Protein sizing and analysis using the Agilent 2100 Bioanalyzer and Protein 200 LabChip® kit

Application

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Introduction

With the Human Genome Project close to completion, attention is now turning to the next step – how to utilize this genetic data to better understand diseases and develop targeted therapeutics more efficiently. Proteins, rather than genes, convey most cellular functions. Understanding protein expression and protein function is crucial to the identification of new targets for drug development. As we now enter this new, post-genomic era, proteins will move into the focus of attention. Although protein analysis technologies are developing fast, many still rely on traditional methods such as SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which are time-consuming and include a number of laborious manual steps. With the increasing focus on proteins, there is a strong demand to automate and speed up protein analysis.
The Agilent 2100 bioanalyzer, utilizing LabChip® technology from Caliper Technologies Corp., is a compact system for rapid and automated analysis of proteins, integrating multiple experimental procedures, such as sample handling, separation staining/destaining, detection and analysis in a single process. The Agilent 2100 bioanalyzer can be used with the Protein 200 LabChip kit to analyze a large variety of protein samples such as cell lysates, column fractions, antibodies and purified proteins. Ten samples can be analyzed in less than 30 minutes.

This Application Note describes the performance of Agilent's Protein 200 LabChip kit in comparison to conventional SDS-PAGE. The Protein 200 LabChip kit offers several advantages over SDS-PAGE analysis. These include:

- significant time savings,
- improved ease of use,
- excellent reproducibility, and
- significant reduction of hazardous waste.

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Experiment

All proteins were purchased from Sigma Aldrich GmbH (Taufkirchen, Germany). Dulbeco's phosphate buffered saline (PBS) was purchased from Life Technologies GmbH (Karlsruhe, Germany). The Agilent 2100 bioanalyzer, Protein 200 LabChip kit, and Protein 200 ladder and upper marker were obtained from Agilent Technologies GmbH (Waldbronn, Germany). All SDS-PAGE reagents and gels were purchased from Invitrogen BV (Groningen, The Netherlands). The digital camera and the imaging software were purchased from Kodak Digital Science, Eastman Kodak Company (Rochester, NY, USA).

Protein 200 assay

The chip-based separations were performed on the Agilent 2100 bioanalyzer in combination with the Protein 200 LabChip kit and the dedicated Protein 200 assay software. All chips were prepared according to the protocol provided with the Protein 200 LabChip kit. The kit includes 25 chips, syringe, spin filters and reagents. In addition, the Protein 200 ladder and upper marker were used. The Agilent 2100 bioanalyzer is controlled by intuitive and user-friendly software, which includes data collection, reporting and interpretation functions.

SDS-PAGE

Gel electrophoresis was performed with 10 % and 4-20 % Pre-Cast Tris-Glycine Gels according to the instructions provided by the manufacturer. An equal volume Tris-Glycine SDS sample buffer (2 x) 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 100 minutes at constant 125 volts. Gels were stained with a Colloidal Blue Stain kit and destained overnight. A Kodak DC 1200 digital camera was used for imaging and analysis was performed with the Kodak 1D Image Analysis software.

Results and discussion

Different protein samples were analyzed to verify the performance of the Protein 200 assay with regard to size range, resolution, sensitivity, linear dynamic range, sizing and quantitation accuracy, and reproducibility. The Agilent's Protein 200 assay was used to determine the size and the relative concentration of each sample. The results for each sample were viewed in real-time as separation when detection was completed. The first result was available in only seven minutes with each subsequent analysis following in 2-minute intervals. The results were displayed in a tabular format, a gel-like image and an electropherogram for each sample (figure 1). In contrast to conventional SDS-PAGE, no additional manual staining, destaining, imaging and analysis steps were necessary.

