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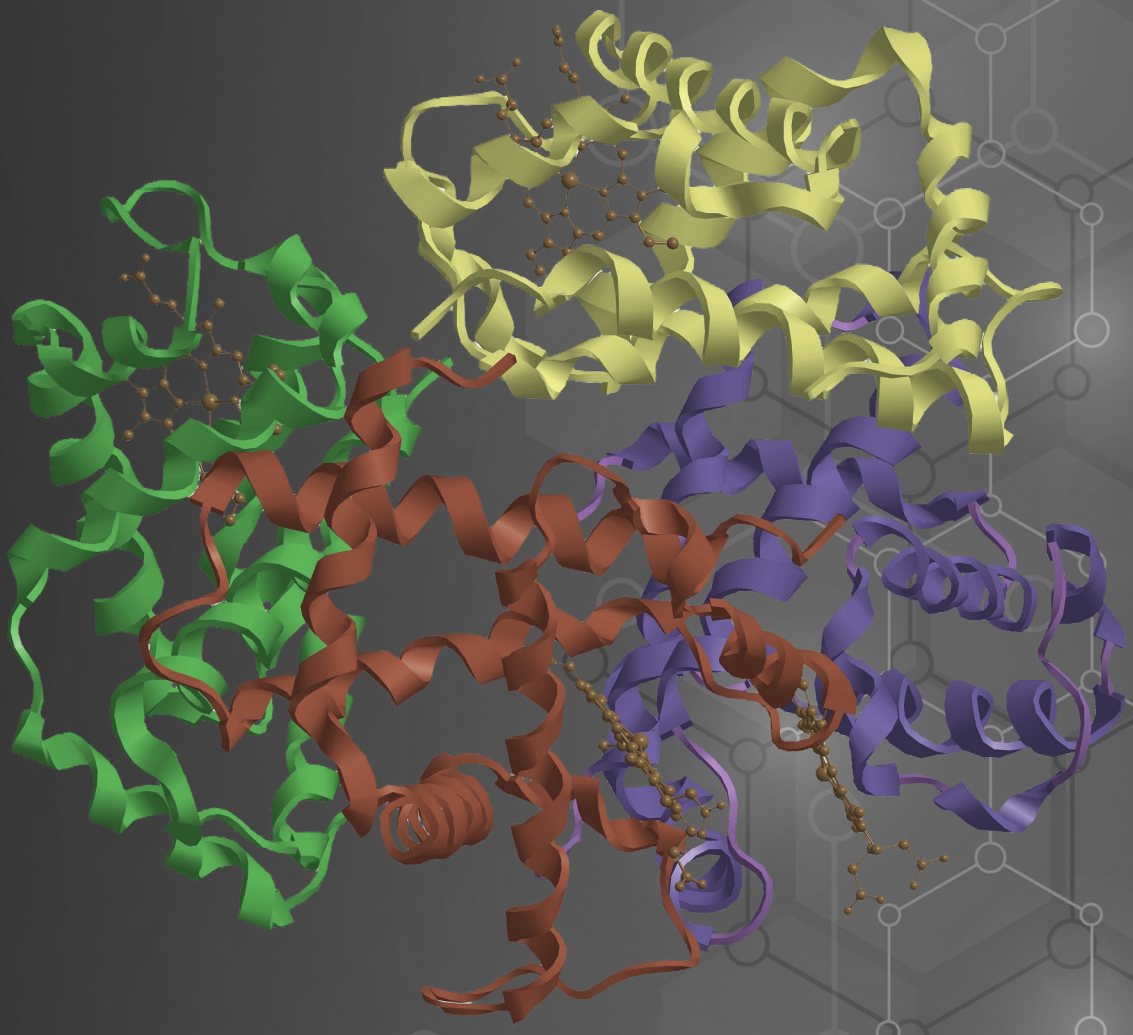
# Protein quantification and qualification

## Application compendium



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# Protein quantification using the NanoDrop One Spectrophotometer

Welcome to protein quantification and qualification e-book. Here you'll find a compendium of useful technical documents, application notes, and protocols for measuring proteins using the Thermo Scientific™ NanoDrop™ One/One$^{C}$ Microvolume UV-Vis Spectrophotometer.

Scientists can use the NanoDrop One/One$^{C}$ instrument to quantify the protein content in their sample using either direct or indirect measurements. An example of a direct measurement is the Protein A280 application, which calculates protein concentration based on the sample absorbance at 280 nm and the protein- and wavelength-specific extinction coefficient. Colorimetric assays are an example of an indirect measurement. The NanoDrop One/One$^{C}$ Software is hardcoded with applications to measure the product of the BCA, Bradford, Lowry, and Pierce™ 660 nm reactions.

Feel free to contact us at **nanodrop@thermofisher.com** if you have any protein quantification or qualification questions.

## Table of contents

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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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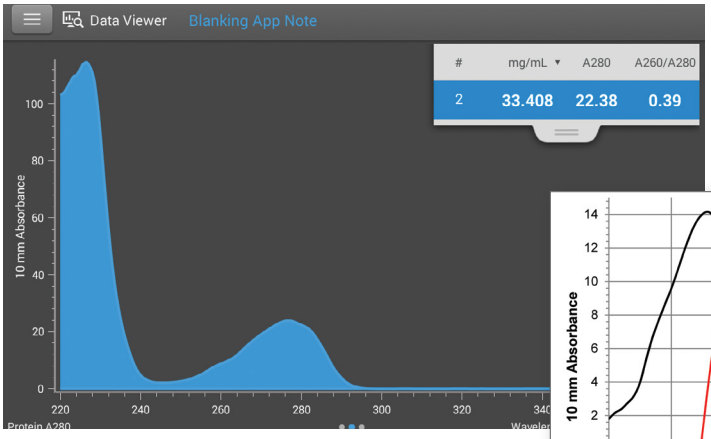
It is generally recommended to blank the instrument with the buffer in which the sample is suspended; however, not all buffers are suitable for absorbance spectroscopy. A good buffer for blanking NanoDrop UV-Vis Spectrophotometers should have no more than ± 0.04 AU (absorbance units at a 10 mm pathlength) 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).



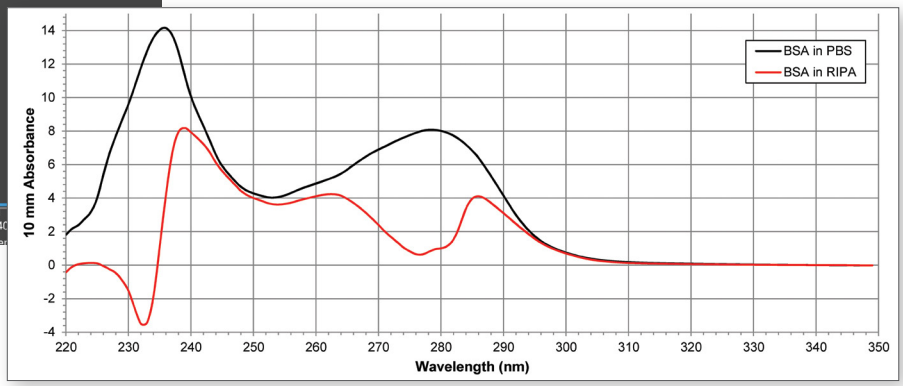

▲ Figure 1: Buffer analysis of RIPA. RIPA absorbs approximately 22 AU at 280 nm, the analytical wavelength of proteins.

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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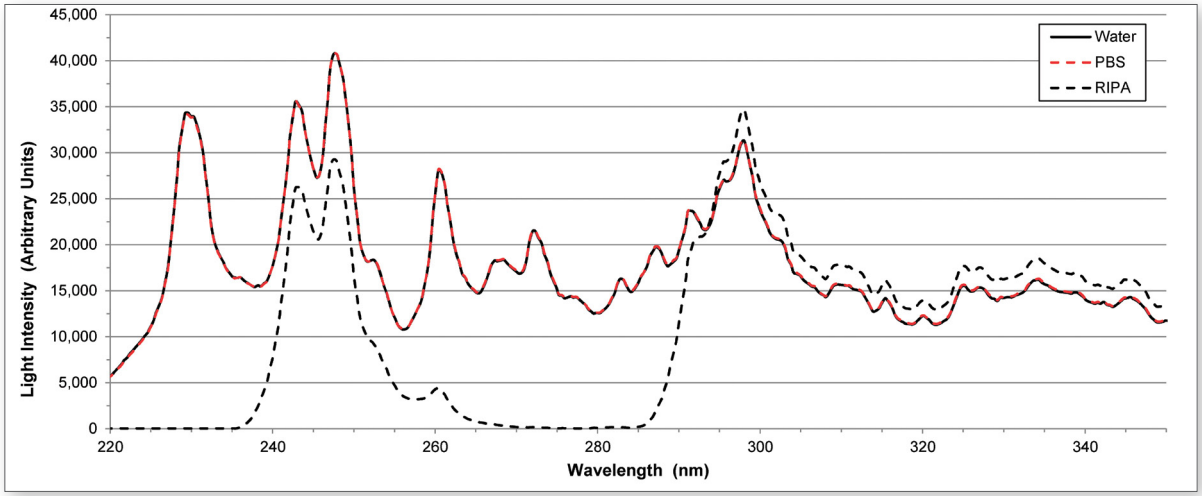
## RIPA buffer limits the light intensity available for a sample measurement

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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# NanoDrop One educational animations

## Quick lessons on quantifying 1-2 µL samples

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APPLICATION NOTE

No. AN52774

# Quantify protein and peptide preparations at 205 nm

## NanoDrop One Spectrophotometer

**Authors**
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Thermo Fisher Scientific, Wilmington, DE

**Abstract**
Life scientists can quantify peptide and protein samples on the Thermo Scientific™ NanoDrop™ One/One$^{C}$ Microvolume UV-Vis Spectrophotometers using the A205 preprogrammed direct absorbance application. The new A205 application offers a choice of methods for peptides that contain Tryptophan and Tyrosine residues in their sequence as well as peptides that completely lack aromatic amino acids. The A205 application offers enhanced sensitivity for peptide quantification in seconds from only 2 µL of sample.


