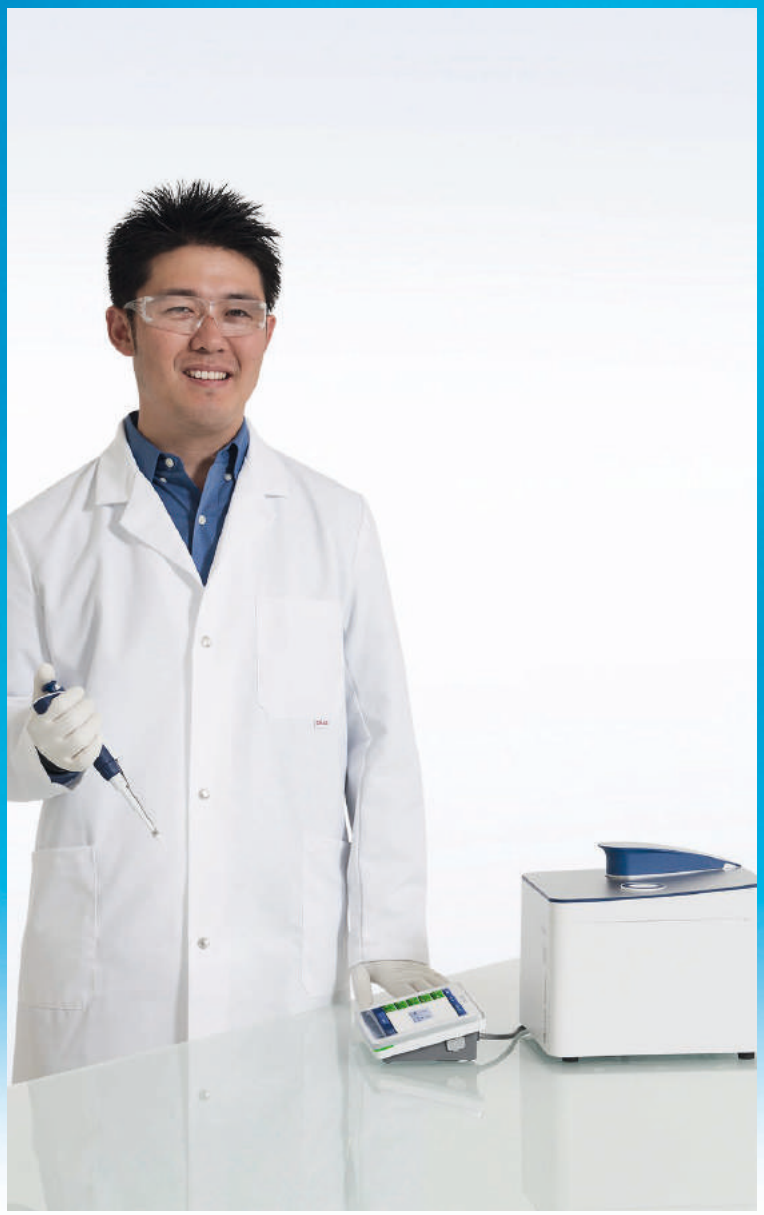
UV/VIS Spectrophotometry



Nucleic Acid Analysis

Protein Analysis

Cell Suspensions

Tips & Hints

Good UV/VIS Practice

UV/VIS Life Science Applications

in a Nutshell

METTLER TOLEDO

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Goal of this guide

This guide aims at giving a well-structured and easy-to-read introduction to the most important UV/VIS spectroscopy applications in the life sciences, as well as provide helpful key information for a successful analysis. Relevant information can be accessed quickly so that it can be used as a handy reference in the lab.

The guide is divided into two sections. The first is dedicated to applications and is classified by sample type. It covers nucleic acid analysis, protein analysis and cell suspensions. The second section gives general tips and hints with the aim to optimize the measurement workflow and improve measurement accuracy.

1. Introduction

UV/VIS Spectroscopy is a very powerful and popular method in life science. It has become a standard method that is used on a daily basis in many laboratories. This is mainly due to its simplicity; it does not require complex sample preparation, it is easy to perform and results are obtained quickly, usually within seconds. A typical measurement requires only a small amount of sample, and as it is a non-destructive method, samples can be used for subsequent analyses. The field of life sciences typically applies UV/VIS spectroscopy in the analysis of nucleic acids, proteins and bacterial cell cultures. The most common applications are the concentration determination of nucleic acids – DNA and RNA – the purity of nucleic acids, the concentration determination of proteins by direct measurement or colorimetric assays, the study of enzymatic reactions, and monitoring growth curves of bacterial cell suspensions.

For an in-depth introduction to UV/VIS spectroscopy we recommend the "UV/VIS Spectrophotometry – Fundamentals and Applications" guide, which is available here: www.mt.com/uv-vis

Beer-Lambert Law:

Many compounds absorb ultraviolet or visible light, a behavior that is described by the Beer-Lambert law. It states that the absorbance of a sample is proportional to its concentration. This is the principle used in simple concentration determinations of samples such as DNA and proteins. To be more precise, the Beer-Lambert law states that the absorbance is proportional to the extinction coefficient ε (epsilon), the path length d and the concentration c.

$$A=\varepsilon \cdot c \cdot d$$

A: photometric absorbance
ε: extinction coefficient
c: concentration of sample
d: path length

1.1 Spectrophotometer Instrument Types

There are essentially two different instrument categories in life sciences; the cuvette based spectrophotometer and the micro-volume spectrophotometer. Hybrid instruments come with an additional cuvette shaft and can be used for both micro-volume and cuvette measurements. However, their accessory range is limited, which also restricts the scope of applications.

Cuvette-Based Spectrophotometers
Conventional UV/VIS spectrophotometers with a large sample area for cuvettes can be used for various applications. They can accommodate many different cuvette types with a wide volume range and allow the usage of many optional accessories such as changers and thermostated holders.

