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AssayWise Letters

Calbryte™: Next Generation Calcium Indicator
Screen Quest™ ELISA cAMP Kits

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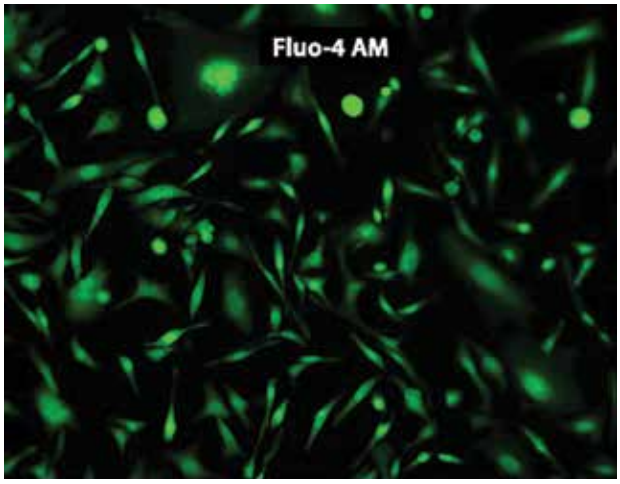
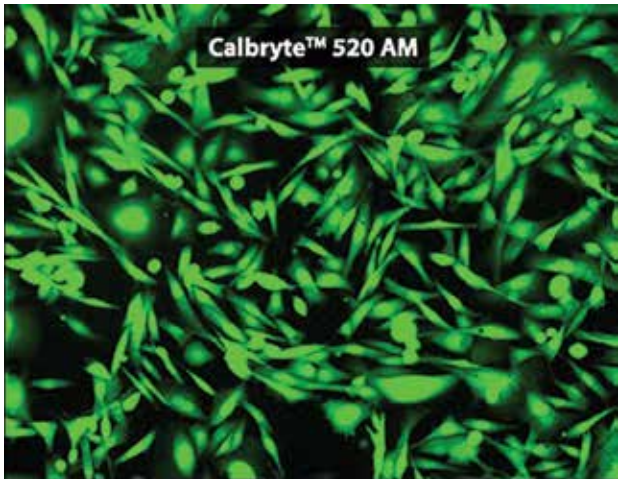




Calbryte™ Series

The Calbryte™ series is a family of fluorescent dyes developed to monitor intracellular calcium. It includes three novel calcium indictors: Calbryte™ 520, Calbryte™ 590 and Calbryte™ 630. These indicators differ primarily in their excitation and emission wavelengths and can be detected using standard fluorescence instruments. The Calbryte™ series has been optimized for use with fluorescence microscopes, fluorescence microplate readers and flow cytometers. They can also be used in high-throughput screening applications.

The Calbryte™ dyes have several key advantages over dyes like Fluo-3 and Fluo-4. Calbryte™ dyes generate much brighter signals, show significantly improved signal-to-background ratio and have greatly enhanced cellular retention. These qualities make the Calbryte™ series a superior choice over older, more traditional calcium indicators.

Can you tell me more about the Calbryte™ dyes?

Dyes in the Calbryte™ series are named roughly according to their maximal emission wavelength. For example, Calbryte™ 520 fluoresces in the green region of the visible spectrum while Calbryte™ 590 and Calbryte™ 630 fluoresce in the red and deep-red region, respectively. All three indicators can be acquired in either AM ester form or salt form (as a potassium salt). The AM ester form is a cell-permeable form that is useful for assaying calcium in live cells. The salt form is primarily used for the calibration of calcium indicators. This step is often required when calculating intracellular calcium concentration from fluorescence signal intensity. Salt forms of Calbryte™ can also be used for microinjection into live cells and tissue.

How do the Calbryte™ dyes work in live cells?

When assaying calcium in live cells, the preferred method is to synthesize Calbryte™ dyes with several acetoxymethyl ester (AM ester) functional groups. The reason for doing so is two-fold.

First, AM esters are lipophilic groups that, when attached to the Calbryte™ core structure, create an overall more hydrophobic compound. This increased hydrophobicity allows the Calbryte™ AM ester to easily penetrate intact lipid membranes and permeate into live cells. This eliminates the need for electroporation, microinjections or other similarly disruptive loading techniques.

Second, Calbryte™ dyes, while in AM ester form, are essentially non-

Figure 1.1 ATP response was measured in CHO-K1 cells with Fluo-4, AM (left) and Calbryte™ 520 AM (right). CHO-K1 cells were seeded overnight at 50,000 cells/100 µL/well in a 96-well black wall/clear bottom costar plate. 100 µL of 10 µg/ml Calbryte™ 520 AM in HH Buffer with probenecid or 10 µg/ml Fluo-4, AM in HH Buffer with probenecid was added and incubated for 45 minutes at 37 °C. Dye loading solution was then removed and replaced with 200 µL HH Buffer/well. ATP (50 µL/well) was added to achieve the final indicated concentration of 10 µM and then imaged with microscope FITC channel (Keyence).

fluorescent and non-activatable. They show minimal response to, for example, trace Ca^{2+} present in extracellular solution. It is only once the Calbryte™ probe permeates into the cell, and only after intracellular esterases cleave off the AM ester function groups, that the Calbryte™ dye becomes activated and responsive to calcium. This two-step activation process is important because it greatly minimizes non-specific, background fluorescence. And by extension, it significantly enhances intracellular calcium signals.

Once the Calbryte™ probe is activated, it detects calcium through a typical chelation process. Unlike other chelators such as Fluo-3 and Fluo-4, however, Calbryte™ dyes show much greater responses upon binding to calcium. In experiments, Calbryte™ probes have shown a greater than 300-fold increase in fluorescence upon chelation. In comparison, Fluo-4 only demonstrates a 100-fold increase. This drastic improvement in fluorescence response allows for an extremely robust detection of calcium not achievable with past calcium indicators.

What are the advantages of using Calbryte™ dyes?

Calbryte™ dyes easily outperform older, more traditional calcium indicators. In live cell experiments, Calbryte™ dyes yield signal-to-background ratios that are an order of magnitude greater than other dyes such as Fluo-4. This is a huge advantage in experimental studies. As many researchers can attest to, poor signal to background ratios are a headache for data analysis. It conflates results with noise, masks potentially significant data and leads to huge assay-to-assay variability. For this reason, when choosing reagents, most researchers prefer ones that have good to great signal-to-background ratios.

Poor signal-to-background ratios can be caused by a multitude of factors that largely fall into two categories. First, there are factors that lead to poor signal intensity. These can include:

1. Low extinction coefficient and/or quantum yield
2. Poor binding of the target
3. Absorbance maximum (λmax) far from excitation source

Calbryte™ dyes were created to address these concerns. For instance, Calbryte™ 520 has a quantum yield three times greater than that of Fluo-3 or Fluo-4. This means that per photon of light it absorbs, Calbryte™ 520 fundamentally emits more fluorescence than either Fluo-3 or Fluo-4 could, resulting in brighter signal intensity. Additionally, all dyes in the Calbryte™ series are designed such that their maximum absorbance occurs near standard excitation sources. For example, Calbryte™ 520 (with λmax = 492 nm) is well excited by the common 488 nm argon ion laser line.

The second reason many probes exhibit poor signal-to-background ratio is due to high background. High background can be a result of:

1. Cell impermeability
2. High autofluorescence
3. Nonspecific activation/binding
4. Poor cellular retention

Many of these factors are related insofar as they concern a probe's ability to enter a cell and localize efficiently in the cytosol. Looking at many past calcium indicators, they often run into one of two problems. 1) Either the probe would be fairly water soluble, but too hydrophilic to permeate live cells or 2) they would be hydrophobic enough to permeate live cells, but have such poor water solubility that they would precipitate out of solution during loading. Neither case is desirable. With the Calbryte™ series, the challenge has been to create a probe which strikes a delicate balance between water solubility and cell permeability. The Calbryte™ series addresses this problem through manipulation of water-soluble functional groups and the aforementioned AM ester groups. By optimizing the presence and quantity of both groups, Calbryte™ dyes are able to obtain drastically higher signal-to-background ratios.

Another reason calcium indicators perform poorly in live cell experiments is because of poor cellular retention. Here, the problem lies with a family of proteins called p-glycoprotein 1 (P-gp). In many cells (such as the well studied HeLa cell line), P-gp acts as an ATP-dependent efflux pump, actively moving a broad range of small molecules from intracellular to extracellular space. With regards to calcium indicators, this poses a problem because activated calcium indicators can "leak" out of the cell through these pumps and

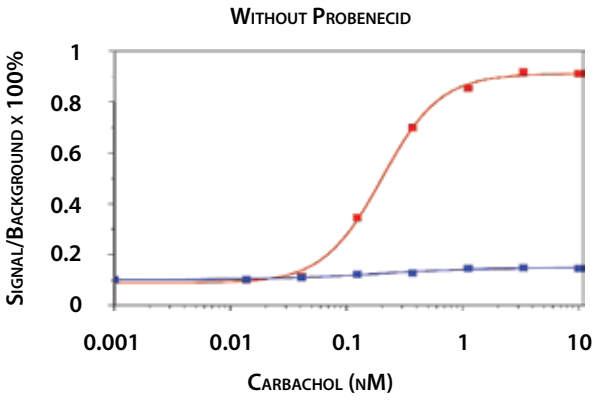


Figure 1.2 Carbachol dose response was measured in CHO-M1 cells with Calbryte™ 520 AM and Fluo-4 AM. CHO-M1 cells were seeded overnight at 50,000 cells/100 µL/well in a 96-well black wall/clear bottom costar plate. 100 µL of 10 µg/ml Calbryte™ 520 AM in HH Buffer or 10 µg/ml Fluo-4 in HH Buffer was added and incubated for 45 minutes at 37 °C. Dye loading solution was then removed and replaced with 200 µL HH Buffer/well. Carbachol (50 µL/well) was added by FlexStation 3 to achieve the final indicated concentrations.

into the extracellular matrix. This type of leakage results in a two-fold problem. First, because the leaked calcium probes have been activated (due to removal of AM esters by intracellular esterases), they will bind to free calcium in the extracellular matrix and fluoresce. This fluorescence is not the signal of interest, however, and thus contributes to what is known is a high background, or noise. Second, the leakage of probes into extracellular space results in a decrease of probes within the cell. This leads to reduced detection of intracellular calcium and lower signal intensity, further exacerbating the problem.

One solution, that has been well documented, is to use probenecid in conjunction with calcium indicator use. Probenecid, a channel blocker, has shown moderate success in reducing probe leakage. However, its use is far from an ideal solution. This is because there are many experimental targets that are quite sensitive to probenecid. For example, the TRPV2 receptors of sensory ganglia have been shown to be activated by probenecid. On the other hand, probenecid seems to have an inhibitory effect on TAS2R receptors, or taste receptors. For many other serum-sensitive or drug-sensitive targets, the effects of probenecid could be largely unknown. This means that by using probenecid, a research risks the introduction of a completely unknown factor into an experimental design.

Noting this critical problem with existing calcium indicators, Calbryte™ has been specifically designed for high performance without probenecid. Calbryte™ dyes accomplish this by carefully balancing ionic charges across the compound. This leads to a negatively charged, hydrophilic molecule that, once inside the cell, shows dramatically reduced cell leakage. Under probenecid-free conditions, Calbryte™ dyes outperform Fluo-4 by an order of magnitude in terms of signal-to-background ratio.

One final advantage of Calbryte™ dyes is that they are well suited for high-throughput screening (HTS) and drug-discovery applications. Since Calbryte™ dyes have such excellent signal-to-background ratios, they can be utilized in a no-wash format (see Cat No. 36317). Moreover, because probenecid addition is not required, Calbryte™ dyes can easily be used in automated setups.

What Calbryte™ dyes are currently available?

At present, Calbryte™ dyes are available in three different wavelengths: Calbryte™ 520, Calbryte™ 590 and Calbryte™ 630.

Calbryte™ 520 is our upgrade for traditional green fluorescent indicators such as Fluo-3 and Fluo-4. This dye has an excitation maximum at 492 nm, which closely matches the standard 488 nm argon ion laser line. Also, with an emission maximum at 514 nm, this dye is compatible with the FITC filter sets found in most fluorescence instruments. Because Calbryte™ 520 has spectral values nearly identical to that of Fluo-4, no additional instrument setup is required. This allows for a seamless and convenient transition between products.

