BERTHOLD

Whitepaper

# DNA QUANTIFICATION TIPS & TRICKS

## Using a microvolume spectrophotometer

### Introduction

DNA quantification is an important pre-analytical method, which is of great importance for many molecular biological analysis methods and can even determine their success. It is also a routine technique in procedures for translational research such as Next-Generation Sequencing (NGS), Polymerase Chain Reaction (PCR) or Real-Time PCR (quantitative PCR; qPCR), cloning or transfection, which initiates the subsequent workflow.

Absorbance has been the method of choice for routine quantification of DNA and RNA since decades. It is simple and convenient to use as no further sample treatment (other than DNA extraction) or reaction with other substances is required. However, it is not very specific (it measures all nucleic acids as a whole) and it is sensitive to contaminants, so it demands very pure DNA to be accurate. The use of fluorescent dyes permits the quantification of DNA with higher sensitivity and specificity, but fluorescence-based quantification of DNA is out of the scope of this whitepaper.

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Absorbance of DNA samples at 260 nm is currently most often measured using a microvolume spectrophotometer (see below), but it is also possible to use a cuvette spectrophotometer or a microplate reader. In this method, the concentration of a substance is calculated according to the Lambert-Beer law based on its absorbance. The following formula can be obtained from the original formulation of the law:

$$A = \varepsilon \cdot b \cdot c$$

Where A = absorbance at a given wavelength, ε = extinction coefficient, b = pathlength of the spectrophotometer, c = concentration of the sample.

Hence, for a pathlength of 1 cm, the concentration is equal to the absorbance at 260 nm (the absorption peak of nucleic acids), divided by the extinction coefficient.

dsDNA has an extinction coefficient of 0.02 (µg/mL)-1 cm-1, hence:

$$c_{dsDNA} = \frac{A}{0.02\ (\mu g/mL)^{-1} \cdot cm^{-1}} = A \times 50\ \mu g/mL\ (\text{or } ng/\mu L)$$

The same formula can be used with the respective extinction coefficients for ssDNA (absorbance x 37 µg/mL) and ssRNA (absorbance x 40 µg/mL). However, it is important to note that the formula is only valid for large nucleic acid molecules with a similar proportion of all nucleotides, such as genomic DNA, plasmids, etc. For oligonucleotides and other short nucleic acid molecules such as miRNAs, the extinction coefficient has to be calculated from the sequence of the oligonucleotide.

## Workflow

DNA quantification involves 6 main steps:

1. Choosing the right equipment and materials
2. Checking measurement settings
3. Pipetting the sample
4. Performing the measurement
5. Assessing sample quality
6. Cleaning sample area

Each step has some influence on the reliability of the quantification and is hence important to performed it correctly to ensure the best results.

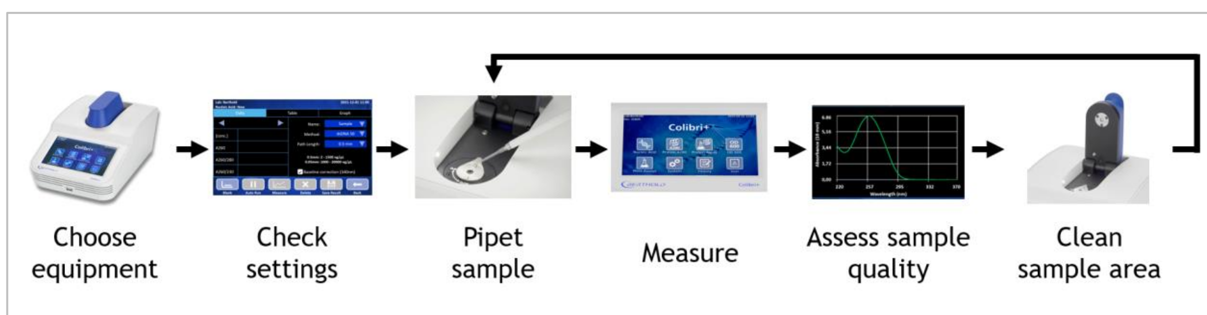


***Figure 1**: graphical representation of the workflow in a typical DNA quantification.*

### 1. Choosing the right equipment

If the laboratory has only recently been set up or is about to start working with nucleic acids, the first step is to select the right equipment for quantifying the DNA. This includes above all the spectrophotometer as well as the pipette and pipette tips.

