

Alternative to 2D gel electrophoresis – OFFGEL electrophoresis combined with high-sensitivity on-chip protein detection

Application Note

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Abstract

Two-dimensional gel electrophoresis (2D-GE) employs isoelectric focusing in the first dimension and a separation of the proteins according to their molecular weights in the second dimension. The gels are then commonly stained using silver stain to visualize the protein pattern. This is a tedious and time-consuming procedure. Here, we demonstrate a combination of two newly developed methods – OFFGEL fractionation based on isoelectric point, and high-sensitivity on-chip electrophoresis with the Agilent 2100 bioanalyzer. This powerful combination performs an analytical 2D-GE-type analysis with excellent sensitivity and reproducibility. The ease of use is significantly improved compared with the traditional 2D-GE method.

Agilent Equipment

- Agilent 3100 OFFGEL Fractionator
- Agilent 2100 bioanalyzer
- High Sensitivity Protein 250 kit

Application Area

- Differential Protein Expression



Agilent Technologies

Introduction

Two-dimensional gel electrophoresis (2D-GE) is a standard protein analysis method that has not changed significantly over the past years. However, the growing interest in proteomics has catapulted 2D-GE to the forefront of modern research techniques.

Generally, isoelectric focusing is used in the first dimension of 2D-GE to separate proteins according to their isoelectric point (pI). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used as a second dimension to separate the proteins according to their molecular weights (kDa). The detection is commonly done with silver stain.

2D-GE is often employed to analyze patterns in global protein expression for an organism or a tissue. In combination with mass spectrometry, it is used for large-scale identification of the proteins present in a complex sample. Furthermore, 2D-GE is used to examine the differential expression of proteins in comparable, related samples, such as healthy tissue versus diseased tissue. 2D-GE is particularly useful for biomarker discovery and drug discovery, development, and evaluation.

2D-GE is a powerful method, but it is technically demanding, tedious, and time-consuming. There is a need for easy-to-use and robust instrumentation that allows reproducible fractionation of complex protein mixtures with high resolution, and allows highly sensitive protein detection.

Here, we present a combination of two instrumental methods that separate proteins according to their isoelectric point (pI) and molecular weight (kDa), in an analogous fashion to 2D-GE. For the first dimension, OFFGEL electrophoresis using the Agilent 3100 OFFGEL Fractionator was employed. This newly developed method takes advantage of the impressive resolving power of immobilized pH gradient-based isoelectric focusing (IPG-IEF).^{1,2} In contrast to conventional isoelectric focusing, OFFGEL fractionation delivers sample in the liquid phase. The main advantages are that there is no need for gel staining and no need to recover sample from the gel. In addition, separations with the Agilent 3100 OFFGEL Fractionator are easy to perform and have the potential for automation.

For the second dimension, a recently introduced assay for high-sensitivity on-chip protein sizing with the Agilent 2100 bioanalyzer was employed. The new Agilent High Sensitivity Protein 250 assay is based on the detection of fluorescently labeled proteins that are electrophoretically separated on microfluidic chips. The assay allows separation of proteins from 10 to 250 kDa, offers a sensitivity equivalent to or better than silver staining, and delivers a linear dynamic range across four orders of magnitude.

Experimental

Protein samples

An *E. coli* protein lysate was used to demonstrate the combination of these two powerful methods. Lyophilized *E. coli* strain B cells (ATCC 11303; Sigma, Taufkirchen, Germany) were resuspended in urea/thiourea buffer (30 mM Tris/HCl, 7 M urea, 2 M thiourea, pH 8.5) and were disrupted with a BeadBeater (Biospec Products, Bartlesville, Oklahoma, USA). Cell debris was removed by centrifugation at 12,000 g at 4 °C for 10 minutes. The protein concentration of the cell lysate was determined with the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, Illinois, USA).