**Figure 1: NanoDrop One Proteins Home screen showing available preprogrammed applications for protein quantitation.**

## Introduction

Researchers have always needed ways to quickly quantify various biomolecules (e.g., protein and nucleic acid preparations) as a routine part of their workflows. This information helps them make informed decisions before proceeding with downstream experiments. There are many protein quantification methods to choose from including gravimetric approaches, colorimetric assays, direct spectrophotometric UV measurements (such as A280), and amino acid analysis. All of these methods have their strengths and weaknesses. Direct spectrophotometric microvolume UV measurements are a popular choice for researchers because they are simple to perform, require no reagents or standards, and consume very little sample. The Thermo Scientific NanoDrop One Spectrophotometer has preprogrammed applications (Figure 1) for direct quantification of proteins using absorbance measurements at 280 nm and 205 nm. This application note specifically describes how to use the Protein A205 application to quantify protein samples.

A protein's peptide backbone absorbs light in the deep UV region (190 nm-220 nm), and this absorbance can be used for protein sample quantitation. The A205 protein quantitation method has several advantages over the direct A280 protein method such as lower protein-to-protein variability (because A205 extinction coefficients are not based on amino acid composition) and higher sensitivity (because of the high molar absorptivity proteins have at 205 nm). However, technical limitations made it difficult to obtain these measurements in the past. Spectrometers' stray light performance, deep UV linearity, and protein buffers containing UV-absorbing components have all added to the challenge of obtaining A205 data. The NanoDrop One patented sample-rentention technology and low stray light performance have simplified quantification of small amounts of protein by A205 methods.

In this application note, we discuss the three A205 measurement options included in the NanoDrop One Protein A205 application and present performance data for each option.

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## A205 extinction coefficients for peptide and protein measurements

The NanoDrop One Protein A205 application allows customers to choose from three different options (Figure 2). The selected option will automatically determine the extinction coefficient that will be used to calculate the protein concentration based on the sample absorbance at 205 nm.

- $\varepsilon_{205}$=31 method
- Scopes method[2]
- Other = custom method $\varepsilon_{205}^{1mg/mL}$

Previous studies showed that most protein solutions at 1 mg/mL have extinction coefficients ($\varepsilon_{205}^{1mg/mL}$) ranging from 30 to 35[2]. The $\varepsilon_{205}$ of 31 mL $mg^{-1}cm^{-1}$ is an extinction coefficient often used for peptides lacking tryptophan and tyrosine residues[1]. The Scopes method gives a more accurate $\varepsilon_{205}$, especially for proteins containing a significant amount of tryptophan (Trp) and tyrosine (Tyr) residues. The increased accuracy of this method takes into account the significant absorbance at 205 nm contributed by the aromatic side chains of Trp and Tyr. This method uses an A280/A205 ratio in its equation to correct for Trp and Tyr side-chain absorbance[3]. Recently, Anthis and Clore proposed the use of a sequence-specific $\varepsilon_{205}$ calculation (e.g., custom/Other method), which is suitable for a wide range of proteins and peptides[1]. This method is appropriate for pure preparations of proteins or peptides whose amino acid sequences are known.

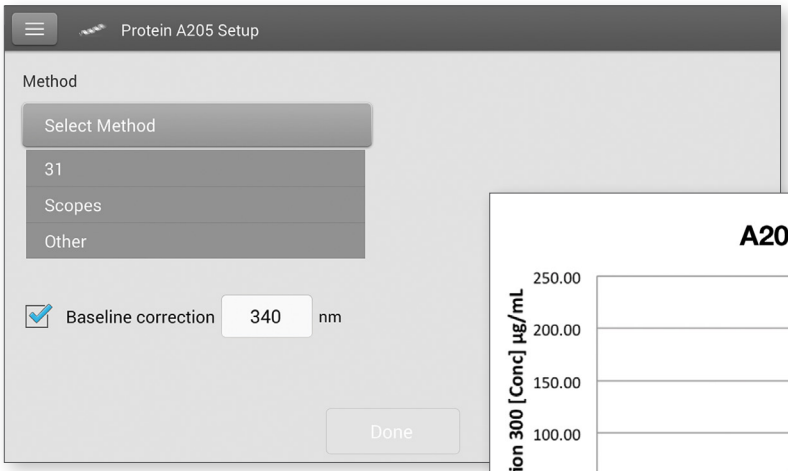


Figure 2: NanoDrop One Protein A205 methods selection screen.

## A205 performance on the NanoDrop One

Preparations of polymyxin, a cationic detergent antibiotic with a peptide backbone, but no Trp or Tyr residues, were made in 0.01% Brij® 35 buffer and measured on the NanoDrop One and the Thermo Scientific™ Evolution™ 300 UV-Vis Spectrophotometers. To ensure the validity of the measurements taken with the Evolution 300 instrument, the polymyxin preparations were diluted in 0.01% Brij buffer to ensure that the measurements taken were within the linear range of the detector. For measurements on the NanoDrop One instrument 2 µL of sample were pipetted directly on the sample pedestal, while a 10 mm quartz cuvette was used for measurements on the Evolution 300 Spectrophotometer. The polymyxin concentration data obtained on both instruments (Table 1) were plotted (Figure 3). Regression line shows that protein concentration results from the NanoDrop One instrument are in good agreement to the results obtained on a traditional high end UV-Vis spectrophotometer using a cuvette.

| Target [Conc] mg/mL | NanoDrop One | | | Evolution 300 |
|---|---|---|---|---|
| | A205 | Std. Dev. | [Conc] mg/mL | [Conc] mg/mL |
| 0 | -0.01 | 0.04 | -0.18 | -0.02 |
| 5 | 0.11 | 0.01 | 3.60 | 5.05 |
| 10 | 0.27 | 0.01 | 8.84 | 10.53 |
| 15 | 0.44 | 0.02 | 14.08 | 17.09 |
| 50 | 1.68 | 0.01 | 54.14 | 55.32 |
| 100 | 3.39 | 0.01 | 109.44 | 108.48 |
| 200 | 6.64 | 0.03 | 214.16 | 222.50 |

Table 1: Various preparations of Polymyxin were measured on the NanoDrop One and Evolution 300 Spectrophotometers. Five(5) replicates of each solution were measured on the NanoDrop One instrument using the 205=31 application. Solutions with absorbance over 1.0A were diluted and measured in triplicate on the Evolution 300 instrument.

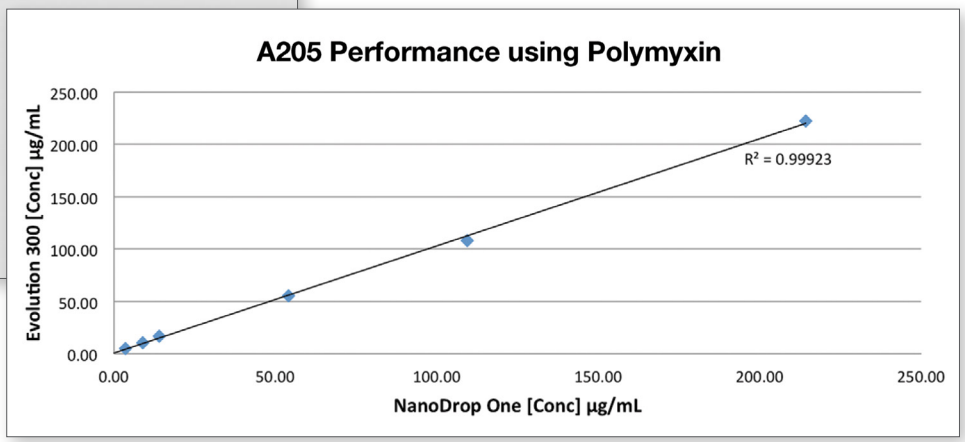


Figure 3: Polymyxin concentrations calculated with the Evolution 300 and NanoDrop One instruments were plotted. Regression line shows that protein concentration measurements on the NanoDrop One instrument are in good agreement to those obtained on a traditional high end UV-Vis spectrophotometer.

