Comparison of different protein quantitation methods

Application

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Abstract

This application note compares the accuracy and reproducibility of absolute protein quantitation of the Agilent 2100 bioanalyzer in combination with the Protein 200 Plus assay to the batch-based methods Lowry and Bradford as well as to denaturing gel-elctrophoresis (SDS-PAGE). The results showed that the Agilent 2100 bioanalyzer provides fast and reliable absolute quantitation data comparable to the other methods investigated.

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Introduction

Determination of protein concentration is a routine procedure in many research laboratories. For example, it is required to calculate and monitor the protein yield after various enrichment or purification processes as well as to optimize and standardize downstream experiments such as protein-protein-interaction studies.

The Agilent 2100 bioanalyzer, developed in collaboration with Caliper Technologies Corp., provides a compact lab-on-a-chip system for the rapid and automated analysis of proteins, DNA, RNA and cells. Lab-on-a-chip technology integrates multiple experimental procedures, such as sample handling, separation staining/destaining, detection and analysis in a single process. Together with the Protein 200 Plus LabChip® kit sizing and analysis of proteins ranging in size from 14 to 200 kDa is possible. In addition, it has the ability to analyze relative quantitation, based on internal standards in each sample, and absolute quantitation based on user-defined calibration standards.

This application note compares the accuracy and reproducibility of absolute quantitation determined using the Protein 200 Plus assay for the following three methods: Lowry assay, Bradford assay and denaturing gel electrophoresis (SDS-PAGE) together with a documentation and analysis system.

Experiment

All proteins were purchased from Sigma Aldrich GmbH (Taufkirchen, Germany). Dulbecco's PBS and distilled water were purchased from Life Technologies GmbH (Karlsruhe, Germany), β-mercaptoethanol was purchased from Fluka (Buchs, Switzerland). The Agilent 2100 bioanalyzer and the Protein 200 Plus LabChip kit were obtained from Agilent Technologies Deutschland GmbH (Waldbronn, Germany). All SDS-PAGE reagents and gels were purchased from Invitrogen GmbH (Karlsruhe, Germany). Coomassie® Stain Solution and Destain Solution were purchased from BIO-RAD Laboratories GmbH (Munich, Germany). The digital camera and the imaging software were purchased from Kodak Digital Science, Eastman Kodak Company (Rochester, NY, USA). The Coomassie Plus Protein Assay Reagent kit and the Modified Lowry Protein Assay were obtained from Pierce / Perbio (Bonn, Germany). The Wallac 1420 Multilabel Counter was purchased from Perkin Elmer Life Science (Turku, Finland).

Protein 200 Plus Assay

The chip-based separations were performed on the Agilent 2100 bioanalyzer in combination with the Protein 200 Plus LabChip kit and the dedicated Protein 200 Plus software assay. All chips were prepared according to the protocol provided with the Protein 200 Plus LabChip kit. The kit includes 25 chips, spin filters and all reagents needed for the experiments including the Protein 200 Plus ladder and the upper and lower marker premixed in the sample buffer. The calibration feature of the software was used to generate a calibration curve with a linear fit, the “unknown” protein concentrations were determined automatically by the software.

SDS-PAGE

Gel electrophoresis was performed with 4-20 % Pre-Cast Tris-Glycine Gels according to the instructions provided by the manufacturer. An equal volume Tris-Glycine SDS Sample Buffer (2x) was added to the samples, and they were denatured for 5 minutes at 95 °C before loading onto the gel. The separation was performed for approximately 120 minutes at constant 125 Volts. Gels were stained with Coomassie Stain Solution for one hour and destained overnight. A digital camera was used for imaging and analysis was performed with the image analysis software. The results were exported to Microsoft® Excel and the data points for the calibration curves were fitted with a linear fit. The resultant equation was used to calculate the “unknown” concentrations

Lowry and Bradford

Both batch-based methods were performed using the Coomassie Plus Protein Assay Reagent kit and the Modified Lowry Protein Assay respectively and a microwell plate according to the instructions of the manufacturer. The absorbance was measured with a fluorescence microwell plate reader at 690 nm for Lowry and 595 nm for Bradford measurements. The results were exported to Microsoft® Excel, the back-

ground signal was subtracted and the data points for the calibration curves were fitted with a second order polynomial fit since no good linear fit was obtained. The equation that resulted was used to calculate the "unknown" concentrations.