Figure 1: Conventional cuvette based spectrophotometer

Micro-Volume Spectrophotometers
Micro-volume instruments are dedicated to those applications where only a small amount of sample is available or when the sample is highly concentrated. The sample volume required for a measurement is typically between 1 and 3 µL. The direct measurement of highly concentrated samples eliminates the tedious dilution step and avoids pipetting errors. It is also convenient that the sample is loaded directly onto the measurement surface, without the need for cuvettes.

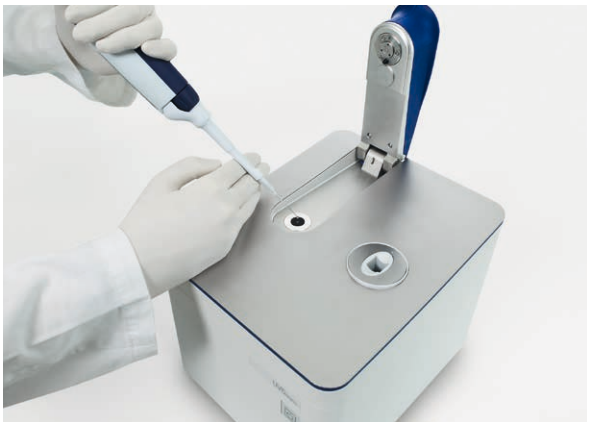


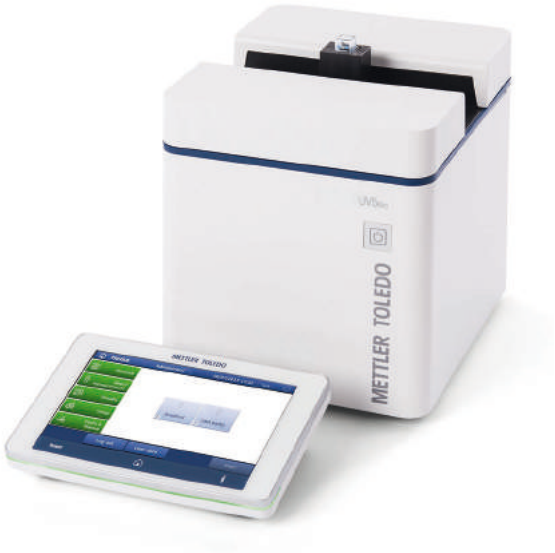
Figure 2: Micro-volume spectrophotometer

Most micro-volume instruments cover a wide concentration range by changing the optical path length of the measurement platform. For example, decreasing the path length by the factor 10 has the same effect as diluting the sample by a factor of 10. This is because reducing the path length also reduces the photometric absorbance. This way very high concentrations can be measured at moderate photometric absorbance levels. In other words, the shorter the path length, the higher the concentrations that can be measured. To achieve accurate results with this approach it is key that the exact path length is known.

1.2 METTLER TOLEDO Solutions for Life Science

METTLER TOLEDO offers two dedicated UV/VIS spectrophotometers for life science applications:

UV5Bio Spectrophotometer for Cuvette Measurements



The UV5Bio spectrophotometer is based on the FastTrack™ technology, which allows fast and accurate measurements. The open sample area on the top makes loading and unloading cuvettes and other accessories uncomplicated. Running an analysis is self-explanatory with the aid of the OneClick™ color touchscreen user interface. Its clear structure and intuitive smartphone-type functions means there is no need for extensive training. Users are expertly guided through each step of their work. The software includes a comprehensive package of life science methods ready to use.

Figure 3: METTLER TOLEDO's UV5Bio spectrophotometer

UV5Nano Spectrophotometer for Micro-Volume Measurements

The UV5Nano spectrophotometer combines innovative technology with intuitive usability. It can be used for small volumes of down to 1 µL, saving time for dilutions and sparing precious samples. The additional cuvette slot allows measurements of large volumes of up to 3 mL. The micro-volume measurements are based on the innovative LockPath™ technology ensuring high accuracy and repeatability. Two independent measurements at two precisely defined path lengths are taken so that a wide concentration range can be covered. The LockPath technology secures the path length at a fixed position, thus excluding path length drift over time. However, some laboratories need documented verification of their procedures. In this case, where utmost certainty is required, a calibration procedure for the path length can be performed.

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Figure 4: METTLER TOLEDO's UV5Nano micro-volume spectrophotometer

2. Nucleic Acid Analysis

2.1 Concentration Determination

The easiest and fastest method for DNA concentration determination is the measurement of absorbance at 260 nm. In this wavelength range, the aromatic ring structures of the bases adenine (A), thymine (T), cytosine (C) and guanine (G) exhibit their highest absorbance (Figure 5).

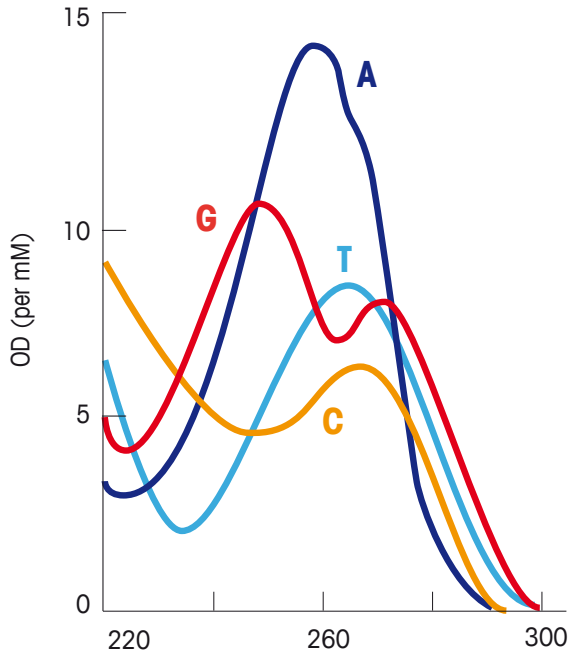


Figure 5: Absorbance spectrum of adenine (A), thymine (T), cytosine (C) and guanine (G)

As shown, the individual bases contribute to the overall absorbance with a maximum at 260 nm. There is, however, a notable difference between the absorbance of dsDNA and ssDNA. Due to the base pairing, double stranded DNA absorbs less light than single stranded DNA (Figure 6). This effect is known as hyperchromicity.

