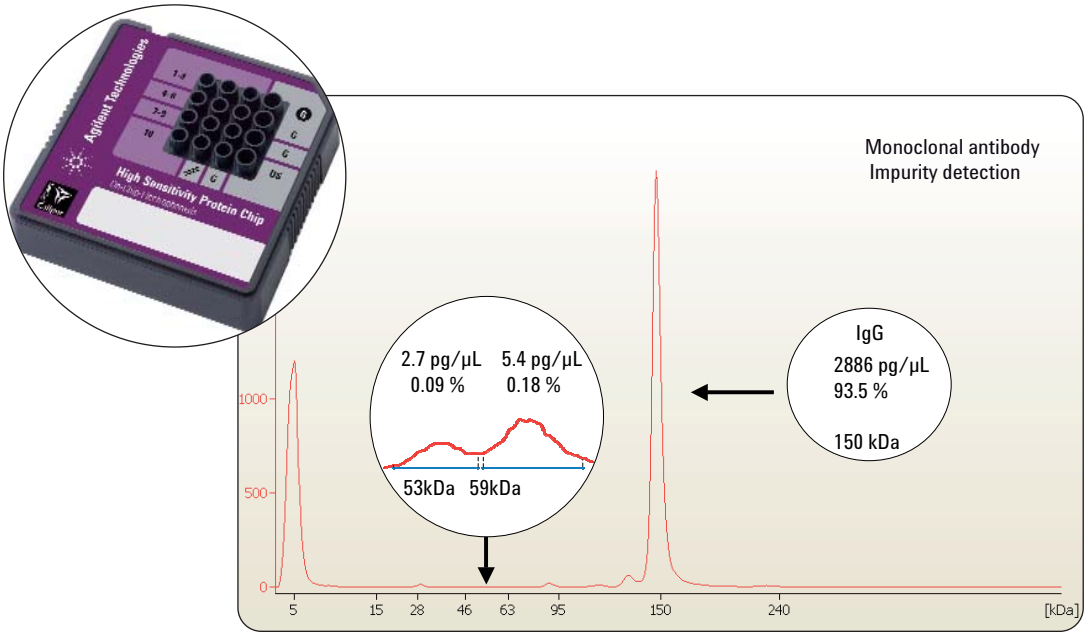
Performance characteristics of the High Sensitivity Protein 250 assay for the Agilent 2100 bioanalyzer

Technical Note



Abstract

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins according to their molecular weights. For highest sensitivity, gels are commonly silver stained using a laborious procedure with low reproducibility, which yields low reproducibility and insufficient quantification capabilities. In this publication we demonstrate the performance of a newly developed Agilent 2100 bioanalyzer method for protein detection based on fluorescent labeling:

- High-sensitivity equivalent to or better than silver staining
- Linear dynamic range up to four orders of magnitude
- Impurity detection, including reliable quantification
- Fast, reproducible results

Agilent Equipment

- 2100 bioanalyzer
- High Sensitivity Protein 250 kit

Application Area

- Protein electrophoresis

Agilent Technologies

Introduction

This publication describes the performance of a recently introduced assay for high sensitivity on-chip protein sizing and quantification – the Agilent High Sensitivity Protein 250 assay. It is a superior alternative to silver staining protocols. The new assay analyzes proteins from 10 to 250 kDa and is based on the detection of fluorescently labeled proteins that are separated electrophoretically on microfluidic chips. It overcomes the critical limitations of traditional silver staining of SDS-PAGE gels by providing a linear dynamic range of four orders of magnitude combined with excellent reproducibility. It extends the existing Agilent 2100 bioanalyzer protein assay portfolio. The existing Protein 80 and Protein 230 assays cover different specific size ranges, providing sensitivity comparable to Coomassie stain and a different protocol.

Experimental

Materials and equipment

Bovine Serum Albumin (BSA) Standard (Pierce, Rockford, IL, USA), 10 kDa ladder (Gibco-BRL), BenchMark ladder and NuPAGE NOVEX 4-12 % Bis-Tris Mini Gels (Invitrogen, Carlsbad, CA, USA), Protein LoBind tubes (Eppendorf GmbH, Hamburg, Germany), Dulbecco's Phosphate Buffered Saline (1X PBS) (Life Technologies GmbH, Karlsruhe, Germany), 2-D Clean-Up kit and PlusOne Silver Staining Kit (GE Healthcare, Freiburg, Germany), the monoclonal IgG was donated by a biopharmaceutical company, Agilent 2100 bioanalyzer, Agilent High Sensitivity Protein 250 kit and Agilent Protein 230 kit.

