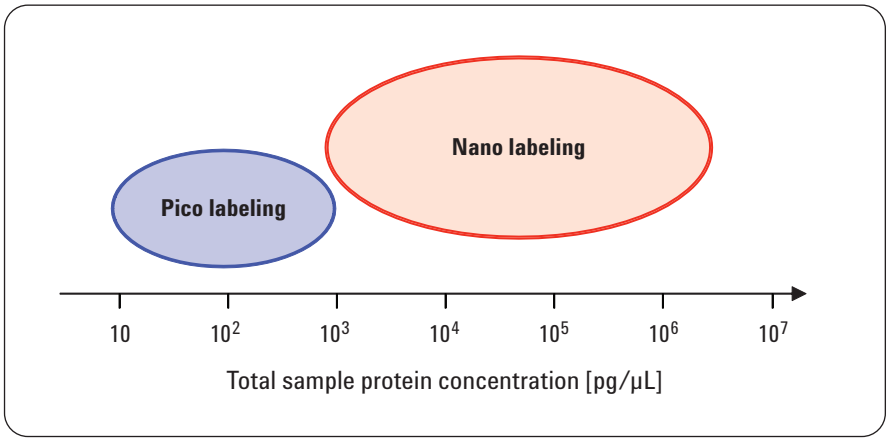
Additional Pico protocol for the High Sensitivity Protein 250 assay with the Agilent 2100 Bioanalyzer

Optimized procedure for lowest concentrated samples

Technical Note

Protein Electrophoresis



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 with a linear dynamic range spanning four orders of magnitude. The sensitivity is superior to silver-stained SDS-PAGE, allowing it to detect concentrations as low as 1 pg/µL labeled bovine serum albumin (BSA) on-chip. However, the fluorescent labeling requires a minimum total protein concentration of 1 ng/µL in the initial sample.

This Technical Note describes a new Pico labeling protocol, which extends the applicability to protein samples with concentrations below 1 ng/µL.

Agilent Technologies

Introduction

The recently introduced High Sensitivity Protein 250 assay for the Agilent 2100 Bioanalyzer allows protein sizing and quantification with sensitivity superior to silver-stained SDS-PAGE and a linear dynamic range spanning up to four orders of magnitude. The performance characteristics of the new assay as well as different options for protein quantification were previously discussed [1, 2].

The High Sensitivity Protein 250 assay consists of two distinct steps: first, a sample preparation step that couples a fluorescent dye covalently to the sample proteins and second, on-chip protein electrophoresis and detection with the 2100 bioanalyzer. It is possible to detect down to 1 pg/µL of protein on-chip. How-ever, the initial labeling step requires a total protein concentration in the sample of at least 1 ng/µL. This technical note introduces an alternative sample labeling procedure, the Pico labeling protocol, which allows analysis of samples with a total protein concentration of less than 1 ng/µL.

Experimental

Materials and equipment

Carbonic anhydrase from bovine erythrocytes (Sigma, Taufkirchen, Germany), 10 kDa ladder and BenchMark ladder (Invitrogen, Carlsbad, CA, USA), Protein LoBind tubes (Eppendorf GmbH, Hamburg, Germany), 2-D Clean-Up kit (GE Healthcare, Freiburg, Germany), 0.5 mL Zeba Desalt Spin Columns and Coomassie Plus Assay Reagent (Pierce, Rockford, IL, USA), Agilent 2100 bioanalyzer and High Sensitivity Protein 250 kit (Agilent Technologies GmbH, Waldbronn, Germany).

High Sensitivity Protein 250 Assay

Sample preparation

The following sample preparation steps were performed to obtain optimal conditions for the labeling reaction. Carbonic anhydrase was dissolved in 1x standard labeling buffer (30 mM Tris/HCl, pH 8.5). The 10 kDa 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). A buffer exchange to 1x standard labeling buffer was done using 0.5 mL Zeba Desalt Spin Columns, and protein concentrations were measured with the Coomassie Plus Assay Reagent. All dilution series prior to sample labeling were prepared with 1x standard labeling buffer.

Nano labeling protocol

Sample labeling and on-chip analysis was performed according to the standard protocol described in the Agilent High Sensitivity Protein 250 Kit Guide [3].