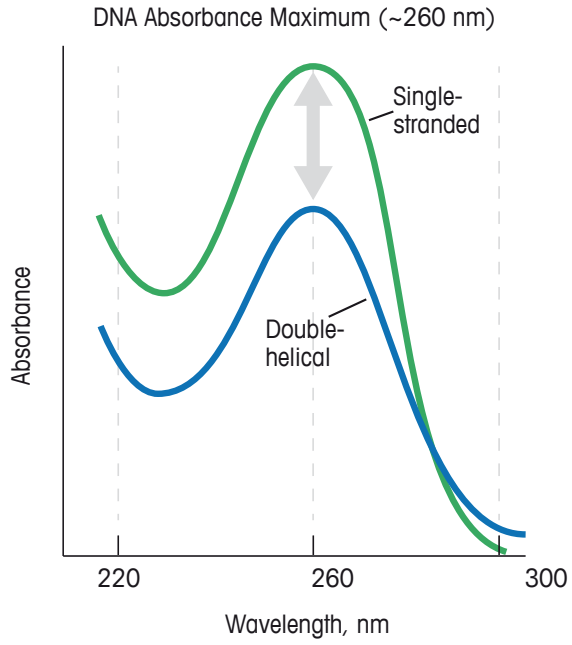


Figure 6: Absorbance spectrum of single stranded and double stranded DNA

The specific extinction coefficient of the nucleic acid must be known for the direct concentration determination at 260 nm. These values are commonly listed in reference tables such as Table 1, which lists the formulas and extinction coefficients of double stranded DNA, single stranded DNA, RNA and single stranded DNA oligonucleotides (Lottspeich and Engels).

Note: Measurement of nucleotide concentration at 260 nm is a good estimate of long, randomized sequences, however, it is less accurate for short sequences with repetitive elements (see "Concentration determination of oligos").

Sample	**Concentration**	**1 A equals**	**Extinction coefficient ε_{260}**
dsDNA	$c\left(\frac{\mu g}{mL}\right) = \frac{A_{260}}{0.02 \left(\frac{\mu g}{mL}\right)^{-1} \times cm^{-1} \times d}$	50 µg/mL	0.02 $(\mu g/mL)^{-1} \times cm^{-1}$
ssDNA	$c\left(\frac{\mu g}{mL}\right) = \frac{A_{260}}{0.027 \left(\frac{\mu g}{mL}\right)^{-1} \times cm^{-1} \times d}$	37 µg/mL	0.027 $(\mu g/mL)^{-1} \times cm^{-1}$
ssDNA Oligo	$c\left(\frac{\mu g}{mL}\right) = \frac{A_{260}}{0.03 \left(\frac{\mu g}{mL}\right)^{-1} \times cm^{-1} \times d}$	33 µg/mL	0.03 $(\mu g/mL)^{-1} \times cm^{-1}$
RNA	$c\left(\frac{\mu g}{mL}\right) = \frac{A_{260}}{0.025 \left(\frac{\mu g}{mL}\right)^{-1} \times cm^{-1} \times d}$	40 µg/mL	0.025 $(\mu g/mL)^{-1} \times cm^{-1}$

Table 1: Formulas for concentration determinations and extinction coefficients of various nucleic acids

2.2 Concentration Determination of Oligos (ssDNA)

Some parameters can influence the analysis of short nucleotide sequences, notably the base composition, sequence context, sequence length and oligo modification. These factors have an effect on the absorbance. In contrast to the simple approach of calculating the concentration with the average extinction coefficient of 33.3 (µg/mL)*cm for ssDNA, better accuracy can be achieved if the nucleotide sequence is known. Many different algorithms exist for calculating the extinction coefficient based on the sequence.

One simple approach is to sum up the individual extinction coefficients of the nucleotides:

$\sum[\varepsilon(\text{dNTP})]$

The molar extinction coefficients can be taken from table 2.

Nucleotide	**ε (molar ext. coeff.)**
dATP	15.4 mM^{-1} cm^{-1}
dCTP	9.0 mM^{-1} cm^{-1}
dGTP	13.7 mM^{-1} cm^{-1}
dTTP	10.0 mM^{-1} cm^{-1}

Table 2: Molar extinction coefficients of nucleotides (Lottspeich and Engels)

A more precise method is the nearest neighbor method, which takes the order of nucleotides into consideration. Using the extinction coefficients of dimers like AG, GC, or CT etc. is a better approximation than summing up the individual nucleotide extinction coefficients.

Most spectrophotometers dedicated to life science applications have a function to calculate the oligo specific extinction coefficient.

METTLER TOLEDO's UV5Nano spectrophotometer uses the nearest neighbor method for the extinction coefficient calculation. The oligo sequence calculator automatically determines the molar extinction coefficient, the molar mass and the extinction coefficient (nucleic acid factor).