Calbryte™ 590 is our upgrade for orange-red fluorescent indicators such as Calcium Orange™ and Rhod-2. This dye has an excitation maximum at 580 nm and is well excited by the 555 nm laser line.

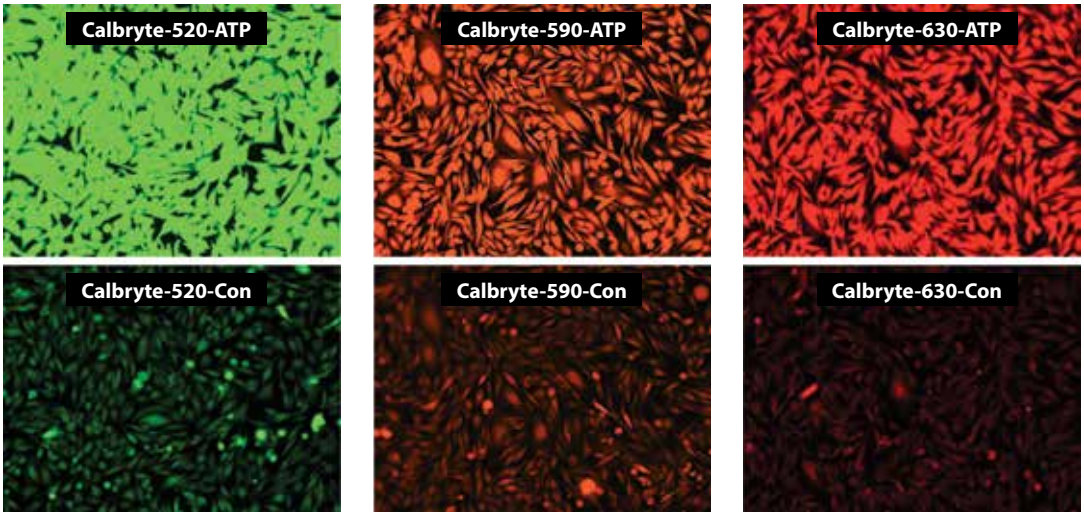


Figure 1.3 Response of endogenous P2Y receptor to ATP in CHO-K cells. CHO-K cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom costar plate. 100 µL of Calbryte™ 520 AM (left), Calbryte™ 590 AM (middle) or Calbryte™ 630 AM (right) in HHBS with 2 mM probenecid were added into the wells, and the cells were incubated at 37 °C for one hour. The dye loading mediums were replaced with 200 µL HHBS, treated with 50 µL of 50 µM ATP, and imaged with a fluorescence microscope (Keyence) using FITC channel (Calbryte™ 520), TRITC channel (Calbryte™ 590) or Texas Red channel (Calbryte™ 630).

It has an emission maximum at 592 nm, making it compatible with TRITC/Cy3 filter sets. Calbryte™ 590 is approximately ten times more sensitive for calcium than Rhod-2, under comparable conditions. Moreover, unlike Rhod-2 which primarily localizes in mitochondria, Calbryte™ 590 retains well in the cytosol of cells.

Calbryte™ 630 is our upgrade for red & deep-red fluorescent indicators such as X-Rhod-1. This dye has an excitation maximum at 608 nm, which aligns well with the 594 nm laser line. This dye has an emission maximum at 624 nm and is compatible with common Texas Red filter sets. Because of its distance from the green region of the spectrum, Calbryte™ 630 is well suited for multiplex with a green fluorescent label such as iFluor™ 488, Alexa Fluor® 488 or GFP. Moreover, Calbryte™ 630's long emission wavelength makes it well suited for study of deep tissue. This is because longer wavelength dyes have an easier time penetrating through many cell layers, whereas short-wavelength dyes cannot.

What other forms do Calbryte™ dyes come in?

Calbryte™ dyes are also available in potassium salt form. These products are designed to help researchers perform calibration of calcium indicators. Calibration of calcium indicators may be required before determining calcium concentration from fluorescence intensity.

What other specifications should I know for the Calbryte™ dyes?

Calbryte™ dyes are shipped lyophilized (desiccated) and can be stored for up to a year if frozen (< -20 °C). During use, Calbryte™ dyes can be reconstituted with a small amount anhydrous DMSO. Reconstituted Calbryte™ dyes can be stored for up to three months if frozen (< -20 °C).

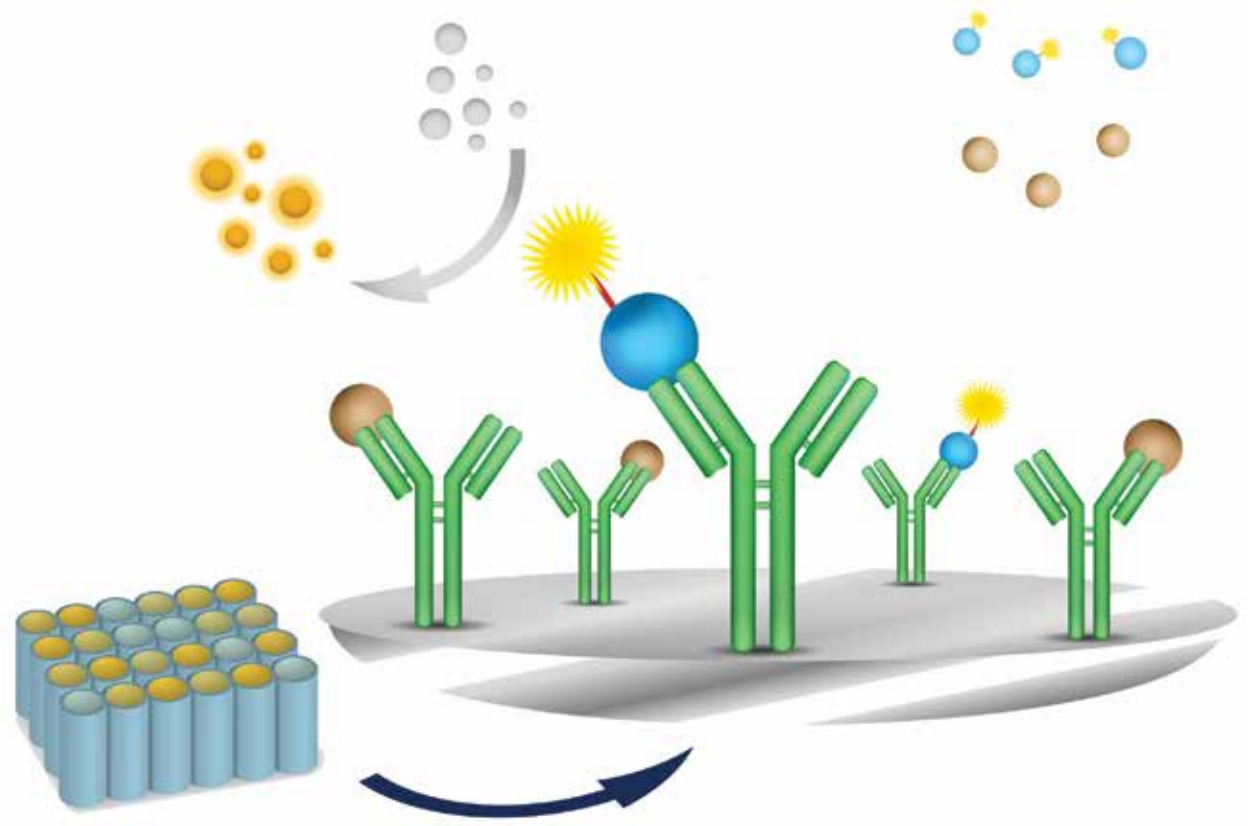
Another specification of interest for some researchers is the dissociation constant (Kd). Please see the table below for Calbryte™ Kd values.

Table 1.1 Spectral properties of Calbryte™ dyes

Ca^{2+} Indicator	Excitation (nm)	Emission (nm)	Kd (µM)
Calbryte™ 520	492 nm	514 nm	1.2
Calbryte™ 590	580 nm	592 nm	1.4
Calbryte™ 630	608 nm	624 nm	1.2

Product Ordering Information For Calbryte™ Probes & Assay Kits

Cat #	Product Name	Unit Size	Excitation (nm)	Emission (nm)
20650	Calbryte™-520 AM	2x50 ug	492	514
20651	Calbryte™-520 AM	10X50 ug	492	514
20653	Calbryte™-520 AM	1 mg	492	514
20656	Calbryte™-520, potassium salt	5x50 ug	492	514
20658	Calbryte™-520, potassium salt	10x50 ug	492	514
20700	Calbryte™-590 AM	2x50 ug	573	588
20701	Calbryte™-590 AM	10X50 ug	573	588
20702	Calbryte™-590 AM	1 mg	573	588
20706	Calbryte™-590, potassium salt	5x50 ug	573	588
20720	Calbryte™-630 AM	2x50 ug	608	626
20721	Calbryte™-630 AM	10X50 ug	608	626
20722	Calbryte™-630 AM	1 mg	608	626
20727	Calbryte™-630, potassium salt	5x50 ug	608	626
36317	Screen Quest™ Calbryte-520™ Probenecid-Free and Wash-Free Calcium Assay Kit	1 Plate	490	525
36318	Screen Quest™ Calbryte-520™ Probenecid-Free and Wash-Free Calcium Assay Kit	10 Plates	490	525
36319	Screen Quest™ Calbryte-520™ Probenecid-Free and Wash-Free Calcium Assay Kit	100 Plates	490	525
36200	Screen Quest™ Calbryte-590™ Probenecid-Free and Wash-Free Calcium Assay Kit	1 Plate	573	588
36201	Screen Quest™ Calbryte-590™ Probenecid-Free and Wash-Free Calcium Assay Kit	10 plates	573	588
36202	Screen Quest™ Calbryte-590™ Probenecid-Free and Wash-Free Calcium Assay Kit	100 Plates	573	588



A Highly Sensitive Direct ELISA of cAMP Without Acetylation

Cyclic AMP (cAMP) is one of the most studied secondary messengers in G protein-coupled receptor (GPCR) signaling pathways (the other being calcium). This is because cAMP can be used to characterize GPCR activity in response to agonist and antagonists. In pharmacology, this is especially important, as GPCRs are a common drug target.

Assays for quantification of cAMP have been proposed since the 1970s. The traditional method involves the use of an anti-cAMP primary antibody in a competitive ELISA format. In recent years, a new class of cAMP assay has been developed. These new assays rely on Förster resonance energy transfer (FRET) in addition to the anti-cAMP primary antibody in order to detect cAMP. While not necessarily more sensitive than the traditional ELISA method, new FRET-based techniques do allow for easier HTS/HCS of cAMP (such as during drug discovery) as these FRET-based assays do not require the labor intensive washing step.

Figure 2.1 Representation of direct ELISA of cAMP principle.

Acetylation of cAMP

During the early development of ELISA-based cAMP detection, an interesting discovery was made. It was found that the anti-cAMP primary antibodies used in the assay actually had a higher affinity for acetylated cAMP than unmodified cAMP. The difference was rather stark. For the same antibody, acetylated cAMP had a dissociation constant of less than 10-10 M. For reference, unmodified cAMP has a dissociation constant of roughly 10-8 M. This represents a more than 100-fold difference in dissociation constants, and consequently, a significant increase in antibody binding affinity for acetylated cAMP over unmodified cAMP. Because of this, most modern ELISA-based cAMP assays will recommend acetylation of the sample cAMP before quantification, as it will result in a more sensitive experiment.

While acetylation does increase the sensitivity of cAMP detection, there are two major drawbacks to such methods. First, many biologically significant macromolecules besides cAMP can be acetylated. For instance, the lysine groups on many proteins are susceptible to acetylation, which can dramatically alter their function. Because many different biomolecules can be acetylated, the impact of acetylation on a given sample cannot be predicted. Second, the acetylation step increases experimental complexity. This not only allows for more experimental error, but deepens the knowledge gap between the assay developer and the end-user.

Here, we introduce a simpler direct ELISA of cAMP that does not require an acetylation step. Even without acetylation, our assay is more sensitive than current assays on the market. It demonstrates both a lower detection limit (0.1 nM) as well as a smaller IC50 (2.6 nM).

