Absolute quantitation with the Agilent 2100 Bioanalyzer and the Protein 200 LabChip® kit

Application

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Abstract

The Protein 200 LabChip® kit allows sizing and quantitation of proteins ranging in size from 14 to 200 kD. Relative quantitation is performed in comparison to the upper marker, used as internal standard in each sample. However, the accuracy of the relative quantitation is affected by the staining efficiency of the protein dye. Absolute quantitation is enabled with the Agilent 2100 bioanalyzer software (revision A.02.01) using the Protein 200 assay in combination with protein standards of known concentrations. Absolute quantitation improves quantitation accuracy by eliminating the differences in staining efficiency among proteins. This Application Note describes how to perform absolute quantitation with the Protein 200 assay and demonstrates the obtained improvement in accuracy.

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Introduction

The Protein 200 assay provides sizing and a relative semi-quantitative concentration determination based on a one-point calibration for proteins ranging in size from 14 to 200 kD[1]. In addition, the new Agilent 2100 bioanalyzer software (revision A.02.01) also allows for absolute quantitation using user-defined calibration standards with known protein concentrations (figure 1). For relative quantitation, the Protein 200 upper marker (myosin) is used as an internal standard in each sample. The Agilent 2100 bioanalyzer software automatically determines the peak area of the unknown proteins and the upper marker in each sample. The relative concentration of the unknown proteins within one sample is then calculated by the software based on the known concentration of the upper marker. The inclusion of the upper marker in each sample corrects for differences in sample injection into the separation channel and allows for reproducible quantitation. Usually a quantitation reproducibility of approximately ±30 % across different chips, instruments and users can be achieved.

The relative quantitation accuracy of the Protein 200 assay depends on the staining efficiency of the protein. Each of the commonly used total protein quantitation methods, such as Lowry, Bradford or bicinchoninic acid (BCA) assays, exhibit some degree of varying color response when assaying different proteins. The staining efficiency depends upon the characteristics of the specific protein and its interaction with the dye. These differences are related to variations among proteins in amino acid sequence, isoelectric point (pI), structure and the presence of certain side chains or prosthetic groups. The variations in staining efficiency can result in the under- or overestimation of protein concentrations. Similar effects are observed when staining polyacrylamide gels with protein stains such as Coomassie or silver stain.

The Agilent 2100 bioanalyzer detection is based on laser-induced fluorescence of an intercalating dye, which interacts with the protein/SDS complexes. The staining efficiency for different proteins also varies with this method. More accurate, absolute protein quantitation can only be

obtained by using a calibration curve generated with identical proteins and known concentrations. This application note describes how to perform absolute quantitation with the Protein 200 assay, and demonstrates the improvement in quantitation accuracy by using the absolute quantitation feature.