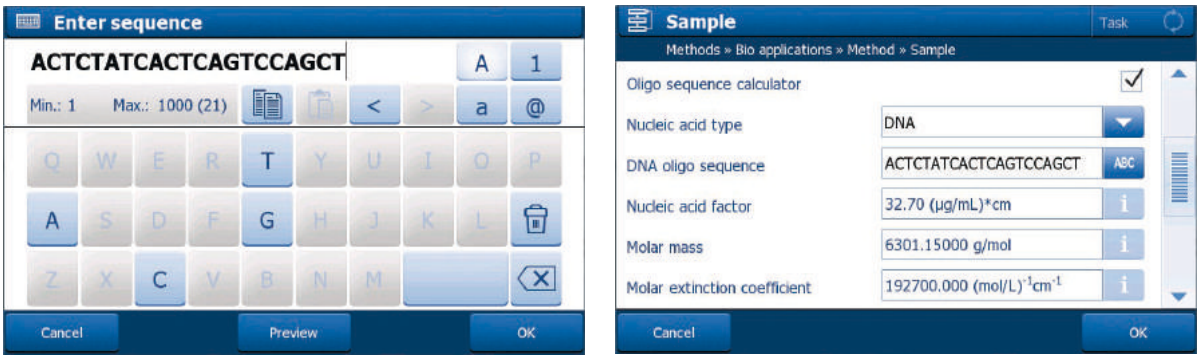


Figure 7: The Oligo Sequence Calculator automatically calculates the extinction coefficient

2.3 Detection Limits of Nucleic Acids

The detection of nucleic acids is constrained by an upper and lower detection limit. This is evident when considering the Beer-Lambert law, which states that photometric absorbance, path length and sample concentration are directly proportional to each other (see Introduction). Hence, the two main limiting factors are the optical capability of the instrument, namely its photometric absorbance range, and the path length chosen for the measurement.

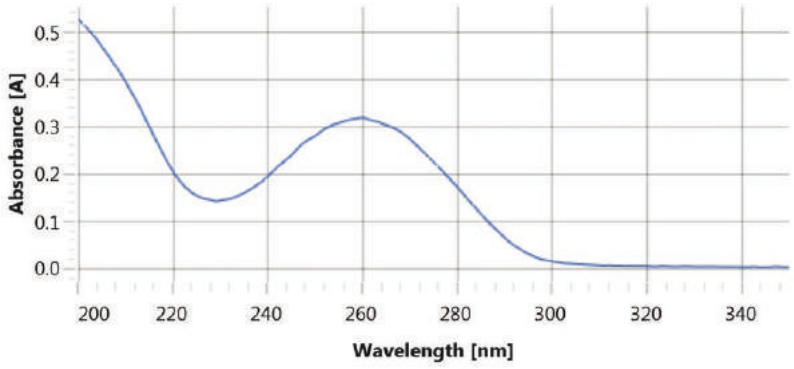
Modern micro-volume spectrophotometers often have an absorbance range of 3 A or higher and can set the path length to 0.1 mm or lower. This is necessary when measuring highly concentrated nucleic acid samples; concentrations of double stranded DNA solutions can be 15'000 ng/µL or higher. However, measuring high DNA concentrations is difficult and should be avoided if possible. Dissolving DNA properly is tricky; as is pipetting such a viscous sample. Furthermore, measurements at very high concentrations are not taken in the linear photometric range of the instrument and the true concentration might be higher than the actual measured concentration.

At the other end of the scale, samples of low DNA concentrations too are often measured in the lab. Various reasons, such as a poor yield, can make this necessary. However, low concentration measurements pose their own set of challenges, as the following example demonstrates. Consider the measurement of two dsDNA solutions of different concentrations (Figure 8). The upper box shows a measurement of a 274 ng/µL dsDNA solution, which results in a clearly defined spectrum. The peak absorbance at 260 nm is between 0.2 and 0.3 absorbance units, an ideal range with very good linearity. In contrast, the lower box shows a measurement of a 5.3 ng/µL dsDNA solution. Here, the signal becomes poorer as the peak absorbance at 260 nm approaches 0.03 absorbance units, close to the detection limit of the instrument. The DNA peak is nevertheless clearly visible.

It has to be noted that the error is bigger for measurements near the detection limit of the instrument for both high and low concentration measurements. Always measure in the optimal absorbance range for best accuracy.

Replicate measurements of a 274 ng/µL dsDNA solution

1	272.4
2	268.1
3	263.1
4	262.8
5	272.1
6	269.3
Mean	268.0
SD	4.22
Srel %	1.57



Replicate measurements of a 5.3 ng/µL dsDNA solution

1	5.7
2	5.4
3	6.3
4	5.6
5	6.1
6	5.8
Mean	5.8
SD	0.30
Srel %	5.26

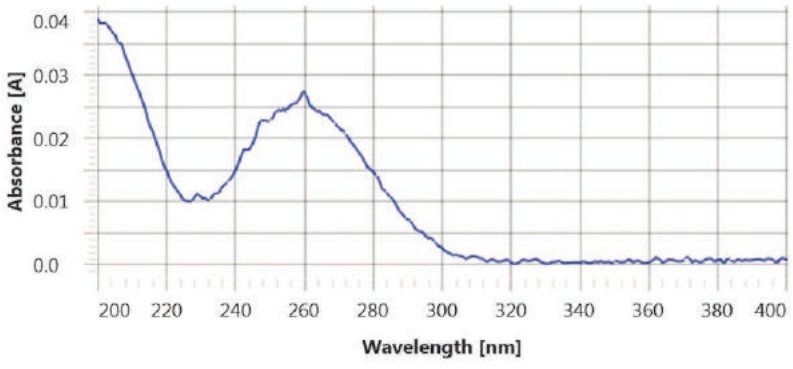


Figure 8: Absorbance measurements of dsDNA solutions with different concentrations at 260 nm. Each sample was measured 6 times.

2.4 Nucleic Acid Purity

In addition to concentration, absorbance measurements can also give an indication of the purity of a nucleic acid. Different wavelengths, depending on the absorbance of the contaminant, can be analyzed (Table 3).

A260/A280
Protein contamination can be detected by measuring the nucleic absorbance maximum (260 nm) and the protein absorbance maximum (280 nm) of a sample. The 260/280 ratio is a known indication of purity in nucleic acid solutions. In a pure solution this ratio should be 1.8 or higher for DNA and 1.9 or higher for RNA. However, ratios can vary from sample to sample, even for pure samples, depending on the base composition of the nucleotide sequences. Another important factor is that proteins show a lower A260 value than nucleic acids of comparable weight. Therefore, a small decrease of the 260/280 ratio can be indicative of severe protein contamination.