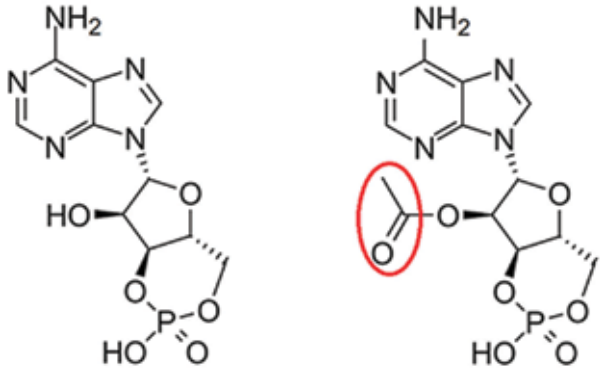


Figure 2.2 Unmodified cyclic AMP (left) compared to acetylated cyclic AMP (right). Acetylation circled in red.

Direct ELISA of cAMP – Principle

Aside from the lack of an acetylation step, our Screen Quest™ Colorimetric ELISA cAMP Assay Kit functions, in principle, as other direct competitive ELISAs on the market. Anti-cAMP primary antibodies are coated onto the surface a microplate well. Then a test sample is added; cAMP in the test sample will bind to the coated antibodies. Afterwards, cAMP-HRP is introduced, which will displace the bound cAMP due to the antibodies' higher affinity for cAMP-HRP over unmodified cAMP. Finally, a chromogenic reagent is added and oxidized by the bound cAMP-HRP, generating a signal proportional to the cAMP-HRP concentration and inversely proportional to the cAMP concentration.

Direct ELISA of cAMP – Methods

1. cAMP standards were prepared with the following concentrations: 10000, 100, 30, 10, 3, 1, 0.1, 0.03, 0.01, 0.003 and 0 nM.
2. 75 µL of cAMP standards was added to respective wells of an anti-cAMP antibody coated 96-well plate.
3. 96-well plate was incubated at room temperature for 10 minutes.
4. 25 µL of cAMP-HRP conjugate was added to each well (both standards and test samples).
5. 96-well plate was incubated at room temperature for 3 hours on a shaker.
6. 96-well plate was aspirated and washed 4 times with 200 µL per well of wash solution.
7. 100 µL of Amplite™ Green solution was added to each well.
8. 96-well plate was incubated at room temperature for 3 hours protected from light.
9. Absorbance was read using a SpectraMax microplate reader at 405 nm, 650 nm and 740 nm.

Direct ELISA of cAMP – Results

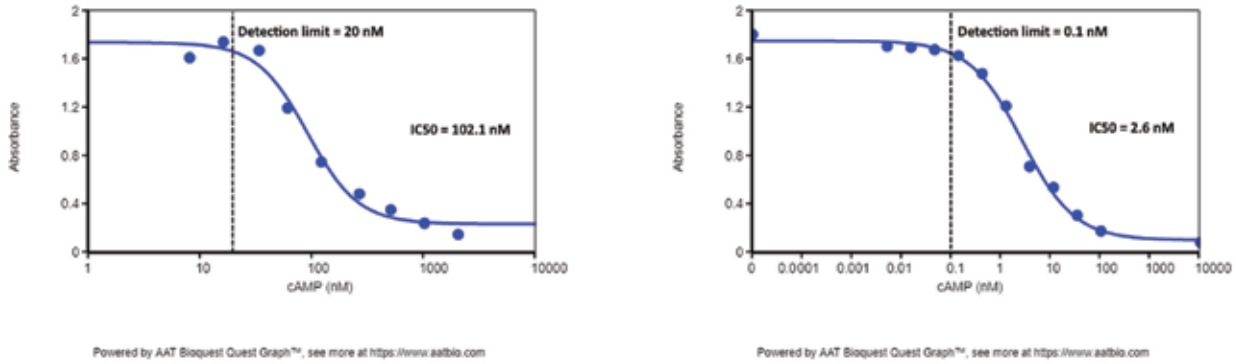


Figure 2.3 Sensitivity of cAMP assay as compared using cAMP standard curves. cAMP Assay Kit from Abcam plc (A, left). Screen Quest™ Colorimetric ELISA cAMP Assay Kit (B, Right).

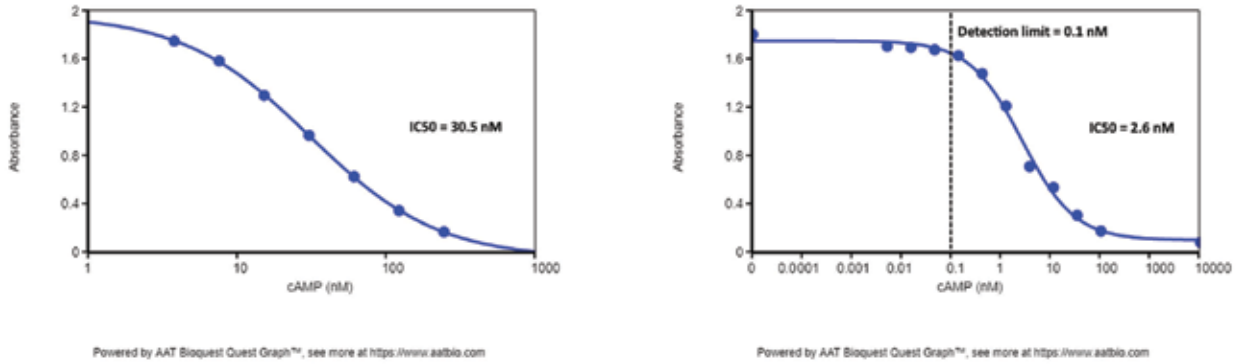


Figure 2.4 Sensitivity of cAMP assay as compared using cAMP standard curves. cAMP Parameter Assay Kit from R&D Systems (A, left). Screen Quest™ Colorimetric ELISA cAMP Assay Kit (B, Right).

As seen from the results, our acetylation-free cAMP assay outperforms other assays that require acetylation of cAMP for high sensitivity. Our acetylation-free cAMP assay also outperforms other mechanistically-similar acetylation-free cAMP assay kits on the market, as seen by the 10-fold difference in IC50 value in the figure above. Compared to assay kits with different assay principles, such as FRET-based or luminescence, our cAMP kit matches or outperforms available assay kits on the market. The table and graphs below shows some of these comparisons using cAMP standards.

Table 2.1 A comparison of available cAMP assays using cAMP standard curves.

Product Name	Supplier	Description	IC50
Screen Quest™ Colorimetric ELISA cAMP Assay Kit	AAT Bioquest	Direct competitive ELISA without acetylation	2.6 nM
cAMP Assay Kit	Abcam	Direct competitive ELISA with acetylation	90.4 nM
cAMP Parameter Assay Kit	R&D Systems	Direct competitive ELISA without acetylation	30.5 nM
Lance® cAMP Assay	Perkin Elmer	Time-resolved FRET with anti-cAMP antibody	3 nM
cAMP-Glo Assay	Promega	Luminescence from ATP-coupled, PKA-dependent luciferase reaction	8.3 nM
GS Dynamic Kit	Cisbio	Time-resolved FRET with anti-cAMP antibody	5.6 nM

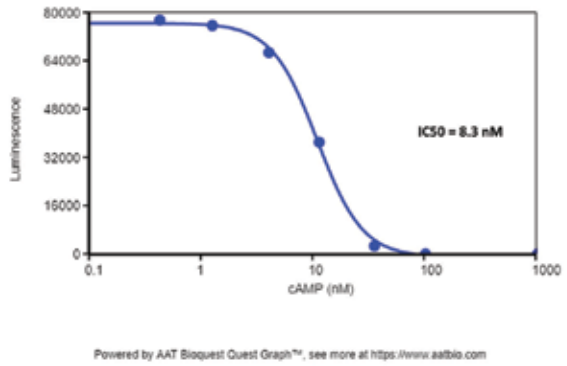


Figure 2.5 cAMP standard curve using Promega cAMP-Glo™ assay with protein-kinase A coupled, ATP-dependent luminescence generation by luciferase.

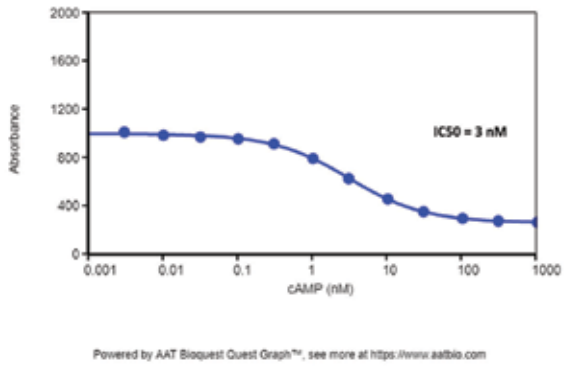


Figure 2.6 cAMP standard curve using PerkinElmer LANCE® cAMP assay with FRET-based absorbance detection.

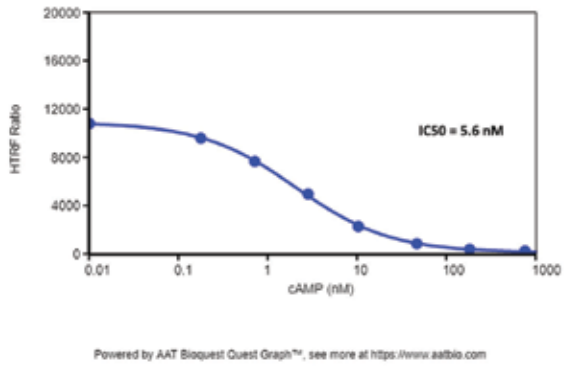


Figure 2.7 cAMP standard curve with CisBio GS Dynamic Kit using HTRF® FRET-based detection.

Our Screen Quest Colorimetric ELISA cAMP Assay Kit also offers a distinct advantage over other assays on the market, namely, the ability to be detected at three different absorbances. Our cAMP assay kit can be read at either 405 nm, 650 nm or 750 nm. No other cAMP assay on the market offers the choice of detection wavelength. Our cAMP assay kit gives researchers the ability to choose the absorbance most suited for their experiment, without any subsequent loss in sensitivity (see figures below, IC50avg ≈ 3 nM). One application may be the selection of a longer wavelength at which to read absorbance (ie. 750 nm). This can help minimize interference from endogenous absorbance signals that typically occurs at <300 nm.

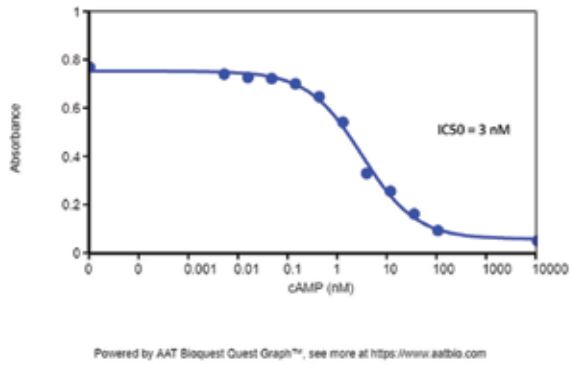


Figure 2.8 cAMP standard curve using Screen Quest™ Colorimetric ELISA cAMP Assay Kit with absorbance reading at 750 nm.

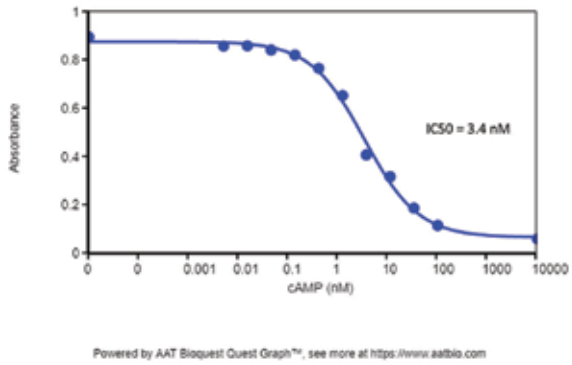


Figure 2.9 cAMP standard curve using Screen Quest™ Colorimetric ELISA cAMP Assay Kit with absorbance reading at 650 nm.

Conclusion

AAT Bioquest's Screen Quest™ Colorimetric ELISA cAMP Assay Kit outperforms assays which require acetylation of cAMP in order to achieve sensitive detection. This allows for a simpler and more controlled protocol. Our cAMP assay also matches or outperforms other detection technologies, such as luminescence and FRET, while offering the distinct advantage of being readable at three different absorbances (405 nm, 650 nm, 750 nm). Based on our comparative data, the Screen Quest™ Colorimetric ELISA cAMP Assay Kit is holistically the best tool for studying cAMP in applications such as drug discovery and GPCR screening.

