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# AssayWise Letters

Buccutite™ Crosslinking Technology
NAADP-AM

# Table of Contents

# Buccutite™ Crosslinking Technology
## A Novel Technology for Protein-Protein Conjugations

### Crosslinkers for Bioconjugation

Crosslinkers are a family of compounds that can be used to join together two or more macromolecules, such as proteins. The modification of proteins through crosslinking techniques provides analysis and insight into complex protein interactions as well as interaction domains. Crosslinking techniques are also an important pre-requisite to affinity purification of proteins and critical to immunological research. To select the best crosslinking method for an experiment, it is imperative to understand the functional and compositional structure of proteins that make them favorable targets for modification.

Complex protein structures are comprised of small amino acid building blocks linked together to form polypeptide chains. These amino acid building blocks contain many basic functional groups, such as amines and thiols, that can be targeted for chemical modification. This targeting mechanism provides the foundation for the development and production of a variety of synthetic crosslinkers.

The most critical feature of a crosslinker is its reactive ends which target specific functional groups on proteins. The most commonly used reactive group, NHS esters, is synthesized into homobifunctional crosslinkers for use in protein conjugations. These crosslinkers serve as powerful tools in protein analysis and detection techniques. Unfortunately, certain disadvantages and adverse side reactions such as self-polymerization are commonly associated with these crosslinkers. To combat this, Buccutite™ crosslinking technology was developed to provide a robust and efficient alternative for protein conjugations.

### Functional Groups and Their Respective Reagents

The most common and simplest functional group targeted for biomolecular modification is primary amines (-$NH_2$) located on the N-terminus of polypeptides and the side chains of lysine residues. Because these primary amines are typically nucleophilic and positively charged, they have an outward facing conformation in physiological conditions. This makes them readily accessible and favorable targets for crosslinkers without denaturing the protein structure. Of the vast synthetic chemical groups targeting primary amines, N-hydroxysuccinimide esters (NHS esters) have been used with the greatest success.

NHS esters are reactive groups formed by EDC activation of carboxylate moieties in alkaline conditions. Activated NHS ester reagents react with primary amines on target proteins forming stable amide bonds. (Figure 1 for mechanism).

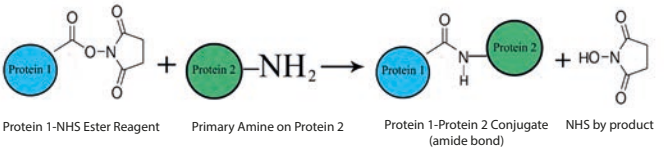


**Figure 1.** NHS esters target primary amines on proteins to form stable amide bonds resulting in a protein conjugate and an NHS by product.

### Disadvantages of NHS Esters

Despite their simplicity and ease of use, homobifunctional NHS ester crosslinkers have various drawbacks, specifically in protein-protein conjugation. These include self-polymerization, hydrolysis and purification.

First, homobifunctional NHS esters have a disadvantage in site specific protein-protein conjugation techniques because of its inability to differentiate between amine groups on the target protein from those on the label protein. This results in an uncontrollable tendency to self-polymerize proteins. For example, the use of homobifunctional crosslinkers when preparing antibody-HRP conjugations is not ideal because of the unfavorable antibody-antibody conjugations that may occur.

To mitigate this problem, AAT Bioquest has developed a convenient ReadiUse™ Preactivated HRP-NHS Ester (Cat# 11025) that contains the mono-NHS ester of HRP (Figure 2 for mechanism). By pre-activating HRP with one NHS ester, the rate of successful protein-HRP conjugation increases while the potential for self-polymerization of the

target protein decreases. Our pre-activated HRP-NHS ester is ideal for labeling antibodies with HRP enzymes in a simple and efficient manner for use in ELISA and other immunoassay applications.

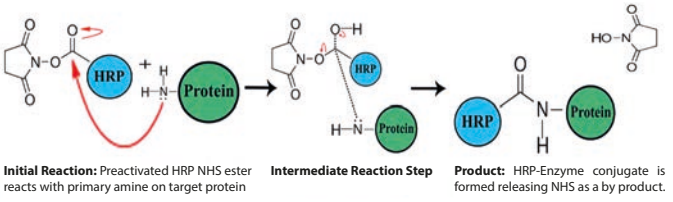


**Figure 2.** Preactivated HRP-NHS esters react with primary amines on target proteins to form stable amide bonds. The resulting product is an HRP-protein conjugate and an NHS by product.

Aside from self-polymerization, hydrolysis of NHS esters and amide bonds is another undesirable drawback associated with NHS ester crosslinking. Hydrolysis competes with the primary amine reaction as the pH of the system increases resulting in less efficient crosslinking of protein targets. Given that optimal conjugation systems occur at pH 8-10, it is clear that this could pose an issue. A possible remedy to minimize the effects of hydrolysis and maximize the amine modification is to use a high concentration of target protein, but this can prove costly for researches.

In addition, after conjugation, purification steps must be taken to remove excess unreactive reagents and proteins when using homobifunctional NHS ester crosslinkers. These final steps present additional complications. First, purification lowers the yield of modified protein conjugates due to the residual loss of modified proteins within purification columns. Secondly, these additional steps prolong the duration it takes to successfully complete conjugation.

The limitations, complications and targeting variability of homobifunctional NHS esters sparked the development of Buccutite™ crosslinking technology. This refined crosslinking technology addresses many problems associated with NHS ester crosslinkers while greatly improving upon the mechanisms and range of applications in which it can be used.

## Buccutite™ Crosslinking Technology

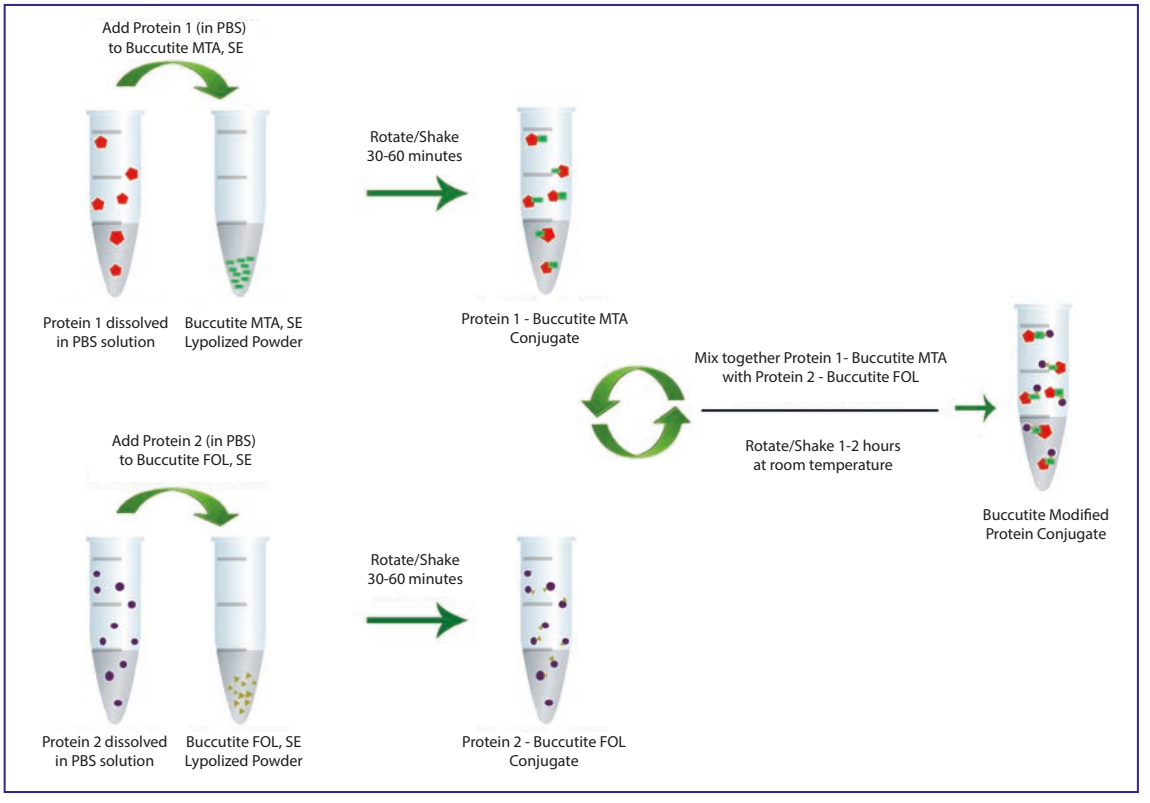


**Figure 3.** The mechanisms for Buccutite™ crosslinking technology for protein-protein conjugation.

Buccutite™ crosslinking technology provides a simplistic and efficient approach to conjugate proteins with another macromolecule such as an antibody or an enzyme. Buccutite™ crosslinking technology utilizes two exclusive linkers, Buccutite™ FOL, SE and Buccutite™ MTA, SE. The unique properties of these Buccutite™ crosslinkers allow them to function separately as two halves of a single homobifunctional crosslinker. Each Buccutite™ crosslinker is engineered to have an amine-reactive group on one end which specifically targets primary amines on the desired protein. The other end contains our proprietary Buccutite™-reactive group, which has a high degree of affinity for only binding its respective Buccutite™ MTA/FOL crosslinker counterpart. When each is present, the two Buccutite™-protein conjugates covalently link together at their Buccutite™-reactive site, "like buckling a seat belt," to successfully create a protein-protein conjugate. (Figure 3 for Buccutite™ Crosslinking Mechanism).

### Sample Protocol

Utilizing a sequential conjugation technique, one can perform protein modifications with Buccutite™ crosslinking technology in three simple steps:

1. Run Protein 1-Buccutite™ MTA reaction by adding Protein 1 solution directly into the vial of lyophilized Buccutite™ MTA, mix and remove free MTA, SE by desalting.
2. Run Protein 2-Buccutite™ FOL reaction by adding Protein 2 solution directly into the vial of lyophilized Buccutite™ FOL, mix and remove free FOL, SE by desalting.
3. Crosslinking Protein 1-Buccutite™ MTA & Protein 2-Buccutite™ FOL conjugates is initiated by mixing the two conjugates together under relatively neutral conditions.

### Advantages of Buccutite™ Crosslinking Technology

Buccutite™ crosslinking technology improves upon amine-reactive conjugation techniques, creating a more efficient and robust two-step conjugation system. Individual preparation of each protein with its respective Buccutite™ crosslinker provides more control over the conjugation process while reducing the possibility of adverse side reactions. Since Buccutite™ crosslinkers are exclusively amine reactive, the use of catalysts and reducing reagents are no longer needed.