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| Protein Preparation | # of Trp of Tyr | | A205 | STDV | [Concentration] $e_{205}$=31 (µg/mL) | [Concentration] Scopes Method (µg/mL) |
|---|---|---|---|---|---|---|
| | Trp | Tyr | | | | |
| BSA 1 | 3 | 21 | 3.960 | 0.013 | 127.73 | 131.80 |
| BSA 2 | 3 | 21 | 37.271 | 0.218 | 1202.30 | 1261.71 |
| BSA 3 | 3 | 21 | 70.044 | 0.239 | 2259.48 | 2387.91 |
| BSA 4 | 3 | 21 | 129.170 | 1.458 | 4166.77 | 4345.20 |
| BSA 5 | 3 | 21 | 271.027 | 0.851 | 8742.81 | 9198.13 |
| Lysozyme 1 | 6 | 3 | 29.069 | 0.169 | 937.71 | 795.95 |
| Lysozyme 2 | 6 | 3 | 53.651 | 0.545 | 1730.68 | 1459.05 |
| Lysozyme 3 | 6 | 3 | 102.713 | 0.668 | 3313.32 | 2814.79 |
| Polymyxin 1 | 0 | 0 | 0.112 | 0.015 | 3.60 | 3.12 |
| Polymyxin 2 | 0 | 0 | 0.274 | 0.014 | 8.84 | 10.12 |
| Polymyxin 3 | 0 | 0 | 0.437 | 0.021 | 14.08 | 16.03 |
| Polymyxin 4 | 0 | 0 | 1.678 | 0.014 | 54.14 | 60.99 |
| Polymyxin 5 | 0 | 0 | 3.393 | 0.014 | 109.44 | 125.16 |
| Polymyxin 6 | 0 | 0 | 6.639 | 0.034 | 214.16 | 244.87 |

**Table 2: Comparison of different A205 methods for various protein and peptide preparations on the NanoDrop One Spectrophotometer.**

To assess the effect that the extinction coefficients used at 205 nm (i.e., Scopes and $\varepsilon_{205}$=31 methods) would have on the result, we prepared dilutions of three different proteins with varied amounts of aromatic residues: bovine serum albumin (BSA, 3 Trp and 21 Tyr residues), lysozyme (6 Trp and 3 Tyr residues) and polymyxin (no Trp, no Tyr). These preparations were measured on the NanoDrop One instrument using the $\varepsilon_{205}$=31 and Scopes methods (Table 2).

## Conclusion

To assess NanoDrop One Spectrophotometer performance at A205, we compared polymyxin concentration results obtained with the NanoDrop One and the Evolution 300 benchtop Spectrophotometers, which have excellent stray light performance. Table 1 shows that the NanoDrop One instrument provided very consistent results between replicate measurements at 205 nm with standard deviations below 0.04A. In addition, the results obtained with both instruments were comparable (Figure 3). Comparison between the A205 methods (Scopes and $\varepsilon_{205}$=31 methods) offered in the NanoDrop One A205 application shows that the number of tryptophan and tyrosine residues has a large effect on the calculated concentration (Table 2). This is because tryptophan is the largest contributor to A280 absorbance, and the Scopes method uses the A280/A205 ratio to correct for aromatic side-chain absorbance at A205.

Our results show that A205 quantification using the $\varepsilon_{205}$=31 method gives comparable results when proteins have only a few tryptophan residues.

One limitation of the A205 method is that many of protein buffers commonly used have absorbance at 205 nm. Before using this technique, we recommend checking the protein buffer for any contribution to the absorbance at 205 nm.

### References


1. Anthis, NJ and Clore, GM 2013. Sequence-specific determination of protein and peptide concentrations by absorbance at 205 nm. *Protein Science* 22:851-858.
2. Goldfarb, AR, Saidel, LJ, Mosovich E 1951. The ultraviolet absorption spectra of proteins. *Journal of Biological Chemistry* 193(1):397-404.
3. Scopes, RK 1974. Measurement of protein by spectrophotometry a 205 nm. *Analytical Biochemistry* 59:277-282.


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# On-demand webinar

## Protein Sample Evaluation using the NanoDrop One UV-Vis Spectrophotometer

**Watch the webinar**

# BCA protein assay

## Introduction

The BCA Protein Assay combines the protein-induced biuret reaction with the highly sensitive and selective colorimetric detection of the resulting cuprous cation ($Cu1^{+}$) by bicinchoninic acid (BCA). A purple colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and the increase in absorbance is linear across a wide protein concentration range. In conjunction with the micro-volume capability of a Thermo Scientific™ NanoDrop™ Spectrophotometer, the assay provides an accurate means of protein quantitation with minimal consumption of sample.

**Note:** All specifications and protocol instructions presented below are specific for the pedestal mode of the Thermo Scientific™ NanoDrop™ One/One$^{C}$ instruments. Please refer to the reagent manufacturer for additional guidance when utilizing the cuvette mode of the NanoDrop One$^{C}$ Spectrophotometer.

## Dynamic range

The micro-assay has a linear range of 20–200 µg/mL using a 1:1 sample to reagent ratio. A higher range of 125–2000 µg/mLmay be obtained using a 1:20 sample to reagent ratio.

Thermo Scientific NanoDrop One$^{c}$
Microvolume UV-Vis Spectrophotometer

## Supplies

**Equipment:**

- NanoDrop One/One$^{C}$ Spectrophotometer
- 0.5–2 µL pipettor (and low retention tips) and 10–1000 µL pipettors

**Materials:**

- Low lint laboratory wipes
- 0.5 mL microcentrifuge tubes or 0.2 mL mini-centrifuge strip tubes and caps

**Reagents:**

- Thermo Scientific™ Pierce™ BCA Protein Assay Kit, product number 23225, 23227, or 23250
- Thermo Scientific™ Pierce™ BSA Standard Pre-Diluted Set, product number 23208, or other commercially available protein standard
- Thermo Scientific™ NanoDrop™ PR-1 Reconditioning Kit, PN CHEM-PR1-KIT

## Assay recommendations

- Measure 2 µL sample aliquots
- All standards and samples should be measured within 10 minutes
- Re-condition pedestals with PR-1 upon assay completion

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## Sample preparation

1. Equilibrate all reagents, unknowns and protein standards to room temperature. Mix thoroughly but gently to avoid introducing micro bubbles.

2. Prepare enough fresh working reagent for all standards and samples to be measured using a 50:1 ratio of the kit reagents A:B.

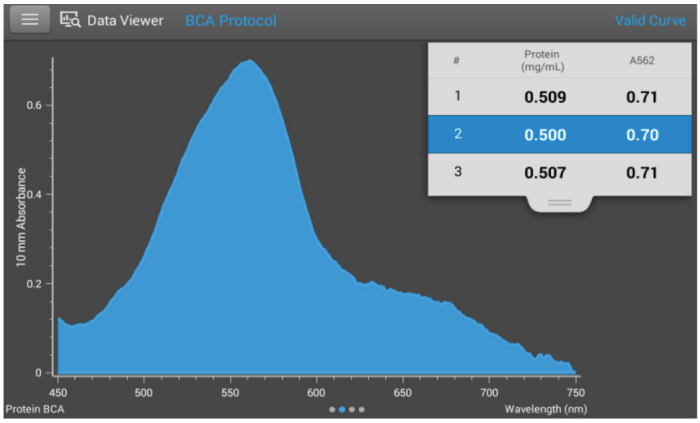
3. Add the appropriate reagent volume to each microcentrifuge tube or PCR strip well.

   **Micro-assay (1:1 sample to working reagent ratio):** Add 10 µL of working reagent to each standard and sample tube/well.

   **High range assay (1:20 sample to working reagent ratio):** Add 200 µL of working reagent to each standard and sample tube/well.

4. Add 10 µL of standards or samples to the appropriate tube. Mix well by gentle vortexing. If necessary, collect the solution at the bottom of the tube by a brief centrifugation.

5. Incubate the standard and sample tubes at either 37° C for 30 minutes or 60° C for ~ 5 minutes. Cool the tubes to room temperature.




**Typical absorbance spectrum for a BCA protein assay sample measurement.**

## Protocol

1. Select the **Protein** tab from the Home screen. Tap the **Protein BCA** application button.

2. Enter the values for each standard concentration in the table on the right. The software allows for the reference and up to 7 additional standards. The zero reference and/or standards can be measured with up to 3 replicates.

   **Note:** The minimum requirement for standard curve generation is the measurement of two standards or the measurement of the zero reference and at least one standard. It is recommended that additional standards be included as necessary to cover the expected assay concentration range.

3. On the left side of the screen, select the Curve Type and number of replicates to measure. We recommend selecting the **Linear** curve type and 3 replicates. Tap **Done**.

   **Note:** If the instrument self-test begins, do not touch the instrument.

4. Establish a blank using diH2O. It is advisable to use the dye reagent and protein buffer without any protein added as the zero reference sample for this assay.

   Pedestal Option: Pipette 2 µL of blank solution onto the bottom pedestal, lower the arm and tap **Blank**.

   Cuvette Option (Model One$^{C}$ only): Insert the cuvette noting the direction of the light path indicated by the etched arrow. The optical beam (2 mm) is directed 8.5 mm above the bottom of the cuvette. Refer to the cuvette manufacturer for volume recommendations.