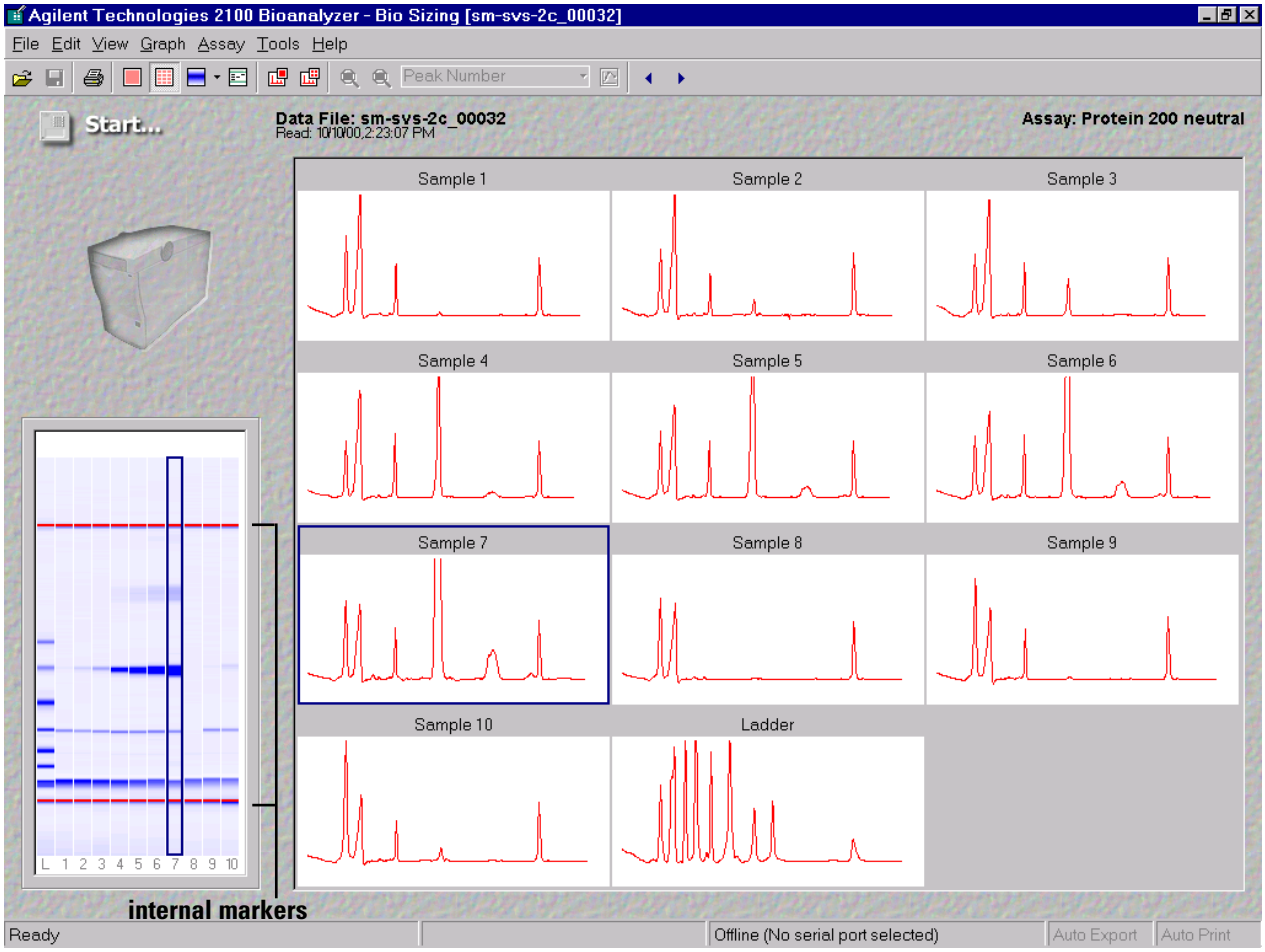

Figure 1
Agilent 2100 bioanalyzer software. Data are displayed as gel-like image as well as electropherograms (samples 1-10 and Protein 200 ladder). Protein samples with carbonic anhydrase (100 ng/µl) and bovine serum albumin in different concentrations (10 to 3000 ng/µl) were analyzed. The Protein 200 ladder (first lane of the gel-like image) and the internal markers are used as reference for sizing and relative quantitation.

Sizing range and resolution

Sizing range and resolution of the Protein 200 assay were evaluated and compared to the results obtained with conventional SDS-PAGE analysis. The Protein 200 ladder, used for sizing with the Protein 200 LabChip kit, was separated and analyzed using both linear and gradient precast polyacrylamide gels, and Agilent's Protein 200 assay (figure 2A). The Protein 200 assay was used to size and analyze proteins ranging in size from 14-200 kD under denaturing conditions in the presence of ß-mercaptoethanol as a reducing agent. The resolution of the Protein 200 assay was comparable or even better than the resolution that was achieved using a 4-20 % polyacrylamide gel. As expected, the resolution of the linear gel was lower compared to the gradient gel. It was also lower than the resolution achieved with the Protein 200 assay. This was especially prominent in the low molecular weight range (figure 2A). Two proteins, lysozyme and b-lactoglobulin cannot be resolved on the 10 % gel, however, they are clearly separated on both the gradient gel and Agilent's Protein 200 assay. In addition, the separation with the Agilent 2100 bioanalyzer resulted in sharper bands compared to the polyacrylamide gels (figure 2A), which allows more accurate sizing. Based on repeated measurements with the Protein 200 ladder (data not shown), it was calculated that a resolution of at least 10 % with a 50 % valley can be achieved through the sizing range from 14-200 kD. Above 100 kD, resolution of 5 % is achievable. The resolution depends on the characteristics of the proteins within a sample and can be limited by several factors. Protein heterogeneity that results in a larger peak width can reduce the resolution. Some proteins, such as bovine serum albumin give relatively broad peaks compared to other proteins. Resolution is also affected by the fact that some proteins do not migrate according to their size. Both effects are also observed using conventional SDS-PAGE.