High Sensitivity Protein 250 assay

The chip-based separations were performed on the Agilent 2100 bioanalyzer in combination with the Agilent High Sensitivity Protein 250 kit. A dedicated assay is available within the Agilent 2100 expert software (revision B.02.06 or higher). If not stated otherwise, the labeling and on-chip electrophoresis was performed according to the kit guide[1]. The kit includes 10 chips for 100 samples, labeling reagents (standard labeling buffer, ethanolamine, DMSO and labeling dye), ladder and reagents for separation (gel matrix, destaining solution and sample buffer) and user documentation (Quick Start Guide and Labeling Protocol).

Labeling reaction

The bovine serum albumin stock (2 mg/mL) was diluted to 0.1, 1.0, 10, 100, and 1000 ng/µL in 1X standard labeling buffer in PBS. Multiple labeling reactions were performed for each BSA concentration. The 10 kDa ladder and the BenchMark ladder were purified with the 2-D Clean-Up kit and resuspended in urea/thiourea buffer (30 mM Tris/HCl, 7 M urea, 2 M thiourea, pH 8.5). Total protein concentrations in the labeling reaction were 60 ng/µL for the 10 kDa ladder and 260 ng/µL for the BenchMark ladder, according to the High Sensitivity Protein 250 assay readout.

The labeling protocol encompassed adding 0.5 µL fluorescent labeling dye (lyophilisate, reconstituted in DMSO) to 5 µL protein sample or High Sensitivity Protein 250 ladder and incubation on ice for 30 minutes. Any excess dye was quenched by the addition of 0.5 µL ethanolamine and 10 minutes incubation on ice (see kit guide[1] for further details.)

High sensitivity protein analysis with the Agilent 2100 bioanalyzer

The labeled protein samples and the ladder were analyzed using the Agilent 2100 bioanalyzer and the High Sensitivity Protein 250 assay. BSA samples labeled at a concentration of 1000 ng/µL were diluted additionally with distilled water (1:10, 1:100, 1:1000, 1:10000) and these dilutions were then treated in the same way as the other labeled samples (control series). Samples were diluted prior to on-chip analysis 1:200 with distilled water as described in the protocol. To prepare labeled samples or ladder for on-chip analysis 2 µL sample buffer (in the absence or presence of DTT as reducing agent) were added to a 4 µL aliquot. The mix was incubated at 95-100 °C for 5 minutes and applied completely to the primed chip.

The labeling reaction and the dilution step in water (5:6 for labeling and 1:200 for dilution) implicated a change in the concentration of the sample. The concentration reported by the software refers to the actual on-chip concentration, which is that prior to sample buffer addition.

SDS-PAGE and silver staining

For comparison, the High Sensitivity Protein 250 ladder was analyzed by SDS-PAGE/silver staining and on the Agilent 2100 bioanalyzer. SDS-PAGE was done on precast NuPAGE NOVEX 4-12 % Bis-Tris Mini gels according to the supplier's instructions. Gels were stained using the PlusOne Silver Staining Kit.

Results and discussion

Development of this new assay successfully implemented the strategy of covalent labeling with a fluorescent dye and subsequent on-chip detection of labeled proteins as an application for the Agilent 2100 bioanalyzer. The assay provides a linear dynamic range of four orders of magnitude combined with excellent reproducibility applicable for proteins from 10 to 250 kDa and besides the detection of high concentration proteins it is ideally suited for two general application areas: detection of minor impurities and detection of lowest protein concentrations. The Agilent High Sensitivity Protein 250 assay measures protein size and relative concentration. The results are available in a digital format, such as gel-like images, a result table and electropherograms.

The figure on the front page of this publication represents typical results for analysis of monoclonal antibodies (IgG) under non-reducing conditions. Labeling was performed at 1 µg/µL. The major band represents the intact antibody at 150 kDa with a purity of 93 %. Various smaller and larger impurities could be quantified such as a 53 kDa peak that might represent a heavy chain fragment at a level of 0.09 % of total protein.

The following sections show different protein samples that were analyzed to verify the performance of the Agilent High Sensitivity Protein 250 assay with regard to sensitivity, linear dynamic range, sizing and quantification.

Linear Dynamic Range

To determine the linear dynamic range of the Agilent High Sensitivity Protein 250 assay, protein samples containing 0.1, 1.0, 10, 100, and 1000 ng/µL bovine serum albumin (BSA) were fluorescently labeled according to the standard protocol. Multiple independent experiments were performed for each BSA concentration to demonstrate the reproducibility of the labeling reaction.

For comparison, a control experiment was performed that excluded any potential influence of the sample protein concentration in the labeling step. For this control series the labeling reactions containing 1000 ng/µL BSA were diluted with distilled water to the respective concentrations. All labeling and dilution series samples, as well as the Agilent High Sensitivity Protein 250 ladder were diluted 1:200 with distilled water (corresponding to on-chip BSA concentrations of 0.4 pg/µL to 4.2 ng/µL) and heat denatured under reducing conditions as described in the standard protocol of the assay. The samples were analyzed on different chips, on four different instruments and by two different users. Results from this experiment are shown in Figures 1 and 2 and Table 1.