Homobifunctional NHS ester crosslinkers randomly target amine groups on proteins, allowing for self-polymerization and making it difficult for site-specific conjugation. The unique reactive property of each Buccutite™ crosslinker provides a more site specific conjugation technique. Each Buccutite™ crosslinker has one amine-reactive end for conjugation to a target protein and one Buccutite™ MTA/FOL-reactive end that binds only to its linker counterpart for successful protein-protein conjugation. Replacing one amine-reactive end with a Buccutite™ MTA/FOL reactive-end eliminates the self-polymerization experienced when using homobifunctional NHS ester, yielding highly stable macromolecular conjugates.

Buccutite™ technology, such as ReadiLink™ antibody labeling kits, provides a more refined approach to protein conjugations by eliminating the purification steps. Buccutite™ crosslinkers eliminate these purification steps because of the high degree of affinity they have to link together proteins only at their Buccutite™ MTA/FOL reactive site. This prevents any unreactive Buccutite™ crosslinkers from self-polymerizing proteins and interfering with the immunoassay of the desired protein-protein conjugation. This distinct feature of Buccutite™ crosslinkers proves useful in sandwich ELISA immunoassays where substrates are captured and detected between two layers of antibodies. Treatment of enzyme-linked secondary antibodies with Buccutite™ limits enzyme-antibody conjugation specifically to the secondary antibody and not to the capture antibody coating the microplate well.

Extensive testing and quality control have also demonstrated that Buccutite™ technology significantly improves conjugation yield over traditional labeling methods. For example the yield of modified proteins increases from 30%, when using NHS ester crosslinkers, to 50-60% when using Buccutite™ crosslinking technology. This makes Buccutite™ a fast and convenient alternative for protein conjugation, particularly when sample protein quantities are limited.

### Applications for Buccutite™ Crosslinking Technology

Protein conjugates are extensively used in the purification and detection of complex biological samples. Commonly, antibodies are popular targets for bioconjugation with enzymes, biotin, and fluorescent labels for use in ELISA assay and flow cytometry. To facilitate the synthesis of protein-protein conjugates for use in immunoassays, AAT Bioquest provides a ReadiLink™ Rapid Protein Crosslinking Kit (Cat# 1315) for robust and efficient crosslinking. This kit comes with all the necessary components to effectively

link two desired proteins. Included are both Buccutite™ MTA and FOL crosslinkers and the necessary reaction buffer needed to perform protein-protein conjugation. This method uses our distinct Buccutite™ crosslinking technology to synthesize protein-protein conjugates at high yields.

***ELISA***

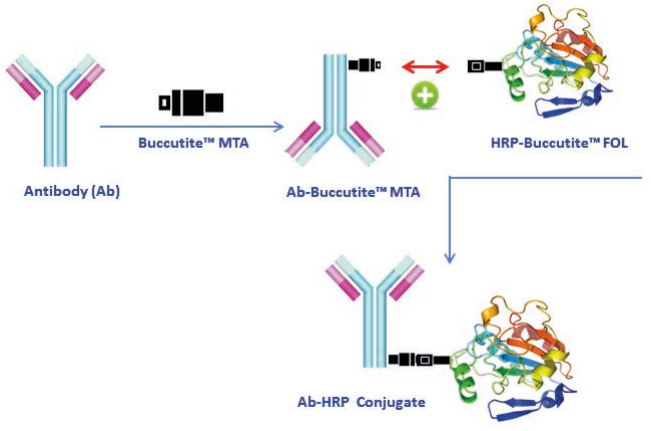


**Figure 4.** The mechanism of Buccutite™ bioconjugation system used for ReadiLink™ Peroxidase Antibody Conjugation Kit (Cat# 5503).

ELISA is a plate-based immunoassay technique that uses a solid-phase to detect antigen-antibody interactions in a biological sample. Antibodies are typically linked to an enzyme, such as horseradish peroxidase (HRP) via amine reactive groups, to amplify detectable colorimetric changes brought about by antibody-antigen interactions. Buccutite™ crosslinking technology provides an easy and robust alternative for antibody-HRP conjugations which, unlike traditional homobifunctional NHS esters, occur without the competing hydrolysis and self-polymerization side reactions. Using homobifunctional NHS esters for this protein-enzyme conjugation would require intermediate purification steps to remove unreactive NHS esters resulting in a low yield of antibody-enzyme conjugates. Replacing NHS esters with Buccutite™ crosslinkers allows for site specific conjugation of antibodies to enzymes without purification, boosting conjugation yields 50-60% modified proteins.

For convenience purposes, AAT Bioquest provides a ReadiLink™ Peroxidase Antibody Conjugation Kit (Cat# 5503) to facilitate an efficient way to conjugate antibodies to HRP. This kit provides HRP pre-activated with our proprietary linker, Buccutite™ FOL, ready for conjugation. Simply prepare the target antibody with the Buccutite™ MTA linker, and then mix the two Buccutite™ conjugated molecules together for protein-protein conjugation (Figure 4 for mechanism). It enables faster and quantitative conjugation of antibody to HRP with higher efficiencies and yields, ready for immediate use in ELISA assays.

***Flow Cytometry***

Another widely accepted application for protein modification is the antibody-fluorophore conjugates used in flow cytometry. This provides researchers with a powerful technique to analyze multiple parameters of individual cells within a heterogeneous sample, giving insight to cell characteristics such as size, structural complexity and phenotype. Common fluorescent probes used in these applications are phycobiliproteins because of the many advantages associated with them. Phycobiliproteins, such as allophycocyanin and r-phycoerythrin, are quench-resistance as a result of their protein backbone and exhibit long-wavelength fluorescence emissions. This makes them preferred fluorescent probes for applications that require high sensitivity or simultaneous multicolor detection.

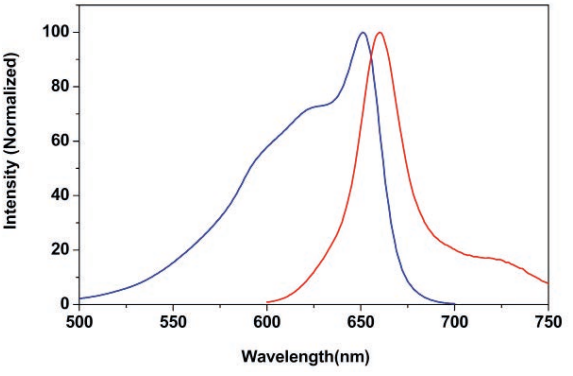


**Figure 5.** The excitation and emission of APC (Allophycocyanin, Cat# 2554).

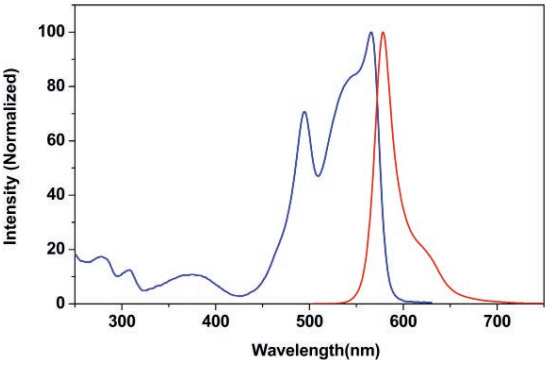


**Figure 6.** The excitation and emission of PE (R-Phycoerythrin, Cat# 2558).

Allophycocyanin (APC, Cat# 2554) and r-phycoerythrin (PE, Cat# 2558) are bright fluorescent proteins with high absorptivity and quantum efficiency that can easily be linked to antibodies for detection in immunoassays. Homobifunctional NHS esters are used to synthesize these antibody-fluorophore conjugates. However, using NHS esters is not the most efficient crosslinking technique for this type of protein conjugation. NHS esters random targeting of amine groups will self-polymerize antibodies lowering the yield of antibody-fluorophore conjugates. This can make it difficult for detection in flow cytometry. For this reason, AAT Bioquest offers a ReadiLink™ Rapid Protein Crosslinking Kit (Cat# 1315) to produce efficient and highly stable antibody-fluorophore conjugates for use in flow cytometry. This kit provides everything necessary to link antibodies and fluorophores together with a high conjugation yield for optimal results in immunoassay applications.

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**Table 1. Buccutite™ Crosslinking Assay Kits**

| Cat # | Product Name | Size | Ex (nm) | Em (nm) |
|---|---|---|---|---|
| 2554 | APC [Allophycocyanin] | 1 mg | 651 | 662 |
| 2558 | PE [R-Phycoerythrin] | 1 mg | 565 | 575 |
| 5503 | ReadiLink™ Peroxidase (HRP) Antibody Conjugation Kit *Optimized for Labeling 100 µg Protein* | 1 labeling | N/A | N/A |
| 1311 | ReadiLink™ Rapid APC Antibody Labeling Kit | 2 labelings | 651 | 662 |
| 1299 | ReadiLink™ Rapid FITC Antibody Labeling Kit | 2 labelings | 492 | 516 |
| 1255 | ReadiLink™ Rapid iFluor™ 488 Antibody Labeling Kit | 2 labelings | 491 | 514 |
| 1230 | ReadiLink™ Rapid iFluor™ 594 Antibody Labeling Kit | 2 labelings | 592 | 614 |
| 1130 | ReadiLink™ Rapid mFluor™ Red 700 Antibody Labeling Kit | 2 labelings | 657 | 700 |
| 1114 | ReadiLink™ Rapid mFluor™ Violet 540 Antibody Labeling Kit | 2 labelings | 405 | 537 |
| 1126 | ReadiLink™ Rapid mFluor™ Yellow 630 Antibody Labeling Kit | 2 labelings | 561 | 630 |
| 1315 | ReadiLink™ Rapid Protein Crosslinking Kit | 2 labelings | N/A | N/A |
| 1300 | ReadiLink™ Rapid trFluor™ Eu Antibody Labeling Kit | 2 labelings | 346 | 617 |
| 1305 | ReadiLink™ Rapid trFluor™ Tb Antibody Labeling Kit | 2 labelings | 330 | 544 |
| 2561 | ReadiUse™ Preactivated APC | 1 mg | 651 | 713 |
| 2586 | ReadiUse™ Preactivated APC-Cy5.5 Tandem | 1 mg | 651 | 700 |
| 2587 | ReadiUse™ Preactivated APC-Cy7 Tandem | 1 mg | 651 | 780 |
| 11025 | ReadiUse™ Preactivated HRP NHS Ester | 300 µg | N/A | N/A |
| 11026 | ReadiUse™ Preactivated HRP Maleimide | 300 µg | N/A | N/A |
| 2580 | ReadiUse™ Preactivated PE-Cy5 Tandem | 1 mg | 565 | 670 |
| 2581 | ReadiUse™ Preactivated PE-Cy5.5 Tandem | 1 mg | 565 | 700 |
| 2583 | ReadiUse™ Preactivated PE-Texas Red Tandem | 1 mg | 565 | 600 |

# NAADP-AM
## A New Player in Calcium Signaling Pathways

Nicotinic acid adenine dinucleotide phosphate (NAADP, Cat# 20999) is a secondary messenger that plays a key role in calcium signaling pathways. First discovered in the early 1980s, this dinucleotide has become the focus of intense research in recent years. It has been proposed as a pharmacological target for a variety of diseases affecting the pancreas, heart and nervous system. Experiments with NAADP have shown it to be an extremely potent calcium mobilizer as well as a modulating agent for other cellular pathways, such as those involving inositol trisphosphate ($IP_3$). The strong interest in NAADP has also led to the discovery and development of several key research tools, namely, NED-19 (a NAADP antagonist) and NAADP-AM (a cell-permeable form of NAADP).

