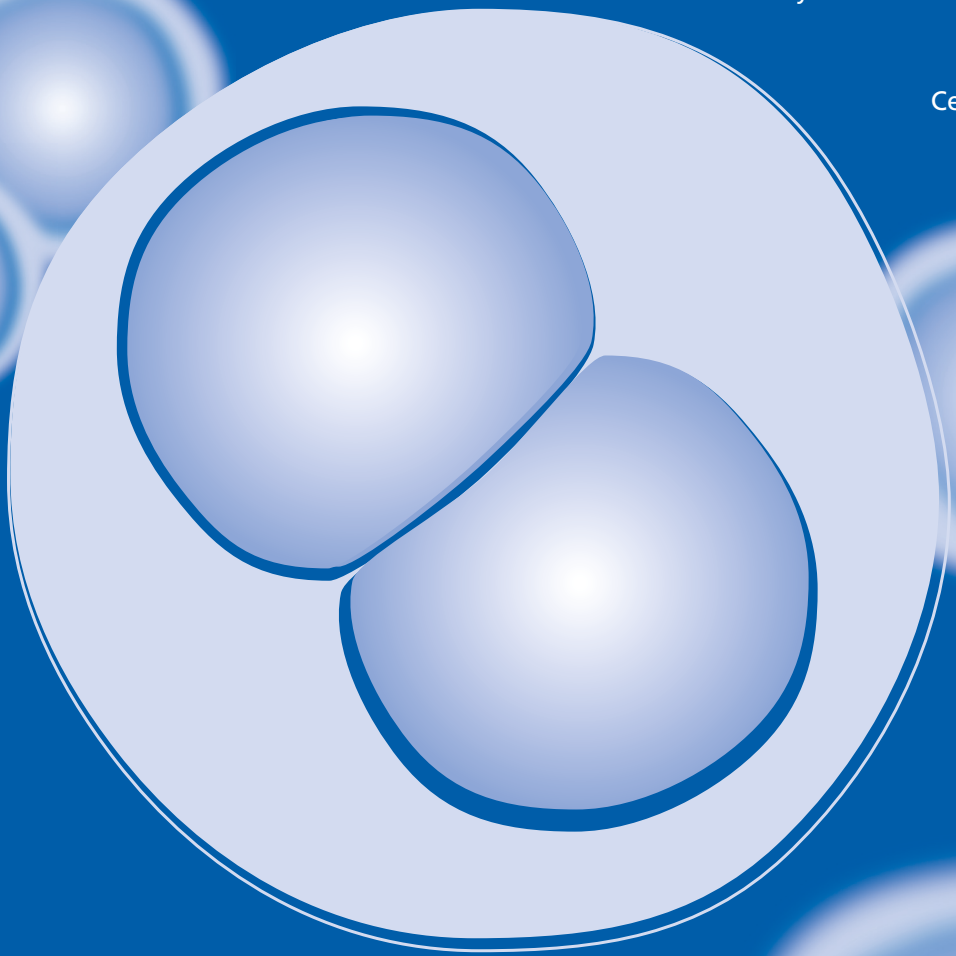
Volume 7 • Issue 2

AssayWise Letters

Novel Products & Tools
Nitrixyte™: Next Generation Nitric Oxide Probe

Featured Product
Cell Meter™ Live Cell Cycle Kits



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Novel Fluorescence Probes for Sensitive Detection of Exogenous and Endogenous Nitric Oxide in Live Cells

Nitric Oxide (NO) is a free radical from the family of reactive nitrogen species (RNS). NO is an essential biological oxidant involved in a broad range of physiological and pathological processes. Dysregulation of NO production can cause damages to a wide array of biomolecules (e.g. proteins, enzymes, lipids and nucleic acids) and can cause various diseases ranging from stroke, heart disease, hypertension, neurodegeneration and erectile dysfunction, to gastrointestinal distress (Rose & Mascharak, 2008). Due to its extremely short lifetime and low steady-state concentration (Mayer, 2000), it remains challenging to selectively and quantitatively detect NO. The lack of quantitative measurements of intracellular NO has limited the understanding of the roles of NO in various biological processes.

To address this limitation, we have developed a series of NO fluorescence probes (Nitrixyte™) to detect NO production with high specificity and sensitivity. Compared to the widely used NO sensor, DAF-2 diacetate, Nitrixyte™ products significantly enhance the signal-to-background ratio in solution assays as well as in live cells. In this comparison study between Nitrixyte™ and DAF-2, two model systems of cell lines were investigated. In the first, NO was introduced exogenously by incubating live HeLa cells with the NO donor DEA NONOate. In the second system, NO was generated endogenously in live RAW 264.7 macrophage cells by drug treatment. As shown in Figure 1 and 2, the detection of exogenous NO in live cells was measured using a fluorescence microplate reader and visualized with a fluorescence microscope, respectively. The microplate reader data demonstrate that compared to DAF-2 diacetate, Nitrixyte™ Orange shows significantly lower background signal in control HeLa cells. More importantly, Nitrixyte™ Orange provides a 6-7 fold increase in signal-to-noise ratio as calculated by dividing the RFU of NONOate treated samples by the corresponding untreated control. The fluorescence images in Figure 1 and 3 also highlight the stronger staining and higher signal-to-noise ratio of Nitrixyte™ Orange over DAF-2 diacetate in detecting both exogenous NO and endogenous NO in live cells.

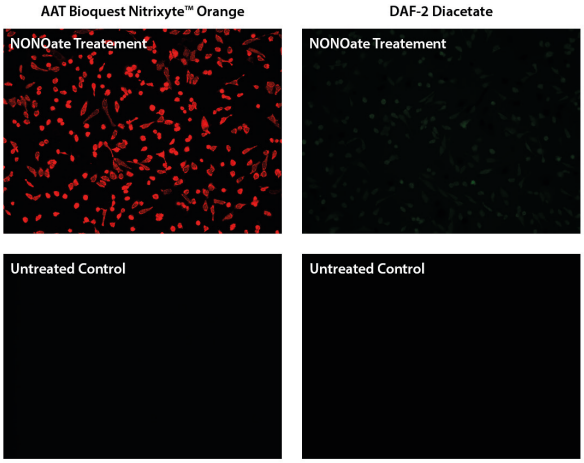

Figure 1 Fluorescence images of exogenous nitric oxide (NO) detection in HeLa cells upon DEA NONOate treatment (NO donor). Cells were incubated with AAT's Nitrixyte™ Orange (Left) or DAF-2 diacetate (Right) at the same concentration for 30 minutes. The fluorescence signals were measured using fluorescence microscope with a TRITC (Left) or FITC (Right) filter sets, respectively. All images were taken using the same exposure time.

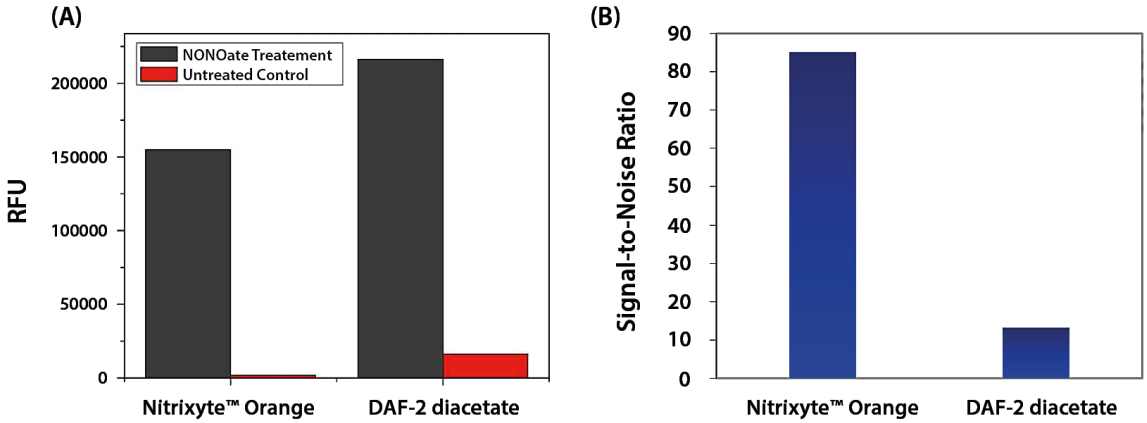

Figure 2 Microplate reader measurement of exogenous nitric oxide (NO) in HeLa cells upon DEA NONOate treatment (NO donor). Cells were incubated with AAT's Nitrixyte™ Orange or DAF-2 diacetate at the same concentration for 30 minutes. The cells were then treated with or without 1 mM DEA NONOate at 37 °C for 30 minutes. The fluorescence signals were measured with bottom read mode at Ex/Em=540/590 nm or Ex/Em=490/530 nm, respectively. The relative fluorescence signal intensity (A) and signal-to-noise ratio (B) were both measured.

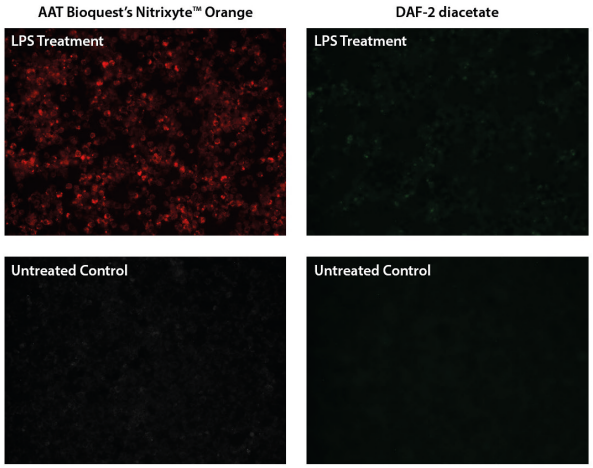

Figure 3 Fluorescence images of endogenous nitric oxide (NO) detection in RAW 264.7 macrophage. Cells were incubated with AAT's Nitrixyte™ Orange (Left) or DAF-2 diacetate (Right) at the same concentration, then treated with or without 20 µg/mL of lipopolysaccharide (LPS) and 1 mM L-arginine (L-Arg) at 37 ºC for 16 hours. The fluorescence signals were measured using fluorescence microscope with a TRITC (Left) or FITC (Right) filter set, respectively. All images were taken using the same exposure time.