   **Note:** The arm must be down for all measurements, except those made with cuvettes. It is recommended that cuvettes be removed from the instrument prior to making a pedestal measurement to ensure that the pedestal arm can move to the proper starting position.

5. Follow the direction at the top of the screen to measure the reference and standards. After each measurement, wipe the upper and lower pedestals using a dry laboratory wipe. After your last standard measurement, you can choose to load more standards or run samples.

6. After all of the Standards have been measured, click on the **Run samples** radio button. Enter a sample ID and load a 2 µL aliquot of sample when using the pedestal. Tap **Measure**.

7. After completing all Standard and Samples measurements it is good practice to re-condition the pedestals using PR-1.

8. It is not necessary to blank the instrument between the standard and the unknown sample measurements.

   **Note:** A fresh aliquot of sample should be used for each measurement.

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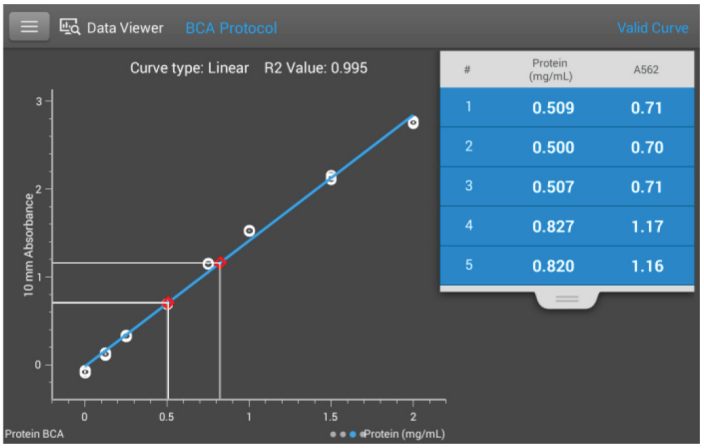
## Standard curve data

| BSA (µg/mL) | A562 (n=3) | Std. dev. | % CV |
|---|---|---|---|
| 0 | -0.077 | 0.016 | NA |
| 125 | 0.120 | 0.013 | NA |
| 250 | 0.327 | 0.010 | NA |
| 500 | 0.691 | 0.002 | 0.3 |
| 750 | 1.148 | 0.005 | 0.4 |
| 1000 | 1.524 | 0.008 | 0.5 |
| 1500 | 2.130 | 0.025 | 1.2 |
| 2000 | 2.759 | 0.013 | 0.5 |




Typical absorbance values for a High Range assay using 1:20 sample to reagent ratio assay using the Pierce BCA reagent.

For additional information regarding the BCA Protein assay and reagents: **thermoscientific.com/pierce**

Toll free (US & Canada): 877-724-7690 • Technical support: **nanodrop@thermofisher.com**

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**T138 Rev 28 April 2020**

# Pierce Rapid Gold BCA Protein Assay

## Introduction

The Thermo Scientific™ Pierce™ Rapid Gold BCA Protein Assay combines the protein-induced biuret reaction with the highly sensitive and selective colorimetric detection of the resulting cuprous cation (Cu1+) by a new, unique BCA chelator. An adaptation of the traditional BCA assay, the Rapid Gold BCA Protein Assay Kit has been optimized to develop in 5 minutes at room temperature. The Rapid Gold BCA Protein Assay produces an orange-gold colored reaction product, which is formed by the chelation of 2 molecules of the chelator with 1 cuprous ion.

This water-soluble complex exhibits a strong absorbance at 480 nm that is very linear with increasing protein concentrations over a broad working range of 20–2000 µg/mL. In conjunction with the microvolume capability of a Thermo Scientific™ NanoDrop™ One/One$^{C}$ Spectrophotometer, the assay provides an accurate means of protein quantitation with minimal consumption of sample.

**Note:** All specifications and protocol instructions presented below are specific the pedestal mode for the NanoDrop One instrument. Please refer to the reagent manufacturer for additional guidance when utilizing the cuvette mode of the NanoDrop One$^{C}$ Spectrophotometer.

## Dynamic range

The Rapid Gold BCA Protein Assay has a linear range of 20–2000 µg/mL using a 1:20 sample to reagent ratio.

Thermo Scientific NanoDrop One$^{c}$
Microvolume UV-Vis Spectrophotometer

## Supplies

### Equipment:

- NanoDrop One/One$^{C}$ Spectrophotometer
- 0.5–2 µL pipettor (and low retention tips), 10–100 µL pipettor, and a 100–1000 µL pipettor

### Materials:

- Low lint laboratory wipes
- 0.5 mL microcentrifuge tubes or 0.2 mL mini-centrifuge strip tubes and caps or 96 well PCR plate (for standards and sample reactions)

### Reagents:

- Pierce Rapid Gold BCA Protein Assay Kit, PN A53225 or A53227
- Thermo Scientific™ Pierce™ BSA Standards Set (pre-diluted PN 23208 or standard ampules PN 23209), Thermo Scientific™ Pierce™ BGG Standards (pre-diluted PN 23213 or standard ampules PN 23212), or other commercially prepared protein standard
- Thermo Scientific™ NanoDrop™ PR-1 Reconditioning Kit, PN CHEM-PR1-KIT

## Assay recommendations

- Measure 2 µL sample aliquots
- Re-condition pedestals with PR-1 upon assay completion

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## Sample preparation

1. Equilibrate all reagents, unknowns and protein standards to room temperature. Mix thoroughly but gently to avoid introducing micro bubbles.

2. Prepare enough fresh working reagent (WR) for all standards and samples to be measured using a 50:1 ratio of the kit reagents A:B.

   **Note:** When Rapid Gold BCA Reagent B is first added to Rapid Gold BCA Reagent A, a pale blue precipitate may be observed, but, upon vortexing or mixing for < 5 seconds, the precipitate should dissolve to yield a clear, green solution. Use fresh working reagent each time.

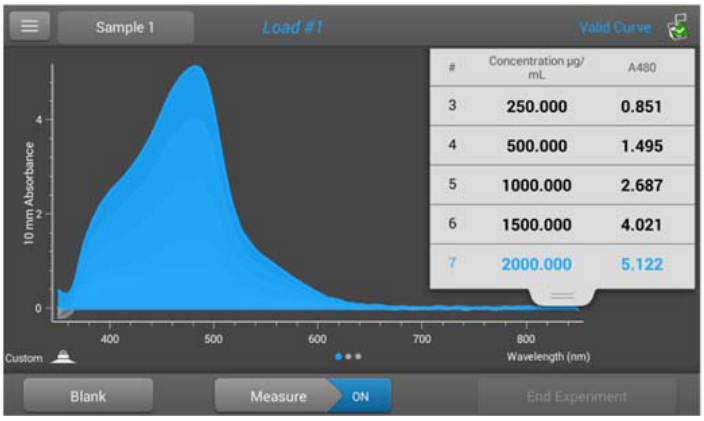
3. Add the appropriate WR reagent volume to each microcentrifuge tube or PCR strip well.

   **Rapid Gold BCA (1:10 sample to working reagent ratio):** Add 100 µL of working reagent to each standard and sample tube/well.

4. Add 10 µL of standards or samples to the appropriate tube. Mix well by gentle vortexing.

5. Incubate the standard and sample tubes at room temperature for 5 minutes.

   **Note:** It is important to measure all standards and samples within 10 minutes or stop the reactions by adding 25 µL 1N HCl.




**Typical absorbance spectrum for a Rapid Gold BCA protein assay sample measurement.**

## Protocol

1. Select the Custom tab from the Home screen. Download Pierce Rapid Gold BCA method file from our **website** and save it on a USB drive. Insert USB drive into the instrument and tap the **Custom** application button.

2. Select USB Drive and the select **Load Method**. Then select the Pierce Rapid Gold BCA method file and tap **Load** button. Then select **Run Method**.

3. Enter the values for each standard concentration in the table on the right. The software allows for the reference and up to 7 additional standards. Tap **Done**.

   **Note:** The minimum requirement for standard curve generation is the measurement of two standards or the measurement of the zero reference and at least one standard. It is recommended that additional standards be included as necessary to cover the expected assay concentration range. Tap **Done**.

   **Note:** If the instruments self-test begins, do not touch the instrument.

4. Prepare a blank solution using 10µL buffer+100 µL WR.

   Pedestal Option: Pipette 2 µL of blank solution onto the bottom pedestal, lower the arm and tap Blank.

   Cuvette Option (Model One$^{C}$ only): Insert the cuvette noting the direction of the light path indicated by the etched arrow. The optical beam (2 mm) is directed 8.5 mm above the bottom of the cuvette. Refer to the cuvette manufacturer for volume recommendations.

   **Note:** The arm must be down for all measurements, except those made with cuvettes. It is recommended that cuvettes be removed from the instrument prior to making a pedestal measurement to ensure that the pedestal arm can move to the proper starting position.

5. Follow the direction at the top of the screen to measure the standards. After each measurement, wipe the upper and lower pedestals using a dry laboratory wipe.

6. After all of the Standards have been measured, enter a sample ID and load a 2 µL aliquot of sample when using the pedestal. Tap Measure.

