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TECHNICAL NOTE

No. T-145

Blanking with high absorbing buffers such as RIPA negatively affects Protein A280 measurements

Abstract

Thermo Scientific™ NanoDrop™ Microvolume UV-Vis Spectrophotometers are useful analytical tools for measuring the absorbance of small volumes of sample. Before making a sample measurement, the instrument must be blanked. The best practice for making a blank measurement is to use the buffer in which the sample is suspended. Doing so allows the software to account for the absorbance of the buffer solution and report an accurate analyte absorbance. The best buffers for UV-Vis spectroscopy have minimal absorbance at the analysis wavelength of the analyte. Here we show that blanking with a buffer that absorbs an appreciable amount of light leads to atypical absorbance measurements by diminishing the amount of light available for the sample measurement.

Thermo Scientific NanoDrop One/One^{C} Microvolume UV-Vis Spectrophotometer

Introduction

The most basic components of a UV-Vis spectrophotometer include a light source, the sample, and a detector. Light is illuminated through the sample and the amount of light transmitted to the detector is quantified. To calculate the absorbance of a sample, the amount of light transmitted through the sample must be compared to the amount of light transmitted through a reference substance called the blank. Given the sample and blank transmittance, one can calculate the absorbance of the sample with the equation:

$$\text{Absorbance} = -\log\frac{\text{Transmittance of Sample}}{\text{Transmittance of Blank}}$$

The function of the blank measurement is to remove the absorbance contributed to the sample measurement by the buffer in which the sample is suspended. Essentially, the blank measurement allows the software to subtract the buffer signal out of the sample signal and accurately report the analyte absorbance. The above equation can be rewritten by expanding the logarithmic term to obtain:

$$\text{Absorbance} = -[\log(\text{Transmittance of Sample}) - \log(\text{Transmittance of Blank})]$$

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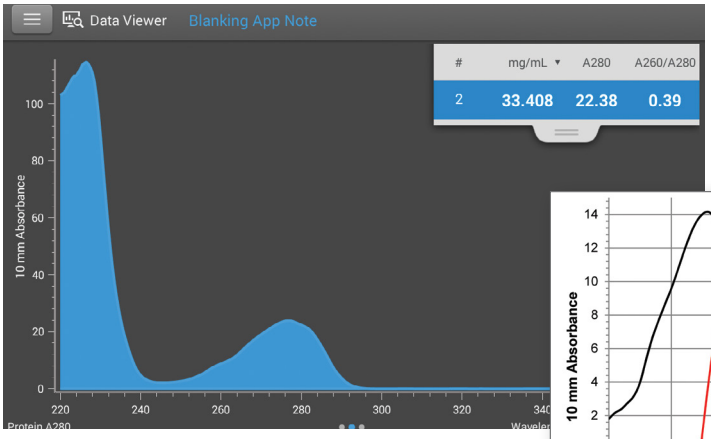
It is generally recommended to blank the instrument with the buffer in which the sample is suspended; however, not all buffers are suitable for absorbance spectroscopy. A good buffer for blanking NanoDrop UV-Vis Spectrophotometers should have no more than ± 0.04 AU (absorbance units at a 10 mm pathlength) at the sample's analytical wavelength. The analytical wavelength refers to the wavelength whose absorbance is used to calculate the analyte concentration. When the software automatically selects the best pathlength for the measurement, it does so using the absorbance at the analytical wavelength.

Radioimmunoprecipitation assay buffer (RIPA) is frequently used in protein lysis preparations, but absorbs a large amount of light near 280 nm. In this application note, we examine the effects of blanking with RIPA on the measurement of protein absorbance at 280 nm.

The absorbance spectrum of RIPA buffer

When preparing to make absorbance measurements of an analyte in a buffer one has never used before, it is recommended to perform a buffer analysis to determine how much light the buffer absorbs. A buffer analysis is very quick to perform. Simply open the application in which you will make analyte measurements, blank the instrument with water, and measure your buffer as if it were a sample. As stated above, you want the buffer to have minimal absorbance.