#### 1.1. Spectrophotometer

In the past, the cuvette spectrophotometer was the only available option for quantifying DNA concentration by absorption measurements. However, its application was very limited due to the large sample volume required and the small sample volume available in molecular biology procedures. Although the sensitivity of a cuvette spectrophotometer is better than that of a microvolume spectrophotometer, the required sample quantity is very large: 300-400 µL in semi-micro cuvettes and 70 µL in ultra-micro cuvettes. A real breakthrough in the application of the method was only achieved with the introduction of microvolume spectrophotometers, which allow the measurement of tiny drops of the sample (typically 1 µL). Therefore, cuvette spectrophotometers for DNA quantification have been largely abandoned, and microvolume spectrometers are the instrument of choice for single sample absorption measurements.

***Definition***: the area where the sample is placed is called pedestal in some instruments, in others

sample compartment, and there are also other names used by different manufacturers. In this paper the term “**sample area**” is used to refer to the part of the instrument which comes in contact with the sample, including the bottom part where the sample is placed and the top part in the detection arm.

There is a broad range of microvolume spectrophotometers available on the market, and most have similar specifications, but there are some important details to consider:

- **Sample volume:** most instruments recommend 1 µL, but some require up to 5 µL, resulting in a larger consumption of precious sample, while others require smaller volumes to quantify samples of high concentration which require short pathlengths. Accurately pipetting volumes under 1 µL can be challenging in some cases.
- **Geometry of sample area:** in some microvolume spectrophotometers the sample area is a small pin, it can be challenging to place the sample in the right position. Also, the sample is exposed to air even when the detection arm is closed. In other instruments the sample is sealed when the arm is closed, which means that the sample can be pipetted more easily and is protected from evaporation. A few other models have a flat surface but lateral pins which make it difficult to clean the sample area between measurements.
- **Lower limit of detection:** most microvolume spectrophotometers have a lower limit of detection for dsDNA of 2 ng/µL. Some models list lower limits of detection in their specifications, but accuracy and reproducibility are generally poor at concentrations close to 2 ng/µL in most instruments.
- **Concentration range:** if you work with high-concentration samples, it is important to pay attention to this specification: if sample concentration falls outside of the linear range of the spectrophotometer, it is necessary to dilute the sample and quantify again. Most microvolume spectrophotometers can measure up to 15.000 ng/µL dsDNA without dilution but, if you work with high-concentration samples, check that the instrument you have or are planning to purchase allows you to measure most of your samples without dilution.
- **Measurement time:** most microvolume spectrophotometers have a measurement time of 5 seconds, but it can go between 2 and 12 seconds, depending on the instrument. If you routinely measure batches of many samples, then measurement time is an important specification for you.
- **Spectrum measurement:** most microvolume spectrophotometers in the market display the absorption spectrum of the sample, but some low-budget models don’t. In addition, instruments lacking spectrum capability usually have very limited wavelength selection capabilities. While purity ratios are often sufficient to assess the quality of samples (see below), the spectrum is much more informative. The flexibility to measure many different wavelengths can be of great benefit in laboratories using a broad range of methods. If you are looking at affordable microvolume spectrophotometers, check that they have all the functions you need.
- **Wavelength range:** all measurements which are relevant for DNA quantification are performed between 230 and 340 nm. This range is covered by almost all microvolume spectrophotometers on the market. However, if the instrument is going to be used for other applications, the broader the wavelength

***Figure 2**: the Colibri+ is an example for a particularly fast measuring system. The measurement of a sample takes less than 3 seconds.*

range, the better. Most instruments can measure up to 850 nm, but some can even analyze up to 1000 nm.

- **Contamination detection:** all microvolume spectrophotometers in the market display the $A_{260}/A_{230}$ and $A_{260}/A_{280}$ ratios, which are a well-established method for assessing the purity of a DNA solution. Some devices also display warnings if ratios are out of a given range, so highly contaminated samples don't go unnoticed. A few instruments on the market use spectral unmixing or other mathematical algorithms instead of purity ratios to flag samples as contaminated: this has the advantage of providing further information on the nature of the contaminant, but results in low sensitivity for detecting contaminants.
- **Cuvette port:** some microvolume spectrophotometers offer the possibility to measure cuvettes in addition to microvolumes. While the cuvette port can sometimes be useful, most laboratories already have a cuvette spectrophotometer or perform colourimetric tests in microplates, which are faster and more convenient than cuvettes, and where sample volume is not critical. **Touchscreen operation:** touchscreen-operated microvolume spectrophotometers have become the norm, but there are still some models on the market which require a computer. The screen should be fairly large, compatible with laboratory gloves, and have a clear user interface that facilitates tapping buttons, entering sample IDs, etc.
- **User/result management:** if the spectrophotometer is going to be used by many different users, it is convenient that the software offers some type of user management, or at least different folders for different users.
- **Other features:** some microvolume spectrophotometers provide special features which are not available in most other instruments: Some allow you to measure multiple samples simultaneously, have a built-in timer or a light that makes pipetting easier and more reliable. Look for the features that are most important for your application and choose the microvolume spectrophotometer accordingly.