7. After completing all Standard and Samples measurements it is good practice to re-condition the pedestals using PR-1.

8. It is not necessary to blank the instrument between the standard and the unknown sample measurements.

   **Note:** A fresh aliquot of sample should be used for each measurement.

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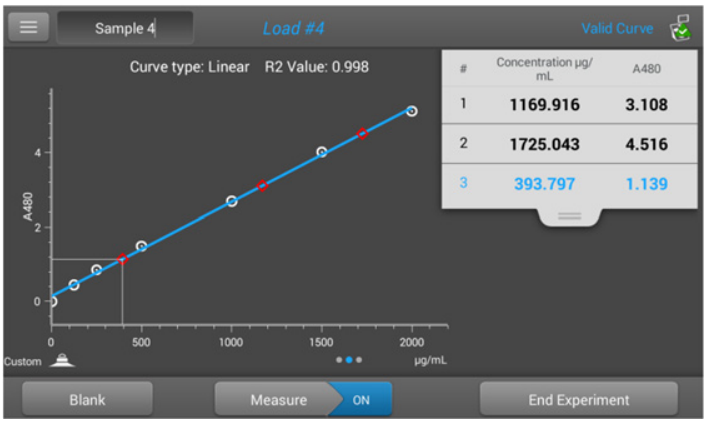
## Standard curve data

| BSA (µg/mL) | A480 |
|---|---|
| 125 | 0.446 |
| 250 | 0.851 |
| 500 | 1.495 |
| 1000 | 2.687 |
| 1500 | 4.021 |
| 2000 | 5.122 |




Typical absorbance values for an assay using 1:20 sample to reagent ratio assay using the Pierce Rapid Gold BCA reagent.

For additional information regarding the Rapid Gold BCA Protein assay and reagents: **thermoscientific.com/pierce**

Toll free (US & Canada): 877-724-7690 • Technical support: **nanodrop@thermofisher.com**

Find out more at **thermofisher.com/nanodrop**

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**Rev 15 April 2020**

# Bradford protein assay

## Introduction

Use of the coomassie G-250 dye in a colorimetric reagent for the detection and quantitation of total protein was first described by Dr. Marion Bradford in 1976. Protein binds to the coomassie dye in the acidic environment of the reagent. This results in a spectral shift from the reddish/brown form of the dye (absorbance maximum at 465 nm) to the blue form of the dye (absorbance maximum at 610 nm). The difference between the two forms of the dye is greatest at 595 nm, the optimal wavelength to measure the blue color from the coomassie dye-protein complex. In conjunction with the micro-volume capability of the Thermo Scientific™ NanoDrop™ Spectrophotometers, the assay provides an accurate means of protein quantitation with minimal consumption of sample.

**Note:** All specifications and protocol instructions presented below are specific to the pedestal mode of NanoDrop One/One$^{C}$ instruments. Please refer to the reagent manufacturer for additional guidance when utilizing the cuvette mode of the NanoDrop One$^{C}$ Spectrophotometer.


Thermo Scientific NanoDrop One$^{c}$
Microvolume UV-Vis Spectrophotometer

## Dynamic range

The Micro assay has a linear range of 15 - 100 µg/mL using a 1:1 sample to reagent ratio. The Standard assay has a higher range of 100 - 1000 µg/mL which may be obtained using a 1:30 sample to reagent ratio.

## Supplies, materials & reagents

- NanoDrop One/One$^{C}$ Spectrophotometer
- 0.5 - 2 µL pipettor (and low retention tips) and 10–1000 µL pipettors.
- Low lint laboratory wipes
- 0.5mL microcentrifuge tubes or 0.2 mL mini-centrifuge strip tubes and caps
- Thermo Scientific™ Pierce™ Coomassie Plus Assay Kit, PN 23236 or 23238
- Thermo Scientific™ Pierce™ BSA Standard Pre-diluted Set, PN 23208 or other commercially prepared protein standard
- Thermo Scientific™ NanoDrop™ PR-1 Reconditioning Kit, PN CHEM-PR1-KIT

## Assay recommendations

- Measure 2 µL sample aliquots
- Making standard and sample measurements in triplicate is good practice, particularity with the limited assay signal obtained with the Bradford Assay.
- Re-condition pedestals with PR-1 upon assay completion

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## Sample preparation

1. Equilibrate all reagents, unknowns and protein standards to room temperature. Mix thoroughly but gently to avoid introducing micro bubbles.

2. Add the appropriate reagent volume to each microcentrifuge tube or mini-centrifuge strip well.

   **Micro assay (1:1 sample to reagent ratio):** add 10 µL of working reagent to each of the standards and sample tubes.

   **Standard assay (1:30 sample to reagent ratio):** add 300 µL of working reagent to each of the standard and sample tubes.

3. Add 10 µL of each standard and sample to each of the reagent tubes. Mix well by gentle vortexing. If necessary, collect the solution at the bottom of the tube by a brief centrifugation.

4. It is advisable to use the dye reagent and protein buffer ("0" reference) without any protein added as the zero reference sample for this assay.

5. Follow reagent manufacturer's recommended incubation time.

## Protocol

1. Select the **Proteins Tab** from the New Experiment Screen. Select the **Protein Bradford** application.

2. On the left side of the screen, select the Curve Type and number of replicates to measure using the drop down menus. The Pierce protocol recommends using a 2nd order polynomial. Please note, the curve type cannot be changed after the assay is in progress.

3. Enter the values for each standard concentration in the table on the right. The software allows for the reference and up to 7 additional standards. The zero reference and standards can be measured with up to 3 replicates. Select **DONE**.

   **Note:** The minimum requirement for standard curve generation is the measurement of two standards or the measurement of the zero reference and at least one standard. It is recommended that additional standards be included as necessary to cover the expected assay concentration range.

4. Establish a blank using $dH_2O$.

   **Pedestal Option:** Pipette 2 µL of blank solution onto the bottom pedestal, lower the arm and select Blank.

   **Cuvette Option (Model NanoDrop One$^C$ only):** Select the cuvette option in the NanoDrop One$^C$ software. Insert the cuvette noting the direction of the light path indicated by the etched arrow. The optical beam (2 mm) is directed 8.5 mm above the bottom of the cuvette. Refer to the cuvette manufacturer for volume recommendations. After loading the cuvette with the appropriate volume, select **Blank**.

   **Note:** The arm must be down for all measurements, except those made with cuvettes. It is recommended that cuvettes be removed from the instrument prior to making a pedestal measurement to ensure that the pedestal arm can move to the proper starting position.

5. Follow the direction at the top of the screen to measure the reference and standards. After each measurement, wipe the upper and lower pedestals using a dry laboratory wipe.

6. After the last standard is measured, you can choose to load more standards or run samples using the window that appears in the lower right corner of the screen. To proceed with sample measurements, select the **Run Samples** radio button, and select **DONE**.

7. Enter a sample ID in the sample ID field located in the upper left corner of the screen. Load 2 µL of sample when using the pedestal. Select **Measure**.

8. After completing all standard and sample measurements, it is good practice to re-condition the pedestals using PR-1.

9. It is not necessary to blank the instrument between the standard and the unknown sample measurements.

   **Note:** A fresh aliquot of sample should be used for each measurement.

10. Cleaning the instrument after a measurement:

    **Pedestal Option:** Simply wipe the upper and lower pedestals using a dry laboratory wipe and the instrument is ready to measure the next sample.

    **Cuvette Option:** Remove the cuvette, and clean according to the manufacturer's recommendations.

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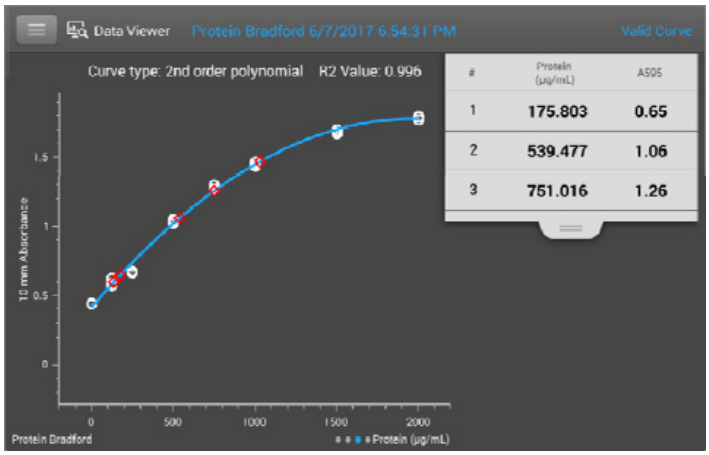
| BSA (µg/mL) | A595 (n=3) | Std. dev. | % CV |
|---|---|---|---|
| 0 | 0.44 | 0.003 | NA |
| 125 | 0.593 | 0.028 | 4.7 |
| 250 | 0.666 | 0.007 | 1.1 |
| 500 | 1.037 | 0.013 | 1.2 |
| 750 | 1.289 | 0.018 | 1.4 |
| 1000 | 1.445 | 0.015 | 1.0 |
| 1500 | 1.683 | 0.017 | 1.0 |
| 2000 | 1.783 | 0.012 | 0.7 |




Typical absorbance values and standard curve for a standard assay using 1:30 sample to reagent ratio assay using the Pierce Coomassie Plus reagent.