To confirm the theoretically calculated resolution of at least 10 %, a protein mixture of eight different proteins, some similar in molecular weight (table 1), was analyzed as shown in figure 2B. For example, carbonic anhydrase II (29 kD) and I (31 kD), which differ in size by only 6.5 % were almost baseline separated using the Protein 200 assay. However, they were only partially separated using a 4-20 % gradient gel (figures 2 B and C).

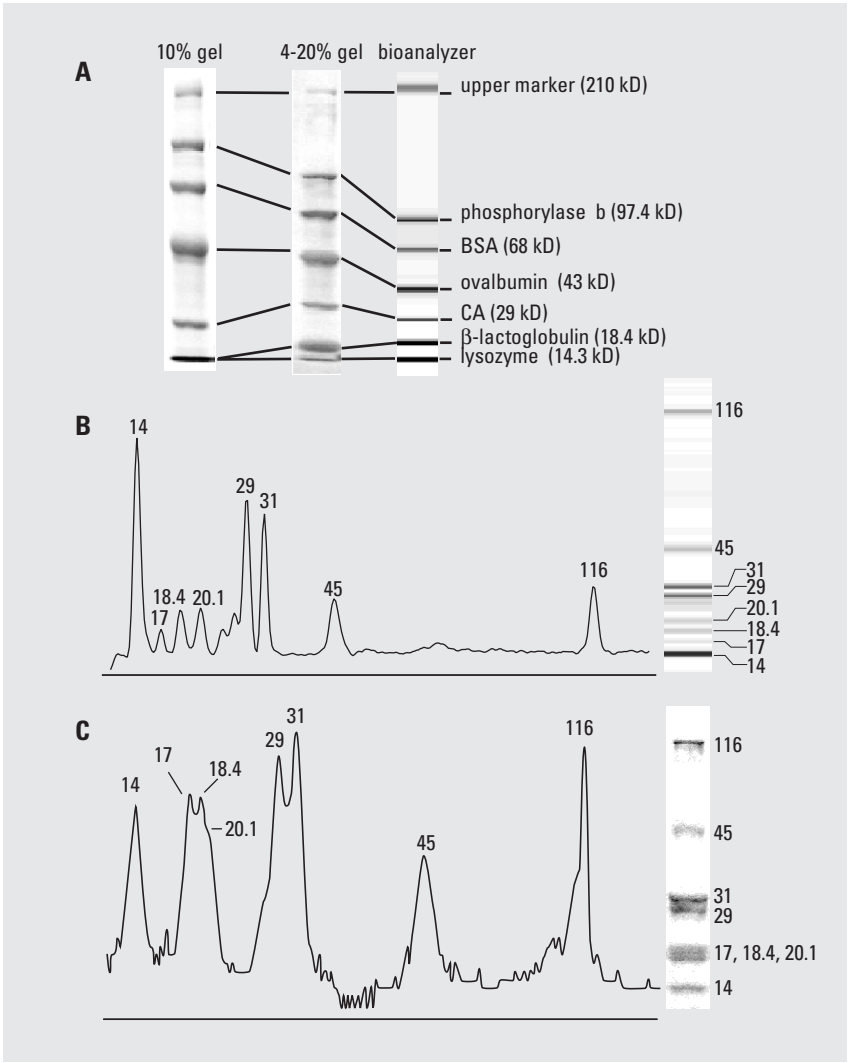

Figure 2
Resolution of the Protein 200 assay.
A: Analysis of the Protein 200 ladder with a linear gel, a gradient gel, and the Protein 200 assay.
B: Electropherogram and the gel-like image of a protein mixture of 8 different proteins (Mw is indicated in kD) demonstrating the resolution of the Protein 200 assay.
C: Analysis of the same protein mixture with a 4-20 % gradient gel (scan and gel image).