Pico labeling protocol

Samples with protein concentrations between 10 pg/µL and 1 ng/µL were labeled with the alternative Pico labeling protocol (Figure 1). One vial of labeling dye was reconstituted in 54 µL dimethyl sulfoxide (DMSO) according to the standard protocol. The reconstituted labeling dye was diluted 100-fold by adding 1 µL dye to 99 µL DMSO. 0.5 µL of this diluted labeling dye was added to 5 µL protein sample in Protein LoBind tubes and the mixture was incubated on ice for 30 minutes. After addition of 2.75 µL sample buffer with or without dithiothreitol (DTT), samples were heated to 95 °C for 5 minutes and immediately analyzed. The on-chip electrophoresis was performed according to the standard method described in the Reagent Kit Guide [3]. The labeled protein samples were not stored. The High Sensitivity Protein 250 ladder was labeled and used according to the standard protocol.

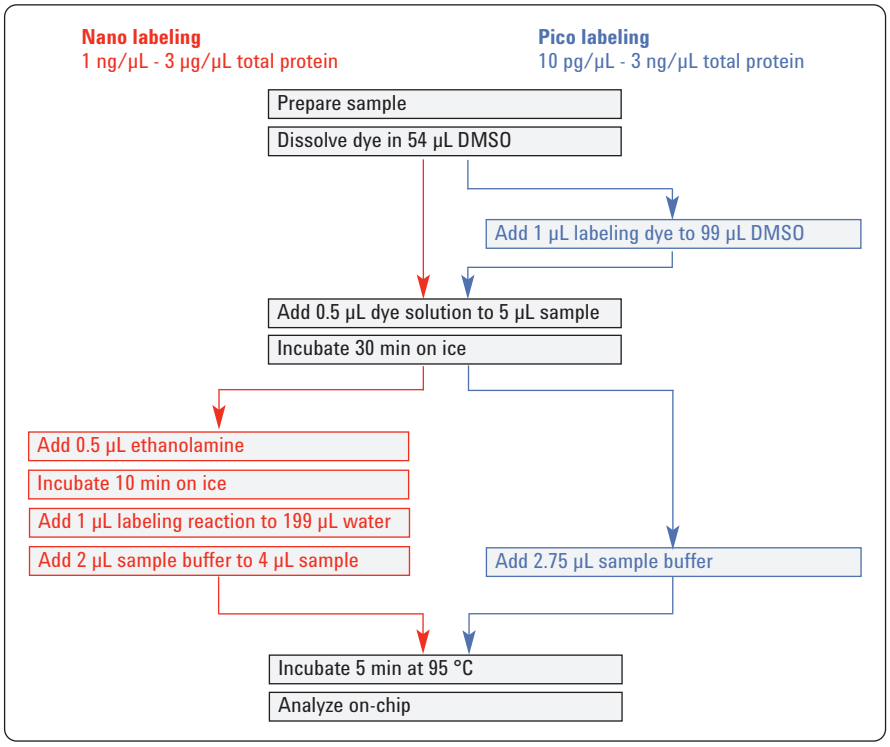


Figure 1
Workflow of the Nano and the Pico labeling protocols.

Results and Discussion

The High Sensitivity Protein 250 assay allows analysis of protein size and quantity with on-chip sensitivity down to 1 pg/µL. The Nano labeling method requires a minimal protein concentration of at least 1 ng/µL in the initial sample. This technical note describes an alternative labeling procedure, the Pico labeling protocol, which allows the analysis of samples with a protein concentration below 1 ng/µL. Both methods, the Nano protocol and the alternative Pico protocol, are compared regarding sensitivity, protein sizing, and quantification.

Figure 2 compares results from the two labeling protocols obtained with the High Sensitivity Protein 250 assay for a carbonic anhydrase sample with a total protein concentration of 1 ng/µL.

In the Nano protocol a 200-fold dilution with water is done after the labeling reaction to ensure that no peak exceeds the measuring range of the 2100 bioanalyzer [3]. In case of very low sample protein concentrations, this dilution is required to reduce the fluorescence of the lower marker peak, which consists mainly of leftover dye from the labeling reaction. If the dilution step is omitted, the lower marker is outside the detection range of the 2100 bioanalyzer and the electropherogram shows rectangular peaks (green electropherogram in Figure 2, arrow). This affects sizing and quantification.