For additional information regarding the Bradford assay and reagents, please refer to the Pierce Website: thermoscientific.com/pierce

Toll free (US & Canada): 877-724-7690 • Technical support: **nanodrop@thermofisher.com**

Find out more at **thermofisher.com/nanodrop**

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**T141 Rev 08 April 2020**

# Lowry protein assay

## Introduction

The Bio-Rad® *DC* Protein Assay[1] is a colorimetric assay for protein concentration following detergent solubilization. The reaction is similar to that described in the "Lowry Assay: Protein by Folin Reaction" by Lowry et al in the *Journal of Biological Chemistry*, 1951.

The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent in a two-step process leading to color development. The first step is the reaction between protein and copper in an alkaline medium and then the reduction of Folin reagent by the copper-treated protein. Color development is due primarily to the amino acids tyrosine and tryptophan, but also to a lesser extent by cystine, cysteine, and histidine. The reaction will have a blue color with maximum absorbance at 750 nm using Thermo Scientific™ NanoDrop™ One/One$^{C}$ Microvolume UV-Vis Spectrophotometer and the Protein Lowry Applications.

## Dynamic Range

The assay has a range of 0.2 to 2.0 mg/mL.

A standard curve should be prepared each time the assay is performed. We recommend choosing a second order polynomial curve type for the standard curve.

## Supplies, Materials & Reagents

- NanoDrop One/One$^{C}$ Spectrophotometer
- 0.5 – 2 µL pipettor tips, low retention tips recommended
- 50 µL pipettor tips, low retention tips recommended
- 400 µL pipettor tips, low retention tips recommended
- Low lint laboratory wipes
- 0.6 mL micro-centrifuge tubes (Fisherbrand™ catalog number 05-408-120)
- *DC* Protein Assay Reagents Package, Bio-Rad PN 5000116
- Thermo Scientific™ Pierce™ BSA Standard Pre-diluted Set, PN 23208 or other commercially prepared protein standards
- Thermo Scientific™ NanoDrop™ PR-1 Reconditioning Kit, PN CHEM-PR1-KIT
- Thermo Scientific™ BupH™ PBS packs, Thermo Fisher PN 28372

## Assay Recommendations

- Measure 2 µL sample aliquots.
- Making standard and sample measurements in triplicate is good practice.
- Re-condition pedestals with PR-1 upon completion of assay.

## Sample Preparation

The sample preparation outlined below is for pedestal measurements on the NanoDrop One/One$^{C}$ Spectrophotometer. Follow the manufacturer's protocol for a standard assay when making measurements in a cuvette.

1. Equilibrate all reagents, unknowns and protein standards to room temperature. Mix thoroughly but gently to avoid introducing micro bubbles.

2. If unknown samples contain detergent, prepare a working Reagent A, by adding 20 µL of Reagent S to each mL of Reagent A that will be required for the run. Label this as "Working Reagent A" with an expiration date of one week post preparation. If unknown samples do not contain detergent, omit this step and use Reagent A as supplied in the kit.

3. Prepare standards to cover the range of the assay (0.2 to 2.0 mg/mL). A standard curve should be prepared each time unknown samples will be tested. If not using pre-diluted standards, for best results, the standards should be diluted in the same buffer as the unknown samples.

4. Label all tubes and pipet 10 µL of standards and unknown samples into appropriately labeled micro-centrifuge tubes. Pipet 10 µL of PBS into the reference or zero standard.

5. Add 50 µL of either Working Reagent A or Reagent A supplied in the kit (see step #2) to each tube. Mix well.

6. Add 400 µL of Reagent B to each tube. Mix well, but not so vigorously as to introduce micro-bubbles into the tubes.

7. Incubate all tubes at room temperature for 15 minutes.

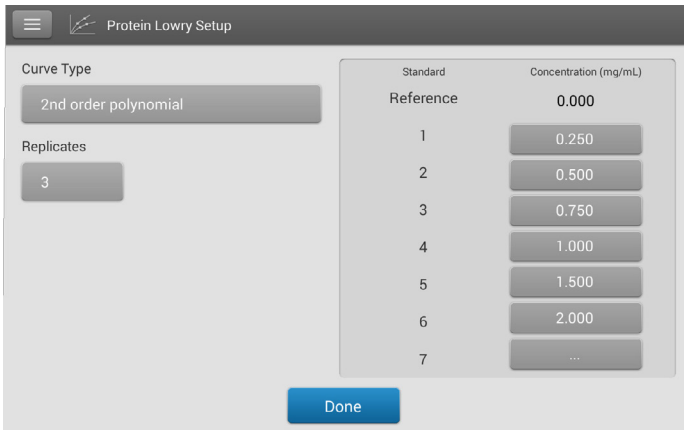
## Protocol

1. From the NanoDrop One/One$^{C}$ *New Experiment* screen, tap the **Proteins** tab and then tap the "Protein Lowry" icon to open the application.

2. Enter the mg/mL concentration values for each standard in the table on the right in the image below.


3. Enter the number of replicates for each standard. We recommend three replicates per standard, including the reference standard.

4. Select the curve type. We recommend a second order polynomial curve type for Protein Lowry Protein Assay.




5. Press *Done* when complete.

6. At the prompt, clean both pedestals and make a blank measurement using water as the blanking solution.

7. Follow the on-screen prompts to measure each replicate of each standard, taking care to wipe the upper and lower pedestal between each measurement. Each replicate measurement of the reference or standards should be made using a fresh aliquot on the pedestal.

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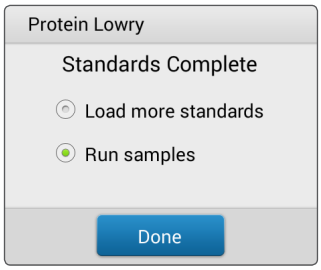
8. You will notice in the upper right corner of the screen the note in red: **Invalid Curve**. When enough data points have been measured to plot the curve type selected, this now will change from red to blue: **Valid Curve**. For a second order polynomial curve, at least three standards, including the reference must be measured before a curve can be constructed. This indication does not address the quality of the curve, it is a notification that enough standards have been measured to construct the curve type selected.

9. When the last replicate of the last standard has been read the following message will appear:




10. At this point, you may choose to run more standards or begin running samples. Please note that once samples are measured, you will not be able to make changes to the standard curve by measuring additional standards.

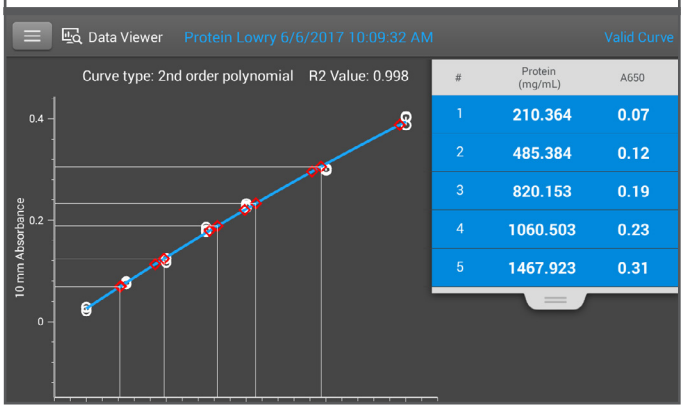
11. It is not necessary to blank the instrument between measurement of the standards and the unknown samples.

12. After each measurement, clean the upper and lower pedestals with a clean, dry laboratory wipe.

13. Upon completion of all measurements, we recommend cleaning and reconditioning the measurement pedestals using PR-1.

## Standard Curve Data

| BSA (µg/mL) | A(595) (n=3) | Std. dev. | %CV |
|---|---|---|---|
| 0 | 0.44 | 0.003 | NA |
| 125 | 0.593 | 0.028 | 4.7 |
| 250 | 0.666 | 0.007 | 1.1 |
| 500 | 1.037 | 0.013 | 1.2 |
| 750 | 1.289 | 0.018 | 1.4 |
| 1000 | 1.445 | 0.015 | 1.0 |
| 1500 | 1.683 | 0.017 | 1.0 |
| 2000 | 1.783 | 0.012 | 0.7 |




The above data is typical of absorbance values and standard curve using the *DC* Protein Assay.

For additional information regarding the Lowry assay and reagents, please refer to the manufacturer's product literature supplied with the *DC* Protein Assay.

### References

1. *DC* Protein Assay Instruction Manual, LIT449 Rev. D Bio-Rad Laboratories.

# Pierce 660 nm protein assay

## Introduction

The Thermo Scientific™ Pierce™ 660 nm Protein Assay reagent is a ready-to-use formulation that offers rapid, accurate and reproducible colorimetric detection of minute amounts of protein in solution. Used in conjunction with the micro-volume capability of the Thermo Scientific™ NanoDrop™ Spectrophotometers, the reagent provides an accurate and rapid means of protein quantitation with minimal consumption of sample. The ability of NanoDrop Spectrophotometers to measure as little as 2 µL of protein samples allows significantly scaled-down reaction volumes, thereby using only a fraction of sample and reagent commonly needed for conventional cuvette-based instruments.

