma S, Conte E, Hochkoeppler A. (2012) Continuous enzyme-coupled assay of phosphate- or pyrophosphate-releasing enzymes. *Biotechniques*, 53, 99.

Ueno A, Terkawi MA, Yokoyama M, Cao S, Aboge G, Aboulaila M, Nishikawa Y, Xuan X, Yokoyama N, Igarashi I. (2013) Farnesyl pyrophosphate synthase is a potential molecular drug target of risedronate in *Babesia bovis*. *Parasitol Int*, 62, 189.

Zaka R, Stokes D, Dion AS, Kusnierz A, Han F, Williams CJ. (2006) P5L mutation in Ank results in an increase in extracellular inorganic pyrophosphate during proliferation and nonmineralizing hypertrophy in stably transduced ATDC5 cells. *Arthritis Res Ther*, 8, R164.

Membrane Potential Measurement

membrane potential measurement at-a-glance*

Class	Probe	Assay Kit
Fast Response	21480, 21482, 21483, 21485, 21487, 21489, 21492, 21493, 21495, 21496, 21497	
Slow Response: Cyanines	22030, 22033, 22034, 22035, 22038, 22039, 22040, 22042, 22044, 22045, 22046, 22050, 22051, 22052, 22054, 22056, 22066, 22070, 22073, 22076, 22077, 22101, 22102, 22103	
Slow Response: Oxonols	22410, 22411, 22412, 22414, 22416, 22417	36000, 36005
Mitochondrial Membrane Potential Dyes	22200, 22204, 22205, 22210, 22211, 22220, 22221, 22225	22800, 22801, 22802, 22803, 22804, 22805

* products listed by catalog number

6.1 Selection of a Potentiometric Probe

Membrane potential is the difference in voltage between the interior and exterior of a cell. The membrane potential allows a cell to function as a battery, providing power to operate a variety of molecular devices embedded in the membrane. In electrically excitable cells such as neurons, membrane potential is used for transmitting signals between different parts of a cell. Opening or closing of ion channels at one point in the membrane produces a local change in the membrane potential, which causes electric current to flow rapidly to other points in the membrane. Ion channels have been identified as important drug discovery targets.

The plasma membrane of a cell typically has a transmembrane potential of approximately -70 mV (negative inside) as a consequence of K^+ , Na^+ and Cl^- concentration gradients that are maintained by active transport processes. Potentiometric probes offer an indirect convenient method of detecting the translocation of these ions although the fluorescent ion indicators can be used to directly measure changes in specific ion concentrations. Potentiometric optical probes enable researchers to perform membrane potential measurements in organelles and in cells that are too small for microelectrodes. Moreover, in conjunction with imaging techniques, these probes can be employed to map variations in membrane potential across excitable cells, in perfused organs and ultimately in the brain *in vivo* with spatial resolution and sampling frequency that cannot be obtained using microelectrodes.

Increases and decreases in membrane potential play a central role in many physiological processes, including nerve-impulse propagation, muscle contraction, cell signaling and ion-channel gating. Potentiometric probes are important tools for studying these processes, as well as for visualizing mitochondria (which exhibit transmembrane potentials of approximately -150 mV, negative inside matrix), for assessing cell viability and for high-throughput screening of new drug candidates.

Potentiometric probes include the cationic or zwitterionic styryl dyes, the cationic carbocyanines and rhodamines, and the anionic oxonols. The class of dye determines factors such as accumulation in cells, response mechanism and toxicity. Selecting the best potentiometric probe for a particular application can be complicated by the substantial variations in their optical responses, phototoxicity and interactions with other molecules. There are two classes of membrane potential probes based on their response mechanisms: fast response and slow response membrane potential dyes.

6.2 Fast Response Membrane Potential Probes

Fast-response probes have their fluorescence in response to a change in the surrounding electric field. Their optical response is sufficiently fast to detect transient (millisecond) potential changes in excitable cells, including single neurons, cardiac cells and intact

brains. However, the magnitude of their potential-dependent fluorescence change is often small, typically 0.02–0.1% fluorescence intensity change per mV.