Results and Discussion

To study the differences in absolute quantitation between the different methods, three proteins were tested: bovine serum albumin (BSA), ovalbumin (OV) and carbonic anhydrase (CA). The quantitation accuracy and reproducibility were determined for each method. Five standards, 25, 100, 250, 500, 1000 and 2000 µg/ml, were diluted from a 5000 µg/ml stock solution for each of the individual proteins. As reference for the 5000 µg/ml stock solution, the concentration supplied by the manufacturer was used. In addition, four so-called "unknown" samples were diluted from the same stock solutions, with concentrations of 40, 200, 750 and 1250 µg/ml. To reduce variability induced by sample preparation, the same dilutions were used for all four quantitation methods.

Protein 200 Plus Assay

The protein samples were analyzed on the Agilent 2100 bioanalyzer using the Protein 200 Plus LabChip kit. This kit allows sizing and quantitatation of 10 protein samples in less than 45 minutes including sample preparation with a size resolution of approximately 10 % or better.

The Agilent 2100 bioanalyzer software provides two functionalities for quantitation. The first possibility is to determine the relative concentration of the individual proteins. This value is determined automatically by the software based on a one-point calibration with the upper marker (myosin), which is used as an internal quantitation standard in every sample. To determine the relative concentration, the peak area of the unknown sample is compared to the peak area of the upper marker with known concentration. The inclusion of the upper marker in each sample corrects for differences in sample injection into the separation channel and allows for reproducible quantitation. However, due to some staining variability between the upper marker and the protein of interest, this value is not as accurate as absolute quantitation, using a pure sample of the target protein as reference. The Agilent 2100 bioanalyzer software supports absolute quantitation, which can be obtained by a user-generated protein quantitation calibration curve. This feature was previously described[1]. Figure 1 shows a screenshot of the Agilent 2100 bioanalyzer software. The data is displayed as gel-like image, as electropherograms for each sample and in a tabular format.

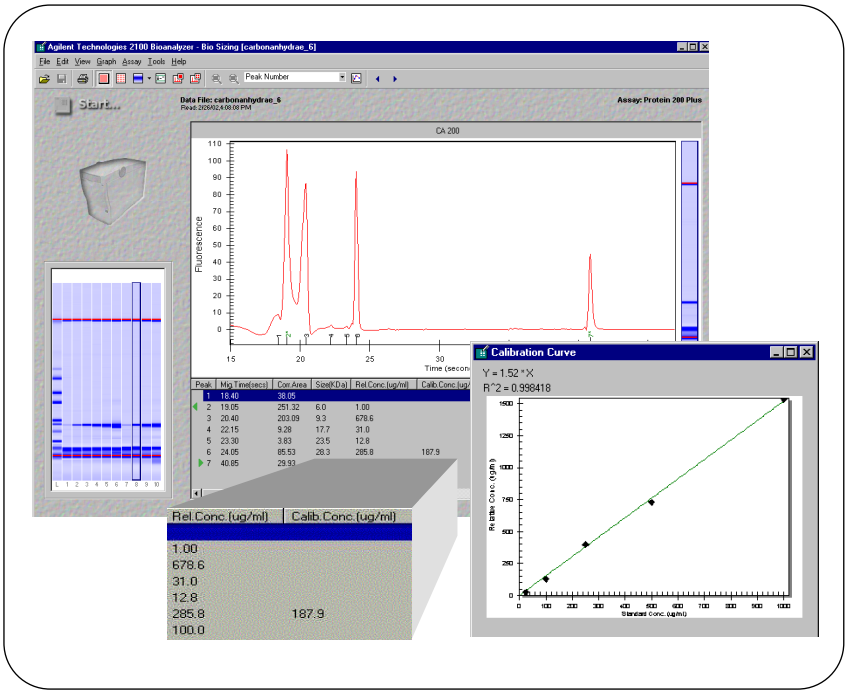

Figure 1
Agilent 2100 bioanalyzer user interface showing an analysis example of carbonic anhydrase (CA) using the Protein 200 Plus assay. The calibration curve was generated using the first six samples loaded on the chip with known concentrations. The equation of the linear regression and the R-squared value is displayed in the calibration curve window. The data table shows the relative concentration and the calibrated concentration for CA sample with a target concentration of 200 µg/ml.

