Microvolume Quantification of Proteins by UV-Vis Absorbance or Fluorescence

Key Words
NanoDrop Spectrophotometers, Absorbance, Colorimetric Assays, Fluorescence, Fluorometric Assays, Quantification

Introduction

Although quantification of proteins using spectrophotometry or fluorometry is commonplace, the choice of technique must be made with several factors in mind. Direct UV A280 absorbance, colorimetric assays, and fluorometric assays are in many cases not interchangeable, and consideration of protein concentration, buffer used, time constraints and sample requirements is necessary. All three types of quantification benefit from the use of the Thermo Scientific™ NanoDrop™ UV-Vis spectrophotometers microvolume capabilities.

Microvolume Measurements

NanoDrop spectrophotometers utilize a revolutionary sample retention technology which retains 1–2 µL samples in place via surface tension between two fiber optic cables. After measurement, samples are quickly and easily removed from the optical surfaces with an ordinary dry laboratory wipe. The final protein concentration, purity ratio, and sample spectra are displayed on user friendly software (Figure 1).

The NanoDrop 2000/2000c spectrophotometer utilizes multiple pathlengths (1.0, 0.2, 0.1 and 0.05 mm) that change in real time while measuring a 2 µL protein sample (Figure 1), resulting in a wide dynamic range capable of measuring 0.1–400 mg/mL of purified BSA protein using the direct UV A280 software module. This automatic pathlength optimization ultimately eliminates the need for sample dilutions, resulting in greater accuracy. In contrast to this, measuring samples with a standard 10 mm quartz cuvette on a conventional spectrophotometer typically has an upper detection limit of ~1.8 mg/mL and requires 500 fold more sample to meet the minimum sample volume of 1 mL. Moreover, the use of cuvettes can potentially lead to cross-contamination from prior samples if not properly cleaned.

The measurement time for the NanoDrop 2000c is less than five seconds, and in cases where higher throughput is desired, the NanoDrop 8000 can measure up to eight samples at a time with a measurement time of just 20 seconds. Moreover, if greater sensitivity is required, microvolume fluorescent protein assays can be accommodated by the NanoDrop 3300 fluorospectrometer which also measures samples as small as 2 µL.

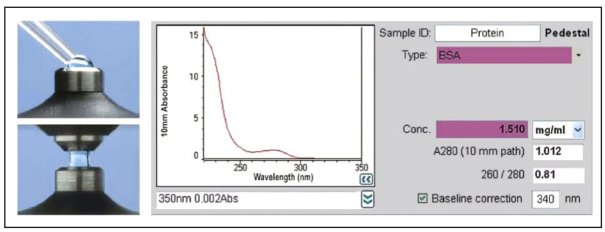

Figure 1: Left: loading of a 2 µL protein sample on the measurement pedestal, Middle: liquid column between pedestals during sample measurement, Right: software output, showing both spectra and numerical data.

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Dynamic Range

The typical upper absorbance limit for a spectrophotometer is approximately 1.5 A, which in turn defines the maximum measurable concentration of protein. Measuring protein samples using a fixed pathlength, typically a 1 cm quartz cuvette, limits the linear range of conventional spectrophotometers, resulting in the need for sample dilutions. Such dilutions consume both time and sample, and promote pipetting errors. Measuring purified protein samples with the microvolume sample retention technology used by NanoDrop spectrophotometers by the direct UV A280 method enables the user to rapidly measure up to 400 mg/mL (BSA protein) directly in a 2 µL volume (Figure 2). The upper detection limit of the NanoDrop spectrophotometer sample retention technology is 200 times greater than that of a standard 1 cm pathlength quartz cuvette.

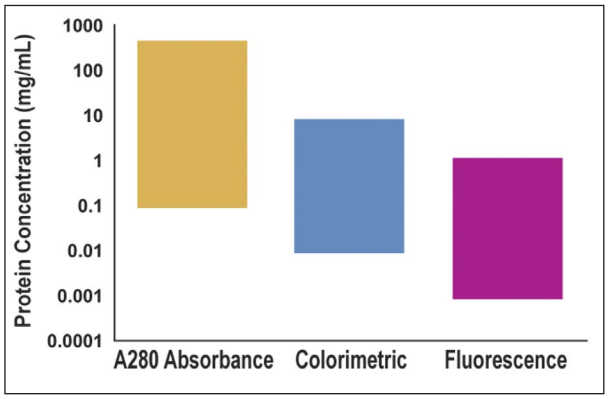

Figure 2: Comparison of the approximate dynamic ranges associated with the various protein quantification methods using a NanoDrop spectrophotometer or spectrofluorometer.

