Spectrophotometry basics

What is spectrophotometry?

Spectrophotometry is a scientific method based on the absorption of light by a substance, and takes advantage of the two laws of light absorption.

Definition

spec·tro·pho·tom·e·ter/spektrōfōˈtämitər/

Noun: An apparatus for measuring intensity of light in a part of the spectrum, as transmitted or emitted by particular substances.

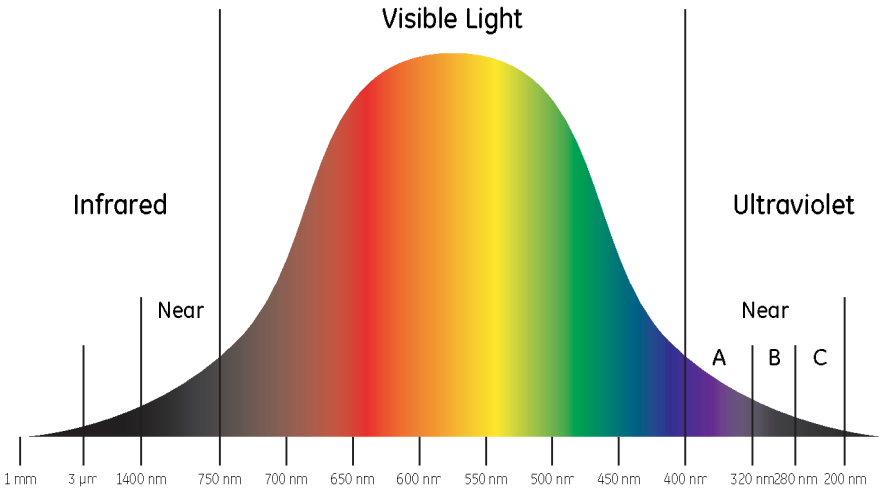

Fig 1. The electromagnetic spectrum. GE Healthcare Life Sciences offers a range of spectrophotometers which operate in the UV, visible and near infrared section of the electromagnetic spectrum.

Lambert's Law (1)

The proportion of light absorbed by a medium is independent of the intensity of incident light. A sample which absorbs 75% (25% transmittance) of the light will always absorb 75% of the light, no matter the strength of the light source.

Lambert's law is expressed as $I/I_o=T$

Where I = Intensity of transmitted light
I_o = Intensity of the incident light
T = Transmittance

This allows different spectrophotometers with different light sources to produce comparable absorption readings independent of the power of the light source.

Beer's Law (2)

The absorbance of light is directly proportional to both the concentration of the absorbing medium and the thickness of the medium. In Spectrophotometry the thickness of the medium is called the pathlength.

In normal cuvette-based instruments the pathlength is 10 mm. Beer's law allows us to measure samples of differing pathlength, and compare the results directly with each other.
GE Healthcare offers a variety of instruments and accessories which allow measurement of pathlengths from 10 cm down to 0.2 mm.

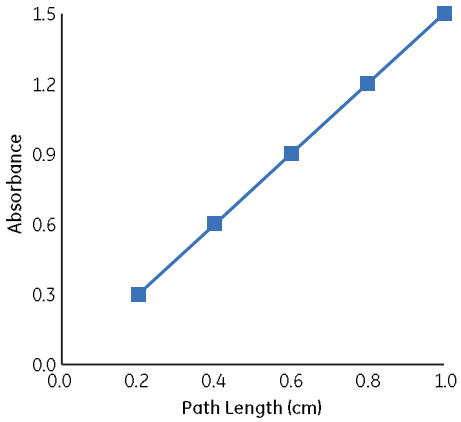

Fig 2. Beer's Law: The absorbance of light is directly proportional to both the concentration of the absorbing medium and the thickness of the medium.

Short pathlength instruments are used when the sample is of limited volume, scarce and maybe requiring recovery, or is very concentrated (e.g. > 50 µg DNA/ml), and the user wishes to avoid the need for dilution. Many samples would traditionally require dilution for two reasons:

1. So that there is enough volume to fill a 10 mm pathlength cuvette.
2. To lower the sample concentration enough to allow accurate measurement by the spectrophotometer.

Dilution introduces an aspect of human error and can also prevent the use of that sample in downstream applications.