The first six wells of the chip were loaded with the carbonic anhydrase standards with known concentrations and the four "unknown" concentrations were loaded into wells seven through ten. A calibration curve was generated by the software, which is depicted in figure 1. The R-squared value of 0.998 clearly shows the excellent linear behavior of the protein assay. The result table shows that the calibrated concentration of 187.9 µg/ml is greatly improved in comparison to the relative concentration of 285.8 µg/ml. The target concentration in this case was 200 µg/ml. All three protein samples, BSA, OV and CA, were analyzed using the same experimental design. They were run on six chips and three different instruments—two chips per instrument. Table 1 gives a summary, showing that quantitation accuracy was greatly improved with the absolute quantitation feature compared to the relative concentration. The quantitation error for CA was reduced from an average error of 42 % to 6 %, from 28 % to 11 % for OV and for BSA from 19 % to 15 %. The quantitation reproducibility was approximately 10 % and comparable between both quantitation methods.

SDS-PAGE

The same standards and samples of the three proteins were also separated on 4–20% gradient gels for two hours at a constant voltage of 125 V. For a better comparison with the bioanalyzer the same amount of starting material (4 µl) was loaded in each gel well. The

	Target	Agilent 2100 bioanalyzer					
	Conc. (µg/ml)	Rel. conc. (µg/ml)	CV (%)	Error (%)	Calib. conc. (µg/ml)	CV (%)	Error (%)
BSA	1250	1523.5	9.8	21.9	1430.8	8.1	14.5
	750	911.6	8.9	21.5	856.1	6.9	14.1
	200	245.2	9.5	22.6	230.2	6.7	15.1
	40	36.4	9.5	9.1	34.2	6.1	14.6
Ovalbumin	1250	985.7	3.4	21.1	1438.2	4.0	15.1
	750	597.6	3.7	20.3	872.7	6.2	16.4
	200	138.5	6.0	30.8	202.4	9.3	1.2
	40	24.3	12.9	39.3	35.6	15.1	11.1
Carbonic	1250	1663.2	11.0	33.1	1142.0	12.0	8.6
anhydrase	750	1083.8	10.0	44.5	742.7	8.6	1.0
	200	315.3	13.2	57.6	215.4	8.9	7.7
	40	53.7	28.8	34.3	36.8	27.7	8.1

Table 1
Comparison of absolute and relative concentration of BSA, OV and CA at 4 different concentrations using the Agilent 2100 bioanalyzer and the Protein 200 Plus assay (n = 6).

