The Protein 200 HT-2 Assay – How it works and how it performs

Technical Note

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Abstract

The Protein 200 HT-2 assay, run on the Agilent 5100 Automated Lab-on-a-chip platform, will size and quantitate proteins ranging in size from 15 to 220 kDa. Analysis is performed under denaturing conditions, in the absence or presence of a reducing agent. Protein samples are analyzed in an automated high throughput mode. This Technical Note describes in detail the technical background, the workflow and the performance of the Protein 200 HT-2 assay. It also describes the protein specific data analysis performed by the 5100 expert HT software.

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Technology introduction

The recently introduced Agilent 5100 Automated Lab-on-a-Chip Platform (5100 ALP) is based on the same principles as the Agilent 2100 bioanalyzer and enables unattended high throughput, sizing and quantitation of DNA and protein samples[1]. Sample handling, electrophoretic measurement and digital data analysis are completely automated, allowing to process more than a thousand samples per day. The system offers unattended and highly reproducible analysis of protein samples in standard 96-well plates.

The depot of the instrument offers storage space for up to 12 sample plates, 2 reagent plates for chip replenishment, as well as room for the chip. The 5100 ALP fills the micro channels of the microfluidic glass chip with a sieving matrix containing a fluorescent dye. The wells on the chip serve as buffer reservoirs and are automatically filled by the robot with reagents needed for the analysis. Once the chip is primed it is moved to the chip environment. The chip environment supplies the needed connections to high voltage, pressure and vacuum. To ensure reproducible results the chip is stored on a carrier, which regulates the temperature during the measurement to maintain a constant viscosity of the gel. In contrast to the Agilent 2100 bioanalyzer chips the Protein 200 HT-2 chip used for the protein assay is equipped with two capillaries allowing for sample loading from well plates as well as for processing and detection of two samples in parallel. The chip is reusable and can analyze up to 2000 samples. By applying vacuum to the chip samples are taken up through the capillaries from the sample plate onto the chip. The samples are electrokinetically injected into a separation channel and are separated through a sieving polymer in the channel. Proteins are then analyzed by laser induced fluorescence after a preceding integrated sample-destaining step (figure 1). Since the reagents are consumed by the electrophoresis, they have to be exchanged if multiple sample well plates are processed in one sequence. This is done by an internal replenishment system that removes used reagents, rinses the chip and aliquots new reagents for further use in the chip channels and wells automatically. An integrated robotic well-plate handler does all movements of sample plates, chip and resources. The 5100 ALP stores the result of the measurement in digital format in an Oracle database for convenient data mining of thousands of samples.

Material and methods

The chip-based separations were performed on the 5100 ALP (Agilent part number G3002A: dual laser system) in combination with

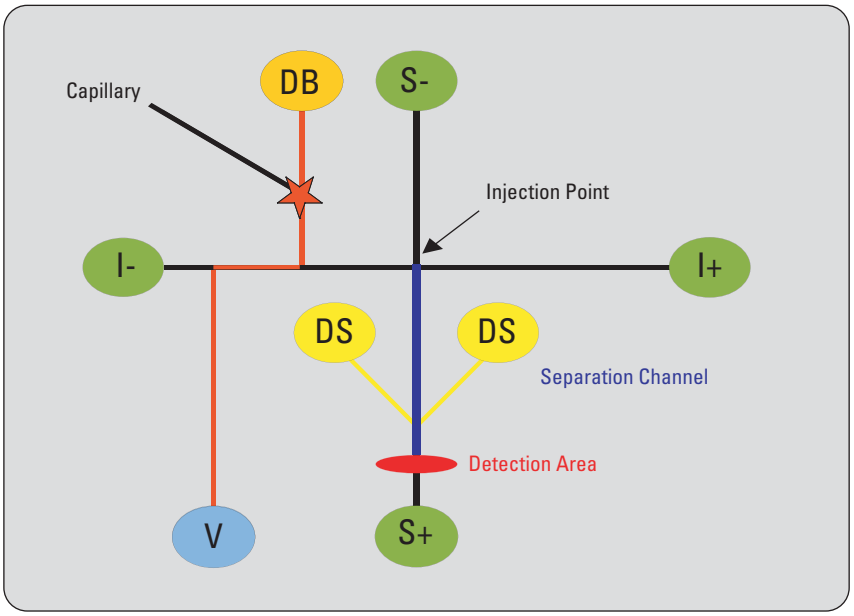

Figure 1
Schematic overview of the principle of analysis. By applying vacuum to well V, samples are drawn through the chip capillaries from the well plate onto the chip where they get mixed with dilution buffer from DB. Then the sample/buffer mix is loaded electro-kinetically across the separation channel (I- to I+), before a sample plug gets injected into and separated inside the separation channel (S- to S+). The Protein 200 HT-2 assay includes sample-destaining on the chip (DS to S+) prior to detection to reduce SDS-induced background fluorescence.