Figure 1 demonstrates the linearity of the Agilent High Sensitivity

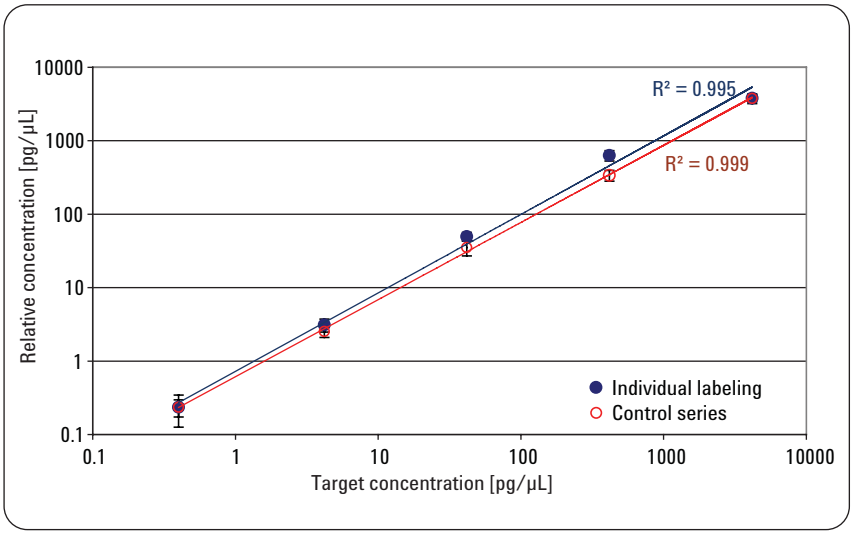


Figure 1

Linear dynamic range of the High Sensitivity Protein 250 assay. Results from individual labeling reactions at various concentrations (filled circles) were compared to serial dilutions (empty circles) starting at the highest concentration (n= 8-12 per concentration). The average relative BSA concentration as determined by the Agilent High Sensitivity Protein 250 assay was plotted against the expected concentration. Standard deviations are indicated.