### $IP_3$, cADPR and NAADP

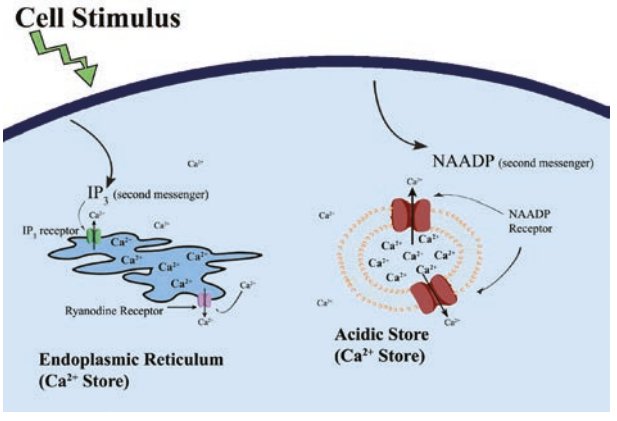


**Figure 7.** Inositol triphosphate ($IP_3$) and NAADP are second messenger molecules that transfer a chemical stimulus received by the cell. $IP_3$ binds to $IP_3$ ligand-gated $Ca^{2+}$ channels causing an influx of $Ca^{2+}$ into the cytosol from the endoplasmic reticulum. NAADP triggers an influx of $Ca^{2+}$ from acidic vesicles into the cytosol.

Without question, calcium ions ($Ca^{2+}$) are one of the most important secondary messengers in biology. They are crucial not only for cellular events such as apoptosis and proliferation, but also for macroscopic functions such as muscle contraction and neuron signaling. Their importance has prompted much investigative effort, as researchers attempt to understand the mechanisms and pathways with which calcium ions exert their influence on cells.

One of the early breakthroughs in this regard was the discovery of inositol triphosphate ($IP_3$). While the precursor of $IP_3$, phosphatidylinositol ($PIP_2$), was first studied in the 1950s, it took an additional 30 years before the link between $PIP_2$, $IP_3$ and $Ca^{2+}$ mobilization was understood. In the early 1980s, extensive experimentation showed that extracellular $PIP_2$ was catabolized by a membrane bound protein called phospholipase C (PLC). This resulted in the formation of intracellular $IP_3$, which acted as a secondary messenger and stimulated $Ca^{2+}$ release from endoplasmic reticulum (ER) stores. This discovery was particularly notable because it demonstrated a mechanism whereby external stimuli (ie. $PIP_2$) could influence intracellular calcium levels, and by extension, the activation of various protein kinases.

With the explication of the $IP_3$-$Ca^{2+}$ relationship, many researchers were satisfied with their understanding of calcium pathways. It was amongst a great deal of surprised individuals then that Hon Cheung Lee and colleagues published their research in 1987, which proposed not one, but two, additional calcium mobilizing secondary messengers. While studying sea urchin eggs, Lee and colleagues discovered that two previously unknown nucleotides, cADPR and NAADP, could stimulate calcium release in cells. Their pivotal study placed much of what was known about calcium pathways at that time into question, as researchers once again scrambled to decipher the complexities of cellular calcium signaling.

### NAADP Structure

While Lee and colleagues first discovered the presence of NAADP in 1987, it was not until 1995, almost a decade later, that its structure was determined. Through a combination of high pressure liquid chromatography (HPLC) and proton NMR, it was discovered that

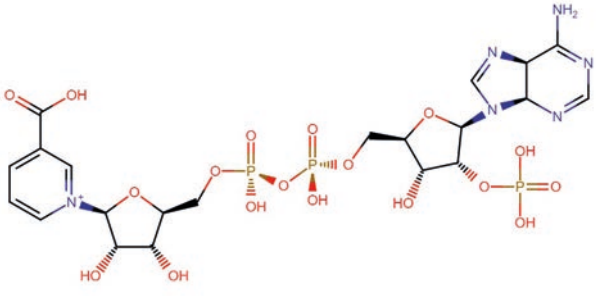

**Figure 8.** The chemical structure of NAADP (Cat# 20999).

NAADP is in fact a derivative of the co-factor NADP, wherein the nicotinamide group is replaced with a nicotinic acid group. This structural determination was somewhat expected, as NAADP was originally discovered as a contaminant in commercial NADP sources. What surprised many researchers, however, was that cellular NAADP did not result from a simple de-amination of existing NADP. Rather, it is a metabolite formed by a base-exchange reaction catalyzed by the same enzyme which forms cyclic ADP-ribose (cADPR). This was a particularly surprising discovery as cADPR and NAADP have very distinct structural and functional properties.

### NAADP Formation

In humans, a glycoprotein called CD38 (cluster of differentiation 38) is responsible for both the formation of cADPR and of NAADP. This discovery, however, was surprising for two reasons.

First, cADPR and NAADP are very different structurally; NAADP is a linear compound whereas cADPR is cyclical. This is an important distinction because it indicates that CD38 catalyzes two discrete reactions, a base-exchange reaction for NAADP and a cyclization reaction for cADPR. While certainly not a unique occurrence in biological systems, CD38 is rather uncommon not only for its discrete catalytic abilities, but also for the specificity with which those catalytic processes occur. That is, despite two different starting compounds, CD38 recognizes and converts NAD to cADPR and NADP to NAADP with a high degree of consistency.

Second, cADPR and NAADP are very different functionally.

### NAADP Targets

As a calcium mobilizer, NAADP is functionally distinct from cADPR and $IP_3$. Unlike the latter, NAADP does not mobilize calcium stores in the ER. Rather, it mobilizes recently discovered acidic calcium stores located throughout the cytoplasm. These acidic calcium stores include subcellular compartments such as endosomes, lysosomes, secretory granules and Golgi bodies. More specifically, recent research suggests that NAADP targets a family of membrane bound ion-channels, called two-pore channels (TPC), in order to stimulate calcium release. The specific mechanisms behind this interaction, however, are still not well understood.

### NAADP Mechanism of Action

There are currently two hypothesis regarding NAADP's role in calcium signaling pathways.

The first proposes that NAADP serves as a signaling primer. The hypothesis is that NAADP causes calcium release from acidic stores, which are then taken up by ER stores. In this way, a cell is primed for an enhanced response upon later stimuli. Research into excitation-contraction coupling (EEC) in atrial myocyte suggests this may be the case, as the presence of NAADP leads to the release of $Ca^{2+}$ from acidic stores, followed by an increase in sarcoplasmic reticulum (SR) $Ca^{2+}$ release.

The second hypothesis is that NAADP acts as a calcium signal amplifier. In this model, NAADP stimulates $Ca^{2+}$ ion release from acidic stores, calcium ions which then interact with calcium sensitive targets to effectuate further calcium mobilization, for example, from the ER. The proposal suggests this can be achieved if the initial calcium release, from acidic stores, can influence additional calcium mobilizing secondary messengers such as cADPR and $IP_3$. Indeed, this appears to be the case in a study conducted on pancreatic acinar cells, in which cADPR and $IP_3$ antagonists were able to block NAADP-dependent $Ca^{2+}$ response.

### Advancements in NAADP Research

As interest in it deepens, scientists have begun looking for better tools to study NAADP. In recent years, the research process has been significantly aided by the development of two separate compounds: NED-19 and NAADP-AM (Cat# 20998).

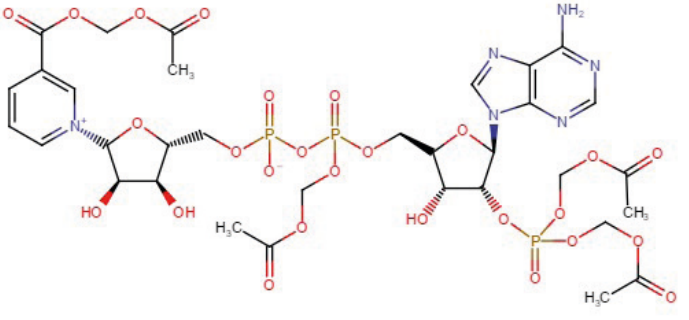

**Figure 9.** The chemical structure of NAADP-AM (Cat# 20998).

NED-19 is a NAADP antagonist that was first developed through virtual chemical screening of NAADP analogs. It acts specifically to block both NAADP-mediated $Ca^{2+}$ response as well as NAADP binding. Interestingly enough, however, by using NED-19 analogs, researchers have been able to show that these two antagonistic effects can be teased apart. Using NED-20, researchers were able to specifically block NAADP binding, while leaving NAADP-mediated $Ca^{2+}$ release untouched. On the other hand, when NED-19.4 was used, NAADP binding could occur, but NAADP-mediated $Ca^{2+}$ release was inhibited. This result suggests that there are actually two different binding sites on NAADP receptors and two different sites with which NED-19 can interact with.