the Protein 200 HT-2 chip (Agilent part number G3011-87300) and the Protein 200 HT-2 reagent kit (Agilent part number G3011-68750). This chip has two external capillaries that enable it to withdraw samples from a sealed titer plate. The reagent kit includes sample buffer, protein standard ladder, storage buffer, and ready-to-use reagent plates. Samples were prepared according to the protocol provided with the kit: 4 µL of each sample were combined with 2 µL of denaturing solution in an Agilent 96-well-plate (Agilent part number 5042-8502). The denaturing solution consists of sample buffer (containing sodium dodecylsulfate for denaturation of the proteins and lower and upper marker as internal standards), and β-mercaptoethanol or dithiothreitol (DTT) as a reducing agent for the chip analysis. For non-reducing conditions the reducing agent was substituted by deionized water. Sample plates were sealed with a Remp plate sealer and heated for 5 minutes to 95 °C using a PCR machine with a heatable lid. The plates were then cooled to room temperature, the foil seal was removed, and 24 microliters of deionized water was added to each well and mixed thoroughly by pipetting. The sample plates were then resealed with foil and inserted together with the Protein 200 HT-2 chip and a reagent plate into the depot of the 5100 ALP. After starting the run the instrument performs analysis of the samples automatically and unattended. For statistical analysis of the assay performance the sample preparation step was scaled up by a factor of 5 to 10 in order to minimize pipetting errors. For statistical data analysis the results generated by the 5100 expert HT software were exported to Spotfire DecisionSite (Spotfire, Inc.).

Principle of data analysis

In contrast to a conventional SDS-PAGE experiment, where proteins are electrophoretically separated in parallel and the separation is stopped at one time point, the Protein 200 HT-2 assay separates proteins sequentially and detection takes place at a fixed migration distance. Similar to SDS-PAGE a protein standard ladder is used to determine the molecular weight of the unknown proteins in the sample. The Protein 200 HT-2 ladder of the Protein 200 HT-2 assay is shown in figure 2A. It consists of 6 protein peaks with known sizes at 15, 28, 46, 63, 95 and 150 kDa and an upper marker protein at 240 kDa. In addition, 2-3 system peaks are visible before the 15 kDa peak, which represent some dodecylsulfate micelles that also incorporate the intercalating dye. The ladder also contains a lower marker (not shown in figure 2A), detected at a different emission wavelength than the proteins and is used for the alignment (see details in chapter “Sample alignment”). The software generates a standard curve by plotting the known sizes of the ladder proteins against their migration time (figure 2B). Molecular weights of unknown proteins are then calculated using the ladder standard curve. Each sample contains a lower and upper marker as internal standards, which are included in the denaturation solution and added to the sample during sample preparation. These internal standards are used to align the sample to the ladder for correct sizing. In addition, the upper marker has a defined concentration and is used for the determination of a relative quantitation of each protein peak within the sample. Figure 2C shows an example of a sample electropherogram. Fluorescence intensities measured using laser-induced fluorescence detection are plotted against the molecular weight, which have been converted from migration times with the ladder standard curve. The lower marker signal is shown in thegreen trace and the sample signal including the upper marker is shown in the black trace. The user can configure whether the software displays the lower marker trace or not. Each electropherogram can also be displayed as a gel-like image, thus gel-like images with several lanes can be generated to conveniently compare multiple samples side by side similar to a SDS-PAGE analysis. For each protein peak results like size [kDa], concentration [µg/mL], purity [%] are reported in a data table. In addition, the total concentration for each sample is calculated.

Sizing

The Protein 200 HT-2 assay allows for sizing of proteins ranging from 15 to 220 kDa. The lower end of the sizing range is limited by the appearance of the system peaks, whereas the upper limit is determined by the upper marker being at 240 kDa. Since all samples are