Another unique advantage of Nitrixyte™ products is their capacity for multiplexing applications in flow cytometric analysis or fluorescence imaging. Through the combination of Nitrixyte™ products with reactive oxygen species (ROS) probes, researchers can simultaneously detect both NO and ROS in live cells. Figure 4 shows an example of applying both Nitrixyte™ Orange (Red) and Amplite™ ROS Green (Green) in macrophage cells. The cells were treated with 20 µg/mL of lipopolysaccharide (LPS) and 1 mM L-arginine (L-Arg) to stimulate endogenous NO. Cells were also treated with 50 µM pyocyanin (Pyo) at the same time to stimulate ROS generation. After 16 hour treatment, the fluorescence signals were measured using a fluorescence microscope. The red fluorescence from Nitrixyte™ Orange indicates the generation of endogenous NO in cells while the green fluorescence of Amplite™ ROS Green shows the increased level of total ROS after treatment. These results highlight the successful application of Nitrixyte™ Orange in multiplexing assay with ROS probes. The distinguished green and red fluorescence signal also indicate the high specificity of both Nitrixyte™ Orange and Amplite™ ROS Green.

AAT Bioquest's Nitrixyte™ fluorescence probes and related Cell Meter™ Fluorimetric Intracellular Nitric Oxide (NO) Activity Assay Kits offer robust and sensitive methods to detect NO in live cells. Compared to the widely used DAF-2 diacetate, Nitrixyte™ products have a lower nonspecific staining in cells which contributes to a significantly higher signal-to-noise ratio. This distinct advantage allows Nitrixyte™ products to be combined with ROS probe for multiplex staining. It has been both challenging and important for researchers to investigate the co-existence and interaction of both RNS and ROS in live cells. Based on our data, Nitrixyte™ products offer the best analytical tools with highest sensitivity and specificity for the thorough understanding of NO in cellular redox regulation. They are applicable in fluorescence imaging, microplate reader and flow cytometer assays, allowing for simple and fast screening tests and will benefit both RNS and ROS chemical biology significantly.

Methods

1. Simple Protocol for Imaging or Plate-reader Assays

1.1. To stimulate endogenous NO, treat cells with 10 µL of 10X test compounds (96-well plate) or 5 µL of 5X test compounds (384-well plate) in cell culture medium or your desired buffer (such as PBS or HHBS). For control wells (untreated cells), add the corresponding amount of medium or compound buffer.

1.2. Add 100 µL/well (96-well plate) or 25 µL/well (384-well plate) of

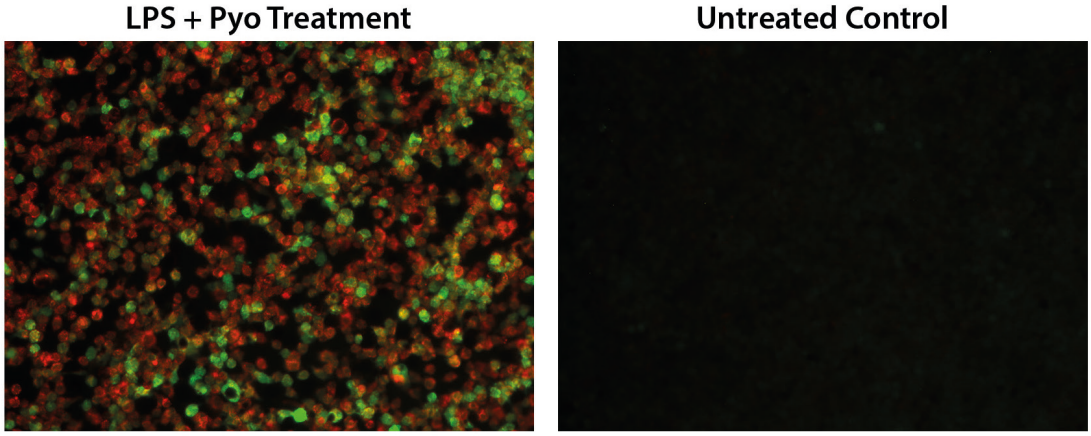

Figure 4 Fluorescence images of simultaneous detection of intracellular nitric oxide (NO) and total ROS in RAW 264.7 macrophage. Cells were co-stained with Nitrixyte™ Orange (Red) and Amplite™ ROS Green (Green) after treatment with lipopolysaccharide (LPS), L-arginine (L-Arg) and pyocyanin (Pho) for 16 hours. The untreated control cells were stained under the same condition but without treatment. The fluorescence signals were measured using fluorescence microscope with TRITC (Nitrixyte™ Orange, Red) and FITC (Amplite™ ROS Green, Green) filter sets, respectively.

Nitrixyte™ working solution in the cell plate. Co-incubate cells with test compound and Nitrixyte™ working solution at 37 °C for desired period of time, protected from light.
Note 1:DO NOT remove the test compounds.
Note 2: Prepare Nitrixyte™ working solution according to the detailed protocol of each kit.

1.3. To induce exogenous NO in living cells, please see examples in 3.

1.4. Remove solution in each well. Add Assay Buffer II 100 µL/well for a 96-well plate or 25 µL/well for a 384-well plate.

1.5. Monitor the fluorescence increase using microplate reader or take images using fluorescence microscope with the filter sets according to each kit.

2. Simple Protocol for Flow Cytometer Assays

2.1. For each sample, prepare cells in 0.5 mL warm medium or buffer of your choice at a density of 5×10^5 -1×10^6 cells/mL.

2.2. Add Nitrixyte™ probe into 0.5 mL cell suspension.
Note: For adherent cells, gently lift the cells with 0.5 mM EDTA to keep the cells intact, and wash the cells once with serum-containing media prior to incubation with Nitrixyte™ probe.

2.3. Incubate cells with test compounds and Nitrixyte™ probe at 37 ºC for a desired period of time to generate endogenous NO.

2.4. To induce exogenous NO in living cells, please see examples in 3.

2.5. Analyze cells with a flow cytometer with the channel setting according to each kit.

3. Examples of Generating Exogenous or Endogenous NO in Cells

3.1 Exogenous NO model: Incubate cells with Nitrixyte™ working solution at 37 ºC for 30 minutes. Remove Nitrixyte™ working solution and incubate cells with 1 mM DEA/NONOate positive control working solution at 37 ºC for 30 minutes to generate exogenous nitric oxide.

3.2 Endogenous NO model: Incubate Raw 264.7 cells with Nitrixyte™ working solution, 20 µg/mL of lipopolysaccharide (LPS) and 1 mM L-Arginine (L-Arg) in cell culture medium at 37 °C for 16 hours.

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Table 1. Product Ordering Information

Product Name	Assay Target	Fluorescence	Cat#	Instrument	Unit Size
Cell Meter™ Fluorimetric Intracellular Nitric Oxide (NO) Activity Assay Kit	Nitric Oxide	Orange	16350	Microplate Reader	200 Tests
			16351	Flow Cytometer	100 Tests
		Red	16356	Flow Cytometer	100 Tests
		NIR	16359	Microplate Reader	200 Tests
			16360	Flow Cytometer	100 Tests

Practical Guide for Live Cell Cycle Analysis in Flow Cytometry

Flow cytometry is a powerful technique routinely used for analyzing complex cell populations based on their physical and chemical characteristics. When combined with fluorescent labels, cellular components can be monitored and quantitatively assessed according to various parameters such as size, internal complexities or through expression of specific cellular markers (e.g. lipids, proteins, or DNA content). In applications utilizing DNA binding stains, flow cytometric analysis has the capacity to screen, characterize and fluorescently sort entire cell populations according to cellular DNA content. This is particularly important in resolving cell distribution within the major phases of the cell cycle, estimating the frequency of apoptotic cells characterized by fractional DNA content and revealing ploidy of the analyzed cell populations [1]. Data collected from this approach has proved significantly useful in fields of research focusing on cell growth and development, proliferation and tumorigenesis, cell cycle regulation, drug discovery and oncology.

Eukaryotic Cell Cycle

The eukaryotic cell cycle includes a series of events that are involved in the growth, replication and division of cells. It describes the progression of a cell through a cycle of division, which consists of cytoplasmic and nuclear events controlled by cyclin-dependent kinases and their cyclin partners [2]. In a given population, cells will be distributed among three major phases of the cell cycle: the G_0/G_1 phase, the S phase and the G_2/M phase. The distribution of cells within these phases of the cell cycle is contingent upon their differences in cellular DNA content, and with the aid of DNA binding stains these variations can be quantitatively assessed with high sensitivity [3].

In flow cytometry, cellular DNA content is referred to as DNA ploidy. In the pre-replicative G_0/G_1 phase, cells induce growth by synthesizing RNA and proteins while the DNA content remains unaltered. At this stage of the cell cycle, cells have a DNA ploidy of 1, and when stained, exhibit fluorescence intensities proportional to its DNA content. Cells that have entered the G_2/M phase of the cell cycle contain double the amount of cellular DNA. As such, these cells will have a DNA ploidy equal to 2 and therefore, exhibit fluorescence intensities twice that of the G_0/G_1 phase cells [4]. In the S phase, cells are undergoing DNA replication and contain varying amounts of DNA (more than G_0/G_1 phase cells but less than G_2/M phase cells). S phase cells are characterized by a DNA ploidy ranging between 1 to 2 and exhibit fluorescence intensities greater than G_0/G_1 phase cells but less than G_2/M phase cells.

Supravital Staining For Cell Cycle Analysis

Analyzing cell cycle progression in live cells requires the use of cell-permeant DNA binding dyes. In this approach, cells are incubated with a dye that binds stoichiometrically to DNA. This means that the dye binds in proportion to the amount of DNA present in the each cell, and when excited by a laser will generate a signal proportional to its cellular DNA content. Traditionally, flow cytometric cell cycle analysis in live cells was performed using Hoechst dyes. Although cell-permeant, Hoechst dyes require ultraviolet excitation which is damaging to cellular DNA and can increase background interference in other channels.