As expected, all peaks are well inside the measuring range of the 2100 bioanalyzer if the Nano protocol, including the 200-fold dilution step, is used. However, not only the lower marker peak but also the sample peak are inevitably reduced about 200-fold compared to the electropherogram without dilution (red and green traces of Figure 2).

If the labeling reaction is performed with 100-fold less fluorescent dye as in the Pico labeling protocol, no dilution of the labeled sample is necessary. The lower

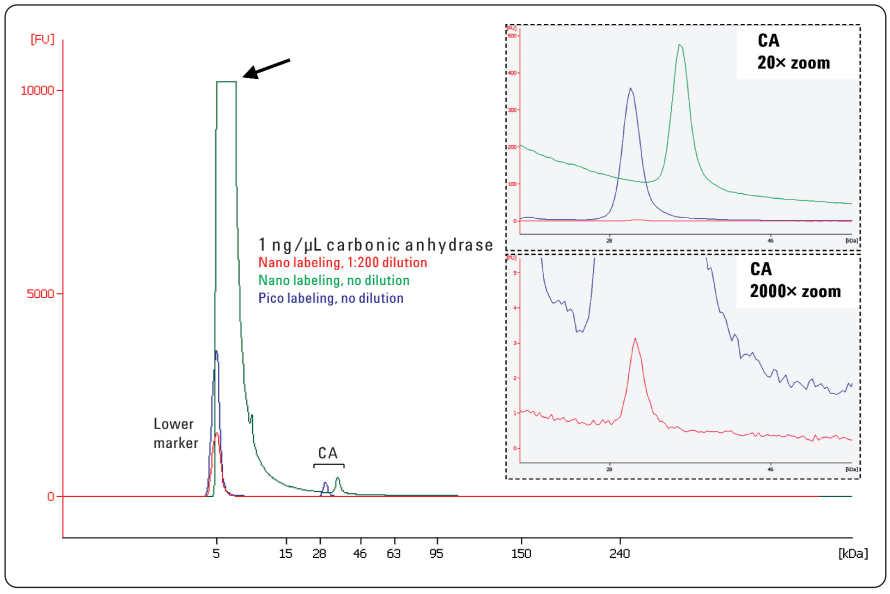


Figure 2
A 1 ng/µL carbonic anhydrase (CA) was labeled according to the Nano (red) and the Pico (blue) labeling protocols, and analyzed on-chip. The result of the Nano sample labeling protocol without the 200-fold dilution in water is shown in green. The arrow marks to the lower marker peak that exceeds the measuring range of the Agilent 2100 bioanalyzer.

marker peak is now well within the measuring range of the 2100 bioanalyzer, whereas the sample peak shows about the same intensity as the one derived from the standard protocol without dilution (green and blue traces of Figure 2). The labeling efficiency for very small total sample concentrations is about the same with the Nano method and the Pico labeling protocol. Since the Pico method uses less labeling dye, the resulting lower marker peak is significantly reduced, and a dilution of the sample prior to on-chip analysis is no longer necessary. This allows analyzing samples with lower initial protein concentrations. The Pico labeling protocol thus extends the applicability of the High Sensitivity Protein 250 assay to samples with total protein concentrations below 1 ng/µL.

The Pico labeling protocol not only required less protein in the original sample, but it is also shortened compared to the standard method (Figure 1). The sample buffer is directly added to the labeling mix after 30 minutes of incubation on ice. No reaction quenching with ethanolamine and no dilution with water are done prior to on-chip analysis.

Sensitivity and linear dynamic range

The alternative pico labeling protocol allows analysis of samples with a total protein concentration between 10 and 1,000 pg/µL (Figure 3). A sample solution of 10 pg/µL carbonic anhydrase still resulted in a peak with a signal-to-noise ratio of 60 that was automatically detected and integrated with standard integration settings. Hence, the limit-of-detection (signal-to-noise > 3) is extrapolated to be around 1 pg/µL of labeled protein on-chip and thus similar to the on-chip detection limit achievable with the Nano method. However, the Nano protocol requires a much higher total protein concentration in the starting original sample.