	Direct UV A280 Absorbance	Colorimetric	Fluorescence
Sample Purity	Samples must be purified as contaminants may interfere with measurement.	No purification necessary.	No purification necessary.
Buffer Compatibility	Buffers with strong UV absorbance may be unsuitable. (see next pane)	Some assays are sensitive to detergents or reducing reagents, which can artificially perturb or enhance color development.	Some assays are sensitive to buffers with primary amines or detergents, which perturb fluorescent signal.
Other Considerations	Knowledge of an E1% value or molecular weight and molar extinction coefficient are required to calculate mg/mL concentration.	Colorimetric signals vary between proteins, therefore standards must be carefully chosen in order to minimize differences in signal between the standard and sample proteins.	Fluorescent assays typically have a lower detection limit than colorimetric assays or direct UV A280 measurement, but are limited by the maximum measurable protein concentration (Figure 2).

Table 1: Sample requirements for direct UV A280 absorbance, colorimetric and fluorescence methods broken down into sample purity, buffer compatibility and other considerations

Microvolume colorimetric assays have shown to be comparable in performance to the same assay performed on a conventional cuvette based spectrophotometer, because even though the absorbance signal is reduced by approximately 10 fold, the limitation of the assay is normally the assay itself and not the spectrophotometer. Colorimetric assays are typically used to negate the presence of contaminants in unpurified protein samples, however these assays have a limited dynamic range when compared to direct UV A280 measurement (Figure 2). Similarly, the NanoDrop 3300 fluorospectrometer has also shown comparable performance versus cuvette based fluorometers for most fluorescent protein assays. Fluorescent quantification assays are also used for unpurified protein samples and have shown to have greater sensitivity than both direct UV A280 and colorimetric methods (Figure 2).

Sample Requirements

The choice of quantification method is heavily influenced by sample requirements (Table 1). Although colorimetric and fluorescent assays may be performed on unpurified protein solutions, direct UV A280 absorbance is only suitable for purified protein solutions. Buffer choice and other considerations, such as sample concentration, are also important. It is always advisable to consult the assay manufacturer for the specific tolerances for buffer components and contaminants for the colorimetric and fluorescent assays.

Buffer Compatibility Example

Introduction

Commonly used protein buffers such as RIPA, produce strong absorbance signals in the UV wavelength range (Figure 3A), therefore negatively influencing the accuracy of direct UV A280 measurements. Accurate quantification is possible, however, by utilizing a compatible colorimetric assay. This experiment uses both A280 and the BCA colorimetric assay to quantify two protein samples with the same concentration; one in PBS and the other in RIPA buffer.

Materials and Methods

A single 2 mg/mL BSA protein standard (Thermo Scientific™ Pierce™ Products) was diluted 1:1 with either PBS or RIPA buffer. Standard curves were then created by preparing serial dilutions using the appropriate buffer diluted to 0.5×. Two BSA samples with the same concentration were prepared, one in 0.5× PBS and the other in 0.5× RIPA buffer. Both samples were then quantified by direct UV A280 measurement and also using a BCA colorimetric assay with the relevant standard curve.

Results

When the protein samples were measured after performing a blank measurement with the appropriate 0.5× buffer, a deviation in sample signal was observed across the monitored wavelength range (Figure 3B). Moreover, the percent difference in concentration derived from the direct UV A280 measurements of the two BSA samples was more than 20%. In addition, precision of sample replication was also compromised for the sample in RIPA buffer (Figure 4). Conversely, quantification of the two protein samples using the BCA colorimetric assay showed the unknown sample to have the same concentration, regardless of buffer (Figure 4).

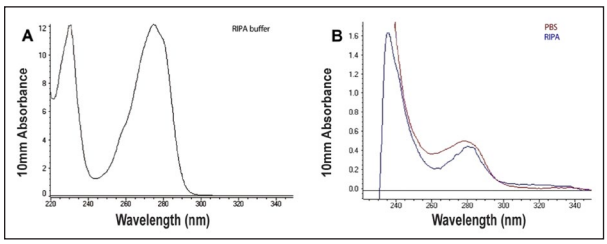

Figure 3: Influence of buffers on direct UV A280 protein measurements, A) Absorbance of 0.5× RIPA buffer (instrument blanked using water), B) Absorbance of the same protein sample in either 0.5× PBS or 0.5× RIPA buffer (blank and sample measurement performed using the same buffer). *Note that even when using appropriate blank, the absorbances of the two samples are not the same.*

