White paper: Protein quantification using spectrophotometer, NanoCuvette™ One and SpectroWorks™ compared to micro-volume system

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Keywords

Protein, UV-Vis Spectrophotometer, NanoCuvette™, SpectroWorks™, Refractive index, Refractive index increment, Bovine Serum Albumin, BSA

Abstract

Accurate quantification of total protein content in a sample or formulated product is important in numerous industries such as biotechnology, pharmaceuticals, chemistry, and foods as well as research. Here we show that quantification of protein can be achieved in seconds using only a cuvette-based UV-Vis spectrophotometer and the novel NanoCuvette™ One with software. The unique cuvette with calibrated optical filter is used to quantify Bovine Serum Albumin (BSA) protein concentrations from 5 mg/mL to 200 mg/mL in aqueous solution using refractive index and the method is compared to industry standards. It is shown that this direct label-free detection method offers enhanced sensitivity and reliability for protein quantification for both microliter and milliliter sample volumes. The approach of upgrading the cuvette sample holder with new technology, rather than instrumentation itself, reduces the need for dedicated micro-volume instrumentation and opens for that existing older equipment may continue to benefit both education, research, and industry.

Introduction

Proteins are the most diverse and abundant molecules and play an important role in almost all types of biological processes, including the delivery of nutrients to cells, balancing of plasma pH, immunological processes, and improvement in the pharmacokinetic properties of drug molecules in biological environments. Proteomics holds the ability to solve the missing pieces of the life science research[1,2]. The vast use of proteomics platform creates the need of precise and quick quantification of proteins. A selection of bioassays including colorimetric assays, qubit assays and others are available for determining the concentration of purified protein. Alternatively, micro-volume absorbance spectroscopy is one of the popular choices for researchers as it is simple, fast and can quantify microvolume samples.

As an alternative to these established methods, NanoCuvette™ One uses an unique optical technology that bends the light beam which make it possible to quantify compounds in

contact with the optical filter[3,4,5,6,7,8].

In addition, the accompanying software, SpectroWorks™, automatically analyses the spectrum, corrects for optical path and cuvette misalignments by accessing 200 million calibration points, and gives back the result to the user in less than 5 seconds. This digital laboratory platform is simple to operate and pre-calibrated, covers sample volume ranging from 0.5 µL to 3 mL, which provides label-free analyte measurement with cloud enabled software solution and secure data storage and tracking. This application notes describes how to use this new technology for protein quantification.

Traditionally, protein concentration measurement is done by color intensity shift *on the y-axis* by absorbance or fluorescence at a fixed wavelength and it is based on Beer-Lambert law. The Beer-Lambert law relates the attenuation of light to the properties of the material through which the light is travelling:

$$A = \varepsilon c l + A_0,$$

where A is the absorbance, ε is molar extinction coefficient, c is the concentration, l is the path length and A_0 is the background absorbance. Label free measurement done with NanoCuvette™ One and software uses a new type of optical filter which is inserted into the light beam and the spectrophotometer measures the refractive index of the analyte by measuring a wavelength color shift *on the x-axis* at fixed intensity that is proportional to the analyte concentration or sample change in the cuvette. SpectroWorks™ is based on Hands law[9] and the linearity is described as:

$$n_{ps} = aC + n_s,$$

where n_{ps} is the refractive index of the protein solution, n_s is the refractive index of the solvent, a is the specific refractive index increment of the protein and C is the concentration of protein solution.

The refractive index increment, *dn/dc*, value of the proteins is one of the important parameters for concentration determination including biophysical characterization of proteins and proteins complexes. Furthermore, the understanding of different optical, properties, structure and function of different proteins are improved by knowing the molecular refractive index[9].