Labeling reaction

Prior to labeling, the *E. coli* lysate (1 mg) was precipitated with acetone (4-fold volume) and the pellet was dissolved in 100 µL of urea/thiourea buffer. The protein concentration was measured and adjusted to 5 mg/mL. Aliquots of the *E. coli* lysate were spiked with 1, 0.1, 0.01 %, or no beta-lactoglobulin (βLG). Prior to spiking, beta-lactoglobulin A (βLG, Sigma, Taufkirchen, Germany) was desalted using Zeba Desalt Spin Columns (Pierce, Rockford, Illinois, USA).

The High Sensitivity Protein 250 kit (Agilent part number 5067-1575) was used for the protein labeling. For each sample, 1 μ L fluorescent labeling dye was added to 10 μ L protein or ladder in urea/thiourea buffer and the mixture was incubated on ice for 30 minutes. Any excess dye was quenched by the addition of 1 μ L ethanolamine, followed by 10 minutes incubation on ice.

OFFGEL electrophoresis

The *E. coli* cell lysate samples were subjected to OFFGEL electrophoresis prior to analysis with the Agilent 2100 bioanalyzer. The Agilent 3100 OFFGEL Fractionator with a 12-well setup and the Agilent 3100 OFFGEL low resolution kit, pH 3-10 (Agilent part number 5188-6425) were used according to the standard protocol. For each sample, the entire labeling reaction (corresponding to 50 μ g of the *E. coli* sample) was diluted in 2 mL of 1X OFFGEL solution. The IPG strips were rehydrated for 15 minutes, and then 150 μ L of sample was loaded per well. The proteins were fractionated based on their pI according to the default method *OG12PR01*.

High sensitivity protein analysis with the 2100 bioanalyzer

The labeled *E. coli* samples were fractionated by OFFGEL electrophoresis as described above and were subsequently analyzed

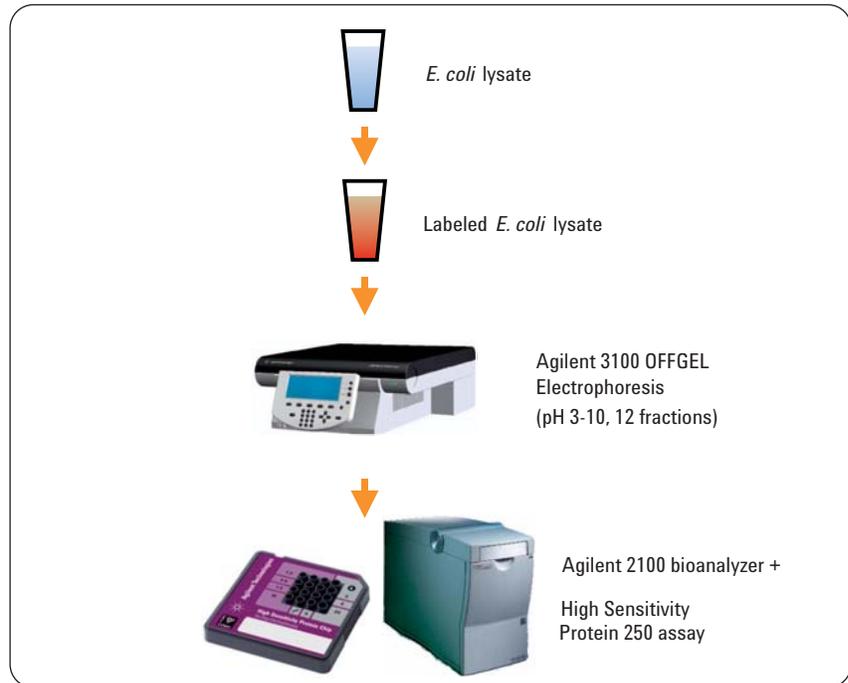


Figure 1
Experimental workflow combining OFFGEL electrophoresis and high-sensitivity on-chip protein analysis.

using the Agilent 2100 bioanalyzer and the Agilent High Sensitivity Protein 250 assay, according to the standard protocol. If the samples were subjected to OFFGEL fractionation prior to the on-chip electrophoresis, the 1:200 dilution step described in the Agilent High Sensitivity Protein 250 protocol was omitted.