A260/A230
Various agents are used during the purification step, which absorb at 230 nm, such as phenol, urea, EDTA and molecules containing peptide bonds or aromatic compounds. An A260/A230 ratio of 1.8-2.2 indicates that samples are free of contaminants.

A320-A340
The range between 320 nm and 340 nm is ideal to detect foreign particles, such as from dirty cuvettes. In this range, nucleic acids show no absorbance and the measurement is often used as background correction.

Absorbance	Expected value	Contaminant
A260/A280	DNA: ≥ 1.8 RNA: ≥ 1.9	Proteins
A260/A230	1.8-2.2	Phenol, Urea, EDTA, molecules containing peptide bonds, aromatic compounds
A320-A340	<0.01	Particles from dirty cuvettes

Table 3: Absorbance values as indicators for nucleic acid purity

3. Protein Analysis

3.1 Protein Concentration Determination

Two types of analysis differentiate the determination of protein concentration: the direct measurement and the indirect method. In a direct measurement, proteins are measured directly at a suitable wavelength. The indirect method in contrast requires the proteins to be labeled with chromophores prior to measurement.
Table 6 summarizes methods for protein concentration determination and their advantages and disadvantages.

3.1.1 Direct Measurement

The direct measurement of proteins is a fast method providing an approximation of the protein concentration. Some proteins absorb light in the visible region, e.g. rhodopsin, chlorophyll, cytochrome and many metalloproteins. The concentration of these proteins can be determined easily by direct measurement provided their extinction coefficients are known (Table 4).

Almost all proteins can be detected in the ultraviolet range where amino acids with aromatic side chains, such as phenylalanine, tyrosine and tryptophan, absorb light. However, proteins are diverse and the proportion of aromatic side chains in proteins varies greatly. The determination of the protein concentration at 280 nm must, therefore, be considered an approximation; only aromatic side chains display absorbance. Proteins with no aromatic side chains show no absorbance and cannot be detected. Nevertheless, the direct method is ideal for quick protein concentration determinations. It also has the additional advantage that the sample can be recovered for further use. Figure 9 shows the absorbance spectra of the aromatic amino acids tyrosine and tryptophan, which contribute to the overall protein absorbance peak at 280 nm.

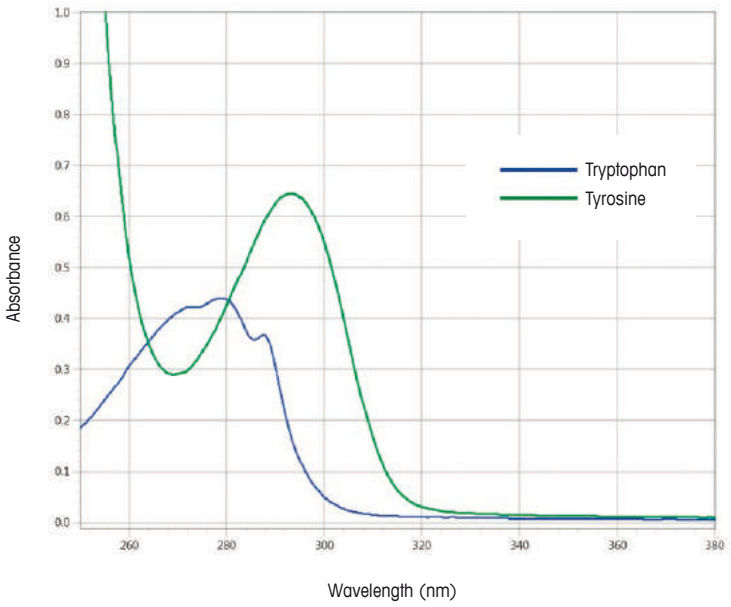


Figure 9: Absorbance spectrum of the aromatic amino acids Tyrosine (Tyr) and Tryptophan (Trp)

It is common to use the extinction coefficient of standard bovine serum albumin, 0.67 $(\mu g/mL)^{-1}cm^{-1}$, for concentration calculations. For more accurate results, it is possible to use the protein specific extinction coefficient, which can be calculated for a known protein sequence.

Nucleic acids too absorb at 280 nm. In fact, they absorb very strongly in the ultra-violet region, so that a small amount of nucleic acids can have a disproportionally large effect on the total absorbance. If the sample is assumed to contain nucleic acids, it is recommended that a colorimetric assay for the concentration determination be used.

Sample	Concentration	1 A equals	Extinction coefficient ε_{260}
Protein	$c\left(\frac{\mu g}{mL}\right) = \frac{A_{260}}{0.67 \left(\frac{\mu g}{mL}\right)^{-1} \times cm^{-1} \times d}$	150 µg/mL	0.67 $(\mu g/mL)^{-1} \times cm^{-1}$

Table 4: Formula for concentration determination and extinction coefficient of proteins

Proteins and peptides (protein fragments) also show absorbance at 230 nm. This is due to their peptide bonds. However, the 230 nm measurement is not suitable to derive their concentration as many other groups absorb in that range as well.

3.1.2 Indirect Methods (Colorimetric Assays)

More precise protein concentration determinations can be attained with colorimetric methods, in which a dye molecule binds to the proteins. The most common colorimetric assays are Bradford, Lowry, Biuret and BCA. All colorimetric assays are based on the same principle, namely that the reagent (dye) binds to a specific part of the protein. The absorbance is proportional to the number of dye molecules that have created a bond with a protein. The difference between these methods is the binding site for the dye. In addition, it has to be taken into account that the intensity of the color reaction depends on the protein sequence, the amino acid composition and amino acid frequency. Different proteins can result in color reactions of differing intensities.
A calibration curve is required for all assays. It is usually obtained from the common protein standard bovine serum albumin (BSA).