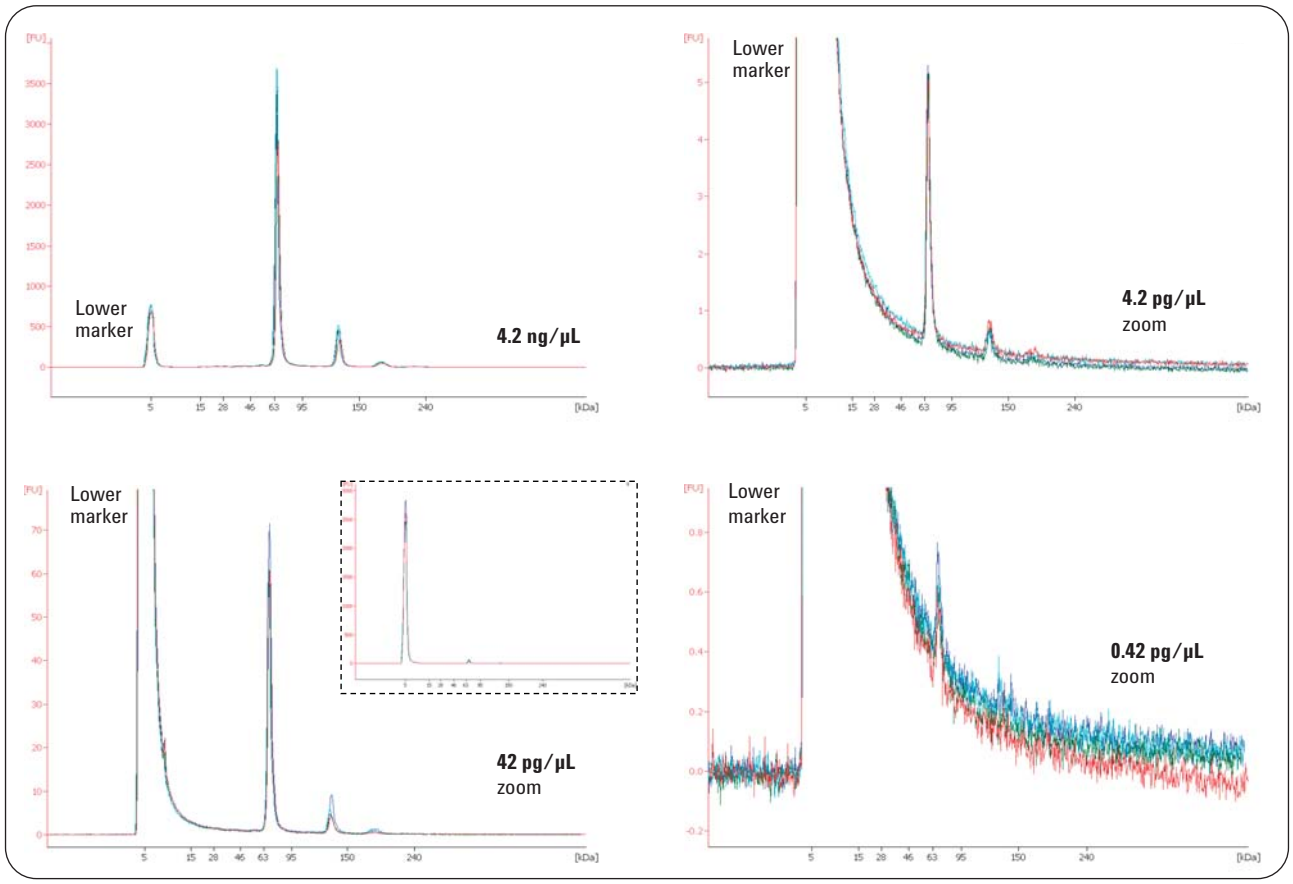


Figure 2
Sensitivity and reproducibility of the High Sensitivity Protein 250 assay. Electropherograms of BSA analyzed at different on-chip concentrations demonstrate the good reproducibility of the method by an overlay of four independent labeling reactions per concentration. The good signal-to-noise ratio allows peak detection even below the specified limit of detection.

Protein 250 assay over four orders of magnitude. Both concentration series show a similar slope and offset in the log/log plot as well as good linearity according to the correlation coefficients (R^2=0.995 and R^2=0.999). This proves the technical suitability of the assay/instrument combination and the reliability of the labeling reaction. Further, for the given concentrations range it demonstrates the independence of the quantification from the protein-to-dye ratio. As a result the excellent linearity of this assay may facilitate, for example, detection of a 0.01 % impurity close to a parent peak.

Sensitivity

The dilution series also demonstrates the excellent sensitivity of the new assay. The respective electropherograms from figure 2 confirm that, even at the lowest BSA concentration, it was possible to detect reproducibly a small BSA peak with an average signal-to-noise ratio of 3.5 ± 0.6 (n = 8; i.e. 0.4 pg/µL BSA on-chip concentration). Be aware that this concentration is below the kit specifications of 1 pg/µL labeled BSA in water on-chip (table 3). The overlay of multiple independent labeling reactions at the various concentrations demonstrates the excellent reproducibility of the labeling method.

The Agilent High Sensitivity Protein 250 assay is affected by the salt concentration of the sample (figure 3). 1 ng/µL BSA was labeled and diluted 200-fold with water or with PBS resulting in an on-chip protein concentration of 4.2 pg/µL, as required for the standard protocol. This concentration was close to the specified lower detection limit of 5 pg/µL labeled BSA in PBS (table 3). The electropherogram overlay demonstrates

the significantly decreased sensitivity for sample dilution in PBS, which contains salt, compared to a dilution with water. For conditions with PBS the amount of injected lower marker was decreased as well and a reduced background due to lower marker tailing was found.

Since on-chip analysis uses electrokinetic injection, a competition for injection into the separation channel exists between the negatively charged protein/SDS micelles and salt ions. High salt concentrations reduce the injection efficiency of the protein/SDS micelles and lower the assay sensitivity. The lower detection limit of the assay was therefore specified to be 1 pg/µL labeled BSA in water and 5 pg/µL labeled BSA in PBS with a signal-to-noise ratio above 3 (table 3). As 4 µL protein sample is required for protein denaturation, this corresponds to a total protein amount of 4 pg BSA in water and 20 pg BSA in PBS.

Silver staining was introduced in 1979 and since then has been continually improved. It is a technique that provides a sensitive tool for protein visualization with reported detection levels down to the 0.2-10 ng level[2]. The sensitivity of protein analysis using the Agilent High Sensitivity Protein 250 assay was compared to the sensitivity obtained with SDS-PAGE followed by conventional silver staining (figure 4). The Agilent High Sensitivity Protein 250 ladder with seven different proteins ranging in size from 15 to 250 kDa was fluorescently labeled to allow on-chip analysis with the Agilent 2100 bio-