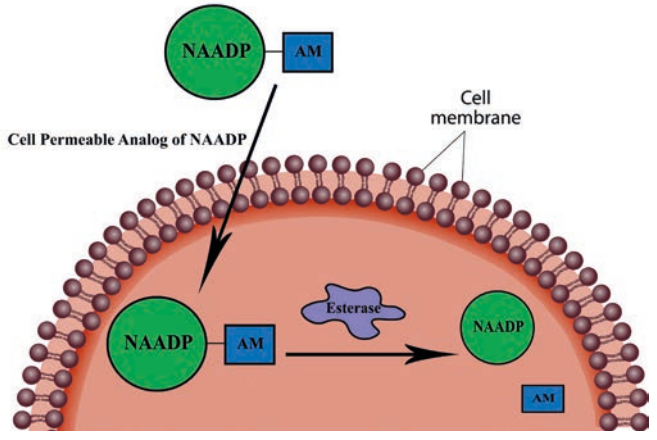


**Figure 10.** NAADP-AM is a cell permeant analog of NAADP. NAADP-AM is taken into a cell's cytosol where it is hydrolyzed by esterase enzymes. The resulting influx of NAADP second messengers induces NAADP-mediated calcium signaling.

The second important development in the study of NAADP is the synthesis of a cell permeable NAADP analog, NAADP-AM. Prior to its development, studies with NAADP had to utilize invasive cellular techniques such as microinjections or electroporation in order to load NAADP into cells. There are several well-documented problems with these methods. At the very least, normal cellular function is disrupted due to the disruption of the cell membrane. In the case of microinjections, the process is very time-intensive as it is limited to single cells. For electroporation, common problems include low loading efficiency and high rates of cell death.

The usage of acetoxymethyl esters (AM esters) resolves many of the problems faced by prior loading techniques. This is particularly true in the case of NAADP because, as a compound, it is negatively charged. What this means is that while NAADP is well-retained in cells, it has an especially difficult time passing through cell membranes. But by chemically adding AM esters to it, thus synthesizing NAADP-AM, NAADP not only loses its negative charge but also becomes hydrophobic. This change in chemical properties allows NAADP-AM to easily pass through the phospholipid membrane of cells. Once inside, the AM ester is cleaved by intracellular esterases, thus returning the compound to its original NAADP form. In this manner, through the use of AM esters, NAADP can be easily loaded into a population of cells without the need for invasive cellular techniques.

### Current Research

NAADP has been the focus of study in a wide range of fields. For example, NAADP has been suggested to play an important role in the onset and progression of diabetes. Studies in pancreatic β cells show that NAADP is involved in glucose-induced $Ca^{2+}$ signaling pathways related to the regulation and release of insulin. In these studies, it was found that intracellular NAADP levels fluctuate as a function of glucose addition. Furthermore, it was found that NAADP acts as a sensitizing agent in pancreatic β cells. That is, at concentrations lower than 100 nM, NAADP leads to an enhancement of cellular calcium responses while levels greater than 1 µM leads to the inactivation of NAADP receptors and downstream pathways.

Another active area of investigation is NAADP's role in Parkinson's disease. In these studies, it is again suggested that NAADP acts as a sensitizing agent, enhancing cell response to additional stressors and stimuli. In particular, focus was placed on the de-regulation of the leucine-rich repeat kinase-2 (LRRK2) gene, wherein mutations are thought to cause late-onset Parkinson's disease characterized by increased autophagy and abnormal protein degradation. It is thought that in the case of Parkinson's disease, the de-regulation of LRRK2 pathways results in over-activation of NAADP and two-pore channels, which in turn over-amplifies calcium signaling in response to external stimuli. NAADP appears to be linked to LRRK2 pathways as the addition of NED-19, an NAADP antagonist, mitigates the effects of LRRK2 de-regulation and the blocking of LRRK2 pathways simultaneously blocks NAADP-dependent autophagy overresponse.

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**Table 2. NAADP Products**

| Cat # | Product Name | Size | Ex (nm) | Em (nm) |
|---|---|---|---|---|
| 20999 | NAADP | 1 mg | N/A | N/A |
| 20998 | NAADP-AM | 250 µg | N/A | N/A |

# Nucleic Acid Staining

Nucleic acid staining is an important tool that gives insight into cellular mechanisms and functionality of macromolecules like DNA and RNA. This is critical as these nucleic acids and their associated organelles are responsible for the development, maintenance and growth of living organisms. The combination of nucleic acid staining with applications such as fluorescence microscopy and flow cytometry also allow for spatiotemporal imaging, providing a powerful means for visualizing stained nucleic components in fixed and live cells.

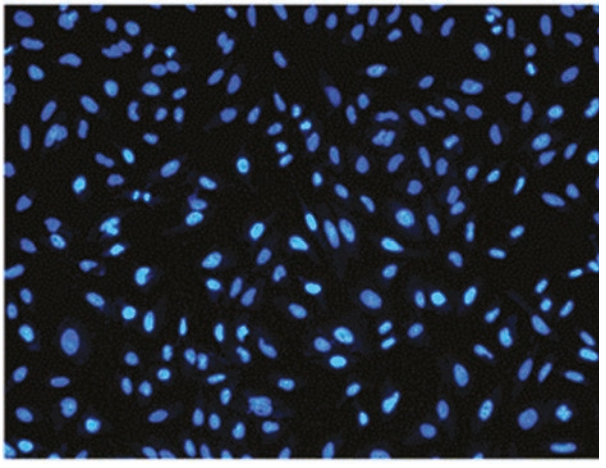

**Figure 11.** Image of dead cells stained with Nuclear Blue™ DCS1 (Cat# 17548). Fixed HeLa cells were plated on a 96-well microtiter plate, incubated with 2.5 µM Nuclear Blue™ DCS1 for 230 minutes and imaged with DAPI channel.

Analyzing nucleic acid stains in cells provides an excellent overview of the cellular localization and organization of nucleic macromolecules and organelles with respect to time. Fixed cell stains in tandem with flow cytometry provide a snapshot of the distribution of nucleic macromolecules in a population of cells with respect to a single temporal time point. For example, DNA staining of fixed cells can be used to analyze the relationship between membrane integrity and the degradation of DNA at specific times during cellular events such as apoptosis. Nucleic acid staining in live cells for fluorescence microscopy aids in visualizing and understanding the spatiotemporal dynamics and organization of nucleic acids and organelles in real time. The observation of dynamic changes provides more insight into the operations of nucleic acids as they move within their environment during specific cellular functions, such as the regulation of gene expression. However, differentiating between nucleic macromolecules is considerably challenging and may prove difficult in studies where selective staining of nucleic acids is imperative for spatiotemporal analysis.

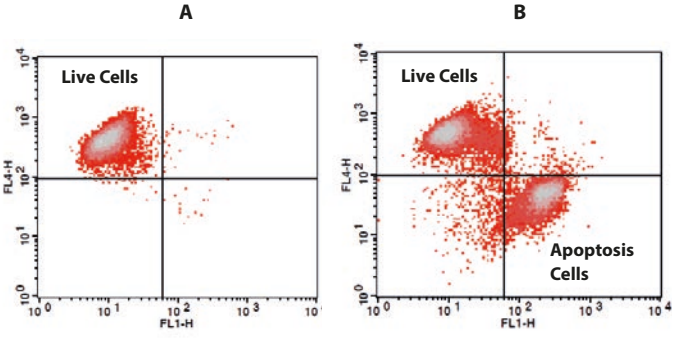


**Figure 12.** The increase in fluorescence intensity of Nuclear Green™ DCS1 (Cat# 17550) with the addition of camptothecin in Jurkat cells. Jurkat cells were treated overnight without (A) or with 20 µM camptothecin (B) in a 37 °C, 5% $CO_2$ incubator, and then dye loaded with Nuclear Green™ DCS1 for 60 minutes. At the end of 15 minutes of Nuclear Green™ DCS1 dye loading, MitoLite™ NIR (Cat# 22802) was added for multicolor analysis. The fluorescence intensity of Nuclear Green™ DCS1 and MitoLite™ NIR was measured with a FACSCalibur (Becton Dickinson) flow cytometer using FL1 channel (Nuclear Green™ DCS1) and FL4 channel (MitoLite™ NIR).

Obstacles associated with selectively staining nucleic acids are the structural and chemical similarities shared between DNA and ribonucleic acid (RNA). Both categories of nucleic acids are nearly identical, and therefore many staining techniques are applicable and synonymous for both. This non-specificity for staining nucleic acids can be detrimental to the signal quality of interest because of the impeding background noise from other nucleic molecules. For example, DNA imaging and localization studies may exhibit impeding background interference by RNA because of the non-specific binding of dye-conjugated nucleic acid probes. To address this issue, research has led to the improvement and development of sensitive tools and alternative techniques optimized to specifically target and stain DNA. A simple improvement to DNA staining involves the removal of RNA by pretreating the nucleic acid sample with RNAse, an enzyme that catalyzes the degradation of RNA into smaller components. Some improvements utilize cell-permeable dyes designed to selectively target specific configura-

tions of DNA, such as the minor groove region of double helix DNA. Other improvements combine applications, such as gel electrophoresis and nucleic acid staining, to separate and stain large double-stranded DNA (dsDNA) macromolecules of a biological sample.

### DNA Imaging

Deoxyribonucleic acid (DNA) is an organic, molecular complex that carries the genetic instruction for all proteins used for the development, functionality and sustainability of living organisms. DNA is comprised of nucleotide molecules containing one of the four nucleobases – cytosine (C), guanine (G), adenine (A) or thymine (T) - a deoxyribose sugar and a phosphate group. These nucleotides covalently bond with one another to form long nucleic acid sequences that have the capacity to store viable information. In normal conditions, DNA molecules form a double helix consisting of two complimentary DNA strands which run antiparallel alongside each other. To better understand the sensitive mechanisms and functionality of DNA, labeling techniques such as DNA staining have been developed to tag and visualize DNA samples.

DNA imaging of cells can provide insight into the spatiotemporal dynamics of DNA during biological functions. For example, imaging can be used to visualize how the components of DNA move in relationship to one another during cell division. The common classes of commercially available nucleic acids stains are intercalating dyes and minor-groove binders.