3.1.3 Bradford Assay

The Bradford protein assay has gained in popularity due to its simplicity, speed and high sensitivity. In this assay, proteins form a complex with the dye Coomassie Brilliant Blue. The dye can change its color from red/ brownish to blue based on the acidity of the environment. The Bradford solution is brownish, and then, upon

Standard Solution	Concentration of BSA [mg/mL]	Absorbance at 595 nm
S1	0.00	0.0009
S2	0.25	0.0959
S3	0.50	0.1822
S4	1.00	0.3814
S5	1.40	0.5315

Table 5: Solutions prepared with common protein standard bovine serum albumin (BSA)

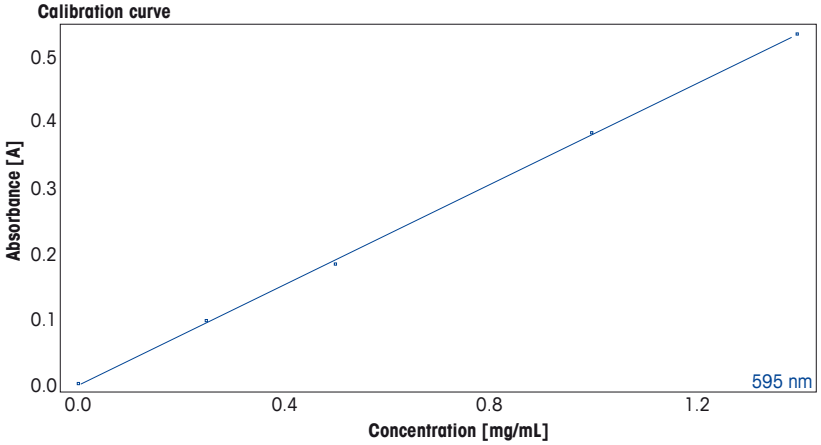


Figure 10: Calibration curve of BSA standard solutions measured at 595 nm.

binding proteins the color is changed to blue. The strength of the Bradford reaction depends on the concentration of basic amino acids (primarily arginine, lysine and histidine). The number of Coomassie dye ligands bound to each protein is approximately proportional to the number of positive charges found on the protein.
The protein concentration of unknown samples is quantified using a calibration curve (Figure 10), typically created from bovine serum albumin (BSA). The standard solutions for the calibration curve are listed in Table 5. This quantification is nevertheless an approximation as the ratio of positively charged amino acids differs from protein to protein.

	Principle	Advantages and Disadvantages
Direct at 280 nm	Detection of aromatic side chains	+ Very simple and fast + Sample can be recovered + No calibration curve required + No reagents required - Errors from contaminants that absorb in that region - Errors due to varying protein absorbance coefficients
Bradford	Reagent binds mainly to positively charged amino acid side chains	+ Simple and comparatively fast + Little interference with other chemicals + Good reproducibility
Lowry	Reagent binds only to tyrosine side chains	+ Good sensitivity - Interference with many agents, like Tris, EDTA, etc.
Biuret	Reagent forms a complex with peptide bonds	+ No amino acid bias + Very few interfering agents - Low sensitivity
BCA	Reagent forms a complex with peptide bonds and cysteine, cysteine, tryptophan and tyrosine amino acid side chains	+ No amino acid bias + High sensitivity - Interference with various buffers and reducing agents

Table 6: Methods for protein concentration determination

3.2 Kinetics

Kinetic assays measure the rate by which an enzyme converts its cognate substrate into product. This is accomplished by measuring the change in absorbance over time. Typically, enzymatic reaction rates are measured either by the rate of substrate consumption or the rate of product generation. As such, either the substrate or the product have to absorb light within the ultra-violet or visible range. In cases where both absorb, a wavelength where only one absorbs should be selected.
It is also possible to monitor the conversion of coenzymes like NAD or NADP. Only the reduced versions NADH or NADPH show absorbance at 340 nm. The amount of coenzyme is proportional to the amount of product so that it can be used for the determination of the reaction rate. The coenzyme can also be used in a subsequent reaction that is coupled to the reaction of interest. Figure 11 shows how the activity of the enzyme phosphofructokinase can be measured indirectly by monitoring the generation of NADH. The amount of generated NADH is directly proportional to the production of Fructose-1,6-biphosphate by PFK.

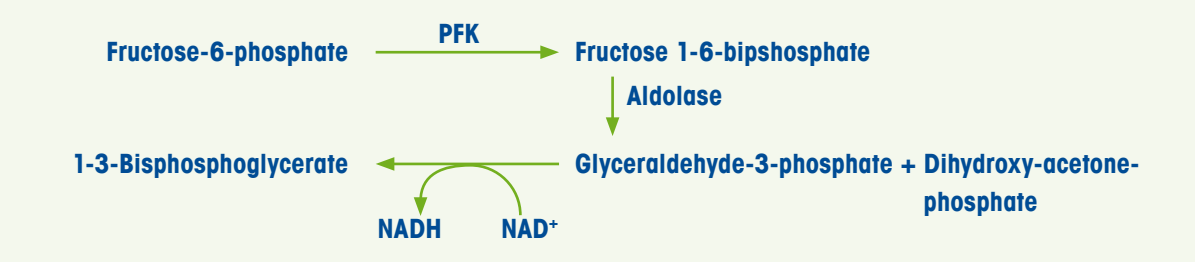


Figure 11: The reaction rate of the enzyme phosphofructokinase (PFK) is determined by the production of NADH in a coupled reaction

Example: Michaelis-Menten Kinetics of β-Galactosidase

The Michaelis-Menten model of enzyme kinetics is often used to study the important enzyme properties K_m and V_{max}. Michealis-Menten kinetics is also used to study enzyme inhibitors, for example how drugs inhibit the active center of an enzyme. In this example, the reaction rate of the enzyme β-Galactosidase was analyzed. The evaluation of the data was done automatically with the LabX software from METTLER TOLEDO, which determines the reaction rate and then derives K_M and V_{max} from the Eadie Hofstee Plot.