The polarizability of the atoms and chemical groups in the molecule determines the refractive index of the particles and therefore the amino acid composition in the proteins are the major determinant for the protein refractive index. This analytical value has been widely used in different refractometric measurement of protein concentration using analytical ultracentrifugation, surface plasmon resonance and other label free analytical methods [9,10]. However, different technical and price limitations made it difficult to obtain easy, quick, and reliable measurements in the past. NanoCuvette™ One and SpectroWorks™ together with the spectrophotometer presents a fast, cost effective, reliable, and simple protein quantification technique over a wide visible wavelength range using refractive index. This application note describes the detail protocol and performance data including the reference nanodrop measurements for Bovine Serum Albumin (BSA).

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Materials required

1. Spectrophotometer
2. NanoCuvette™ One
3. SpectroWorks™
4. Bovine Serum Albumin
5. Cover slips
6. Triton X

Note: Handling, storage, and the use of NanoCuvette™ One and SpectroWorks™ should be performed in accordance with the product specification sheet and introduction videos supplied by cphnano.

Experimental protocol

1. Microvolume concentration measurements

1. Preparation of diluted Bovine Serum Albumin

Dissolve Bovine serum albumin (BSA) in Deionized (DI) water at a concentration of 200 mg/mL. Use the same diluent used in stock solution to prepare the series of diluted BSA standard solution as described in table 1.

2. Treatment of NanoCuvette™ One and Cover slips with Triton X solution

0.001% of Triton X solution has been used to reduce the non-specific binding of proteins with optical filter and cover slip. Soak the NanoCuvette™ One and cover slips in 0.001% Triton X solution for five minutes and leave it to dry out completely.

3. Protocol for running protein sample using NanoCuvette™ One and Spectrophotometer

1. Turn on the spectrophotometer and stabilize it by letting it sit for at least 15 minutes before running any samples. Prepare your samples during this warmup time.
2. Pipette 0.5µL of reference solution, DI water in the bottom of the 0.001% Triton X-soaked optical filter. Apply the cover slip above the reference solution drop and align it using pipette tip. Please see the instruction video https://nanocuvette.cphnano.com/
3. Insert the reference sample dropped cuvette into the sample chamber of spectrophotometer. Run the B side measurement first and save the spectra, then turn the cuvette 90 degree and record the A side measurement. See B and A side confirmation details here. https://nanocuvette.cphnano.com/
4. Remove the cover slip after reference measurement and drop 0.5µL protein sample in the bottom of the optical filter using well calibrated pipette. Apply the cover slip above the protein solution drop and align it using pipette tip.

5. Insert the sample cuvette into the sample chamber of spectrophotometer. Run and save the B side measurement spectra first, then turn the cuvette 90 degree and record the A side measurement.

4. Analysis of data in SpectroWorks™

1. Create a user profile and log in at https://cphnano.com/spectroworkscom/ . See the detailed instruction on using SpectroWorks™ here. https://knowledge.cphnano.com/en/knowledge/how-to-use-nanocuvette-one-with-spectroworks
2. Create the project folder and select important parameters including protein concentration. Enter the specific refractive increment value for protein and refractive index of reference liquid. 0.1650mL/g is used for Bovine Serum Albumin[11].
3. Go to create option and select cuvette type NanoCuvette™ series. Enter the box code and cuvette number and click next.
4. Reference refractive index measurement: Choose the reference liquid which is water in most of the case. Drag and drop the reference water B side measurement and click next and drag and drop sample B side which is water for reference measurement, then drag and drop A side measurement and finish.
5. Repeat step 3 again.
6. Protein concentration measurement: Choose the reference liquid and drag and drop the reference water B side measurement and click next. Drag and drop sample B side and A side respectively which is Bovine serum albumin in this case and finish. Read more about microvolume measurements here. https://knowledge.cphnano.com/en/knowledge/droplet-application-for-nanocuvette-one.

2. 3mL protein sample volume concentration measurements

1. Preparation of diluted Bovine Serum Albumin

Prepare 0.001% Triton X solution. Dissolve Bovine serum albumin (BSA) in 0.001% Triton X solution at a concentration of 200mg/mL. Use the same diluent used in stock solution to prepare the series of diluted BSA standard solution as described in table 2.