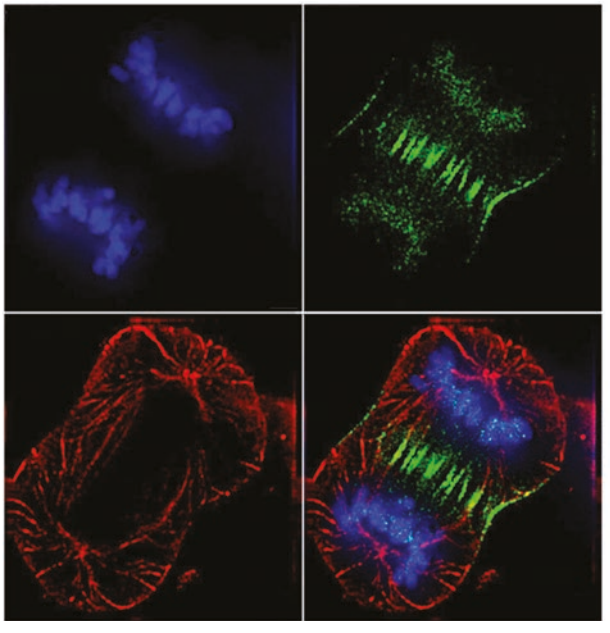

**Figure 13.** Image of a dividing (anaphase stage) human cancer cell taken with an epifluorescence microscope. The cancer cell was fixed and the DNA was stained with DAPI (blue), INCENP stained with GFP (green) and Tubulin stained red.

### Intercalating Dyes and Gel Electrophoresis

Agarose gel electrophoresis is an analytical method that uses an electrical current to separate DNA molecules according to molecular size. Because DNA is colorless, the use of intercalating dyes and loading buffers are combined with electrophoresis for their tagging and fluorescent properties. Intercalating dyes tag DNA for visualization, while the loading buffer increases the density of the DNA sample, impeding DNA solubilization and allowing the sample to be successfully loaded into the wells of the gel.

Ethidium bromide (EtBr) is a commonly used intercalating agent in molecular biology as a fluorescent dye for tagging and visualizing nucleic acids in gel electrophoresis. Its flat structure allows EtBr to intercalate, or insert, between nitrogenous bases in DNA molecules. Upon exposure to UV light, EtBr fluoresces, providing a means to visualize DNA molecules. EtBr's intercalating properties makes it a considerably potent mutagen that can interfere with the functionality of DNA molecules. Because of its high toxicity and mutagenic properties, many labs shy away from using EtBr as a DNA stain. To address toxicity concerns, AAT Bioquest offers a safer set of DNA dyes, Cyber Green™ (Cat# 17590) and Cyber Orange™ (Cat# 17595), that are excellent for nucleic acid gel stains. These dyes are commercially available individually as well as conveniently packaged in


**A: Cyber Green™**

**B: SYBR® Green**

**Figure 14.** DNA molecular weight ladders stained with Cyber Green™ (Cat# 17590) and SYBR® Green I Nucleic Acid Gel Stain.

AAT Bioquest's Gelite™ Green and Gelite™ Orange Nucleic Acid Gel Staining Kits (Cat# 17589 and Cat# 17594). Gelite™ kits include all the necessary components, proprietary dyes and loading buffers combined with an optimized and robust protocol to effectively visualize nucleic acid gel stains.

Cyber Green™ and Cyber Orange ™ are highly stable and sensitive stains capable of detecting double-stranded DNA (dsDNA) in agarose gels. Their high sensitivity for dsDNA is useful in assays where the presence of RNA or single-stranded DNA (ssDNA) may obscure results. Upon binding to dsDNA, Cyber Green™ emits a fluorescent intensity far greater than EtBr. The quantum yield of the DNA/Cyber Green™ complex is more than 5 times greater than DNA/EtBr complex. The DNA/Cyber Green™ complex has a convenient excitation of 497 nm and emission of 520 nm making it compatible with instruments capable of detecting fluorescein. The DNA/Cyber Orange™ complex has a similar excitation of 495 nm and emission of 540 nm and can also be used in a variety of instrumentations. The major benefit of Cyber Orange™ is its ultrasensitive detectability of dsDNA on gels as little as a picogram. The efficiency and sensitivity of Cyber Green™ and Cyber Orange™ makes them robust alternatives to EtBr for nucleic acid gel staining.

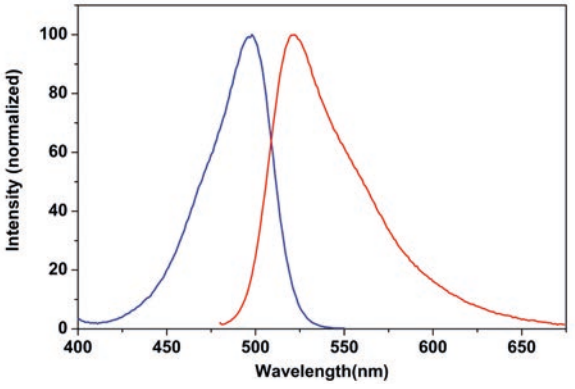


**Figure 15** The excitation and emission spectra of Cyber Green™ Nucleic Acid Gel Stain (Cat# 17590) in the presence of calf thymus DNA.

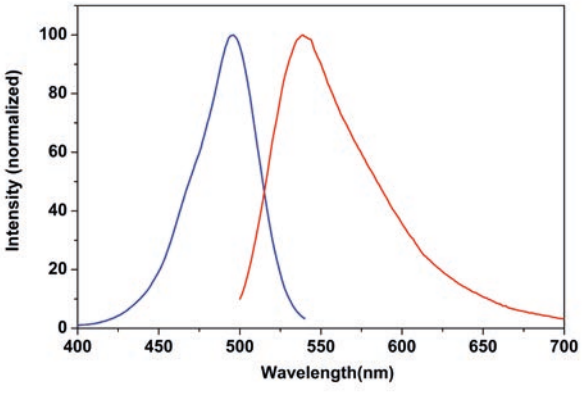


**Figure 16.** The excitation and emission spectra of Cyber Orange™ Nucleic Acid Gel Stain (Cat# 17595) in the presence of calf thymus DNA.

### Minor-Groove Binders and Fluorescence Microscopy

The structural significance of double stranded DNA provides another avenue for nucleic acid staining. Double helix DNA molecules have distinct chemical features on their molecular surface that act as recognition sites for small molecules and binding proteins. These sites are characterized by either shallow and wide major grooves,

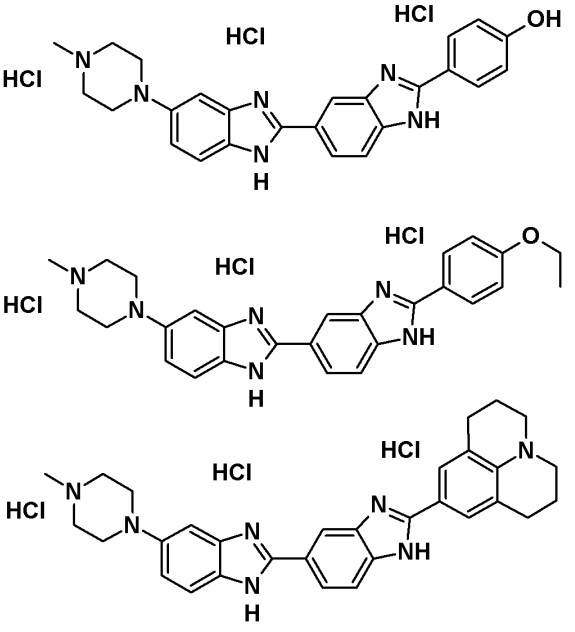


**Figure 17.** The chemical structures of Hoechst 33258 (top, Cat# 17520), Hoechst 33342 (middle, Cat# 17530) and Hoechst 34580 (bottom, Cat# 17537).

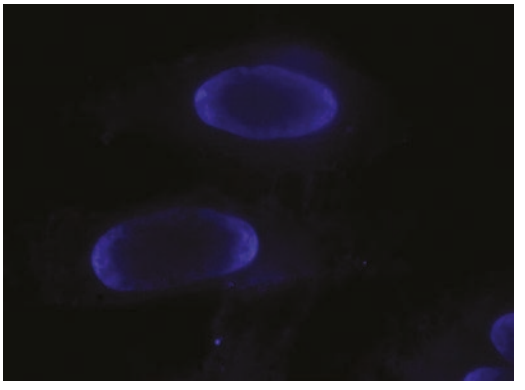

**Figure 18.** Nuclei containing DNA was stained for fluorescence microscopy using Hoechst 33342 (Cat# 17530). Hoechst 33342 can be excited by UV light at 350 nm and will emit cyan fluorescence light with an emission max at 461 nm.

or deep and narrow minor grooves. Transcription factor proteins, essential for the regulation of gene expression, bind to the base pairs of the major grooves in dsDNA. Compounds that bind to the minor grooves in double helix DNA molecules are small fluorescent stains such as DAPI (Cat# 17507) and Hoechst stains (Cat# 17520, Cat# 17530 and Cat# 17537).

DAPI (4′, 6-diamidino-2phenylindole) and Hoechst stains are cell permeable fluorescent dyes for labelling and visualizing DNA in fluorescence microscopy and flow cytometry. After permeating the cell membrane, these dyes have a strong affinity for A-T rich regions of the minor grooves in DNA. DAPI has an excitation of 358 nm and an emission of 461 nm, and Hoechst stains have a nearly identical excitation of 352 nm and emission of 461 nm. The blue emission of both dyes is convenient for multiplexing assays due to the minimal fluorescent overlap between DAPI or Hoechst stains with traditional green-fluorescent molecules.

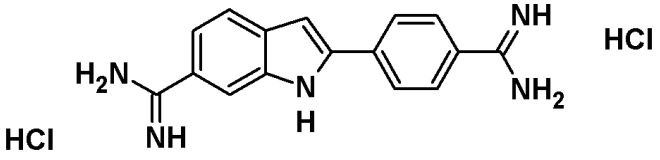


**Figure 19.** The chemical structure of 4',6-diamidino-2-phenylindole (DAPI, Cat# 17507).

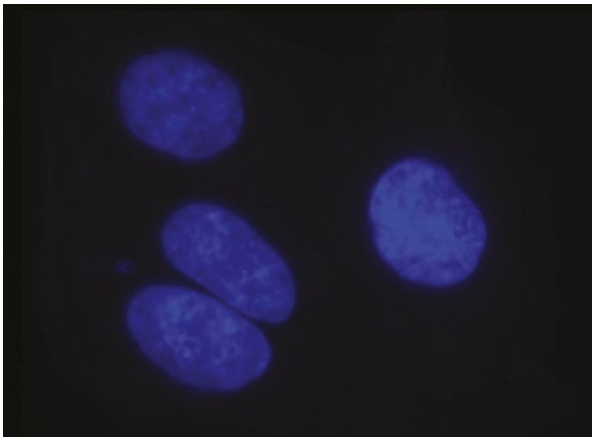

**Figure 20.** DAPI (Cat# 17507), a minor groove binding dye which binds to A-T rich regions of dsDNA, was used to stain and visualize cell nuclei containing DNA.