Two μ L of sample buffer containing dithiothreitol (DTT) was added to 4 μ L of each fractionated sample. Samples were then incubated at 95-100 $^{\circ}$ C for 5 minutes. The wells and the channels on the

chip were filled with both gel matrix and destaining solution. The samples and the ladder were loaded and the chip run was started immediately.

Results and discussion

Figure 1 demonstrates the experimental workflow that was followed. The *E. coli* cell lysate was fluorescently labeled and subjected to OFFGEL electrophoresis, followed by high-sensitivity protein analysis with the Agilent 2100 bioanalyzer.

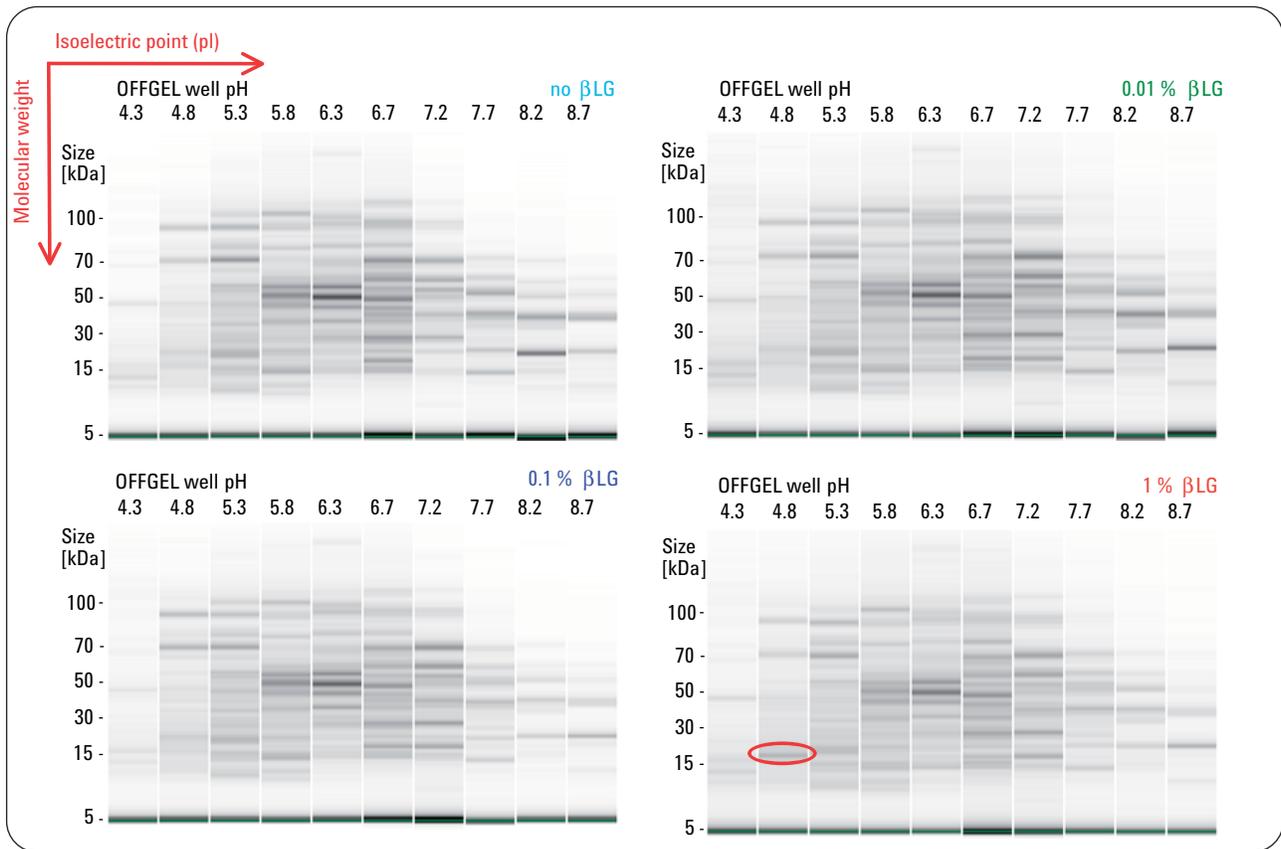


Figure 2
E. coli cell lysate in the absence and presence of βLG was subjected to OFFGEL fractionation followed by high-sensitivity on-chip electrophoresis. A red circle indicates the position of the βLG.