Sensitivity and linear dynamic range

To determine the sensitivity and the linear dynamic range of the Protein 200 assay, protein samples containing 100 ng/µl carbonic anhydrase and 10 to 3000 ng/µl bovine serum albumin in phosphate buffered saline (PBS) were analyzed as shown in figure 3. The lower detection limit of the Protein 200 assay was 20 ng/µl BSA in PBS (80 ng in 4 µl sample) with an approximate signal to noise ratio of 3. Vendors state a sensitivity of 50 ng and 10 ng for standard Coomassie stain (R-250) and colloidal Coomassie stain (G-250), respectively. Therefore, the sensitivity of the Protein 200 assay was, comparable to the detection with a standard Coomassie stain. However, the sensitivity of the Protein 200 assay was effected by the salt concentration of the sample buffer. If the sample buffer contains salt concentrations lower than PBS a larger amount of protein will be injected into the separation channel, enhancing the sensitivity. Similarly, increasing the salt concentration in the sample buffer will decrease the sensitivity of the Protein 200 assay, for example, 40 ng/µl of BSA in 0.5 M NaCl can be detected with a signal-to-noise ratio of 3.

In addition, the sensitivity of protein detection is also affected by the staining efficiency and the width of the separated protein band or peak.

The Protein 200 assay is linear over two orders of magnitude, for example, from 20 ng/µl to 2000 ng/µl BSA in PBS. A correlation coefficient of R^2=0.968 was determined analyzing samples from 10 to 3000 ng/µl BSA in PBS as shown in figure 3B. For example, this allows detection a 1 % impurity close to a parent peak, when checking the purification progress in column fractions.

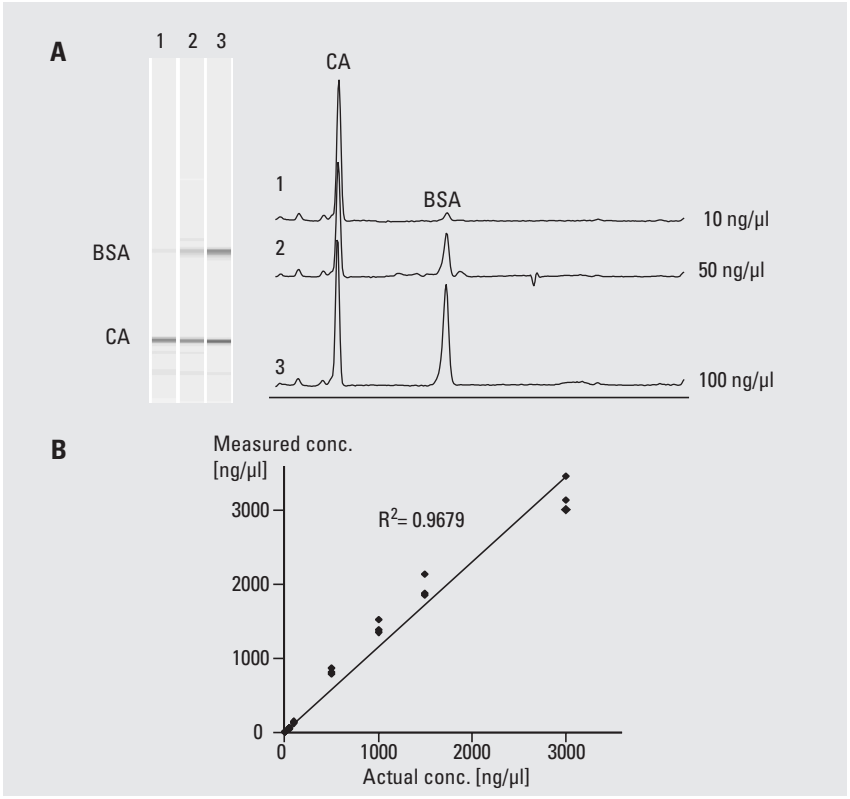

Figure 3
Sensitivity and linear dynamic range of the Protein 200 assay.
A: Gel-like image and electropherogram showing the analysis of a protein sample with 100 ng/µl carbonic anhydrase and 10 to 3000 ng/µl BSA in PBS buffer.
B: Graph showing the linear dynamic range of the Protein 200 assay.