The linearity of the modified assay is good, with a correlation coefficient (r^2) of 0.998 (Figure 3b).

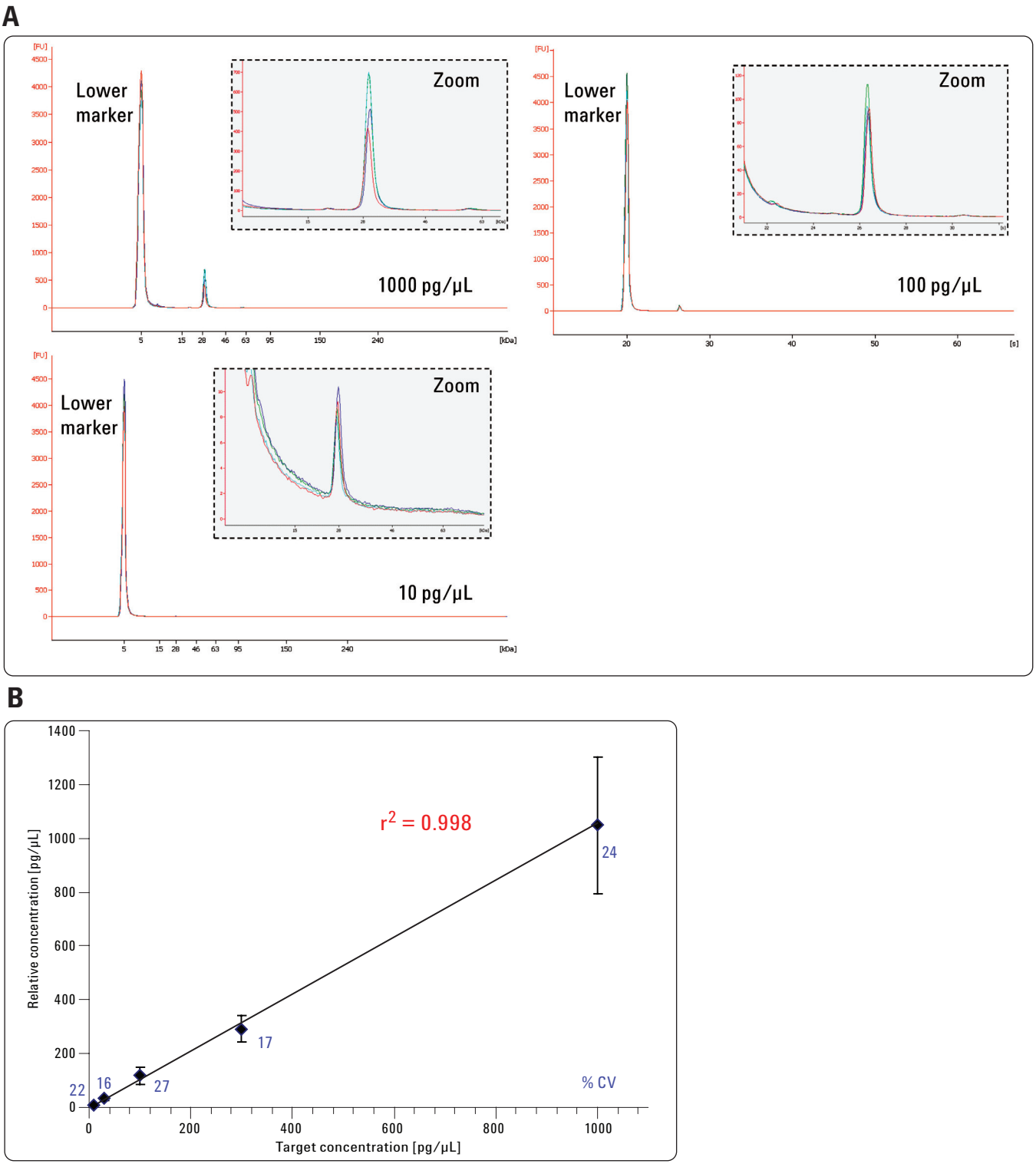


Figure 3

Sensitivity, reproducibility, and linear dynamic range of the High Sensitivity Protein 250 assay employing the Pico labeling protocol. 10 to 1,000 pg/µL CA was labeled and analyzed on-chip. (A) The overlays of electropherograms from four independent labeling reactions for every concentration demonstrate the reproducibility of the method. (B) Average relative CA concentrations with standard deviations from eight independent labeling reactions were plotted against the expected concentrations; % CV data for each concentration are shown in blue.