To measure concentrated samples using a 10 mm pathlength would require a very powerful light source to give transmittance that is high enough to be detected reliably. A shorter pathlength reduces the absorbance – increasing the transmittance – hence reducing the incident light required to achieve a reliable result. This removes the need to dilute the sample, or to have a larger, more powerful or more expensive instrument.

When using short pathlengths (less than 10 mm), results are generally normalized to that of a 10 mm pathlength, e.g. In the case of a 0.2 mm pathlength, the absorbance results are multiplied by 50. However, at the same time any error from the system of absorption by the cuvette is also multiplied by 50, increasing the possible effect on the result.

In basic terms: Absorbance = Concentration × Pathlength

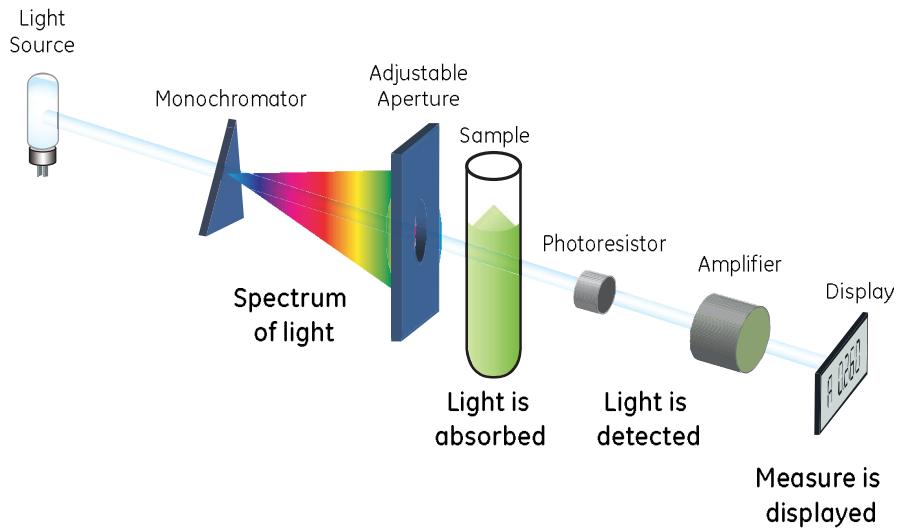

Fig 3. General schematic of a spectrophotometer.

Nucleic acid applications

Spectrophotometry can be used to estimate DNA or RNA concentration and to analyze the purity of the preparation. Typical wavelengths for measurement are 260 nm and 280 nm. In addition measurements at 230 nm and 320 nm can provide further information.

Purines and pyrimidines in nucleic acids naturally absorb light at 260 nm. For pure samples it is well documented that for a pathlength of 10 mm, an absorption of 1A unit is equal to a concentration of 50 µg/ml DNA and 40 µg/ml for RNA. For oligonucleotides the concentration is around 33 µg/ml but this may vary with length and base sequence.

So for DNA: Concentration (µg/ml) = $Abs_{260} \times 50$.

These values are known as conversion factors.

A number of other substances which also absorb light at 260 nm could interfere with DNA values, artificially increasing the result calculated from the absorption readings. To compensate for this a selection of ratios and background corrections have been developed to help eliminate false readings.