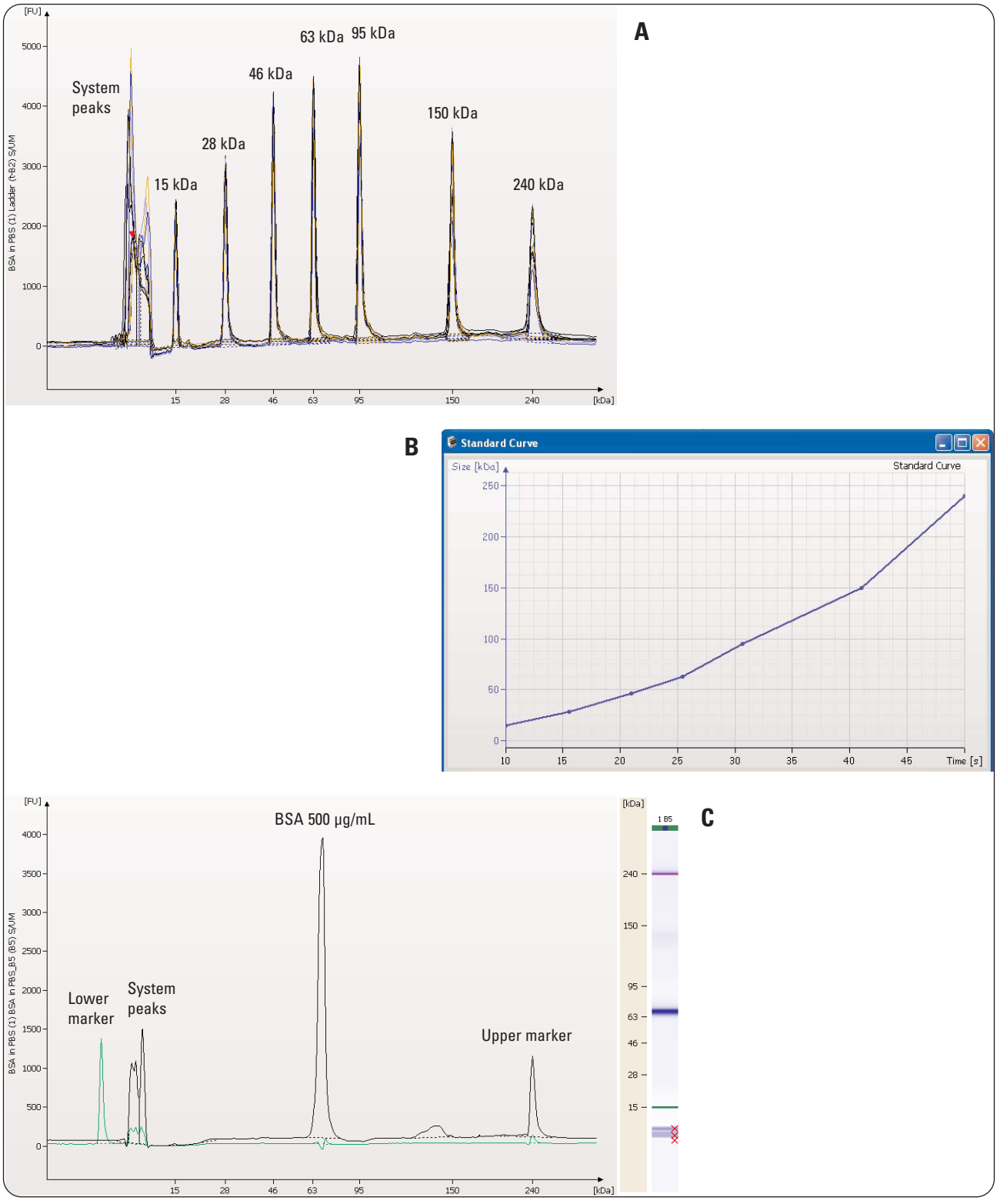

Figure 2

A) Electropherogram overlay of 18 analysis of the Protein 200 HT-2 ladder. B) Standard curve: plot of size [kDa] versus migration time [s] of the ladder protein peaks. C) Electropherogram and gel-like image of a BSA sample in PBS.

aligned to the ladder using the internal standards, changes of the migration behavior (e.g. due to ion depletion during electrophoresis) can be corrected. This enables a very reproducible size determination. Table 1 shows the results of the sizing analysis of a protein mixture including 7 different proteins. Results are based on 1200 individual analyses of the protein mixture using different Protein 200 HT-2 chips and different 5100 ALP instruments. The sizing reproducibility is excellent and between 0.4 – 2.6 % and therefore well below the assay specification of 3 % CV sizing reproducibility. Sizing accuracy can (normally the ionic strength and charge of the SDS neutralizes the proteins inherent properties) depend on the protein characteristics such as amino acid sequence, pI value, structure and the presence on certain side chains or prosthetic groups and may therefore vary for particular proteins. This however is also true for other methods such as SDS-PAGE or size exclusion chromatography[2].

Protein	Theoretical size [kDa]	Average size [kDa]	StDev of size	CV [%]
A	15	15.1	0.4	2.6
B	28	28.1	0.7	2.5
C	46	46.3	0.62	1.3
D	63	63.7	0.69	1.1
E	95	95.8	0.58	0.6
F	150	151.6	0. 91	0.6
G	192	197.3	0.73	0.4

Table 1
Sizing analysis of a protein mixture including 7 different proteins using the Protein 200 HT-2 assay (n=1200).

Protein	Theoretical size [kDa]	Average rel. conc. [µg/mL]	StDev of rel. conc.	CV [%]
A	15	34.5	6.8	19.7
B	28	38.3	7.5	19.6
C	46	50.0	9.2	18.4
D	63	43.5	6.8	15.6
E	95	59.9	9.6	16.0
F	150	49.7	7.2	14.5
G	192	40.0	3.6	9.0

Table 2
Quantitation analysis of a protein mixture including 7 different proteins using the Protein 200 HT-2 assay (n = 1200).

Quantitation

In addition to the size determination the Protein 200 HT-2 assay provides also quantitation information for each protein within a sample. The concentration calculation is based on a comparison to the upper marker concentration as internal standard in each sample. Since the ratio of sample to denaturing solution is fixed in the sample preparation protocol and the concentration of the upper marker is known, the concentration of each sample peak can be calculated using the rule of proportion with the sample and upper marker areas. The areas are corrected by dividing them by migration time to adjust for the fact that larger proteins pass the detector more slowly than smaller proteins, a common procedure also used in capillary electrophoresis. The big advantage of using the upper marker as an internal standard is, that differences in sample injection to the separation channel of the chip can be corrected. Since the injection is performed electrokinetically the amount of sample injected is highly dependent on the ionic strength of the buffer the sample is in, also a known effect from capillary electrophoresis. Using the upper marker therefore increases the robustness of the Protein 200 HT-2 assay with respect to sample buffer variability. Since the concentration determination also depends on the staining efficiency of each individual protein the determined concentration is a relative concentration - relative to the upper marker. Table 2 shows the results of the quantitation analysis of the same protein mixture as shown in table 1. The results are based on 1200 individual analysis of the protein mixture using different Protein 200 HT-2 chips and different 5100 ALP instruments. The quantitation reproducibility is well below the assay specification of 20 % quantitation reproducibility, the best result was achieved for Protein G with 9 % quantitation reproducibility. Quantitation accuracy however strongly depends on the protein quantitation method. In most cases a dye is used to stain the proteins and the staining efficiency varies for different proteins which are stained with the same dye or for the same proteins stained with different dyes. Therefore it is difficult to measure the absolute correct concentration of a protein. Similar limitations exist for other methods, a UV measurement at 280 nm, which is a dye independent protein

quantitation method, is biased by certain amino acid sequences. In addition proteins need to be very pure for determining their concentration. Because of these difficulties the Protein 200 HT-2 assay has no specification on quantitation accuracy, but the possibility to analyze calibration standards.