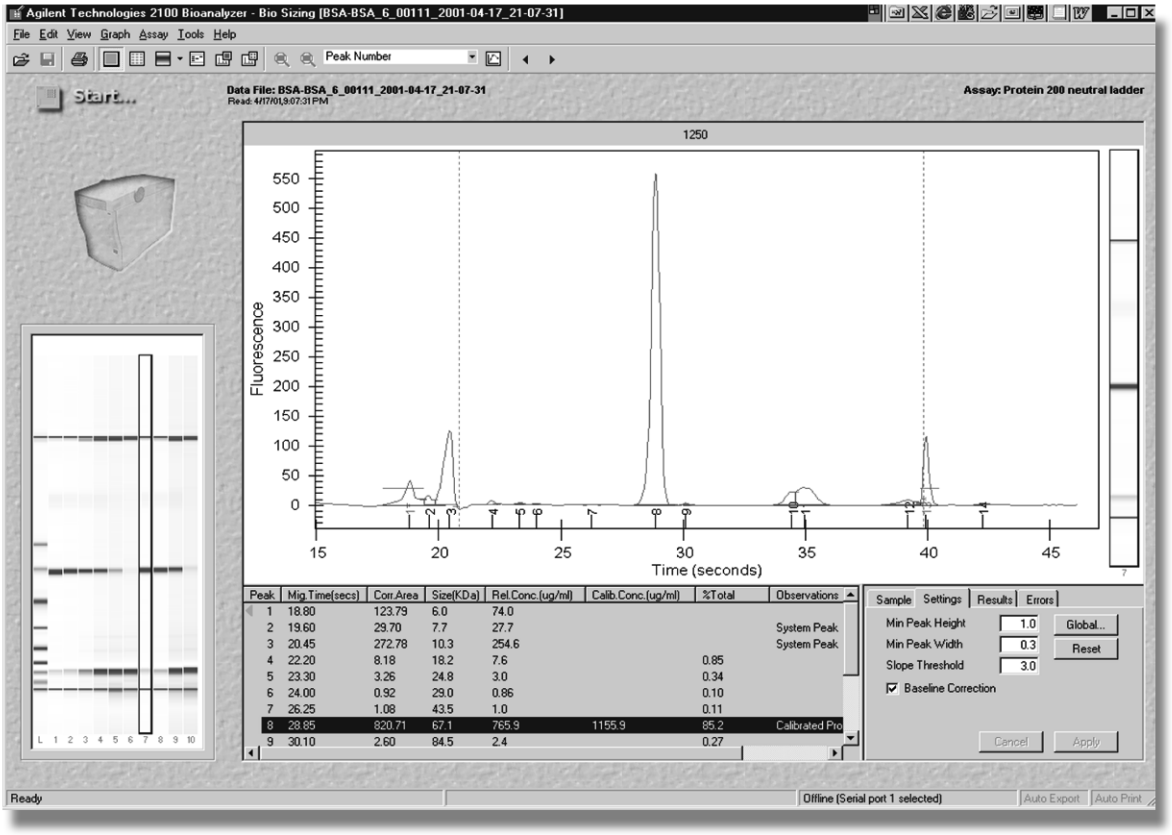

Figure 1
Agilent 2100 bioanalyzer software (revision A.02.01)

Results and Discussion

The Agilent 2100 bioanalyzer software allows for both relative and absolute quantitation of proteins. Most assays for protein quantitation currently available, i.e. Bradford, Lowry, BCA, also allow relative or absolute quantitative analysis. Samples are mixed with specific reagents and a colorimetric change is read. These changes are compared to a standard curve generated with BSA or any other protein, against which the total concentration of the unknown protein sample is determined. As described earlier, the Agilent 2100 bioanalyzer calculates the relative concentration of the unknown sample relative to the upper marker (myosin).

To attain a greater level of accuracy the Agilent 2100 bioanalyzer software (revision A.02.01) allows for the absolute quantitation of a given protein. A calibration curve is generated using a pure protein standard with known concentration of the protein of interest. The absolute concentration of the same protein in the sample is determined from the calibration curve.

The Agilent 2100 bioanalyzer software provides a *General Chip Information* dialog box where sample information can be entered. This dialog box can also be used to define samples as standards for calibration (figure 2). The software will automatically generate a calibration curve of the standards and calculate the absolute calibration concentration of the samples. The calibration curve is generated by plotting the known concentration of the user-defined standards against the relative concentration measured by the Agilent 2100 bioanalyzer. A linear regression is calculated based on the data points and an R squared value (coefficient of determination) is determined to indicate how good the fit is (figure 2). Both relative and calibrated concentrations are reported in the data table (figure 3). Calibration proteins (standard proteins), and calibrated proteins (sample proteins) are identified under the column *Observations* in the result table.

