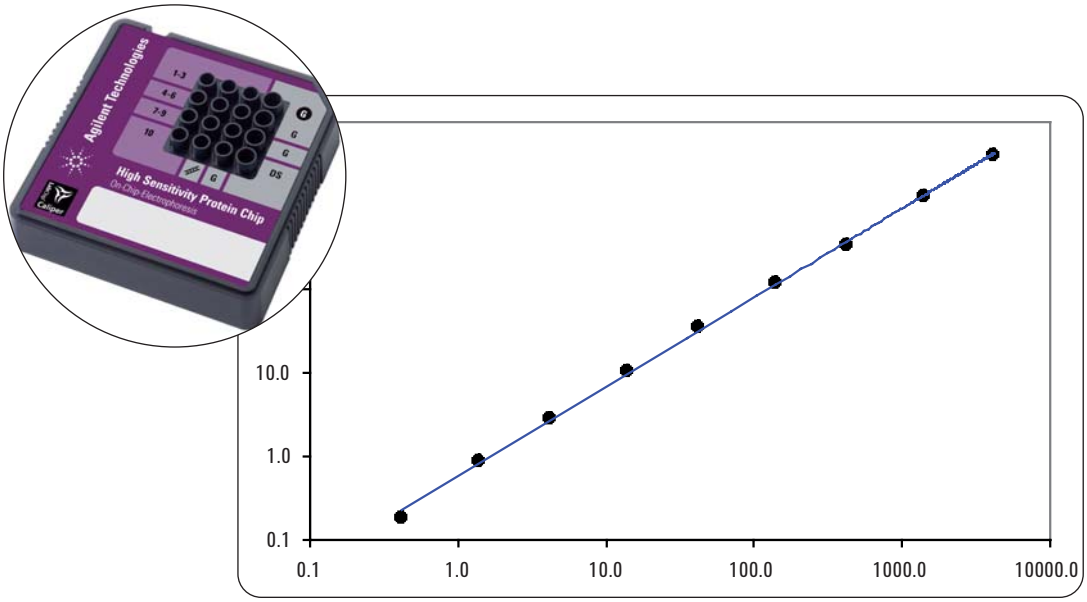
Quantification Strategies Using the High Sensitivity Protein 250 Assay for the Agilent 2100 Bioanalyzer

Technical Note



Abstract

The Agilent High Sensitivity Protein 250 assay for the Agilent 2100 bioanalyzer analyzes proteins from 10 to 250 kDa. This assay is based on the detection of fluorescently labeled proteins that are separated electrophoretically on microfluidic chips. It provides sizing and quantification of proteins over a large concentration range, including concentrations as low as 1 pg/µL labeled BSA on-chip. The quantification and sizing of sample peaks is done relative to a ladder as an external standard on the same chip. This Technical Note describes options for the utilization of the assay:

- How to do an absolute quantification
- How to do quantification with a user-defined internal standard
- How to customize the quantification, for example, in order to correct for sample matrix effects

Agilent Equipment

- 2100 Bioanalyzer
- High Sensitivity Protein 250 Kit

Application Area

- Protein Electrophoresis

Agilent Technologies

Introduction

The Agilent High Sensitivity Protein 250 assay for the Agilent 2100 bioanalyzer offers a sensitivity equivalent or better than silver staining and a linear dynamic range spanning up to four orders of magnitude[1]. Quantification is done relative to a ladder as an external standard in a different well on the same chip. This is in contrast to the Agilent Protein 80 and Protein 230 assays for the Agilent 2100 bioanalyzer that deploy an internal upper marker for relative quantification. The upper marker is added to every sample through the sample buffer and also serves for alignment. The purpose of a quantification strategy that includes an upper marker is to correct for varying injection efficiencies for individual samples. For the Agilent High Sensitivity Protein 250 assay samples applied to the chip are in a well-defined matrix (standard labeling buffer and water from dilution steps) and an injection control is not mandatory. Further, the elimination of an upper marker is beneficial because

- overlap between marker and sample peaks,
- detection of artificial impurities related to the marker, and
- quantification offsets relating to incompatibilities between sample matrix and the marker protein (for example, precipitation of the upper marker) are avoided.

