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SHINING THE LIGHT  
ON A LABORATORY  
WORKHORSE

ABSORBANCE DETECTION  
AND ITS APPLICATION  
WITH MICROPLATES



# Shining the Light on a Laboratory Workhorse

## Absorbance detection and its application with microplates

For over half a century, instruments measuring absorbance of light have served as standard equipment in every biological laboratory—a routine clinical lab is as likely to own such an instrument, as is a university lab engaged in cutting-edge research. Even as researchers find more and more molecules of interest in biological systems—cytokines, viral markers, tumor agents—and an unprecedented number of biological assays become commercially available, it is interesting to note that the detection of these molecules is still mainly conducted using the tried and tested techniques of absorbance measurement.

Figure 1: iMark – Bio Rad's Newest High-performance Absorbance Microplate Reader



Absorbance is by no means the only spectroscopic technique available to detect molecules or reactions in a sample—although it is the cheapest and the best known one. **Absorption spectroscopy** measures how much light of a particular wavelength or color incident upon a sample gets absorbed by it. The absorption of specific wavelengths (which gives rise to the perception of color if it happens in the visible spectrum) can be correlated with the presence and/or structure of a particular chemical. Colorimetric measurements are often easy and inexpensive to make, thus absorbance spectroscopy is widely used to determine the presence of a chemical, measure concentrations, detect conformational changes and binding of ligands, and follow enzyme reactions in a wide range of fields. The molecule, or part of a molecule responsible for giving rise to the color, is called a *chromophore*.

The other widely used spectroscopic techniques fall under **emission spectroscopy**, which measures the intensity of light of different wavelengths emitted by a substance. If the emission of light occurs following a molecule's interaction with electromagnetic radiation as it transitions to a lower energy state, the phenomenon is referred to as **fluorescence**. Light can also be emitted as the result of a chemical reaction, as in the case of **chemiluminescence**, or **bio-luminescence** if the chemical reaction occurs in a living organism. For this article, both chemiluminescence and bioluminescence will be referred to simply as **luminescence**. Measurements of these emissions give important information about the presence of molecules, and their structures. Fluorophores and luminophores are the components of a molecule or a chemical compound that causes it to fluoresce or luminescence respectively.

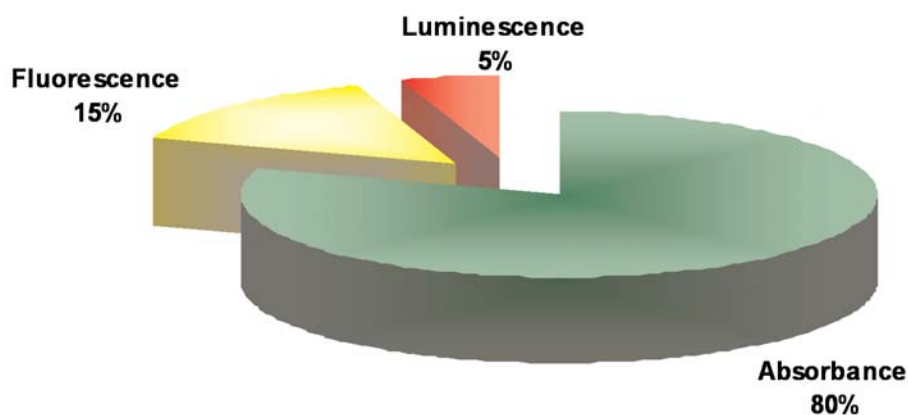
## Large and Still Growing: The Market for UV-Vis Absorbance Readers

At \$700 Million per annum, the market for absorbance detection instrumentation is one of the largest laboratory equipment markets, and is still growing, albeit modestly<sup>1</sup>. A significant part of this market is made of microplate absorbance readers—which come in a variety of configurations and functionalities. While absorbance only detectors still greatly outnumber the fluorescence based microplate readers, there is a growing trend towards multi-modality readers. It is expected that approximately half of all microplate readers in the U.S. are dedicated absorbance-based readers. While multi-mode spectroscopy has the potential to have a huge impact on the traditional UV-Visible (absorbance) spectroscopy market, the cost of this technique compared to traditional techniques, along with its overall performance, will be a crucial factor influencing the decision to switch technologies.