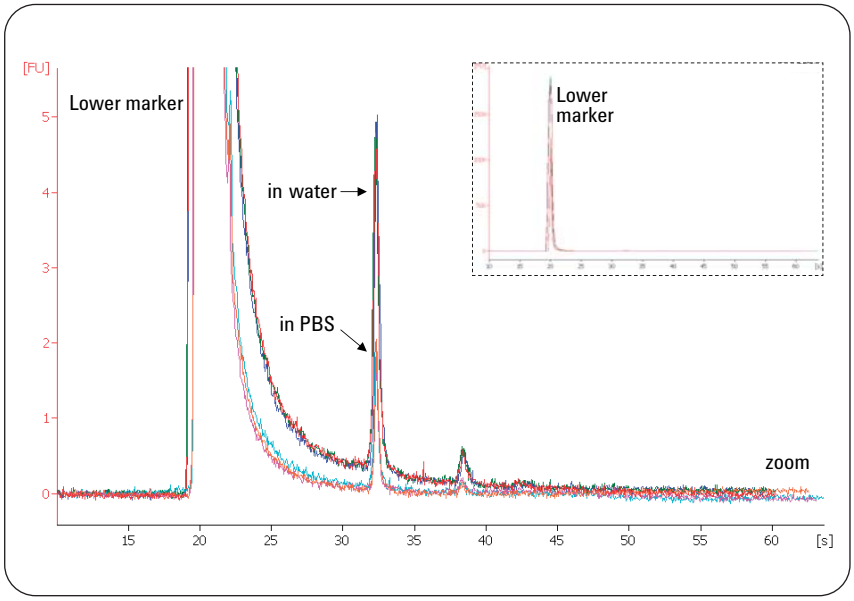


Figure 3
Analysis of labeled BSA in water or PBS. The on-chip BSA concentration was 4.2 pg/µL. Shown is an overlay of three electropherograms for each condition.

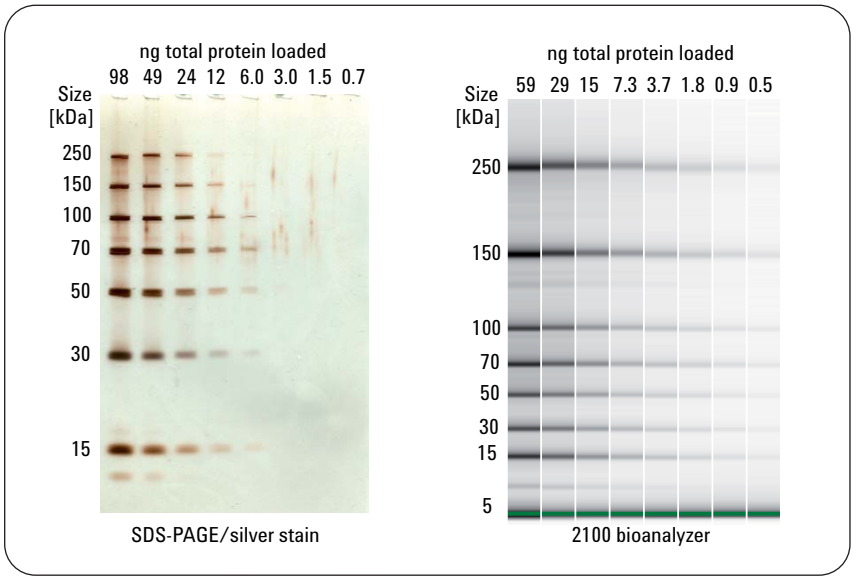


Figure 4
Comparison of the Agilent High Sensitivity Protein 250 assay and SDS-PAGE/silver stain. Analysis of the ladder provided with the kit with 4-12 % SDS-PAGE followed by silver stain and with the Agilent 2100 bioanalyzer at identical concentrations. The figure shows a scanned image of an SDS-PAGE gel and the gel-like image from the Agilent 2100 expert software.

analyzer. For a direct comparison, the labeled sample was run on 4-12% SDS-PAGE followed by silver staining. Silver staining is not affected by the applied label since less than 10 % of the proteins carry the modification.

Two-fold serial dilutions of the labeled ladder were prepared to obtain samples with 8 different protein concentrations (0.1 up to 15 ng/µL) and 6.5 µL with a total protein amount of 0.7 to 98 ng protein were loaded per well on the SDS-PAGE gel (figure 4). For comparison, the protein analysis with the 2100 bioanalyzer was performed with the same dilutions, but using 4 µL sample representing 0.5 to 59 ng total protein. Using SDS-PAGE and silver staining it was possible to detect all ladder proteins down to the total protein amount of 6 ng (about 0.9 ng/µL). This corresponds to approximately 0.9 ng protein per band. At this dilution the 250 kDa band is already very faint. At 3 ng total protein (about 0.5 ng/µL), none of the expected bands appeared. In contrast, the Agilent 2100 bioanalyzer facilitates detection of the individual protein bands with a signal-to-noise ratio above 20 down to a total protein amount of 0.5 ng (about 0.13 ng/µL), which corresponds to approximately 0.07 ng per protein band. For this tested sample, the Agilent High Sensitivity Protein 250 assay demonstrated an about 50-fold higher sensitivity compared to SDS-PAGE and silver staining.