Resolution

The Protein 200 HT-2 assay can separate proteins with a 10 % difference in molecular weight. The gel-matrix used for the assay is non-crosslinked and has dynamic pores with variable size. The composition, length and concentration of the polymer have been adjusted to obtain optimal resolution within the 15-220 kDa size range. Figure 3A shows a plot of % molecular weight resolution behavior of the gel-matrix versus size. The data has been generated using a standard protein mix with the assumption of a resolution value R=1, which corresponds to a separation until one third of the peak height. The plot clearly shows that the assay is well within the specification of 10 % molecular weight resolution. The resolution depends also on the protein characteristics within samples and can be limited by the fact that proteins migrate differently than expected or possess heterogeneity that result in larger peak width. Figure 3B shows an example of two proteins: Carbonic Anhydrase (CA) 29 kDA and Lactate Dehydrogenase (LDH) 32.5 kDa. The peaks are baseline separated.

Linear dynamic range and sensitivity

To determine sensitivity and linear dynamic range several proteins have been analyzed in various dilutions. Figure 4A shows an example of protein phosphorylase B at concentrations of 3.5, 7 and 35 µg/mL. The concentration values were taken from the vendor's specification of that purified protein. As can be seen, the signal-to-noise ratio is still greater than 3 for the lowest concentration tested. The minimal detectable concentration of a specific protein depends on its staining behavior as well as on adhesion effects to the plastic wall of the sample plate, which show significant contribution at low concentrations. The minimal detectable concentration for Bovine Serum Albumin (BSA) in PBS with a signal to noise of 3 was found at 10 µg/mL (40 ng in 4 µL sample). Vendors state a sensitivity of 50 ng BSA and 10 ng BSA for standard Coomassie stain (R-250) and col-

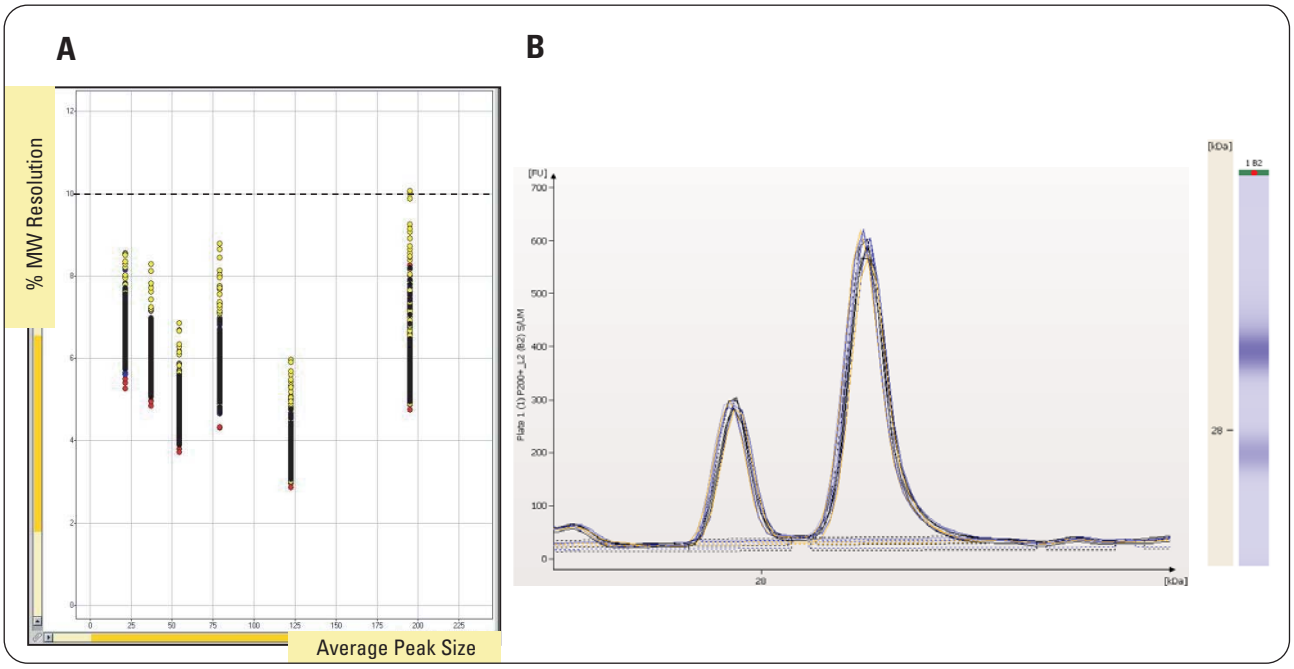

Figure 3
A) Plot of % molecular weight resolution behavior of the gel-matrix versus average peak size. B) Example of two proteins, which are baseline resolved: Carbonic Anhydrase (CA), 29 kDa and Lactate Dehydrogenase (LDH), 32.5 kDa.