AAT Bioquest also offers a comprehensive line of exceptional dyes with various spectral properties that can be combined with other stains for multicolor analysis in fluorescence microscopy. Our Nuclear dye series includes Nuclear Yellow, Nuclear Green™, Nuclear Orange™, Nuclear Red™, and Nuclear Violet™. These dyes are available in cell-permeable forms for staining live cells as well as cell-impermeable forms for staining fixed cells.

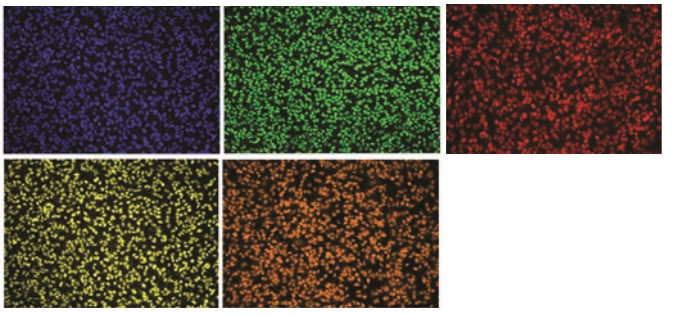

**Figure 21.** A composition of fluorescence images of HeLa cells stained with AAT Bioquest's Nuclear LCS1 dye series. HeLa cells were stained with Nuclear Violet™ (17543), Nuclear Green™ (Cat# 17540), Nuclear Red™ (Cat# 17542), Nuclear Yellow (Cat# 17539) and Nuclear Orange™ (Cat# 17541) LCS1 dyes in a Costar black wall/clear bottom 96-well microtiter plate.

Minor groove binders and intercalating dyes, discussed previously, are conventional approaches for imaging and analyzing localization of nucleic acids in cells. Utilization of these dyes in conjunction with imaging and localization studies has confirmed DNA organization and concentration to mitochondrial organelles and to the cell nucleus. For a more comprehensive understanding of the functionality of nucleic macromolecules and organelles, RNA staining is crucial. However, differentiating between RNA and DNA is difficult because of their similar composition. As a result, there has been an increase in interest regarding the research and development of RNA staining techniques. Modifications to dye-conjugated probes and DNA staining techniques can be altered to selectively target and stain cytoplasmic RNA molecules and organelles. Imaging and localization of RNA can provide vital information regarding its functionality in the cellular process of transcription and translation required for protein synthesis.

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**Table 4. Nucleic Acid Staining Reagents and Assay Kits**

| Cat # | Product Name | Size | Ex (nm) | Em (nm) |
|---|---|---|---|---|
| 17590 | Cyber Green™ Nucleic Acid Gel Stain [Equivalent to SYBR® Green] | 1 mL | 497 | 520 |
| 17595 | Cyber Orange™ Nucleic Acid Gel Stain | 1 mL | 495 | 540 |
| 17507 | DAPI [4,6-Diamidino-2-phenylindole, dihydrochloride] | 2 mL | 358 | 461 |
| 17589 | Gelite™ Green Nucleic Acid Gel Staining Kit | 1 kit | 497 | 520 |
| 17594 | Gelite™ Orange Nucleic Acid Gel Staining Kit | 1 kit | 495 | 540 |
| 17520 | Hoechst 33258 *CAS# 23491-45-4* | 100 mg | 352 | 461 |
| 17530 | Hoechst 33342 *CAS# 23491-52-3* | 100 mg | 350 | 461 |
| 17537 | Hoechst 34580 *CAS# 911004-45-0* | 5 mg | 368 | 437 |
| 17548 | Nuclear Blue™ DCS1 | 0.5 mL | 350 | 461 |
| 17550 | Nuclear Green™ DCS1 | 0.5 mL | 503 | 526 |
| 17540 | Nuclear Green™ LCS1 | 0.5 mL | 503 | 526 |
| 17551 | Nuclear Orange™ DCS1 | 0.5 mL | 528 | 576 |
| 17541 | Nuclear Orange™ LCS1 | 0.5 mL | 514 | 555 |
| 17552 | Nuclear Red™ DCS1 | 0.5 mL | 642 | 660 |
| 17542 | Nuclear Red™ LCS1 | 0.5 mL | 622 | 645 |
| 17543 | Nuclear Violet™ LCS1 | 0.5 mL | 401 | 459 |
| 17539 | Nuclear Yellow [Hoechst S769121] *CAS# 74681-68-8* | 25 mg | 355 | 495 |

# Quantitative Analysis of Thiols and Maleimides

Quantitative analysis is an important tool in analytical chemistry that is used to determine the concentration of one or several particular substances present in a sample. In proteomic studies, quantitative analysis can be used to detect the amount of thiol functional groups and maleimide-reactive sites in a sample protein. This analysis can be used to characterize a protein over the course of an experiment and in bioconjugation it can help determine which crosslinking techniques are most desirable. Quantitation of thiol groups also provides analysis and insight into thiol-disulfide interconversions and the relationship it has on the oxidative-stress of a protein. Before discussing the specifics of thiol and maleimide quantification, however, it is perhaps worth discussing spectroscopy as it broadly applies to quantitative analysis.

## Spectroscopy

One common approach for quantitating thiols and maleimides is through spectroscopic analysis. Spectroscopic analysis, or spectroscopy, is a powerful quantitative tool in analytical chemistry. It can be used to determine the presence of a particular substance in a sample by measuring the absorption of radiation, as a function of wavelength. This absorption spectrum in combination with Beer-Lambert's Law reveals a directly proportional relationship between absorbance and the concentration of the attenuating moieties, or functional groups, in the material sample. As the max absorbance of a particular substance increases so does the concentration of that same substance.

The combination of spectroscopic analysis with colored reagents has led to the development of a quantitative technique known as colorimetry. In colorimetric assays, a colored reagent causes a solution to undergo a measurable color change while in the presence of its target analyte. The intensity of this color change is directly proportional to the concentration of the substance; the more intense the color, the higher the concentration of the analyte. With the help of a colorimeter, such as a colorimetric microplate reader, the concentration of the particular substance in the solution can be determined by measuring the absorbance of that solution at a specific wavelength of light known as lambda max ($\lambda_{max}$).

Colorimetry is a commonly accepted quantitative approach due to its simplistic protocol and the ability to easily detect a color change with the naked eye, allowing for quick results. The equipment necessary to perform a colorimetric assay is relatively cheap, and there is extensive documentation and validation of colored reagents in the scientific literature. Despite the long-standing use of colorimetric assays, however, they are susceptible to several key problems. For example, background interference by protein absorbance at the same wavelength (namely, 280 nm) can be detrimental to signal quality. Additionally, colorimetric assays suffer from poor dynamic range, leading to poor substrate sensitivity in some cases as well as easy signal saturation. If these problems are of concern, the use of a fluorimetric quantitative approach is recommended.

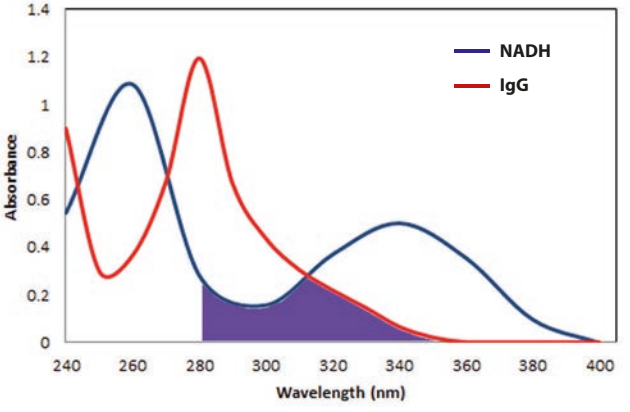


**Figure 22.** UV absorption suffers from poor selectivity and high background interference because many biological substances have a strong UV absorption from 250 nm to 400 nm. The NADH probe absorption above experiences background interference from protein absorption such as IgG at a wavelength of 280 nm (as highlighted by the shaded region below the absorption curves).

Fluorimetric assays are chosen for their extraordinary sensitivity and high specificity. This sensitive technique allows for accurate detection of fluorescent material in a relatively minute sample size which is perfect when the protein of focus is relatively expensive or available in small quantities. Fluorimetric assays can detect and quantitate moieties in a similar manner as colorimetric assays, albeit with the use of a fluorescent dye rather than a colored reagent. In fluorimetric spectroscopy, a fluorimeter excites the sample of interest and measures the intensity of the emitted light, which is

proportional to the concentration of the analyte.

Fluorimetry is an advantageous quantitative tool because it produces minimal background interference and a brighter signal. It has a significantly broader dynamic range, which allows for assay linearity across many orders of magnitude. It is typical for a fluorimetric assay, for example, to have a significantly lower detection limit than a colorimetric assay of the same substrate. The greater assay linearity of fluorimetric assays allows them to detect fluorescent signals of different magnitude, all within one measurement. This makes fluorimetry a highly sensitive and efficient technique for measuring an extensive number of samples at one time. Furthermore, fluorimetric assays can easily be multiplex through use of different fluorometric probes, allowing for complex studies of numerous substrates simultaneously.

### Chemistry of Thiols and Maleimides

The thiolate anion is intrinsically one of the strongest nucleophiles, making thiol groups one of the most reactive functional groups found in proteins. Thiols or sulfhydryl groups (-SH) are located in the side chains of cysteine residues. They participate in thiol-disulfide exchange reactions to introduce and remove protein disulfide bonds. These thiol-disulfide interconversions contribute to protein stability and reduce oxidative-stress. Protein disulfide bonds are involved in many cellular functions from the regulation of enzyme activity and signal transduction to protein folding. They also play a critical role in maleimide-based bioconjugation systems. The importance of thiol groups and disulfides in these functions of proteins has led to the development of many methods for the quantitative determination of thiol groups and disulfide compounds.