To address this limitation, AAT Bioquest® has developed three Cell Meter™ Fluorimetric Live Cell Cycle Assay Kits for use with the common 405 nm violet laser or the 488 nm blue laser. These assays are ideal tools for flow cytometric analysis of DNA content in live cells as they progress through various phases of the cell cycle. Utilizing the same approach as aforementioned, cell populations are incubated with a stoichiometrically binding, cell permeable DNA dye. Upon DNA binding, the dye will undergo a significant fluorescence enhancement, emitting a signal that is proportional to cellular DNA mass. The percentage of cells in a given sample that are in G_0/G_1, S and G_2/M phases, as well as the cells in the sub-G1 phase prior to apoptosis can be determined by flow cytometry. The collected fluorescence data can be used to generate a histogram illustrating the distribution of cells in each phase of the cycle (Figure 5).

Protocols for labeling live cell populations with Cell Meter™ Fluorimetric Live Cell Cycle Assays are simple and robust. Because these dyes are DNA selective, RNAse treatment of cell populations is not required to reduce background interference. Additionally, Nuclear Violet™, Nuclear Green™ CCS1 and Nuclear Red™ CCS2, the dye component of each kit, are provided as pre-formulated solutions and therefore do not have to be reconstituted. Simply incubate cells with the dye and then monitor the cell population using a flow cytometer, without washing.

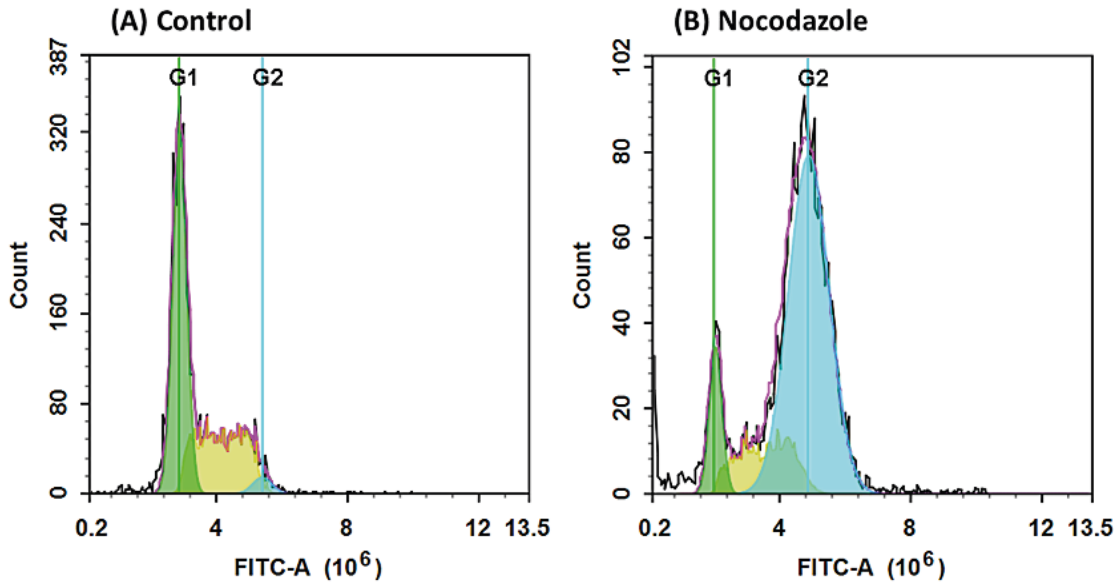

Figure 5 DNA profile in growing and nocodazole treated Jurkat cells. Jurkat cells were treated without (A) or with 100 ng/mL nocodazole (B) in a 37 °C, 5% CO2 incubator for 24 hours, and then dye loaded with Nuclear Green™ CCS1 for 30 minutes. The fluorescence intensity of Nuclear Green™ CCS1 was measured with ACEA NovoCyte flow cytometer using the channel of FITC. In growing Jurkat cells (A), nuclear stained with Nuclear Green™ CCS1 shows G_1, S, and G_2 phases. In nocodazole treated G_2 arrested cells (B), frequency of G_2 cells increased dramatically and frequencies of G_1 and S phases decreased significantly.

Another distinct advantage of Cell Meter™ Fluorimetric Live Cell Cycle Assays is the capacity for multiplexing applications in flow cytometric analysis. Cell Meter™ Fluorimetric Live Cell Cycle assays are available in three distinct color choices for use with violet or blue laser. These options allow researchers the flexibility to designate other channels on their flow cytometers for analyzing different parameters. The Nuclear Violet™ stains take advantage of the commonly available 405 nm excitation source, while Nuclear Green™ CCS1 and Nuclear Red™ CCS2 stains utilize the 488 nm excitation source. This enables simultaneous co-staining of the cell population for other parameters such as analysis of GFP cells, CFSE cell tracing, cell sorting and immunophenotyping. A fluorescence spectrum viewer can assist in determining the ideal dye combination for multiplex analysis. Fluorophores under consideration should have minimal spectral overlap to reduce any bleed-through or spill-over effects.

While flow cytometric analysis of DNA content may seem like a simple application, many factors influence the measurement. Instrument performance, sample preparation and acquisition, and the manner in which data is analyzed can have significant effects on measurement [5]. Prior to cell cycle analysis, ensure that the flow cytometer is properly calibrated, and optimize the conditions for cell preparation, labeling acquisition and analysis.

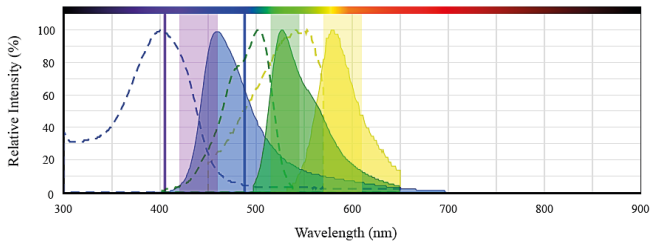

Figure 6 Excitation and emission spectra for Nuclear Violet™, Nuclear Green™ CCS1 and Nuclear Red™ CCS2. Nuclear Violet™ is efficiently excited by the 405 nm violet laser and can be visualized using the DAPI channel. Nuclear Green™ CCS1 is efficiently excited by the 488 nm blue laser and can be visualized using the FITC channel. Nuclear Red™ CCS2 is efficiently excited by the 488 nm blue laser and can be visualized using the Cy3/TRITC channel.

Additional Resources

Cell Sample Preparation: Flow cytometry assays

Each cell line should be evaluated on the individual basis to determine the optimal cell density. For detaching adherent cells from the plate, 0.5 mM EDTA is recommended. Enzymatic reagents

(e.g. trypsin, Accutase™) can be considered but need to be tested to make sure the receptor of interest on the cell surface is not affected.

Adherent Cells

1. Plate cells at 400,000 to 800,000 cells/mL in cell growth medium on the day prior to use.

Non-adherent Cells

1. Centrifuge the cells and carefully discard the supernatant (i.e., the culture medium).
2. Re-suspend the cell pellet in 500 µL – 1 mL cell growth medium or HHBS at 500,000 to 1,000,000 cells/mL .

Sample Protocol for Flow Cytometric Live Cell Cycle Analysis

1. For each sample, prepare cells in 0.5 mL of warm medium or buffer of your choice at a density of 5×10^5 to 1×10^6 cells/mL.
   a. Note: Each cell line should be evaluated on an individual basis to determine the optimal cell density for apoptosis induction.
2. Treat cells with test compounds for a desired period of time to induce apoptosis or other cell cycle functions.
3. Add 2.5 µL of 200X Nuclear Green™ CCS1 (Component A) into the cells containing the growth medium, and incubate the cells in a 37 °C, 5% CO_2 incubator for 30 to 60 minutes.
   a. Note: For adherent cells, gently lift the cells with 0.5 mM EDTA to keep the cells intact, and wash the cells once with serum-containing media prior to incubation with Nuclear Green™ CCS1.
   b. Note: The appropriate incubation time depends on the individual cell type and cell concentration used. Optimize the incubation time for each experiment.
   c. Note: It is not necessary to fix the cells before DNA staining since the Nuclear Green™ CCS1 is cell-permeable.
4. Optional: Centrifuge the cells at 1000 rpm for 4 minutes, and then re-suspend cells in 0.5 mL of assay buffer (Component B) or the buffer of your choice.
5. Monitor the fluorescence intensity by flow cytometry using the 488 nm blue laser (Ex/Em = 490/525 nm). Gate on the cells of interest, excluding debris.

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Table 2. Product Ordering Information

Cat#	Product Name	Unit Size	Ex/Em (nm)	Instrument
22841	Cell Meter™ Fluorimetric Live Cell Cycle Assay Kit *Green Fluorescence*	100 Tests	503/536	Flow Cytometer
22845	Cell Meter™ Fluorimetric Live Cell Cycle Assay Kit *Optimized for 405 nm Violet Laser Excitation*	100 Tests	401/459	
22860	Cell Meter™ Fluorimetric Live Cell Cycle Assay Kit *Red Fluorescence*	100 Tests	488/615	

NAD^+ Metabolism – A Link to Age-Associated Pathologies

Cellular metabolism involves a host of complex biochemical reactions which are essential for cell survival and energy homeostasis. Research in metabolism has contributed to the understanding that perturbations in these metabolic pathways, particularly mitochondrial dysfunction, have major implications on the onset and progression of metabolic disorders, neurodegenerative diseases and other age-associated pathologies. This has sparked interest in the discovery and development of therapeutic solutions aimed at recovering metabolic functionality to treat such disorders.