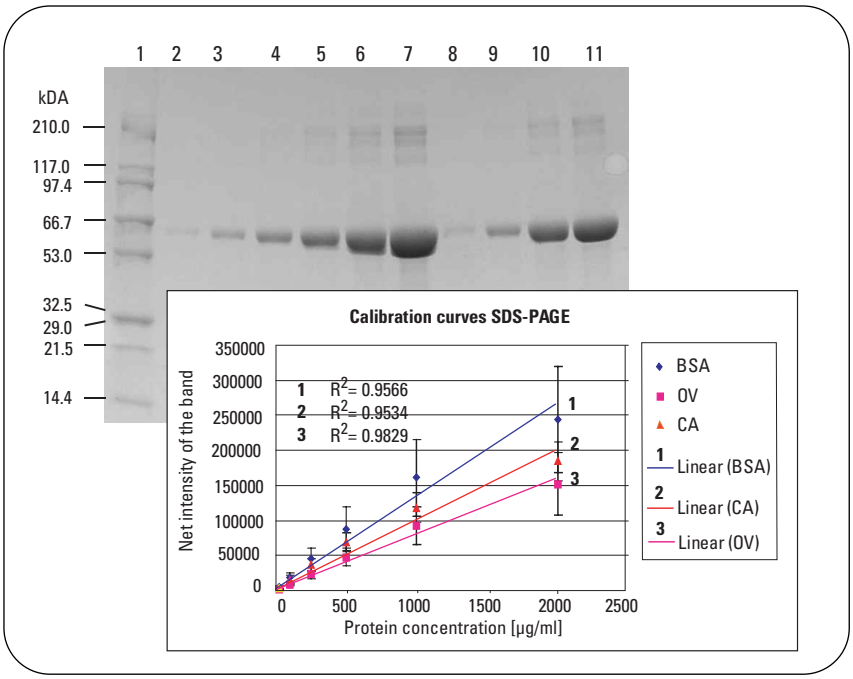

Figure 2
The top panel shows an example of a Coomassie stained 4-20% gradient SDS-PAGE gel loaded with the BSA samples. Lane 1: Protein 200 Plus ladder, lane 2-7: standards, lane 8-11: "unknown" samples. The lower panel shows the averaged calibration curves of the three different proteins evaluated with the image analysis software (n = 5). The R-squared values of the linear regressions are displayed in the chart.

gels were stained with Coomassie for one hour and destained overnight. After that, a digital image was taken (figure 2, top panel) and the data was evaluated with the gel electrophoresis analysis software.

The net intensity, which gives the sum of the background-subtracted pixel values in the band rectangle, of the corresponding band from the standards, was used to generate a calibration curve with a linear fit. Using a polynomial fit did not improve data accuracy. The lower panels of figure 2 shows the averages of the different calibration curves, which were calculated. The displayed R-squared values are between 0.95 and 0.98 indicating linear behavior, however it is not as good as the linear fit of the bioanalyzer. Table 2 shows the quantitation results for the four “unknown” target concentrations determined with this method. Relative standard deviations (CVs) of around 13 % on average were achieved for the samples with concentrations between 200 and 1250 µg/ml. The quantitation of all protein samples with a concentration of 40 µg/ml resulted in CVs of 40 % or worse and also showed a high deviation from the target concentration. The relatively high standard deviations reflect the low staining and destaining reproducibility from gel to gel.

The best results were obtained for the highest target concentration of 1250 µg/ml showing good quantitation reproducibility and an error of 10 % or better. In contrast the 2100 bioanalyzer results are much more consistent for all of the analyzed target concentrations, providing good and comparable quantitation accuracy and reproducibility across the tested concentration range.

Lowry

Analysis with the Lowry method was performed using the Pierce modified Lowry protein assay reagent. The microwell plate version of the protocol was followed. Therefore, a volume of 40 µl of

	Target	SDS-PAGE (linear fit)		
	Conc. (µg/ml)	**Calib. conc. (µg/ml)**	**CV (%)**	**Error (%)**
BSA	1250	1308.6	11.6	4.5
	750	994.5	13.3	24.6
	200	303.4	18.5	34.1
	40	81.8	46.3	51.1
Ovalbumin	1250	1212.1	12.8	3.1
	750	883.7	10.0	15.1
	200	236.2	13.3	15.3
	40	55.6	44.7	28.0
Carbonic	1250	1389.2	7.5	10.0
anhydrase	750	962.4	6.1	22.1
	200	313.1	24.3	36.1
	40	54.9	79.2	27.2

Table 2
Quantitation results of the four "unknown" target concentrations achieved for the three individual proteins using 4-20 % gradient gels, Coomassie stain and the electrophoresis analysis system (n = 5).