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4. Goldberg, Melvin L. "Radioimmunoassay for adenosine 3', 5'-cyclic monophosphate and guanosine 3', 5'-cyclic monophosphate in human blood, urine, and cerebrospinal fluid." *Clinical chemistry* 23.3 (1977): 576-580.
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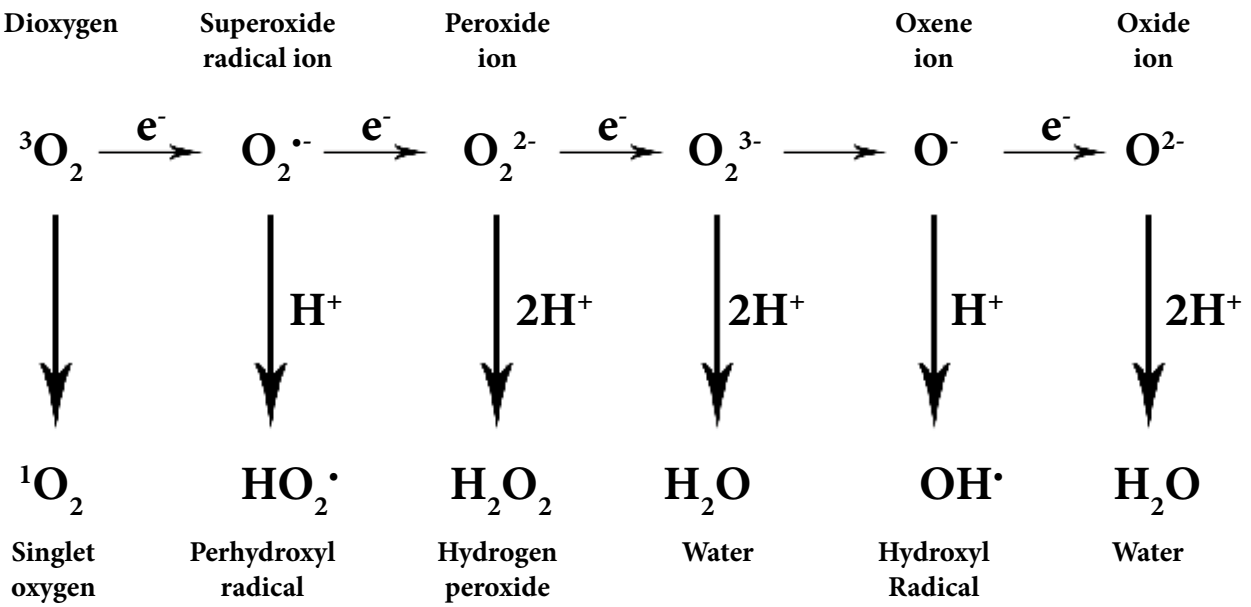
6. Sutherland, Earl W., G. Alan Robison, and Reginald W. Butcher. "Some aspects of the biological role of adenosine 39, 59-monophosphate (cyclic AMP)." *Circulation* 37.2 (1968): 279-306.

Product Ordering Information For Screen Quest™ cAMP Assay Kits

Cat #	Product Name	Unit Size	Excitation (nm)	Emission (nm)
36370	Screen Quest™ Colorimetric ELISA cAMP Assay Kit	1 plate	650	None
36371	Screen Quest™ Colorimetric ELISA cAMP Assay Kit	10 plates	650	None
36373	Screen Quest™ Fluorimetric ELISA cAMP Assay Kit	1 plate	571	585
36374	Screen Quest™ Fluorimetric ELISA cAMP Assay Kit	10 plates	571	585
36379	Screen Quest™ FRET No Wash cAMP Assay Kit	1 plate	390	650
36380	Screen Quest™ FRET No Wash cAMP Assay Kit	10 plates	390	650
36381	Screen Quest™ FRET No Wash cAMP Assay Kit	50 plates	390	650



Selecting the Right ROS Probe

Reactive oxygen species (ROS) are chemically reactive, oxygen-containing chemical species that are generated as byproducts of cellular metabolism. In animal cells, generation of ROS typically occurs in the mitochondria. In plant cells, ROS can also be generated in chloroplasts. More recently, ROS has been shown to be generated in peroxisomes and from plasma membrane oxidases of various cell types.

When generated in excess, ROS has long been thought to result in damage of cellular macromolecules such as DNA, lipids and proteins. Holistically, this is represented by a cell's oxidative stress state. Such states of stress have been linked to cellular processes such as apoptosis, and more macroscopically, play a role in the pathogenesis of many human diseases.

Because of its damaging effects, cells have several carefully regulated systems for managing excess ROS. The most well studied system is the glutathione-ascorbate cycle, which detoxifies H_2O_2 into H_2O, using NADH and NADPH as electron donors. Other systems include enzymes such as superoxide dismutase, which catalyzes the dismutation of the superoxide anion (O_2^-) into O_2 or H_2O_2, and catalase, which catalyzes the decomposition of H_2O_2 into H_2O and O_2.

While ROS has been extensively studied for their detrimental effect on cells, it is only more recently that studies have looked at the role of ROS in cell signaling. In controlled amounts, ROS has been shown to regulate gene activation. The specific mechanism by which this occurs is, however, still up for debate. It is possible that ROS binds to special receptors which initiate a signaling cascade leading up to gene regulation. It is also possible that ROS directly modifies the proteins in such a signaling cascade, perhaps by regulation of protein phosphorylation.

Due to its importance in biological systems, a plethora of tools have been developed to study ROS both and *in vivo*. The table below provides a summary of the most common tools as well as their targets.

Figure 3.1 Reduction of oxygen and its byproducts

Table 3.1 AAT Bioquest® reactive oxygen species (ROS) probes and assay kits.

Target	Application	Tools	Catalog Number	Principle
Hydrogen peroxide (H_2O_2) a product of many enzymatic ROS scavenging pathways. The most well studied is superoxide dismutase activity, which catalyzes the reduction of superoxide anion to hydrogen peroxide.	live cell	DCFH-DA	15204	Probe enters cell wherein esterases cleave off diacetate group. Then DCFH is oxidized by hydrogen peroxide to DCF and emits green fluorescence upon excitation.[4]
		Dihydrorhodamine 123	15206, 15207	Probe passively permeates cell membrane. Oxidation by hydrogen peroxide yields rhodamine 123, which fluoresces blue upon excitation.
		OxiVision™ Blue	11504, 11505	Probe permeates cell and is oxidized by intracellular hydrogen peroxide. Generates fluorescence upon excitation.
		OxiVision™ Green	11503, 11506	
	cell extract; solutions	Amplite™ Fluorimetric Hydrogen Peroxide Assay Kit (Red)	11501	Hydrogen peroxide dependent oxidation of ADHP (synonyms: 10-acetyl-3,7-dihydroxyphenoxazine, Amplex® Red) by horseradish peroxidase (HRP) converts ADHP to resorufin. Resorufin can be detected using colorimetric or fluorimetric methods.
		Amplite™ Colorimetric Hydrogen Peroxide Assay Kit	11500	
		Amplex™ Red Hydrogen Peroxide/ Peroxidase Assay Kit	Not available	
		Amplite™ Fluorimetric Hydrogen Peroxide Assay Kit (Infrared)	11502	Hydrogen peroxide dependent oxidation of Amplite™ IR by horseradish peroxidase (HRP) generates activated probe. Excitation emits a near-infrared fluorescent signal
Superoxide anion (O_2^-) a by-product of aerobic metabolism, such as mitochondrial respiration (particularly Complex I and Complex III).	live cell; cell extract; solutions	Lucigenin	21259	Lucigenin is activated by conversion to lucigenin cation radical. Lucigenin cation radical reacts with superoxide anion to produce dioxetane intermediate, which decomposes to N-methylacridone. High energy electrons in N-methylacridone fall to lower energy state, resulting in luminescence.[5]
		Coelenterazine	21150	Oxidation by superoxide anion results in an excited electron state. Upon decay to ground state electron configuration, photons are released as luminescence.[8]
		Luminol	11050	Superoxide-dependent enzyme catalyzed oxidation of luminol results in luminescence.[6]
		Hydroethidine	15200	Probe passively permeates intact cells and localizes in the mitochondria. Probe is activated through oxidation by superoxide. The activated probe intercalates with DNA and, upon excitation, fluoresces. DNA binding is necessary for strong fluorescence signal. For hydroethidine, activated probe is ethidium (ie. same active species as DNA stain ethidium bromide).[3,7,12]
		MitoSox™ Red	Not available	
		MitoROS™ 580	16052, 22970, 22971	
		MitoROS™ 520	16060	Probe readily passes through intact cell membranes whereupon it localizes in mitochondria. It is then oxidized by superoxide. Upon excitation, it releases a green fluorescence.
Hydroxyl radical (•OH) can be generated when superoxide anions react with transition metals. Extremely reactive. Can react with hydrogen on DNA backbone, resulting in strand breakage.	live cell; cell extract; solutions	MitoROS™ OH580	16055	Probe is able to freely enter live cells wherein it becomes oxidized specifically by free hydroxyl radicals. Upon excitation, oxidized probe fluoresces red.
Total ROS includes hydrogen peroxide, superoxide anion, hydroxyl radical, singlet oxygen, nitric oxide, butyl peroxide and hypochlorous acid	live cell; cell extract; solutions	ROS Brite™ 570	16000, 22902	Probe passively permeates intact cell membranes. Once inside the cell, probe is oxidized by intracellular ROS. Probe can also be oxidized by ROS in solution for cell extract assays. Upon excitation, probe emits a fluorescent signal.
		ROS Brite™ 670	16002, 22901	
		ROS Brite™ 700	16004, 22903	
		Amplite™ ROS Green	22900	

Table 3.2 AAT Bioquest® reactive oxygen species (ROS) probes and assay kits.

Target	Tool	Catalog Number	Principle
SOD dismutase (SOD) catalyzes conversion (ie. dismutation) of superoxide into oxygen and hydrogen peroxide	Amplite™ Colorimetric Superoxide Dismutase (SOD) Assay Kit	11305	First uses xanthine oxidase (XO) to convert xanthine into hydrogen peroxide and uric acid while simultaneously catalyzing the reduction of molecular oxygen (O_2) into superoxide anion (O_2^-). Then uses competitive inhibition of superoxide dismutation by ReadiView™ SOD560 to quantify superoxide dismutase activity. Decrease in absorbance of ReadiView™ SOD560 is directly proportional to SOD activity.
Catalase nearly ubiquitous antioxidant enzyme that converts hydrogen peroxide to water and molecular oxygen	Amplite™ Fluorimetric Catalase Assay Kit (Red)	11306	Competitive inhibition assay. Amplite™ Red probe is activated through oxidation by hydrogen peroxide. Thus, probe competes with catalase for hydrogen peroxidase substrate. Amplite™ Red absorbance is inversely proportional to catalase activity.
Glutathione (GSH) very important redox compound. It is a major component of the glutathione-ascorbate cycle which converts hydrogen peroxide into water	Thiolite™ Green	10055, 22810	Probe becomes activated after reaction with glutathione. Upon excitation, probe releases a green fluorescence.
	Amplite™ Fluorimetric Glutathione GSH/ GSSG Ratio Assay Kit	10056, 10060	Uses Thiolite™ Green probe, which becomes activated after reaction with glutathione (GSH), to quantify glutathione. Oxidized glutathione (GSSG) is determined by measuring GSH concentration before and after enzyme-catalyzed reduction of GSSG to GSH. GSSG concentration is calculated by subtracting initial GSH (before enzyme reaction) from total GSH (after enzyme reaction).
Glutathione peroxidase (GPx) catalyzes the oxidation of GSH to GSSG and the conversion of hydrogen peroxide to water	Amplite™ Fluorimetric Glutathione Peroxidase Assay Kit (Blue)	11560	Enzymatic cycling assay. Glutathione peroxidase (GPx) catalyzes the oxidation of glutathione from GSH to GSSG. Glutathione reductase (GR) then catalyzes the reduction of GSSG back into GSH with the coenzyme NADPH, which is oxidized to $NADP^+$. The Quest Fluor™ NADP Probe then quantifies the level of $NADP^+$ which is directly proportional to the original GPx activity.
Ascorbate also called ascorbic acid or vitamin C, it is a major component of the glutathione-ascorbate cycle which converts hydrogen peroxide to water	Amplite™ Fluorimetric Ascorbic Acid Assay Kit (Blue)	13835	Relies on the dehydrogenation of ascorbic acid to dehydroascorbic acid (DHA) by an enzyme-catalyzed reaction. Resulting DHA is quantified by Ascorbrite™ Blue probe.
NAD^+/NADH major component in ATP synthesis that occurs in mitochondria. This coenzyme is thought to be involved in superoxide production, the rate of which is dependent on the NAD+/ NADH ratio.	Amplite™ NAD/ NADH Kits (assorted)	15273, 15258, 15275, 15280, 15263, 15257, 15261, 15262, 15291, 15290, 15259, 15271	Typically, uses a NAD^+ specific probe to quantify NAD^+ concentration. NAD^+/NADH ratio is determined by enzymatic cycling assay.
NADPH acts as an electron donor in the glutathione-ascorbate cycle that converts hydrogen peroxide to water	Amplite™ NADPH Kits (assorted)	15274, 15272, 15260, 15276, 15264, 15262, 15259, 15291, 15290	Typically, uses a NADPH specific probe to quantify NADPH concentration. NADP/NADPH ratio is determined by enzymatic cycling assay.