The manipulation of intracellular NAD^+ is emerging as a promising therapeutic focus. Studies in yeast, worms and mice have shown that an increase in intracellular nicotinamide adenine dinucleotide (NAD^+) by dietary supplementation with NAD^+ precursors boosted sirtuin activity and subsequently improved metabolic efficiency by enhancing mitochondrial functionality and biogenesis [1,3,5]. Picciotto et al. 2016 discovered that supplementation with nicotinamide mononucleotide (NMN), a key NAD^+ intermediate, restored arterial SIRT-1 activity and ameliorated endothelial dysfunction and arterial stiffening in aged mice. This provides evidence that increasing intracellular NAD^+ levels can reverse vascular dysfunction and oxidative stress in aging mice, advocating the therapeutic potential of NAD^+ supplementation.

What is NAD^+ and why is it important?

NAD^+ is a key coenzyme found in all living cells. NAD^+ was initially discovered over a century ago as a cofactor in yeast fermentation (Harden et al., 1906) and since then, the roles of NAD^+ and its metabolite have been expanded. Research has shown NAD^+ is a critical coenzyme for oxidoreductases and dehydrogenases in cellular respiration pathways that generate adenosine triphosphate (ATP) from nutrient breakdown. NAD^+ is a major degradation substrate, key signaling molecule and rate-limiting cofactor in enzymatic reactions involving sirtuins, poly-ADP ribose polymerases (PARPs) and cyclic ADP ribose synthases (CD38/CD157) [2,10]. These NAD^+ dependent proteins consume NAD^+ in order to execute posttranslational modifications, such as protein deacetylation, which influence many vital physiological processes including DNA repair, mitochondrial biogenesis and functionality, and calcium signaling [10].

NAD^+ Bioavailability and Metabolic Homeostasis

Among the NAD^+-consuming proteins, sirtuins play a fundamental role in the regulation of cellular metabolism and adaptation. Mammals have seven sirtuin homologs and each has distinct catalytic activities, biological functions and subcellular localizations [9]. Sirtuins utilize a collection of metabolic targets to translate stress induced changes, such as caloric restriction and oxidative stress, into metabolic adaptations. For example, a well-studied nuclear sirtuin, SIRT-1, targets various transcriptional co-activators (e.g. PGC-1α) and cofactors (e.g. FOXO1) during times of fasting and caloric restriction. PGC-1α acts as the primary regulator of mitochondrial biogenesis and functionality, while FOXO1 modulates mitochondrial fatty acid metabolism and protects against oxidative stress [5].

SIRT-1 is involved in promoting fat mobilization, fatty acid oxidation, glucose production, and insulin secretion in response to nutrient availability in mammals. Moynihan et al., 2005 demonstrated BESTO mice at 3 and 8 months of age have enhanced glucose-stimulated insulin secretion (GSIS) and improved glucose tolerance. This phenotype was found to be due to SIRT-1 mediated repression of UCP2 which causes increased ATP levels in response to glucose stimulation thus resulting in GSIS. Further research by Ramsey et al., 2008, demonstrated that aged mice lose their glucose-responsive phenotypes with age (18-24 months) even though SIRT-1 expression remained high. Isolation of islets of aged BESTO mice notably, no longer showed SIRT-1-mediated repression of UCP2 expression or increased ATP levels. Further investigation showed the depletion in NAD^+ levels, suggesting that SIRT-1 activity is contingent upon NAD^+ bioavailability. NAD^+ levels were replenished using dietary supplementation of a NAD^+ precursor, nicotinamide mononucleotide (NMN). The supplementation was followed by a restoration of GSIS and an improved resistance to diabetes in aged mice. This suggests that sirtuin activation can be therapeutically stimulated to ameliorate age-associated disorders and improve health through NAD^+ replenishment. This paves new therapeutic avenues for increasing subcellular NAD^+ levels through NAD^+-precursor supplementation, increased activation of de novo or salvages NAD^+ synthetic pathways, or reduced competition for NAD^+ via pharmacological inhibition of PARPs and cADPR synthases.

Amplite™ Quantitative Tools for NAD⁺ and NADH Determination

Quantification of the generation or consumption of NAD⁺ and its reduced form, NADH, is important for monitoring NAD⁺/NADH enzyme-mediated reactions or for screening for modulators and substrates of these reactions. Unfortunately, traditional NAD⁺ and NADH determination assays are not without their caveats. Many of the existing methods monitor changes in NADH using absorption at an ultraviolet wavelength of 340 nm. At this wavelength, biological samples and plastics generate high levels of interference due to light scattering and autofluorescence. These interfering artifacts significantly decrease assay sensitivity and may require the use of counter-assays to identify potential compounds that interfere with the detection method. In addition, assays done in the UV range require the use of expensive quartz cuvettes and microplates. To remedy this, AAT Bioquest® has developed an array of colorimetric and fluorimetric assays for the determination of NAD⁺ and NADH in the visible spectrum (400 nm – 700 nm). Our fluorimetric assays offer improved sensitivity and require the use of a fluorescence microplate reader or analogous setup. While colorimetric assays are simple to handle and can be read with standard absorbance microplate readers.

NAD⁺ Determination

While several assays are commercially available for quantifying NADH as well as the total or ratio of NAD⁺/NADH within a biological sample, very little exists for the quantification of NAD⁺. This is due to the intrinsic properties of NAD⁺. Upon peak absorption of ultraviolet light at 259 nm, NAD⁺ is non-fluorescent, making its measurement impractical. However, AAT Bioquest® has developed a fluorimetric NAD⁺ determination assay that utilizes a proprietary fluorimetric sensor, Quest Fluor™ NAD, which specifically measures NAD⁺. Our assay can detect as little as 30 nM NAD⁺ in a 100 µL assay volume. The Quest Fluor™ NAD probe has little to no response towards NADH and upon association with NAD⁺ generates a fluorescence signal proportional to the concentration of NAD⁺. The simple add-mix-read protocol makes this assay amenable for high-throughput screening in 96-well or 384-well microtiter plate formats.

NADH Determination

The determination of NADH, the reduced form of NAD⁺, can be measured colorimetrically or fluorimetrically. Amplite™ Colorimetric NADH Assay Kit utilizes a novel chromogenic sensor that is superior to traditional NADH probes. Compared to traditional probes, which maximally absorb at 340 nm, our chromogenic sensor has a maximum absorbance at 460 nm upon NADH reduction. From absorbance increase at 460 nm, a concentration of as little as 3 µM of NADH in a 100 µL assay volume can be easily calculated, as these two values are proportional. Amplite™ Fluorimetric NADH Assay Kit is a homogenous single-reagent-addition assay that specifically recognizes NADH in an enzyme cycling reaction. This enzyme cycling reaction significantly enhances assay sensitivity detecting as little as 1 µM of NADH in a 100 µL assay volume.

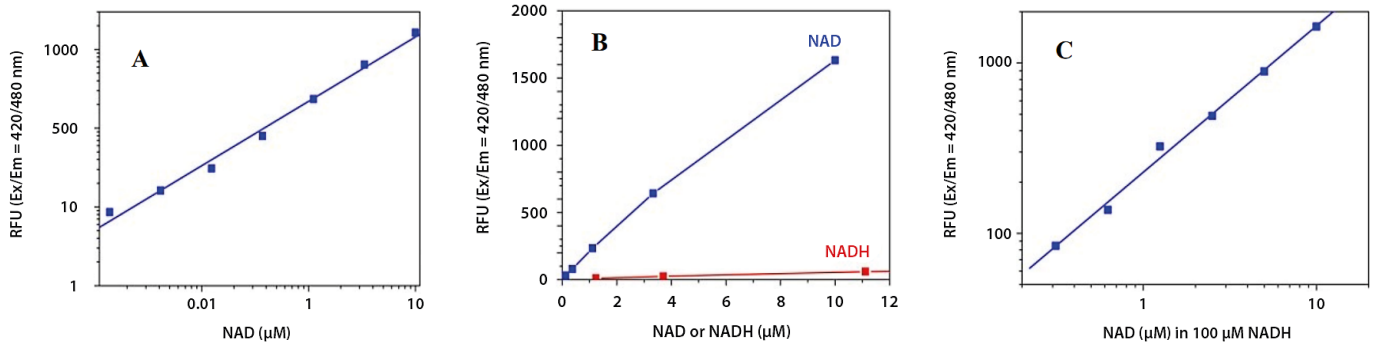

Figure 7 NAD⁺ dose response was measured with Amplite™ Fluorimetric NAD⁺ Assay Kit (Cat# 15280) in a 96-well black/solid bottom plate using a Germini microplate reader (Molecular devices). A: NAD⁺ standard curve, as low as 30 nM of NAD⁺ can be detected with 20 min incubation (n=3). B: Comparison of NAD⁺ and NADH response. C: NAD⁺ standard curve with 100 µM NADH in presence in the solution. As low as 0.3% of NAD⁺ (~300 nM) converted from NADH can be detected with 20 min incubation (n=3).

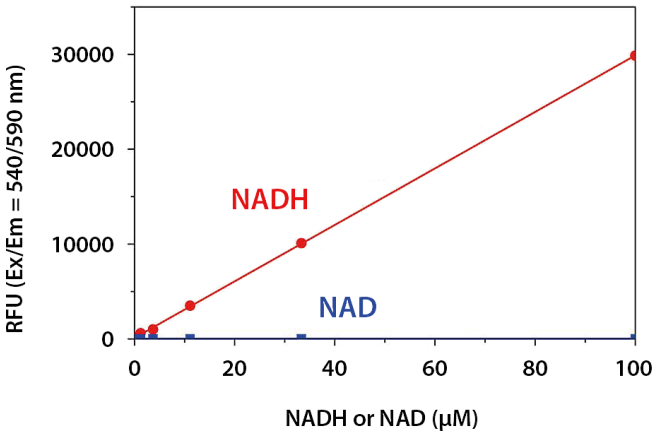

Figure 8 NADH dose response was measured with Amplite™ Fluorimetric NADH Assay Kit (Cat# 15261) in a 96-well black plate using a NOVOStar microplate reader (BMG Labtech). As low as 1 µM (100 pmols/well) of NADH can be detected with 1 hour incubation (n=3) while there is no response from NAD+..