***Tip*: if your samples frequently contain concentrations below 5 ng/µL dsDNA, using fluorescent dyes for quantification is more suitable for your application.**

### 1.2. Pipette and pipette tip

Since small sample volumes (typically 1 µL) have to be pipetted in microvolume spectrophotometers, choosing the right pipette and pipette tip is of great importance. Air displacement pipettes are the most commonly used. These are recommended to pipet aqueous solutions such as purified DNA samples. Mechanical pipettes are well suited, but electronic pipettes offer greater consistency and improved ergonomics. Most 10 µL pipettes have a volume range of 1-10 µL or 0.5-10 µL. They are therefore suitable for pipetting 1 µL. However, if your microvolume spectro-photometer requires to pipet smaller volumes (e.g., to use short pathlengths), it's recommended to use a smaller pipette (0.2-2 µL or similar). Pipettes should be calibrated at regular intervals (at least once a year).

Tips used to quantify DNA should be nuclease-free and have a filter to block aerosols from the liquid sample contaminating the shaft, and subsequently contaminating later samples. Low retention tips can be beneficial to reduce the volume of sample remaining inside the tip after dispensing and help to ensure that the entire volume is dispensed.

***Tip***: The pipette and pipette tip combination should be considered as a single instrument, as the same pipette may work differently when using different tips. Some pipette manufacturers even recommend recalibrating the pipette when switching to tips of a different model.

## 2. Checking measurement settings

Settings of a DNA quantification protocol using absorbance at 260 nm are quite straightforward: normally picking the correct DNA species (dsDNA or ssDNA) sets the conversion factor needed for the quantification, and that's it. But, depending on the software, some additional settings might be available:

- **Pathlength:** long pathlengths are used for low-concentration samples, short ones for high-concentration ones. On some instruments this is set manually, on others it is automatic, and in some you can choose between manual and automatic. Select the recommended pathlength for the expected absorbance of your samples. Be aware that the automatic mode takes longer on most devices, as the sample is measured with several pathlength settings and the optimal one is selected.
- **Baseline correction:** it corrects absorbance values for the contribution of particles in suspension (most often salt precipitates which appear when the sample is frozen). This is normally active by default at the wavelength recommended by the manufacturer (usually 320 or 340 nm), but it's always a good idea to verify that it is active.

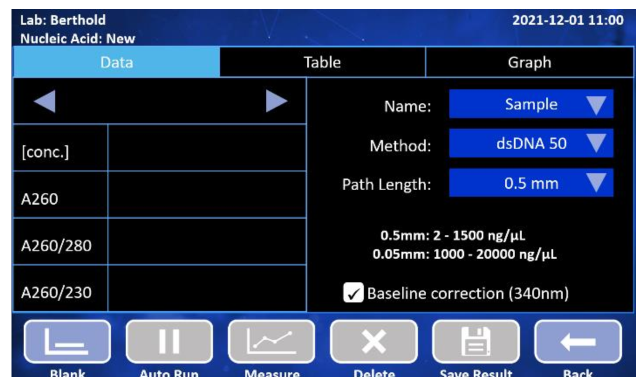


***Figure 3***: *typical parameters in DNA quantification*

## 3. Pipetting the sample

A reliable quantification starts with reliable pipetting. No matter how good the spectrophotometer is: if the wrong amount of DNA is pipetted, the wrong amount of DNA will be quantified. Thus, it is always good to keep in mind good pipetting techniques:

- Mix the sample tube gently but thoroughly, so the aliquot measured is representative of the content of the tube.
- Immersion angle of the tip should be as perpendicular to the liquid as possible, and immersion depth for small volumes should be around 1-2 mm.
- Aspiration should be careful and consistent from sample to sample.
- Some pipette manufacturers recommend forward pipetting technique to pipet small volumes, while others recommend reverse pipetting. If unsure, check the pipetting handbook of your pipette's manufacturer, or try both.
- Be careful when placing the sample: the drop has to be in the centre of the sample area of the microvolume spectrophotometer. Use one finger of your free hand to stabilize the pipet if needed.