Di-8-ANEPPS

The ANEP dyes are among the most sensitive of the fast-response probes. They are essentially nonfluorescent in aqueous solutions and exhibit spectral properties that are strongly dependent on their environment. When bound to phospholipid vesicles, di-8-ANEPPS has absorption/emission maxima of $\sim 467/631$ nm as compared with $\sim 498/713$ nm in methanol. Di-8-ANEPPS responds to increases in membrane potential (hyperpolarization) with a decrease in fluorescence excited at approximately 440 nm and an increase in fluorescence excited at >530 nm. These spectral shifts permit the use of ratiometric methods to correlate the change in fluorescence signal with membrane potential. Zwitterionic di-8-ANEPPS exhibits fairly uniform 10% per 100 mV changes in fluorescence intensity in a variety of tissue, cell and model membrane systems. The millisecond-range temporal characteristics of the ANEP dyes compensate for this modest response amplitude.

RH Dyes

RH dyes are predominately used for functional imaging of neurons. There is no single dye that can provide the optimal response under all experimental conditions. Currently, the most widely used RH dyes are RH 237, RH 421 and RH 795. Like the ANEP dyes, the RH dyes exhibit varying degrees of fluorescence excitation and emission spectral shifts in response to membrane potential changes. Their absorption and fluorescence spectra are also strongly dependent on the environment. Spectra of RH 414 bound to phospholipid vesicles are similar to those obtained on neuronal plasma membranes. The RH dyes (e.g., RH 237) might be used in conjunction with fluorescent Ca^{2+} indicators (e.g., Rhod-2 AM and Rhod-4™ AM) for the simultaneous optical mapping of membrane potential and intracellular calcium in cardiomyocyte monolayers.

Table 6.1 Fast Response Membrane Potential Probes

Cat #	Product Name	Size	Ex (nm)	Em (nm)
21496	Di-2-ANEPEQ	5 mg	517	721
21497	Di-8-ANEPPS	5 mg	498	713
21483	MM 1-43	1 mg	510	626
21489	MM 2-10	1 mg	506	620
21487	MM 4-64	1 mg	558	734
21493	RH 155	25 mg	650	N/A
21480	RH 237	5 mg	528	782
21485	RH 414	5 mg	532	716
21482	RH 421	5 mg	515	704
21495	RH 795	1 mg	530	712
21492	TMA-DPH	5 mg	355	430

6.3 Slow Response Membrane Potential Probes

Slow-response probes exhibit potential-dependent changes in their transmembrane distribution that are accompanied by a fluorescence change. The magnitude of their optical responses is much larger than that of fast-response probes, typically a 1% fluorescence change per mV. Slow-response probes, which include cationic carbocyanines and rhodamines and anionic oxonols, are suitable for detecting changes in average membrane potentials of nonexcitable cells caused by respiratory activity, ion-channel permeability, drug binding and other factors.

DiI, DiS and DiO Derivatives

The cationic carbocyanine dyes accumulate on hyperpolarized membranes and are translocated into the lipid bilayer. Aggregation within the confined membrane interior usually results in decreased fluorescence, although the magnitude and even the direction of the fluorescence response are strongly dependent on the concentration of the dye and its structural characteristics. DiOC₆(3) and DiOC₅(3) have been the most widely used carbocyanine dye for membrane potential measurements. In flow cytometry measurements, the detected intensity of carbocyanine fluorescence is dependent not only on the membrane potential, but also on cell size. In some cases, measurements of forward light scatter have been used to normalize the optical changes for cell size variability.