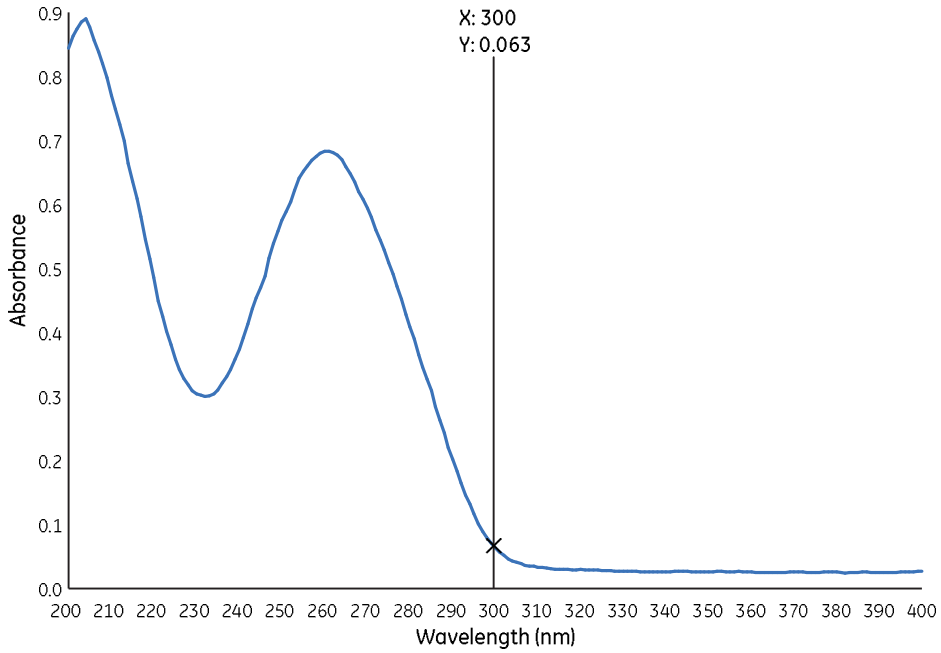

Fig 4. A typical wavelength scan for a pure DNA sample.

There is a wide absorbance peak around 260 nm preceded by a 'dip' at 230 nm. Therefore to measure the DNA absorption, the 260 nm DNA peak must be distinguishable from the 230 nm reading.

If the readings at 230 nm are too similar to those at 260 nm, DNA cannot be measured accurately. Higher 230 nm readings can indicate contaminants in the sample. There should also be a rapid tail-off from 260 nm down to 320 nm. For this reason, 320 nm is often used to measure background (see background correction).

Direct UV measurement

A_{260}/A_{280} Ratio

The most common purity check for DNA and RNA is the A_{260}/A_{280} ratio. Any protein contamination will have maximum absorption at 280 nm. Measurements are taken at both 260 nm and 280 nm and compared to give a ratio. For DNA the result of dividing the 260 nm absorption by the 280 nm needs to be greater or equal to 1.8 to indicate a good level of purity in the sample. For RNA samples this reading should be 2.0 or above. Results lower than this are indicative of impurities in the sample.

A_{260}/A_{230} Ratio

An increase in absorbance at 230 nm can also indicate contamination, which may in turn affect the 260 nm reading for DNA and RNA. A number of substances absorb at 230 nm, as this is the region of absorbance of peptide bonds and aromatic side chains. Several buffer components exhibit strong absorption at 260 nm and therefore can alter the results of photometric quantification. One example of such a component is EDTA in concentrations above 10 mM. Contaminants in a sample, such as proteins, phenol, or urea, can result in absorption at 230 nm. Phenol contamination also increases a sample's absorption at 280 nm and therefore can be identified through a lower A_{260}/A_{280} ratio. An A_{260}/A_{230} ratio of 2 or above is indicative of a pure sample.

A_{320} Background correction

Background correction is a process whereby the absorption at a point on the spectrum unrelated to the sample being analyzed is also measured, and the reading subtracted from the peaks. Absorption at 320 nm may be due to light scatter caused by particles, or to a precipitate in the sample. Dirty or damaged cuvettes can cause absorption at 320 nm. Contaminations with chaotropic salts, such as NaI, can also lead to increased light scatter.

Measuring and correcting for the reading at 320 nm therefore removes any interference from light scatter, from the cuvette, or in cases where a blanking plate is used to target the light beam though the sample.

Background correction is particularly useful when using small volume cells or specialist small volume spectrophotometers.

Nucleic acid measurements with low volume instruments

To get meaningful results, as a rule of thumb, the following two criteria must be met:

- Abs_{260} > approx. twice Abs_{230} (A_{260}/A_{230} Ratio=2)
- Abs_{260} = 0.1 or more (indicative of a high enough concentration, ie solution not too dilute)

Typically the only measurements checked for DNA measurement are concentration and A_{260}/A_{280} ratios. Unexpected results can often be explained by looking at the underlying A_{230}, A_{260}, A_{280}, A_{320} values.