Figure 2 shows the gel-like images obtained with the Agilent 2100 bio-analyzer. The images show the separation of OFFGEL fractions of *E. coli* cell lysate and the same lysate spiked with different beta-lactoglobulin (βLG) concentrations. All samples were fluorescently labeled prior to the pI-based separation in the first dimension. Ten OFFGEL fractions corresponding to pH 4.3 to 8.7 were

separated according to their molecular weight (kDa) in the second dimension using the High Sensitivity Protein 250 assay.

All gel-like images in figure 2 show the highly reproducible typical protein pattern from an *E. coli* lysate. The position of the additional βLG peak is indicated in the fraction that corresponds to pH 4.8 at 1 % βLG. The pI of βLG is 5.1; therefore, it is expected to be

recovered in the fraction with pH 4.6 to 5.1. The signal at 5 kDa seen in all gel-like images corresponds to the internal lower marker of the assay, which is used together with the ladder for accurate protein sizing.

By comparing the gel-like images in figure 2, the addition of 1 % βLG can be easily detected. However, it is difficult to see any difference between the control (no βLG) and

the addition of 0.01 or 0.1 % β LG. Such small changes in the protein profile can be much better detected when overlaying the electropherograms, as shown in figure 3. This demonstrates that the combination of both technologies (OFFGEL and on-chip separations) allows detection of a small change in protein pattern, comparable to the absence and presence of 0.1 % β LG relative to the total protein concentration.

The OFFGEL electrophoresis reduces sample complexity, making it possible to detect a small difference in the protein pattern. The upper panel of figure 3 clearly shows that it would not be feasible to detect a 0.1 % addition of β LG without the additional OFFGEL fractionation step. The addition of 1 % β LG can hardly be detected in the electropherogram, and it is impossible to detect the addition of 0.1 % β LG. The lower panel of figure 3 shows that when the same samples are OFFGEL fractionated before analysis with the 2100 bioanalyzer, a small peak at 0.1 % β LG can be noticed and the addition of 1 % β LG is easily detected by the appearance of a prominent peak at 17.5 kDa. Here, a fractionation into 12 fractions from pH 3-10 was performed. A fractionation into 24 samples would have reduced sample complexity even further, returning a more prominent β LG peak in the sample with 0.1 % β LG.