- Ensure lighting conditions are good and allow you to clearly see the drop and sample area.
- Before closing the arm, observe if the drop is round and regular. If it flattens, the hydrophobic coating of the sample area needs reconditioning. Failing to recondition the coating of the sample area can lead to improper column formation and cross-contamination. Depending on the specific model of microvolume spectrophotometer and the way it's used, coating may need to be reconditioned often, or very seldom.
- As the drop to be measured is so small, depending on the laboratory conditions,, there may be considerable evaporation of the drop and thus concentration of the sample. If sample is exposed to air in your microvolume spectrophotometer (see above), measure as quickly as possible after placing the drop.
- Use a fresh tip for each sample.

***Figure 4**: pipetting in the sample area of the Colibri+*

## 4. Performing the measurement

Before measuring the first sample you have to blank the instrument. Blanking means setting a reference absorbance value of zero to the buffer in which the sample is dissolved. Blanking correctly is extremely important for the reliability of the quantification: blanking with the wrong buffer or a dirty sample area will result in wrong quantification.

To perform the measurement, refer to the documentation of your microvolume spectrophotometer, as the exact blanking procedure could differ depending on its software. The typical procedure is as follows:

1. Clean the sample area with deionized or distilled water.
2. Pipette the same buffer in which the samples are dissolved (usually TE buffer for nucleic acid samples).
3. Lower the detection arm and tap the "Blank" button.
4. Wait for the measurement to complete.
5. Wipe the sample area with lint-free wipe paper.
6. Before measuring the first sample, it can be helpful to verify the blank: place a new drop of the buffer used for blanking and measure it. Check that the absorbance at 260 nm is close to zero (usually under 0.020). If it's not, clean the sample area and blank again.
7. Wipe the sample area with lint-free wipe paper.
8. Pipette the first sample.
9. Lower the detection arm and tap the "Measure" button (if necessary).
10. If the spectrum looks unusual, clean the sample area and blank again.
11. Wipe the sample area with lint-free wipe paper.
12. Continue with subsequent samples.

***Tip***: if you are measuring many samples in a row, verify the blank at regular intervals

## 5. Assessing sample quality

In addition to measuring the DNA concentration of the sample, it is also important to assess its quality, as contaminated samples might be unsuitable for some downstream methods. This can be performed just after measuring each sample, or after finishing the full batch of samples. However, it is recommended to at least check the spectrum after measuring each sample, as this could reveal a bad blank that would affect all quantified samples until the instrument is re-blanked. Many of the most frequent contaminants can be estimated by measuring the absorption of the sample at wavelengths other than 260 nm: mainly 280 and 230 nm. A low ratio between the absorbance at 260 nm and the absorbance at 280 ($A_{260}/A_{280}$) or 230 nm ($A_{260}/A_{230}$) is a sign of contamination.

### 5.1. $A_{260}/A_{280}$ ratio

**Proteins** have a higher absorption at 280 nm compared to260 nm. The ratio between the absorbances at 260 ($A_{260}$) and 280 nm ($A_{280}$) is an accepted tool for assessing protein contamination in a sample of purified DNA. The $A_{260}/A_{280}$ ratio of a sample containing pure DNA with no protein contamination should be around 1.8, with values below 1.8 indicating contamination by protein, and higher ratios indicating contamination by **RNA**.

The sensitivity of the $A_{260}/A_{280}$ ratio for the detection of protein contamination is low: a ratio of 1.75 (only 0.05 below the “ideal” 1.80) could already indicate a protein content of about 50% in the sample. While some systems offer a corrected DNA concentration based on the deviation of the sample's spectrum from the theoretical spectrum of pure DNA, the high amounts of protein required for the difference to be measurable make the reliability of this correction questionable.

A low $A_{260}/A_{280}$ ratio may also be indicative of the presence of **phenol**, an additive used in some DNA purification methods.

Even small changes in the **pH** of the solution can also modify the $A_{260}/A_{280}$ ratio by up to 0.3, downwards in acidic solutions, and upwards in basic solutions. For example, DNA samples which have been prepared with TE buffer but are diluted afterwards with water will have a more acidic pH and hence a lower $A_{260}/A_{280}$ ratio, even if the DNA is completely pure.