2. Protocol for running 3mL protein sample using NanoCuvette™ One and Spectrophotometer.

1. Turn on the spectrophotometer and stabilize it by letting it sit for at least 15 minutes before running any samples. Prepare your samples during this warmup time.
2. Pipette 3 mL of reference DI water solution in NanoCuvette™ One.
3. Insert the reference sample cuvette into the sample chamber of spectrophotometer. Run the B side measurement first and save the spectra, then turn the cuvette 90 degree and record the A side measurement.
4. Remove the reference liquid after reference measurement and pipette 2.5 mL of 0.001% Triton X solution in the same cuvette using well calibrated pipette.
5. Insert the 0.001% Triton X filled cuvette into the sample chamber of spectrophotometer. Run and save the B side measurement spectra first, then turn the cuvette 90 degree and record the A side measurement.

6. Pipette 3 mL of protein sample in the same cuvette. Run and save the B side protein sample measurement spectra first, then turn the cuvette 90 degree and record the A side protein sample measurement.

3. Analysis of data in SpectroWorks™

1. Create a user profile and log in at https://cphnano.com/spectroworkscom/
2. Create the project folder and select important parameters including protein concentration. Enter the specific refractive increment value for protein and refractive index of reference liquid which is 0.001% Triton X in this case. 0.1650mL/g is used for Bovine Serum Albumin.
3. Go to create option and select cuvette type NanoCuvette™ series. Enter the box code and cuvette number and click next.
4. Reference water refractive index measurement: Choose the reference liquid which is water in most of the case. Drag and drop the reference water B side measurement and click next and drag and drop sample B side which is water for reference measurement, then drag and drop A side measurement and finish.
5. Repeat step 3 again.
6. Reference 0.001% refractive index measurement: Choose the reference liquid which is water in this case. Drag and drop the reference water B side measurement and click next and drag and drop sample B side which is 0.001% Triton X for reference measurement, then drag and drop A side measurement and finish.
7. Protein concentration measurement: Go to edit project option and add the obtained refractive index of 0.001% Triton X in reference refractive index column. Choose the reference liquid and drag and drop the reference 0.001% Triton X B side measurement and click next. Drag and drop protein sample B side and A side respectively and finish.

3. Nanodrop measurement

Nanodrop measurement was done at the Technical University of Denmark, Department of Chemistry using Nanodrop 1000. Nanodrop manual was followed for the protein quantification using the mass extinction coefficient of 6.7 at 280 nm for a 1% (10 mg/mL) BSA solution. Dilution series of Table 1 and 2 were used for BSA concentration quantification.

Table 1: Dilutions for microvolume protein concentration measurements.

Tube number	Stock volume	Added Diluent	Final volume	Final BSA concentration mg/mL
1.	100 µL	0	100 µL	200mg/mL
2.	75 µL	25 µL	100 µL	150 mg/mL
3.	50 µL	50 µL	100 µL	100 mg/mL
4.	40 µL	60 µL	100 µL	80 mg/mL
5.	30 µL	70 µL	100 µL	60 mg/mL
6.	20 µL	80 µL	100 µL	40 mg/mL
7.	10 µL	90 µL	100 µL	20 mg/mL
8.	5 µL	95 µL	100 µL	10 mg/mL
9.	0	100 µL	100 µL	0 mg/mL

Table 2: Dilutions for 3mL protein concentration measurements

Tube number	Stock volume	Added Diluent	Final volume	Final BSA concentration mg/mL
1.	3 mL	0	3 mL	200 mg/mL
2.	2.25 mL	0.75 mL	3 mL	150 mg/mL
3.	1.5 mL	1.5 mL	3 mL	100 mg/mL
4.	1.2 mL	1.8 mL	3 mL	80 mg/mL
5.	0.9 mL	2.1 mL	3 mL	60 mg/mL
6.	0.6 mL	2.4 mL	3 mL	40 mg/mL
7.	0.3 mL	2.7 mL	3 mL	20 mg/mL
8.	0.15 mL	2.85 mL	3 mL	10 mg/mL
9.	0.075 mL	2.925 mL	3 mL	5 mg/mL
10.	0	3 mL	3 mL	0 mg/mL