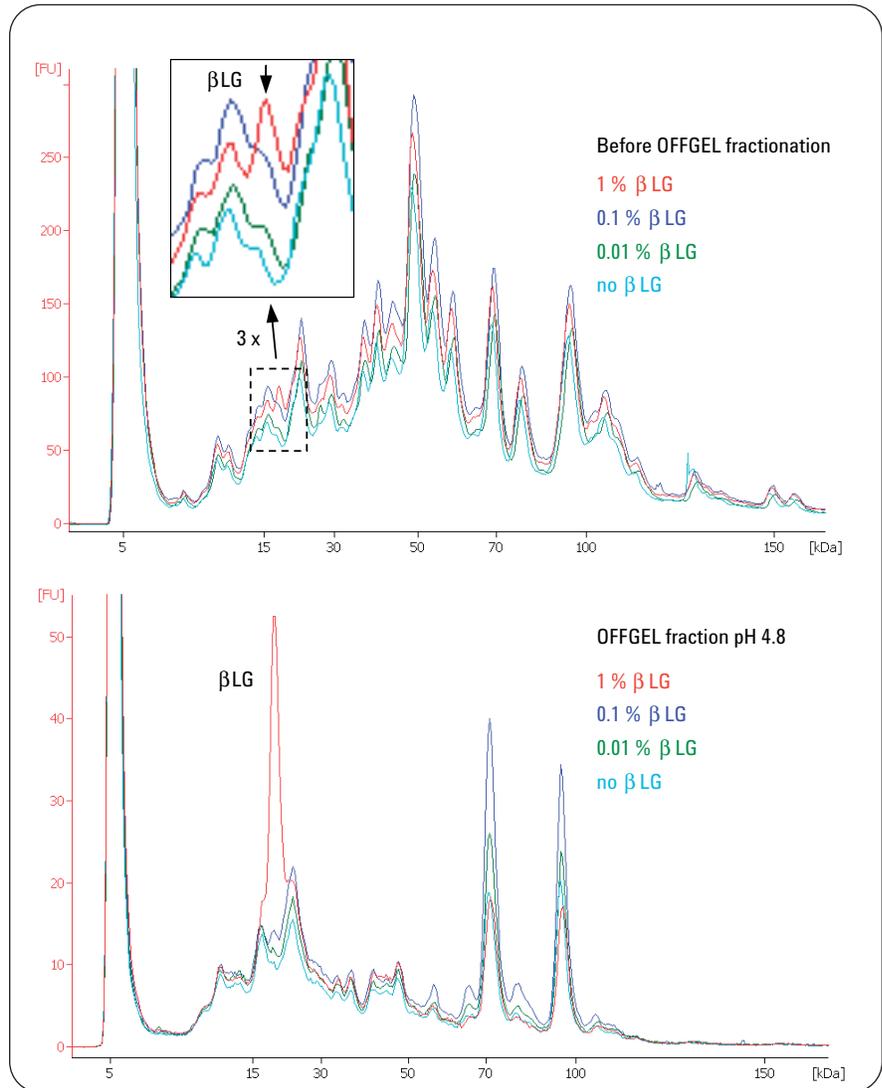


Figure 3
Overlays of electropherograms obtained by analyzing *E.coli* lysates before and after OFFGEL fractionation. The different traces show the effect of the addition of different concentrations of β LG.

For this type of application, it must be considered that the fluorescent protein labeling affects the pI. The negatively charged fluorescent dye is bound to lysine residues and therefore changes the net charge of the protein at physiological pH by -2 per attached dye molecule. Hence, the labeling should be performed at high protein concentrations (≥ 1 mg/mL) to keep the number of introduced labels per protein to a minimum.

By combining the resolving power of OFFGEL electrophoresis in the first dimension with the high-sensitivity on-chip electrophoresis in the second dimension, it is possible to obtain a highly reproducible pseudo-2D-gel image, significantly improving the ease of use compared with conventional 2D-GE.

Both of these instrumental methods provide diagnostic features that permit a quick and easy check of the fractionation quality or on-chip electrophoresis performance. The Agilent 3100 OFFGEL

Fractionator records voltage, power, and current during fractionation, which provides a diagnostic indicator for the quality of each individual sample fractionation. The Agilent 2100 bioanalyzer provides online diagnostics, and the analytical results can be reviewed during the chip run. When performing traditional 2D-GE, the outcome of the tedious and time-consuming experiments is only visualized after the silver-stain procedure.

The 2D-GE-type analysis achieved by combining OFFGEL electrophoresis and on-chip protein analysis delivers reproducible and reliable results with a significant improvement in ease of use and automation compared with traditional 2D-GE. The labeling reaction takes approximately 45 minutes, the OFFGEL fractionation is done overnight, and the on-chip electrophoresis takes only 35 minutes, which includes protein separation, staining, detection, and data analysis.

Conclusion

The combination of the OFFGEL fractionation with the high-sensitivity on-chip electrophoresis of the Agilent 2100 bioanalyzer provides an analytical tool with excellent reproducibility and sensitivity. The combination enables 2D-GE-type analyses with the high resolution and high sensitivity that are suitable for differential gene expression applications. The data demonstrates that it is possible to detect a 0.1 % change in protein expression pattern. The OFFGEL fractionation provides resolution and reduces the sample complexity. The Agilent High Sensitivity Protein 250 kit adds additional separating power, delivers sensitivity that is comparable or superior to silver stain, and provides a linear dynamic range of up to four orders of magnitude.

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