	Target Concentration [pg/µL]				
	4200	420	42	4.2	0.42
CV (individual labeling)	11%	16%	14%	20%	26%
CV (control series)	15%	16%	23%	17%	46%

Table 1
The quantitation reproducibility was determined for the analysis of BSA samples that were labeled at different concentrations (individual labeling) or from the control series.

Quantification reproducibility

Relative concentrations are calculated automatically by the Agilent 2100 expert software. This calculation is based on the total time corrected area of the Agilent High Sensitivity Protein 250 ladder and the area integrated under the sample peak. Assuming equal labeling and injection efficiencies, the rule of three can be applied on sample peak areas to calculate concentrations. The known concentration of the ladder (4167 pg/µL on-chip concentration, a customizable setpoint) is taken into account as external quantification standard from the ladder lane. The Agilent 2100 expert software also facilitates absolute quantification based on calibration with known samples and other quantification strategies[3].

Quantification results for relative quantification from figure 1 are summarized in table 1. The quantification reproducibility for BSA for on-chip concentrations larger than 1 pg/µL was in average 17 %. Lowest concentrations such as 0.42 pg/µL were below the specified detection limit and yielded a CV above 20 %. This data include day-to-day, instrument-to-instrument and user-to-user variability for the labeling reaction and the on-chip analysis. For BSA the quantification reproducibility of the Agilent High Sensitivity Protein 250 assay is specified to be typically below 20 % CV for on-chip concentrations above 1 pg/µL (table 3).

Sizing range and resolution

The 10 kDa and the BenchMark ladder were analyzed under reducing conditions with two Agilent 2100 bioanalyzer assays, the Agilent High Sensitivity Protein 250 and the Agilent Protein 230 assay[4] (figure 5). The BenchMark ladder comprised 15 recombinant proteins ranging in molecular weight from 10 to 220 kDa. The 20 and 50 kDa proteins were more prominent for easy orientation and to ensure proper identification of each protein. The 10 kDa ladder contained 13 recombinant proteins ranging in size from 10 to 200 kDa. The 50 kDa band was more prominent for easier identification.

The sizing range of the Agilent High Sensitivity Protein 250 assay is extended compared to the Agilent Protein 230 assay. The Agilent Protein 230 assay covers a size range from 14 to 230 kDa, whereas the Agilent High Sensitivity Protein 250 assay covers a size range from 10 to 250 kDa. The upper limit is 250 kDa, which

represents the largest sizing standard from the ladder. Visualization and evaluation of proteins beyond 250 kDa is possible. The 10 kDa peak of both sizing standards is outside the size range of the Agilent Protein 230 assay because they collide with the system peak and can be analyzed with bias only.

The peaks of the 10 kDa ladder were all well resolved. The 20 kDa protein comprised two overlapping peaks in the Agilent High Sensitivity Protein 250 assay compared to two separate peaks in the Agilent Protein 230 assay, which were not specified by the manufacturer. The BenchMark ladder 25 kDa protein was not resolved from the 20 kDa protein – it formed a shoulder in both assays. The 70/80 kDa proteins were baseline separated in the Agilent Protein 230 assay but not resolved in the Agilent High Sensitivity Protein 250 assay.

In summary, the resolution of the Agilent High Sensitivity Protein 250 assay was slightly decreased for the given set of proteins in comparison to the Agilent Protein 230 assay, but it featured a significantly extended size range. The typical sizing resolution that can be achieved with the Agilent High Sensitivity Protein 250 assay is specified to be 10 % (table 3).