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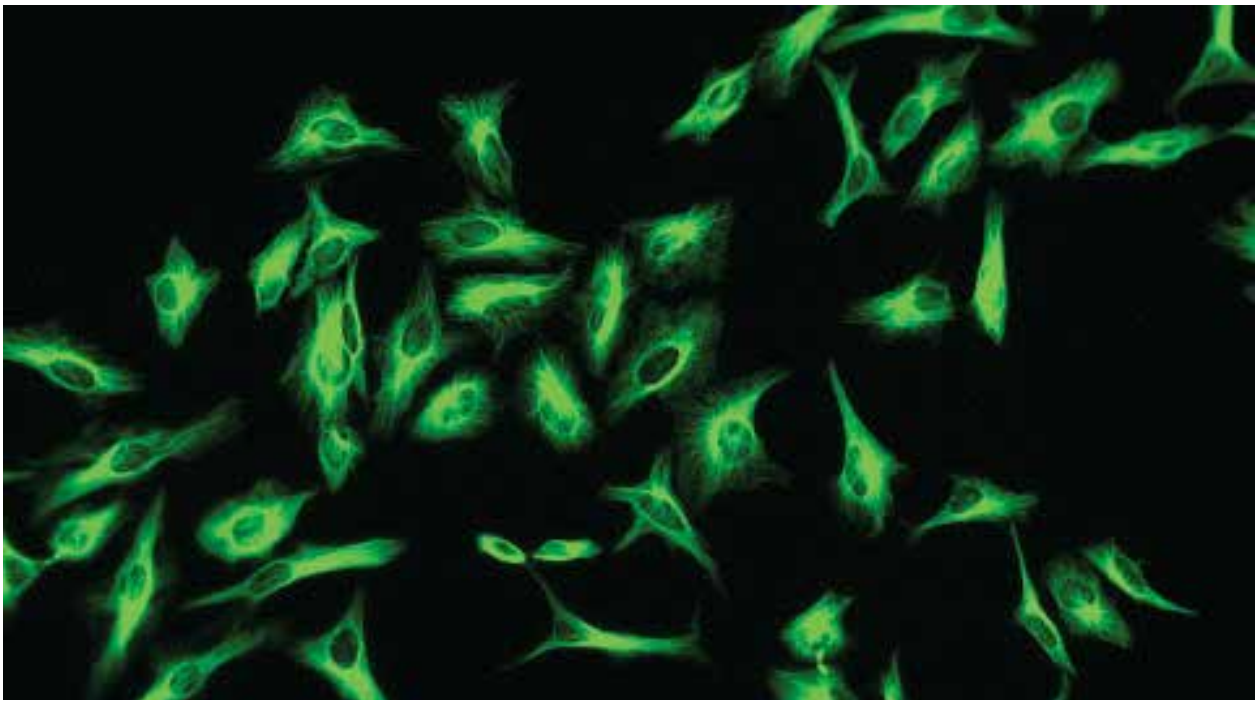
Product Ordering Information For ROS Probes & Assay Kits

Cat #	Product Name	Unit Size	Excitation (nm)	Emission (nm)
15204	DCFH-DA [2',7'-Dichlorodihydrofluorescein diacetate] *CAS 4091-99-0*	25 mg	504	529
15206	Dihydrorhodamine 123 *CAS 109244-58-8*	10 mg	507	529
15207	Dihydrorhodamine 123 *CAS 109244-58-8*	5X1 mg	507	529
11504	Cell Meter™ Intracellular Fluorimetric Hydrogen Peroxide Assay Kit *Blue Fluorescence*	100 Tests	405	450
11505	Cell Meter™ Intracellular Fluorimetric Hydrogen Peroxide Assay Kit *Blue Fluorescence Optimized for Flow Cytometry*	100 Tests	405	450
11503	Cell Meter™ Intracellular Fluorimetric Hydrogen Peroxide Assay Kit *Green Fluorescence*	200 Tests	492	515
11506	Cell Meter™ Intracellular Fluorimetric Hydrogen Peroxide Assay Kit *Green Fluorescence Optimized for Flow Cytometry*	100 Tests	490	530
11501	Amplite™ Fluorimetric Hydrogen Peroxide Assay Kit *Red Fluorescence*	500 Tests	575	590
11500	Amplite™ Colorimetric Hydrogen Peroxide Assay Kit	500 Tests	650	None
11502	Amplite™ Fluorimetric Hydrogen Peroxide Assay Kit *Near Infrared Fluorescence*	500 Tests	647	670
21259	lucigenin [Bis-N-methylacridinium nitrate]	10 mg	455	505
21150	Coelenterazine *CAS#: 55779-48-1*	250 ug	429	466
11050	Luminol [3-Aminophthalhydrazide] *CAS 521-31-3*	1 g	355	411
15200	Hydroethidine [Dihydroethidium] *CAS 38483-26-0*	25 mg	518	605
16052	MitoROS™ 580 *Optimized for Detecting Reactive Oxygen Species (ROS) in Mitochnodria*	500 Tests	510	580
22970	Cell Meter™ Fluorimetric Mitochondrial Superoxide Activity Assay Kit*Optimized for Flow Cytometry*	100 Tests	540	590
22971	Cell Meter™ Fluorimetric Mitochondrial Superoxide Activity Assay Kit*Optimized for Microplate Reader*	200 Tests	540	590
16060	Cell Meter™ Fluorimetric Mitochondrial Superoxide Activity Assay Kit *Green Fluorescence*	200 Tests	509	534
16055	Cell Meter™ Mitochondrial Hydroxyl Radical Detection Kit *Red Fluorescence*	200 Tests	576	598
16000	ROS Brite™ 570 *Optimized for Detecting Reactive Oxygen Species (ROS)*	1 mg	556	566
22902	Cell Meter™ Fluorimetric Intracellular Total ROS Activity Assay Kit*Orange Fluorescence*	200 Tests	556	566
16002	ROS Brite™ 670 *Optimized for Detecting Reactive Oxygen Species (ROS)*	1 mg	658	675
22901	Cell Meter™ Fluorimetric Intracellular Total ROS Activity Assay Kit*Red Fluorescence*	200 Tests	520	605
16004	ROS Brite™ 700 *Optimized for in Vivo Imaging*	1 mg	680	706
22903	Cell Meter™ Fluorimetric Intracellular Total ROS Activity Assay Kit*Deep Red Fluorescence*	200 Tests	658	675
22900	Cell Meter™ Fluorimetric Intracellular Total ROS Activity Assay Kit*Green Fluorescence*	200 Tests	492	520
11305	Amplite™ Colorimetric Superoxide Dismutase (SOD) Assay Kit	1 kit	560	None
11306	Amplite™ Fluorimetric Catalase Assay Kit *Red Fluorescence*	1 kit	571	585
10055	Amplite™ Fluorimetric Glutathione Assay Kit *Green Fluorescence*	200 Tests	510	524
22810	Cell Meter™ Intracellular GSH Assay Kit *Optimized for Flow Cytometry*	1 kit	490	515

Product Ordering Information For ROS Probes & Assay Kits

Cat #	Product Name	Unit Size	Excitation (nm)	Emission (nm)
10056	Amplite™ Fluorimetric Glutathione GSH/GSSG Ratio Assay Kit *Green Fluorescence*	200 Tests	510	524
10060	Amplite™ Rapid Fluorimetric Glutathione GSH/GSSG Ratio Assay Kit *Green Fluorescence*	200 Tests	510	524
11560	Amplite™ Fluorimetric Glutathione Peroxidase Assay Kit *Blue Fluorescence*	200 Tests	420	480
13835	Amplite™ Fluorimetric Ascorbic Acid Assay Kit	200 Tests	340	430
15273	Amplite™ Colorimetric NAD/NADH Ratio Assay Kit	250 Tests	460	None
15258	Amplite™ Colorimetric Total NAD and NADH Assay Kit	400 Tests	575	None
15275	Amplite™ Colorimetric Total NAD and NADH Assay Kit *Enhanced Sensitivity*	400 Tests	460	None
15280	Amplite™ Fluorimetric NAD Assay Kit *Blue Fluorescence*	200 Tests	422	466
15263	Amplite™ Fluorimetric NAD/NADH Ratio Assay Kit *Red Fluorescence*	250 Tests	571	585
15257	Amplite™ Fluorimetric Total NAD and NADH Assay Kit *Red Fluorescence*	400 Tests	571	585
15261	Amplite™ Fluorimetric NADH Assay Kit *Red Fluorescence*	400 Tests	571	585
15291	Cell Meter™ Intracelluar NADH Flow Cytometric Analysis Kit	100 Tests	540	590
15290	Cell Meter™ Intracelluar NADH Fluorescence Imaging Kit	100 Tests	540	590
15259	Amplite™ Fluorimetric Total NADP and NADPH Assay Kit *Red Fluorescence*	400 Tests	571	585
15271	Amplite™ Colorimetric NADH Assay Kit	400 Tests	460	None
15274	Amplite™ Colorimetric NADP/NADPH Ratio Assay Kit	250 Tests	460	None
15272	Amplite™ Colorimetric NADPH Assay Kit	400 Tests	460	None
15260	Amplite™ Colorimetric Total NADP and NADPH Assay Kit	400 Tests	575	None
15276	Amplite™ Colorimetric Total NADP and NADPH Assay Kit *Enhanced Sensitivity*	400 Tests	460	None
15264	Amplite™ Fluorimetric NADP/NADPH Ratio Assay Kit *Red Fluorescence*	250 Tests	571	585
15262	Amplite™ Fluorimetric NADPH Assay Kit *Red Fluorescence*	400 Tests	571	585
15281	Amplite™ Fluorimetric NADP Assay Kit *Blue Fluorescence*	200 Tests	422	466



Quantitative Comparison of iFluor™ 488- and FITC-Conjugated Antibodies For Use In Cell Labeling

The use of fluorophore labeled antibodies has been a powerful tool for the study of biological systems. These fluorophore-antibody conjugates have found important applications in ELISA, Western blotting, FACS and fluorescence microscopy. Their importance is attributed to the sensitivity with which they detect a substrate and the ease by which that detection event can be translated into a quantifiable signal (ie. fluorescence). Because of their importance in biological assays, a great deal of research has been conducted to identify and synthesize the ideal fluorophore for a given application. While subject to some debate, ideal fluorophores typically possess one or more of the following qualities: (1) strong fluorescence as characterized by a high extinction coefficient and quantum yield (2) high photostability and resistance to photobleaching (3) resilience under different environmental conditions, such as pH (4) minimal background interference or auto-fluorescence and (5) simplicity and consistency in experimental usage.

Here, we describe a novel fluorophore for cell labeling applications, iFluor™ 488. This fluorophore is part of the iFluor™ family that spans the spectrum from ultra-violet to infrared. iFluor™ 488 absorbs light at 491 nm and emits at 518 nm, making it spectrally similar to the conventional fluorophore fluorescein isothiocyanate (FITC), which absorbs light at 495 nm and emits at 521 nm. However, iFluor™ 488 is far superior to FITC for cell labeling applications, particularly when comparing fluorescence intensity and photostability. In the present study, iFluor™ 488 and FITC were conjugated to goat anti-mouse polyclonal antibodies (GAM). The resulting conjugates were used to perform cell stains for fluorescence microscopy. Comparisons were made for fluorescence intensity, degree of labeling (ie. the number of fluorophores per antibody) and photostability.

Figure 4.1 Fluorescence image of HeLa cells stained with iFluor™ 488-goat anti-mouse IgG conjugate (Green, Cat#16448).