Absorbance detection has lasted for decades and is still the most affordable, accessible and flexible detection technology. When polled in an online survey<sup>2</sup>, respondents indicated that they the maximum growth potential for UV-Vis spectroscopy was in the fields of protein research and clinical analysis. The main end-users of absorbance microplate readers fall into 5 categories: academic labs, government labs, clinical labs, pharma and biotech companies. Approximately half the instrumentation dollars are coming from pharma and biotech companies, which have a tendency to purchase more expensive models. Clinical labs face the most severe market pressure and generally opt for standard, inexpensive readers.

The wide usage of absorbance readers is in no small measure due to the broad range of assays available for this technology. According to market experts, approximately 80 % of assays used in the U.S. are absorbance-based, while only 15 % are fluorescence-based and 5 % are luminescence-based.

Figure 2: Assay Development Distribution in the U.S. according to Detection Modality



### Absorbance, Fluorescence and Luminescence: Why and Why Not?

Absorbance measurements are routinely used to measure lab samples; however, newer techniques such as fluorescence and luminescence spectroscopy also hold out a lot of promise in the biology lab. The ubiquity of absorbance-based assays can be understood on the basis of

<sup>1</sup> Spectroscopy Magazine Online – August 8, 2007

<sup>2</sup> Spectroscopy Magazine Online – August 28-September 18, 2006

the many strengths of absorbance detection today, but its advantages or disadvantages as compared to newer spectroscopic techniques are not always fully appreciated.

#### Absorbance based detection:

Absorbance is the most affordable, accessible, simple and flexible of the spectroscopic technologies currently available. It is hard to beat absorbance in cost-effectiveness—the reader and the reagents for an absorbance based assay are typically a fraction of the comparable costs in fluorescence and luminescence. It is possible, for instance, to purchase adequate absorbance readers in the range of \$5-10,000 today; however, the cheapest line of fluorescent based microplate readers are in the range of \$16-18,000, with the range extending to \$50-100,000.

Another advantage of using absorbance based readers is simply that it is ubiquitous. Almost every lab has equipment readily available for reading microplates using absorbance. There are far more companies manufacturing assays and kits for absorbance, and there is a variety of absorbance readers available in the market. Absorbance detection systems are easy to operate and require minimal operator training to deliver results the user can trust. Moreover, the results can usually be observed with the eye, which provides an easy way for quality control.

The nondestructive nature of absorbance detection provides flexibility for the sample material to be measured by complimentary techniques as well. The signal is clean and robust, and after the reaction is completed, chromophores are stable and can be stored. This allows for repeat readings of the reaction without having to redo the assay. Modern absorbance readers are also quite fast, completing readings in 6-7 seconds, as compared to 2 minutes per sample in fluorescence readers.

The main disadvantage in absorbance detection is its relatively narrow dynamic range, being limited to two orders of magnitude. It has a limited standard curve range, which makes it necessary to dilute some samples many times in order to read them on the curve.

#### Fluorescence

The main advantage of using fluorescence is its higher dynamic range. The standard curve is much broader than that of absorbance, and the samples are likely to fall on the curve without undergoing multiple dilutions, saving on time, processing and reagents.

Homogeneous assays, which are used by screening labs in large pharmaceutical companies, usually require fluorescence based detection methods.

Fluorescent assays are much more expensive than absorbance based assays. The probes, the readers, the reagents—everything about fluorescence tends to cost more. However, for higher density formats—say 384 or 1536 well plate formats, additional costs for automated platforms for liquid handling, pipetting, washing etc. tend to proportionally reduce the disparities between absorbance and fluorescence-based platforms.

Compared to other methods, it is also just a bit more difficult to make fluorescence work—and there are a number of steps which can go wrong. Fluorophores are not as stable as chromophores—the fluorescent tags are photolabile and tend to be fairly sensitive to the

*"It is hard to be cheap, and absorbance is cheap. It has been around forever, it's very simple, it's very durable, it's robust, and it has a reasonably wide dynamic range."*  
-assay development manager in a biotech firm

environment e.g. pH, oxidation, temperature etc. One cannot simply pull the fluorescent probes off the shelf and expect them to work—one always has to budget time for troubleshooting and calibrations.