Sizing and quantitation accuracy and reproducibility

The Protein 200 ladder is run on each chip from a designated ladder well. Following the analysis of the Protein 200 ladder the software generates a calibration curve of the migration time versus the molecular weight of each protein contained in the ladder. This calibration curve is then used to determine the size of each of the detected proteins in the 10 samples. The lower and upper markers, which are run with each of the 10 samples, correct for small drifts in migration time and ensure accurate sizing (figure 1). Following each analysis, the size and the relative concentration of the proteins within the sample are immediately displayed in real-time in the data table.

The same protein mixture shown in figure 2B was used to verify the sizing accuracy and reproducibility of the Protein 200 assay. As shown in table 1, the sizing of the eight different proteins analyzed with the Agilent 2100 bioanalyzer was comparable to both the expected molecular weight and the molecular weight determined using SDS-PAGE. The sizing accuracy of SDS-PAGE and chip-based analysis depend on the protein characteristics and may therefore vary for particular proteins. Some proteins may not migrate according to their molecular weight. In general, the sizing reproducibility of the Protein 200 assay is excellent, commonly achieving a sizing reproducibility of 5 % or better.

The concentrations of the proteins within this protein mix were determined with the Agilent 2100 bioanalyzer using a one-point calibration, by comparing the peak area of the protein of interest to the peak area of the Protein 200 upper marker (table 2). The quantitation reproducibility was below 30 %. The Protein 200 upper marker is used for correction of different injection efficiencies due to varying salt concentrations. As indicated before, the amount of material injected into the separation channel depends on the conductivity of the sample buffer. Because all proteins within a certain sample are affected to the same degree, the use of an internal marker allows for correction of this variability and permits determination of the relative concentration of the proteins independent of the sample matrix. In contrast to batch-based assays for quantitation, based on methods developed by Lowry or Bradford, the Protein 200 assay is compatible with most of the commonly used protein sample buffers, including a wide variety of reagents such as detergents, dithiothreitol or imidazole.

Since absolute quantitation is only possible comparing individual results to a standard curve generated with the protein of interest, the Protein 200 assay allows relative quantitation only.

However, as observed with other protein dyes, the relative quantitation accuracy can vary from protein to protein due to the different staining efficiencies.

Protein	Theoretical Size [kD]	SDS-PAGE Size [kD]	StDev	% CV	Bioanalyzer Size [kD]	StDev	% CV
lysozyme	14.0	14.0	0.5	3.3	13.8	0.4	2.9
myoglobin	17.0	17.6	0.5	3.0	16.2	0.6	3.7
ß-lactoglobin	18.4	18.4	0.6	3.2	18.1	0.5	2.9
soybean trypsin inhibitor	20.1	19.1	0.6	3.4	20.7	1.0	5.0
carbonic anhydrase II	29.0	25.3	0.7	2.6	27.5	0.5	1.9
carbonic anhydrase I	31.0	27.2	0.6	2.1	30.7	0.5	1.5
ovalbumin	45.0	46.1	1.1	2.3	42.4	0.5	1.2
ß-galactosidase	116.0	116.6	7.8	6.7	118.3	0.6	0.5

Table 1
Sizing analysis of the same protein mixture including eight different proteins with the Protein 200 assay (based on 5 chips with n=15), and SDS-PAGE (based on 2 gels with n=20) as shown in figures 2B and C.

Protein	Bioanalyzer Rel. conc. [ng/µl]	StDev	% CV
lysozyme	117.4	12.7	10.8
myoglobin	9.4	2.3	24.8
ß-lactoglobin	23.7	4.9	20.8
soybean trypsin inhibitor	22.5	3.8	17.0
carbonic anhydrase II	70.6	8.6	12.1
carbonic anhydrase I	57.6	15.7	27.3
ovalbumin	34.6	2.2	6.3
ß-galactosidase	26.2	1.5	5.9

Table 2
Reproducibility of the relative quantitation of eight different proteins with the Protein 200 assay and (based on 5 chips with n=15).

Conclusion

The Agilent 2100 bioanalyzer is an ideal tool for quick and easy sizing and analysis of proteins. For example, the Protein 200 LabChip kit allows researchers to more efficiently monitor the purification process from cell lysates to the purified protein, to study protein expression, or to monitor antibody production, purification and quality. The use of internal and external markers allows the analysis of multiple samples with excellent reproducibility and reliability. Separation performance and data precision is comparable or superior to gel-based analysis, while analysis times are greatly reduced. Automation of separation and data analysis also makes the Agilent 2100 bioanalyzer versatile and easy to use. In addition to the protein analysis the Agilent 2100 bioanalyzer can be used in combination with a variety of other LabChip kits for the analysis of DNA and RNA samples.

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