Table 6.2 Slow Response Membrane Potential Probes

Cat #	Product Name	Size	Ex (nm)	Em (nm)
22030	DiA	25 mg	491	613
22033	DiD	10 mL	644	663
22101	DiI iodide	100 mg	549	565
22102	DiI perchlorate	100 mg	549	565
22103	DiI triflate	100 mg	549	565
22056	DiIC ₅ (5) iodide	25 mg	638	658
22050	DiIC ₁₂ (3)-DS	5 mg	555	570
22035	DiIC ₁₂ (3) perchlorate	25 mg	549	565
22051	DiIC ₁₂ (5)-DS	5 mg	650	670
22044	DiIC ₁₆ (3) perchlorate	25 mg	549	565
22052	DiIC ₁₈ (3)-DS	5 mg	555	570
22054	DiIC ₁₈ (5)-DS	5 mg	650	670
22066	DiO perchlorate	25 mg	484	501
22038	DiOC ₂ (3) iodide	25 mg	482	497
22039	DiOC ₃ (3) iodide	25 mg	482	497
22045	DiOC ₅ (3) iodide	25 mg	482	504
22046	DiOC ₆ (3) iodide	25 mg	482	504
22040	DiOC ₇ (3) iodide	25 mg	482	504
22042	DiOC ₁₆ (3) perchlorate	25 mg	484	501
22070	DiR iodide	25 mg	748	780
22073	DiSC ₂ (3)	25 mg	560	571
22077	DiSC ₂ (7)	25 mg	770	790
22076	DiSC ₅ (5)	25 mg	660	675

Oxonol V and Oxonol VI

The anionic bis-isoxazolone oxonols accumulate in the cytoplasm of depolarized cells by a Nernst equilibrium-dependent uptake from the extracellular solution. Their voltage-dependent partitioning between water and membranes is often measured by absorption rather than fluorescence. Oxonol VI gives the largest spectral shifts, with an isosbestic point at 603 nm. In addition, oxonol VI responds to changes in potential more rapidly than oxonol V.

DiBAC and DiSBAC Dyes

DiBAC and DiSBAC dyes are usually ionized, hydrophobic and cell-permeable. When incubated with cells, these hydrophobic probes are intended to move from the aqueous phase (culture medium) to the lipid phase (plasma membrane). The negatively charged inner side of a resting cell membrane prevents the further movement of these ionized dyes. Upon the depolarization of the membrane potential, they translocate to the interior of the cells where they bind to intracellular proteins or lipids that enhance DiBAC and DiSBAC fluorescence. Typically, the time constant for the redistribution of the fluorescent anion is from millisecond to second, which is in the time scale for measuring the rapid cellular electrical events. Increased depolarization results in more influx of the anionic dye and thus an increase in fluorescence. Conversely, hyperpolarization is indicated by a decrease in fluorescence. In contrast to cationic carbocyanines, anionic DiBAC and DiSBAC₃(3) dyes are largely excluded from mitochondria and are primarily sensitive to plasma membrane potential. Potential-dependent fluorescence changes generated by DiBAC₄(3) are typically ~1% per mV.

Table 6.3 Slow Response Oxonol Membrane Potential Probes

Cat #	Product Name	Size	Ex (nm)	Em (nm)
21411	DiBAC ₄ (3)	25 mg	493	516
21410	DiBAC ₄ (5)	25 mg	590	616
21414	DiSBAC ₃ (3)	25 mg	535	560
21416	Oxonol V	25 mg	610	639
21417	Oxonol VI	25 mg	599	634

6.4 Mitochondrial Membrane Potential Probes

Rhodamine 123, TMRM and TMRE

Highly selective, potential-dependent staining of mitochondria is obtained by setting the extracellular K⁺ concentration close to intracellular values (~137 mM), thereby depolarizing the plasma membrane. Rhodamine 123 is widely used as a structural marker for mitochondria and as an indicator of mitochondrial activity. TMRM and TMRE are closely related to rhodamine 123. They are primarily mitochondrial membrane potential sensors. TMRM and TMRE cross the plasma membrane more rapidly than rhodamine 123, and their strong fluorescence allows the use of low probe concentrations, thus avoiding aggregation. Because their fluorescence is relatively insensitive to the environment, spatially resolved

fluorescence of TMRM and TMRE presents an unbiased profile of their transmembrane distribution that can be directly related to the plasma membrane potential via the Nernst equation.

JC-1 and JC-10™

JC-1 exists as a green-fluorescent monomer at low concentrations or at low membrane potentials. However, at higher concentrations (aqueous solutions above 0.1 μM) or higher potentials, JC-1 forms red-fluorescent "J-aggregates," which exhibit a broad excitation spectrum and a very narrow emission spectrum. Because J-aggregate formation increases linearly with applied membrane potential over the range of 30–180 mV, this phenomenon can be exploited for potentiometric measurements. JC-1 is more specific for mitochondrial versus plasma membrane potential and more consistent in its response to depolarization than some other cationic dyes such as DiOC₆(3) and rhodamine 123.