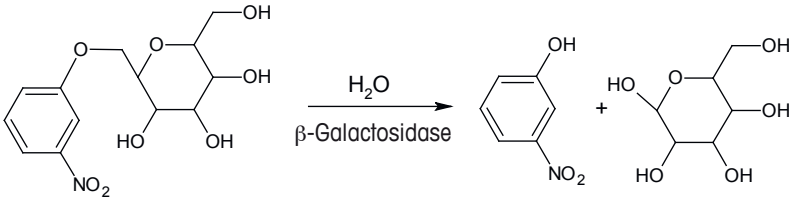


Figure 12: The substrate o-nitrophenol-β-D-galactoside (ONPG) is hydrolyzed by the enzyme β-galactosidase to o-nitrophenol and galactose

Reaction Rate (OD/sec) at 100 sec	[ONPG] (mM)	v/[s] (OD/(sec*mM))
0.00108	0.0556	0.0194
0.00139	0.1113	0.0124
0.00184	0.167	0.0110
0.00243	0.5060	0.0048
0.00253	0.835	0.00302

Table 7: Initial rate constants and v/[s] for different concentrations of ONPG

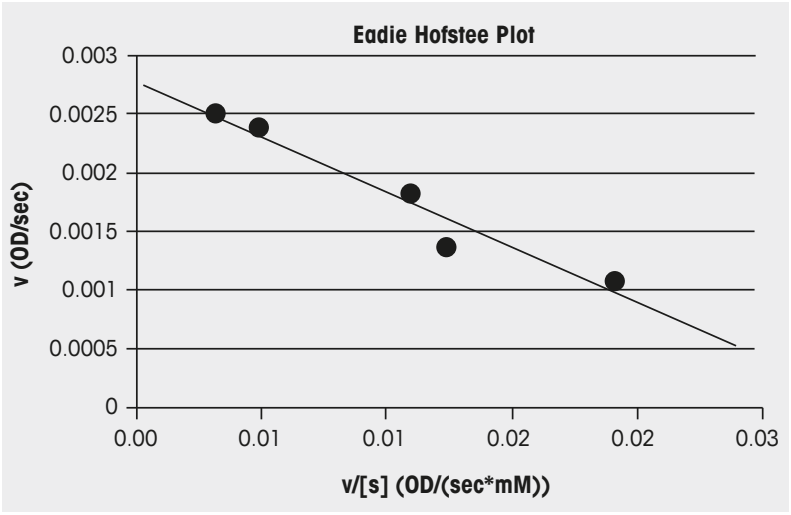


Figure 13: Eadie Hofstee Plot of the β-Galactosidase kinetics with ONPG as substrate

4. Concentration of Cell Suspension

Under certain conditions, UV/VIS spectroscopy can be used to measure the concentration of cell suspensions. In principle, it is a turbidity measurement and based on the fact that cell suspensions scatter light with increasing concentrations. Assuming optimal conditions, the decrease of light intensity is directly proportional to the number of cells in suspension.
The method can be used for bacteria, yeast and spores. Advantages of this method are that it is simple, fast and non-destructive. It is also suitable for continuous control of growth curves.

For ideal measurements the following points should be considered (Bast):
- A suitable wavelength has to be selected, where the cells do not absorb light. Usually a wavelength between 400 and 600 nm is chosen.
- The cell size should not be too small and not too large, approximately between 0.1 and 5 μm^3.
- The cell concentration should be low.
- The measurement should be taken only up to approximately 0.5 A, because the discrepancy from the linear relationship is otherwise too big.

5. Tips and Hints

Accurate and repeatable measurement results are achievable provided a few key points are considered. This chapter explores the tips and hints considered necessary for an ideal measurement workflow.

5.1 General Tips and Hints

5.1.1 Cuvette Selection and Handling

- Path length:
  According to the Beer-Lambert law, absorbance is directly proportional to the cuvettes' path length and sample concentration. Selecting an ideal path length (e.g. from 1 mm to 5 cm) can eliminate the need for dilutions. Absorbance that lies in the range of 0.2 A to 1.5 A generates the most accurate results. A micro-volume instrument with a very short path length, (1 mm or 0.1 mm) is ideal for highly concentrated samples.
- Transmittance range:
  High precision fused quartz cells (QS grade) are recommended for all UV/VIS analyses. For the visible range (>400 nm), disposable PMMA or PS cuvettes are frequently used. Nowadays, disposable plastic cuvettes for the ultra-violet range which can be used for DNA, RNA and protein analysis are also available.
- Positioning:
  Position the cuvette with the transparent side in the light beam, ensuring that the blank and sample cuvette label is pointing in the same direction.
- Cuvette handling:
  Be careful not to leave finger prints on the cuvette. Hold cuvettes only on the frosted sides.
- Filling:
  Mix the sample well before use, especially when re-suspending nucleic acids or proteins. Avoid using glass pasteur pipettes to fill the cuvette, as they could scratch the optical surface. Pipettes with disposable plastic tips are ideal.
- Cleaning:
  For thorough inner and outer cleaning use a 60% isopropanol/water solution and wipe with an optical cleaning cloth or lint-free tissue.

5.1.2 Sample Preparation

- Solvent selection and transmittance range:
  Select a suitable solvent that is transparent throughout the applied region.
- Concentration:
  Adjust the sample concentration for good absorbance (see section on path length selection above)
- Side reactions:
  Beware of possible side reactions between analyte and solvent molecules, which may affect the spectrum. Polar solvents (i.e. water, ketones, alcohols, etc.) dissolving polar samples can influence the electronic environment of the absorbing chromophore, thus lowering the spectral resolution.