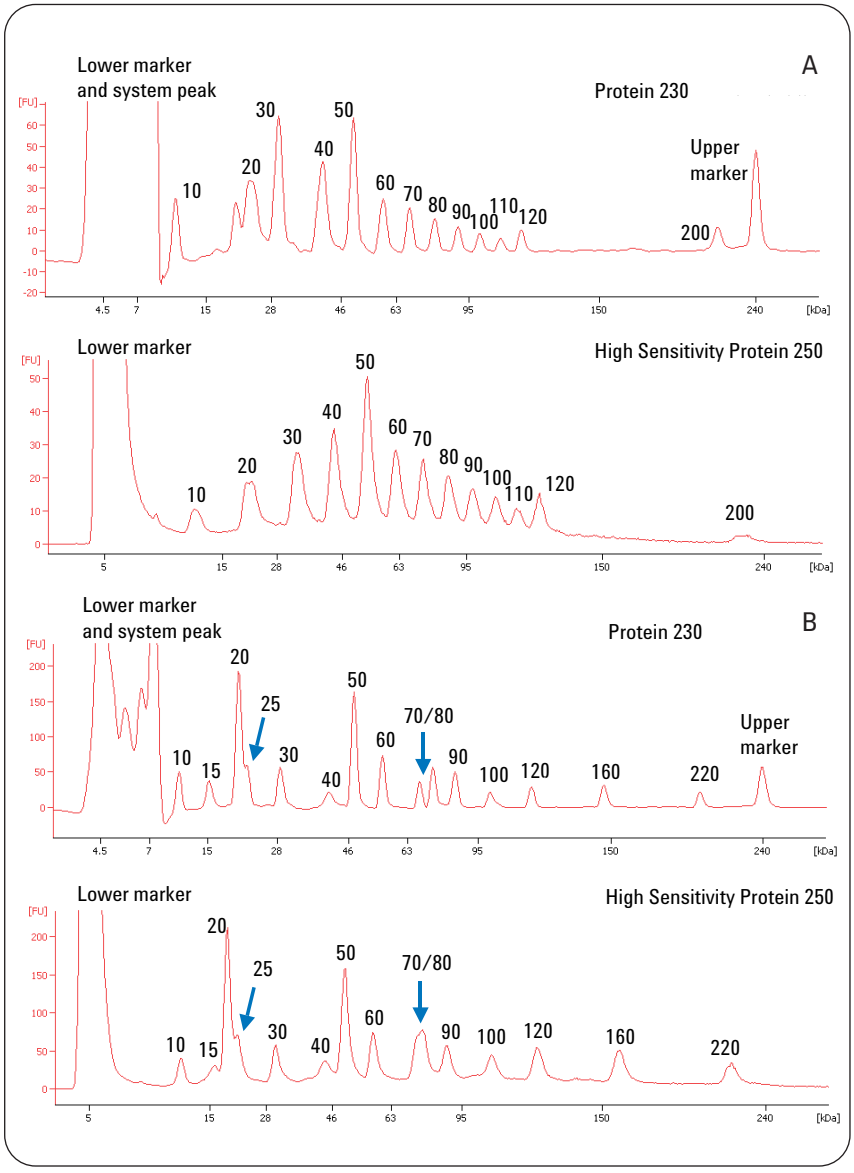


Figure 5
Sizing range and resolution. Electropherograms of the 10 kDa (A) and the BenchMark (B) ladder obtained with the Protein 230 and the High Sensitivity Protein 250 assay. The sizes of the standard proteins are indicated in kDa.

Sizing accuracy and reproducibility

The Agilent High Sensitivity Protein 250 ladder is run on each chip from a designated ladder well. Following the analysis of the ladder the 2100 expert 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 protein in the 10 samples. The lower marker is run with each of the samples for alignment in migration time and ensures accurate sizing. It comprises dye present in the sample buffer and left over dye from the labeling reaction. The 10 kDa and the BenchMark ladder were analyzed under reducing conditions. The sizing accuracy and reproducibility for both sizing standards were determined under two conditions, dilution after labeling in water or in PBS.

Table 2 summarizes the results from figure 6. It shows sizing accuracy (% deviation from the target size) and the average sizing reproducibility (% CV), which is on average 1 % or below independently if water or PBS was used for dilution. The assay is specified to have a typical sizing reproducibility of 3 % CV or better based on BSA as sample. The average sizing accuracy for the 10 kDa and the BenchMark ladder proteins was determined to be below 10 % deviation from the expected size. The typical sizing accuracy of the High Sensitivity Protein 250 assay is specified with

Sample	Diluent	Sizing reproducibility % CV	Sizing accuracy % Dev. from target
10 kDa ladder	water	1.0	8.6
	PBS	1.1	8.8
BenchMark ladder	water	0.6	5.5
	PBS	0.6	5.7

Table 2
Average sizing reproducibility (% CV) and accuracy (% deviation from the target size) of the Agilent High Sensitivity Protein 250 assay with the 10 kDa and BenchMark ladder in water or in PBS.