Materials and Methods

Conjugation of FITC and iFluor™ 488 to GAM IgG

FITC and iFluor™ 488 (AAT Bioquest, Sunnyvale, CA) were conjugated to goat anti-mouse IgG (AAT Bioquest, Sunnyvale, CA) according to manufacturer's specifications. Final bioconjugates were purified using size exclusion spin columns (Bio-rad, Hercules, CA).

Determination of degree of labeling (DOL)

Degree of labeling was determined using absorbance measurements and Degree of Labeling Calculator (https://www.aatbio.com/tools/degree-of-labeling-calculator/, AAT Bioquest, Sunnyvale, CA).

Preparation of fixed cells

HeLa cells were seeded in a 96-well black wall/clear bottom plate (10,000 cells/well) in culture medium overnight. Growth medium was removed, cells were washed once with phosphate buffered saline (PBS) and then PBS was aspirated. 100 µL 4% paraformaldehyde in PBS (pH 7.4) was added to each well and incubated with cells for 15 minutes at room temperature to fix the cells. Cells were then washed with 200 µL PBS/well for three times. After fixation, 100 µL of blocking solution (PBS + 1% BSA) was added to each well to block for 30 minutes at room temperature. Blocking solution was then replaced with cell permeabilization buffer (PBS + 1% BSA + 0.1% TritonX-100 cells) and cells were incubated for 15 minutes at room temperature. Cells were then washed with 200 µL PBS/well for three times. 200 µL PBS was added to each well and the plate was stored at 4˚C until staining.

Tubulin monoclonal antibody staining

Storage PBS buffer in each well was aspirated and 1 µg/mL alpha tubulin monoclonal antibody (Thermo Fisher, Waltham, MA) in PBS + 0.02% Tween-20 was added and incubated at room temperature for 30 minutes. For experimental control, cells were stained with 1 µg/mL mouse IgG. After incubation, cells were washed with 200 µL PBS/well for three times.

GAM IgG-FITC and GAM IgG-iFluor™ 488 staining

10 µg/mL of previously prepared GAM IgG-FITC was added to wells stained with alpha tubulin monoclonal antibody or stained with mouse IgG (control) for 30 minutes at room temperature. Cells were then washed with 200 µL PBS/well for three times. After washing, 100µL PBS was added to each well. The same procedure was repeated for GAM IgG-iFluor™ 488. Cells were imaged using a Keyence X710 fluorescence microscope with FITC channel.

Test of photobleaching and photostability

Cells were continuously illuminated using a Keyence X710 fluorescence microscope. Images of the cells were captured and saved every 2.5 seconds for the duration of 300 seconds. Final intensity values were determined using ImageJ software's integrated density (National Institutes of Health, Bethesda, MD).

Results

Degree of labeling (DOL)

The degree of labeling (DOL) was calculated using AAT Bioquest's Degree of Labeling Calculator (https://www.aatbio.com/tools/degree-of-labeling-calculator/). The DOL represents the average moles of fluorophore per mole of antibody. For GAM IgG-FITC, the DOL was determined to be 4.5 fluorophores/antibody. For GAM IgG-iFluor™ 488, the DOL was determined to be 6.5 fluorophores/antibody.

Photostability

Measurements of total fluorescence intensity (TFI) showed that HeLa cells labeled with GAM IgG-FITC had an initial intensity of 449.33 units, as determined by ImageJ analysis. After 300 seconds of exposure (5 minutes), the TFI was 144.51 units. This represented a 68% decrease in TFI.

Measurements of TFI showed that HeLa cells labeled with GAM IgG-iFluor™ 488 had an initial intensity of 1213.87 units, as determined by ImageJ analysis. After 300 seconds of exposure (5 minutes), the TFI was 885.29 units. This represented a 27% decrease in TFI.

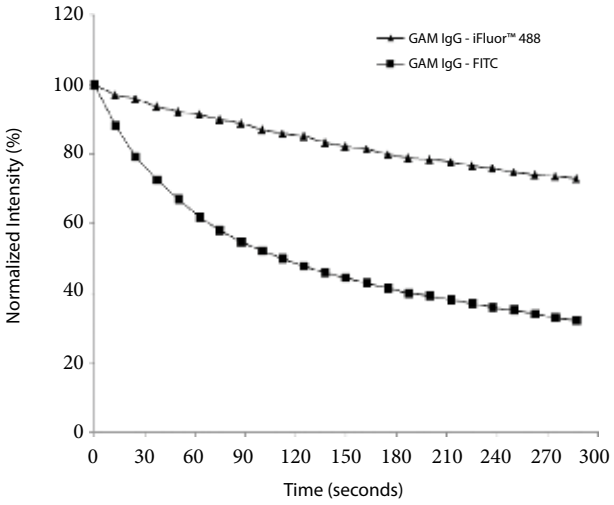


Figure 4.2 Comparison of fluorescence intensity between HeLa cells labeled with GAM IgG-FITC and GAM IgG-iFluor™ 488.

Discussion

One of the key motivators for this experiment was to determine if iFluor™ 488 had the potential to replace FITC as a green fluorescent dye for cell labeling applications. From this experiment, it was shown that iFluor™ 488 had much better photostability compared to FITC when conjugated to IgG and used to label tubulin. The fluorescence intensity loss of FITC was more than double that of iFluor™ 488 (68% vs 27%) over the same period of time. Thus, for applications which require photostable dyes or long exposure times, iFluor™ 488 is clearly superior.

Another quality to note is the dramatic difference in fluorescence intensity when comparing FITC labeled cells to iFluor™ 488 labeled cells. In captured images shown in Figure 2, iFluor™ 488 appears much "brighter". This notion is supported by the intensity values, which demonstrates significantly greater intensity values for iFluor™ 488 than for FITC. This stark contrast was perhaps most evident in the image slice taken at 300 seconds, wherein the FITC labeled cells were barely visible whereas the iFluor™ 488 labeled cells were still clearly visible.

Conclusion

In a direct comparison of FITC and iFluor™ 488, it was shown that iFluor™ 488 has a superior fluorescence intensity. Furthermore, iFluor™ 488 is significantly less susceptible to photobleaching, allowing for stained cells to be detectable for a much longer period of time. Based on these findings, iFluor™ 488 can serve as a superior alternative to FITC for cell labeling applications.

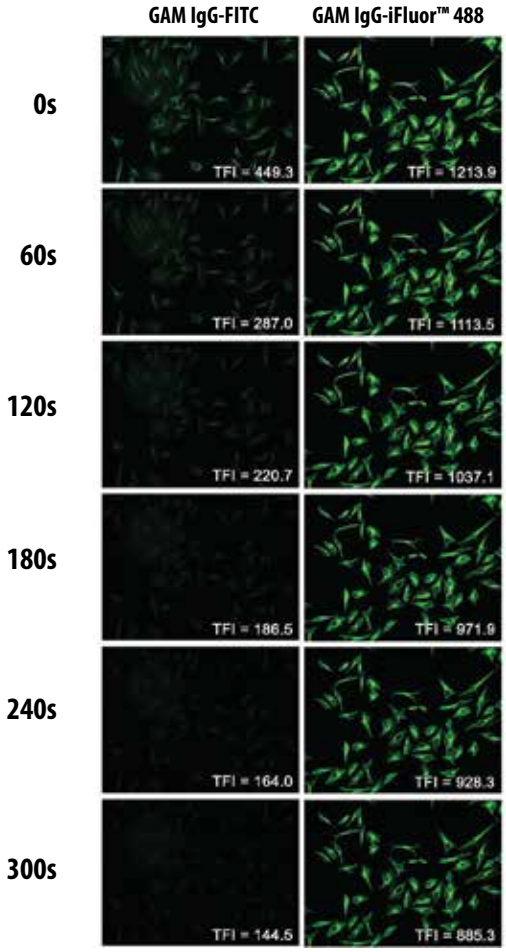
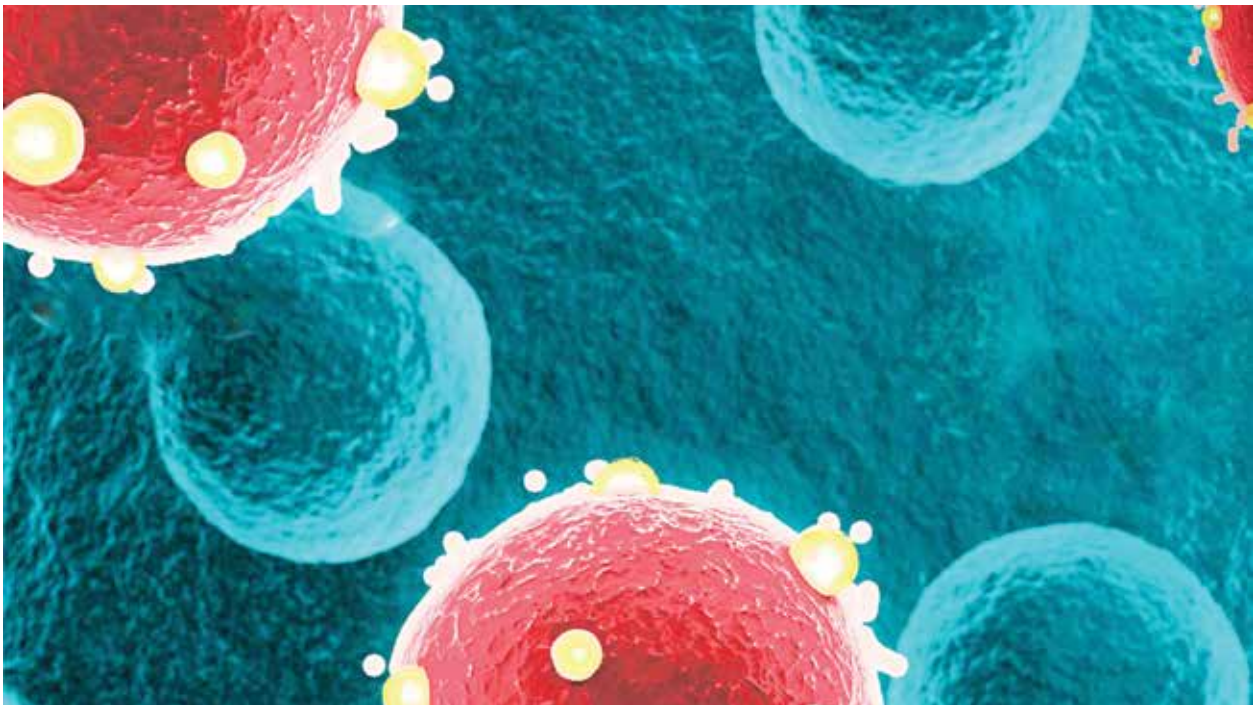


Figure 4.3 Images of HeLa cells captured using Keyence X710 fluorescence microscope. TFI represents total fluorescence intensity as calculated by ImageJ software.

Product Ordering Information For iFluor™ 488 & FITC - Labeled Secondary Antibody Conjugtaes

Cat #	Product Name	Unit Size	Excitation (nm)	Emission (nm)
16852	FITC goat anti-mouse IgG (H+L)	1 mg	492	515
16860	FITC goat anti-mouse IgG (H+L) *Cross Adsorbed*	1 mg	492	515
16868	FITC goat anti-rabbit IgG (H+L)	1 mg	492	515
16876	FITC goat anti-rabbit IgG (H+L) *Cross Adsorbed*	1 mg	492	515
1072	iFluor™ 488 amine	1 mg	491	514
1000	iFluor™ 488 azide	1 mg	491	518
16735	iFluor™ 488 goat anti-mouse IgG (H+L)	1 mg	491	514
16773	iFluor™ 488 goat anti-mouse IgG (H+L) *Cross Adsorbed*	1 mg	491	514
16800	iFluor™ 488 goat anti-rabbit IgG (H+L)	1 mg	491	514
16828	iFluor™ 488 goat anti-rabbit IgG (H+L) *Cross Adsorbed*	1 mg	491	514
1082	iFluor™ 488 hydrazide	1 mg	491	514
1062	iFluor™ 488 maleimide	1 mg	491	518
1023	iFluor™ 488 succinimidyl ester	1 mg	491	518
11060	iFluor™ 488 tyramide	1 mg	491	514



A Practical Guide For Use of PE and APC In Flow Cytometry

Flow cytometry and fluorescence activated cell sorting (FACS) have enabled an unprecedented level of analysis in cell biology. With these tools, entire cell populations can be screened and characterized on a cell-by-cell basis for specific, biologically-significant traits. It has become trivial to determine the percentage of viable cells in a given population, or to quantify the presence of a particular protein or marker. Indeed, flow cytometry provides a degree of specificity unachievable by traditional microplate reader platforms and with a practicality not afforded to microscopy. But perhaps the greatest advantage of flow cytometry is the ease by which multi-factor analysis can be accomplished.