Note: All specifications and protocol instructions presented below are specific to the pedestal mode for NanoDrop One/One$^{C}$ instruments. Please refer to the reagent manufacturer for additional guidance when utilizing the cuvette mode of the NanoDrop One$^{C}$.

## Dynamic range

The assay has a linear range of 50–2000 µg/mL using a 1:15 sample to reagent ratio. The sensitivity of the assay may be increased by using a 1:7.5 sample to reagent ratio yielding a linear range of 25–1000 µg/mL.

Thermo Scientific NanoDrop One$^{c}$
Microvolume UV-Vis Spectrophotometer

## Supplies

**Equipment:**

- NanoDrop One/One$^{C}$ Spectrophotometer
- 0.5-2 µL pipettor (and low retention tips) and 10-1000 µL pipettors

**Materials:**

- Low lint laboratory wipes
- 0.5 mL microcentrifuge tubes or 0.2 mL mini-centrifuge strip tubes and caps

**Reagents:**

- Thermo Scientific™ Pierce™ 660 nm Protien Assay Reagent, Pierce PN 22660
- Thermo Scientific™ Pierce™ BSA Pre-Diluted Standards Pre-diluted Set, PN 23208 or other commercially prepared protein standard
- Thermo Scientific™ NanoDrop™ PR-1 Reconditioning Kit, PN Chem-PR1-KIT

## Assay recommendations

- Measure 2 µL sample aliquots.
- Re-condition pedestals with PR-1 upon assay completion.

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## Sample preparation

1. Equilibrate all reagents, unknowns and protein standards to room temperature. Mix thoroughly but gently to avoid introducing micro bubbles.

2. Prepare a zero reference (0 mg/mL protein).

   **For a 1:15 sample to working reagent ratio:** Add 10 µL of the assay buffer to 150 µL of the Pierce 660 reagent

   **For a 1:7.5 sample to working reagent ratio:** Add 20 µL of the assay buffer to 150 µl of the Pierce 660 reagent.

   **Note:** The zero reference solution is used as the 'blank'. This is unlike the other colorimetric assays run on NanoDrop instruments where water is used for the 'blank' measurement.

3. Prepare standards and samples.

   **For a 1:15 sample to working reagent ratio:** Add 10 µL of each standard and sample to 150 µL of the Pierce 660 reagent.

   **For a 1:7.5 sample to working reagent ratio:** 20 µL of each standard and sample to 150 µL of the Pierce 660 reagent.

4. Mix each standard and unknown sample thoroughly by gently pipetting up and down several times. If necessary, collect the solution at the bottom of the tube by a brief centrifugation.

5. Incubate at room temperature for 5 minutes.

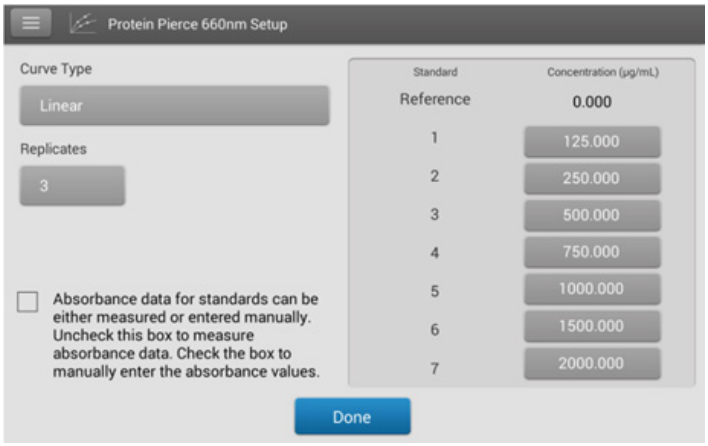
## Protocol

1. Tap the **Protein** tab from the Home screen. Tap the **Protein Pierce 660 nm** application button.

2. On the left side of the screen, select the Curve Type and number of replicates to measure. We recommend selecting the **Linear** curve type and measuring 3 replicates of each standard.

   **Optional:** The user has the option to measure the absorbance of each standard or enter the manufacturer supplied standard absorbance values manually. In the bottom left pane, select the box to enter the manufacture supplied standard absorbance values manually. Deselect the box to measure the absorbance of each standard. See image below.

3. In the table on right side of the screen, enter the values for each standard concentration. The software allows for the reference and up to 7 additional standards. The zero reference and standards can be measured with up to 3 replicates. Tap **Done**.




   **Note:** The minimum requirement for standard curve generation is the measurement of the zero reference and at least one standard. It is recommended that enough standards be included to cover the expected assay concentration range.

4. Establish a blank using the appropriate buffer. It is advisable to use the dye reagent and protein buffer ("0" reference) without any protein added as both the blank and zero reference sample for this assay.

   Pedestal Option: Pipette 2 µl of blank solution onto the bottom pedestal, lower the arm and tap **Blank**.

   Cuvette Option: (Model NanoDrop One$^{C}$ only): Insert the cuvette noting the direction of the light path indicated by the etched arrow. The optical beam (2 mm) is directed 8.5 mm above the bottom of the cuvette. Refer to the cuvette manufacturer for volume recommendations.

   **Note:** It is recommended that cuvettes be removed from the instrument prior to making a pedestal measurement to ensure that the pedestal arm can move to the proper starting position.

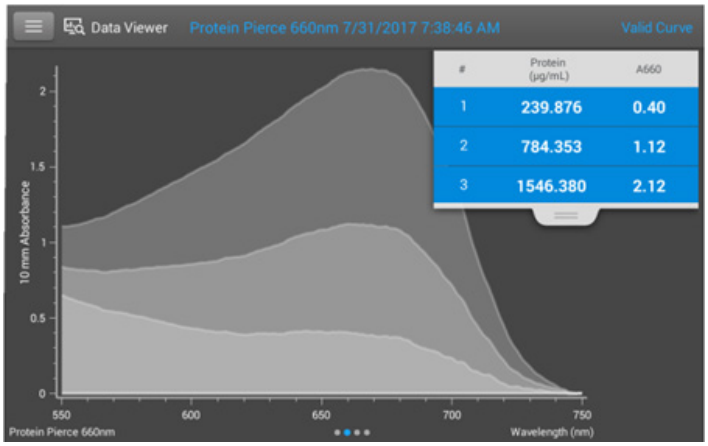
5. A message at the top of the screen will prompt you to, "**Clean both pedestals and Load – Reference 0.000,**" if you are using the pedestal mode. If you are using the cuvette mode, a message will prompt you to, "**Insert Cuvette and Load - Reference 0.000.**" Load the reference sample in the manner described for loading the blank.

1. After each measurement, wipe the upper and lower pedestals using a clean, dry laboratory wipe.
2. Once the reference and at least one standard have been measured, the message **Invalid Curve** in red at the top right of the screen will switch to **Valid Curve** in blue.
3. After all standard measurements have been made a pop-up box will indicate **Standards Complete**. Select **Load more standards** to enter and measure additional standards or **Run samples** if standard measurements are complete.
4. After selecting **Run samples**, enter a sample ID at the top of the screen. Load 2 µL of sample when using the pedestal. Tap **Measure**.
5. It is not necessary to blank the instrument between the standard and the unknown sample measurements.

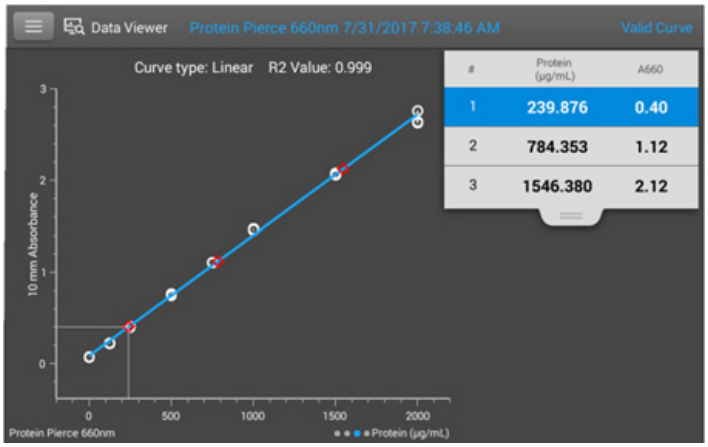
   **Note:** A fresh aliquot of sample should be used for each measurement.
6. After completing all Standard and Sample measurements, it is good practice to re-condition the pedestals using PR-1.

## After the measurements:

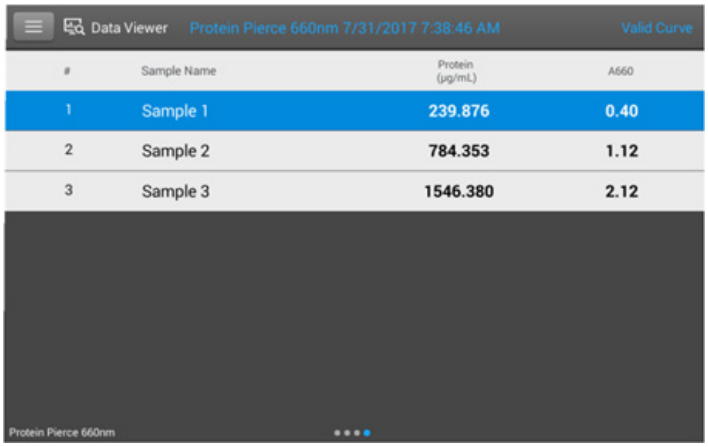
1. Select each sample to display multiple spectra at a time.