The first part of this publication describes an example of an absolute quantification performed with the Agilent High Sensitivity Protein 250 assay. The labeling or staining efficiencies can generally vary from protein to protein. To compensate for this bias the Agilent 2100 expert software provides for absolute protein quantification using a standard curve. This is generated with the protein of interest and applied to individual sample results. The second part describes how quantification relative to a user-defined internal standard analogous to the Agilent Protein 80 and Protein 230 assays is supported by the Agilent High Sensitivity Protein 250 assay. The final part of this publication illustrates a straightforward procedure of how to check and correct for sample matrix effects using the ladder concentration setpoint of the Agilent 2100 expert software. Using the same setpoint, the assay can be customized to account for different dilution factors for ladder and samples or, if the reported relative concentration should reflect, for example, the initial sample concentration prior to the labeling step.

Experimental

Materials and equipment

Bovine Serum Albumin (BSA) Standard and Zeba™ Desalt Spin Columns were from Pierce, Rockford, IL, USA, beta Lactoglobulin A (ßLG) from Sigma, Taufkirchen, Germany, Protein LoBind tubes from Eppendorf GmbH, Hamburg, Germany, Dulbecco's Phosphate Buffered Saline (PBS) from Life Technologies GmbH, Karlsruhe, Germany, Agilent 2100 bioanalyzer and Agilent High Sensitivity Protein 250 kit, from Agilent Technologies, Germany.

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 and the dedicated assay of the Agilent 2100 expert software according to the kit guide[1]. Sample preparation prior to on-chip analysis was done in Protein LoBind tubes. Prior to labeling, ßLG was desalted using Zeba Desalt Spin Columns. Exported values were plotted with Microsoft Excel. Minor peaks were integrated manually using the respective Agilent 2100 expert software feature.

Results and discussion

Principle of relative quantification

Quantification of sample peaks is done in the Agilent High Sensitivity Protein 250 assay rela-

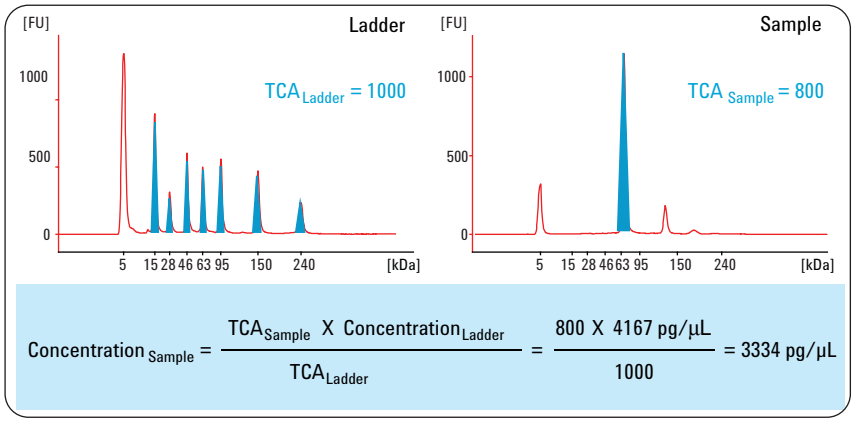


Figure 1
Quantification relative to the Ladder. The concentration of a sample peak returning a time corrected peak area (TCA) of 800 was calculated relative to the sum of all ladder peaks.

tive to the ladder (figure 1). Equal labeling and injection efficiencies for ladder and sample proteins are a prerequisite for correct peak quantification. Time-corrected areas are used for all calculations. The sum of the seven identified ladder peak areas is compared to every sample peak. The lower marker peak is used for alignment of raw data and does not result from protein but from a fluorescent dye, so it is not taken into consideration for quantitation. The initial concentration of the ladder is 1 mg/mL or 10^6 pg/µL. Following the standard protocol, after 5/6 dilution during labeling and 1:200 dilution with water, the final ladder concentration on-chip is 4167 pg/µL. By default, this on-chip concentration is used to calculate the concentration of each sample peak by the rule of three. Therefore, the relative concentration returned by the Agilent 2100 expert software refers to the concentration of the sample after labeling and dilution but before the addition of sample buffer. The relative concentration multiplied with 6/5 and 200 yields the concentration of the initial sample solution.