loidal Coomassie stain (G-250), respectively. Therefore, looking at this example the sensitivity is comparable to standard Coomassie stain. However, due to the principle of electrokinetical injection into the separation channel the sensitivity of the Protein 200 HT-2 assay can be influenced by the ionic strength of the buffer of the sample. If the ionic strength of the buffer is lower than PBS the sensitivity can be enhanced, in contrast high salt buffers can cause a slight loss in sensitivity. The Protein 200 HT-2 assay has been tested with a 10µg/mL BSA sample in 1M NaCl and the signal to noise ratio was still 3. In addition to buffer effects, the sensitivity of protein detection is also affected by the staining efficiency or the width of a protein peak. The Protein 200 HT-2 assay is linear over 2 orders of magnitude, for example from 10 µg/mL to 2000 µg/mL BSA in PBS. This allows, for example, a detection of 0.5 % impurity in column fractions if the parent peak has a concentration of 2 mg/mL. Figure 4B shows a plot of quantitation results using BSA standards (Pre-Diluted Protein Assay Standards: BSA Set, Pierce, #23208). Protein concentrations determined with the Protein 200 HT-2 assay are plotted against the target concentrations specified by the vendor. Each data point reflects the average of 860 sample analyses. A linear regression resulted in correlation coefficient of $R^2 = 0.9737$.

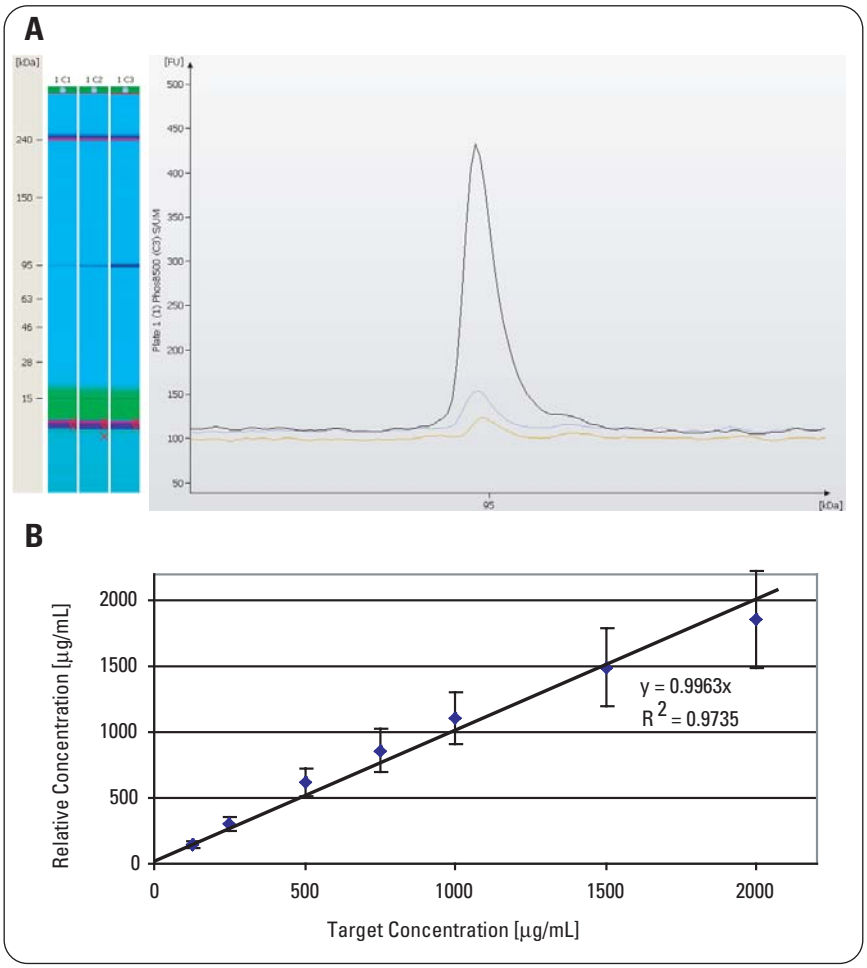

Figure 4
Sensitivity and linear dynamic range of the Protein 200 HT-2 assay A) Gel-like image and electropherogram showing the analysis of phosphorylase B sample at 3.5, 7, and 35 µg/mL. B). Plot of protein concentrations determined with the Protein 200 HT-2 assay against target concentrations specified by the vendor (n = 860).

Carry-over

Since the Protein 200 HT-2 assay uses a reusable chip, several hundreds of samples are analyzed through the same separation channel. Therefore, the question whether there is carry-over is a valid one. The detection of carry-over is of course also associated to assay sensitivity. The lower the sensitivity the lower is the probability to detect carry-over even if it is there. In order to estimate the probability of carry-over several analysis of a sample containing 4000 µg/mL BSA in PBS was performed followed by a PBS blank analysis. The PBS analysis was then inspected for peaks in the sizing range of BSA. Only in 5 cases out of a total number of 500 samples tested carry-over was detected. In those cases where carry-over was detected the average amount of BSA found was only 1.1 µg/mL, which is 0.03 % of the amount of BSA analyzed prior to the blank. These results support that carry-over is almost not detectable. The assay is therefore specified to have a carry-over less than 0.1 % when working with a maximal loadable protein amount of 4000 µg/mL per protein.