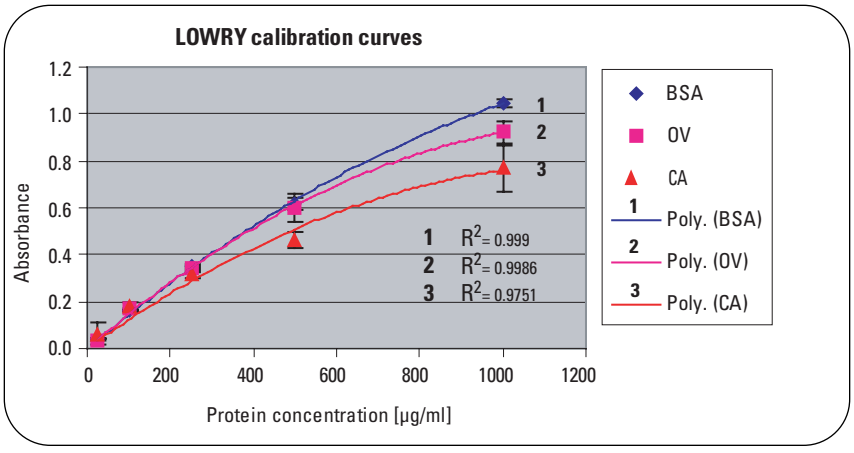

Figure 3
Averaged calibration curves of the three different proteins obtained with the Lowry method using the fluorescent plate reader for analysis (n = 5). The data points were fitted with a second order polynomial fit, the R-squared values are displayed on the chart.

each sample was consumed for every measurement. Figure 3 shows the calibration curves achieved with the six different standards of each individual protein.

Since non-linear color response curves are typically obtained the data points were fitted with a second order polynomial fit. Both R-squared values for BSA and OV are 0.99 reflecting that the fit is nearly perfect. In addition, standard deviations are very narrow indicating a good reproducibility of the method for these proteins. The calibration curve for carbonic anhydrase is not optimal (R2 = 0.975) and also shows a relatively high standard deviation compared to the other two proteins tested. This could be a hint that impurities in the protein sample interfere with the assay since it was not observed with the other three methods. The quantitation results for the "unknowns" are shown in table 3a.

As expected from the calibration curve the CVs for the carbonic anhydrase samples are very high ranging up to 55 % for the 40 µg/ml sample. For BSA and OV the CV values and the error values around 10 % are comparable to the bioanalyzer quantitation data.

A.	Target	LOWRY (polynomial fit)		
	Conc. (µg/ml)	Calib. conc. (µg/ml)	CV (%)	Error (%)
BSA				
	1250	1158.2	5.4	7.3
	750	748.7	4.0	0.2
	200	187.1	9.5	6.5
	40	30.6	20.4	23.4
Ovalbumin				
	1250	1335.8	4.3	6.9
	750	708.9	4.3	5.5
	200	222.6	8.9	11.3
	40	40.2	13.9	0.5
Carbonic anhydrase				
	1250	1402.7	13.1	12.2
	750	885.1	28.6	18.0
	200	149.1	29.5	25.4
	40	43.7	54.8	9.4

B.	Target	BRADFORD (polynomial fit)		
	Conc. (µg/ml)	Calib. conc. (µg/ml)	CV (%)	Error (%)
BSA				
	1250	1413.7	4.8	13.1
	750	894.9	6.1	19.3
	200	123.5	11.2	38.3
	40	3.0	30.3	92.5
Ovalbumin				
	1250	1434.1	6.2	14.7
	750	774.4	5.9	3.2
	200	135.2	10.1	32.4
	40	9.8	34.0	75.5
Carbonic anhydrase				
	1250	1625.0	6.6	30.0
	750	943.9	5.1	25.9
	200	135.6	7.2	32.2
	40	11.5	26.6	71.2

Table 3
Quantitation results of the four "unknown" target concentrations achieved for the three individual proteins using the Lowry (A) and the Bradford (B) method. Absorbance was measured with the fluorescence plate reader at 690 nm for Lowry and 595 nm for Bradford (n = 5).