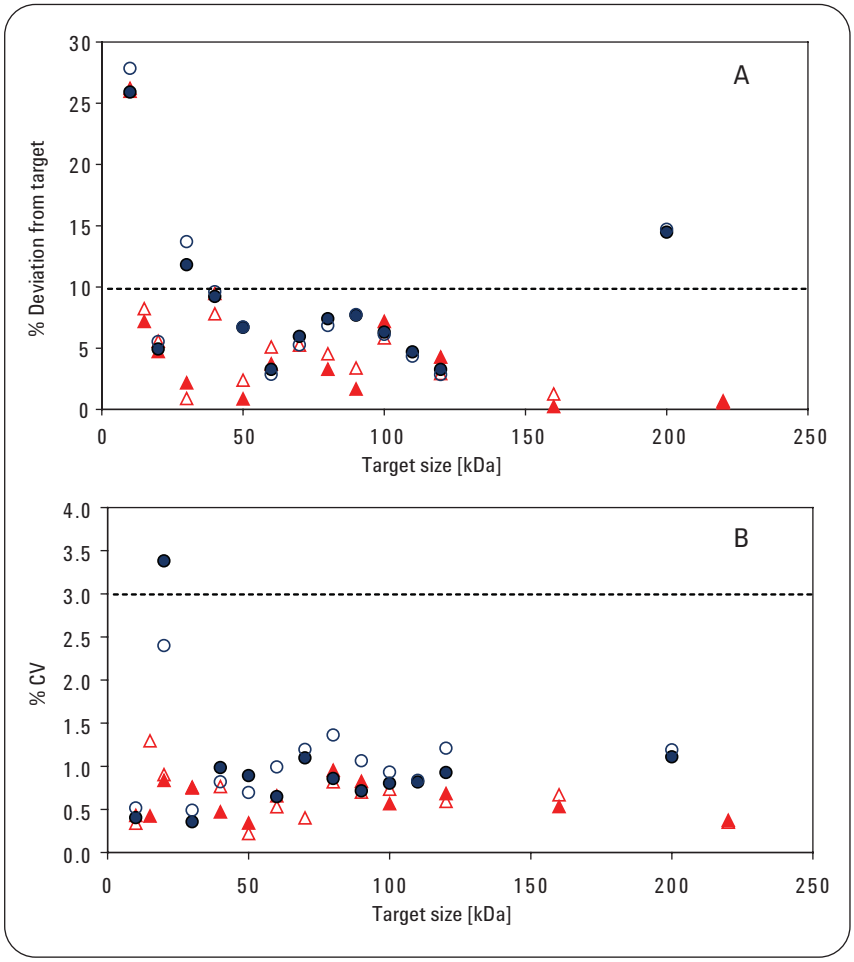


Figure 6
Sizing accuracy and reproducibility of the High Sensitivity Protein 250 assay. The 10 kDa (circles) and the BenchMark ladder (triangle) were analyzed in water (filled) or in PBS (empty). Deviation from target (A) refers to the manufacturers' size statement. The % CV calculation (B) is from n = 4 measurements. Assay specifications are based on BSA and represented by dashed lines.

10 % CV for BSA as test sample. In general, the sizing accuracy of the on-chip electrophoresis or SDS-PAGE depends on the individual protein characteristics and may therefore vary for particular proteins, for example, glycosylated proteins[5]. Some proteins may not migrate according to their molecular weight, depending on charge or structure, for example. Nevertheless, for nearly all of the 27 protein peaks analyzed the analytical specifications based on BSA apply as well.

Labeling specific	
Sample type	Lysates, extracts, column fractions, purified proteins, lyophilized proteins
Concentration	1 ng/µL to 3 µg/µL total protein
Volume	5 µL per labeling reaction
pH	Value adjustment to pH 8.0 - 9.0
Optimal matrix	Standard labeling buffer, supplied with kit
Interferences	All components with primary amino or thiol groups
Separation and detection specific	
Sizing range	10-250 kDa
Typical sizing resolution	10 %, based on ladder
Typical sizing accuracy	10 % CV (BSA)
Sizing reproducibility	3 % CV (BSA)
Quantitative range	up to 4 orders of magnitude (0.3 to 3000 ng/µL BSA)
Sensitivity	1 pg/µL labeled BSA in water on chip with labeling reaction performed at 1 ng/µL total Protein; 5 pg/µL for labeled BSA in PBS
Quantification reproducibility	20% CV BSA, for sample concentration greater 1 ng/µL labeled protein

Table 3
Prerequisites for the labeling procedure and specifications for the analysis of labeled proteins with the Agilent High Sensitivity Protein 250 assay

Conclusion

The Agilent High Sensitivity Protein 250 assay complements the existing Agilent 2100 bioanalyzer protein assays. It is suitable for detection, sizing and quantification of a broad range of samples from protein expression, purification, or quality control applications. The existing Agilent Protein 80 and 230 assays are quick and easy-to-use tools for analysis of small and medium sized to large proteins with a sensitivity equivalent to Coomassie staining. The Agilent High Sensitivity Protein 250 assay deploys an extra labeling step prior to on-chip analysis and facilitates the analysis of minute protein amounts with sensitivity equivalent or better than silver staining. Further, it offers outstanding quantification performance with a linear dynamic range spanning four orders of magnitude enabling the analysis of low level impurities, for example, for QA/QC applications. Automation of separation and data analysis makes the Agilent 2100 bioanalyzer versatile and easy to use. In addition to protein analysis, the Agilent 2100 bioanalyzer can be used in combination with a variety of other kits for the analysis of cells, DNA and RNA samples.

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