Total NAD⁺/NADH and NAD⁺/NADH Ratio Determination

The NAD⁺/NADH ratio (balance between the oxidized and reduced forms) is an important indicator of a cell's redox state and when measured provides insight to the metabolic activities and health of a cell. The Amplite™ colorimetric and fluorimetric total NAD⁺/NADH and NAD⁺/NADH ratio assays are a series of homogenous assays for the sensitive detection of total oxidized and reduced NAD⁺ as well as their ratio in biological samples. These assays rely on a system of enzyme cycling reactions that specifically recognize NAD⁺/NADH with superior sensitivity. In addition, the fluorimetric assays have been shown to have reduced interference from biological samples due to its spectral properties, which lie in the red visible range. For researchers concerned about usability, these kits are simple and convenient. Purification of NAD⁺/NADH from the sample prior to use is not necessary and each assay can easily be adapted for automation without a separation step. Depending on detection format, simply mix-and-read using either an absorbance microplate reader at 460 nm or a fluorescence microplate reader at Ex/Em = 540/590 nm.

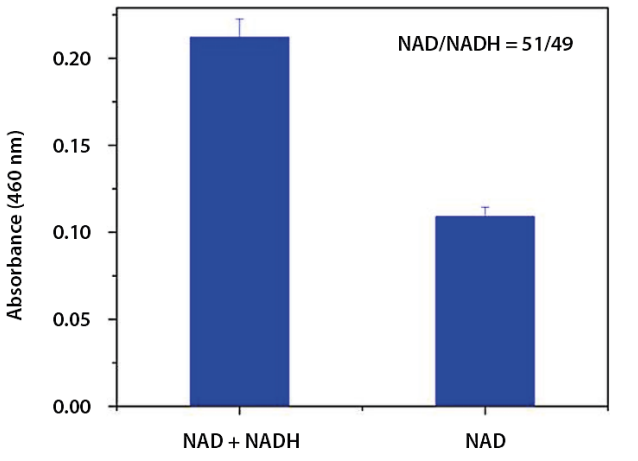

Figure 9 Amplite™ Colorimetric NAD⁺/NADH Ratio Assay Kit (Cat# 15273) was used to measure NAD⁺/NADH ratio in a 96-well white wall/clear bottom microplate using a SpectraMax® microplate reader (Molecular Devices). Equal amount of NAD⁺ and NADH mixtures were treated with or without NAD⁺ extraction solution for 15 minutes, and then neutralized with extraction solution at room temperature. The signal was read at 460 nm. NAD⁺/NADH ratio was calculated based on the absorbance shown in the figure.

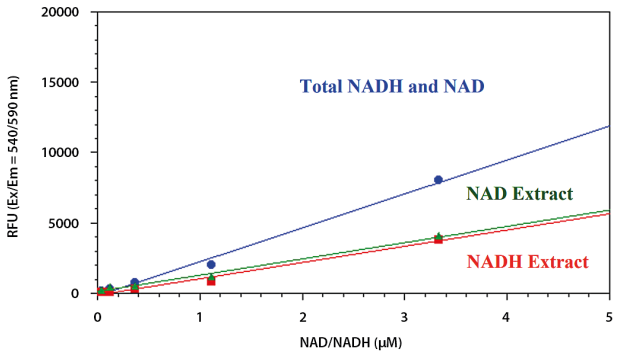

Figure 10 Total NADH/NAD⁺ and their extract dose responses were measured with Amplite™ Fluorimetric NAD⁺/NADH Ratio Assay Kit (Cat# 15263) in a 96-well black plate using a Gemini microplate reader (Molecular Devices). 25 µL of equal amount of NAD⁺ and NADH was treated with or without NADH or NAD⁺ extraction solution for 15 min, and then neutralized with extraction solutions at room temperature. The signal was read at Ex/Em = 540/590 nm 30 min after adding 75 µL of NADH reaction mixture. The blank signal was subtracted from the values for those wells with the NADH reactions.

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Table 3. Product Ordering Information

Cat#	Product Name	Unit Size	Ex/Em (nm)	Detection Mode
15280	Amplite™ Fluorimetric NAD Assay Kit *Blue Fluorescence*	200 tests	422/466	Fluorescence
15271	Amplite™ Colorimetric NADH Assay Kit	400 tests	460/none	Absorption
15261	Amplite™ Fluorimetric NADH Assay Kit *Red Fluorescence*	400 tests	571/585	Fluorescence
15258	Amplite™ Colorimetric Total NAD and NADH Assay Kit	400 tests	575/none	Absorption
15275	Amplite™ Colorimetric Total NAD and NADH Assay Kit *Enhanced Sensitivity*	400 tests	460/none	Absorption
15257	Amplite™ Fluorimetric Total NAD and NADH Assay Kit *Red Fluorescence*	400 tests	571/585	Fluorescence
15273	Amplite™ Colorimetric NAD/NADH Ratio Assay Kit	250 tests	460/none	Absorption
15263	Amplite™ Fluorimetric NAD/NADH Ratio Assay Kit	250 tests	571/585	Fluorescence

Novel Esterase Substrate for the Quantification of Bacterial Viability

The determination of bacterial viability is important in the fields of microbiology, medicine, and food technology. Bacterial viability assays are commonly used in research for evaluating antimicrobial properties, for determining viability of environmental species and for assessing fermentative microbe efficacy in brewing applications [1,2]. Since a diverse range of microorganisms and pathogenic microbes grows on food sources and environmental reservoirs (e.g. water, air and soil), the accurate detection and quantification of bacterial viability is important for monitoring contamination. Traditionally, microbial viability assessment is determined utilizing plating and counting techniques, which involve careful sample dilution, plate-spreading, lengthy incubation times and manual counting [3]. Although these methods are inexpensive, interpretation of viable count data must be considered carefully because it is unable to account for viable but non-culturable bacteria. As such, fluorescence-based techniques for measuring bacterial viability have been developed. These techniques quantify and detect viable bacteria more efficiently and with greater sensitivity, and are amenable to be used in conjunction with assays for analysis of membrane integrity, respiratory activity or metabolic enzyme activity [4].

A frequently used fluorescence technique for monitoring metabolically active bacteria is based on measuring intracellular esterase activity using esterified fluorogenic substrates. Esterification of fluorogenic substrates is optimal for two reasons. First, it alters the charge of the substrate making it more neutral. This improves cell permeability allowing the substrate to passively cross intact cell membranes. Secondly, esterified substrates are non-fluorescent. The substrate does not become fluorescent until it has entered the cell and intracellular esterases cleave off the ester functional groups.

Carboxyfluorescein diacetate (CFDA), a derivative of fluorescein diacetate (FDA), is used in both flow cytometric and fluorescent microscope applications for the rapid detection and quantification of bacterial viability relative to esterase activity [5]. Although CFDA staining curtails many of the disadvantages of the traditional colony counting assays, pitfalls still remain. The poor cellular retention exhibited by CFDA contributes to a low signal-to-background ratio and a decrease in assay sensitivity. To address these limitations of CFDA, AAT Bioquest has developed MycoLight™ 520, a novel replacement with superior staining performance.

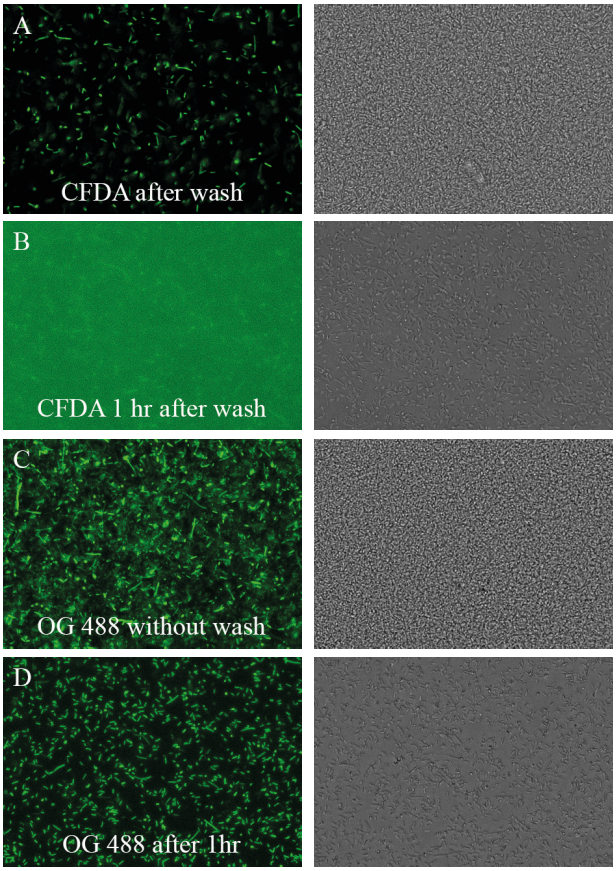

Figure 11 E. coli were cultured to late-log phase in LB medium. After removing the medium, 108 cells/mL were stained with CFDA for 30 min at 37 °C (Fig 11A, Fig11B), or stained with MycoLight™ 520 for 5 minutes at 37 °C. CFDA staining was washed out before imaging and MycoLight™ 520 was not.

Brighter Staining of Viable Bacteria using MycoLight™ 520

In our comparison studies, Escherichia coli (E. coli) bacteria treated with MycoLight™ 520 and CFDA were evaluated using fluorescence microscopy. As illustrated in Figure 11D, MycoLight™ 520 exhibited

improved intracellular retention and a significantly higher signal-to-background ratio compared to CFDA. A notable drawback of CFDA was the continuous dye leakage after 1 hour which generated high levels of background interference to the extent that individual cells were indistinguishable (Figure 11B). As for MycoLight™ 520, no significant change in the signal-to-background ratio was exhibited after 1 hour. Another benefit not illustrated by the fluorescence images, is that MycoLight™ 520 can be rapidly loaded into viable bacteria. MycoLight™ 520 only requires a 5 minute incubation time at 37 °C. Additionally, washing steps are not required, but an enhancer may be used to further improve the signal-to-background ratio of MycoLight™ 520. CFDA, however, requires a 30 minute incubation period and multiple washes after staining to reduce background interference.