As an example:

Sample 1

Concentration 10.7 µg/ml

A_{230}	A_{260}	A_{280}	A_{320}
9.27	0.244	0.129	0.030

A_{260}/A_{280}	A_{260}/A_{230}
2.162	0.023

Sample 3

Concentration 11.0 µg/ml

A_{230}	A_{260}	A_{280}	A_{320}
9.32	0.326	0.211	0.107

A_{260}/A_{280}	A_{260}/A_{230}
2.106	0.024

Sample 2

Concentration 11.1 µg/ml

A_{230}	A_{260}	A_{280}	A_{320}
9.33	0.323	0.206	0.101

A_{260}/A_{280}	A_{260}/A_{230}
2.114	0.024

Sample 4

Concentration 10.5 µg/ml

A_{230}	A_{260}	A_{280}	A_{320}
9.30	0.303	0.192	0.094

A_{260}/A_{280}	A_{260}/A_{230}
2.133	0.023

In this example, the instrument is giving concentration values which are very close together (<2% variation). So the reproducibility for concentration values is within the expected values. However, looking at the underlying absorbance readings shows that the 230 nm reading is very high compared to other readings making the ratio A_{260}/A_{230} very low. A value < 1.8 indicates potential contamination.

Note that in this example

- The background at A_{320} nm is provided for information & must be subtracted from the A_{260} nm reading to arrive at the absorbance
- For information: Not all low volume instruments display the A_{320} nm subtraction in the calculation.

Protein applications

As with DNA, proteins absorb light at a specific wavelength, allowing direct measurement using a spectrophotometer. The amino acids Tyrosine and Tryptophan have a very specific absorption at 280 nm, allowing direct A_{280} measurement of protein concentration. Direct UV measurement at 280 nm has many advantages, since the protein solution alone is used, without the addition of reagents, and it is not modified or inactivated during the process. No incubation period is required, so measurements are quick and highly reproducible.

The chemical composition of the protein will affect the absorption: the number as well as the type of amino acids will cause variation. How much a protein absorbs at 280 nm is dependent on the amount of the amino acids Tyrosine and especially Tryptophan: the aromatic ring of Phenylalanine absorbs well at 260 nm, but not 280 nm. So proteins of similar molecular weight can have quite different absorbances, since they can have completely different Tryptophan and Tyrosine content. UV absorbance of aromatic side chains is also affected by protein structure. Therefore conditions which affect structure, such as temperature, pH, ionic strength, or the presence of detergents, can affect the ability of aromatic residues to absorb light at 280 nm, and change the value of the protein's extinction coefficient.

As with nucleic acids each protein has its own conversion factor. The common standard protein bovine serum albumin (BSA) has a factor of 1.551.

$$\text{Concentration (µg/ml)} = \text{Abs}_{280} \times \text{Factor}$$

The A_{260}/A_{280} ratio can be used as a guide to the purity of the sample.

Some instruments also contain factors for other common proteins such as BSA or IgG which allow users to choose the protein closest in type to their sample, if the factor for the sample of protein is unknown.

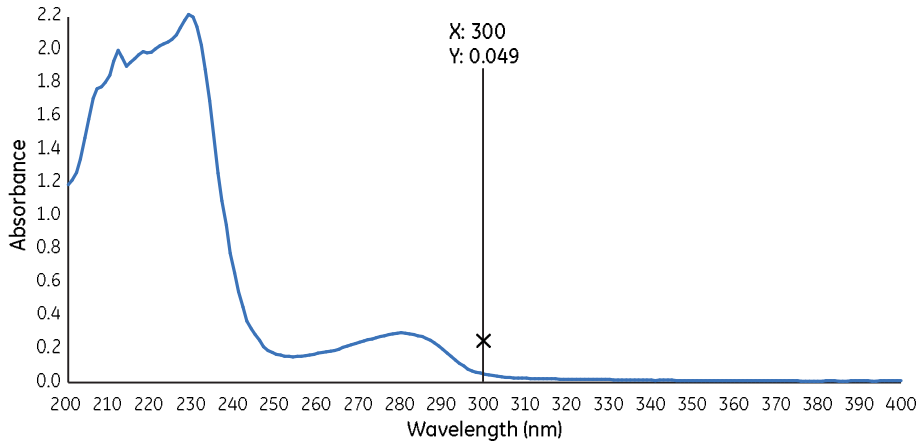

Fig 5. A typical wavelength scan for a protein sample.