### Luminescence

Luminescent assays are mainly used as reporter assays and have fewer applications when compared to fluorescence. Luminescence shares the same advantage as fluorescence when it comes to having a higher dynamic range. It also has some of the same disadvantages in terms of luminophores being unstable, although they are more stable than fluorophores. However, there is still a limited window of time in which measurements can be made, as they decay and change with time. Since luminescence is a chemical reaction, it is very sensitive to temperature. There are a fewer number of companies that offer fluorescence and luminescence assays commercially, thus limiting the choices for end-users.

### Weighing the Pros and Cons: What Factors to Consider When Choosing a Detection Platform

While the sections above detail some of the advantages and disadvantages inherent in all these detection techniques, it is important to consider how these can affect some choices in the laboratories.

Figure 3: Comparison of Absorbance, Fluorescence and Luminescence-Based Assays

	<b>Absorbance</b>	<b>Fluorescence</b>	<b>Luminescence</b>
<b>Cost of Reagents</b>	Inexpensive	Expensive	Expensive
<b>Cost of Detector</b>	\$5,000-10,000	\$16,000 and above	\$13,000 and above
<b>Ease of use</b>	Simple to use	Difficult	Difficult
<b>Stability of reagents</b>	Stable chromophores	Photolabile fluorophores	Luminophores photobleach, but are more stable than fluorophores
<b>Commercial Availability</b>	Easily available, many vendors	Fewer vendors	Few vendors
<b>Operator Training</b>	Minimal	Some training required	Some training required
<b>Multiplexing</b>	Limited to 2-3 chromophores	5-6 fluorophores commonly used	Multiple luminophores used along with fluorophores
<b>Environmental sensitivities</b>	Robust, less sensitive to environment	Sensitive to oxidation, pH, temperature	Very sensitive to temperature
<b>Dynamic Range</b>	2-3 logs	5-7 logs	5-8 logs, but is non-linear at higher ranges
<b>Sensitivity</b>	pg/ml and ng/ml levels	Below pg/ml levels	fg/ml levels

Cost (dyes, substrates, readers, automation etc.): Absorbance is, by far, the cheapest technology, all the way around. The absorbance-based readers are at least four fold less expensive than the basic fluorimeters or luminometers. Even though antibodies, conjugates and plastics for fluorescence assays are getting cheaper as the technique gains popularity, they still add up to a significant cost. The substrates remain the most cost prohibitive part of fluorescence and luminescence based assays--the order of difference in cost between a colorimetric substrate and a luminescent substrate is 10-20 fold. Although in some cases, the same enzyme system can be used for absorbance, fluorescence or luminescence, more work is required to develop fluorescence and luminescence based assays, adding to their overall cost.

*“As an assay developer, you go for the “low hanging fruit” first--you go for the cheapest, easiest way possible i.e. absorbance. If that does not work, that is when you start getting more exotic technologies, such as fluorescence and luminescence.”*  
 -manager of new technologies in a pharmaceutical company

It has to be remembered though, that eventually, it is the cost per data point that is most important. While absorbance based assays are usually the cheapest, in some cases, other detection techniques might provide a cheaper cost per measurement. For example, in the case of a very expensive antibody conjugate system, a technique like fluorescence which can detect very small concentrations might end up as a less expensive system overall.

*Robustness and Reliability:* Absorbance has a very consistent signal, and the protocols are well-developed and robust. This makes it easier to train people to use an absorbance based assay. Given the high environmental sensitivities of fluorescence and luminescence based assays, as well as the tendency of the tags to photobleach, these assays present significant challenges to a development of a reliable lab protocol, that can be repeatedly and consistently used.

*Dynamic Range and Background:* Fluorescence and luminescence are considered more sensitive methods, largely because they have a higher dynamic range over which they operate. For most assays, however, the dynamic range offered by absorbance of 2-3 orders of magnitude is adequately broad.

Fluorescence and luminescence are also able to detect lower concentrations of the reaction product, but the sensitivity of an absorbance-based assay is often comparable to that of fluorescence or luminescence assays. Most assay protocols include a step of enzymatic amplification, which greatly increases the sensitivity of the system. In other cases, such as proliferation assays, fluorescence detection can theoretically lead to higher sensitivity, but that depends upon all other extraneous factors being held constant—something that is not practical. In practice, there is great variability in cells from one well to the next, which ultimately makes both fluorescence- and absorbance-based assays equally sensitive for this application.