MycoLight™ 520 Staining For Flow Cytometry

Furthermore, we investigated the efficacy of MycoLight™ 520 at quantifying live and dead bacteria utilizing flow cytometry. Accuracy was evaluated using different proportions of live and dead E. coli suspensions treated with MycoLight™ 520. Figure 12A illustrates the easily distinguishable staining patterns of live and dead bacterial populations treated with MycoLight™ 520. When measured in the FITC channel, peaks generated at higher intensities were representative of live bacteria while peaks generated at lower intensities illustrated the dead bacteria. The count of each sample was plotted against the percentage of live bacteria in order to generate a standard curve which can be used to accurately quantify the viability proportions of unknown sample (Figure 12B).

Conclusion

Our direct comparison of CFDA and MycoLight™ 520 demonstrates that MycoLight™ 520 is a more robust and sensitive tool for staining and differentiating live and dead bacteria. MycoLight™ 520 dye exhibited a more rapid cellular uptake, with an incubation time of 5 minutes at 37 °C. Furthermore, MycoLight™ 520's improved intracellular retention resulted in a significantly higher signal-to-background ratio, and allowed for staining to be detected for much longer periods of time. Based on these finding, MycoLight™ 520 is a superior alternative to CFDA in fluorescence microscope and flow cytometric applications for assessing bacterial viability.

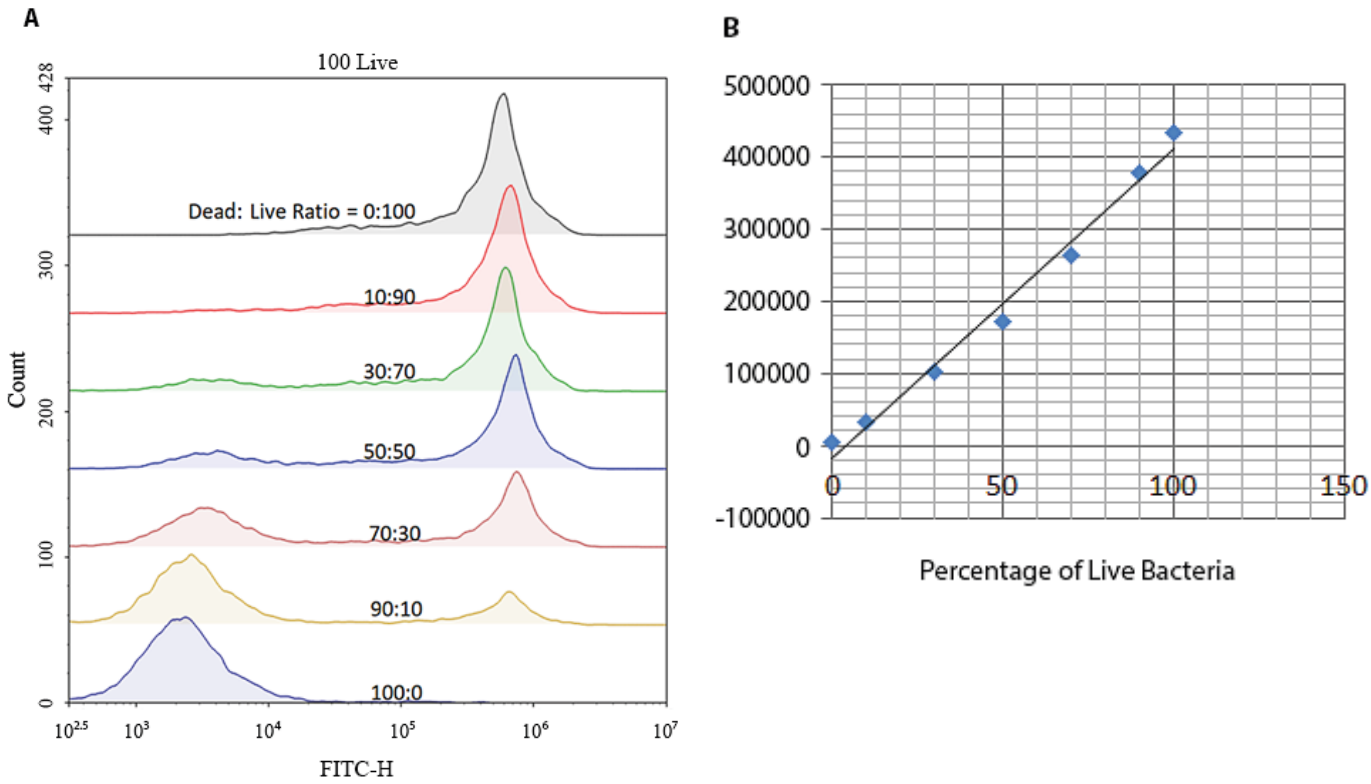

Figure 12 Relative viability of E.coli suspension was analyzed using the FITC channel of Flow Cytometer. The readings (Count (%)) were obtained from various live/dead E.coli mixtures (A). The live and dead population in each mixture can be easily distinguished by the two distinct peaks. The count of each sample was plotted against the percentage of live bacteria to generate a standard curve (B).

Sample Protocol for Microscopic Imaging or Flow Cytometer Assay

1. Bacteria should be cultured in appropriate medium to late-log phase to allow optimum detection.
2. For each sample, prepare bacteria in 0.5 mL of staining buffer at a density of $1X10^6$ – $1X10^8$ cells/mL.
3. Add MycoLight™ 520 into 0.5 mL of bacteria suspension, for microscope imaging, the addition of an enhancer is recommended.
4. Incubate bacteria with MycoLight™ 520 at 37 ºC for 5 min.
5. Image bacteria using fluorescence microscope or analyze cells with a flow cytometer using the FITC filter set

Note: To exclude debris, it is recommended to set the threshold of the flow cytometer as the following: FSC >10,000, SSC>5,000.

Note: The efficiency of MycoLight™ 520 is highly strain dependent and the staining conditions should be optimized accordingly.

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Table 4. Product Ordering Information

Cat#	Product Name	Unit Size	Ex/Em
22407	MycoLight™ Flow Cytometric Live Bacteria Assay Kit	100 tests	496/524
22409	MycoLight™ Live Bacteria Fluorescence Imaging Kit	100 tests	496/524

Intracellular pH Measurement with Dual Excitation Fluorescence Sensor BCFL

Intracellular pH (pH_i) plays an important modulating role in many cellular events, including cell proliferation and apoptosis, cell volume regulation, cellular metabolism, calcium regulation, receptor-mediated signal transduction, ion transport and endocytosis. Under physiological conditions, the pHi of intracellular fluid ranges between 6.8 and 7.4. This is important for many cellular functions such as protein synthesis, enzymatic activity, contractile efficiency in muscle cells and ion channel conductivity. However, neutral pH levels are not characteristic of all cellular components as some organelles require acidic environments. For example, lysosomes maintain an acidic environment (pH 4.5-6.0) to facilitate the degradation of proteins during cellular metabolism. Additionally, monitoring pH_i is important for studying intracellular signaling pathways. It has been reported that the dysregulation of pHi can affect the concentration of intracellular messengers like calcium and cAMP and thus adversely influence cellular signaling.

Over the past decades various techniques have been developed for measuring pH_i. These include partitioning of weak acids and bases, pH-selective microelectrodes, nuclear magnetic resonance and pH-sensitive fluorescent probes. Compared to the other pHi measurement methods, pH-sensitive probes combined with fluorescence techniques have the capacity to observe pH_i changes in a spatial and temporal context. Moreover, fluorescent pH probes have high sensitivities, are non-destructive to cells and convenient to handle. A widely used fluorescent pH sensor, BCECF (2′, 7′-bis-(2-carboxyethyl)-5-(and-6) - carboxyfluorescein) was first introduced in 1982 by Roger Tsien and company (Tsien et al. 1982). It has a pKa value of 6.98 which is well within the physiological pH range of 6.8 to 7.4 making it ideal for pHi measurements. Furthermore, BCECF has the capacity to measure pH ratiometrically due to its pH-dependent dual-excitation profile. However, BCECF is not without its disadvantages. BCECF, AM (the cell permeable from) is a complex mixture of three isomers with ratios that vary between batches, and this variability can lead to complications in certain applications potentially resulting in inaccurate pHi measurements.

To overcome the isomeric variability concerning BCECF, AM, AAT Bioquest® introduces a proprietary fluorescent pH sensor, BCFL. BCFL shares an identical pKa value and spectral profiles with BCECF making it an ideal replacement for pHi applications. Ratiometric imaging with BCFL makes pHi determination significantly more accurate because variable parameters such as dye concentration, optical path length, cellular leakage and rate of photobleaching are made irrelevant. In the sections below we discuss the spectral characteristics of BCFL and experimental procedures of BCFL, AM. In two common cell lines, HeLa and HL-60, we demonstrate pH_i calibration and measurement using both a flow cytometer and microplate reader. Finally, we apply BCFL in an experiment quantifying cell apoptosis based on intracellular acidification.