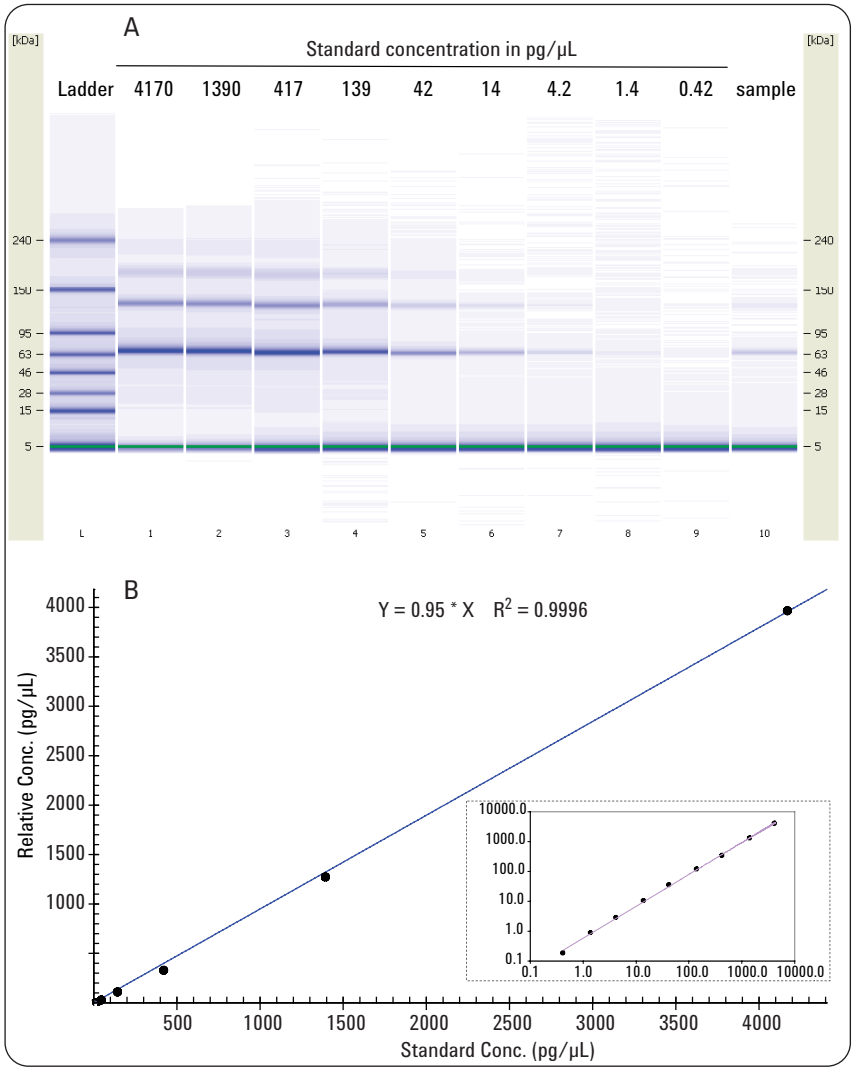


Figure 2
Absolute quantification with the High Sensitivity Protein 250 assay. BSA was labeled at a concentration of 1000 ng/µL. A series of calibration standards with concentrations from 4170 to 0.42 pg/µL were prepared by dilution with water and analyzed together with a BSA sample of "unknown" concentration on two chips. A) Gel-like image, B) Calibration curve. The inset in B) shows a double logarithmic plot of the calibration curve. Manual peak integration was done for the two lowest concentrations of the standard curve.

Absolute quantification

Absolute quantification can be done in the same way as with the Agilent Protein 80 and Protein 230 assays. The unknown samples and reference samples for the protein of interest are measured on the same chip. In the example in figure 2, a standard curve spanning 4 orders of magnitude was prepared with a BSA standard. The same samples were measured on two chips. In both cases the correlation coefficient for the linear regression (R^2) was better than 0.999. By default the Agilent 2100 expert software calculates linear calibration curves only (figure 2b). However, for better visualization of the calibration data a double logarithmic plot is shown (inset in figure 2b). The calibrated concentration of the "unknown" sample was found to be 10.0 ± 0.4 pg/µL and closer to the target of 8.8 pg/µL than the relative concentration of 6.9 ± 0.1 pg/µL (N=2; deviation from target was 14 % compared to 21 %). For the given sample the deviation from absolute to relative quantification was minor. However, absolute quantifi-

cation can be a valuable tool for proteins with deviating labeling performance.

Peaks from samples showing the same size as the calibration peak or manually assigned peaks are quantified using the calibration curve. By default only one peak per lane fitting in size is recognized as calibrated protein. In case all sample peaks must be evaluated with the calibration curve, select the function “calibrate all” under advanced global setpoints from the setpoint explorer (see also Kit Guide[1]).