Figure 1 shows the absorbance spectrum of RIPA after running a buffer analysis. RIPA absorbs approximately 22 AU at 280 nm, the analytical wavelength of proteins. By comparison, phosphate buffered saline (PBS) absorbs less than 0.01 AU at 280 nm (not pictured).



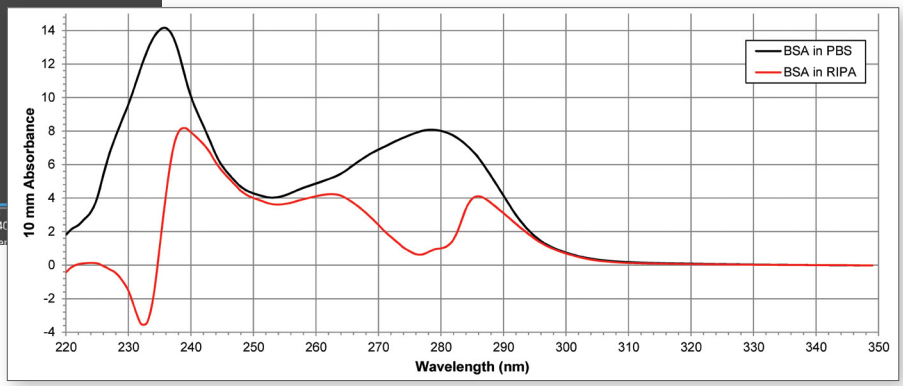
▲ Figure 1: Buffer analysis of RIPA. RIPA absorbs approximately 22 AU at 280 nm, the analytical wavelength of proteins.

The Thermo Scientific™ NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer operating software is built with the Thermo Scientific™ Acclaro™ Sample Intelligence Technology. One of the new technologies is the blank absorbance verification feature. The software will not let you proceed if you try blanking with a buffer that has increased absorbance at the analysis wavelength. Instead the software will display the message "Error: Blank solution absorbance too high. Clean both pedestals and blank again." This check is in place because blanking with a buffer such as RIPA can negatively affect the absorbance measurement.

Measuring protein suspended in PBS and RIPA

The Protein A280 application measures the absorbance peak at 280 nm and calculates a protein concentration using the protein-specific extinction coefficient. The absorbance peak at 280 nm appears due to the presence of tryptophan, tyrosine, and cysteine double bonds in the protein. Two solutions were prepared with the same weight of BSA and the same volume of solvent – one with PBS and the other with RIPA. Theoretically, the software should calculate the BSA concentration in both samples to be equivalent. Figure 2 displays the two resulting absorbance spectra. The black spectrum shows BSA suspended in PBS after the instrument was blanked with PBS. The red spectrum shows the result of blanking with RIPA and measuring BSA suspended in RIPA. While the PBS spectrum appears as we would expect, the RIPA spectrum is quite distorted. The BSA sample suspended in PBS displays a peak at 280 nm, yet the BSA sample suspended in RIPA displays a trough at 280 nm. The difference in the absorbance at 280 nm between these two samples is approximately 88%.

▼ Figure 2: Absorbance spectra of BSA suspended in PBS and RIPA. The black spectrum shows a pure BSA sample suspended in PBS after the instrument was blanked with PBS. The red spectrum was generated by blanking the instrument with RIPA and measuring BSA in RIPA buffer.