Protein sizing

Figure 4 compares the results obtained with the Nano and the Pico labeling protocols for two different protein size standards, the BenchMark ladder and the 10 kDa ladder. All peaks were detected with both variants of the High Sensitivity Protein 250 assay. No difference was seen in terms of sizing resolution, accuracy, or reproducibility. Data obtained with the Pico labeling protocol were within limits of error identical to those obtained with the Nano method (data not shown and [1]).

Protein quantification

The quantification reproducibility of the Nano method for samples with total protein concentrations between 1 and 3,000 ng/µL is significantly better compared to the Pico labeling protocol for samples with concentrations between 10 and 1,000 pg/µL. Typical CV values for protein quantification obtained with the standard protocol are 5 to 20 % [1], whereas the corresponding values for the Pico labeling method range between 15 and 30 % (Figure 3b). In addition, the new assay variant should not be used for total protein exceeding the indicated concentration range since the labeling efficiency levels off at above 1 ng/µL because the amount of dye becomes limited (data not shown).

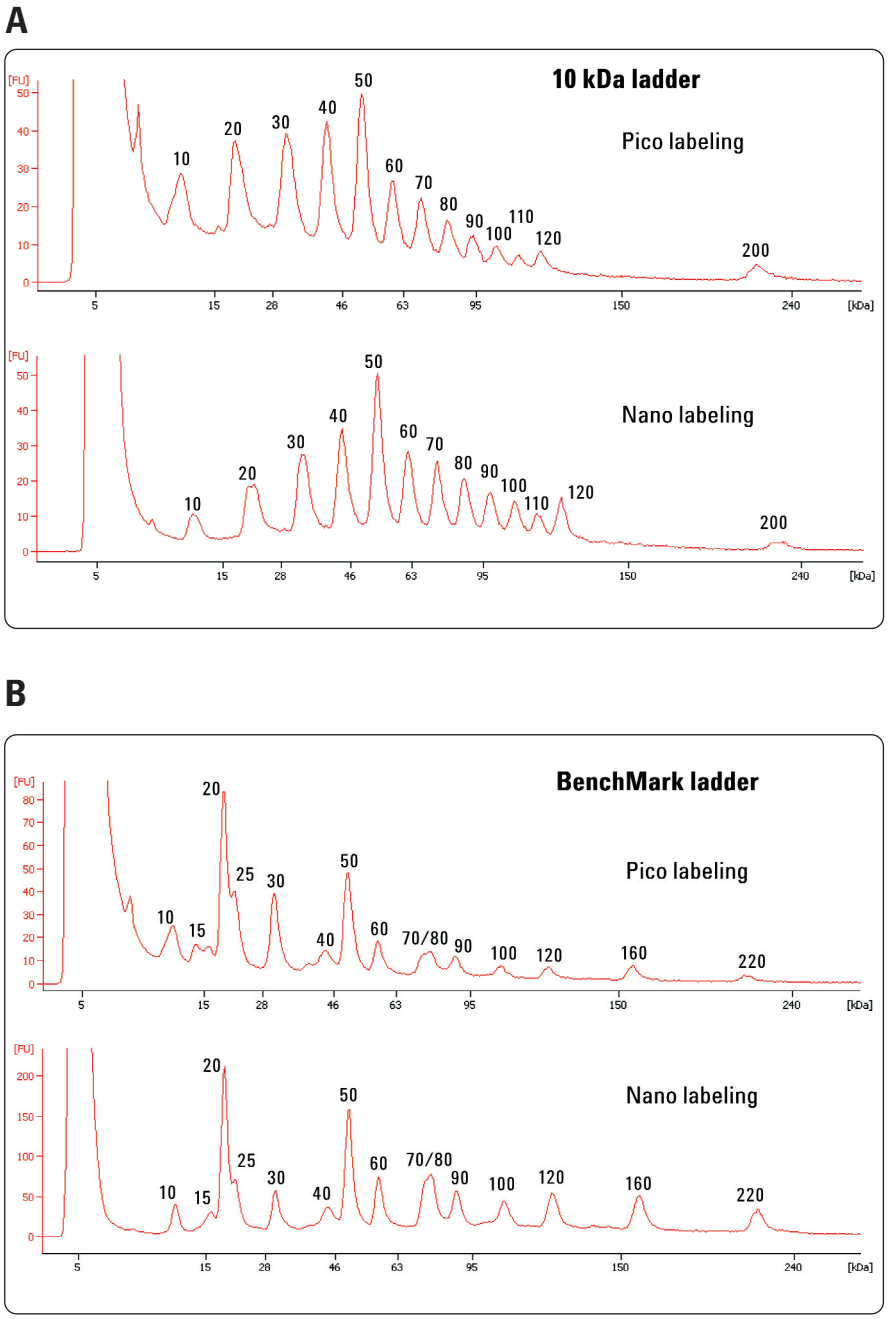


Figure 4
Analysis of protein sizing standards with the two labeling variants of the High Sensitivity Protein 250 assay. (A) The 10 kDa ladder was labeled at 150 ng/µL with the Nano method and at 1 ng/µL with the Pico labeling protocol. (B) The BenchMark ladder was labeled at 240 ng/µL with the Nano method and at 1 ng/µL with the Pico labeling protocol.

Furthermore, a quantification bias with the Pico labeling protocol is observed in Figure 4 that favors proteins below 40 kDa. A more quantitative description of this effect is presented in Figure 5. A reason for the enhanced variability of the assay employing the Pico labeling protocol could be the increased loss of protein at plastic surfaces like microcentrifuge tubes or pipette tips. At protein concentrations lower than 1 ng/µL in nonphysiological buffers, like the standard labeling buffer (30 mM Tris/HCl, pH 8.5), large proteins especially are prone to denature and expose hydrophobic patches to the solvent and stick to plastic surfaces. Along these lines of evidence is the observation that the addition of salt to the labeling buffer can attenuate this bias to some extent, however, at the expense of sensitivity (data not shown).

Electrophoretic effects related to the sample matrix pose another potential pitfall for the Pico protocol in combination with the High Sensitivity Protein 250 assay. With the sample being directly analyzed on-chip without dilution matrix components like salt and detergents are not diluted out and may therefore affect the analysis. Please refer to the High Sensitivity Protein 250 Kit Guide[3] for a list of potential matrix effects. Given such considerations, the Pico labeling protocol should be regarded as semi-quantitative.