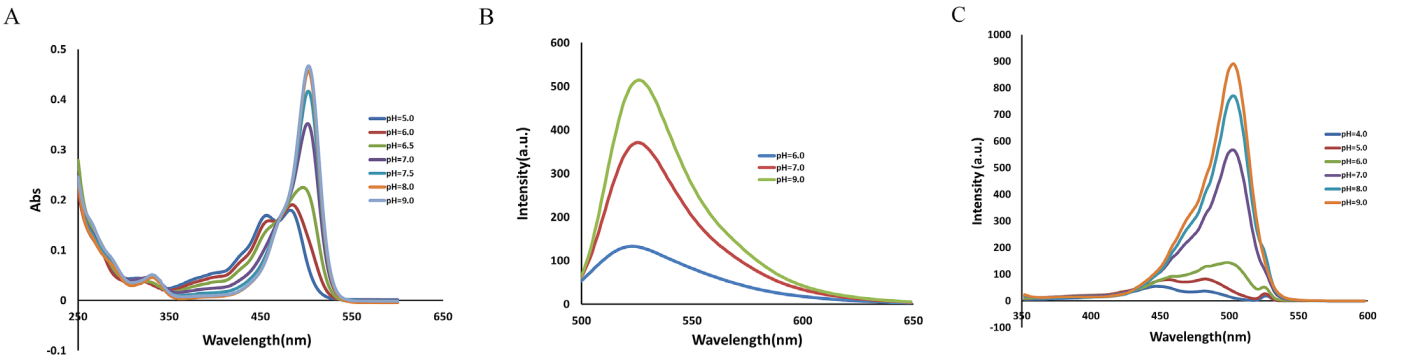

Figure 13 The pH dependent spectra of BCFL: a) absorbance spectra, b) emission spectra, and c) excitation spectra. The fluorescence excitation spectra on the panel c is enlarged 10x to show the isosbestic point. And the isosbestic point in excitation spectra is different from the absorption spectra.

Spectra Characteristics of BCFL

The spectra characteristics of BCFL were measured using buffers with pH values ranging from 4.5 to 9.0. Figure 13a shows that the absorption of BCFL red shifts (450 nm to 505 nm) from pH 4.5 to 9.0 and its molar absorptivity increases to approximately 90,000 $cm^{-1}M^{-1}$. This demonstrates the pH-sensitive absorption of BCFL. Figure 13b demonstrates the emission profile of BCFL in the physiological range (pH 6-8). Similar to BCECF, BCFL has a 526 nm emission wavelength in the physiological range. Finally, Figure 13c illustrates BCFL as a dual-excitation ratiometric pH indicator with an excitation isosbestic point at 430 nm.

In vitro pH Calibration and Measurement using BCFL

As a ratiometric excitation pH indicator, the measurement of pH is dependent upon the ratio of emission intensities of BCFL at two excitations, 440 nm and 505 nm (Figure 14). Ratiometric pH measurements are advantageous for its ability to correct for variations such as dye concentration, cell number, and photobleaching effects. Figure 14 illustrates that BCFL, similar to BCECF, can accurately determine pH with a standard deviation as low as 5%. Compared to the pH value of 7.33 which was measured using a pH electrode, the pH determined from the BCFL calibration curve was 7.30, demonstrating a 2% error from 8 replicates. Additionally, the calibration curve shows BCFL has a pKa value of ~6.8 which is comparable to the pKa of BCECF.

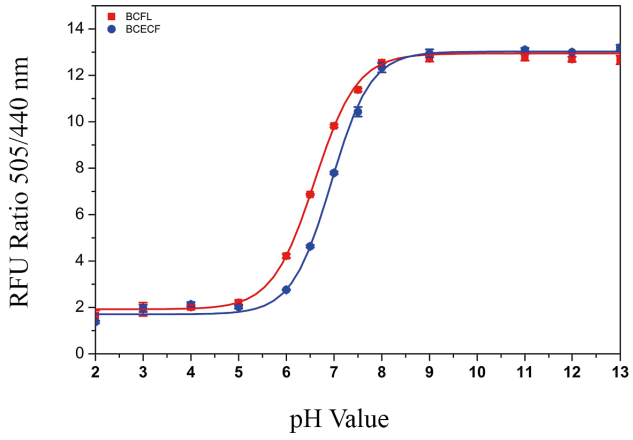

Figure 14 The pH dependent standard curve of BCFL using buffers with varying pH values. An average of 3 data points was plotted and a sigmoidal trendline was fitted to get the pH standard curve. +/- error bars represent the standard deviation.

Intracellular pH Measurement by Plate Reader

The non-fluorescent acetoxymethyl ester of BCFL (BCFL, AM) is cell membrane-permeant probe that can be applied to in situ experiments measuring pHi. After BCFL, AM noninvasively enters cells, AM ester functional groups are removed by intracellular esterase hydrolysis. This improves the cellular retention of BCFL by restoring its negative charge and simultaneously activates the pH-sensitive fluorescent properties of BCFL. BCFL, AM's pHi calibration curve is sigmoidal from 4.5 to 9.0 with its linear range falling in the cytosolic physiological pH range of 6 - 8 (Figure 15). The pKa from this calibration curve is ~6.8 which is almost identical to the in vitro calibration curve illustrated in Figure 14.

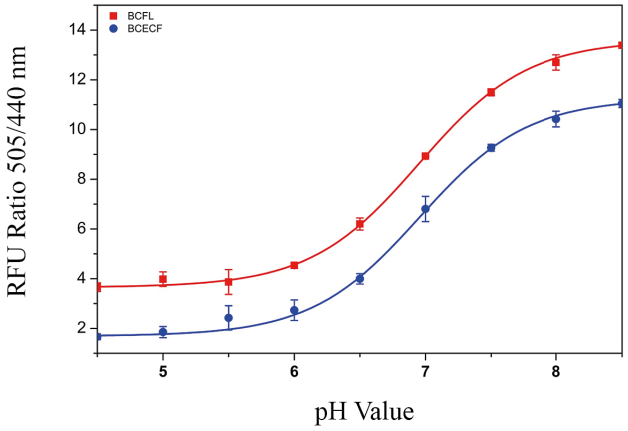

Figure 15 Standard curve created using BCFL, AM (Cat# 21190) with Intracellular pH Calibration Buffer Kit. HeLa cells were incubated with 5 uM BCFL, AM for 30 minutes 37 °C. The Intracellular pH Calibration Buffer Kit (Cat#21135) was used to clamp the intracellular pH with extracellular buffers at pH 4.5 to 9.0. An average of 4 data points was plotted and a sigmoidal trendline was fitted to get the pH standard curve from 5 to 8.

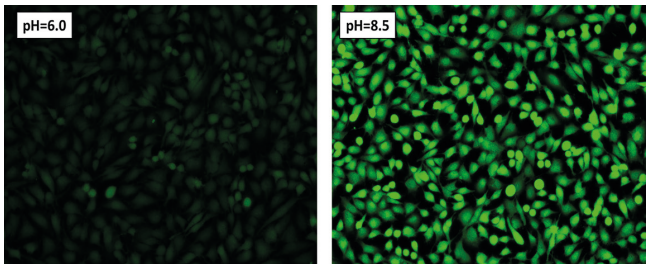

Figure 16 HeLa cells labeled with BCFL, AM Intracellular pH Indicator. HeLa cells were incubated with 5 µM of BCFL, AM Intracellular pH Indicator (Cat#21190) for 30 minutes at 37 °C. Images were captured using a microscope FITC filter. Incubation of BCFL, AM solution with Hela cells showed a homogenous uptake of BCECF (Fig. 18) and stained cell cytosol.

Intracellular pH Measurement by Flow Cytometry

Intracellular pH measurement by flow cytometry is a generally procedure that can be done using any instrument equipped with a 488-nm argon laser. But ratio measurement is not easily performed with flow cytometers because of the limitation of the excitation laser. Although there is no universal calibration curve for internal pH calibration, the pH change could be easily straightforwardly determined with flow cytometers.

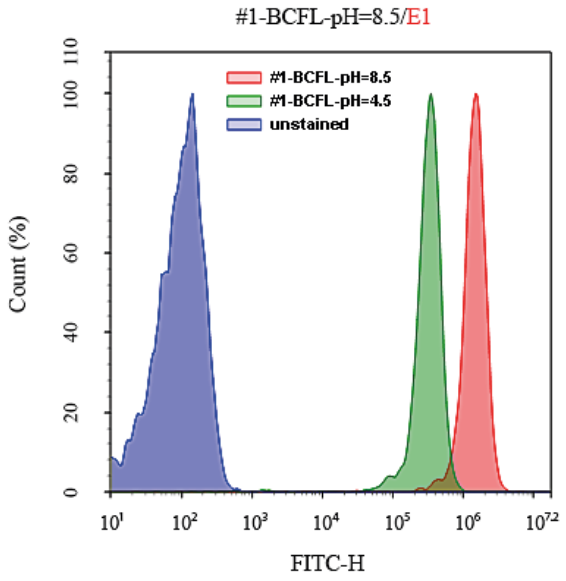

Figure 17 HL-60 cells incubated with BCFL, AM (Cat#21190) and clamped with intracellular pH calibration buffer (pH=4.5 & pH=8.5). HL-60 cells were incubated with 100nM BCFL, AM for 30 minutes at 37 °C. The Intracellular pH Calibration Buffer Kit (Cat. 21235) was used to clamp the intracellular pH with extracellular buffer at pH 4.5 (Green area) and pH 8.5 (Red area). Unstained cells are represented by the Blue area. Samples were acquired and analyzed using Acea Novocyte Cytometer.

Application of BCFL in Cell Apoptosis

Under normal conditions, pHi is actively maintained within a very narrow pH range of 6.0 – 8.0. However, during certain important cellular processes, such as cell proliferation or apoptosis, pHi values change and therefore serve as a cell signaling factor. During apoptosis, cells induce intracellular acidification to pH values which activate enzyme reactions necessary for programmed cell death.

Intracellular acidification was observed during apoptosis in Jurkat cells after treatment with staurosporine for 4 hours. In Figure 6 staurosporine treated Jurkat cells show lower fluorescence intensities in the FITC channel, indicating the intracellular acidification of Jurkat cells during apoptosis. Acidification was also noted in the population of smaller size cells. In the earlier stage of apoptosis, the pHi shows no significant change, but as the process of apoptosis progresses, the cells decrease in size and become more acidic (Figure 18). This decrease in pH_i can serve as a practical indicator of cellular deterioration and cell death.

Conclusion

Overall, we demonstrate BCFL is an ideal dual excitation fluorescence pH sensor for quantifying pHi and a suitable replacement for BCECF. We demonstrated that with BCFL, the physiological pH_i can be determined from calibration curves derived from flow cytometer and microplate reader formats. Most importantly, the single isomer of BCFL, AM makes pH measurements much more reproducible than BCECF, AM which suffers from isomeric variability.