Various types of ratio measurements are possible by combining signals from the green-fluorescent JC-1 monomer (Ex/Em = ~514/529 nm) and the red-fluorescent J-aggregate (Ex/Em = ~585/590 nm), which can be effectively excited anywhere between 485 nm and its absorption maximum. JC-1 is widely used for detecting mitochondrial depolarization in apoptotic cells and for assaying multidrug-resistant cells. It is also frequently employed for mitochondrial function assessment in cell-based high-throughput assays.

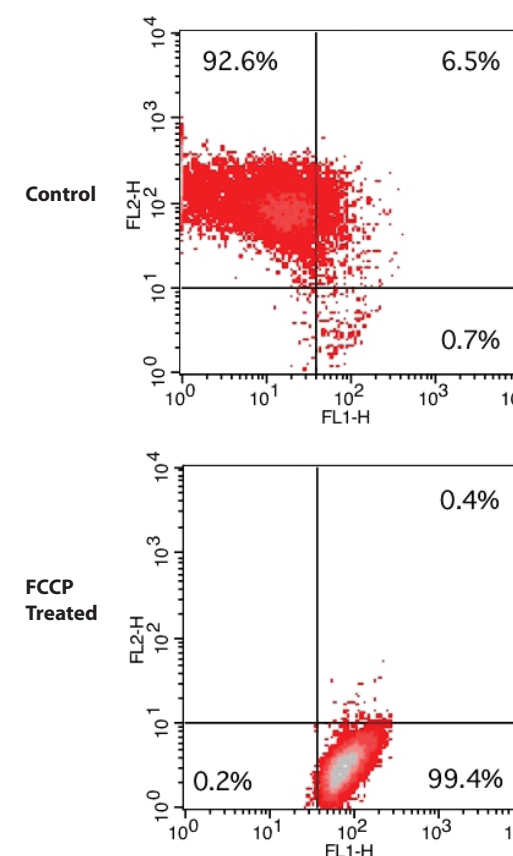


Figure 6.1. FCCP-induced mitochondrial membrane potential changes in Jurkat cells. Jurkat cells were dye loaded with JC-10™ dye-loading solution along with DMSO (Top) or 20 μM FCCP (Bottom) for 10 minutes. The fluorescence intensities for both J-aggregates and monomeric forms of JC-10™ were measured with FACSCalibur™ flow cytometer (Becton Dickinson) using FL1 and FL2 channels after compensation.

AAT Bioquest has developed JC-10™ to be a superior alternative to JC-1. JC-10™ has potential-dependent spectroscopic properties similar to those of JC-1 for detecting mitochondrial depolarization in apoptotic cells. JC-10™ is superior and more convenient to use than JC-1 due to its higher sensitivity and improved water solubility. The poor water solubility of JC-1 makes it hard to use for some applications. Even at 1 μM concentration, JC-1 tends to precipitate in aqueous buffer. When high dye concentration is desired, JC-10™ is capable of entering selectively into mitochondria, and changes reversibly its color from green to orange as membrane potentials increase. This property is due to the reversible formation of JC-10™ aggregates upon membrane polarization that causes shifts in emitted light from 520 nm (i.e., emission of JC-10™ monomeric form) to 570 nm (i.e., emission of J-aggregate). When excited at 490 nm, the color of JC-10™ changes reversibly from green to orange as the mitochondrial membrane becomes more polarized. Both colors can be detected using the filters commonly mounted in all flow cytometers, so that green emission can be analyzed in fluorescence channel 1 (FL1) and orange emission in channel 2 (FL2). Besides its use in flow cytometry, JC-10™ can also be used in fluorescence imaging. For the first time, we have developed a protocol to use JC-10™ in fluorescence microplate platform. In some cell lines JC-10™ has far superior performance to JC-1.