5.1.3 Measurement Considerations

- Blank correction:
  The blank must consist of a fresh solvent, as used for the sample.
- Background correction:
  The background correction subtracts the measured absorbance value, typically at a wavelength where the analyte has no absorbance, from the measured absorbance value at the wavelength of interest. As an example for direct DNA and protein measurements in the UV range, a background correction is commonly applied at 320 nm or 340 nm as these molecules do not absorb light in this wavelength range. Therefore, measuring and correcting the background removes any possible interference due to light scattering by particles, air bubbles or a precipitate in the sample.
- Measurement at absorbance peak:
  For optimal results, measurements should be performed at the wavelength corresponding to the absorbance peak. The error that may be introduced is shown in figure 14. If the measurement takes place at the peak, a small shift in wavelength due to an improper instrument setting or instrument calibration has little effect on the absorbance value at the peak, but it has a great effect on the absorbance value at the shoulder of the peak.
- Measure in the linear range:
  The accuracy of measurements can be improved by running analyses in the linear range of the instrument; typically between 0.2 and 1.5 absorbance units.
  Avoid very high or very low absorbance values.

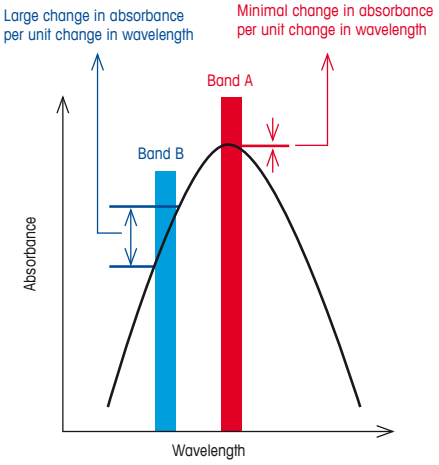


Figure 14: Measurements at the absorbance peak minimize errors

5.1.4 Effects of pH, Temperature and Ambient Air

- pH:
  The effects of pH on absorbance spectra can be very large if the conjugated acid or base has a different color (pH indicators Phenolphthalein, methyl red/orange etc.). If the spectrum of the sample under study is found to be affected by pH, a buffer should be used as a control.
- Temperature:
  Expansion of the solvent, mainly organic solvents, may change the apparent absorbance causing temperature dependency. For samples or solvents with high temperature dependency use a thermostatic sample holder.
- Lid:
  In order to limit solvent evaporation or water absorption from ambient air in case of hydrophilic samples use the lid normally provided with the cuvette.

5.1.5 Instrument Maintenance

- Periodic maintenance:
  Perform the instrument maintenance procedure regularly as recommended by the manufacturer.
- Check the performance of the instrument, ideally with certified reference materials at regular intervals. The CertiRef module for the UV5Bio instrument is a convenient accessory that runs performance verification test with certified reference materials, complete with automatic evaluation and pass/fail report. It checks all important optical parameters automatically such as photometric accuracy, wavelength accuracy, resolution and stray light, and eliminates the need for manual standard preparation and manual data analysis.

5.2 Tips and Hints for Micro-Volume Measurements

5.2.1 Sample Handling

- Use a suitable pipette for transferring a small sample volume.
- Use fresh tips for every pipetting step.
- Pipetting technique:
  Pipette the sample smoothly onto the micro volume platform to prevent the formation of air bubbles. Use a sufficient amount of sample to fill the area between the upper and lower platform. The sample should form a nice droplet shape as shown in figure 15. Air bubbles in the measurement gap can lead to false measurements. If required clean the surface with distilled water.

Figure 15: Droplet shape on the micro-volume platform

5.2.2 Cleaning

- The optical parts of the micro-volume platform, window on the lower side and mirror on the upper side must always be perfectly clean to achieve accurate and repeatable results. Clean the window and mirror twice with distilled water after finishing the measurement series or at the beginning of a measuring sequence prior to the blank measurement. This prevents the new measurement from being contaminated by the remains of an old sample. Use distilled water, analytical grade isopropanol/ethanol or a specific cleaning agent for cuvettes (e.g. Hellmanex III), applied with non-scratching, optical grade wipes.
- Clean both lower and upper sample area with a clean, dry, lint-free lab wipe between measurements.
- For thorough cleaning (e.g. if sample has dried on the platform) first clean the sample area with the solvent that is used to dissolve the sample, or 3 µL of 0.5 M HCl, 100% isopropyl alcohol, and then clean with water.

5.2.3 Instrument Maintenance

- Periodic maintenance:
  Perform the periodic maintenance procedure according to the manufacturer's guidelines.
- Performance verification:
  A regular instrument performance verification with certified reference materials can confirm the proper functioning of the instrument.
  For example, METTLER TOLEDO offers a calibration service for the UV5Nano spectrophotometer which checks both path lengths using certified reference materials from an independent manufacturer. The path lengths measured in this procedure are stored in the instrument and can be retrieved from the user interface for reference.

Figure 16: The certificate of calibration is issued after testing the instrument with certified reference materials

6. Good UV/VIS Practice

The Good UV/VIS Practice program by METTLER TOLEDO offers comprehensive guidance throughout the complete life cycle of your UV/VIS spectrophotometer. It helps to minimize risks and optimize the quality of measurements in order to obtain reliable results. The program includes the following 5 steps, which can also be considered individually.

- Step 1: Evaluation
  The first step is to determine what is needed, evaluating the current and future requirements of the laboratory.
- Step 2: Selection
  An informed proposal of the best suitable instrument including accessories and software based on the determined requirements from the evaluation process.
- Step 3: Installation and Qualification
  Installation and instrument qualification plays an ever more important role, especially in highly regulated environments. The GUVP EQPac for equipment qualification complies with the strictest regulatory requirements for installation, operational and performance qualification.
- Step 4: Training
  Appropriate instrument and software training should not be underestimated. Competent users make fewer mistakes saving time and money.
- Step 5: Routine Operation
  It is important to verify instrument performance from time to time during routine operation. This is best achieved using materials for which the expected values are precisely known, ideally with certified reference materials as used in the CertiRef module.

Figure 17: The GUVP life cycle for UV/VIS spectrophotometers

7. References

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More information on UV/VIS

www.mt.com/uv-vis

Learn more about Good Measuring Practices program

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