2. Swipe the screen to the left to view the curve.




3. Swipe the screen again to the left to view your measurement results.




## Standard curve data

| BSA (µg/mL) | A660 (n=3) | Std. dev. | % CV |
|---|---|---|---|
| 0 | 0.072 | 0.003 | NA |
| 125 | 0.223 | 0.004 | 1.6 |
| 250 | 0.403 | 0.002 | 0.5 |
| 500 | 0.757 | 0.013 | 1.7 |
| 750 | 1.107 | 0.002 | 0.2 |
| 1000 | 1.467 | 0.006 | 0.4 |
| 1500 | 2.065 | 0.012 | 0.6 |
| 2000 | 2.676 | 0.074 | 2.8 |

Table 1. Typical absorbance values for a 1:15 sample to reagent ratio assay using the Pierce 660 nm Protein Assay.

For additional information regarding the Pierce 660 nm assay and reagents: thermoscientific.com/pierce






**T139 Rev 01 April 2020**

# A custom method for hemoglobin measurements

## Using the NanoDrop One Spectrophotometer

### Abstract

Thermo Scientific™ NanoDrop™ Microvolume UV-Vis Spectrophotometers have been used for many years to reliably measure nucleic acids and proteins using as little as 1–2 µL of sample. However, NanoDrop spectrophotometers may also be used for many other applications. This application note discusses how the NanoDrop One/One$^{C}$ Spectrophotometer can be used to measure hemoglobin using a custom method.


### Introduction

Hemoglobin is a tetrameric protein found in erythrocytes that transports oxygen to tissues throughout the body and carries $CO_2$ away from tissues [1]. Hemoglobin can reversibly bind oxygen and typically contains iron in the reduced ferrous state ($Fe^{2+}$). When the iron is oxidized to the ferric state ($Fe^{3+}$), hemoglobin is converted to methemoglobin and can no longer bind oxygen [2].

A spectrophotometer may be used to quantitate various forms of hemoglobin using the appropriate extinction coefficient and peak absorbance value. Oxyhemoglobin contains bound oxygen and exhibits absorbance peaks at 414 nm, 541 nm, and 576 nm [3]. Deoxyhemoglobin does not contain bound oxygen and exhibits an absorbance peak at 431 nm. Methemoglobin cannot bind oxygen due to containing iron in the ferric state, and exhibits an absorbance peak at 406 nm [4].

A spectrophotometer may also be used to assess a plasma or serum sample for hemolysis. Hemolysis occurs when the red blood cell membrane breaks down and releases hemoglobin and other intracellular components into the surrounding serum or plasma [5]. The estimation

of hemolysis is important as it can affect the accuracy of many laboratory assays. A spectrophotometer may be used to evaluate hemolysis using the absorbance of a plasma or serum sample at 414 nm. An elevated absorbance at 414 nm is associated with increased free oxyhemoglobin [3, 6].

It is important to keep in mind that spectrophotometers measure the total absorbance of a sample, and cannot distinguish the analyte of interest from any other component in the sample that absorbs at the same wavelength. Spectrophotometric hemoglobin measurements in plasma may be subject to interference from increased bilirubin levels, plasma proteins, albumin, lipids, and other absorbing components [5].

If you would like to quantitate hemoglobin in whole blood, a colorimetric assay may be performed at 540 nm using Drabkin's reagent. A colorimetric assay is beyond the scope of this application note, but users can set up a custom method for the assay on the NanoDrop One/One$^C$ Spectrophotometer.

## Experimental Procedures

A custom method was created for the NanoDrop One/One$^C$ instrument to measure the absorbance of hemoglobin at 406 nm, 414 nm, 431 nm, 541 nm, and 576 nm. The method includes an analysis wavelength at 414 nm to calculate the concentration of oxyhemoglobin using an extinction coefficient of 524,280 $M^{-1}$ $cm^{-1}$ and molecular weight of 64.5 kDa [7]. The method also includes an additional calculation to determine the concentration of deoxyhemoglobin using the absorbance at 431 nm, an extinction coefficient of 552,160 $M^{-1}$ $cm^{-1}$, and molecular weight of 64.5 kDa [7]. The method includes wavelengths 541 nm and 576 nm to monitor, as oxyhemoglobin exhibits absorbance peaks at these wavelengths [5]. The method includes wavelength 406 nm to monitor for methemoglobin. A baseline correction at 750 nm was included for the entire spectrum, as well as an analysis wavelength correction based on the sloping baseline correction from 360–500 nm.

The hemoglobin custom method was validated using hemoglobin obtained from MP Biomedicals, LLC (catalog number 100714, lot number 7541J). The hemoglobin preparations available through MP Biomedicals may be primarily methemoglobin, which cannot bind oxygen, as the iron in hemoglobin is easily oxidized by air.

A hemoglobin stock was prepared at 8 mg/mL using reagent grade water. Serial dilutions were then performed to create standards at 4 mg/mL, 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, and 0.125 mg/mL. The instrument was blanked with water, and five replicates of each standard were measured using the Oxy-hemoglobin Custom Method on the NanoDrop One/One$^C$ Spectrophotometer.

### Custom Method Download

1. Navigate to www.thermofisher.com/nanodrop
2. On the left, select "NanoDrop Software Download"
3. Choose the "NanoDrop One/One$^C$" tab
4. Select "Local Control Software Download Instructions"
5. Scroll to "How to add a NanoDrop One/One$^C$ Custom Method file" and click on "Oxy-hemoglobin Method"
6. Unzip the custom method file and copy the .method file to a USB device and then follow the online "Instructions for uploading a Custom Method to the instrument from a USB device".

## Results

Each hemoglobin standard was measured in replicates of five on the NanoDrop One instrument using the hemoglobin custom method. The standard deviation based on five replicates of each solution ranged from 0.015–0.552A. The coefficient of variation for each solution ranged from 0.61–2.79%.

| | Average A406 n=5 | Standard Deviation n=5 | %CV |
|---|---|---|---|
| **Solution 1** | 44.493 | 0.487 | 1.09 |
| **Solution 2** | 22.846 | 0.552 | 2.41 |
| **Solution 3** | 11.127 | 0.068 | 0.61 |
| **Solution 4** | 5.549 | 0.058 | 1.05 |
| **Solution 5** | 2.660 | 0.019 | 0.71 |
| **Solution 6** | 1.245 | 0.035 | 2.79 |
| **Solution 7** | 0.579 | 0.015 | 2.66 |

**Table 1. Results for hemoglobin standards measured using the hemoglobin custom method on the NanoDrop One/One$^C$.**

## Conclusion

The hemoglobin custom method described in this application note can be used to determine oxyhemoglobin and deoxyhemoglobin concentrations on the NanoDrop One/One$^C$ Spectrophotometer. Depending on your needs, the method may be used to monitor the

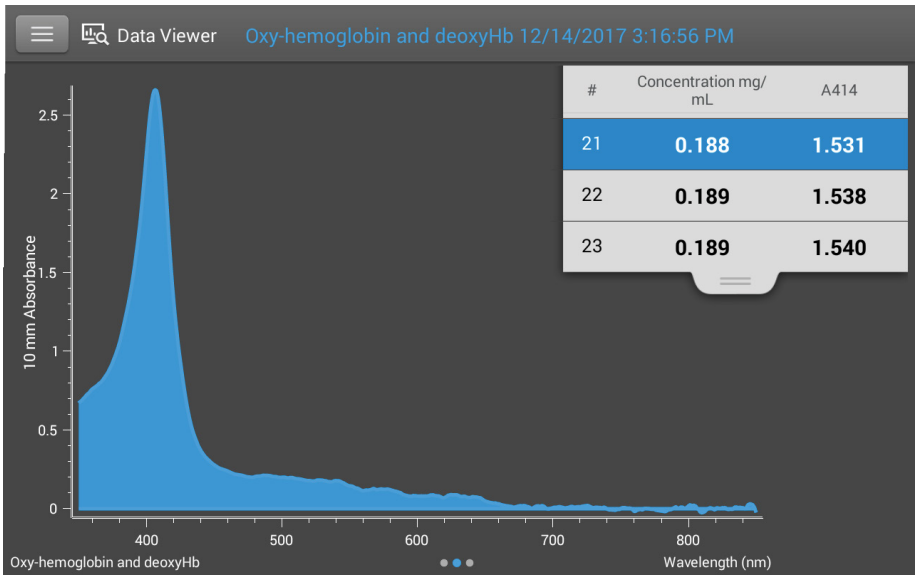



**Figure 1. Typical absorbance spectrum for methemoglobin measured on the NanoDrop One with a peak at 406 nm.** The spectra also lacks peaks at 541 nm and 576 nm which are present in oxyhemoglobin.

absorbance at 414 nm to determine if the sample may be hemolyzed. If measuring oxyhemoglobin, the method may be used to monitor the absorbance at 541 nm and 576 nm. If measuring methemoglobin, the method also may be used to monitor the absorbance at 406 nm.

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