*Multiplexing* allows the user to test for multiple analytes in the same sample. While multiplexing is possible with absorbance as well as with fluorescence/luminescence, there are greater possibilities with fluorescence-based detection. Multiplexing is possible only if different optically active moieties that are being used as tags have clearly differentiated, non-overlapping peaks in their absorbance/emission spectra. Typically, chromophores have wider wavelength ranges of high absorbance, than fluorophores have for emission. Multiplexing in absorbance based assays is usually limited to 2 chromophores.

Fluorophores have sharper peaks, and fluorescence based multiplex assays commonly utilize 4-6 tags. However, even with fluorescence based assays, there is crosstalk between the different tags, and the complexity of multiplexed systems can be considerable. Moreover, these assays do not work at the analytical standards as they would, if they were run separately. Currently, and for the foreseeable future, results obtained with single assays are more robust and reliable than multiplexed assays, and hence clinical assays are typically not multiplexed.

*Commercial availability:* There are fewer manufacturers of luminescent and fluorescent based assays. Many times the method of choice is dictated by what kit can be bought off the shelf.

*High Density Microplates:* 96-well plates are by far the most common type of microplates being used today. Higher density well plates, especially 1536 well plates increase the complexity of the system considerably. For one, they necessitate the use of robotic liquid handling systems since it is nearly impossible to manually pipette such minute volumes. Additionally, these are more prone

*“Absorbance is the easiest to train, and fluorescence is probably the most difficult. It needs the right emission wavelength, right excitation wavelength, the exact pH, an oxygen scavenger, just the right buffer etc. Similar things are also needed for luminescence, leaving open the possibility of many things going wrong.”*  
-director of technology in a life science provider company

*“Luminescence is probably the most sensitive detection technology, but many assays e.g. ELISA, include a step of enzymatic amplification. If you have a fluorescent label, you’re measuring just one fluorescent molecule. But if you have an enzyme label, the enzyme converts thousands of molecules of substrate into chromophores which will get detected. In this case, the latter situation gives you more sensitivity.”*  
-director of technology in a life science provider company

to errors due to meniscus effects and small air bubbles, which interfere with path length and optical signal. In absorbance, readings are made using a parallel beam and a meniscus or particulate matter can distort path length, introducing errors. In luminescence and fluorescence, optics are designed to produce 'sensed volume', and asymmetric meniscus, air bubbles etc. will tend to scatter light, changing the size and shape of the sensed volume.<sup>3</sup> However, neither end-users nor manufacturers are particularly concerned with any of these potential problems. There are several ways in which these errors are overcome: plate centrifugation, adding a drop of alcohol to the top of the well, or just eliminating that data point and repeating it. For absorbance, measurements done with dual wavelengths are particularly advantageous because the errors resulting from volume fluctuations may be eliminated by taking the ratio of readings at two wavelengths.

### **Absorbance Spectroscopy: Numerous Applications in Microplate Assays**

Given the inherent advantages of absorbance spectroscopy—its inexpensive nature, its long history of use, its stability and robustness—it is the detection technology that is most commonly used with microplate assays. Indeed, as one assay manufacturer called it, “absorbance is the bread and butter of assay development”. Most commonly, absorbance based microplate readers are employed for the following applications:

- **ELISAs (Enzyme Linked ImmunoSorbent Assays)** are the vast majority of absorbance based assays. Indirect ELISAs are used to quantify the amount of an antibody to a specific antigen, while sandwich ELISAs are used to indicate the quantity of antigen in a sample.
- **Protein assays** to measure concentrations of unknown proteins—these are usually performed by measuring absorbance at 280nm (and in some cases, by measuring absorbance at 205 nm), or by colorimetric methods such as Lowry's assay<sup>4</sup> or the Bradford assay.<sup>5</sup>
- **Cell based assays:** These include cell viability assays, proliferation and apoptosis assays, which are mainly based on colorimetric reagents. Examples include the MTT Cell Proliferation Assay<sup>6</sup>, XTT<sup>7</sup> and WST-1 proliferation assays, p53 sandwich ELISA<sup>8</sup> etc.
- **Nucleic acid quantification** has traditionally been done using absorbance measurements at 260 nm. Nucleic acid contamination of protein samples is measured using the ratio of absorbances at 260 and 280 nm.<sup>9</sup>

<sup>3</sup> Petersen, J. and Nguyen, J. Comparison of Absorbance and Fluorescence Methods for Determining Liquid Dispensing Precision. JALA 2005, pp.82-87.