A carry-over of small molecules for example reducing agents such as β-mercaptoethanol or DTT has not been detected. Therefore, it is possible to analyze protein samples under reduced or non-reduced conditions in the same analysis sequence. Figure 5 shows an example of an antibody analysis under non-reduced conditions (figure 5A) and under reduced conditions (figure 5B). Both electropherograms show an overlay of 6 individual analyses with excellent reproducibility. The size of the intact IgG was determined to be 154.5 ± 1.5 kDa, the size of the light chain (LC) was 26.6 ± 0.1 kDa and the size of the heavy chain was 57.4 ± 0.2 kDa (n=36).

Sample matrix effects

The Protein 200 HT-2 assay is compatible with a large number of different buffers and buffer components. As already discussed, high ionic strength buffers can influence sensitivity of the assay, however 1 M NaCl was tested without loss of assay performance. Components like acetonitrile, guanidine or methanol, which are known to precipitate SDS can decrease the upper marker area and therefore affect quantitation reproducibility at higher concentrations. Some detergents show an increased signal for the system peaks since they also form micelles and intercalate with the fluorescent dye of the gel-matrix. In addition, detergents potentially alter the shape of the protein-SDS micelles, which could result in a slight sizing offset compared to a buffer without detergents. A detailed list of compatible buffers can be found in the Protein 200 HT-2 reagent kit. In addition, samples containing particles or cell debris have to be handled with care, since these can clog the capillaries of the chip or the micro channels. However, filtering of samples or a centrifugation step prior to the analysis can avoid possible problems effectively.

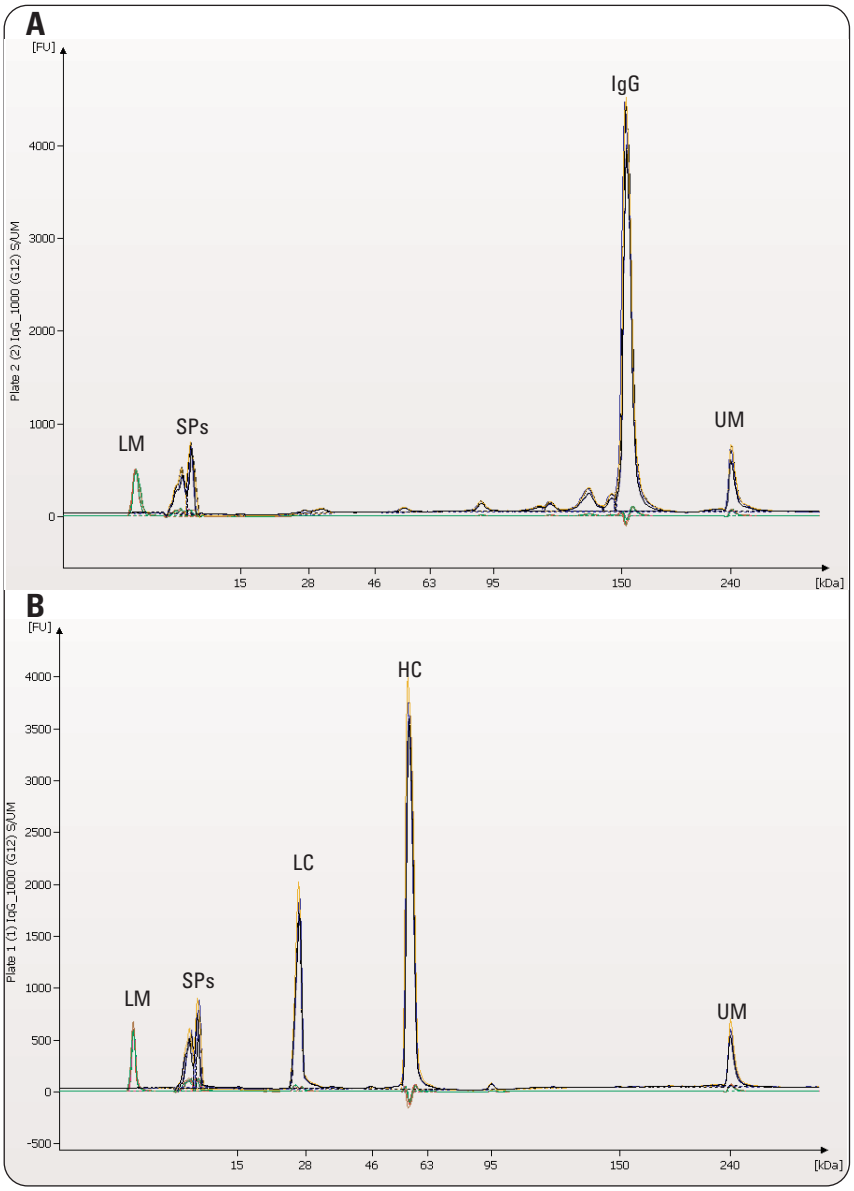

Figure 5
A) Analysis of an intact antibody under non-reduced condition, electropherogram overlay of 6 analyses. B) Analysis of light chain (LC) and heavy chain (HC) of an antibody under reduced condition, electropherogram overlay of 6 analyses. Lower marker (LM), upper marker (UM), system peaks (SPs).