Results

Using the protocol, Bovine serum albumin concentration was measured using NanoCuvette™ One, SpectroWorks™ on a spectrophotometer. Furthermore, the validation measurement was done at Technical University of Denmark, Department of Chemistry using a Nanodrop 1000. With the use of NanoCuvette™ One and SpectroWorks™ the data is following the theoretical concentration of BSA without any further data processing, both for 0.5 µL and 3 mL measurements (Fig.1 A and B). However, for protein concentration measurement using a NanoDrop 1000 with its built-in BSA extinction coefficient, the concentrations are considerably lower by the factor of almost 1.5 for both microvolume and 3mL concentration measurements compared to the theoretical concentrations. This is a common and know issue for the older Nanodrop models. However, the data has been processed by making the standard curve and calculating the correction factor of 1.4 (data not shown) using the standard deviation, which gives more trustworthy results when compared with the theoretical concentration. The lack of accuracy and precision of the older Nanodrop models is clearly visible when the error of the measurements is compared to the well calibrated scale measurements. The error fluctuates between 20-40% for the measurements done with the built-in BSA extinction coefficient for the Nanodrop 1000, and the error of the processed data ranges between 2-20% (Fig.1 C and D). However, concentration measurements using NanoCuvette™ One with SpectroWorks™ gives the error less than 5% for 3mL measurements and less than 8% for microvolume concentration measurements from the theoretical concentration (Fig.1 C and D). Noteworthy, the error for 0.5 µL measurements remains considerable stable. The error for both 3 mL and 0.5µL concentration measurements remain consistently under Nanodrop 1000 measurements. Furthermore, the reproducibility of the measurements done with smart cuvette and online software are in agree with the Nanodrop for higher concentration (Fig.1 E and F). With this we demonstrate a direct protein concentration measurement, together with an online automatically data processing and data saving cloud-based software, with low and reliable error measurements from the theoretical concentration with a high reproducibility.