$A_{280}$ values approach the lower detection limit of the instrument of the instrument faster than $A_{260}$ values: for example, for a concentration of dsDNA of 4 ng/µL, $A_{260}$ would be 0.080, and $A_{280}$ for a “pure” dsDNA sample would be 0.044, and the closer to 0, the higher the variability and error of the measurement. This means that the $A_{260}/A_{280}$ ratio is in most cases not reliable for low DNA concentrations (under 10 ng/µL). Hence, an abnormal $A_{260}/A_{280}$ ratio may NOT indicate contamination problems if the DNA concentration is very low.

### 5.2. $A_{260}/A_{230}$ ratio

Some common contaminants cause a relative increase in absorbance at 230 nm compared to 260 nm, and the $A_{260}/A_{230}$ ratio is hence also used to assess DNA purity. The $A_{260}/A_{230}$ ratio of pure DNA is typically between 2.0 and 2.2, with values up to 2.4 not being uncommon. A lower ratio indicates contamination by **protein, phenol, EDTA, guanidine or carbohydrates**. Hence, the presence of protein or phenol will cause a decrease in BOTH the $A_{260}/A_{280}$ and the $A_{260}/A_{230}$ ratios.

The information can be summarized in the following table:

| **Purity ratio** | **$A_{260}/A_{280}$ < 1.80** | **$A_{260}/A_{280}$ 1.80** | **$A_{260}/A_{280}$ >1.80** |
|---|---|---|---|
| **$A_{260}/A_{230}$ < 2.0** | Protein<br>Phenol | EDTA<br>Guanidine<br>Carbohydrates | Possible combination of RNA or high pH with other contaminants |
| **$A_{260}/A_{230}$ > 2.0** | Low pH | **Pure DNA** | RNA<br>High pH |

***Table 1***: *typical causes of deviations in purity ratios.*
*Color code: orange = contaminated, green = pure DNA, white = unclear*

### 5.3. Other quality problems visible in the spectrum

As summarized above, purity ratios are very informative about the type of contaminants present in the sample, and the presence of contaminants which alter $A_{260}/A_{280}$ or $A_{260}/A_{230}$ ratios is also visible in the spectrum:

- The presence of guanidine usually produces a shift of the valley, which should be visible around 230 nm, to longer wavelengths (usually around 240 nm).
- Phenol/Trizol and protein contamination also produce a shift of the valley at 230 nm, but also the peak at 260 nm, towards longer wavelengths.
- Unusual spectra (negative values in some parts of the spectrum, ragged spectrum…) usually mean the blank was not performed properly or that the sample area was is dirty.

Some instruments perform spectral unmixing or other mathematical algorithms to the spectral information to flag samples as contaminated, as this allows sometimes to identify the contaminant, but this method is often less sensitive to detect contamination than purity ratios.

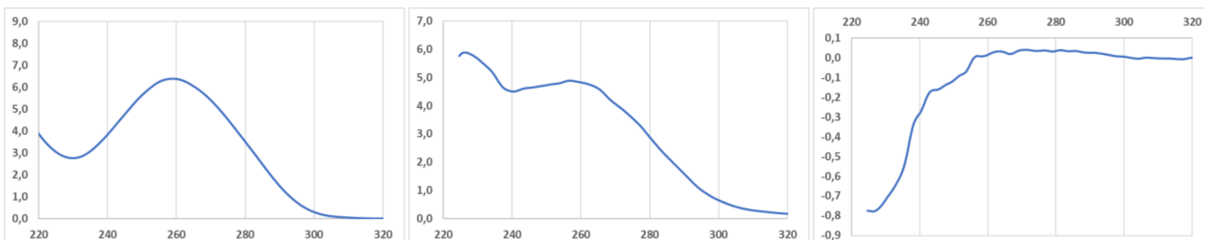


***Figure 5***: *typical spectra of a high-purity DNA sample (left), a DNA sample contaminated with protein (center) and a sample measured after a wrong blanking procedure (right).*

### 5.4. DNA integrity

Assessing the integrity of your samples is also very important, as degraded DNA is unsuitable for many downstream methods. Unfortunately, while the spectrum of the sample measured in a microvolume spectrophotometer is very informative about the purity of the extracted nucleic acids, it does not provide any information about its integrity. This is due to the absorption spectrum of free nucleotides being identical to that of nucleotides belonging to a large DNA or RNA molecule. To assess the integrity of nucleic acids, other methods have to be used (for example, running a gel electrophoresis and visualizing the nucleic acid using an intercalating dye).

## 6. Cleaning sample surface

The last step is simple, but it is very important, as any contamination of the sample area could lead to an overestimation of the DNA concentration.