Staining efficiency

Staining efficiency depends on the characteristics of a specific protein and its interaction with the dye. The Protein 200 HT-2 assay is based on laser induced fluorescence detection of an intercalating fluorescent dye, which binds to protein-SDS-micelles. Similar to other protein detection methods such as Bradford, Lowry or SDS-PAGE the Protein 200 HT-2 assay is affected by staining efficiency as well. These variations can result in over- or underestimation of protein concentrations since the protein of interest might have a different staining efficiency than the upper marker used for relative quantitation. In order to correct for concentrations biased by staining efficiency and to attain a greater level of quantitation accuracy the 5100 expert HT software provides the possibility to generate a calibration curve with the protein of interest at different known concentrations. The calibration needs to be done for each separation channel by pipetting dilution series of the target protein into each sample plate. The software then calculates one calibration curve per plate and separation channel and uses the slope of the curve to convert the relative concentration of the unknown samples to a calibrated concentration. Figure 6 shows an example of a BSA dilution series used for calibration. The red dots correlate the known concentration (calibration or "absolute" concentration) value that was provided by the user to the relative concentration, which has been calculated using the upper marker. The blue line is the calibration curve, which is a least square fit going through the origin for this data points and is used for calculating the calibrated concentration for the samples. Table 3 compares quantitation results determined with a one-point calibration fit using the upper marker (relative concentration) with the calibrated concentration calculated based on a calibration curve. In this example the calibration curve was generated with three different BSA concentrations, and "unknown" concentrations at 125, 500, 1000 µg/mL were evaluated. The target concentration value was taken from the supplier (Pierce). The results clearly demonstrate that quantitation accuracy was greatly improved with the absolute quantitation feature compared to relative concentration. The quantitation error was reduced for the 125 µg/mL sample from 14.0 % to 3.6 %, for the 500 µg/mL sample from 30.2 % to 17.2 % and for the 1000 µg/mL sample from 24.2 % to 12.8 %. The quantitation reproducibility was approximately 13 % and comparable between both quantitation methods.

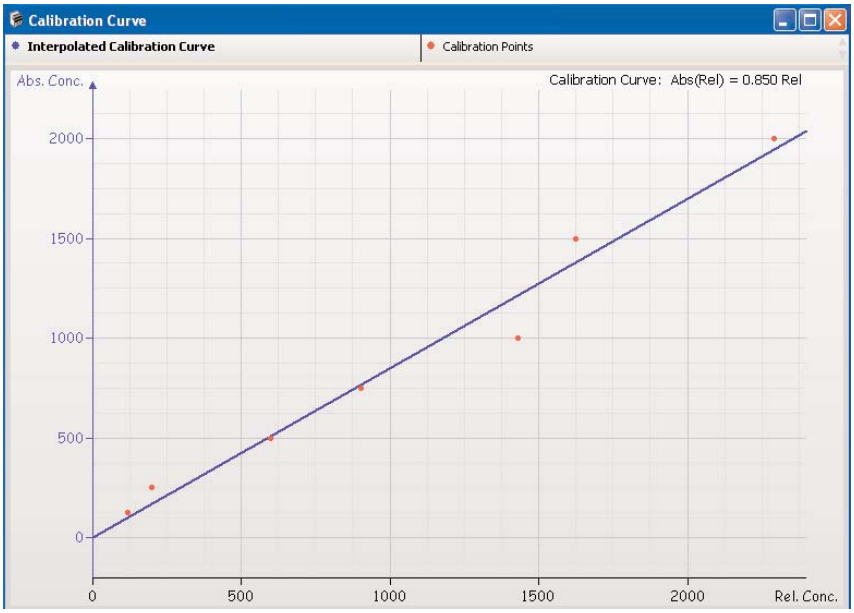

Figure 6
Calibration curve of a BSA dilution series.

Target concentration [µg/mL]	Relative concentration [µg/mL]	CV [%]	Error [%]	Calibrated concentration [µg/mL]	CV [%]	Error [%]
125	142.6	7.4	14.0	129.5	14.6	3.6
500	651.1	12.4	30.2	588.2	14.5	17.7
1000	1242.4	14.7	24.2	1122.8	16.6	12.8

Table 3
Comparison of relative and absolute concentration of BSA at 3 different "unknown" concentrations (n = 100).

Sample alignment

The Protein 200 HT-2 assay is capable of analyzing 2000 samples per Protein 200 HT-2 chip. Due to slight changes in electrophoretic boundary conditions over time, migration times of identical proteins differ slightly from analysis to analysis. This effect is similar to a smiling effect on SDS-PAGE gels, where the outer lanes of the gel face slightly different electrophoretic conditions than the inner lanes. To correct for this, the software performs an alignment of the samples using the lower and upper marker from the samples and the ladder. Therefore, the Protein 200 HT-2 ladder is measured before and after the measurement of a series of 12 sample measurements, a process called ladder bracketing. Figure 7 explains the ladder bracketing mechanism in detail. Figure 7A shows the results when the analysis in the software is turned off. It demonstrates that the upper marker migration behavior is slightly shifting in both ladders and sample over time. In addition, identical proteins in the sample show the same effect. Figure 7B shows how the software corrects for this effect when the data analysis is switched on. Both ladder measurements from well t-A1 are shifted to standardized values. Then all sample measurements between these two ladders are aligned to the ladder measurements using the marker peaks of the sample and ladder measurements. As a result, all identical proteins now also have the same corrected migration time and thus the same calculated size.