Since its inception, researchers have been interested in flow cytometry's capability for multi-factor analysis. Due to the nature of flow cytometry (ie. single-cell analysis), it becomes possible to co-monitor several cellular events simultaneously within any given cell. For example, a cell can be screened for both apoptosis and surface marker expression. Because many biological processes are co-dependent and occur in tandem, the ability to study multiple variables in parallel is clearly advantageous.

In flow cytometry, multi-factor analysis, or multiplexing, is accomplished through the use of several fluorescent probes simultaneously. These fluorescent probes differ in their "color", that is, the wavelength at which they emit fluorescent light. Fluorescent probes are typically chosen for their brightness, expressed as the stain index, and their spectral separation from other probes in the multiplex panel. Of potential fluorophores, PE and APC have found great success in flow cytometry, both due to their individual brightness as well as their ability to create spectral separation when implemented as tandem dyes.

Figure 5.1 Representation of two covalently attached fluorescent molecules that make up a tandem dye. One fluorescent molcule serves as the donor and the other as an acceptor. A tandem dye behaves as a unique fluorophore with excitation properties of the donor and the emission properties of the acceptor.

PE & APC

Phycoerythrin (PE) and allophycocyanine (APC) are members of a protein family called phycobiliproteins. This family of proteins is present in photosynthetic organisms, such as algae and cyanobacteria. In nature, they typically act as accessory proteins to chlorophyll pigments in photosynthesis. Structurally, phycobiliproteins are comprised of a protein complex covalently bound to phycobilins, the part which captures light energy. For use in flow cytometry, PE and APC are extracted from red algae and purified.

Phycoerythrin (PE) is available in two forms: R-PE and B-PE, though R-PE has found more use in flow cytometry (because of this, the shorthand PE is often used interchangeably with R-PE). PE has a molecular weight of 240,000 Daltons, with an extinction coefficient of 1,500,000 cm-1 M-1 and a quantum yield (Φ) of 0.84. PE is maximally excited at 565 nm (with a secondary maximum at 495 nm) and emits at 573 nm, which is in the yellow-orange region of the visible spectrum. For comparison, free Cy3, which also fluoresces in the yellow-orange spectrum region, has an extinction coefficient of 150,000 cm-1 M-1 and a quantum yield of 0.20 (Φ ≈ 0.40 when bound to DNA). Thus, compared to Cy3, PE is much "brighter". In fact, PE is, across most experimental conditions, the brightest fluorophore available for flow cytometry.

Allophycocyanine (APC) is a 105 kDa protein, with an extinction coefficient of 700,000 cm-1 M-1 and a quantum yield (Φ) of 0.68. APC is maximally excited at 652 nm (with a secondary maximum at 625 nm) and emits at 658 nm. Its fluorescence falls in the red region of the visible spectrum. Compared to PE, APC is not as "bright". This is due in part to its lower extinction coefficient and quantum yield. It is also because APC is red-shifted compared to PE. Thus, the light source used to maximally excite APC will contain less energy than the one used to maximally excite PE. Compared to other red fluorophores such as Cy5, however, APC is still significantly brighter and an excellent choice for use in flow cytometry.

Preparation of PE and APC For Use In Flow Cytometry

In order to be used for flow cytometry, PE or APC must first be conjugated to an antibody. The antibody serves as the detector for the target of interest, while the PE or APC acts as the reporter which translates the detected event into a quantifiable signal. Traditional methods of PE or APC conjugation to antibodies involve activation of PE or APC with SMCC. While not detailed here, the general process is as follows:

1. Activate the reporter (PE or APC) with SMCC
2. Reduce the detector (antibody or protein) with DTT
3. Mix activated reporter with reduced detector
4. Purify bioconjugate using dialysis

Though an established method, SMCC-based conjugation does suffer from several drawbacks. First, the conjugation efficiency is typically low, with a good yield resting at approximately 30% recovery. A second drawback is the need to reduce the antibodies with DTT, which may significantly impact their function. Finally, because the efficiency of SMCC-based reactions are typically low, very high concentrations of reactants (ie. PE and antibody) are required for adequate reactivity.

There are several alternatives to SMCC chemistry. These alternatives typically rely on first conjugating the reporter and detector independently, each with a different tag. These tags are typically chosen for their high affinity and specificity for each other and only each other, such that when the tagged reporter and detector are

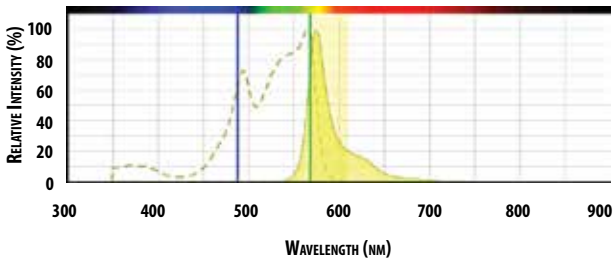


Figure 5.2 Absorption and emission spectrum of PE (Cat#2556). Exciations at 488 nm (Blue laser line) and 568 nm (Green laser line). PE read with emission filter Cy3/TRITC (Yellow band).

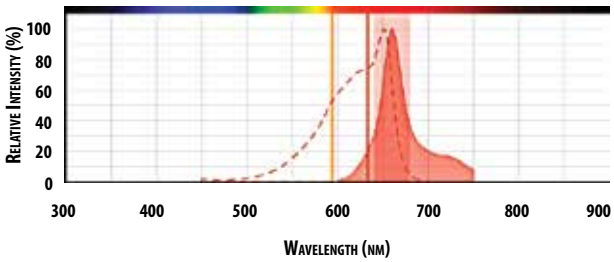


Figure 5.3 Absorption and emission spectrum of APC (Cat#2554). Exciations at 594 nm (Orange laser line) and 633 nm (Red laser line). PE read with emission filter Cy5 (Red band).

mixed, the tags bind, forming a bioconjugate between the reporter and the detector.

One fairly established usage of this type of tagging is the biotin-streptavidin conjugation system. In this system, an antibody is tagged, or labeled, with biotin. The detector, for example PE, is then labeled with streptavidin. Because of biotin's and streptavidin's high affinity for each other, they form a strong covalent bond, ultimately resulting, in this case, in an antibody-PE bioconjugate.

A second alternative to SMCC chemistry is AAT Bioquest's Buccutite™ technology. Similar to the biotin-streptavidin conjugation system, Buccutite™ technology relies on two separate tags: MTA and FOL. These two tags are independently labeled onto the reporter and detector, respectively, and when mixed will bind strongly to each other, resulting in a reporter-detector bioconjugate. Compared to other conjugation chemistries, Buccutite™ offers several unique advantages. First, Buccutite™ reactions typically have much higher yields, with final recovery more than double (>60%) that of SMCC methods. Moreover, because the reaction between MTA and FOL is very efficient, reactions can occur at very low concentrations of reactants (ie. <1 mg/mL of antibody).

Usage of PE and APC in flow cytometry

While the actual experimental procedure will differ depending on the experiment, there are some general guidelines to follow when using PE and APC for flow cytometry.

1. Determine the equipment specifications

The successful usage of PE and APC depends on the available laser and filter sets of the flow cytometer. For PE, this is typically a non-issue. This is because, while PE has a maximal excitation at 565 nm, it also has a secondary maximum at 495 nm. Thus, if the 568 nm krypton laser is not available, the extremely common 488 argon laser will suffice. For emission filter selection, PE can be read using the Cy3/TRITC filter (590/40).

For APC, the excitation source can vary depending on the available lasers. The 594 nm or 633 nm laser line are suitable. For emission filters, the Cy5 filter (660/40) is recommended.

2. Determine if compensation is necessary

For multicolor analysis, compensation may be required before analyzing data. This is because two or more fluorophores may have overlapping emission spectra. In such cases, any signal read may in fact be due to the combination of multiple individual signals. Compensation accounts for this overlap by mathematically removing signals that "spillover" into the main signal of interest. Consider the following flow cytometry panel:

Here, there is a portion of spectral overlap between the PE emission and the propidium iodide (PI) emission. To account for, and remove the signal overlap, is the basis of compensation. Most modern instrumentation and software can automatically perform compensation if given the control parameters. In the case of the above example, this would entail first performing a run with just PE stained cells, then performing a run with just PI stained cells, before finally performing the two-color analysis.

While modern technology has made the compensation process much easier, choosing the right fluorophore can also minimize the need to perform compensation to begin with. Here, advancements in tandem dyes play a big role.

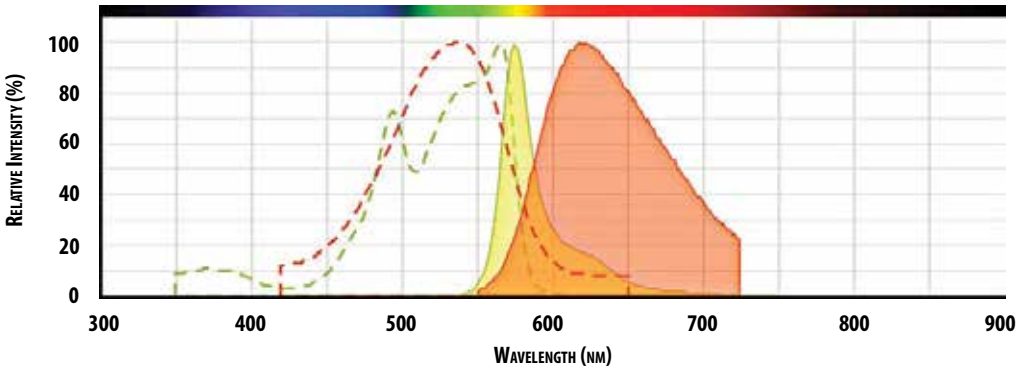


Figure 5.4 Representation of overlapping emission spectra of PE (Yellow, Cat#2556) and PI (Red, Cat#17516). The portion of spectral overlap between the PE emission and PI emission is represented by the orange shaded region between the yellow and red curves. Multicolor analysis may require compensation prior to data analysis. Compensation accounts for this overlap by removing signals that "spillover" into the main signal of interest.

Use as tandem dyes

Tandem dyes refer to compounds in which two different fluorophores have been covalently linked. In this conformation, the excitation of one fluorophore (termed the "donor") results in the transfer of energy to, and the fluorescing of, the other (termed the "acceptor"). This transfer of energy from donor to acceptor occurs through Förster resonance energy transfer, or FRET. Though not elaborated upon here, the mechanism of FRET and its application in biological probes is well documented. In particular, FRET has been applied very successfully in studies of nucleotides and peptides. More recently, however, there has been an explosion of interest in FRET-based flow cytometry applications, particularly with PE and APC based tandem dyes.

The reason PE and APC based tandem dyes are particularly useful in flow cytometry is because of their unusually large Stoke's shifts. The Stoke's shift refers to the distance between the maximal excitation and the maximal emission. For a typical fluorophore, this difference is small, ranging between 10 to 50 nm on average. PE and APC based tandem dyes can achieve Stoke's shifts in the hundreds, depending on the fluorophore pair used. The large Stoke's shifts of tandem dyes is extremely valuable when design multicolor flow cytometry panels.

Consider the following three flow cytometry panels:

Panel A has good spectral separation amongst the three fluorophores; the three emission peaks are fairly far apart. This means that minimal compensation is required. However, because the three fluorophores have varying excitation maximums, an optimal experimental setup will require three different laser sources. This in practice is both expensive and inconvenient to set up.

Panel B has three fluorophores that can all be well excited by a singular laser source. In this case, the three fluorophores can all be excited by the extremely common 488 nm argon-ion laser. Thus in practice, the instrumentation for such a panel is fairly straightforward. However, panel B suffers from extensive spectral overlap; the emission peaks are too close. Such a panel will require very complex compensation calculations, if such calculations can be done at all.

Panel C demonstrates the power of tandem dyes (PE-Cy5 and PE-Cy7). In this panel, all three fluorophores can be well excited by the same laser line, as in panel B. However, unlike panel B, the three fluorophores demonstrate excellent spectral separation. This, as mentioned prior, is due to the large Stoke's shifts of the tandem dyes (171 nm and 283 nm). In panel C, the advantage of tandem dyes becomes evident, namely, tandem dyes allow for the use of multiple fluorophores that share an excitation maximum but do not require extensive compensation.