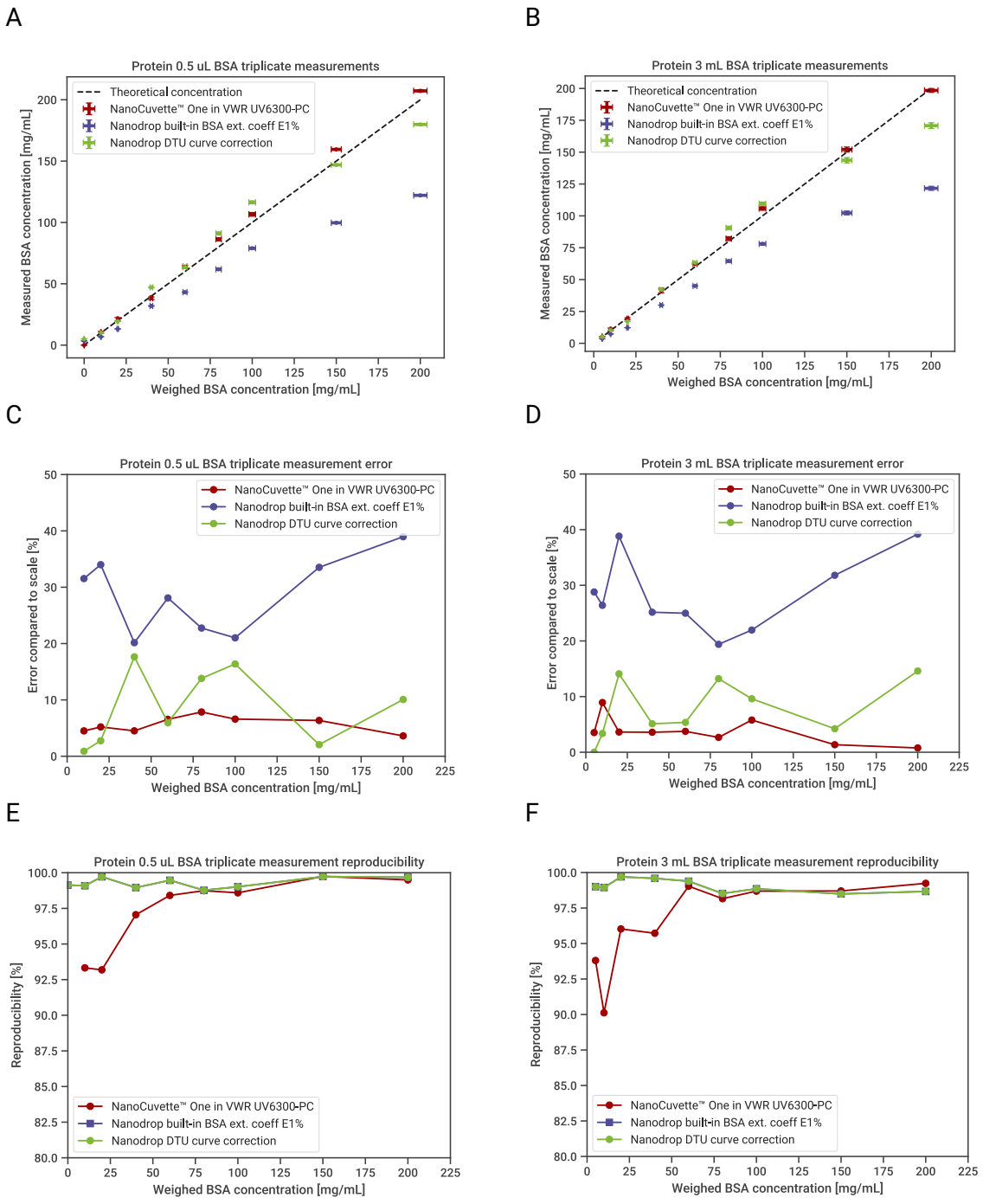

Figure 1: A. Microvolume concentration measured by NanoCuvette™ One and SpectroWorks™ together with spectrophotometer and Nanodrop 1000. B. 3 mL concentration measured by NanoCuvette™ One and SpectroWorks™ together with spectrophotometer and Nanodrop 1000. C. Error percentage plotted for microvolume concentration measurement. D. Error percentage plotted for 3 mL concentration measurement. E. Reproducibility rate plotted for microvolume system. F. Reproducibility rate plotted for 3 mL system.

Conclusion

We have successfully achieved the excellent sensitivity and dynamic range of NanoCuvette™ One and SpectroWorks™ in protein quantification. The cuvette-based UV-Vis spectrophotometer achieves its superior functionality by use of optical filter in the cuvette and first online based software. The optical filter inserted in the cuvette upgrades the analytical capability and offers the state of art performance by UV-VIS spectrophotometer. The superior performance of NanoCuvette™ One and SpectroWorks™ is assessed by comparing the results of protein quantification done with Nanodrop 1000. Optical filter inserted cuvette and software together with spectrophotometer provided very consistent results between replicate measurements with error of less than 5% and reproducibility rate of above 93% in both microvolume and 3 mL protein concentration measurements. In addition, the results obtained with both instruments are comparable. Label free analyte measurement, cloud enabled software, excellent sensitivity, superior performance, cost effective, ease of use with flexible on-site operation and secure data storage package makes NanoCuvette™ One and SpectroWorks™ an ideal consumable for today's life science laboratory.

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