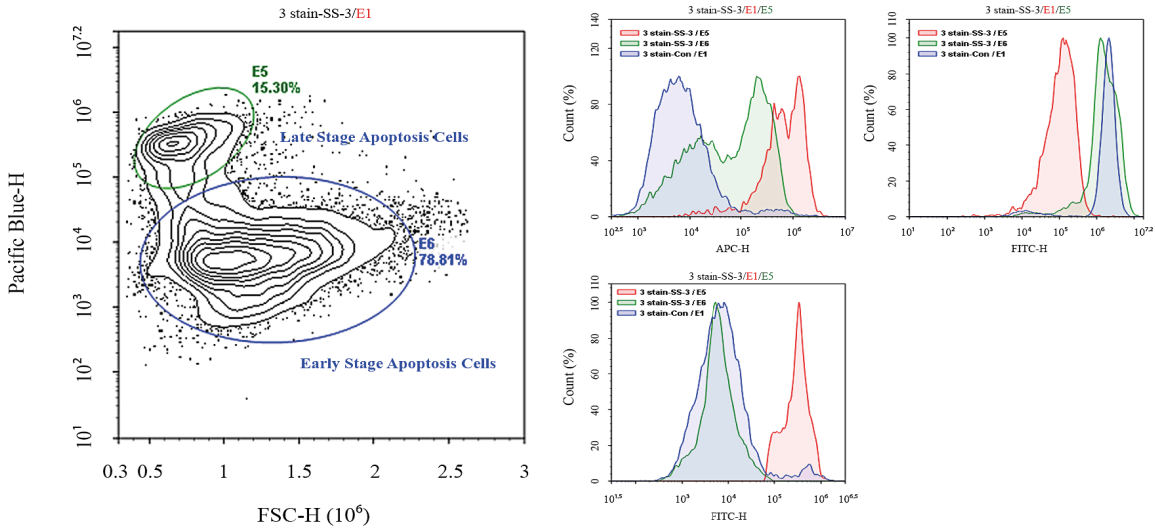

Figure 18 BCFL, AM & AnnexinV-iF647 Staining of Apoptosis Jurkat cells. Junket cells were treated with/without 1uM staurosporine in growth medium for ~4 hours. After treatment, cells were stained with BCFL, AM & AnnexinV-iF647 and DAPI cocktail in HH Buffer for 30 minutes, and dye loading solution was then replaced with HH buffer and analyzed with ACEA NovoCyte Flow Cytometer with DAPI, FITC and APC channels.

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MATERIALS AND METHODS

Materials

BCFL (Cat# 21189) and BCFL, AM (21190) were used for in vitro pH calibration and intracellular pH measurement, and BCECF (Cat#21201) and BCECF, AM (Cat#21202) were also used as a reference. Internal pH calibration buffers were from Spexyte™ Intracellular pH Calibration Buffer Kit (Cat# 21235). The 4-parameter curve pH calibration curves were plotted with AAT Bioquest's Four Parameter Logistic (4PL) Curve Calculator.

Cell Culture

HeLa cells were cultured in DMEM medium with 10% FBS and 1% Penicillin-Streptomycin-Glutamine. HL-60 and Jurkat cells were cultured in RPMI medium with 10% FBS and 1% Penicillin-Streptomycin-Glutamine. When indicated, the cells were treated with 1 µM staurosporine in growth cells directly, and followed by culturing for ~4 hours to induce cell apoptosis.

Instruments

The fluorescence intensities was measured by FlexStation 3 (Molecular Devices) bottom reading mode with Ex/Em=505 nm, 440 nm/535 nm (cutoff=515 nm). Flow cytometry data were acquired and analyzed with NovoCyte Flow Cytometry (ACEA) using FITC channel. All cell images were captured with a BZ-X710 fluorescence microscope using FITC filter set.

Sample Protocol for intracellular pH nigericin calibration

1. HeLa Cells were plated in 100 µL culture medium in 96-well clear bottom black plates at 50,000 cells per well.
2. Medium was removed and 100 µL HH buffer with 0.04% PF127 with 5 µM BCFL, AM was added to each well and cells were incubated at 37 °C for 30 min to 60 min.
3. The dye loading solution was removed.
4. 100 µL Intercellular pH Calibration (Cat# 21235) with 10 µM Nigerincin & 10 µM Valinomycin were added to each well and then incubated at room temperature for 10-30 min.
5. The fluorescence was measured by FlexStation 3 (Molecular Devices) bottom reading mode with Ex/Em=505 nm, 440 nm/530 nm (cutoff=515 nm).

Intracellular pH Calibration and Measurement with Flow Cytometry Platform:

Sample Flow Cytometry Protocol for Intercellular pH calibration:

1. HL-60 cells were grown in culture medium overnight
2. The next day, 5 mL cells were prepared in a tube
3. Centrifuge to remove growth medium
4. Add 1 mL HH buffer with 0.02% PF127 and 100 nM BCFL,AM
5. Incubate for 30 min at 37 °C
6. Centrifuge to remove dye loading solution
7. Resuspend cells in 1 mL HH buffer
8. Prepare 1.0 mL of each intracellular pH buffer with 10 µM Nigerincin & 10 µM Valinomycin
9. Add 100 µL cells to each intracellular pH calibration buffer
10. Incubate for 10-30 min at room temperature, and then analyze with a flow cytometer using FITC channel

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Table 5. Product Ordering Information

Cat#	Product Name	Unit Size	Ex/Em (nm)
21190	RatioWorks™ BCFL, AMt	1 mg	503/528
21191	RatioWorks™ BCFL, SE	1 mg	503/528
21189	RatioWorks™ BCFL, Acid	1 mg	503/528

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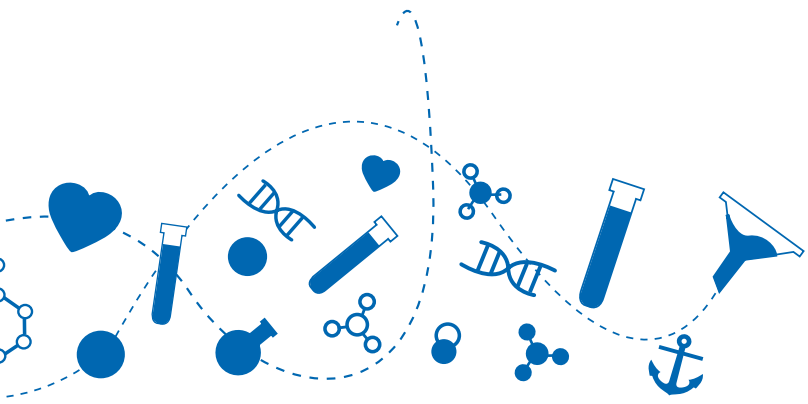
Fax: 0800-246 66 52



Notes

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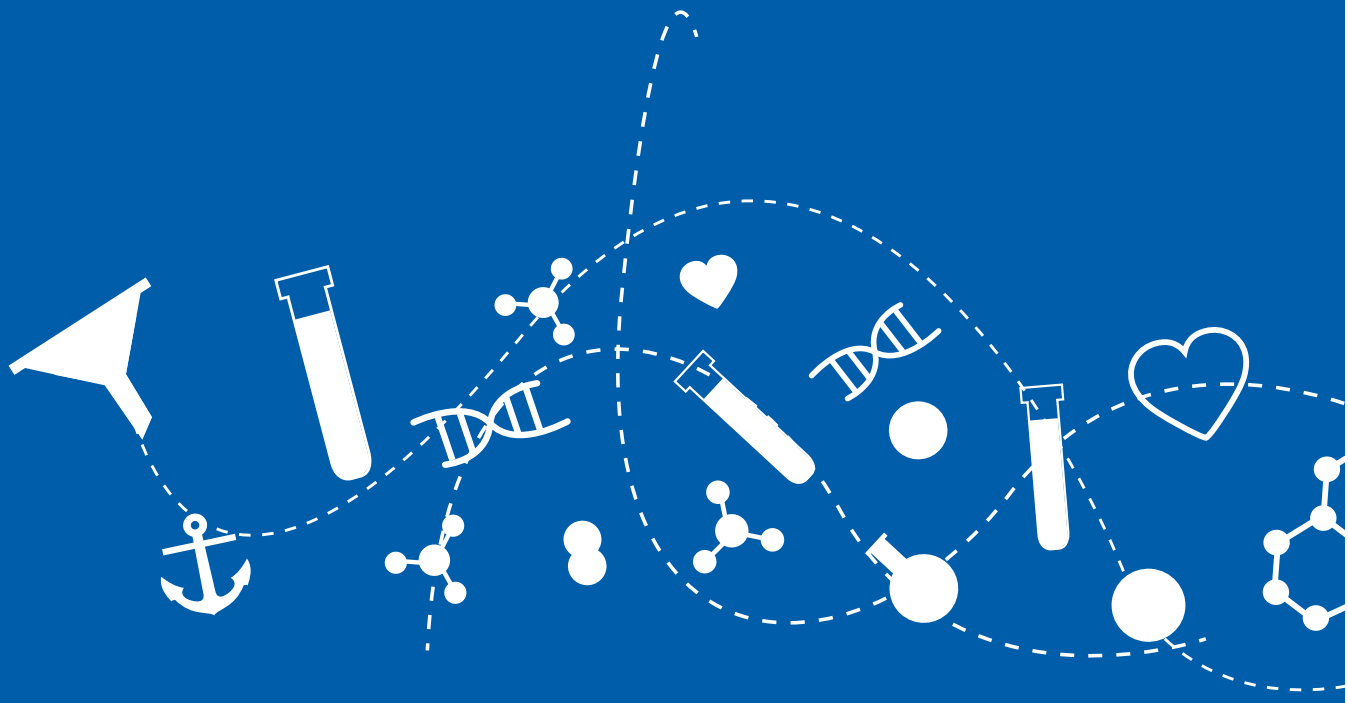

biomol
SIE HABEN DIE VISION,
WIR HABEN DIE SUBSTANZ.

2018