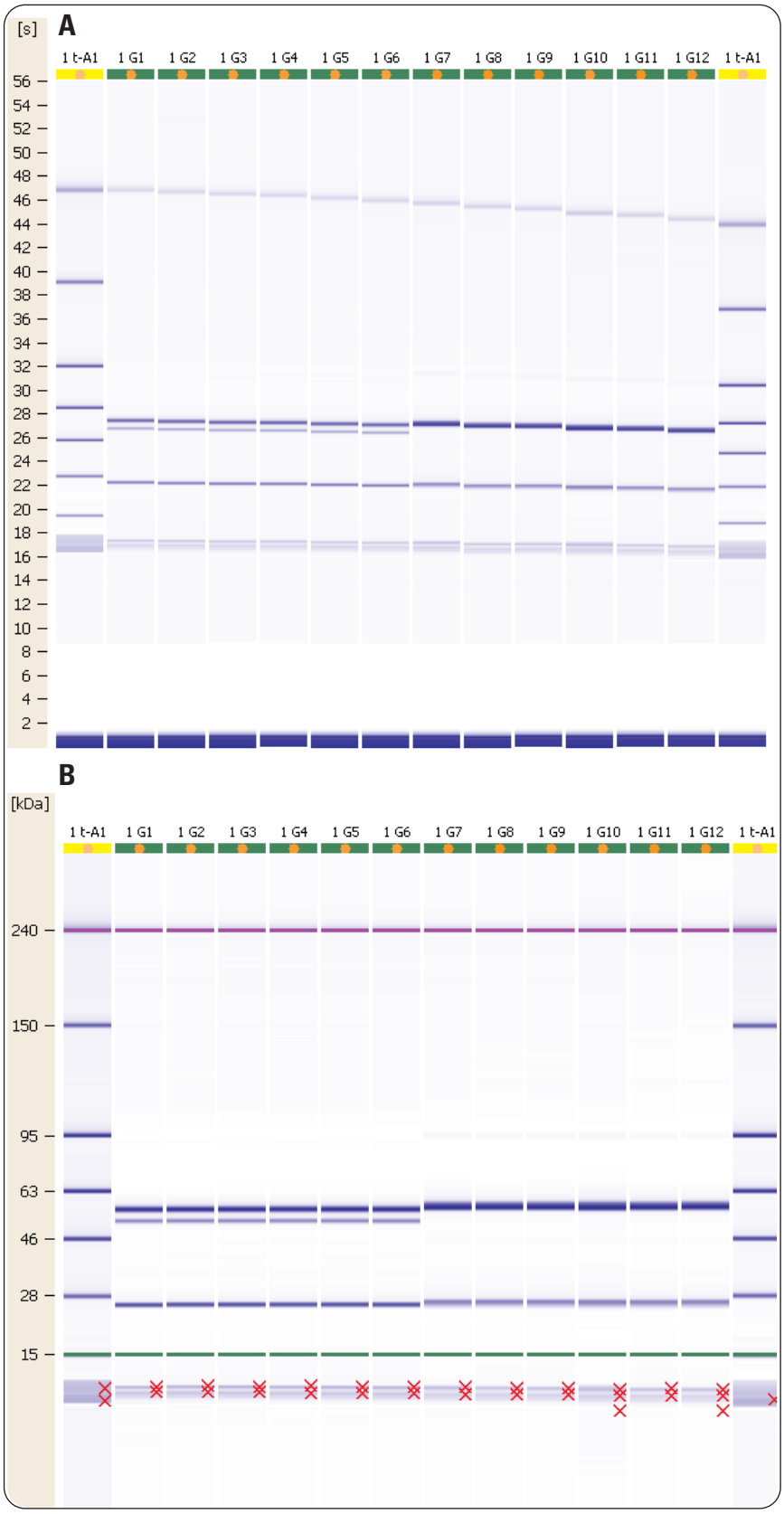

Figure 7
Alignment is part of the data analysis. A) Analysis off: no alignment between samples and bracketing ladders. B) Analysis on: samples are aligned to the bracketing ladders.

Conclusion

The Protein 200 HT-2 assay in combination with the 5100 ALP instruments provides a new tool for sizing and quantitation of proteins in a high throughput mode. It allows researchers to streamline their processes for parallel protein expression and purification studies, for example, to address the higher demand for pure and correctly folded target proteins in the areas of target validation and high throughput screening[3, 4]. The use of internal and external markers allows for the analysis of hundreds of samples with excellent reproducibility and reliability. A summary of the discussed assay specification can be found in table 4. In addition to protein analysis the 5100 ALP instrument can also be used for the analysis of DNA samples, which makes the instrument versatile for other applications[5]. More assays will be developed on this platform in the future.

	Specification
Sizing range	15 - 220 kDa
Sizing reproducibility	3 % CV
Sizing resolution	± 10 % difference in MW
Min. detectable conc. (S/N = 3)	10 µg/mL (BSA in PBS)
Max. loadable amount of protein	4000 µg/mL
Linear dynamic range	10 - 2000 µg/mL
Quantitation reproducibility	20 % CV
Carry-over	<0.1 %
Sample Matrix Compatibility	common protein buffers (see kit guide)
Throughput	80 - 150 seconds per samples (depending on job setup)
Well plate format	96 (max. 12 plates per job)
Chip Lifetime	2000 samples
Minimum sample volume	4 µL
Reagent and chip stability	minimum 3 months in customer's hands

Table 4
Summary of Protein 200 HT-2 Assay specifications.

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