<sup>4</sup> Lowry, OH, NJ Rosbrough, AL Farr, and RJ Randall. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 1951, 193, 265.

<sup>5</sup> Bradford, MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 1976, 72, 248-254.

<sup>6</sup> Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983, 65(1-2), 55-63.

<sup>7</sup> Scudiero, P.A., et al. Evaluation of a Soluble Tetrazolium/Formazan Assay for Cell Growth and Drug Sensitivity in Culture Using Human and Other Tumor Cell Lines. Cancer Res. 1988, 48, 4827-4833

<sup>8</sup> Apoptosis Assay Methods. Product information. Roche Applied Science: Indianapolis, Indiana.

<sup>9</sup> Sambrook and Russell. Molecular Cloning: A Laboratory Manual. 3rd ed.; Cold Spring Harbor Laboratory Press, 2001.

*“...if you're doing a new assay, you start going through catalogues, you see what is commercially available and if it ends up being an absorbance based assay which is \$50 dollars cheaper than the fluorescence based kit, then you're going to try the colorimetric one first.”*  
-manager of new technologies in a pharmaceutical company

- **Biochemical/activity assays:** Biochemical assays measuring activity of enzymes such as beta-galactosidase, or levels of creatinine, nitric oxide or bilirubin are all popular assays that measure the optical density of samples, which could be from blood, urine or saliva.

Due to the wide variety of applications and types of samples that can be measured, UV/Vis instrumentation is used in almost every life science end-market. Some of the markets and applications are listed in Figure 4.

Figure 4: Microplate Applications of Absorbance Spectroscopy



In pharmaceuticals and biotech industry, many of the high throughput assays for **drug discovery** are using absorbance-based detection systems to test for protease, kinase and cytokine activities.

These high throughput assays are also helping out in clinical trials where they are used for tracking specific biomarkers.

The **Food and Drink Industry** is a heavy user of absorbance-based assays. While these are routinely used for ensuring the safety of food products (checking for endotoxins and pesticide residues), they are also used for checking food quality. One such example is the phenolics assay in a winery laboratory that allows the wine-makers and enologists to monitor the fermentation, pressing and blending of different wines.<sup>10</sup> A similar phenolic assay is also used to determine the bitterness in virgin olive oil for quality control purposes.<sup>11</sup>

Biochemical and ELISA assays are routinely used in **Clinical Diagnostic Laboratories**, to test samples for many different types of biological biomarkers—from cardiac markers to neonatal screens. Allergy panels and autoimmune diseases are commonly diagnosed through ELISA tests. Serological and infectious diseases screening also utilizes absorbance based testing. Some of the more recent threats to public health—such as the concern about bioterrorism using Anthrax<sup>12</sup>, or the spread of West Nile virus<sup>13</sup> and Avian flu—are diagnosed using ELISA assays. Testing for lead in blood, another public health concern following the recent news of use of lead in some children's toys—is also conducted using absorbance based assays.

**Forensic Testing**, which is testing of biological samples for law enforcement, and **Therapeutic Drug Monitoring** (TDM, monitoring for drug levels in the blood), both utilize absorbance based assays. Forensic Testing is mainly done on government and law enforcement labs, and include testing for blood alcohol levels, as well as for the presence of illegal drugs (anabolic steroids) in the blood levels. TDM is often used to monitor the progress of drug treatment and check the metabolism of different drugs by the body. This is used to monitor the dosage for antidepressants, sedatives and other drugs—whose metabolism shows a large amount of variance in the patient population.

Researchers, both in academia as well as in pharma/biotech industry are using many of these assays to further their own **Research**—monitoring cellular processes, finding drug targets, manipulating biological systems. The assays most commonly used by them include cytokines, chemokines and cell surface markers, angiogenesis and tumor metastasis, DNA/ RNA and protein quantification, protein binding assays etc.