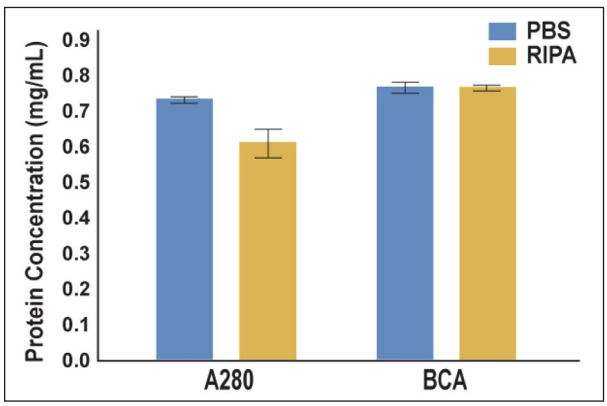

Figure 4: Quantification of the same protein sample in either 0.5× PBS or 0.5× RIPA buffer. Quantification was performed using both A280 (blank performed using the appropriate buffer) and the BCA colorimetric assay (standard curve prepared using the same buffer). *Note that the A280 measurement of the sample in RIPA buffer was not only inaccurate, but also showed poor reproducibility.*

n = 3 for all; error bars represent standard deviations

Buffer Compatibility Conclusions

The discrepancy in concentration measurements of the two protein samples when measured by direct UV A280 absorbance measurement is most likely due to the NP-40 or Triton X-100 content of the RIPA buffer, as surfactants such as these strongly absorb UV light. Choice of quantification method is crucial when working with these surfactants, as blanking on any spectrophotometer when using the direct UV A280 method may not fully compensate for the absorbance of the buffer.

Time to Result

The time required to complete an assay is influenced by three major steps post sample extraction: preparation, incubation and measurement cycle. The direct UV A280 absorbance method is by far the fastest, as the time required to obtain a result is solely based on the time required to complete the measurement cycle. Table 2 compares the time required to perform the three different assay types, showing how aspects of each assay contribute to the overall time needed to complete the assay.

	Direct UV A280 Absorbance	Colorimetric	Fluorescence
Preparation	None	Production of working solutions is required in some assays; fresh standards need to be made for a calibration curve.	Production of working solutions is required in most assays; fresh standards need to be made for a calibration curve.
Incubation	None	Time required to stabilize colorimetric signal is between 10 and 60 minutes, depending on the assay used (Figure 5).	Typical time required to stabilize fluorescent signal is between 10 and 30 minutes, depending on the assay used.
Measurement	~5 seconds using a NanoDrop spectrophotometer	Colorimetric assays require more time than the direct UV A280 method as a calibration curve must be established prior to unknown sample quantification.	Fluorescent assays require more time than the direct UV A280 method as a calibration curve must be established prior to unknown sample quantification.

Table 2: Time requirements for direct UV A280 absorbance, colorimetric and fluorescence methods broken down into preparation, incubation and measurement times

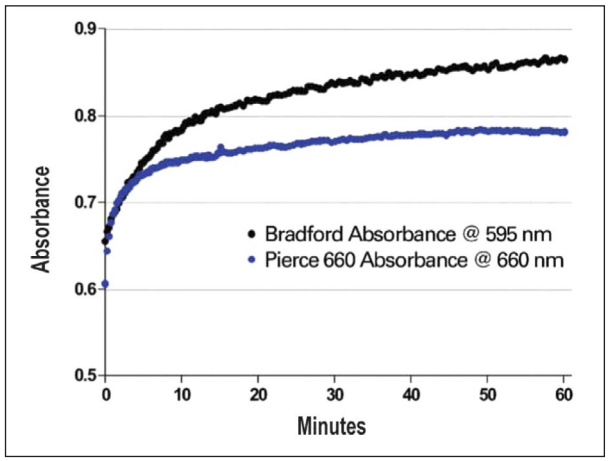

Figure 5: Colorimetric assay development time for Bradford and Pierce 660 assays. *Note that both time for color development and stability of color vary between assays.*

Conclusions

As molecular techniques used to interrogate proteins evolve to require smaller amounts of starting material, the need for microvolume quantification measurements must follow in parallel. Several options exist for both absorbance and fluorescent measurements to determine the concentration of protein samples post-extraction. Care should be taken, however, in selecting the quantification method. Important considerations include sensitivity requirements, buffers used, time constraints and sample purity. In addition to this, a NanoDrop spectrophotometer or fluorometer can be used to further speed measurement, increase measurable concentration range, and save money on consumables and reagents. Consequently, with the advent of the sample retention technology of the Thermo Scientific NanoDrop product line it is now possible to perform scaled down protein quantification measurements efficiently with a high degree of accuracy. Eliminating sample dilutions necessary to measure protein samples on conventional spectrophotometers plays a major role in determining protein concentrations with minimal error.

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