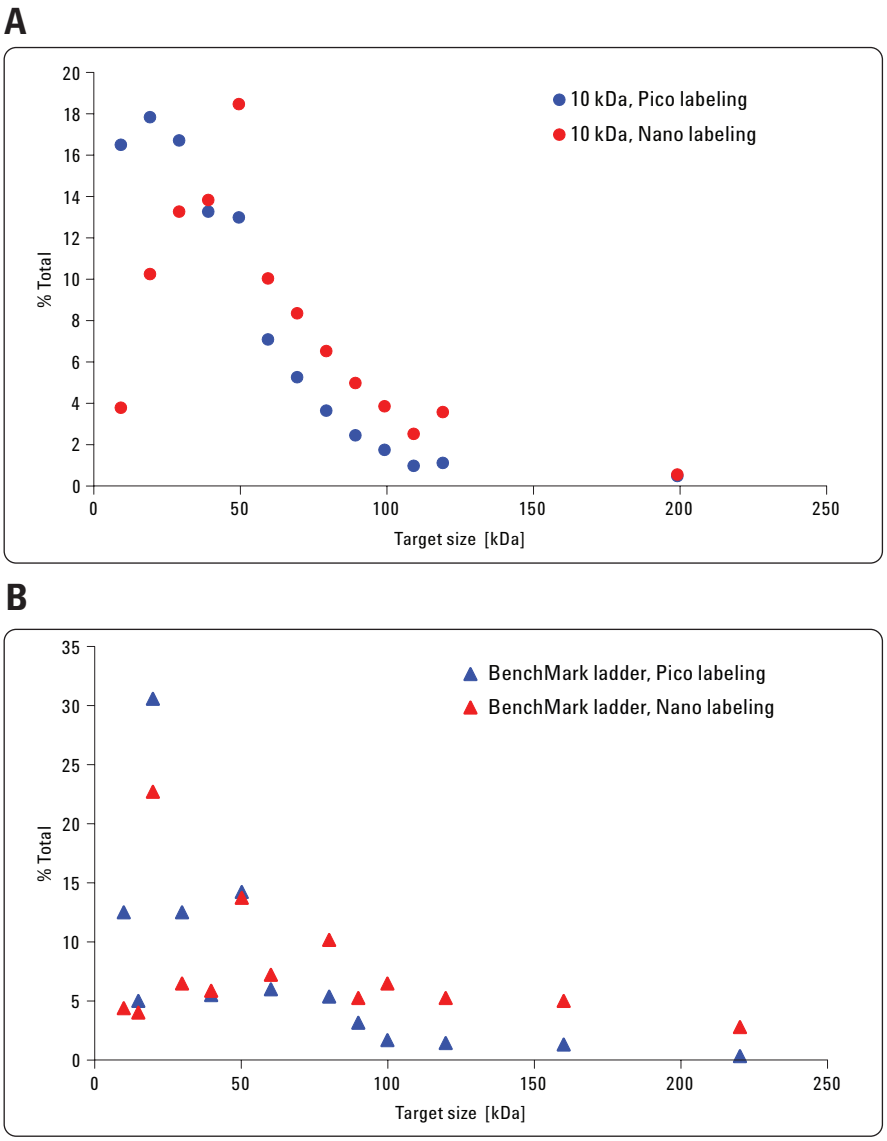


Figure 5
Bias favoring small proteins with the Pico sample labeling protocol. The data are derived from the experiment shown in Figure 4. % Total values given by the 2100 expert software are plotted against the target protein size; (A) for the 10 kDa ladder and (B) for the BenchMark ladder. Averages of four independent labeling reactions are shown. The data from the Nano protocol is plotted in red and from the Pico protocol in blue.

Conclusion

This technical note introduces an alternative Pico labeling protocol for the High Sensitivity Protein 250 assay that is suited for the semiquantitative analysis of samples with a total protein concentration from 10 pg/µL to 1 ng/µL. Table 1 summarizes the performance of the Pico labeling in comparison to the standard Nano labeling protocol. The Pico labeling protocol for the High Sensitivity Protein 250 assay and the 2100 bioanalyzer will prove extremely useful for the analysis of highly diluted proteins, such as secreted proteins in culture media, or in the analysis of particularly small amounts of protein derived from laser micro dissections.

	High Sensitivity Protein 250 Assay	
	Nano labeling	**Pico labeling**
Sizing range	10 – 250 kDa	
Typical sizing resolution	10%	
Typical sizing accuracy	10% CV	
Sizing reproducibility	3% CV	
Protein conc. required for labeling	1 ng/µL – 3 µg/µL	10 pg/µL – 1 ng/µL
Protein conc. detected on-chip	≥ 1 pg/µL	
Quantitative range	Up to 4 orders of magnitude	
Quantification reproducibility	Quantitative 5% to 20% CV	Semi-quantitative 15% to 30% CV
Compatible buffers	See list of compatible buffers[3]	More sensible towards potential matrix effects
Labeling protocol	See Figure 1 and Kit Guide[3]	See Figure 1, shortened by approximately 15 min

Table 1
Comparison of the High Sensitivity Protein 250 performance for samples labeled with the Nano and the Pico labeling protocol.

References

1.
Performance characteristics of the High Sensitivity Protein 250 assay for the Agilent 2100 bioanalyzer, *Agilent Technologies Technical Note*, publication number 5989-8940EN, **2008**.

2.
Quantification strategies with the High Sensitivity Protein 250 Assay, *Agilent Technologies Technical Note*, publication number 5989-8941EN, **2008**.

3.
Agilent High Sensitivity Protein 250 Kit Guide, *Agilent Technologies Manual*, reference number G2938-90310, **2008**.

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