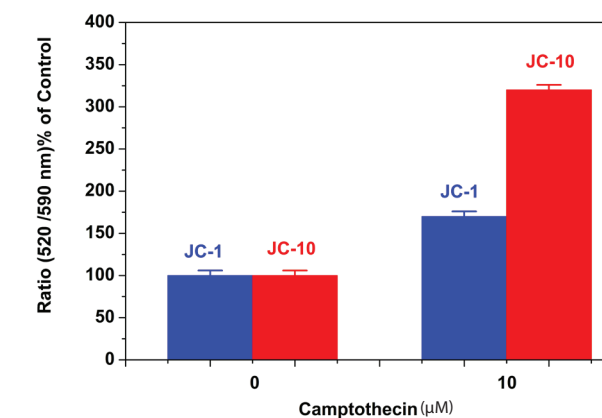


Figure 6.2. Camptothecin induced mitochondrial membrane potential changes were measured with JC-10™ and JC-1 in Jurkat cells. After Jurkat cells were treated with camptothecin (10 μM) for 4 hours, JC-1 and JC-10™ dye loading solutions were added to the wells and incubated for 30 minutes. The fluorescence intensities for both J-aggregates and monomeric forms of JC-1 and JC-10™ were measured at Ex/Em = 490/525 nm and 540/590 nm with NOVOstar microplate reader (BMG Labtech).

Mitochondrial Membrane Potential Assay Kits

Our Cell Meter™ Mitochondrial Membrane Potential (MMP) Assay Kits provide all the essential components with an optimized assay method. These fluorimetric assays use our proprietary cationic mitochondrial probes for the detection of mitochondrial membrane potential changes. In normal cells, the red fluorescence intensity is increased when mitochondrial probes are accumulated in the mitochondria. However, in apoptotic cells, the fluorescence intensity of mitochondrial dyes is decreased following the collapse of MMP. The collapse of mitochondrial membrane potential coincides with the opening of the mitochondrial permeability transition pores, leading to the release of cytochrome C into the cytosol, which in turn

triggers other downstream events in the apoptotic cascade. These kits are individually optimized for screening apoptosis activators and inhibitors with a flow cytometer, a fluorescence microscope or a fluorescence microplate reader.

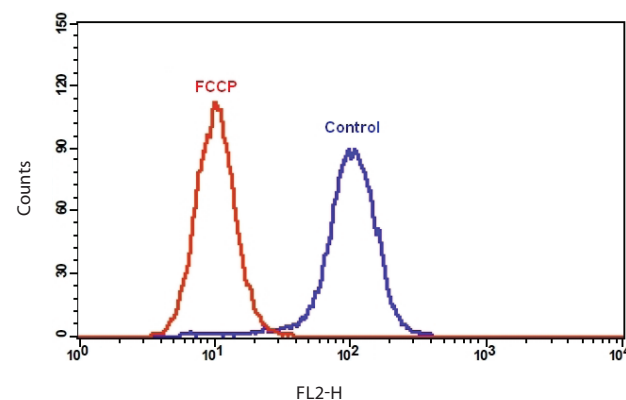


Figure 6.3. The decrease in fluorescence intensity of MitoLite™ Orange with the addition of FCCP in Jurkat cells. Jurkat cells were loaded with MitoLite™ Orange alone (Blue) or in the presence of 30 μM FCCP (Red) for 15 minutes. The fluorescence intensity of MitoLite™ Orange was measured with a FACSCalibur™ (Becton Dickinson) flow cytometer using FL2 channel.