In **Veterinary Sciences**, absorbance-based kits are used as diagnostic tools in poultry, livestock and pets, and ensure that herds maintained by commercial operations are free of deadly diseases. These assays have been invaluable in face of the recent concerns about avian flu to check the health of poultry flocks, and also to screen large cattle herds for Bovine Spongiform Encephalitis (Mad Cow Disease).

These absorbance assays are also used extensively in **Toxicology**—for cell viability and

<sup>10</sup> Harbertson et al., Evaluation of a Comprehensive Red Wine Phenolics Assay Using a Microplate Reader. *Am. J. Enol. Vitic.* 2006, 57, 497-502.

<sup>11</sup> Garcia-Mesa, J.A., et al. Direct Spectrophotometric Determination of Bitterness in Virgin Olive Oil without Prior Isolation by pH Gradient. *J. Agric. Food Chem.* 2005, 53(24), 9615-9619.

<sup>12</sup> Cirino, N.M., et al. Rapid generation of an anthrax immunotherapeutic from goats using a novel non-toxic muramyl dipeptide adjuvant. *J Immune Based Ther Vaccines.* 2007, 5:11.

<sup>13</sup> Prince and Lapé-Nixon. Evaluation of a West Nile Virus Immunoglobulin A Capture Enzyme-Linked Immunosorbent Assay. *Clin. Diagn. Lab. Immunol.* 2005, 12(1), 231-233.

cytotoxicity assays, in **Agriscience**—for plant virus diagnostics, and in **Instrumentation**—for quality control and volume calibration of multichannel liquid handling systems.<sup>14</sup>

## Trends in Instrumentation

Applications for multimodal spectroscopy are expected to increase because of its flexibility for different assay technologies. However, wide adoption will mostly depend upon cost of ownership.

The market for absorbance detection instrumentation for microplate readers will continue to grow at a moderate rate despite expected declines in instrument prices. The differentiation in the market is along the lines of costs of different products as well as specialized features such as user interface, specific automation capabilities, differences in terms of speed, etc.

The configurations of absorbance microplate readers most valued by users are:

- **Wavelengths:** Multiple wavelengths readers, or at least dual wavelength readers are preferred in order to correct for possible fluctuations in light path length. Also, multiple wavelengths are needed for multiplexing. There are microplate readers that go 200 to 1000 nm wavelengths. However, there are few applications that need absorbance at the lower end of the scale (since a polystyrene microtiter plate starts to absorb at those wavelengths), or at the higher end of the range (water starts absorbing over 900 nm). The most commonly used wavelengths are 260, 280, 340, 405, 450, 605 and 690 nm.
- **Monochromator** instruments with variable wavelengths for characterization of unknown compounds are preferred to filter-based type instruments, which are more limited. However, there is a market for both types. Filter based are more likely to be used by laboratories performing routine testing at fixed wavelengths, and assay development groups, like a pharmaceutical company would need an instrument with a monochromator.
- **Versatility** on plate formats i.e. ability to read 24, 96, 384-well plates

## Absorbance Spectroscopy: Here to Stay

Absorbance spectroscopy is an older, well-developed technology, and as described earlier—there is a multitude of applications where it is routinely used. Newer technologies that are being developed, such as time resolved fluorescence, fluorescence polarization anisotropy etc. occupy a certain niche because of their strengths in one area or another. However, these newer technologies are not likely to have wide adoption until they become at least as inexpensive, flexible, and easy to use as the technology they hope to replace—absorbance.

All technologies eventually grow obsolete, and no doubt, absorbance detection will one day too—however, that is not going to happen unless there is a newer technology which enables laboratory users to do the task better, cheaper and faster. Given the robustness, flexibility and inexpensive nature of absorbance detection, it will remain the workhorse of the industry for a long time to come. It will continue to be viewed as a valuable tool that can be used in tandem with a variety of other techniques.

<sup>14</sup> Hafeman, D.G., et.al. Verification of Multichannel Liquid Dispenser Performance in the 4-30  $\mu$ l Range by Using Optical Pathlength Measurements in Microplates. Clin. Chem. 1998, 44(10), 2206-2208.

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