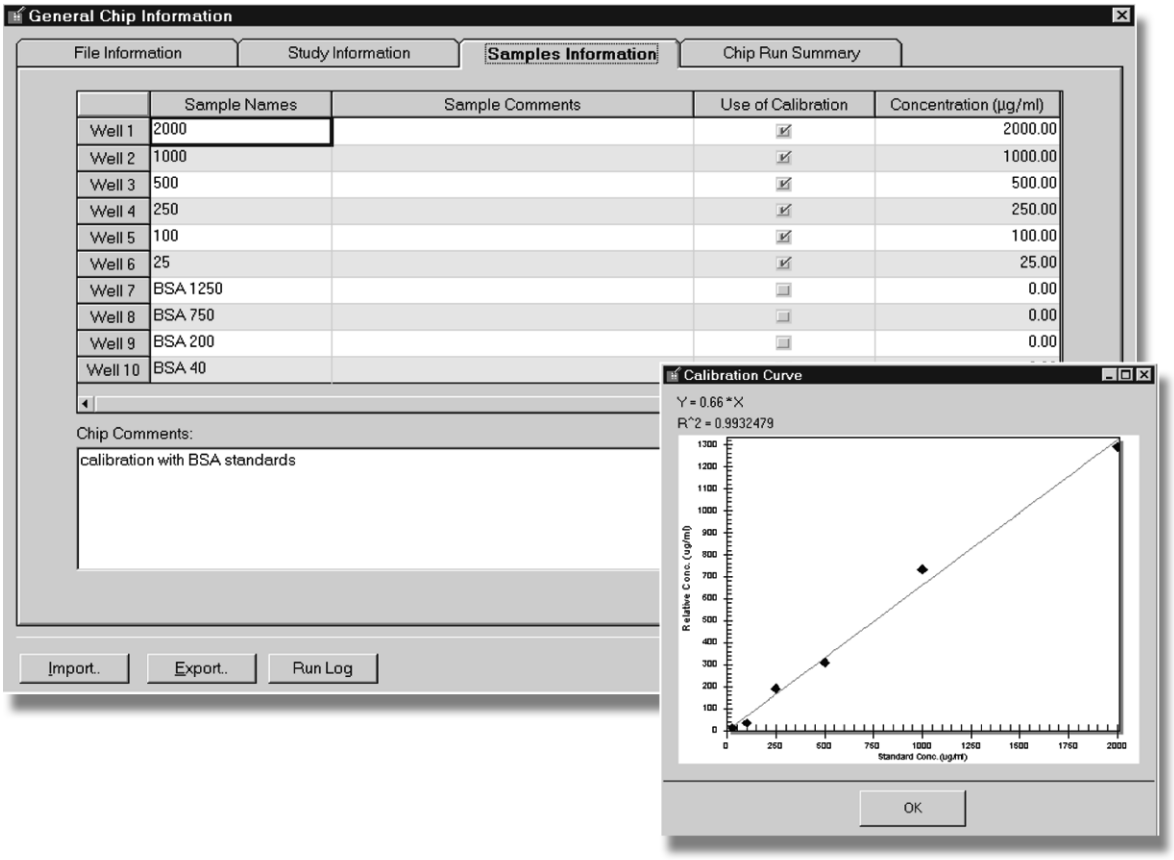

Figure 2
General Chip Information **table and** ***Calibration Curve*** **used for absolute quantitation**

Peak	Mig.Time(secs)	Corr.Area	Size(KDa)	Rel.Conc.(ug/ml)	Calib.Conc.(ug/ml)	%Total	Observations
1	18.80	123.79	6.0	74.0			
2	19.60	29.70	7.7	27.7			System Peak
3	20.45	272.78	10.3	254.6			System Peak
4	22.20	8.18	18.2	7.6		0.85	
5	23.30	3.26	24.8	3.0		0.34	
6	24.00	0.92	29.0	0.86		0.10	
7	26.25	1.08	43.5	1.0		0.11	
8	28.85	820.71	67.1	765.9	1155.9	85.2	Calibrated Pro
9	30.10	2.60	84.5	2.4		0.27	

Figure 3
Data table showing Relative Concentration and Calibrated Concentration for 1250 mg/ml BSA sample

If no standards of the target protein are available, BSA or another protein can be substituted to generate the calibration curve. However, this will only allow relative quantitation based on the calibration curve in contrast to the relative concentration determined by the software, which is based on a one-point calibration with the upper marker. This is not as accurate as using a pure sample of the target protein due to variability in the interactions of the protein, SDS and dye.