Quantification relative to a user defined internal standard

An internal upper marker as it is used in the Agilent Protein 80 and Protein 230 assays is not included in the Agilent High Sensitivity Protein 250 assay. However, a suitable internal standard can be added. Neither the standard itself nor its impurities should interfere with the quantification of sample peaks or give rise to an increased background. A good choice would be a small standard protein such as ß-Lactoglobulin (ßLG, 18.4 kDa). In the example in figure 3, ßLG was spiked into a BSA solution prior to the labeling reaction and the analysis was performed according to the standard protocol.

Peak		Size [kDa]	Rel. Conc. [pg/µL]	% Total
bLG	AVERAGE	16.9	184	3.3
	%CV	1.4	2.3	1.5
BSA monomer	AVERAGE	68.7	4191	75.7
	%CV	0.8	3.1	0.3

Table 1
Quantification data for the experiment shown in figure 3. Sizing and quantification data for the ßLG and BSA monomer peaks with averages and %CV are shown (N=10).

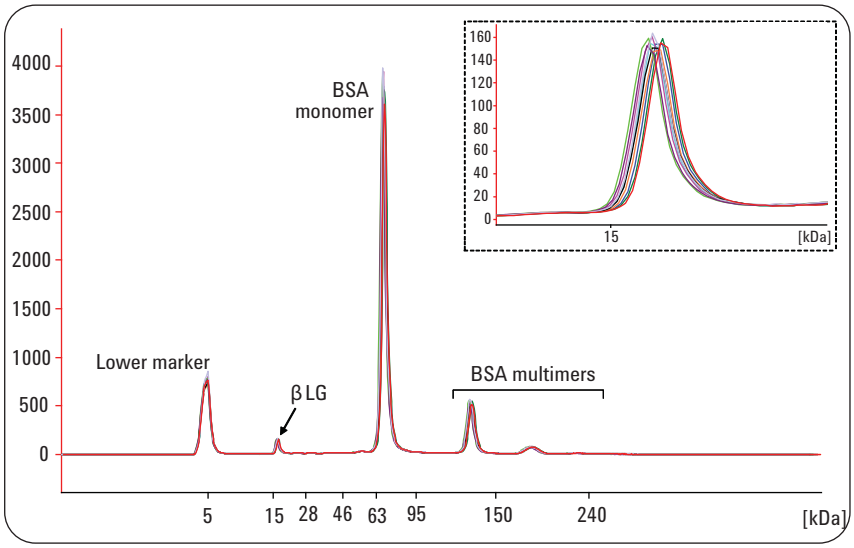


Figure 3
Using an internal standard with the High Sensitivity Protein 250 assay. BSA (900 ng/µL) and a small amount of ßLG were mixed, labeled and analyzed on-chip. The electropherogram shows an overlay of 10 runs and a zoom on the ßLG peak in the inset. See table 1 for quantification data.

As shown in figure 3 and table 1, the quantification reproducibility of the concentrations calculated relative to the ladder was well below the assay specification of 20 % CV. However, reproducibility of the “% Total” values was even better. This is due to minute variations in injection efficiency. If the concentrations are calculated relative to other peaks from the same well, as it is the case for an approach with internal standard, these well-to-well variations for sample injection are excluded from the quantification calculations. On the other hand, if concentrations are calculated relative to the ladder as in the standard modus for relative quantification, these well-to-well variations are reflected in the quantification results and thus cause a slightly elevated % CV. Therefore, evaluating data based on “% Total” results returned by the Agilent 2100 expert software are advantageous, for example, for the quantification of impurities besides major components.

Another useful option to increase the reproducibility of the assay is to increase the volumes for sample preparation steps. For example, doubling the volume used for the labeling reaction from 5 to 10 µL avoids pipetting volumes lower than 1 µL and should therefore decrease the pipetting error significantly. Reagents can be purchased separately from the entire kit for this purpose.