Bradford

The analysis with the Bradford method was performed using the Pierce Coomassie Plus protein assay reagent kit. As for the Lowry method, the microwell plate version of the protocol was followed, thus using 10 µl of each sample for every measurement. The data points of the calibration curves were fitted with a second order polynomial fit. All R-squared values were 0.99 (not shown). Standard deviations are very tight indicating a good reproducibility of the method. Table 3b summarizes the results of the quantitation of the target concentration. Since the working range specification for this protocol given by the manufacturer is only 100 to 1500 µg/ml, the extremely high CV's and errors for the lowest concentration of 40 ng/µl are explainable. All the other values show comparable CV's to the Lowry method and to the data obtained with the 2100 bioanalyzer. However, the average error of quantitation for the samples within the specified concentration range is around 28 % and therefore a factor of two to three higher compared to the 2100 bioanalyzer analysis and to the Lowry method (figure 4).

Comparison of the methods

Summarizing the data obtained, all four methods show similar behavior in terms of absolute quantitation accuracy. For better direct comparison of the percent CV and error values, a statistic is shown in figure 4.

With respect to reproducibility the 2100 bioanalyzer is comparable to the batch-based methods Lowry and Bradford and superior to SDS-PAGE. Also the percent error is comparable to Lowry and even better than Bradford and SDS-PAGE. However, the 2100

bioanalyzer has two big advantages over Lowry and Bradford. The batch-based methods allow only for total protein quantitation, whereas the 2100 bioanalyzer quantitates each protein in a mixture. In addition, the sample consumption is 40 µl per sample for Lowry and 10 µl using the Bradford method, whereas the 2100 bioanalyzer only needs 4 µl of sample. Analyzing protein mixtures is also possible with SDS-PAGE, but this method is very time-consuming, labor- intensive, and produces hazardous waste in larger quantities. Furthermore, the researcher requires extra equipment such as an imaging system in order to evaluate the data. In contrast the 2100 bioanalyzer offers a speedy analysis of ten samples within 45 minutes. No additional staining or destaining is needed, the data is evaluated automatically by the software on the same instrument and the hazardous waste is significantly reduced.

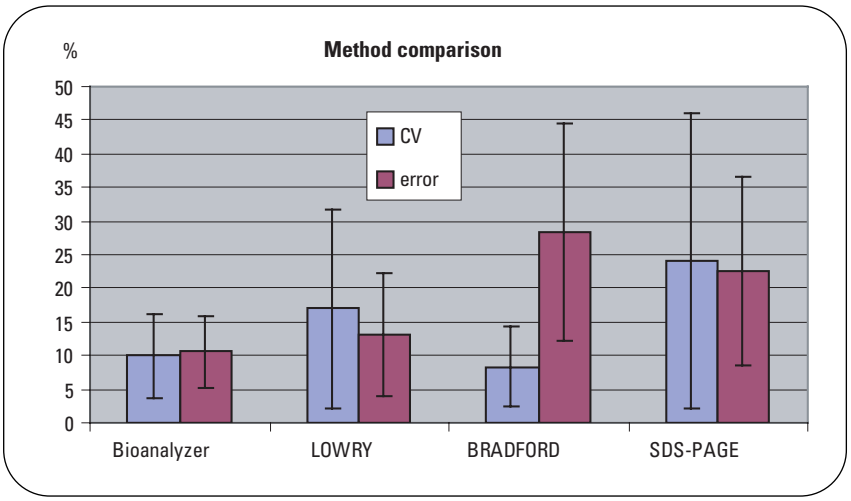

Figure 4
For overall comparison of the four different quantitation methods the average CV and % error were compared. For the 2100 bioanalyzer, Lowry and SDS-PAGE methods all the data points were used for the statistics (n = 12), for the Bradford method the 40 µg/ml values were excluded (n = 9).

Conclusion

The Agilent 2100 bioanalyzer in combination with the Protein 200 Plus LabChip kit and the absolute quantitation feature of the software provides fast and reliable absolute quantitation data. The reproducibility and accuracy of the method is comparable to Lowry, Bradford and SDS-PAGE measurements. However, the 2100 bioanalyzer displays several additional advantages, which include speed, automated detailed protein analysis and significant reduction of hazardous waste.

References

1.
Barthmaier P., "Absolute quantitation with the Agilent 2100 Bioanalyzer and the Protein 200 LabChip kit", Application Note (**2001**) Agilent publication number 5988-4021EN.

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