Table 6.4 Mitochondrial Membrane Potential Probes and Assay Kits

Cat #	Product Name	Size	Ex (nm)	Em (nm)
22801	Cell Meter™ JC-10™ Mitochondrial Membrane Potential Assay Kit *Optimized for Flow Cytometry Assays*	1 kit	510	525
22800	Cell Meter™ JC-10™ Mitochondrial Membrane Potential Assay Kit *Optimized for Microplate Assays*	1 kit	510	525
22804	Cell Meter™ Mitochondrial Membrane Potential Assay Kit *Orange Fluorescence Optimized for Flow Cytometry*	1 kit	546	575
22805	Cell Meter™ Mitochondrial Membrane Potential Assay Kit *Orange Fluorescence Optimized for Microplate Reader*	1 kit	546	575
22802	Cell Meter™ NIR Mitochondrial Membrane Potential Assay Kit *Optimized for Flow Cytometry*	1 kit	646	659
22803	Cell Meter™ NIR Mitochondrial Membrane Potential Assay Kit *Optimized for Microplate Reader*	1 kit	646	659
22225	DASPEI (2-(4-(dimethylamino)styryl)-N-ethylpyridinium iodide)	100 mg	461	589
22200	JC-1	5 mg	515	529
22204	JC-10™	5x100 μL	510	525
22205	Nonyl acridine orange	25 mg	495	519
22210	Rhodamine 123	25 mg	507	529
22211	Rhodamine B, hexyl ester, perchlorate	10 mg	556	578
22220	TMRE	25 mg	549	574
22221	TMRM	25 mg	549	573

6.5 FLIPR® Membrane Potential Assay Kits

AAT Bioquest Screen Quest™ Membrane Potential Assay Kits are a homogeneous assay with fast read time. They use our proprietary long wavelength membrane potential indicators to detect the membrane potential change that is caused by the opening and closing of the ion channels. The orange or red fluorescent membrane potential indicators used in the kits have enhanced fluorescence upon entering cells and minimizes the interferences resulted from the screening compounds and/or cellular autofluorescence. These kits have been successfully used for screening chloride, potassium and sodium channel targets in new drug development.

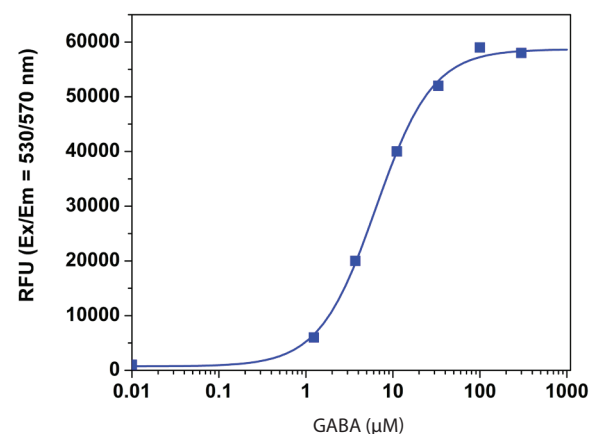


Figure 6.4. GABA dose responses in WSS-1 cells measured with Screen Quest™ Membrane Potential Assay Kit. WSS-1 cells were seeded overnight in 50,000 cells per 100 μL per well in a 96-well black wall/clear bottom Costar plate. The cells were incubated with 100 μL of Screen Quest™ Membrane Potential Assay kit for 30 minutes at room temperature. GABA (50 μL/well) was added by FlexStation® (Molecular Devices) to achieve the final indicated concentrations.

Table 6.5 FLIPR® Membrane Potential Assay Kits

Cat #	Product Name	Size	Ex (nm)	Em (nm)
36000	Screen Quest™ Membrane Potential Assay Kit *Orange Fluorescence*	1 kit	535	560
36005	Screen Quest™ Membrane Potential Assay Kit *Red Fluorescence*	1 kit	635	660

References for FLIPR® Membrane Potential Assays

Benjamin ER, Skelton J, Hanway D, Olanrewaju S, Pruthi F, Ilyin VI, Lavery D, Victory SF, Valenzano KJ. (2005) Validation of a fluorescent imaging plate reader membrane potential assay for high-throughput screening of glycine transporter modulators. *J Biomol Screen*, 10, 365.

Vasilyev DV, Shan QJ, Lee YT, Soloveva V, Nawoschik SP, Kaftan EJ, Dunlop J, Mayer SC, Bowlby MR. (2009) A novel high-throughput screening assay for HCN channel blocker using membrane potential-sensitive dye and FLIPR. *J Biomol Screen*, 14, 1119.

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BIOMOL GmbH

Waidmannstraße 35 · 22769 Hamburg · Germany

info@biomol.de · www.biomol.de

Fon: +49 (0)40-853 260 0 · Fax: +49 (0)40-853 260 22

TOLL FREE IN GERMANY:

Fon: 0800-246 66 51 · Fax: 0800-246 66 52