On the topic of conjugation systems, thiol detection can serve as a useful tool to determine if maleimide crosslinkers are appropriate for protein bioconjugation. Maleimides are crosslinkers that selectively target free thiols in proteins at near neutral conditions to form irreversible, stable thioether linkages. They are extremely beneficial in developing stable protein conjugates. The distinct location of thiol groups in cysteine residues and their relatively low abundance on proteins make them advantageous for site-specific bioconjugation. This is particularly important for labeling small proteins in applications where the activity or binding affinity of the conjugate is paramount. When conjugating antibodies with other proteins such as an enzyme, it is critical to avoid conjugation at or near the epitope-binding site because of the potential risk of blocking this site and rendering the conjugate inactive. The use of maleimides for this application eliminates this risk. Reduction of disulfide bonds found in the hinge and heavy chain region of antibodies, opposite the epitope-binding sites, generate free thiol groups which maleimides can target without compromising the functionality of the newly conjugated protein. Unfortunately, a key challenge in maleimide crosslinking is the lack of rapid and sensitive assay kits available to quantify the number of maleimide groups in a biopolymer.

### Quantitation of Thiols

Analysis of free thiols and disulfide bonds in proteins can be quantitatively determined by colorimetric and fluorimetric assays and by a variety of reagents. Colorimetric quantitation of thiols utilizes a color reagent to determine the concentration of a particular moiety in a sample by measuring the absorption of the colored solution. This quantitative approach is great for quantifying thiols in proteins and other high molecular weight molecules. Fluorimetric quantitation of thiols is a highly sensitive approach that can detect fluorescent materials in relatively small sample sizes and small molecules. Fluorimetric assays are highly specific and less susceptible to interferences because fewer materials absorb and also emit light (ie. fluoresce). Until recently, however, not many fluorimetric assays were commercially available to quantitate thiol groups on sample proteins.

Two common colorimetric approaches for thiol detection use two distinct chromogenic thiol reagents. The first approach uses the thiol reagent DTNB (5, 5′-dithiobis (2-nitrobenzoic acid)), also known as Ellman's reagent. DTNB is an aromatic disulfide that reacts with thiols by an exchange reaction to form a mixed disulfide of the protein and one molecule of TNB, which ionizes to the TNB2-dianion in water, producing a yellow color. TNB is then quantified using a spectrophotometer with a max absorption at 412 nm and an

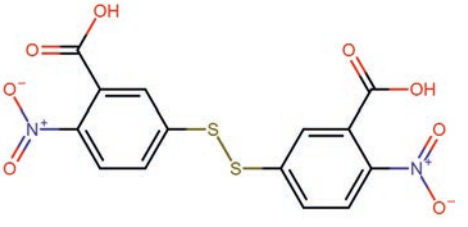


**Figure 23.** The chemical structure of Ellman's reagent, a chemical used to quantify the concentration of thiol groups in a sample.

extinction coefficient of 14,100 $M^{-1}cm^{-1}$. However, the disadvantage of DTNB as some studies have shown, is that it at times underestimates the concentration of thiol groups in a protein due to incomplete reaction of TNB.

A second, more promising alternative to Ellman's reagent is the use of 4, 4' dithiodipyridine (DTDP). DTDP is smaller, more hydrophobic and mostly uncharged at neutral conditions, with a max absorption at 324 nm and an extinction coefficient of 19,800 $M^{-1}cm^{-1}$. This makes it a great reagent for protein sulfhydryls in their native environment. DTDP reacts with thiols in the same way as does Ellman's reagent.

Although these reagents are useful in quantitative analysis, limitations are associated with both. DTDP and Ellman's reagent are fairly sensitive to hydrolysis at elevated temperatures and pH values greater than 7. In these conditions, decomposition of the activated disulfide of DTNB impedes quantitative analysis. While DTDP has improved upon the sensitivity of DTNB, it experiences higher background interference by protein absorbance. This is because its max absorbance is at 324 nm, which is fairly close to the 280 nm absorbance of proteins. Furthermore, colorimetric assays of this sort are in general hindered by the need for frequent calibration steps, which increase the duration of this quantitative approach as well as provide more opportunities for human error.

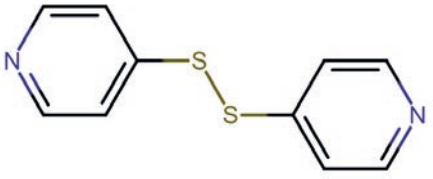


**Figure 24.** The chemical structure of 4, 4' dithiodipyridine (DTDP).

## Quantitation of Thiols with Amplite™ Technology

A key goal in colorimetric thiol quantitation has been the development of a rapid and accurate method to quantify the amount of free thiols in a specific protein. AAT Bioquest's Amplite™ Rapid Colorimetric Protein Thiol Quantitation Kit (Cat# 5529) addresses this challenge with the uses of its proprietary thiol sensor, Thiol Blue™. Thiol Blue™ reacts with a protein sample that contains free thiol groups. Upon completion of the reaction, the resulting thiol adduct runs through a single spin column to remove the excess Thiol Blue™. The absorption spectrum of the purified product can then be measured at a max absorbance of ~680 nm with use of a colorimeter. The thiol to protein ratio is calculated from the ratio of absorbances at 680 nm (sensor $\lambda_{max}$) and 280 nm (protein $\lambda_{max}$).

For small molecule thiol quantification, such as cysteine and glutathione, AAT Bioquest offers a fluorimetric quantitative analysis kit called Amplite™ Fluorimetric Total Thiol Quantitation Assay Kit (Cat# 5524). This kit utilizes a proprietary dye which, while initially non-fluorescent, emits a strong fluorescence upon reacting with thiols. It can detect as little as 1 picomole of cysteine. The absorption and emission spectra of the thiol adduct are pH-independent, making this assay kit highly robust.

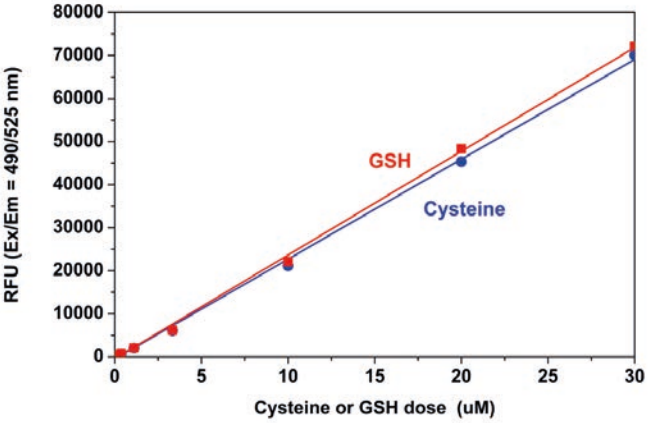


**Figure 25.** GSH and cysteine dose responses were measured in a 96-well black plate with Amplite™ Fluorimetric Thiol Quantitation Assay Kit (Cat# 5524) using a NOVOstar microplate reader (BMG Labtech).

## Applications for Thiol Quantitation

One of the key applications for quantification of thiols is in relation to reactive oxygen species. Reactive oxygen species (ROS) are highly reactive and naturally occurring intracellular byproducts of cellular metabolism. Increases in the production of ROS in cells are related to the overconsumption of oxygen. When the production of ROS in cells far exceeds its neutralization by antioxidants, the cell experiences oxidative stress. Prolonged or excessive oxidative stress is detrimental, causing significant damage to DNA or RNA, deactivation of enzymes, and damage to cell structures which can lead to apoptosis. The natural production of an antioxidant, glutathione (GSH), in cells acts as a defense mechanism to counteract the adverse effects of elevated cellular ROS. Amplite™ Thiol Quantitation Kits can be used to detect thiol groups in glutathione and monitor its rate of production in cells as a response to elevated levels of oxidative

stress from cellular activities. GSH also plays an important role in the detoxification of a variety of compounds and regulation of protein and gene expression via thiol-disulfide exchange reactions. AAT Bioquest's Amplite™ Thiol Quantitation Kits provide a rapid and sensitive approach for the detection and analysis of thiols resulting from thiol-disulfide exchange reactions. This can be a powerful tool in studying the effects of thiol oxidative-stress in areas such as immunochemistry.

In immunochemistry, phagocytic cells have been shown to regulate redox by producing antioxidants. For example, murine macrophages and dendritic cells secrete cysteine, which are taken up by T cells and converted to intracellular GSH. Furthermore, these phagocytic cells utilize GSH precursors, such as N-acetyl-L-Cysteine (NAC), to increase surface protein thiol expression. Through colorimetric and fluorimetric quantitative analysis, these increases in intracellular and surface-expressed thiols can be detected in mononuclear cells. This analysis allows for the study of thiol concentration and its effects on T cell reactivity and proliferation. AAT Bioquest's Amplite™ Rapid Colorimetric Total Protein Thiol Quantitation Kit (Cat# 5529) can be used to investigate the role of protein surface thiol groups during redox regulations. To investigate the role of intracellular, small molecule thiols during redox regulations, AAT Bioquest offers a convenient and ultrasensitive kit- Amplite™ Fluorimetric Total Thiol Quantitation Kit (Cat# 5524).

### Quantitation of Maleimides

For a long time, the challenge of quantifying maleimide groups was the lack of commercially available colorimetric and fluorimetric quantification tools. A rudimentary approach for the quantitation of maleimides can be performed by spectrophotometrically assaying maleimides at a wavelength of 302 nm with an extinction coefficient of 620 $M^{-1}cm^{-1}$. Unfortunately, this approach is a very insensitive assay technique complicated by the interference of protein absorbance at a nearby wavelength (ie. 280 nm). To eliminate this complication, AAT Bioquest invested in developing the most comprehensive set of solutions for maleimide quantification. Utilizing proprietary technology, AAT Bioquest provides two highly sensitive colorimetric maleimide quantitation kits that provide a rapid and robust colorimetric method for quantifying maleimides as well as an ultrasensitive fluorimetric maleimide quantitation kit.

### Quantitation of Maleimides with Amplite™ Technology

AAT Bioquest's Amplite™ colorimetric and fluorimetric quantitation kits are designed using proprietary technology to ensure rapid, robust and efficient quantitative analysis of maleimide groups. The quantitation of maleimides is an excellent intermediate step to verify the successful initial linkage of maleimides to the sample protein prior to bioconjugation with its target.