- Wiping the sample area with a dry lint-free wipe paper is normally enough to clean the sample surface between measurements.
- If the samples measured have high concentration, some instrument manufacturers recommend wetting the wipe paper with distilled water to ensure proper cleaning.
- Wiping the sample area with a wet lint-free wipe paper is also recommended after the last sample is measured.

# Troubleshooting

Even when choosing the most suitable equipment, pipetting correctly, and following all recommendations, problems can still occur. Some frequent problems and their solutions are summarized here.

***Tip:*** the source of many frequent problems is improper blanking. If you are getting bad results, clean thoroughly the sample area, check that you are using the correct buffer for blanking and blank again before performing any further troubleshooting steps.

| Problem | Possible Cause | Solution |
|---|---|---|
| Measurement too high | Blanking not properly performed | Clean the sample area, check buffer used for blanking and blank again |
| | Surfaces of the sample area polluted | Clean reflector and sample area according to the description in the user manual |
| | Wrong normalization settings | Check normalization settings |
| | Wrong sample type selected | Select the right sample type |
| | Sample has air bubbles | Remove air bubbles from sample or pipet again |
| | Sample area not correctly closed | Open detection arm, check sample area and close detection arm |
| | Evaporation of sample drop increases concentration with time | Clean the sample area, pipet the sample again and measure immediately |

| Problem | Possible Cause | Solution |
|---|---|---|
| Measurement too low | Blanking not properly performed | Clean the sample area, check that the buffer used for blanking is correct and blank again |
| | Wrong normalization settings | Check normalization settings |
| | Wrong sample type selected | Select the right sample type |
| | The solutions are not homogenous and well-mixed prior to sampling | Ensure all solutions are homogenous and well-mixed prior to sampling, heat up samples if needed (high concentration) |
| | Sample concentration too high | Dilute sample and measure again |
| | Wrong pipetting of the sample | Clean sample area, pipette sample and measure again |
| Low accuracy or reproducibility | The solutions are not homogenous and well-mixed prior to sampling | Ensure all solutions are homogenous and well-mixed prior to sampling, heat up samples if needed (high concentration) |
| | Wrong pathlength selected | Check that the pathlength settings are suitable for the OD of the sample and measure again |
| | Sample concentration too low (close to detection limit of the instrument) | Quantify using a different method (cuvette spectrophotometer, fluorescent dye) |
| | Sample concentration too high | Dilute sample and measure again |
| | Sample has air bubbles | Remove air bubbles from sample or pipet again |
| | Surfaces of the sample area polluted | Clean reflector and sample area according to the description in the user manual |
| | Wrong pipetting of the sample | Clean sample area, pipette sample and measure again |
| | Scratched surfaces of sample compartment | Call for service |
| | Instrument calibration invalid | Calibrate instrument |
| | Hardware malfunction (lamp, optics, alignment…) | Call for service |
| Low $A_{260}/A_{280}$ ratio | Sample contaminated with protein or phenol | Troubleshoot extraction and purification procedure |
| | Low pH | Check that the buffer to resuspend or dilute the sample is correct |
| High $A_{260}/A_{280}$ ratio | Contamination with RNA | Troubleshoot extraction and purification procedure |
| | High pH | Check that the buffer to resuspend or dilute the sample is correct |
| $A_{260}/A_{280}$ ratio different to other spectrophotometers | Different wavelength accuracy of the compared instruments | No action required in most cases. If difference is large ($<0.4$), check calibration of both instruments. |
| Low $A_{260}/A_{230}$ ratio | Sample contaminated with phenol, protein, EDTA, guanidine or carbohydrates | Troubleshoot extraction and purification procedure |
| Unusual spectrum | Sample contaminated | Troubleshoot extraction and purification procedure |
| | Dirty sample area | Clean thoroughly the sample area, check buffer used for blanking and blank again |
| | Sample used for blanking instead of buffer | Clean the sample area, check buffer used for blanking and blank again |
| | Sample area needs reconditioning | Recondition sample area |
| | Sample volume too low | Increase sample volume |

| Problem | Possible Cause | Solution |
|---|---|---|
| Sample drop flattened | Sample area needs reconditioning | Recondition sample area |
| Detection time too long | Pathlength set to Auto | Set a specific pathlength which is suitable for the concentration of most of your samples |
| | Hardware malfunction | Call for service |
| Other problems | Probably instrument-specific problem | Check the user manual of your microvolume spectrophotometer. Contact Technical Service if needed. |

Not for use in diagnostic procedures.

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