The utilization of the value "% Total" of a spiked internal standard allows quantification relative to this customized standard by manual calculation. The "% Total" result of the corresponding peak can be used together with the known concentration to calculate the concentration of any sample peak by the rule of three using formula 1. The "% Total" value can be read out from the results table within the Agilent 2100 expert software. The use of an internal standard as spike also rules out labeling inhibition from the sample matrix. Such a spike will be affected in the labeling reaction, by a wrong pH or remaining DTT, in the same way as the target proteins and potential inhibition becomes evident by comparison to a spike-only analysis.

$$\text{Concentration}_{\text{SamplePeak}} = \%\text{Total}_{\text{SamplePeak}} * \frac{\text{Concentration}_{\text{internalStandard}}}{\%\text{Total}_{\text{internalStandard}}}$$

Formula 1

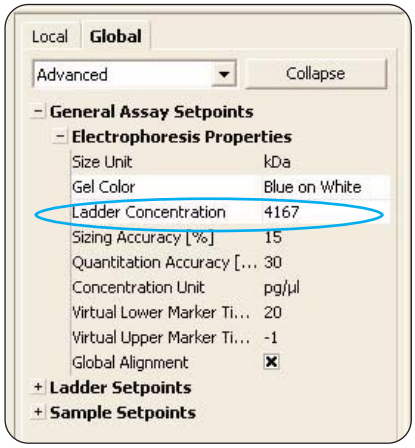


Figure 4
The ladder concentration setpoint in the 2100 expert software. This setpoint can be found under Global/Advanced/General Assay Setpoints/Electrophoresis Properties. The default value is 4167 pg/µL.

Customizing the quantification relative to the ladder

A significant contribution to the high sensitivity and reproducible quantification of the Agilent High Sensitivity Protein 250 assay is the implementation of on-chip stacking effects. Sample stacking is based on differences in electrical resistance between the sample and the gel matrix. It thus depends on the low conductivity of the matrix of the sample after labeling and dilution. The salt concentration of the initial sample only makes a small contribution to the conductivity of the finally applied sample. This is true when the user adheres to the standard protocol that employs a 200-fold dilution of the sample in water prior to on-chip analysis. For example, an initial salt concentration of 1 M NaCl will be reduced to only 4 mM for the on-chip concentration by the different dilution steps of the standard protocol. However, if variant dilution buffers or alternative workflows are used, the matrix effect due to elevated conductivity of the loaded sample can be substantial. If the same amount of labeled BSA is alternatively analyzed in PBS or water an approximately fivefold lower signal is obtained with PBS[2].

Prior to usage, every sample matrix for dilution different from water should be tested. A straightforward way to calibrate the system is to measure a standard protein in the sample matrix of choice and in water. Comparison of results should yield a factor that can be used to adjust the ladder concentration setpoint in the Agilent 2100 expert software (figure 4). For example, for samples diluted in PBS instead of water this factor is roughly five. It is possible to measure samples in PBS along with the ladder prepared in the standard way, i.e. in water, if the ladder concentration setpoint is increased fivefold. Then, the software will return relative concentrations that reflect the sample

concentrations as if prepared in the same way as the ladder (figure 5). An alternative to avoid quantification bias due to matrix effects is always to prepare the ladder and the samples in exactly the same matrix. A comprehensive list of tested sample matrices with known effects can be found in the Agilent High Sensitivity Protein 250 Kit Guide[1] (see section "List of Known Effects from Dilution Buffers"). Another useful option is to use the ladder concentration setpoint to obtain from the Agilent 2100 expert software protein concentrations that refer to other stages of the workflow such as the very initial sample. As mentioned above, the relative concentrations refer by default to the sample concentration after labeling and after dilution. It is the concentration before addition of sample buffer which implies the need to multiply with 6/5 and 200 to calculate the concentration reading for the initial sample solution. In order to get directly concentration readings that refer to the sample concentration prior to the labeling reaction, the value of the ladder concentration setpoint should be set to 10^6 pg/µL. A consequence of such customization is that, for example, printed results from the Agilent 2100 expert software directly report the required concentrations without the need for additional calculations.