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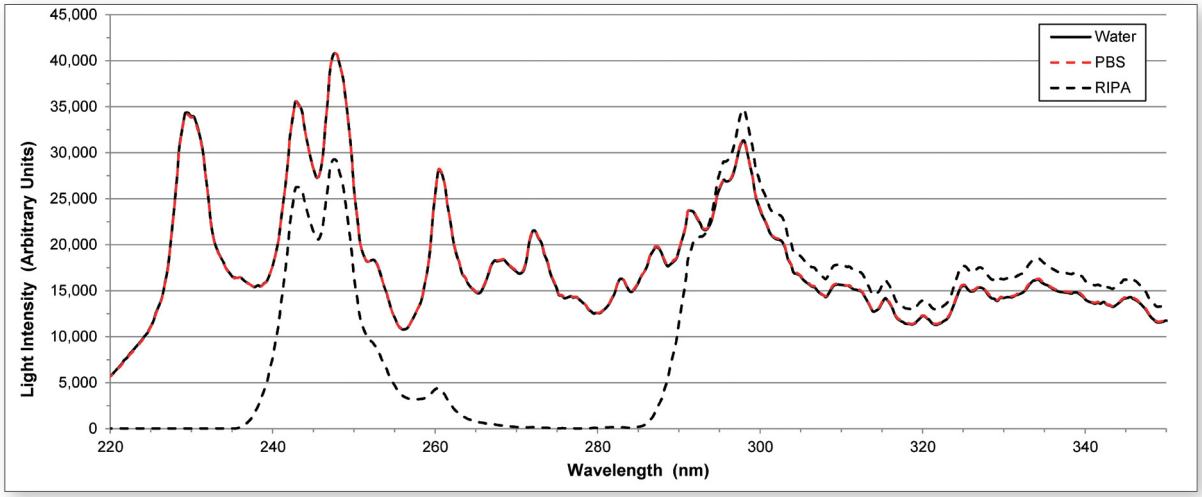
RIPA buffer limits the light intensity available for a sample measurement

The intensity check diagnostic is designed to confirm the flash lamp and spectrometer are working within specifications. When running the intensity check, the user leaves the pedestals dry and the detector measures the amount of light produced by the lamp at a 1 mm pathlength. The software verifies the peaks in the xenon spectrum appear at their NIST-traceable locations. Three intensity checks were performed – one with water on the pedestals, one with PBS on the pedestals, and one with RIPA on the pedestals. Figure 3 shows the resulting light intensities from 220 – 350 nm when different solvents were present on the pedestals.

Deionized water is highly transparent across the UV-Vis spectrum. Conversely, RIPA absorbs a large amount of light, especially in the low UV region. Data presented here show the water (black solid line) and PBS (red dashes) intensities overlap. This tells us the light signal is not attenuated by PBS buffer from 200 – 350 nm. In contrast, the intensity of RIPA (black dashes) shows a complete attenuation of light with wavelengths 220 – 235 nm and 270 – 285 nm.

Conclusion

It is recommended to blank NanoDrop UV-Vis Spectrophotometers using the same buffer in which the sample is suspended. To make accurate analyte measurements, the blank buffer should have minimal absorbance near the analyte analytical wavelength. This is true with the Protein A280 application as well as with other applications, such as Nucleic Acids. Researchers can determine the buffer absorbance by performing a buffer analysis. In the case of RIPA buffer, it absorbs a large amount of light at the analytical wavelength of proteins. Absorbing such high amounts of light limits the light available for the analyte measurement. This characteristic makes RIPA a poor buffer choice for quantifying protein concentration using a direct 280 nm measurement. Rather than using the Protein A280 application, it is recommended to use a colorimetric assay application in the NanoDrop Spectrophotometer Software to measure the concentration of proteins in RIPA buffer. The operating software for full spectrum NanoDrop UV-Vis Spectrophotometers are hardcoded with applications to read the results of the Bradford, BCA, and Thermo Scientific™ Pierce™ 660 nm Protein Assays. This is discussed in detail in the technical document T112 – Influence of Buffer on Choice of Protein Quantification Method.



▲ **Figure 3: Flash lamp intensities with different solvents on the pedestal. The intensity of the lamp with PBS on the pedestals (red dashes) is the same as the intensity with water on the pedestals (black solid line). The signal with RIPA on the pedestals (black dashes) is attenuated near 280 nm and at wavelengths 220 – 235 nm.**

For further assistance, contact NanoDrop technical support at **nanodrop@thermofisher.com** or visit **thermofisher.com/nanodrop**

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