Colorimetric maleimide quantitation can be used as an intermediate step in bioconjugation involving the use of heterobifunctional crosslinker. Heterobifunctional crosslinkers are designed to have two different reactive-moieties at respective ends of the crosslinker. The bioconjugation of an antibody with an enzyme can be facilitated by heterobifunctional crosslinkers containing a NHS-ester and a maleimide moiety. Following the heterobifunctional-antibody reaction, the rate of successful linkage can be estimated by quantitative analysis of excess maleimides. AAT Bioquest offers an Amplite™ Colorimetric Maleimide Quantitation Kit (Cat# 5525) that may be used for such purposes.

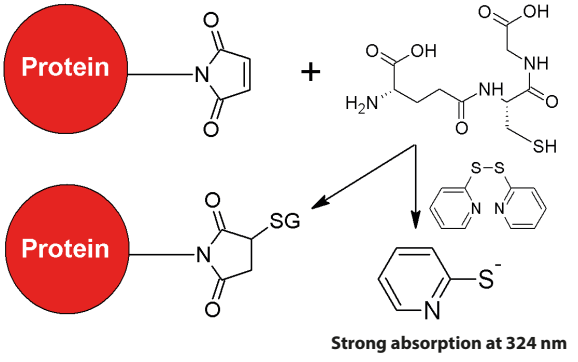


**Figure 26.** 4,4'-DTDP assay principle for quantifying maleimides.

Amplite™ Colorimetric Maleimide Quantitation Kit is a robust spectrophotometric assay that quantifies maleimide-reactive groups by reverse glutathione (GSH) assay with use of DTDP. This kit takes advantage of the high reactivity of GSH thiols with the maleimide moiety. GSH with a known amount of excess thiol is reacted with the sample to form stable thiosuccinimidyl linkages with GSH. The excess unreactive thiols of GSH are reacted with a DTDP and then assayed with a max absorption at 324 nm and a molar extinction coefficient of 19,800 $M^{-1}cm^{-1}$.

For a faster colorimetric maleimide assay, AAT Bioquest offers an Amplite™ Rapid Colorimetric Maleimide Quantitation Kit (Cat# 5526). This kit utilizes our proprietary Maleimide Blue™ probe, which significantly simplifies the maleimide quantification process. The target sample simply needs to be incubated with the Maleimide Blue™ sensor and passed through a spin column. The elutant can then be directly read in a colorimeter at a max absorbance of about 780 nm. The large separation between sensor $\lambda_{max}$ and protein $\lambda_{max}$ ensures minimal spectral interference and clean assay results, and the simple protocol allows for quick, convenient maleimide quantification.

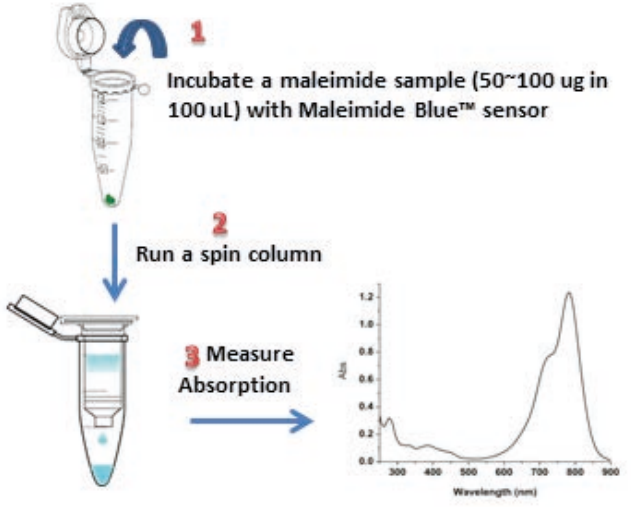


**Figure 27.** The assay principle of Amplite™ Rapid Colorimetric Maleimide Quantitation Kit (Cat# 5526).

Both Amplite™ colorimetric assays can be used to draw observations or make changes that will improve the success of bioconjugation. For example, a low max absorbance indicates a low concentration of maleimide moieties. This can be visualized with little to no color change in the assay solution. Since antibodies contain amine and thiol groups, a little to no color change can be indicative of self-polymerization, protein precipitation or antibody aggregation. This would indicate that changes should then be made to the bioconjugation protocol to in order to improve conjugation yield. An intense color change indicates a high concentration of maleimide-reactive sites after the initial crosslinking, and the antibody is ready for further bioconjugation with an enzyme or other protein.

In sensitive and selective situations where the protein of interest is expensive or only available in relatively small amounts and successful bioconjugation is paramount, use of fluorimetric quantitative analysis is recommended. AAT Bioquest's Amplite™ Fluorimetric Maleimide Quantitation Kit (Cat# 5523) is a convenient and ultrasensitive approach that can detect as little as 10 picomoles of maleimides. It can be used in a broad array of applications where the quantification of maleimide groups is essential. This fluorimetric assay has a wide dynamic range allowing for the detection of very weak and very strong signals in a large sample at one time. These benefits make fluorimetric analysis a sensitive and efficient approach to quantify maleimides.

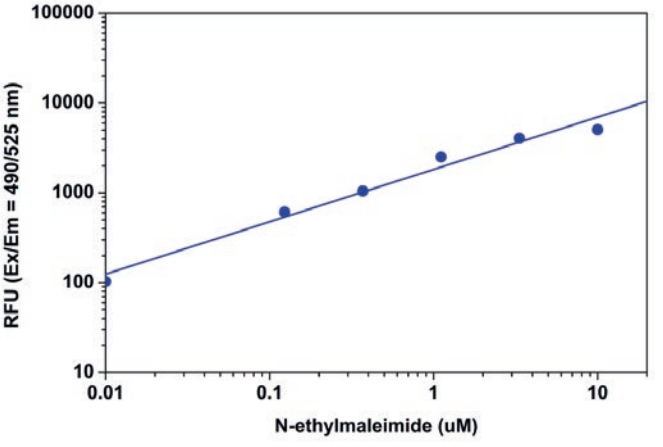


**Figure 28.** N-ethylmaleimide dose response was measured in a 96-well black plate with Amplite™ Fluorimetric Maleimide Quantitation Assay Kit (Cat# 5523) using a NOVOstar microplate reader (BMG Labtech). As little as 0.1 µM (10 picomol/well) maleimide can be detected with 10 minutes incubation time (n=3).

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**Table 4. Thiols and Maleimides Quantitation Reagents and Assay Kits**

| Cat # | Product Name | Size | Ex (nm) | Em (nm) |
|---|---|---|---|---|
| 5525 | Amplite™ Colorimetric Maleimide Quantitation Kit | 100 tests | 324 | N/A |
| 5523 | Amplite™ Fluorimetric Maleimide Quantitation Kit *Green Fluorescence* | 200 tests | 490 | 515 |
| 5524 | Amplite™ Fluorimetric Total Thiol Quantitation Assay Kit *Green Fluorescence* | 200 tests | 510 | 524 |
| 5526 | Amplite™ Rapid Colorimetric Maleimide Quantitation Kit | 2 tests | 780 | N/A |
| 5529 | Amplite™ Rapid Colorimetric Total Protein Thiol Quantitation Assay Kit | 2 tests | 680 | N/A |
| 5528 | Amplite™ Rapid Fluorimetric Total Thiol Quantitation Assay Kit *Green Fluorescence* | 200 tests | 510 | 524 |
| 21507 | Thiolite™ Blue | 5 mg | 335 | 460 |
| 21506 | Thiolite™ Blue AM | 1 mg | 335 | 460 |

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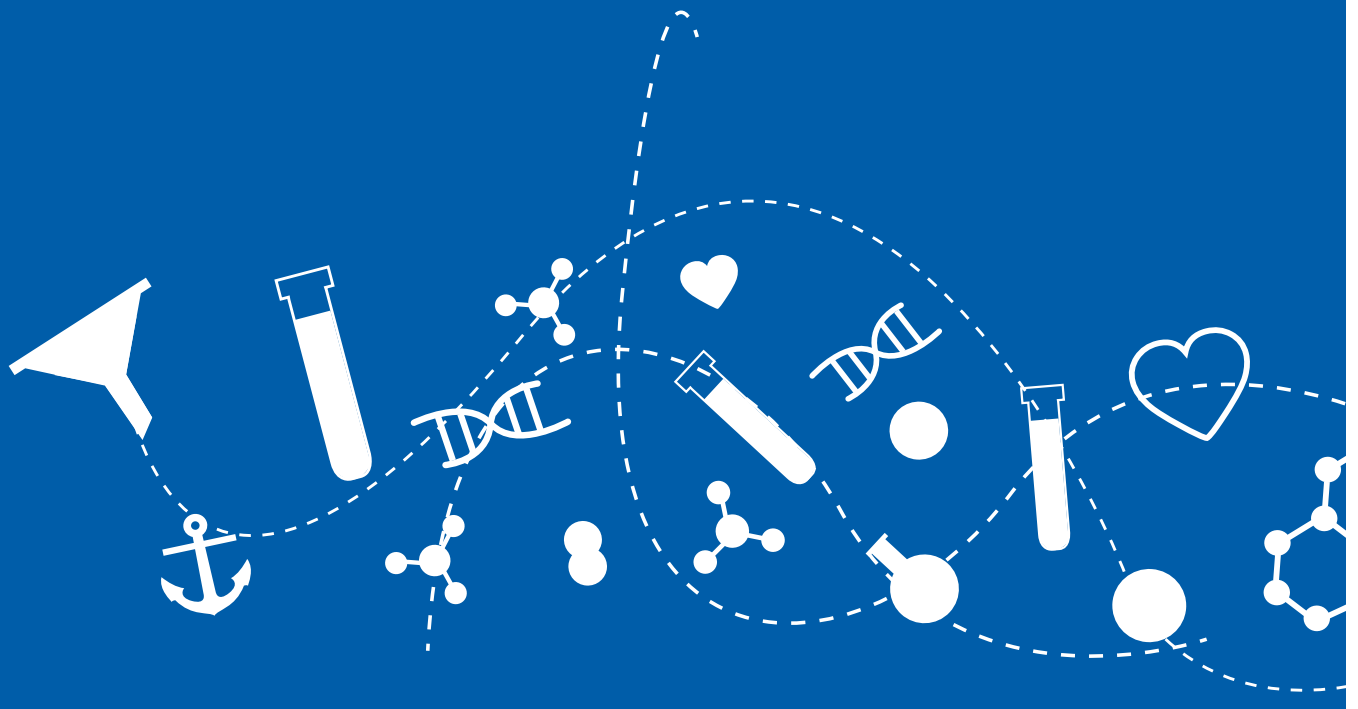

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