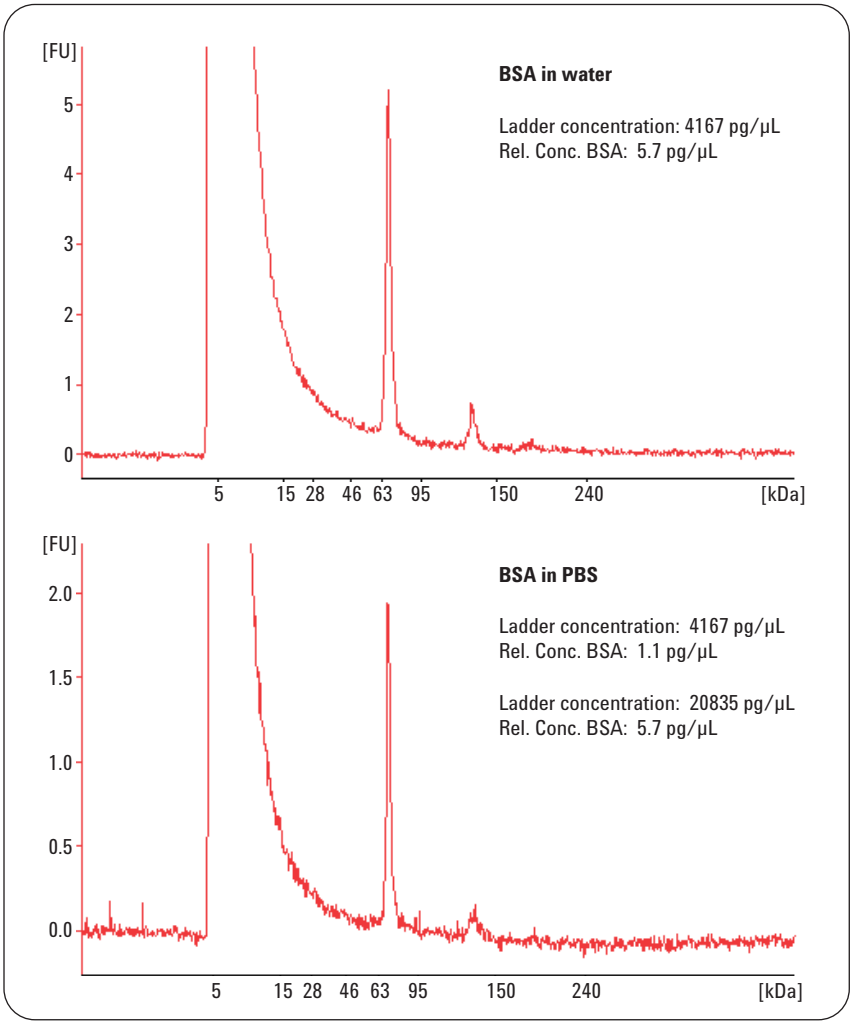


Figure 5
Correcting for sample matrix effects using the ladder concentration setpoint. An example is shown for labeled BSA that was diluted either in water or PBS. A fivefold lower relative concentration is measured for BSA in PBS compared to water. However, if the ladder concentration setpoint is increased fivefold, from the standard value of 4167 pg/µL to 20835 pg/µL, the Agilent 2100 expert software will return for samples measured in PBS relative concentrations that reflect the corresponding concentrations in water.

The ladder concentration setpoint can also be used to account for different dilution factors for ladder and samples. This makes sense especially in case when following alternative workflows. Alternative workflows may represent any preparative separation technique performed with the labeled sample proteins. Here the dilution step can be adapted or even omitted since the left over dye from the labeling reaction, which contributes to the lower marker peak height, is depleted. The dilution for alternative workflows must be estimated or determined experimentally. It should be sufficient to avoid saturation of the Agilent 2100 bioanalyzer's detector. If the samples are diluted, for example, only 1:50 (deviating from the standard protocol) and the ladder still 1:200, the value of the setpoint has to be decreased fourfold to obtain correct results.

Conclusion

The Agilent High Sensitivity Protein 250 assay facilitates accurate and reproducible sizing and quantification of protein samples with sensitivity comparable to or better than silver staining of PAGE gels over a linear dynamic range of four orders of magnitude. Options for sample quantification are available to customize the assay, for example, to correct for sample matrix effects. The reproducibility of the assay can be enhanced by employing a user defined internal standard or by increasing sample volumes to reduce the pipetting error. Quantification accuracy of the assay may be increased by employing a calibration standard for a target protein. The outstanding quantification features make this assay an ideal tool to tackle challenging analytical problems such as the analysis of minute protein amounts or the quantification of low level impurities.

References

1.
"Agilent High Sensitivity Protein 250 Kit Guide", *Agilent Technologies Manual, reference number G2938-90310*

2.
"Performance Characteristics of the High Sensitivity Protein 250 Assay for the Agilent 2100 Bioanalyzer", *Agilent Technologies Technical Note, publication number 5989-8940EN*, **2008.**

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