To demonstrate the new functionality of the software three individual proteins were tested: bovine serum albumin (BSA), ovalbumin and myoglobin (all purchased from Sigma-Aldrich Co., St. Louis, Missouri, USA). Five standards were diluted in phosphate buffered saline (PBS) from a 2000 µg/ml stock solution for each of the individual proteins. Calibration standards with concentrations of 2000, 1000, 500, 250, 100 and 25 µg/ml were loaded into the first six wells of each chip. Four "unknown" concentrations of the same protein were loaded into wells seven through ten. The "unknown" samples were diluted from the same stock solutions in PBS. The concentrations of the "unknown" samples were 1250, 750, 200 and 40 µg/ml.

Using the *General Chip Information* dialog box the first six lanes were designated as calibration standards. Six chips were run for each protein. Three separate instruments were used, two chips per instrument. Table 1 summarizes the data. As reference for the ovalbumin and the BSA quantitation accuracy the concentration given by the manufacturer was used. For myoglobin the stock solution was prepared by weighing the exact amount of myoglobin. The new Agilent 2100 bioanalyzer software, with absolute quantitation, greatly improved the quantitation accuracy compared to the relative quantitation. The quantitation error for ovalbumin was reduced from an

	Target	**Agilent 2100 bioanalyzer**					
	Conc. (µg/ml)	Rel. conc. (µg/ml)	RStDev (%)	Error (%)	Calib. conc. (µg/ml)	RStDev (%)	Error (%)
BSA	1250	863.4	7.3	30.9	1265.6	4.4	1.2
	750	672.9	11.9	10.3	986.7	10.7	31.6
	200	152.2	11.0	23.9	223.1	8.8	11.5
	40	26.7	19.0	33.3	38.9	14.8	2.7
Ovalbumin	1250	546.3	10.4	56.3	1110.8	10.2	11.1
	750	299.5	13.5	60.1	609.6	14.7	18.7
	200	99.9	8.9	50.1	200.4	13.4	0.2
	40	16.8	7.7	58.1	34.1	10.1	14.7
Myoglobin	1250	547.7	15.1	56.2	1553.8	14.1	24.3
	750	310.7	9.9	58.6	881.6	8.6	17.5
	200	91.8	10.7	54.1	260.9	10.7	30.4
	40	11.0	33.4	72.6	31.2	33.5	22.0

Table 1
Comparison of absolute and relative quantitation using the Agilent 2100 biolanalyzer

average error of 56 % to 11 %, from 25 % to 12 % for BSA and for myoglobin from 60 % to 24 %. The quantitation reproducibility was approximately 13 % and comparable for both relative and absolute quantitation methods.

Samples with the lowest concentration showed a slightly increased variation. For the 750- µg/ml BSA sample a smaller error for the relative quantitation and a larger error for the absolute quantitation was observed, indicating that this error may have been introduced when diluting the stock solution to this concentration by a pipetting error.
Both Bradford and Lowry assays measure total protein content within a sample. If the sample is contaminated with other proteins they too will contribute to the overall protein concentration and inflate the apparent protein concentration. The Agilent 2100 bioanalyzer provides an electropherogram, a gel-like image and the relative quantitation data for all of the proteins in the sample. The added absolute quantitation feature in the Agilent 2100 bioanalyzer software allows for a more accurate absolute concentration measurement of the protein of interest.

Conclusions

The Agilent 2100 bioanalyzer software (revision A.02.01) enables absolute quantitation of protein samples. Using the absolute quantitation feature for the Protein 200 assay allows sizing of proteins from 14-200 kD and determines the absolute concentration in a single experimental step. No other assay currently on the market can offer the speed and accuracy of the Agilent 2100 bioanalyzer, providing information on size, purity and concentration simultaneously.

1.
Kuschel M., “Protein sizing and analysis using the Agilent 2100 Bioanalyzer and Protein 200 LabChip® kit” (**2000**), Application Note, Agilent publication number 5988-0975EN

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