Panel A

Fluorophore	Excitation (nm)	Emission (nm)	Stoke's shift (nm)
FITC	489	515	26
Cy5	554	568	14
Cy7	650	669	19

Panel B

Fluorophore	Excitation (nm)	Emission (nm)	Stoke's shift (nm)
FITC	489	515	26
GFP	489	508	19
Alexa Fluor® 488	499	520	21

Panel C

Fluorophore	Excitation (nm)	Emission (nm)	Stoke's shift (nm)
FITC	489	515	26
PE-Cy5	495	666	171
PE-Cy7	495	778	283

Preparation of PE and APC tandem dyes

The common method of synthesizing tandem dyes is through use of succinimidyl esters (also called NHS esters). This is a common method to label small molecule fluorophores onto proteins and antibodies. In this method, the small molecule fluorophore becomes reactive due to the addition of the succinimidyl ester (SE) functional group. The fluorophore-SE then reacts to free amine groups on PE or APC, forming a covalent bond. Once the PE or APC tandem dye is synthesized, it can then be conjugated to an antibody using SMCC or related chemistries as mentioned previously.

Reactive succinimidyl esters of common small molecule fluorophores such as FITC, Cy dyes, Alexa Fluors and iFluors can be directly purchased. For convenience purposes, conjugated tandem dyes (such as PE-Cy5) and activated, conjugated tandem dyes (such as Buccutite™ PE-Cy5) are also available for purchase.

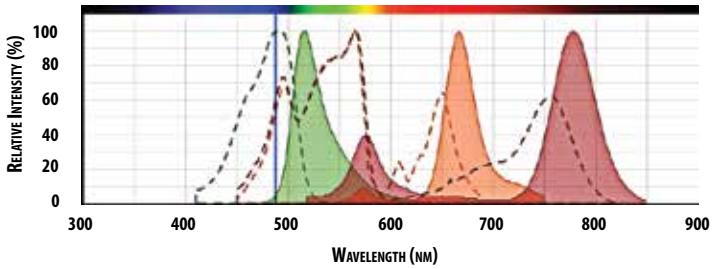


Figure 5.5 Abosrption and emission spectra of FITC (Green), PE-Cy5 (Orange) and PE-Cy7 (Red). All three fluorophores can be well excited by the 488 nm laser line (Blue) with excellent spectral seperation.

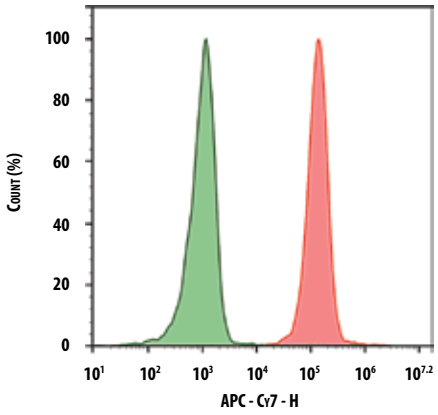


Figure 5.6 Flow cytometry analysis of HL-60 cells stained with 1ug/ml Mouse IgG2a control (Green) or with 1ug/ml Anti-Human HLA-ABC (Red) and then followed by Goat Anti-Mouse IgG-APC-Cy7 conjugate prepared with Buccutite™ Rapid APC-Cy7 Tandem Antibody Labeling Kit (Cat#1321).

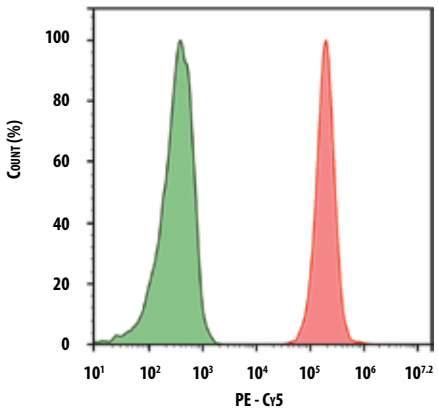


Figure 5.7 Flow cytometry analysis of HL-60 cells 1ug/ml stained with Mouse IgG2a Control or with 1ug/ml Anti-Human HLA-ABC and then followed by Goat Anti-Mouse IgG-PE-Cy5 conjugate prepared with Buccutite™ Rapid PE-Cy5 Tandem Antibody Labeling Kit (Cat#1315).

Table 5.1 Some common PE and APC tandem dyes

Tandem Dye	Excitation (nm)	Emission (nm)	Stoke's shift (nm)
PE-Cy5	495	666	171
PE-Cy5.5	495	672	177
PE-Cy7	495	778	283
PE-Alexa Fluor 647	495	666	171
PE-iFluor™ 700	495	722	227
PE-Texas Red	495	613	118
PE-Alexa Fluor 750	495	779	284
APC-Cy5.5	625	700	75
APC-Cy7	625	779	154
APC-Alexa Fluor 750	625	785	160
APC-iFluor™ 700	625	710	85
APC-iFluor™ 750	625	791	166

Additional Resources

The Spectrum Viewer is a web-based tool for comparing a comprehensive library of fluorophores. This proves useful when designing flow cytometry panels. It can be used to determine if two fluorophores have spectral overlap and if compensation will be required. It can also be used to determine which of similar fluorophores is most suited for an experimental instrumentation.

The Degree of Labeling Calculator is a web-based tool used for determining the number of dyes conjugated to a particular protein. The DOL is an important quantity when trying to optimize an experiment. Typically, too many dyes labeled onto a protein will result in a quenching effect, reducing the fluorescence of the conjugate. On the other hand, too few dyes will also result in decreased fluorescence intensity. The DOL Calculator can be used to monitor the optimal conjugation ratio.

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Product Ordering Information For APC, PE and Tandem Dyes

Cat #	Product Name	Unit Size	Excitation (nm)	Emission (nm)
2554	APC [Allophycocyanin]	1 mg	651	662
2622	APC-Cy5.5 Tandem	1 mg	651	700
2625	APC-Cy7 Tandem	1 mg	651	780
1311	Buccutite™ Rapid APC Antibody Labeling Kit *Microscale Optimized for Labeling 100 ug Antibody Per Reaction*	2 Labelings	651	662
1320	Buccutite™ Rapid APC-Cy5.5 Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 100 ug Antibody Per Reaction*	2 Labelings	651	700
1350	Buccutite™ Rapid APC-Cy5.5 Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 25 ug Antibody Per Reaction*	2 Labelings	651	700
1321	Buccutite™ Rapid APC-Cy7 Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 100 ug Antibody Per Reaction*	2 Labelings	651	780
1351	Buccutite™ Rapid APC-Cy7 Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 25 ug Antibody Per Reaction*	2 Labelings	651	780
1319	Buccutite™ Rapid APC-iFluor™ 700 Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 100 ug Antibody Per Reaction*	2 Labelings	651	713
1347	Buccutite™ Rapid APC-iFluor™ 700 Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 25 ug Antibody Per Reaction*	2 Labelings	651	713
1310	Buccutite™ Rapid PE Antibody Labeling Kit *Microscale Optimized for Labeling 100 ug Antibody Per Reaction*	2 Labelings	565	575
1340	Buccutite™ Rapid PE-Cy5 Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 25 ug Antibody Per Reaction*	2 Labelings	565	674
1316	Buccutite™ Rapid PE-Cy5.5 Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 100 ug Antibody Per Reaction*	2 Labelings	565	700
1341	Buccutite™ Rapid PE-Cy5.5 Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 25 ug Antibody Per Reaction*	2 Labelings	565	700
1317	Buccutite™ Rapid PE-Cy7 Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 100 ug Antibody Per Reaction*	2 Labelings	565	780
1342	Buccutite™ Rapid PE-Cy7 Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 25 ug Antibody Per Reaction*	2 Labelings	565	780
1318	Buccutite™ Rapid PE-Texas Red Tandem Antibody Labeling Kit *Microscale Optimized for Labeling 100 ug Antibody Per Reaction*	2 Labelings	565	600
2558	PE [R-Phycoerythrin] *CAS 11016-17-4*	1 mg	565	575
2610	PE-Cy5 Tandem	1 mg	565	670
2613	PE-Cy5.5 Tandem	1 mg	565	700
2616	PE-Cy7 Tandem	1 mg	565	780
2619	PE-Texas Red Tandem	1 mg	565	600

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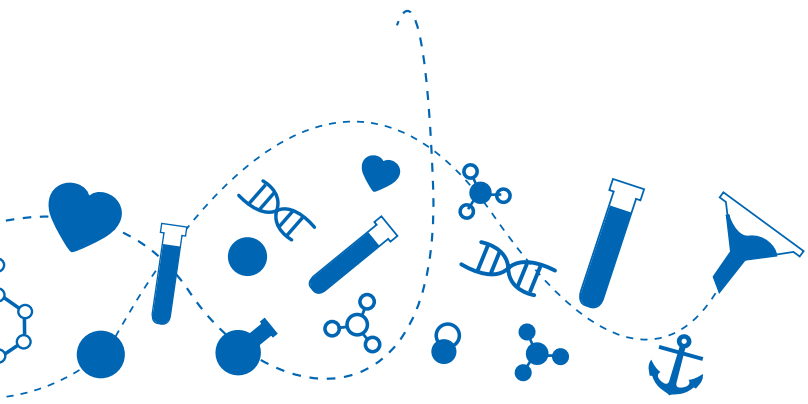

Notes

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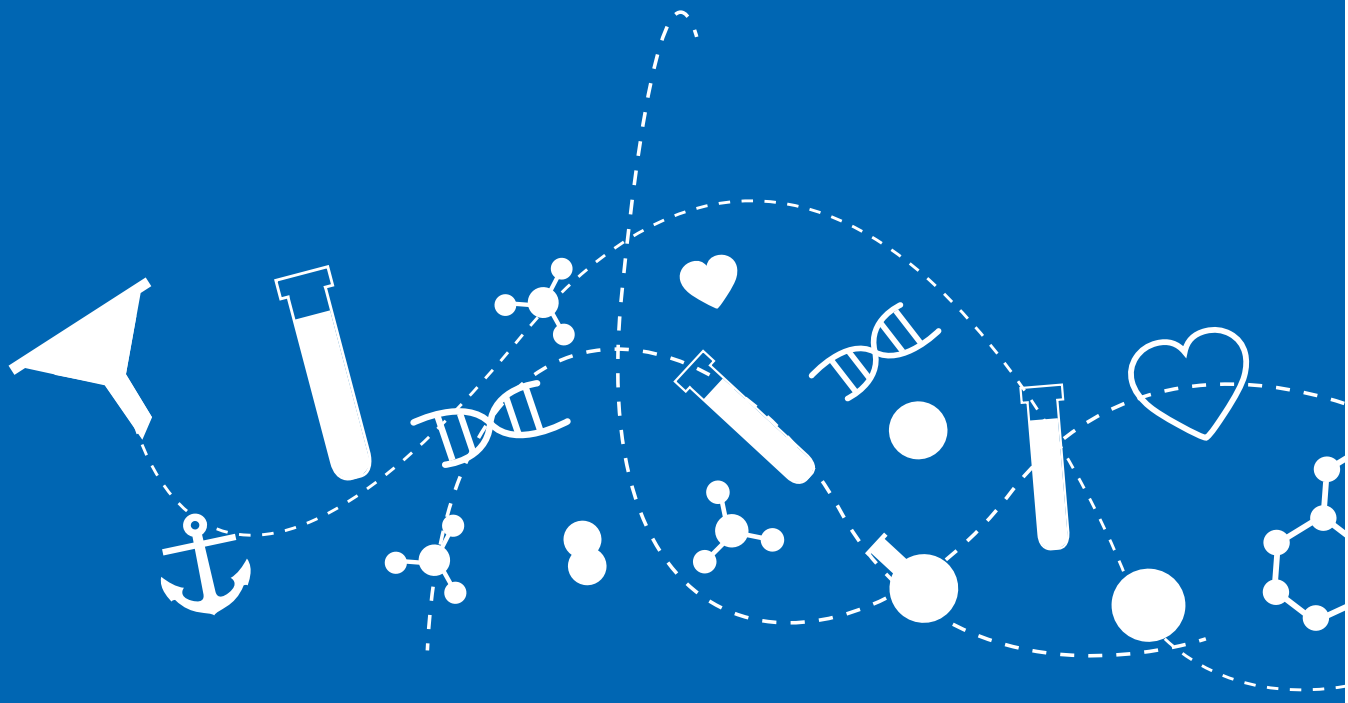

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